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NATURAL COLORING MOLECULES FROM MARINE FUNGAL BIODIVERSITY OF REUNION ISLAND: Optimization of production, extraction and characterization of polyketide pigments from Talaromyces albobiverticillius 30548

Thesis for the degree of Doctor of Philosophy

Reunion Island, November, 2017.

THÈSE

Présenté pour l'obtention du titre de Docteur de l'Université de La Réunion

Spécialité - **Biotechnologie microbienne**

Par

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Molécules colorantes naturelles de la biodiversité fongique marine La Réunion:
Optimisation de la production, extraction et caractérisation du polyketide pigments de Talaromyces albobiverticillius 30548

Soutenue publiquement le 17 Novembré 2017

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DOCTORAL THESIS

Presented By

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To the Faculty of Sciences and Technologies

Université de La Réunion

For The Award Of

Doctor of Philosophy (Ph.D.) in Sciences

On The Research Project Entitled

**Natural coloring molecules from marine fungal biodiversity of Reunion Island:
Optimization of production, extraction and characterization of polyketide pigments of *Talaromyces albobiverticillius* 30548**

Discipline – **Microbial Biotechnology**

Thesis will be publicly defended at Amphithéâtre Charpak

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STEPPING STONE INTO RESEARCH

Nothing wrong with not knowing as long as you are willing to find out
                                              ~ *Lawrence Krauss*

FOR A TRAINED RESEARCHER

If you can't explain it simply, you don't understand it well enough

                                              ~ *Albert Einstein*

PREFACE

The present PhD study describes the results from the research work conducted from January 2014 to June 2017. This research has been primarily carried out at Laboratoire de Chimie des Substances Naturelles et des Sciences des Aliments (LCSNSA), Université de La Réunion. Few experiments were performed in collaboration with Technical University of Denmark (DTU) with the help of Prof. Kristian Fog Nielsen and also at University of Messina, Italy under the guidance of Prof. Daniele Giuffrida.

This entire research work has been supervised by Prof. Laurent Dufossé and co-supervised by Dr. Mireille Fouillaud from Université de La Réunion. This research project was made possible with the grant, DIRED/20131527 from Conseil Régional de la Réunion, France and FEDER action of the European Union.

This study focuses on the production of natural polyketide pigments from the marine-derived fungal isolate, *Talaromyces albobiverticillius*. Reading this dissertation will give an understanding of market of natural food colors; microbial colorants and its applications; morphological characters and pigments produced from the studied strain *Talaromyces albobiverticillius*; Optimization of Production and extraction of pigments; HPLC and NMR analysis of identified pigment fractions; upscaling from lab scale to fermenter.

Since from my early days, I wish to pursue my research studies abroad especially in the field of food science and technology. Though our world is awash in colors, I have always had a passion towards natural colors. Being engaged in food related studies; I had developed a strong interest to continue my scientific research on natural food colors. With this in mind, I undertook this "tailor-made" PhD project and thoroughly enjoyed working in the intriguing field of fungi. Societies have used fungi for centuries in a wide variety of ways that have been exploited industrially and commercially. Among them, fungal pigments serve as sustainable natural food colorants that have largely gained the attention of food scientists to explore the fundamentals. I was so glad to work in this project as it helped me to understand and enhance my knowledge on fungal world and pigments produced by them. In addition to that, I came to know the importance of teamwork and role of dedication in the field of scientific research.

Mekala Venkatachalam

Reunion Island, October, 2017

ACKNOWLEDGEMENTS

Abstract

It is well known that the vast majority of food colorants used in food and beverage applications comes from the pigments synthesized by plant materials. Besides, stability of many plant-derived colors can create formulation problems. Factors such as the region, the climate, the environment, the cultivar all impact colors shade, strength and overall stability in the final product. As an alternate, fungi of the genus *Monascus, Penicillium* and *Talaromyces* are known as excellent producers of red pigments. These red pigments are of industrial interest as they are stable and non-toxic and can be used as food colorants.

This present research deals with the selection of high throughput red pigment producing *Talaromyces albobiverticillius* as a source of polyketide based natural food colorants. Design of Experiments (DoE) and Response Surface Methodology (RSM) have been used to optimize culture conditions and media formulation of fermentation process. Using Box Behnken Design (BBD), the influence of different physical factors on pigment and biomass production was studied using potato dextrose broth as culture media. The best optimal conditions were found to be with initial pH of 6.4, temperature of 24 °C, agitation speed of 164 rpm and fermentation time of 149 h gave 47.93 ± 0.58 mg /L of orange pigment, 196.28 ± 0.76 mg / L of red pigment and 12.58 ± 0.41 g /L of dry biomass. With the application of Plackett- Burman Design (PBD), 16 different media formulations were optimized using various carbon and nitrogen sources. When Sucrose and Yeast extract was used as a basal medium at 24° C, high pigment yield was observed: 695.93 ± 0.29 mg /L of orange pigment, 738.28 ± 0.51 mg / L of red pigment and 6.80 ± 0.37 g /L of dry biomass.

Twelve different compounds were detected from the HPLC-PDA-ESI/MS analysis of intracellular and extracellular pigmented extracts. In particular, N-threonine-monascorubramine, N-glutaryl-rubropunctamine and PP-O were tentatively identified among these twelve compounds; further, this work reports for the first time on the PDA, MS and NMR characterization of the here named as N-GABA-monascorubramine derivative (6-[(Z)-2-Carboxyvinyl]-N-GABA-monascorubramine) pigment bearing a cis configuration at the C10-C11 double bond, in *Talaromyces albobiverticillius* 30548. Attempts were made to study the effects of sea salts on pigment synthesis; sustainable green extraction methods for pigments; upscaling of fermentation from shake flasks to laboratory fermenter. All these experiments with their results were discussed briefly as individual chapters. Overall, these findings bring out the potential of marine-derived red pigment producing fungi and its possibility of obtaining tailor made food colorants.

Résume

Il est bien connu que la grande majorité de colorant alimentaire, utilisé dans l'application des aliments comme dans les boissons, provient des pigments **synthétisés** des matériels végétaux. De plus, plusieurs couleurs dérivées de plantes peuvent entraîner des problèmes de **formulation.** Des éléments, comme par exemple, la région, le climat, l'environnement**, la variété cultivée,** ont un effet, de nuances de couleurs, de résistance et surtout de stabilité dans le produit final. Par ailleurs, les champignons de genus monascus, Penicillium et Talaromyces sont connus comme des excellents producteurs de pigments rouges. Ces pigments intéressent aussi les industries car ils sont stables, non-toxiques et peuvent être utilisés comme un colorant alimentaire.

La recherche actuelle concerne la sélection des atouts du pigment rouge qui produit le talaromyces albobiverticillius, comme étant une source de polycétide basé sur les colorants alimentaires. Le plan d'expérience (DOE) et de méthodologie des surfaces de réponses (MSR), a été utilisé pour optimiser la condition de culture et de la formulation du milieu du procès de fermentation. En utilisant la Box Behnken design (BBD), l'influence des différents facteurs physiques sur le pigment et de la production de biomasse, ont été étudiés en utilisant le bouillon de dextrose de pommes de terre comme milieu de culture. La meilleure condition optimale trouvée avec l'initial pH de 6.4, température de 24°C Vitesse agitation de 164 rpm et le temps de fermentation de 149 heures, a donné 47.93 ± 0.58mg/L de pigment orange, 196.28 ± 0.76mg/L de pigment rouge et 12.58 ± 0.41 g/L de biomasse sèche. Avec l'application one-variable-at-a-time, les 16 différents milieux de la formulation ont été optimisés en utilisant diverses sources de carbone et nitrogène. En utilisant le sucrose et l'extrait de levure comme milieu de base à 24°C, un haut rendement de pigmentation a été observé : 695.93 ± 0.29mg/L de pigment orange, 738.28 ± 0.51 mg/L de pigment rouge et 6.80 ± 0.37g/L de biomasse sèche.

Douze différents composants ont été détectés, du HPLC-PDA-ESI/MS analyse d'intracellulaire et d'extracellulaire d'extrait pigmenté. En particulier, N-thréonine-monascorubramine, N-glutaryl-rubropunctamine and PP-O, avec une tentative d'identification parmi les 12 composants ; de plus, ce rapport travaillé pour la première fois sur la PDA, MS et RMN qui qualifie aussi le nom de N-GABA-monascorubramine dérivé de (6-[(Z)-2-Carboxyvinyl]-N-GABA-monascorubramine) pigment portant une configuration de cis au C10-C11 double liaison, dans *Talaromyces albobiverticillius* 30548.

Des essais ont été faits, pour étudier les effets du sel marin sur la synthèse de pigment ; les méthodes viables d'extraction de vert des pigments ; l'amélioration de fermentation des fioles agitées des laboratoires fermenteurs. Toutes ces expériences, aussi bien que leurs résultats ont été discutées brièvement en tant que chapitre individuel. Globalement, ces résultats font ressortir le potentiel des champignons marins produisant le pigment rouge et sa possibilité d'obtenir les colorants alimentaires adaptés.

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CHAPTER: I

THE POWER OF COLOR

Color plays a vital role in the world in which we live and is irreplaceable.

"Everything that you can see in the world around you presents itself to your eyes only as an arrangement of patches of different colors." *~ John Ruskin*

Since ancient times, natural dyes obtained from plants, animals and minerals have been used to color fabrics in the textiles, as paints and coatings to color various inanimate objects, as printing ink, coloration of plastics, in cosmetics and food products [1]. The study on history of food colors dates back to remember the ancient civilizations of Egyptians or Romans where they used saffron as a food coloring agent. Later on, other natural coloring agents like turmeric, indigo, marigold, parsley, spinach, flower petals started to gain popularity not only act as colorants but also as medicinal substances [2].

Figure 1.1 Application of colorants in different fields such as textiles, foods cosmetics and pharmaceuticals

Color is an important quality attribute of food products which makes food more appealing thereby perceiving its taste and acceptance. Beyond appearance, adding colors to food

products influence a person's ability to identify flavors and also makes an impact on purchasing decisions [3]. According to FDA, color additives have a specific set of roles in foods and beverages as mentioned below

> ➢ To compensate color loss by cause of light, air, humidity, temperature variations and storage conditions
> ➢ To balance the natural variations in color
> ➢ To enrich the color that has been lost during processing
> ➢ To provide color to the colorless or fun foods

However, there is a growing trend among the manufacturers especially for natural food colors due to increasing consumer demands.

1.1   UNDERSTANDING COLOR CONCEPT: COLOR MATTERS

VISIBLE SPECTRUM AND COLORS

Without light there is no color. The concept of color is bound to visible wavelengths. Therefore, we turn at first toward the typical properties of natural and man-made light sources because the spectral power distribution of an illuminant affects the color impression. Due to ever-present changes in natural daylight, such a source is unsuited for producing a consistent color sensation with an unchanging non-self-luminous color. On account of this uncertainty, we are forced to rely on man-made sources of constant and reproducible light emission – normally in the visible range.
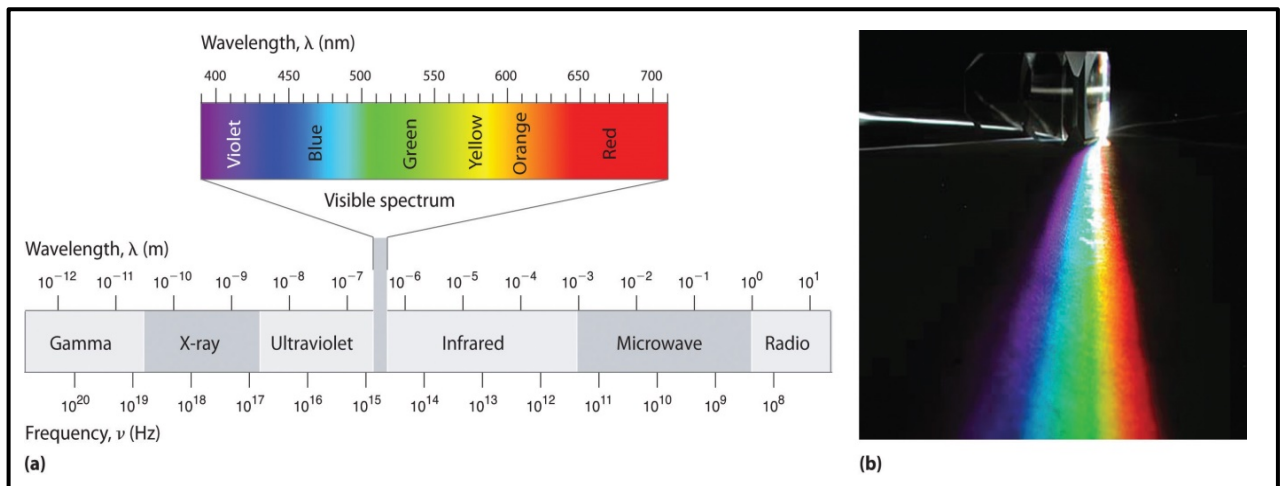
Figure 1.2: **Electromagnetic spectrum** (a) wavelength and frequency of electromagnetic radiation with narrow visible region of wavelengths between 400 and 700nm. (b) When white light is passed through a prism, it is split into light of different wavelengths, whose colors correspond to the visible spectrum (Violet, Indigo, Blue, Green, Yellow, Orange, and Red abbreviated as VIBGYOR)

The electromagnetic spectrum covers an enormous range of wavelengths λ from, for example, values such as λ ≈ 1 fm (1 fm corresponds to 10^{-15} m) for cosmic radiation to λ ≈ 10 km for radio waves, therefore a range of around 19 orders of magnitude; see Fig 1.2. On the other hand, the visible range of humans is only a small part of the spectrum of electromagnetic waves. Merely wavelengths in the very small interval from 380 to 780 nm are normally perceived by humans as visible light. Wavelengths at the left end of the range between 380 and 440 nm are perceived as violet. With increasing wavelength, the color impression changes to blue, green, yellow, orange, and finally red. Red is perceived at wavelengths above 600 nm (figure 1.2) [4].

The associated wavelengths are subject to individual variations in color perception. The so-called spectral colors are the purest producible colors. They are characterized by a wavelength width of less than 1 nm (i.e., with a laser). On the other hand, if the radiation contains nearly all wavelengths of the visible spectrum and of equal intensity, the resulting color impression is white light (e.g., white clouds). For the entire range of visible light between about 380 and 780 nm to be perceived, there must be sufficiently high intensity. Under normal illumination conditions, the wavelength interval that can be perceived by humans is restricted between 400 and 700 nm. This concept is explained in figure 1.3, by taking two different colors of food dyes as examples.
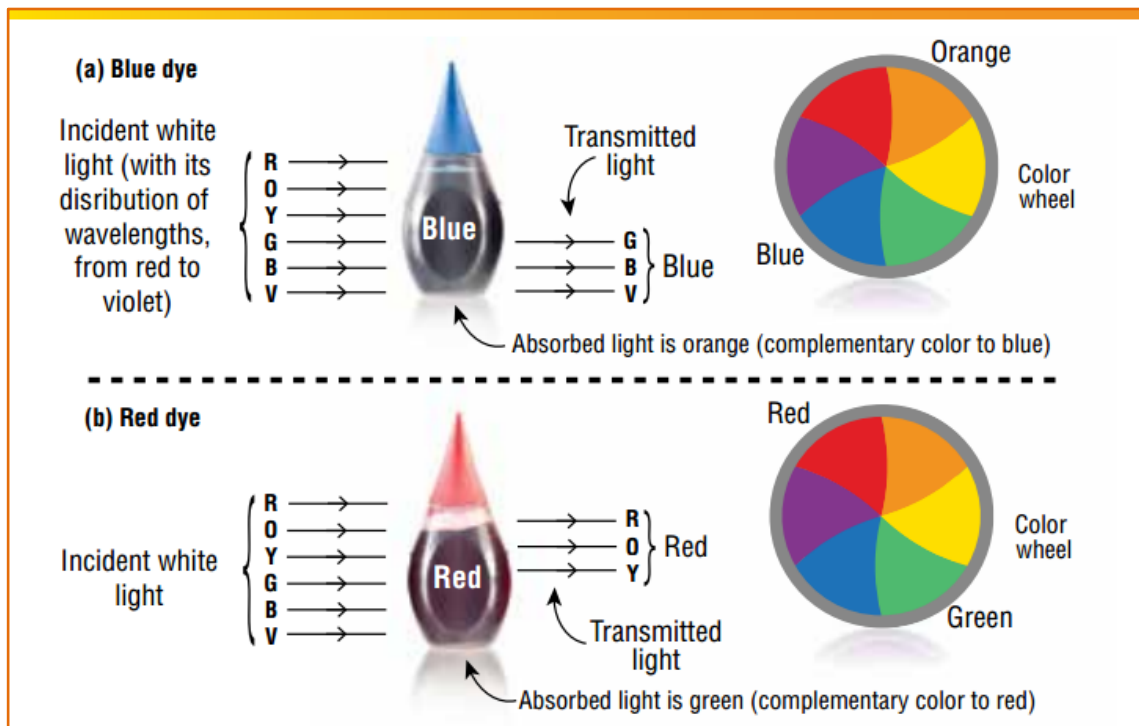
Figure 1.3: A

food dye will appear a particular color because it absorbs light whose color is complementary to the food dye's color (a) blue dye (b) red dye

1.1.1   Electronic structure of pigments

Certainly, the color of a biological molecule is determined by its structure, particular electrons, its size, solubility and elemental composition. Almost in all biological molecules, just four elements, H, C, N and O predominate. Most of the pigmented molecules are relatively large and contain either N or O, or often both. Among the more common pigmented compounds, molecular weights range from about 200 (anthraquinones), 300 (anthocyanidins), 400 (betalaines), 500 (carotenoids) to 800 (chlorophylls).

The electrons in the outer shells hold a finite number of electrons to occupy the least energy demanding orbits and are in a resting state. When irradiated with sufficient amount of energy, electrons will be raised to an exciting state. After the excitation or decay, the electrons will come back to their resting state and so the energy will be released in a less energetic form commonly as heat, light or shorter wavelengths. [6]
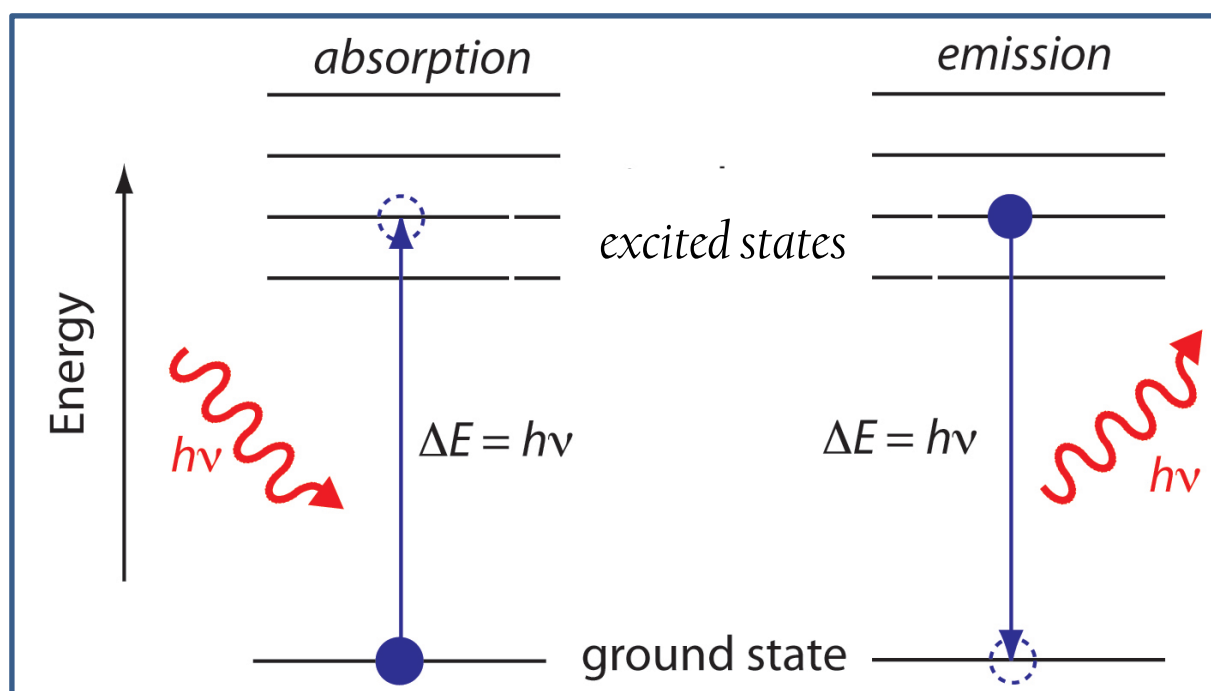
Figure 1.4: Absorption happens when incident light has enough energy to excite an electron from its lowest energy. Emission also possible and release energy via heat, light.

The three electronic orbitals designated as d, p and n are participating in coloring of compounds. On excitation, one or more electrons within these orbitals may be raised respectively to d* and p* orbitals. Transitions from d to d*, which are characteristic of saturated hydrocarbons requires more energy than that provided by the visible part of the spectrum and so appear colorless. Transitions from p to p* as in unsaturated hydrocarbons are less energy demanding but the providing energy is beyond the visible part of the spectrum. Transition from n to d* also appear in the visible range as pale yellow compounds as seen in many fungal pigments. The least energy demanding is n to p* transitions characteristic of unsaturated hydrocarbons with N or O substitutions provides the greatest range of pigmented compounds. The expected color with this transition appears as red, blue and green.

1.2   COLORANTS AND THEIR CLASSIFICATION

Colorants are used in many industries – to color clothes, paints, plastics, photographs, prints, ceramics, etc. Colorants are also now being used in novel applications and are termed as functional (high technology) for special purposes, example in surgery. Colorants can be either dyes or pigments based on their solubility in media [8].

Dyes are soluble colored organic compounds that usually forms a solution in water imply that do not require any fixatives. They are usually added to textiles that are designed to bond strongly to the polymer molecules that makes a textile fiber.

Pigments are insoluble organic compounds applied by dispersion in a suitable medium that requires a fixative. They are used in paints, printing inks, ceramics, foods and plastics.

The colorants were classified based on four criteria and each are discussed in detail and defined as follows

1.2.1. Based on Origin and legislation

Pigments can be classified based on their origin as natural, nature-identical, synthetic and inorganic [9].

1.2.1.a.   **Natural colorants** are the pigments produced by living organisms such as plants, animals, fungi and microorganisms. That includes colorants such as saffron, vegetable carbon, paprika, anthocyanins, chlorophyll, etc.

1.2.1.b.   **Nature-identical colorants** are obtained through man made chemical synthesis, replicates molecular structure to become identical to the natural derived coloring. Examples are β carotene, β -Apo-8' carotenal, canthaxanthin, riboflavin.

1.2.1.c.   **Synthetic colorants** are obtained by chemical synthesis and are not found in nature. Chemically synthesized colors were easier to produce, less expensive, and superior in coloring properties but has negative health hazards. Examples are carmoisine, tartrazine, sunset yellow, etc.

1.2.1.d.   **Inorganic pigments** can be obtained from various naturally mineral sources or minerals and these minerals are mainly oxides, sulphides of one or more than one metals.

1.2.2. Based on the structure of chromophore

The colorants are sometimes classified according to the chemical structure of main chromophore. In simple terms, it can be considered that the organic dye molecules contain three main components such as chromogen, chromophore and auxochrome. The chromophore is a chemical group that is responsible for the appearance of color in compounds (the chromogen) where it is located along with auxochrome as a substituent group (figure 1.5). Unlike most organic compounds, colorants possess color because they:

- absorb light in the visible spectrum (400–700 nm),
- have at least one chromophore (color-bearing group)

Conjugated systems forms the basis of chromophores and are found in organic pigments including azo dyes, compounds in fruits and vegetables (carotenoids, anthocyanins, betalains, caramel) **[10].**
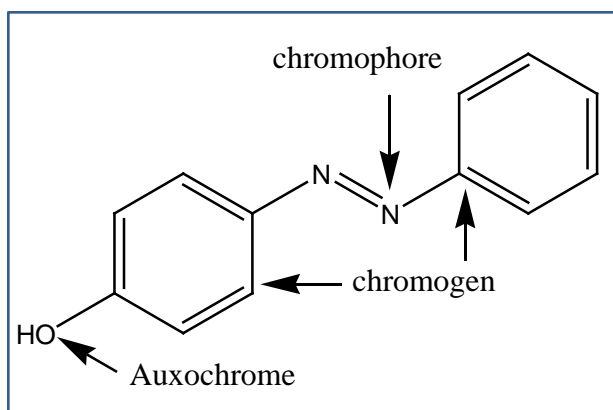
Figure 1.5: The components of 4-Hydroxyazobenzene pointing chromogen, chromophore responsible for its yellow color

Image source: reference [11]

1.2.3. Based on application as food additives

A color additive is any dye, pigment or substance that can impart color when added or applied to a food. All color additives permitted for use in foods are classified as "certifiable" or "exempt from certification" by US FDA.

Certifiable color additives are man-made and sub divided as synthetic pigments and lakes, with each batch being tested by the manufacturer and FDA. One example is FD&C Yellow No. 6, which is used in cereals, bakery goods, snack foods and other foods [12].

Color additives that are **exempt from certification** include pigments derived from natural sources such as vegetables, minerals or animals, and man-made counterparts of natural derivatives. Examples include β carotene, annatto extract, titanium dioxide and so on. Certification exempt color additives must comply with the identity and purity specifications and use limitations described in their listing regulations. Most are straight colors but one exception is carmine which is derived from an insect [13].

1.2.4. Based on structural characteristics of the natural pigments

Moreover, natural pigments derived from a variety of sources can also be classified based on their structural characteristics and grouped into five important classes [14].

- ≈ Tetrapyrrole derivatives (chlorophylls and heme colours),
- ≈ Isoprenoid derivatives (carotenoids),
- ≈ N-heterocyclic compounds different from tetrapyrroles (purines, pterins, flavins, phenazines, phenoxazines and betalains),
- ≈ Benzopyran derivatives (anthocyanins and other flavonoid pigments),
- ≈ Quinones (benzoquinone, naphthoquinone, anthraquinone),
- ≈ Melanins (Eumelanins, Allomelanins)

1.3 NATURAL AND SYNTHETIC COLORS: SPOTLIGHT

Recognizable though they may be, colorants from fruits and vegetables aren't natural. That's because the Code of Federal Regulations (CFR) doesn't classify colors as natural or artificial, but rather lists them as either subject to certification or exempt from certification. Those that gets certified are the synthetic azo dyes listed with their FD&C numbers in title 21, CFR, part 74. The fame carmine red coming from the cochineal insect and the humble beet appear in the part 73 of CFR. Exempt colorants cover the spectrum of red, yellow and orange used to add in foods and beverages. Red hues from anthocyanins, tomato lycopene, carmine; colors in the yellow range are turmeric, carrot and blend of turmeric with annatto; Orange hues are based on carotenoid pigments from paprika and annatto [15]. Relatively, in European Union, the European Parliament and Council Directive

94/36/EC that lays down detailed rules on colors and is given by E-numbers depending on the category [16].

Ultimately, many consumers are concerned about the possible adverse health effects of artificial colors (certified colors), even though a number of governmental agencies including FDA, have confirmed their safety. The "Southampton six" study compelled the European Food safety Authority (EFSA) to carry warning label on foods and beverages containing the suspect colors [17, 18]. These colors include allura red AC (FD&C red 40), ponceau 4R, tartrazine (FD&C Yellow 5), sunset yellow FCF/orange yellow S (FD&C Yellow 6), quinolone yellow and carmoisine. If any of these six colors additives are used in a food or beverage for the European Union market, the warning "May have an adverse effect on activity and attention in children" must be printed on the product label [19]. All the six colors are linked to cause hyperactivity, few may bring on allergic reactions (tartrazine, allura red), sunset yellow is linked to cause stomach upsets and swelling of skin.

Most of the food colors in use were initially evaluated and approved a long time ago. To bring assessments up to date, the European Commission asked EFSA to re-evaluate, by 2020, all color additives authorized before 20 January 2009, taking into account any new evidence. With the relevant scientific studies as well as data on toxicity and human exposure, the re-evaluation of most food colors was carried out and their safety was assessed by 2012. In light of new information, in 2012, the European Commission lowered the ADI for three colors (E 104, E 110, E 124) in food uses. Another significant impact was the market withdrawal of the color Red 2G (E 128) in 2007. EU decision-makers agreed with EFSA that this color could not be regarded as safe for humans and it was subsequently suspended from use in the EU [20].

1.3.1 A new day for naturals

In the past 20 years, exempt colors have gone through a lot of improvements in efficient extraction technology, advances in stabilization, improved process development and availability in different user friendly forms with wide ranges of shades. As an example, "A lot of pigments, especially the red ones are water soluble by nature. It was therefore very difficult in the past to apply in the food products where the water activity was very low. It was overcome by the dispersion of water soluble pigments in an oil phase by using other ingredients"[21].

Obviously, there are several challenges when working with natural and synthetic colors in terms of pH range, application base, and exposure to heat, light, temperature. Through extensive research and testing, some natural colors have been developed which will replace the synthetic ones in food and

beverage applications. The listed chart below (Table 1.1) shows few examples of synthetic colors that are now available in natural form.

Table 1.1 Examples of synthetic colors in wide ranges which was replaceable by natural ones and applications

Color	Application	E No	Synthetic color used in the past	Natural solution	Comments
Red	Beverages (alcoholic and non-alcoholic)	E129	FD&C red n°4	Stabilized anthocyanins	The selection of anthocyanins is an essential parameter to ensure stability. The addition of natural antioxidants can help
Yellow	Beverages (alcoholic and non-alcoholic)	E102	FD&C yellow n°4 and E110 FD&C yellow n°5	Carotenes – β carotenes	Carotenoids cover yellow to orange colors. Adding ascorbic acid as an antioxidant in the final beverage as recommended.
Red	Fruit preparations	E129	FD&C red n°4	Mix of anthocyanins and paprika extract	Mixing natural pigments can yield different colors
Orange	Chocolate	E129	FD&C red n°4 and E102 FD&C yellow n°4	Paprika extract	The color stability can be improved with natural antioxidant

Table source : [22]

1.4   MARKET WATCH: A RAINBOW OF POSSIBILITIES

In the current trend, market for natural colors has grown tremendously owing to the toxicity of synthetic colors   [23]. Consumers are looking for products made with more natural ingredients, and colors are the top in hit lists on their ingredients concern [24]. Manufacturers are taking note to increase adding natural counterparts as being preferred by consumers although market for synthetic colors remains strong. The natural and synthetic colors market is projected to reach $2.3 billion by 2019, growing at a compound annual rate (CAGR) of 4.6 percent, according to a 2014 MarketsandMarkets report (figure 1.6). In terms of revenues, the global market for natural colors was estimated to be worth approximately $0.7 billion in 2011 and is expected to reach $1.3 billion by 2017, representing a CAGR of 10.4 percent from 2014 to 2017, MarketandMarkets reported [25].
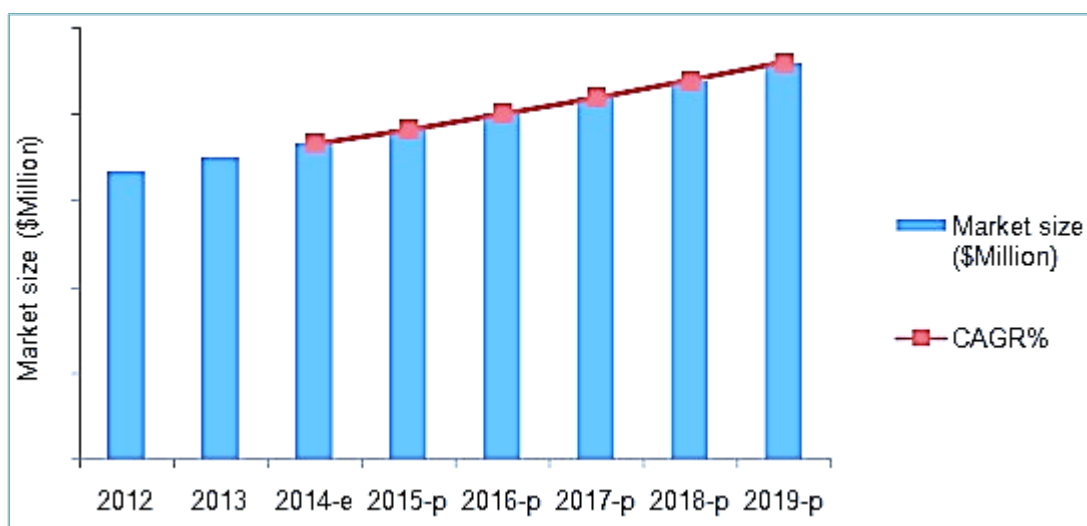
Figure 1.6: Food Colors Market Size, 2012-2019 ($Million)

Natural & Synthetic Food Color Market worth $2.3 Billion By 2019

e- estimated; p- projected (without values, full report is subjected to purchase only)

Source: Government Authorities, Related Associations/Institutes, Related Research Publication, Government Publication, Company Press Release, Company Annual Report, Company Website, Company Publication, and MarketsandMarkets Analysis [25]

Bottom-up and top-down approaches were used to derive the market value and volume of the food colors market. The natural food color market will witness a major contribution from colors such as yellow-orange-red and pink from carotenoids and anthocyanin [26]. Currently, North America is dominating player in natural food colors market followed by Europe. Subsequently, the market of Asia Pacific is projected to grow at a CAGR of 7.5% from 2015 to 2022 considering as the fastest growing and most attractive market. This is because of increasing consumer preference towards natural ingredients and rising disposable income, offer a high growth potential for the development of natural food colors market in economies such as India, China, South Korea and Indonesia. Japan, China and Australia are the largest market in APAC region and projected to witness highest growth rate. RoW region is projected to grow at a moderate CAGR of 6.9% from 2015 to 2022 [27]. Figure 1.7 representing the growth of food colors market segmented by geographic locations and forecasted to the period of 2019 without increasing values as the report was subjected to purchase only. Economies such as Brazil and Saudi Arabia offer huge untapped market opportunities.
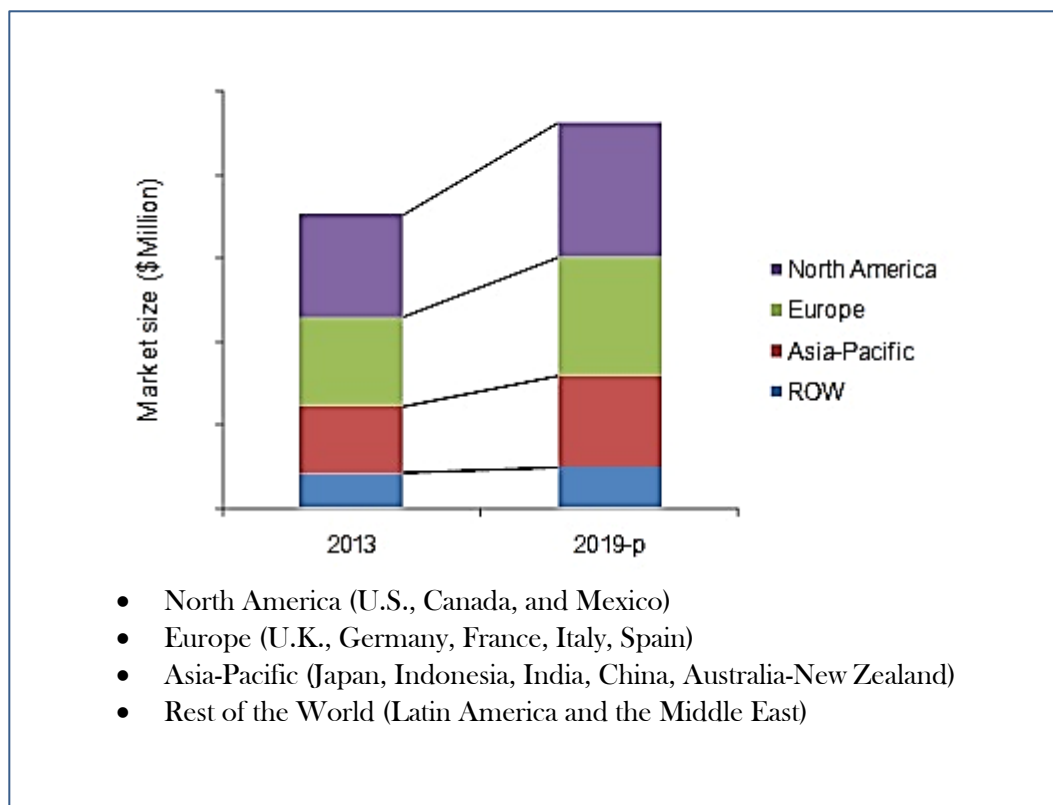
Figure 1.7: Market size of food colors, by geography, 2013 vs. 2019 ($million)

Image source: reference [27]

More specifically, the food additives market in the United States will reach $5.5 billion by 2018, with natural colors driving the growth, according to the "Food Additives: The U.S. Market" report by Packaged Facts, Rockville, Maryland. The major drivers of the market are increasing awareness regarding the ill-effects of artificial food colors & flavors in food products and strict regulations regarding the inclusion of synthetic colors & flavors. Despite at times, the major restraining factors include lesser stability and higher cost as compared to artificial food colors and flavors [28].
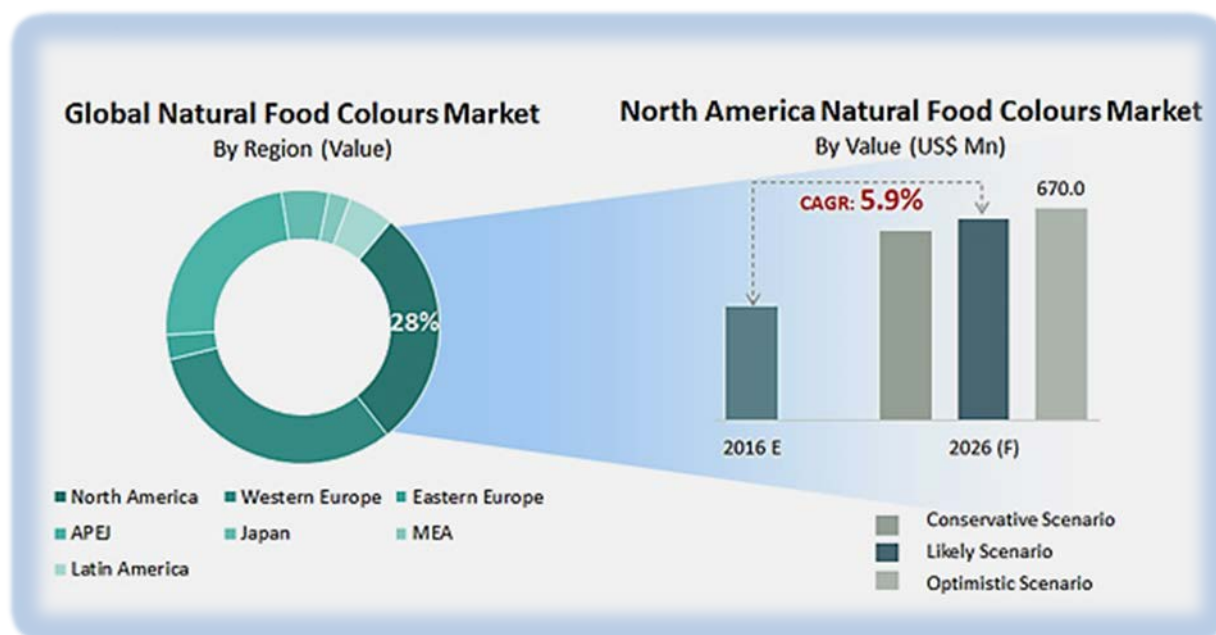
1.5   NATURAL COLORS AND THEIR USE IN FOODS

Ever since pre historic times, natural food colors are extracted from traditional sources such as fruits, vegetables, seeds and roots. Plant pigments, by virtue of their natural occurrence considered to be harmless. Those colorants possess brilliant color ranges ex: water soluble pigments such as anthocyanins, betanins and fat soluble carotenoids and chlorophyll [29]. Other sources of colorants include scale insects such as cochineal and lac, microorganisms such as cyanobacteria, filamentous fungi and microalgae. The microbial production of colorants would have the advantage of producing higher yields when compared to the plant cell growth rate [30].

1.5.1   Extraction and Formulation:

Generally water soluble pigments such as carminic acid and anthocyanins are extracted with simple solvents such as alcohols and water whereas lipid soluble pigments like chlorophyll and carotenoids are extracted with organic solvents. Particularly, lipid soluble pigments are extracted along with other compounds such as triglycerides, wax, sterols, etc [31]. Nowadays, the extraction is carried out by different techniques using solvents such as water, ethyl acetate, acetone, isopropanol, methanol, ethanol and butanol. Besides the extraction steps, formulation is an essential process in which the extracted colorant is mixed with other components to serve a number of purposes. This is a challenge

for food processors or manufacturers to find additives that must withstand the rigors of processing, storage period, pH of the finished product, stability under environmental conditions [32].

1.5.2   Plant pigments: improving applications

Throughout the years, ingredient suppliers developed naturally derived colorings from a variety of sources that have improved stability under certain conditions in certain applications. For example, to counter oxidation of annatto pigments, antioxidants can be added to the color formulation or food application. Anthocyanins isolated from fruits and vegetables are sensitive to pH changes, heat, and light, so they are acylated (adding an acyl group to a compound) to produce anthocyanins that are more stable [33] . Other example is the coloring, which is lycopene containing Tomat-O-Red offered in a ready to-use powder or liquid formulation and as an oleoresin. Light and oxygen affect lycopene on its own. To counter this, the company Sensient has coated lycopene crystals in its ready-to-use formulations to increase stability to light and oxygen and reduce the occurrence of color change during processing. The oleoresin formulation is standardized to 8% lycopene and is used to color foods after it is emulsified or solubilized to form a transparent micro-emulsion or an opaque emulsion. This is principally used in confectionaries like panned sweets where other naturally derived colorings are unable to provide a stable red color [34].

1.5.3   Discovering new alternatives: microbial pigments

In the last years, much focus is given to colors produced by microorganisms such as bacteria, microalga, mainly from filamentous fungi. The production of pigments from microbes is closely related to the bioprocess conditions with well-defined nutrient concentrations which were proved by many literature studies [35-40]. For the industrial perspective, the colorants produced by microorganisms are considered to be of great interest due to their production under controlled conditions, regardless of external factors and supply of raw materials, also minimal batch to batch variations [41]. Along with the pigment producing ability of microorganisms they often demonstrate bioactive properties such as antimicrobial, antioxidant, and anticancer properties as noted in carotenoids, melanins, flavins, quinones, monascines, violaceins, phycocyanins, and indigo [42-45].

Dharmaraj et al. (2009) found that *Streptomyces* strain (AQBWWS1) isolated from a marine sponge and fermented under fluorescent white light produced carotenoid pigments that could be used as food-grade pigments. The researchers say that the next step is to determine if the process can be scaled up to mass produce the pigments [46]. While studying the antioxidant properties of a pigment found in another marine creature, a microalga, Pouvreau et al. (2008) learned that the pigment, called

marennine, had good light and heat stability and may show promise as a naturally derived, blue-green coloring [47].

"The success of any pigment produced by fermentation depends upon its acceptability in the market, regulatory approval, and the size of the capital investment required in bringing the product to market," report the researchers [48].

1.6 FORMULATING FOR A CLEAN LABEL

In today's market, consumers are highly aiming for products that offer recognizable ingredients. In case of foods and beverages, color has always played a key role in creating identity and value. The term clean label provides access to information with product labelling, ingredient statement and front of pack claims. When formulating a product to achieve a clean label, it is important to consider the regulations of the country where that product will be sold.

Any color additive added to food in the USA must be approved by US FDA (Food and Drug Administration) and EFSA (European Food Safety Authority) in the European Union. The permitted color additives are of two categories and listed in 21 Code of Federal Regulations (21CFR) Parts 73- 82 **(Table 1.2).** They are commonly referred as natural or exempt from certification colors and certified/synthetic or FD&C colors. Whereas in European Union, it uses E numbers representing codes for chemicals which can be used as food additives. The policy and quality approach in the US and Europe are dramatically different [49]. For example, in the US, both carmine and β carotene need only be listed by name but in the EU, these require E numbers to mention. On the other hand, colors made from edible fruits and vegetables provide global regulatory acceptance and consistent opportunities for a clean label. A color made form pumpkin may be labelled "vegetable juice in the United States" and "pumpkin concentrate in the European Union" which mentions the color as natural to the end consumer [50].

Table 1.2: Summary of Color Additives approved for Use in the United States added to foods Part 73

Color additives	Color	US CFR No	EEC#	Year Approved	Uses and Restrictions
Annatto extract	Orange	§73.30	E160b	1963	Foods generally
Canthaxanthin[1]	Violet	§73.75	E161g	1969	Foods generally, NTE [2] 30 mg/lb of solid or semisolid food or per pint of liquid food; May also be used in broiler chicken feed.
Caramel	Brown	§73.85	E150a-d	1963	Foods generally
Carrot oil		§73.300	----	1967	Foods generally

Cochineal extract	Crimson	§73.100	E120	1969	Foods generally
				2009	Food label must use common or usual name "cochineal extract"; effective January 5, 2011
Dehydrated beets (beet powder)	Red	§73.40	E162	1967	Foods generally
Fruit juice [1]		§73.250	----	1966	Foods generally
				1995	Dried color additive
Grape color extract [1]	pH dependent (Red, green and purple ranges)	§73.169	E163?	1981	Non beverage food.
Grape skin extract (enocianina)	pH dependent (Red, green and purple ranges)	§73.170	E163?	1966	Still & carbonated drinks & ades; beverage bases; alcoholic beverages (restrict. 27 CFR Parts 4 & 5).
Mica-based pearlescent pigments		§73.350	----	2006	Cereals, confections and frostings, gelatin desserts, hard and soft candies (including lozenges), nutritional supplement tablets and gelatin capsules, and chewing gum.
				2013	Distilled spirits containing not less than 18 % and not more than 23 % alcohol by volume but not including distilled spirits mixtures containing more than 5 % wine on a proof gallon basis.
				2015	Cordials, liqueurs, flavored alcoholic malt beverages, wine coolers, cocktails, nonalcoholic cocktail mixers and mixes and in egg decorating kits.
Paprika	Red	§73.340	E160c	1966	Foods generally
Paprika oleoresin	Red	§73.345	E160c	1966	Foods generally
Riboflavin	Yellow orange	§73.450	E101	1967	Foods generally
Saffron	Yellow-orange-red	§73.500	E164	1966	Foods generally
Sodium copper chlorophyllin [1]	Green	§73.125	E141	2002	Citrus-based dry beverage mixes NTE [2] 0.2 % in dry mix; extracted from alfalfa.
Spirulina extract		§73.530	----	2013	Candy and chewing gum.
				2014	Coloring confections (including candy and chewing gum), frostings, ice cream and frozen desserts, dessert coatings and toppings, beverage mixes and powders, yogurts, custards, puddings, cottage cheese, gelatin, breadcrumbs, and ready-to-eat cereals (excluding extruded cereals).

Synthetic iron oxide [1]	Brown	§73.200	E172	1994	Sausage casings NTE [2] 0.1 % by weight.
				2015	Hard and soft candy, mints and chewing gum.
				2015	For allowed human food uses, reduce lead from ≤ 20 ppm to ≤ 5 ppm.
Titanium dioxide	White	§73.575	E171	1966	Foods generally; NTE [2] 1 % by weight
Toasted partially defatted cooked cottonseed flour		§73.140	----	1964	Foods generally
Tomato lycopene extract; tomato lycopene concentrate [1]	Bright to deep red	§73.585	E160e	2006	Foods generally
Turmeric	Yellow orange	§73.600	E100	1966	Foods generally
Turmeric oleoresin	Yellow orange	§73.615	E100	1966	Foods generally
Vegetable juice [1]		§73.260	----	1966	Foods generally
				1995	Dried color additive, water infusion.
β-Apo-8'-carotenal	Yellow-orange to brown	§73.90	E160e	1963	Foods generally, NTE [2]: 15 mg/lb solid, 15 mg/pt liquid.
β-Carotene	Yellow-orange to brown	§73.95	E160a	1964	Foods generally

Table source: FDA/For Industry/Color Additives [51]

[1] - Petitioned for use after the 1960 amendments; not provisionally listed.
[2] - NTE – Not To Exceed

BACK TO NATURE: A ROAD TO THE FUTURE?

The range of natural colorants currently used in the food industry appears narrow worldwide with some limitations such as instability to light, heat or adverse effects, relying on large scale cultivation for colorants from plant sources. In this kind of situation, to replace conventional plant growing techniques, some experts promoted plant cell and tissue cultures. However, the plant cell growth is rather slow when compared to microbial growth. Also, for the sole purpose of pigment production, it is doubtful to use such techniques though it is of huge economic investment [30]. Microbes, particularly filamentous fungi from non- conventional sources seems to be more promising producers of biosynthesized pigments because of their chemical and color versatility, easier large scale controlled cultivation and produced compounds with bioactivities.

This thesis is mainly focused on the fermentation, optimization, extraction and identification of red pigments produced by the filamentous fungus *Talaromyces albobiverticillius* 30548, a new strain

isolated from the marine environment of La Réunion Island, Indian Ocean area which has not been previously studied.

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CHAPTER 2

MICROBIAL PIGMENTS: STATE-OF-THE ART

2.1 Microbial Pigments:

Nature is rich in colors and color is normally present in plants, minerals, microbes. In foods, colors serves as the primary identification and indicates its freshness, safety, aesthetic and sensorial values [1]. Currently, the vast majority of food colorants permitted in the European Union and the United States are derived from plant sources which has several limitations such as dependence of raw materials, influenced by agro climatic conditions, notably batch to batch variations. These drawbacks could be easily tackled by using synthetic pigments, such as azo dyes and its derivatives. Synthetic pigments are cheaper to produce, offers a vast range of new colors, brighter than natural ones. However, in the last few decades, there has been an increasing trend towards replacement of synthetic colorants with natural pigments because of the strong consumer demand for more natural products. Most of the synthetic pigments cause considerably environmental pollution and adverse toxicological side effects. Also, the controversy of synthetic dyes was amplified in 2007 with the Southampton study linking hyperactivity in young children consuming mixtures of some artificial food colors [2].

Though many natural colors are available, microbial colorants play a significant role as food coloring agent, because of their production and easy down streaming process. Industrial production of natural food colorants by microbial fermentation has several advantages such as massive production, easier extraction, higher yields through strain improvement, no lack of raw materials and no seasonal variations [3]. Microorganisms could be made to produce colorants in high yield by inserting genes coding for the colorant, even colorants not naturally produced by microorganisms could be made in this way [4]. Colorants made in this way will probably face some hurdles, at least in Europe where genetically modified food is generally viewed with a large degree of skepticism by the consumers. These pigments are looked upon for their safe use as natural food colorants and will not only benefit human health but also preserve the biodiversity, as harmful chemicals released into the environment while producing synthetic colorants could be stopped [5].

2.1.1 Generalities

2.1.1.a   Bacterial pigments

Pigments produced by bacteria are of great interest and intensively studied in the present because of its potential for applications in food, feed, textile and pharmaceutical industries [6-8]. The pigmented bacteria can be sourced from various environmental sources which can be cultured and purified. The pigments produced by bacteria can be separated using solvent extraction in a relatively pure and concentrated form to further characterized using various instrumental and analytical techniques such as TLC, UV-Vis, FTIR, ESI-MS, NMR, HPLC and Gel Permeation Chromatography [9]. Bacteria produce different molecules such as carotenoids, melanins, flavins, phenazines, quinones, bacteriochlorophylls, more specifically monascins, violacein or indigo with distinct colors and are used as color additives or as supplements [10, 11].

Among carotenoids, specifically astaxanthin (pink) produced by bacteria such as *Agrobacterium aurantiacum, Paracoccus carotinifaciens* or *Halobacterium salinarum* [12]. To overcome high commercial price of astaxanthin and to improve the production, metabolic engineering of bacteria (*Escherichia coli*) recently allowed production of astaxanthin at >90% of the total carotenoids, providing the first engineered production system capable of efficient astaxanthin production [13]. Considering ketocarotenoid, canthaxanthin (violet) was mainly known from *Bradyrhizobium sp* and a gram-positive bacterial strain *Gordonia jacobea* (CECT 5282). To improve the production of canthaxanthin in an industrial scale, a hyper pigment mutant (MV-26) was isolated after several rounds of mutation. It produced six times more canthaxanthin (1-13.4 mg/L) than the wild type strain by varying the culture medium. In industrial scale, production of zeaxanthin (orange) was noted in *Flavobacterium multivorum* by utilizing the alternative deoxyxylulose (DXP) pathway [14].

2.1.1.b   Algal pigments

Among microalgae, efficient production of carotenoids such as β-carotene using *Dunaliella* and astaxanthin using *Haematococcus* was observed [15]. Regarding phycobiliproteins, the red microalgae *Porphyridium* yields approximately 200 mg of colorant/L of culture after 3 days and concentration up to 30% under optimal conditions along with the co-production of zeaxanthin [16]. Relating to blue colored phycocyanin, yields from the algal extract of *Porphyridium aerugineum* reached up to 60% of the dry matter and the quantity required for coloring was 140 – 180 mg of color/kg of blue food or drink [17]. Along with that, phycocyanin has been recently reported to exhibit antioxidant, anti-inflammatory, neuroprotective and hepatoprotective effects by experimental studies [18].

2.1.1.c   Fungal pigments

Filamentous fungi are currently playing a crucial role in the production of food grade pigments and gaining attention for their remarkable color range, chemical profile and easy large scale cultivation [19]. Besides food applications, colors extracted from mushrooms and lichens has a long history which was apparently first used for textile dyeing [20]. The pigment produced by fungi generally falls under two classes, carotenoids and polyketides.

In textile dyeing, red anthraquinones from *Fusarium oxysporum* and *Dermocybe sanguinea*, as well as yellow pigments from *Trichoderma viride* and *Fusarium oxysporum*, green tints from *Bankera violascens*, melanin pigments from *Curvularia lunata* were exhibited as potential dye on cellulosic, wool and silk fibers with good colorfastness and rub fastness [21, 22]. Howbeit, food colorants from ascomycetes fungi have known to be produced using biotechnological approach in a relatively easier way massed with high yield. The yellow food colorant, riboflavin (vitamin B2) can be synthesized in large amounts by the fungi *Ashbya gossypii* and *Eremothecium ashbyii* [23-25].

Carotenoids such as β-carotene and lycopene are functional pigments and having a successful market for industrial production. The main organism used for β-carotene production is the fungi *Blakeslea trispora* by co fermentation of two type strains (+) and (-) [25]. The ingredients company, DSM (The Netherlands) was the first to produce β-carotene in 2000 and today other companies are producing in Russia, Ukraine and Spain (Leon) [26]. Concerning polyketide azaphilone pigments, various strains of *Monascus spp.* produces three main types of color compounds (yellow, orange and red) counting up to 63 compounds and few with bioactivities [27]. So far, more than fifty *Monascus* species have been isolated, identified and serves as a colorant in processed meats, marine products like surimi, fish paste and tomato ketchup [28, 29]. Over the past few years, it was identified that several species of *Penicillium* and *Talaromyces* produces Monascus like red pigments without the co- production of mycotoxin citrinin but it is produced in case of *Monascus spp.* [30].

Polyketide anthraquinone pigments belong to quinone family and some of their derivatives also exhibit coloring properties. A lot of strains belonging to the genus *Fusarium, Curvularia, Drechslera, Cordyceps, Paecilomyces spp* seem to produce pigmented anthraquinones [31]. Arpink red™, now called Natural red ™ was the first fungal anthraquinoid food colorant manufactured by Ascolor Biotech s.r.o located in Czech Republic and got temporary approval to use as a food additive in 2004 inside the country. The colorant was produced using the strain *Penicillium oxalicum var. Armeniaca* CCM8242, sourced from soil by the fermentative and bioprocessing approach [29, 32]. In 2009, a new patent was filed by the Czech company Biomedical s.r.o., using the genetically modified strain (site-directed mutagenesis applied to former strain) *Penicillium oxalicum* var. Armeniaca CCM 8374 producing an exogenous red pigment of anthraquinone type with improved production of pigment up to 5-10g/L [33].

Table 1: Various shades of pigments produced by different microorganisms, their bioactivities and regulatory status in the market.

S.No	Pigment	Color	Microorganism	Bioactivities	Status	References
			Bacteria			
1	Astaxanthin	Pink-red	*Agrobacterium aurantiacum* *	Antioxidant, photoprotectant, Anti-cancer, Anti-inflammatory	RP	[34]
2	Canthaxanthin	Orange	*Bradyrhizobium Spp.* *	Antioxidant, Anti-cancer	RP	[29, 35-37]
3	Cycloprodigiosin	Red	*Pseudoalteromonas denitrificans*	Anti-plasmodial, Anti-cancer	DS	[38, 39]
4	Granadaene	Orange-red	*Streptococcus agalactiae*	Antioxidant, detoxify ROS	DS	[40, 41]
5	Heptyl prodigiosin	Red	*α Proteobacteria*	Anti-plasmodial	DS	[42]
6	Indigoidine	Blue	*Corynebacterium insidiosum*	Anti-microbial, Phaeobacter sp	DS	[43, 44]
7	Prodigiosin	Red	*Serratia marcescens, Pseudoalteromonas rubra*	Anti-cancer, DNA Cleavage, Immunosuppressant	IP	[45-48]
8	Pyocyanin	Blue, green	*Pseudomonas Spp.* *	Cytotoxicity, Neutrophil apoptosis, Ciliary dysmotility, Pro-inflammatory	IP	[49]
9	Rubrolone		*Streptomyces echinoruber* **		DS	[50, 51]
10	Scytonemin		*Cyanobacteria*	Anti-inflammatory, Anti-proliferative	-	[52]
11	Staphyloxanthin	Golden	*Staphylococcus aureus*	Antioxidant, detoxify ROS	-	[53-55]
12	Tryptanthrin		*Cytophaga/Flexibacteria AM13, 1Strain*	-	-	[56]
13	Undecylprodigiosin	Red	*Streptomyces spp*	Anti-bacterial, anti-oxidative, UV-protective, Anti-cancer	-	[53, 54, 57, 58]
14	Violacein	Purple	*Janthinobacterium*	Antioxidant, detoxify		[59-61]

			lividum, Pseudoalteromonas tunicate, Pseudoalteromonas spp, Chromobacterium violaceum	ROS		25
15	Xanthomonadin	Yellow	Xanthomonas oryzae	protection against photodamage	-	[62]
16	Zeaxanthin	Yellow	Staphylococcus aureus, Flavobacterium spp. **, Paracoccus zeaxanthinifaciens, Sphingobacterium multivorum	-	DS	[63]
Fungi						
17	Ankaflavin	Yellow	Monascus spp. *	Anti-tumor, Anti-inflammatory	IP	[64]
18	Anthraquinone	Red	Penicillium oxalicum *	Anti-fungal, virucidal	IP	[9, 65, 66]
19	Canthaxanthin	Orange, Pink	Monascus roseus	Antioxidant, Anti-cancer	-	[36, 37, 67, 68]
20	Lycopene	Red	Fusarium Sporotrichioides *, Blakeslea trispora *	Antioxidant, Anti-cancer	RP/DS	[69, 70]
21	Monascorubramin	Red	Monascus spp. *	Anti-micrbial, Anti-cancer	IP	[71]
22	Naphthoquinone	Deep blood red	Cordyceps unilateralis *	Anticancer, Anti-bacterial, Trypanocidal	RP	[72-74]
23	Riboflavin	Yellow	Ashbya gossypi *	Anti-cancer, anti-oxidant, protection against cardiovascular diseases, in vision	IP	[75-77]
24	Rubropunctatin	Orange	Monascus spp. *	Anti-cancer	IP	[78, 79]
25	β-carotene	Yellow-orange	Blakeslea trispora *, Fusarium sporotrichioides, Mucor circinelloides,	Anti-cancer, Antioxidant, suppression of cholesterol synthesis	IP	[68, 80-83]

			Neurospora crassa, Phycomyces blakesleeanus			
			Algae			
26	Astaxanthin	Red	*Haematococcus pluvialis*	Antioxidant, photoprotectant, Anti-cancer, Anti-inflammatory	-	[80, 84]
27	ß-carotene	Orange	*Dunaliella salina*	Anti-cancer, Antioxidant, suppression of cholesterol synthesis	-	[85-87]
			Yeast			
28	Astaxanthin	Red, Pink-red	*Phaffia rhodozyma*, Xanthophyllomyces dendrorhous**	Antioxidant, photoprotectant, Anti-cancer, Anti-inflammatory	DS	[88-90]
29	Melanin	Black	*Saccharomyces neoformans*	-	-	[91]
30	Torularhodin	Orange-red	*Rhodotorula spp.*	Antioxidant, Anti-microbial	-	[92, 93]
			Archea			
31	Canthaxanthin	Orange	*Haloferax alexandrines*	Antioxidant, Anti-cancer	-	[29, 35-37]
			Protozoan			
32	Hemozoin	Brown–black	*Plasmodium spp.*	-	-	[41, 94]

DS – Development Stage, IP – Industrial Production, RP – Research Project

Table source: according to reference [29, 95]

2.2 BOOK CHAPTER

Pigments and Colorants from Filamentous Fungi:
Fungal metabolites

This book chapter deals with the pigment producing fungal species of different families as well as the pigments from marine- derived fungi. Also explains the biosynthetic pathway of pigment production and this forms a part of this chapter

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Pigments and Colorants from Filamentous Fungi

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Abstract

With the impact of globalization on research trends; the search for healthier lifestyles; the increasing public demand for natural, organic, and "clean labelled" products; as well as the growing global market for natural colorants in economically fast-growing countries all over the world, filamentous fungi started to be investigated as readily available sources of chemically diverse pigments and colorants. The formulation of recipes containing fungal pigmented secondary metabolites has steadily increased over recent years. For all of these reasons, this chapter highlights exciting findings, which may pave the way for alternative and/or additional biotechnological processes for industrial applications of fungal pigments and colorants. The fungal biodiversity from terrestrial and marine origins is first discussed as potential sources of well-known carotenoid pigments (e.g., β-carotene, lycopene) and other specific pigmented polyketide molecules, such as *Monascus* and *Monascus*-like azaphilones, which are yet not known to be biosynthesized by any other organisms like higher plants. These polyketide pigments also represent promising and yet unexplored hydroxy-anthraquinoid colorants from Ascomycetous species. The putative biosynthetic pathways of the carotenoids and polyketide-derivative colored molecules (i.e., azaphilones, hydroxyanthraquinones, and naphthoquinones) in pigment-producing fungal species are investigated herein. As an additional aspect, this chapter describes biotechnological approaches for improving fungal pigment production and identifying new clean opportunities for the future. Alternative *greener* extraction processes of the fungal colored compounds are also further explored. The current industrial applications along with their limits and further opportunities for the use of fungal pigments in beverage, food, pharmaceutical, cosmetic, textile, and painting areas are, then, presented.

Abbreviations

ADI	Acceptable daily intake
ASE	Accelerated solvent extraction
ATPS	Aqueous two-phase system
BIK	Bikaverin polyketide synthase
CoA	Coenzyme A
CWD	Cold-water-dispersible
DOE	Design of Experiment
EAE	Enzyme assisted extraction
EFSA	European Food Safety Authority
EU	European Union
FDA	Food and Drug Agency
GMO	Genetic Modified Organism
GMP	Good Manufacturing Practices
GRAS	Generally Recognized As Safe
HPLC	High performance liquid chromatography
IL	Ionic liquids
IPP	IsoPentenyl-pyrophosphate
JECFA	Joint FAO/WHO Expert Committee on Food Additives
MAE	Microwave assisted extraction
PCR	Polymerase chain reaction
PKS	Polyketide synthase
PLE	Pressurised fluid extraction
PUFAs	Polyunsaturated fatty acids
SFE	Subcritical fluid extraction
SWE	Subcritical water extraction
TLC	Thin layer chromatography
UAE	Ultrasound assisted extraction
UV	Ultraviolet

1   Introduction

Molecules and ingredients derived from microbial fermentation are steadily gaining ground in industry. Thickening or gelling agents (e.g., polysaccharides such as xanthan, curdlan, gellan), flavor enhancers (yeast hydrolysate, monosodium gluta-mate), polyunsaturated fatty acids (PUFAs), flavor compounds (gamma-decalactone, diacetyl, methyl-ketones), vitamins, essential amino acids, and acidulants (lactic acid, citric acid) are some examples illustrating this trend. Efforts have been made

and continue to be made in order to reduce the production costs of pigments produced by fungal fermentation, since synthetic pigments or those extracted from natural plant sources can often be produced more economically [1]. The successful marketing of natural pigments such as β-carotene, lutein, and astaxanthin derived from algae (i.e., nonconventional sources) or extracted from flowering plants (conventional sources), both as food colorants and nutritional supplements, reflects the presence and importance of niche markets in which consumers are willing to pay a premium for "natural healthy ingredients."

Among other nonconventional sources, filamentous fungi are known to produce an extraordinary range of pigments that include several chemical classes such as carotenoids, melanins, azaphilones, flavins, phenazines, quinones, and more specifically, monascins, violacein, and indigo [2]. The success of any class of pigment produced by fermentation depends on its acceptance by the consumers, regulatory approval, and the capital investment required to bring the product onto the market. Twenty years ago, influential representatives from industry expressed doubts about the successful commercialization of algae-derived and fermented food grade pigments due to the high investment required for open ponds, photobioreactors and fermentation facilities, and the extensive and lengthy toxicity studies requested by the regulatory authorities. Nonexistent or poor public perception of fungal-derived products for food use had also to be taken into account. Nowadays, some food grade pigments obtained by fermentation exist on the market worldwide. Among them, fungal *Monascus* pigments, Arpink red™ (now Natural Red™) produced by *Penicillium oxalicum*, microalgal phycocyanin from *Arthrospira* (*Spirulina*) *platensis*, riboflavin from the mold fungus *Ashbya gossypii*, lycopene and β-carotene from the tropical mold *Blakeslea trispora*, β-carotene from the microalgae *Dunaliella salina*, and astaxanthin from the bacterium *Paracoccus carotinifaciens* and microalgae *Haematococcus pluvialis*, respectively. As an example, the production yield of β-carotene may be as high as 17 g/L of the *Blakeslea trispora* culture medium [3].

Thus, the present chapter emphasizes the crucial role that fungi are currently playing and are likely to continue to play in the future as microbial cell factories for the production of pigments for the industry. This is due to the versatility in their pigment color and chemical profile, amenability for easy large-scale cultivation, and a long history of production by well-investigated production strains.

## 2	Natural Polyketide Pigments Produced by Filamentous Fungi

Among nonconventional sources, filamentous fungi are known to produce an extraordinary range of fungal pigments that are often more stable and soluble than plant-derived pigments [3, 4]. Fungal secondary metabolites like fungal pigments can be grouped into four different classes depending on their structural properties: terpenes, polyketides, nonribosomal peptides, and amino acid-derived compounds. These fungal secondary metabolites, also known as exometabolites, are small molecules produced during morphological and chemical differentiation that are

outward directed, i.e., secreted or deposited in or on the cell wall, and accumulated in contrast to endometabolites (primary metabolites) that are fluctuating in concentration, and either transformed into other endometabolites or feeding into exometabolites, exoproteins, and exopolysaccharides. While endometabolites can be found in almost all species of fungi, exometabolites are taxonomically restricted, being produced in species-specific profiles [5].

Biosynthetically, many exometabolites produced by ascomycetous filamentous fungi are polyketides. Polyketides are typically synthesized by multifunctional polyketide synthases (PKS) from small carboxylic acid derivatives (acetyl-coenzyme A (CoA) and malonyl-CoA) in a manner similar to the synthesis of fatty acids. Nonreducing polyketide synthases synthesize polyketides in which carbonyl groups are not reduced, and reducing polyketide synthases synthesize polyketides in which the carbonyl groups are partially or fully reduced [6, 7]. Polyketides represent an array of often structurally complex natural products and include such classes as anthraquinones, hydroxyanthraquinone pigments, naphthalenes, naphthoquinone pigments, flavonoid pigments, macrolide antibiotics, polyenes antibiotics, tetracyclines, and tropolones. Azaphilone pigments, namely pigments with pyrone-quinone structures and a chiral quaternary center, can also be considered as polyketide derivatives. Polyketide-based pigments with different shades (red, yellow, orange, brown) have been found abundantly in ascomycetous filamentous fungi as exemplified in case of pigments produced by species belonging to *Monascaceae, Trichocomaceae, Pleosporaceae,* and *Nectriaceae* families [3, 8].

2.1   Species of the *Monascaceae* Family Producing Pigments

Monascus has been used to produce natural colorants and food supplements for more than one thousand years in Asia, and approximately more than one billion Asian people consume *Monascus*-fermented products with their daily diet. The first known source reporting the use of these red colorants was a recipe for the preparation of red pot-roast lamb, in which meat was simmered with *hong qu* (red rice koji, made with *Monascus purpureus*), as handed down in the Qing Yilu in AD 965. *Monascus* species are known to produce six major azaphilone pigments, namely the yellow monascin and ankaflavin, the orange monascorubrin and rubropunctatin, and the red monascorubramine and rubropunctamine. To date, more than 50 different chemical structures have been identified as azaphilones easily combine with nitrogen-containing compounds [9]. Using next-generation sequencing and optical mapping approaches, a 24.1-Mb complete genome of a *Monascus purpureus* YY-1 industrial strain has been described for the first time and this will allow huge improvements in the process in the coming years [9]. It consists of 8 chromosomes and 7491 genes. *M. purpureus* should belong to the *Aspergillaceae*, mainly comprising the genera *Monascus, Penicillium,* and *Aspergillus*. Phylogenetic analysis at the genome level provides the first comprehensive prediction of the biosynthetic pathway for *Monascus* pigments. Comparative genomic analyses demonstrated that the genome of *M. purpureus* is 13.6–40 % smaller than that of closely related filamentous fungi

and has undergone significant gene losses, most of which likely occurred during its specialized adaptation to starch-based foods. Some polyketide synthases (PKS) are expressed at high levels under high pigment-yielding conditions. The citrinin PKS C6.123 has also been found in the genome [9], paving the way for research aiming at non-mycotoxin-producing strains, if suppression of the citrinin gene does not change the ability of the strain to produce pigments, which seems to be feasible, as described by Fu et al. [10]. The latter group has shown that monascorubrin and citrinin are synthesized by two separate pathways, because, when the PKS gene responsible for synthesis of citrinin was disrupted, red pigment production from the fungus was not affected. Comparative transcriptome analysis revealed that carbon starvation stress, resulting from the use of relatively low-quality carbon sources, contributed to the high yield of pigments by suppressing central carbon metabolism and augmenting the acetyl-CoA pool. As for other pigments produced by biotechnology, the problem is to have enough carbon oriented in the correct pathway, i.e., the pigment pathway.

Woo et al. [11] investigated another filamentous fungus, *Penicillium marneffei*, for the production of azaphilones exhibiting black, yellow, and red hues. The polyketide gene cluster and biosynthetic pathway were reported for monascorubrin in this red pigment-producing, thermal dimorphic fungus, taking advantage of available genome sequence and faster growth rate compared to *Monascus* species [11]. The red pigment of *P. marneffei* has been shown to consist of a mixture of more than 16 chemical compounds, which are amino acid conjugates of monascorubrin and rubropunctatin, as amino acids can be conjugated under specific conditions without enzymatic catalysis, i.e., by Schiff base formation (Fig. 1) [11].

The aforementioned polyketide gene cluster and pathway have been shown to be also responsible for the biosynthesis of ankaflavin and citrinin, the latter being a mycotoxin exerting nephrotoxic activity in mammals [12]. Twenty-three putative PKS genes and two putative PKS-nonribosomal peptide synthase hybrid genes were identified in the *P. marneffei* genome [11]. Woo et al. [11] systematically knocked out all 25 PKS genes of *P. marneffei*. They also knocked out genes located up and downstream of the PKS gene responsible for red pigment production and character-ized the pathway for biosynthesis of the red pigment. However, it is still questionable whether it will be possible to produce mevinolin/lovastatin-free (a cholesterol-lowering drug that is undesired in normal foods) and citrinin-free red pigments from *P. marneffei*, as the latter, a mycotoxin, appears to be an early by-product of the biosynthetic pathway.

2.2 Species of the *Trichocomaceae* Family Producing Pigments

2.2.1 Pigments from Penicillium and Talaromyces Species

Fungi belonging to the genera *Penicillium* and *Talaromyces* are cosmopolitan, classified under the family *Trichocomaceae* and contain 354 and 88 species, respec-tively. Species of the genera are commonly found in soil, decaying organic materials, animal feed, stored grains, and other materials [13]. Species of *Penicillium* and *Talaromyces* are extraordinarily productive concerning exometabolites. They are
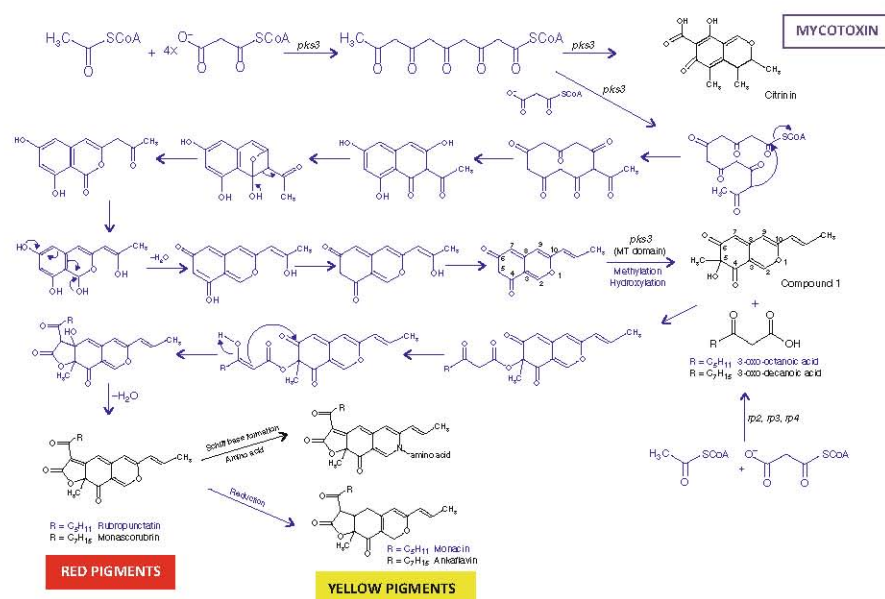
Fig. 1 Hypothetical pathway of monascorubrin, ankaflavin, and citrinin biosynthesis in *P. marneffei* (Adapted from Woo et al. [11])

among the most chemically inventive of all fungi. A comparison with other genera shows that most exometabolites have been reported from *Aspergillus* (1984 exometabolites), next-most from *Penicillium* (1338), and fifth-most by *Talaromyces* (316), with only *Fusarium* (507) and *Trichoderma* (438) producing more exometabolites [14]. These fungal genera produce various compounds, which are beneficial to the society, but amongst all, they are potential producers of natural pigments, thus they could be used for various industrial purposes. Many workers have studied the pigment production of various species of *Penicillium* and *Talaromyces* isolated from different environments. In addition, some researchers have made experiments to optimize the pigment production from these fungal species using different media by modifying the intrinsic and extrinsic factors. The most common hues produced by both genera include yellow, red, orange, and reddish-brown. According to Teixeira et al. [15], it was found that the yellow pigment is predominant in most of the *Penicillium* species. Strains collected from Amazon forest such as *P. simplicissimum* DPUA 1379, *P. melinii* DPUA-1391, and *P. atrovenetum* yielded yellow pigment with antibiotic activity [15]. As a first commercial fungal red colorant, the Arpink red™ pigment (now Natural red™) has been claimed to be produced by fermentation and bioprocess engineering using the strain *Penicillium oxalicum* var. armeniaca CCM 8242 obtained from soil. On the second day of cultivation in liquid broth containing carbohydrates, zinc sulfate, and magnesium sulfate, a hydroxyanthraquinone red colorant is released in the liquid medium, and its concentration keeps increasing up to 1.5–2.0 g/L of broth after 3–4 days [2, 16, 17].

In *Penicillium* and *Talaromyces* species, polyketide-based pigments are very common, and particularly the azaphilone (hexaketide) ones, like the derivatives of monascorubrin and rubropunctatin from *P. marneffei* [11]. In similar lines, two azaphilone pigments, monascorubramine homologues PP-V [(10Z)-12-carboxyl-monascorubramine] and PP-R [(10Z)-7-(2-hydroxyethyl)-monascorubramine], are isolated from a strain of *Penicillium* sp. AZ [18]. PP-V and PP-R are slight modifications of monascorubramine. Over the past 5 years, very few reports have been published on the *Monascus*-like azaphilone pigments produced by non-mycotoxigenic strains of *Talaromyces* species (formerly *Penicillium sp.*) [19–24] (Fig. 2). *Penicillium purpurogenum* is an important species in biotechnology for its ability to produce enzymes and pigments, which are used as natural colorants [24–27]. Recent revision of the taxonomy of *P. purpurogenum* showed that this species is a complex consisting of four taxa: *T. purpurogenus, T. ruber* (syn. *P. rubrum*), *T. amestolkiae*, and *T. stollii* [28]. From a biotechnological point of view, it is recommended to use *T. ruber* for enzyme production, because *T. purpurogenus* produces four types of mycotoxins and *T. amestolkiae* and *T. stollii* are potentially pathogenic to immunocompromised individuals [23]. As reported by Méndez et al. [20], the strain *P. purpurogenum* GH2 can produce *Monascus*-like pigments with no coproduction of toxic citrinin, which paves the way for producing water-soluble red pigments at an industrial level to be used in food industry [19–21]. *N*-glutarylmonascorubramine and *N*-glutarylrubro-punctamine were the water-soluble *Monascus*-like polyketide azaphilone pigments discovered in the extracellular pigment extract obtained from the liquid medium of *P. purpurogenum* [29] (Table 1). More recently, an European patent has been granted for a submerged cultivation method for some of the non-mycotoxigenic strains of *Talaromyces* sp. whereby the concentration of pigments was significantly enhanced, and the number of pigmented constituents was significantly reduced with the polyketide azaphilone purple pigment PP-V being the major compound [30].

Talaromyces is the telemorph genera, which comprise a monophyletic clade that is distinct from *Penicillium*. Some species of *Talaromyces* produce red pigments while a few other synthesize yellow pigments of azaphilone series [31]. Studies have shown that *Monascus*-like azaphilone red pigments and/or their amino acid derivatives are naturally produced by *Talaromyces aculeatus, T. pinophilus, T. purpurogenus,* and *T. funiculosus. Talaromyces amestolkiae, T. ruber,* and *T. stollii* also produce azaphilone pigments, as recently described by Yilmaz et al. [23], but in those three species the pigments are not diffusing into the growth medium. *T. atroroseus* can secrete large amounts of *Monascus*-like azaphilone red pigments, without the production of any known mycotoxins. On the other hand, *T. purpurogenus* produces mycotoxins such as rubratoxins A and B, rugulovasins, and luteoskyrin [23]. These factors limit the use of these species for biotechnological production of azaphilone pigments. However, some *Talaromyces* species can be used to produce pigments at industrial scale if no coproduction of toxins has been concluded. Red pigment producers, such as *T. atroroseus, T. albobiverticillius, and T. purpurogenus*, produce rubropunctatin and other *Monascus* pigments while other
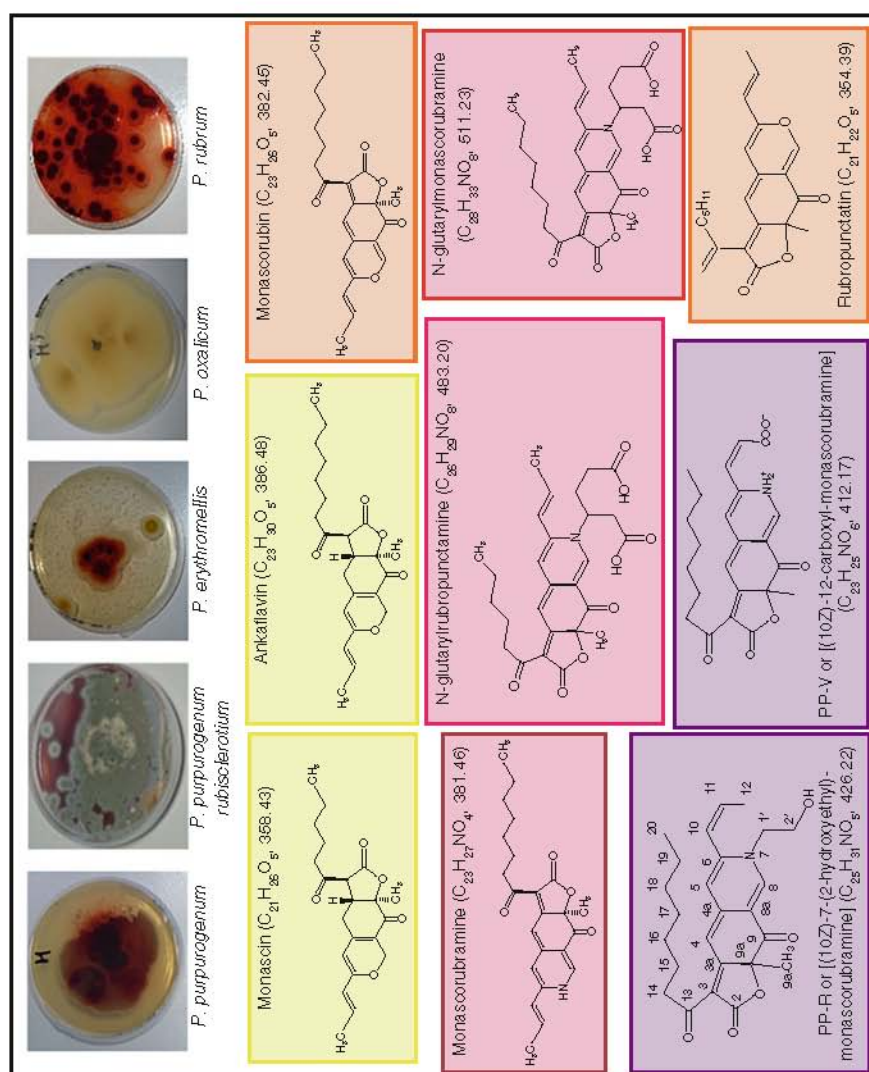
Fig. 2 Main *Monascus*-like azaphilone pigments produced by *Penicillium* and *Talaromyces* species

Table 1 Polyketide pigments produced by *Penicillium* and *Talaromyces* species

Fungal species	Pigment composition (colour) (Toxic colored compounds in bold)	Major known mycotoxic uncolored metabolites
P. atramentosum	Uncharacterized dark brown	Roquefortine C
		Rugulovasine A and B
P. atrosanguineum	Phoenicin (red)	Unknown
	Uncharacterized yellow and red	
P. atrovenetum	Atrovenetin (yellow)	beta-nitropropionic acid
	Norherqueinone (red)	
P. aurantiogriseum	Uncharacterized	Nephrotoxic glycopeptides
		Penicillic acid
		Verrucosidin
P. brevicompactum	Xanthoepocin (yellow)	Botryodiploidin
		Mycophenolic acid
P. chrysogenum	Sorbicillins (yellow)	Roquefortine C
	Xanthocillins (yellow)	
P. citrinum	Anthraquinones (yellow)	Unknown
	Citrinin (yellow)	
P. cyclopium	**Viomellein (reddish-brown)**	Penicillic acid
	Xanthomegnin (orange)	
P. discolor	Uncharacterized	Chaetoglobosin A, B, and C
P. echinulatum	Uncharacterized (yellow)	Territrems
P. flavigenum	Xanthocillins	Unknown
P. freii	**Viomellein (reddish-brown)**	Unknown
	Vioxanthin	
	Xanthomegnin (orange)	
P. herquei	Atrovenetin (yellow)	Unknown
	Herqueinones (red and yellow)	
P.oxalicum	Arpink red™- anthraquinone derivative (red)	Unknown
	Secalonic acid D (yellow)	
P. paneum	Uncharacterized (red)	Botryodiploidin
		Patulin
		Roquefortine C
P. persicinum	Uncharacterized cherry red	Roquefortine C
P. viridicatum	**Viomellein (reddish-brown)**	Penicillic acid
	Vioxanthin	Viridic acid
	Xanthomegnin (orange)	
T. macrosporus	Mitorubrin (Yellow)	Duclauxin
		Islanditoxin
P. aculeatum	Uncharacterized	Unknown
P. crateriforme	Uncharacterized	Rubratoxin
		Rugulovasine A and B
		Spiculisporic acid

Table 1 (continued)

Fungal species	Pigment composition (colour) (Toxic colored compounds in bold)	Major known mycotoxic uncolored metabolites
P. funiculosum	Uncharacterized	Unknown
P. islandicum	**Emodin (yellow)**	Cyclochlorotine
	Erythroskyrin (orange-red)	Islanditoxin
	Luteoskyrin (yellow)	Rugovasin A and B
	Skyrin (orange)	
	Rugulosin (yellow)	
P. marneffei	Monascorubrin (red)	Unknown
	Rubropunctatin (orange)	
	Mitorubrinol	
	Monascorubramine (purplered	
	Secalonic acid D (yellow)	
P. pinophilum	Uncharacterized	Unknown
P. purpurogenum	Mitorubrin (yellow)	Unknown
	Mitorubrinol (orange-red)	
	Purpurogenone (yellow-orange)	
	PP-R (purple red)	
P. rugulosum	**Rugulosin (yellow)**	Unknown
P. variabile	**Rugulosin (yellow)**	Unknown

species such as *T. cnidii* and *T. coalescens* produce red soluble pigments in some isolates (Table 1).

2.2.2 Pigments from Aspergillus and Emericella Species

Aspergillus and *Emericella* are two genera consisting of a few hundred mold species found worldwide in various climates. They are well known to produce different secondary metabolites such as polyketide-based pigments in solid and liquid cultures. The most important colored metabolites produced by *Aspergillus* and *Emericella* species are, respectively, hydroxyanthraquinone (octaketide) pigments and azaphilone (hexaketide) ones (Fig. 3). From the genus *Aspergillus*, 18 different hydroxyanthraquinone pigments, at least, were identified. Some strains of this genus, such species as *A. glaucus*, *A. cristatus*, and *A. repens*, share many common secondary metabolites and are found to produce hydroxyanthraquinone pigments such as emodin (yellow), physcion (yellow), questin (yellow to orange-brown), erythroglaucin (red), catenarin (red), and rubrocristin (red) (or dimer of emodin and physcion) [17, 32]. Other hydroxyanthraquinone pigments, such compounds as averufin, norsolorinic acid, versicolorin, variecolorquinone, ascoquinone A, averantin, chrysophanol (orange), cynodontin (bronze), and tritisporin (brownish-red), are rarer products revealed from this genus. Presently, *A. glaucus* (group) seems to be the best producer according to the diversity of hydroxyanthraquinone compounds produced (11 different) [17, 32]. In addition, other polyketide-based

Fig. 3 Main azaphilone, hydroxyanthraquinone and naphthoquinone pigments produced by *Aspergillus* and *Emericella* species

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pigments, e.g., viomellein (reddish-brown), viopurpurin (purple), xanthomegnin (orange), and rubrosulfin (red), are produced by *A. ochraceus*, *A. melleus*, *A. sulphureus*, and *A. westerdijkiae* [25]. The fungus *Aspergillus nidulans* is also known to produce dark-brown melanin pigments. Melanin pigments appear to be essential for the protection of the fungus against environmental stress [33]. The conidial pigment of *Aspergillus fumigatus* contains the 1,8-dihydroxynaphthalene-like melanin pigment (a complex aggregate of polyketides). This pigment plays a major role in the protection of the fungus against immune effector cells; for example, it is able to scavenge reactive oxygen species generated by alveolar macrophages and neutrophiles [34]. From studies performed on *A. niger*, secondary metabolite profiling of the color mutants revealed a close relationship between polyketide synthesis and conidial pigmentation in the fungus [35]. The production of the 1,8-dihydroxynaphthalene, precursor of melanin pigment, and the naphtho–γpyrone subclass of polyketides (commonly found in significant quantity in *A. niger* culture extracts) were dependent on polyketide synthases [35, 36]. More recently, six novel compounds belonging to the family of azaphilones, azanigerones A-F, were isolated and characterized from culture of *A. niger* [37], which indicates the presence of an azaphilone biosynthetic pathway in *Aspergillus* species.

The genus *Emericella* comprises 34 species and the name *Emericella* refers to the sexual phase (teleomorph) of these fungal species. Some of these fungi are well known to produce yellow azaphilone pigments. As example, eight azaphilone-based pigments, named falconensins A-H (yellow), have been isolated from the mycelium of both *Emericella falconensis* and *Em. fructiculosa,* along with other yellow pigments, falconensones A1 and B2 (i.e., cyclopent-2-enone derivatives), and hopane-type triterpene, zeorin (yellow) [25, 38]. Three dicyanide derivatives, epurpurins A to C (yellow) were also isolated from *Emericella purpurea* [39, 40]. The yellow pigment sterigmatocystin, a carcinogenic polyketide compound, has been reported in several *Emericella* species (e.g., *Em. rugulosa*, *Em. parvathecia*, and *Em. nidulans*) and also in the fungus *Aspergillus versicolor* [25, 41] (Fig. 3). The effect of different wavelength of light (daylight, darkness, blue 492–455 nm, green 577–492 nm, yellow 597–577 nm, and red 780–622 nm) on growth, intracellular and extracellular pigment production by the fungus *Emericella nidulans* has been reported by Velmurugan et al. [42], and total darkness was concluded to favor biomass, extracellular and intracellular pigment productions.

2.2.3 Pigments from Eurotium and Paecilomyces Species

The common genus *Eurotium* consists in teleomorphic often xerophilic species, usually related to *Aspergillus* anamorphs, especially from *A. glaucus* group. The genus *Eurotium* contains several species and is also an important polyketide producer. Some species of *Eurotium* including *E. amstelodami*, *E. chevalieri,* and *E. herbariorum* are found to produce hydroxyanthraquinone pigments. The pigments most frequently identified are physcion (yellow) and erythroglaucin (red); however, the strains produce in addition two benzaldehyde colored compounds, e.g., flavoglaucin (yellow) and auroglaucin (orange-red), and the mycotoxin echinulin (colorless) [17, 43]. Sixteen more species of *Eurotium* are able to synthesize

hydroxyanthraquinone pigments. Physcion and erythroglaucin are the most widespread pigments throughout the *Eurotium* studied strains [17, 32]. From Anke study [44], within this genus, *E. rubrum* and *E. cristatum* produce the highest diversity of compounds regarding hydroxyanthraquinones: erythroglaucyn, physcion, catenarin (red), rubrocristin (red), and emodin (orange) were identified in their cultures. *E. umbrosum, E. spiculosum, E. glabrum, E. echinulatum, and E. chevalieri* synthesize the first four compounds out of the five mentioned. *E. tonophilum, E. acutum, E. herbariorum, E. intermedium,* and *E. leucocarpum* produce only either physcion or erythroglaucin (Fig. 4). The study also demonstrated that within a same species there was a great variability toward hydroxyanthraquinones production, as some
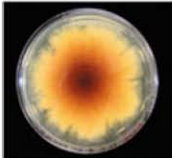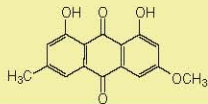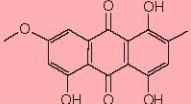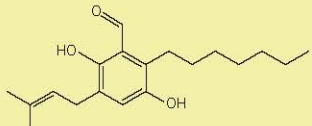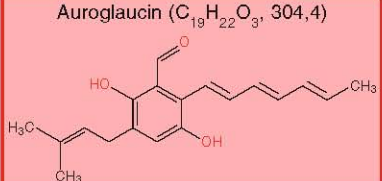
Fig. 4 Main hydroxyanthraquinone and hydroquinone pigments produced by *Eurotium* and *Paecilomyces* species

strains of *E. rubrum*, *E. niveoglaucum*, *E. leucocarpum*, *E. intermedium*, *E. herbariorum*, *E. pseudoglaucum*, *E. appendiculatum*, *E. echinulatum*, and *E. acutum* behaved differently when grown under the same culture conditions [44]. Moreover, some of the strains of *E. amstellodami*, *E. heterocaryoticum*, and *E. montevidensis* included in this study did not produce any hydroxyanthraquinone pigments under the conditions of the experiment [44]. Another *E. rubrum* strain (QEN-0407-G2) isolated from the inner tissue of the stem of the marine mangrove plant *Hibiscus tiliaceus* synthesized the pigment questin (orange), a glycosyled derivative of questin [6, 3-*O*-(α-D-ribofuranosyl)- questin; orange], and three other fungal metabolites (eurorubrin, and two *seco*-anthraquinone derivatives [3, 2-*O*-methyl-9-dehydroxyeurotinone and 4, 2-*O*-methyl-4-*O*-(α-D-ribofuranosyl)-9-dehydroxyeurotinone]) [45].

The genus *Paecilomyces* may be distinguished from the closely related genus *Penicillium* by forming colonies that show various color shades. Colonies are fast growing, powdery or suede-like, gold, yellow-brown, lilac, or tan, but never green or blue-green as in *Penicillium*. A red uncharacterized pigment has been isolated from the fungus *Paecilomyces sinclairii* and is certainly an amino group linked to a hydroxyanthraquinone structure [46] (Fig. 4).

2.3   Species of the *Nectriaceae* Family Producing Pigments

Fusarium is a diverse group of fungi of the *Nectriaceae* family. The name *Fusarium* refers to the asexual phase (anamorph) of the fungus. In accordance with their genetic potential, *Fusarium* species have been found to produce a wide range of fungal pigments that are diverse in structure and biological activity. However, among the *Fusarium* secondary metabolites, numerous toxic compounds have been identified (e.g., fumonisins, zearalenone, fusaric acid, fusarins, and beauvericins) [25, 47, 48]. Numerous terpenes have been characterized from *Fusarium* species, and a representative example is *Fusarium fujikuroi*, which is able to produce orange carotenoids (neurosporaxanthin) [49, 50]. Among the *Fusarium* secondary metabolites, numerous polyketide pigments have also been identified, such as the naphthoquinone pigments which form the most abundant group [51–62] and the hydroxyanthraquinone ones [17, 63]. Previously, Cajori et al. [64] have isolated the red naphthoquinone pigment, bostrycoidin, produced by a *F. oxysporum* (formerly *F. bostrycoides*) strain. Tatum et al. [51] reported that six napthtoquinone pigments of the naphthazarin structure were produced by *F. oxysporum*. According to the authors, the major pigment isolated was the 9-*O*-methylfusarubin (formerly 8-*O*-methylfusarubin), with 5-*O*-methyljavanicin (formerly 8-*O*-methyljavanicin), 8-*O*-methylbostrycoidin, 1,4-naphthalenedione-3,8-dihydroxy-5,7-dimethoxy-2-(2-oxopropyl) (formerly 8-*O*-methyl-13-hydroxynorjavanicin), 5-*O*-methylsolaniol (formerly 8-*O*-methylsolaniol), and 9-*O*-methylanhydrofusarubin (formerly 8-*O*-methylanhydrofusarubin) in decreasing concentration. All of these pigments are red, except 8-*O*-methylanhydrofusarubin, which is purple (Fig. 5). Then Baker and Tatum [63] have isolated two yellow/orange hydroxyanthraquinone pigments among
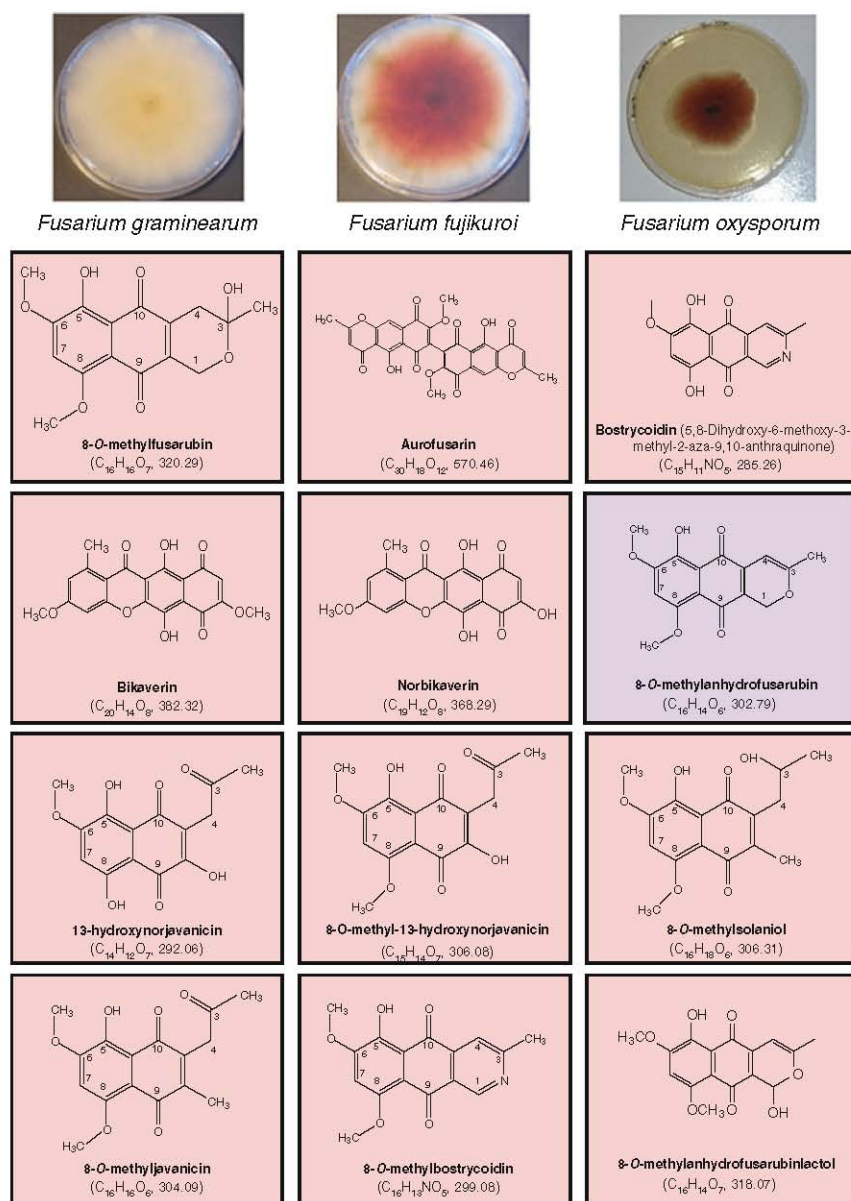
Fig. 5 Main naphthoquinone pigments produced by *Fusarium* species

the secondary metabolites produced by *F. oxysporum*. Nowadays, the most thoroughly studied *Fusarium* polyketide pigments are the red dimeric naphthoquinone pigment, aurofusarin, from *F. graminearum*, the red naphthoquinone pigment, bikaverin, and its minor coproduct, nor-bikaverin, from *F. fujikuroi* [52–58]. Over the past decade, few reports have been published on the aurofusarin and bikaverin

biosynthetic pathways. Their polyketide nature was confirmed through identification of polyketide synthase genes responsible for their biosynthesis. Aurofusarin was first described as a *Fusarium culmorum* pigment by Ashley et al. [65]. The color of aurofusarin is dependent on the pH value of the solvent, ranging from golden yellow in acidic solvents to red/purple in alkaline solvents [65]. The pigment is produced in high quantities, continuously during mycelium development, resulting in the increasing staining of both mycelium and medium (the mycelium shifts from white to yellow and finally to a deep red color). Known producers include *Fusarium acuminatum, F. avenaceum, F. crookwellens, F. culmorum, F. graminearum, F. poae, F. pseudograminearum, F. sambucinum, F. sporotrichioides,* and *F. tricinctum* [66]. Aurofusarin is produced under various suboptimal conditions, such as high or low pH, high temperatures, and phosphate starvation. The genes responsible for the production of aurofusarin could be regulated by a global pH regulatory factor. The ambient pH is the most critical parameter, as many naphthoquinones are cytostatic at neutral pH [67].

Concerning the red naphthoquinone pigment, bikaverin, it has been shown that its production is strongly dependent on culture conditions and its regulation has been investigated in detail in *F. fujikuroi*. The bikaverin production is repressed in culture media containing high nitrogen levels and under alkaline conditions; aeration also stimulated its production [54–56]. Bikaverin was first discovered as a pigment in cultures of *F. lycopersici* and *F. vasinfectum* [55, 68]. Limón et al. [55] have reported no negative incidence of bikaverin-contaminated products on human health, even if bikaverin is often considered a mycotoxin today [53]. Biological effects of bikaverin differ largely between different organisms, and bikaverin was not genotoxic according to a DNA synthesis assay [52]. Bikaverin is a fungal polyketide-based pigment with antibiotic activity against fungi [69] and antitumor action [70]. Some reports on isolation of aurofusarin and bikaverin have also been published for other *Fusarium* species such as *F. solani* [51] and *F. verticillioides* [42, 61]. More recently, Sørensen et al. [71] have discovered a medium with low nitrogen content that partially redirects the aurofusarin and bikaverin biosynthetic pathways to produce the lactones citreoisocoumarin and SMA93, respectively, in some aurofusarin and bikaverin producing *Fusarium* species; the redirection seems to be regulated by the same mechanism, which is triggered by some organic nitrogen source (glutamine, arginine) and acidic conditions (with an optimum at pH 5–6). According to Medentsev et al. [72], who have studied the biosynthesis of colored naphthoquinone metabolites by *Fusarium decemcellulare, F. graminearum,* and *F. bulbigenum* fungi, the biosynthesis of naphthoquinone pigments in *Fusarium* species was shown to be the main response of the fungi to stress, observed under conditions of growth inhibition or arrest. Depending on the conditions of cultivation, *F. bulbigenum* and *F. graminearum* synthesized bikaverin and aurofusarin, respectively, whereas *F. decemcellulare* synthesized soluble extracellular naphthoquinones of the naphthazarin structure (javanicin, anhydrojavanicin, fusarubin, anhydrofusarubin, bostrycoidin, and novarubin) or extracellular dimeric naphthoquinone aurofusarin [72] (Fig. 5).

Concerning the *Fusarium* perithecial pigments, a violet pigment that accumulates in the walls of sexual fruiting bodies (perithecia) has been isolated from both cultures

of *F. verticillioides* and *F. graminearum* [60, 73]. In contrast, the pigment that accumulates in the perithecial walls of *F. solani* is red and results from the activity of a different polyketide synthase [59]. The fungus *F. fujikuroi* has been reported to produce particular naphthoquinone pigments, i.e., red fusarubins [62]. Five main red fusarubin-like naphthoquinone pigments have been isolated: 8-*O*-methylfusarubin, as the main product (interestingly, with the same structure of the major pigment isolated by Tatum et al. [51] in culture of *F. oxysporum*), 8-*O*-methylnectriafurone, 8-*O*-methyl-13-hydroxynorjavanicin, 8-*O*-methylanhydrofusarubinlactol, and 13-hydroxynorjavanicin that are produced under specific culture conditions, which are different from those for red bikaverin [62] (Fig. 5). Naphthoquinone pigment related to fusarubin was initially isolated from the fungus *F. solani* [74]. Although the functional characterization of most of the cluster in *F. fujikuroi* provides strong evidence that fusarubins (red) are the precursors of the perithecial pigment (violet), the structure of the violet perithecial pigment has yet to be determined [62, 75]. Thus, studies are in progress to determine the relationship between fusarubin pigments and the violet perithecial one.

2.4   Species of the *Hypocreaceae* Family Producing Pigments

Fungal strains of the genus *Trichoderma* from the *Hypocreaceae* family are well-known producers of secondary metabolites with antibiotic activity [76] and are important biocontrol agents successfully applied as biopesticides worldwide. Strains of *Trichoderma aureoviride*, *T. harzianum*, *T. polysporum*, and *T. viride* all produce the hydroxyanthaquinone pigments pachybasin (yellow) and chrysophanol (orange-red). The fungus *T. viride* also synthesizes emodin (yellow), 1,3,6,8-tetrahydroxyanthraquinone, and 2,4,5,7- tetrahydroxyanthraquinone. Only emodin is known from culture of *T. polysporum* [17] (Fig. 6). In 2012, Lin et al. [77] reported that the pachybasin and emodin compounds are secreted by *T. harzianum*. In the genus *Trichoderma*, the increase of the concentration of excreted emodin or pachybasin increases the concentration of cyclic AMP indicating that these hydroxyanthraquinone pigments are key substances in the regulation of this secondary messenger and also suggest that pachybasin and emodin play roles in the biocontrol mechanism of *Trichoderma sp.* [32, 77]. The major secondary metabolites produced by *Trichoderma harzianum* T22 and T39, two commercial strains successfully used as biopesticides and biofertilizers in greenhouse and open field production, are hydroxyanthraquinones (e.g., 1-hydroxy-3-methyl-anthraquinone and 1,8-dihydroxy-3-methyl-anthraquinone), azaphilone (T22azaphilone), and three other metabolites (T39-butenolide, harzianolide, and harzianopyridone) [78].

2.5   Species of the *Pleosporaceae* Family Producing Pigments

In the *Pleosporaceae* family (*Alternaria*, *Curvularia*, *Drechslera*...), both hyphae and conidia are heavily pigmented. Hydroxyanthraquinone pigments are produced
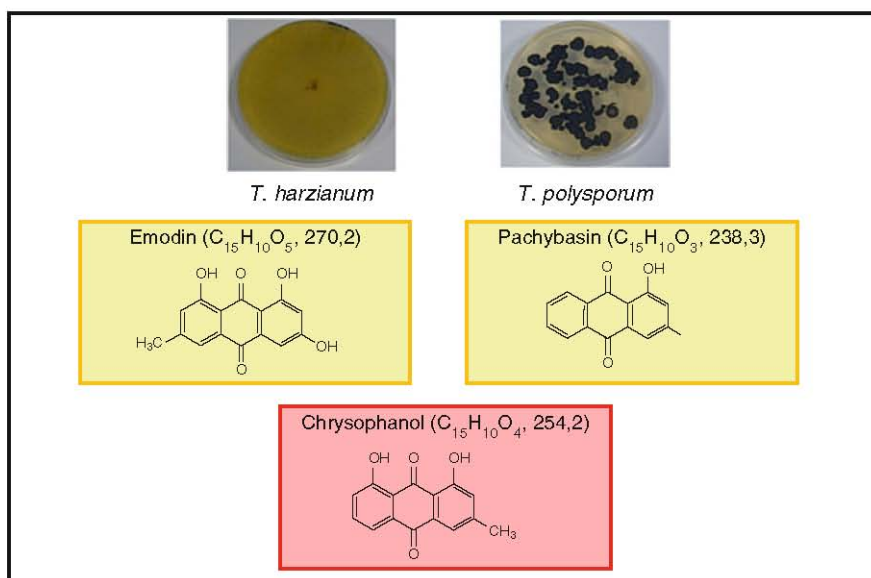
Fig. 6 Main hydroxyanthraquinone pigments produced by *Trichoderma* species

by a broad range of *Alternaria*, *Curvularia, and Drechslera* species. As example, the main hydroxyanthraquinone pigments characterized from the fungus *Curvularia lunata* are chrysophanol (orange-red), cynodontin (bronze), helminthosporin (maroon), erythroglaucin (red), and catenarin (red) [17, 25]. Cynodontin extracted from the biomass of *C. lunata* has been converted successfully to two anthraquinone biodyes (Disperse blue 7 and Acid Green 28). The properties of these biodyes applied to knitten polyamides were compared with those of conventional dyes and found to be identical to all-important aspects. Several species of *Drechslera* (e.g., *D. teres*, *D. graminea*, *D. tritici-repentis*, *D. phlei*, *D. dictyoides,* and *D. avenae*) produced the following hydroxyanthraquinone pigments: catenarin (red), cynodontin (bronze), helminthosporin (maroon), tritisporin (reddish-brown), and erythroglaucin (red), without coproduction of known mycotoxins [17, 79]. In similar lines, three species of *Alternaria*, e.g., *Alt. solani*, *Alt. porri,* and *Alt. tomatophila,* can produce a yellow-orange polyketide pigment, altersolanol A, without myco-toxin's production [80] (Fig. 7). These fungal strains can be investigated as a possible potentially safe source of pigments [25].

2.6 Species of the *Cordycipitaceae* Family Producing Pigments

Fungal strains belonging to the *Cordydipitaceae* family are often characterized as insect pathogens, and some of them can be used as biological control against crop pests, such as aphids [81]. From the 18 genera classified in this family, shades of
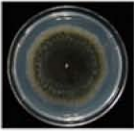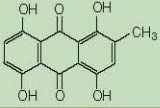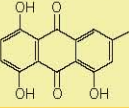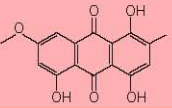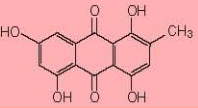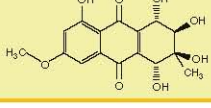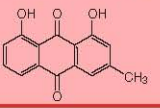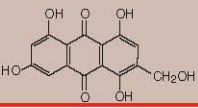
Fig. 7 Main hydroxyanthraquinone pigments commonly and specifically produced by *Curvularia, Dreschlera* and *Alternaria* species

orange to deep-red pigments have been described, and mainly found in the 5 following clades: *Beauveria, Cordyceps* (the sexual states (teleomorphs) of *Beauveria* species), *Hyperdermium, Isaria* (formely *Paecilomyces*), *and Lecanicillium* (former *Verticillium*). The fungus *Cordyceps unilateralis* can produce six extracellular red naphthoquinone pigments, with erythrostominone as the major one, followed by 4-*O*-methyl erythrostominone, deoxyerythrostominol, deoxyerythrostominone, epierythrostominol, and in a smaller proportion 3,5,8-trihydroxy-6-methoxy-2-(5-oxohexa-1,3-dienyl)-1,4-naphthoquinone (shortened to 3,5,8-TMON) [25, 82] (Fig. 8). Interestingly, the 3,5,8-TMON presents the most intense red hue and the lowest cytotoxic properties among the six naphthoquinones mentioned, rendering this latter promising for food and cosmetic applications. Moreover, it has been shown that erythrostominone can be chemically converted to 3,5,8-TMON by heating up the fermentation broth (100 °C) under acidic conditions (pH 4).

A blood-red dibenzoquinone mycotoxin, oosporein, was isolated in *Beauveria bassiana* [83] and *Lecaniccilium aphanocladii* [84]. This molecule has a wide range of bioactivities from antifungal, antimicrobial, and phytotoxic effects to growth inhibition in plants. Additionally, kidney damage and even death were noticed in poultry exposed to oosporein. Yellow pigments 2-pyridone tenellin [85] and

Fig. 8 Main naphtoquinone, dibenzoquinone and hydroxyanthraquinone pigments produced by *Cordyceps*, *Beauvaria* and *Hyperdermium* species

bassianin [86] have been described in *Beauveria sp.* (Fig. 8). Little has been investigated on these components. Tenellin have also been used as biocontrol agent for agrochemical pests, such as thrips, bollworms, whiteflies, mealybugs, and mites. Similarly, the known orange-red anthraquinone dimer, skyrin, was found in *Hyperdermium bertonii*, and was demonstrated to have selective toxicity toward insect cells, which suggests potentialities for agrochemical applications of such pigments [87]. Other alkaloid-type yellow pigments, torrulbiellone A, as well as brownish isocoumarine glucoside compounds, were described in *Torrubiella sp.* [88, 89], and were concluded to have strong activity against human cancer cell lines, in particular regarding breast cancer and epidermoid carcinoma.

A red pigment produced by a strain of *Isaria farinosa* (formerly *Paecilomyces farinosus*) was elucidated as a chromophore of the hydroxyanthraquinone type [17, 42]. This pigment was excreted in the fermentation broth and was relatively easily extracted with a mixture of water and ethanol (1:1, v/v). The ready availability of this pigment along with its heat, pH, and temperature resistance offer new insights for food coloring applications.

Thus, the *Cordycipitaceae* family displays promising fungal strains producing relatively high concentrations of reddish bioactive pigments. Most of them are also mycotoxins, whose activities against insects have been widely demonstrated, suggesting that further toxicity tests would be required to ensure they would remain harmless to human health whatsoever their final industrial application and discard.

2.7 Species of the *Xylariaceae* Family Producing Pigments

The *Xylariaceae* is one of the largest families of filamentous fungi isolated from plant material. This is due to the fact that many members, as *Hypoxylon* or *Daldinia*, can develop an endophytic stage during their life cycle. Nowadays, around 1300 species are accepted in this family. They essentially grow under the form of mycelial structure, as their fruiting bodies (stromata) seem to form only when their host is stressed or unhealthy. Thereby, the elucidation of their life cycle and their phylogeny have been recently achieved, thanks to molecular studies. They are known to produce several novel and interesting secondary metabolites such as antiparasitic agents, enzyme inhibitors, immunomodulators, antimicrobial substances, or pigments, mainly extracted from their fruiting stages [90].

The subfamily *Hypoxyloideae* includes many genera whose stromata show bright colors, depending on their cycle stage. The stromata of many *Xylariaceae*, above all the genera *Hypoxylon*, *Daldinia*, and *Annulohypoxylon*, are rich in characteristic pigments that also serve as chemotaxonomic marker molecules [91, 92]. Many of them are azaphilones containing a highly oxidized pyrone–quinone bicyclic core with a chiral quaternary center. *Hypoxylon lechatii*, collected in French Guiana, produced vermelhotin and three novel congeners hypoxyvermelhotins A–C. Like vermelhotin, these compounds constitute orange-red pigments and a preliminary biological characterization revealed rather strong cytotoxic and moderate to weak antimicrobial effects [93–95]. High-performance liquid chromatography (HPLC)

profiling of *H. fragiforme* (a common fungus associated with *Fagus sp.* in the Northern hemisphere) by Stadler et al. [96] revealed changes in the pigment composition during stromatal development. The white cytotoxic mycotoxin, cytochalasin H, and two new cytochalasins (fragiformins A–B) were identified as major constituents of the young, maturing stromata, whereas mature, ascogenous material yielded large amounts of mitorubrin-type azaphilones. Indeed, the red color of mature *H. fragiforme* as well as in *H. howeanum* is due to four mitorubrin azaphilones (mitorubrin, (+)-mitorubrinol, mitorubrinol-acetate, and (+)-mitorubrinic acid) which are concentrated in orange-brown granules located beneath the stromatal surface [97]. Mellein derivatives and the green pigment hypoxyxylerone have also been isolated from cultures of this fungus [98, 99]. Two apparently specific mitorubrinol derivatives were, moreover, identified from the stromata of a strain of *H. fulvo-sulphureum*, belonging to the *H. rubiginosum* complex [100]. Bodo et al. [101] identified a novel naphthyl-naphthoquinone: 5-hydroxy-2- (1',8'-dihydroxy-4'-naphthyl)-1,4-naphthoquinone, named hypoxylone (orange), in *H. sclerophaeum*. From *H. rickii*, rickenyl B and D (red and brown) were obtained. These compounds belong to the class of parasubstituted terphenyls, which are widespread in the kingdom Fungi without any preferences for specific taxonomic groups [94]. Seven new pigmented azaphilones: lenormandins A–G, were also extracted from several strains of *H. lenormandii*, and *H. jaklitschii* sp. nov. They seem specific for these species or closely related taxa [102].

In the comprehensive review from Stadler et al. [103], it is mentioned that *H. fuscum* and its allies have greenish, olivaceaous or isabelline pigment colors due to daldinins (*H. fuscum* chemotype). *H. rubiginosum* and allies are typically orange, orange brown, or yellowish green from the presence of mitorubrin (*H. fragiforme* chemotype), rubiginosin (*H. rubiginosum* chemotype), or hypomiltin (*H. hypomiltum/ perforatum* chemotype). *H. macrocarpum* contains different olivaceous to purplish brown pigments named macrocarpones.

Anyway many members of this genus show very diverse colors and hues: vinaceous (strains of *Hypoxylon rubiginosum*), bright orange (*H. petriniae*), deep orange or sienna (*H. subticinense*), orange–red (*H. rutilum*), amber, ochreous yellow or greenish-yellow (*H. perforatum*), lilaceaous gray to purple (*H.* cf. *vogesiacum*) [104]. These different colors clearly reflect the presence of several different metabolites bound to be identified through additional studies [105].

The diversity and complexity of secondary metabolites in this subfamily (*Xylariaceae: Hypoxyloideae*) has been demonstrated through several studies. In 2010, Laessoe et al. [106] identified the yellowish lepraric acid, and derivatives from *Hypoxylon aeruginosum*, but also from *Chlorostroma subcubisporum* and *C. cyaninum* sp. nov. (species close to *Thuemenella* sp.). Similar substituted chromones were also known from lichenized ascomycetes or plants (see review by Ellis [107]).

The production of specific azaphilones like cohaerin A, multiformin A, and sassafrins D recently allowed to place some strains formerly identified as *Hypoxylon sp.* in a new genus, namely *Annulohypoxylon* [108–110]. Additional studies of *A. cohaerens* further established two subtypes, one of which contains cohaerin variants A and B [109], while the other contains the cohaerins C-F [111]. Additional

cohaerins G-K in yellow hues were obtained from this strain showing evidence of the coexistance of several cohaerin's structural variations, produced by the fungus *A. cohaerens*, through the combination of single minor changes [112]. Various *Entonaema spp.* (Pyrenomycetes) as *Entonaema splendens* also contain mitorubrins variants such as entonaemins, rubiginosins, or hypomiltin [113, 114]. Sassafrins A–D, four new azaphilones, were also extracted from the stromata of *Creosphaeria sassafras*. Their apparently unique occurrence in *C. sassafras* supports the status of this fungus as a member of a distinct genus within the *Xylariaceae*, coinciding with molecular and morphological traits [108].

Up to date the genus *Daldinia* includes about 20 angiosperm associated species. Some of them have recently been clustered with the genera *Xylaria* and *Hypoxylon* [115]. They develop a wide range of hues from yellow to purple, essentially due to the pigments produced in their stromatal structures but also during the mycelial growth. The main metabolites detected strongly differ according to the culture conditions [116, 117]. However, all the experimented species (*D. bambusicola, D. caldariorum, D. childiae, D. clavata, D. concentrica, D. eschscholzii, D. fissa, D. grandis, D. lloydi, D. loculata, D. cfr loculata, D. petriniae, D. singularis, D. sp. "Scania"*) produce the yellow BNT (1,1'-Binaphthalene-4,4'-5,5'-tetrol) and daldinol in their stromata (Fig. 9). The yellowish daldinal A, daldinal B, and daldinal C were also produced by *D. concentrica* and *D. eschscholzii*. The colored 8-methoxy-1-napthol and 2-Hydroxy-5-methylchromone were found from the liquid cultures of all strains. The yellow azaphilones and benzophenones found in *D. childiae* were lacking in species with purple stromatal colors. Most cultures of *Daldinia spp.* then produced naphthalene and chromane derivatives, differing from allied genera by the absence of mellein. Stromata of *Daldinia spp.* did not produce mitorubrin but generally contained binaphthyls.

2.8 Species of the *Chaetomiaceae* and *Sordariaceae* Families Producing Pigments

Chaetomiaceae and especially the genus *Chaetomium* is a commoun fungal genus from soil and environment. *Chaetomium cupreum* amongst other exhibit antagonistic activities against many fungi. Ketomium®, a commercial product, has been developed from this species, and is being widely used as broad spectrum bio-fungicide for disease control in various crops [118]. *C. cupreum* abundantly produces a red pigment identified as oosporein (6,6'-tetrahydroxy-2,2' dimethyl-5,5'-bi-p-benzoquinone), known to have antifungal effects against *Rhizoctonia solani, Botrytis cinerea, Pytium ultimum* and many pathogenic fungi. More recently three new azaphilones named rotiorinols A − C (red), two new stereoisomers, (−)-rotiorin (red) and epi-isochromophilone II (yellow), and a known compound, rubrorotiorin (red), were isolated from *Chaetomium cupreum* [119]. This rubrorotiorin is also known from *Penicillium hirayame* [120]. Most of these compounds are active mycotoxins. *C. globosum* (*C. globosum* var. *flavo-viridae*) is also producing yellow azaphilones named chaetoviridins (A, B, C, D) [121].
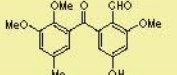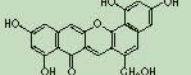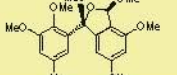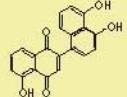
Fig. 9 Main azaphilone and naphthoquinone pigments produced by *Hypoxylon*, *Daldania* and *Annulohypoxylon* species

Chaetoviridin A is clearly involved in the induction of chlamydospores-like cells and also inhibits the growth of other fungi. Other strains of *C. globosum*, moreover, produce the cytochalasan alkaloids chaetoglobosins A–G, J, Q, R, T, U arising from a mixed polyketide-amino acid biosynthetic pathway, and with different hues (F is a pale yellow compound also found in *Chaetomium subaffine*). Strains also produce chaetoglobin A–B, chaetomugilins A–F, I, M, seco-chaetomugilins A–D, and three new nitrogenous azaphilones; 4′-epi-N-2-hydroxyethyl-azachaetoviridin A (dark

red), N-2-butyric-azochaetoviridin E (orange), and isochromophilone XIII (orange) [122–124]. *C. globosum* additionally synthesizes a purple pigment called cochliodinol [125].

In the same family, the thermophilic genus *Thielavia Zopf* (*Chaetomiaceae*, Sordariales) (anamorphic genus in the human pathogenic *Myceliophthora sp.*) is also widespread as plant endophytes. Many species are characterized by darkly pigmented ascospores containing melanin and/or by pigments exuded in the culture media. *T. intermedia* or *T. rapa-nuiensis sp. nov.* f. i. excrete orange-yellow pigments. *T. terrestris* colonies are yellow to orange in reverse and sometimes exude a diffusible reddish brown pigment [126, 127]. Several nor-spiro-azaphilones (thielavialides A − D), and a bis-spiroazaphilone (thielavialides E), have been identified from the cultures, but they appear as unpigmented [128]. The human pathogenic *Achaetomium sp.*, is known to produce the orange hydroxyanthraquinone pigment, parietin [129]. Such agents, sometimes causing osteomyelitis or fatal cerebral mycosis are generally difficult to isolate, identify, and also treat effectively. The pigments production in this family is not the first skill to be studied (Fig. 10).

Neurospora crassa is a well-known *Sordariaceae* originating from tropical or subtropical countries. It produces several yellow-orange carotenoids identified as phytoene, ß-carotens, γ-carotene, lycopene, neurosporen, neurosporaxanthin or spirilloxanthin. Overaccumulation of carotenoids has extensively been studied in this genus, and overexpression has frequently been generated by photoinduction [130–132].

3   Natural Carotenoid Pigments Produced by Filamentous Fungi

For several decades, carotenoids have been commercially produced by chemical synthesis or sold as plant extracts or oleoresins, e.g., of tomato and red pepper. Some unicellular green algae, under appropriate conditions, become red due to the accumulation of high concentrations of "secondary" carotenoids. Two examples, *Dunaliella* spp. and *Haematococcus pluvialis*, are cultured extensively as sources of β-carotene and (3S,3'S)-astaxanthin, respectively.

Nonphotosynthetic microorganisms – fungi, bacteria, yeasts – are also often strongly pigmented by carotenoids, so commercial production by these organisms is an attractive prospect. Fermentation processes for pigment production at commercial scale are now in use in the food industry, such as the production of β-carotene from the fungus *Blakeslea trispora*, in Europe. Efforts have been made to reduce the production costs so that pigments produced by fermentation can be competitive with synthetic pigments or with those extracted from natural sources, i.e., plant or microalgal. There is scope for innovations to improve the economics of carotenoid production by isolating new microorganisms, creating better ones, or improving the processes. The fungal carotenoid products may be used as color additives in many

Fig. 10 Main azaphilone, hydroxyanthraquinone and carotenoid pigments produced by *Chaetomium*, *Achaetomium* and *Neurospora* species

industries, including food and feed, and are now under consideration for use as health supplements.

3.1 β-Carotene

β-Carotene (Fig. 11) is produced on a large scale by chemical synthesis, and also from plant sources such as red palm oil, as well as by fermentation and from microalgae.

3.1.1 Blakeslea Trispora

Blakeslea trispora is a commensal microorganism associated with tropical plants. The fungus exists in (+) and (−) mating types; the (+) type synthesizes trisporic acid, which is both a metabolite of β-carotene and a hormonal stimulator of its biosynthesis. On mating the two types in a specific ratio, the (−) type is stimulated by trisporic acid to synthesize large amounts of β-carotene [133, 134]. The production process proceeds essentially in two stages. Glucose and corn steep liquor can be used as carbon and nitrogen sources. Whey, a by-product of cheese manufacture, has also been considered, with strains adapted to metabolize lactose. In the initial fermentation process, seed cultures are produced from the original strain cultures and subsequently used in an aerobic submerged batch fermentation to produce a biomass rich in β-carotene. In the second stage, the recovery process, the biomass is isolated and transformed into a form suitable for isolating the β-carotene, which is then extracted with ethyl acetate, suitably purified and concentrated, and crystallized. The final product is either used as crystalline β-carotene (purity >96 %) or is formulated as a 30 % suspension of micronized crystals in vegetable oil. The production process is subject to Good Manufacturing Practices (GMP) procedures and adequate control of hygiene and raw materials. The biomass and the final crystalline product comply with an adequate chemical and microbiological specification and the final crystalline product also complies with the JECFA (Joint FAO/WHO Expert Committee on Food Additives) and European Union (EU) specifications as set out in Directive 95/45/EC for coloring materials in food.
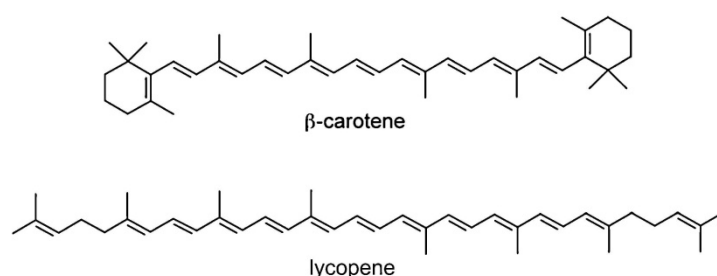
Fig. 11 Chemical structures of the carotenoids ß-carotene and lycopene

The first β-carotene product from *B. trispora* was launched in 1995. The mold has shown no pathogenicity or toxicity in standard pathogenicity tests in mice, by analysis of extracts of several fermentation mashes for fungal toxins, and by enzyme immunoassays of the final product, the β-carotene crystals, for four mycotoxins. HPLC analysis, stability tests and microbiological tests showed that the β-carotene obtained by co-fermentation of *Blakeslea trispora* complies with the EU specification for β-carotene (E 160 aii), listed in Directive 95/45/EC, including the proportions of *cis* and *trans* isomers, and is free of mycotoxins or other toxic metabolites and free of genotoxic activity. In a 28-day feeding study in rats with the β-carotene manufactured in the EU no adverse findings were noted at a dose of 5 % in the diet, the highest dose level used. The EU Scientific Committee considered that "β-carotene produced by co-fermentation of *Blakeslea trispora* is equivalent to the chemically synthesized material used as food colorant and is therefore acceptable for use as a coloring agent for foodstuffs."

There are now other industrial productions of β-carotene from *B. trispora* in Russia, Ukraine, and Spain. The process has been developed to yield up to 170 mg of β-carotene/g dry mass or about 17 g/L. *Blakeslea trispora* is now also used for the production of lycopene (see Sect. 3.2.).

3.1.2   Phycomyces Blakesleeanus

Another fungus, *Phycomyces blakesleeanus*, is also a potential source of various chemicals including β-carotene [135]. The carotene content of the wild type grown under standard conditions is modest, about 0.05 mg per g dry mass, but some mutants accumulate up to 10 mg/g. As with *Blakeslea trispora*, sexual stimulation of carotene biosynthesis is essential and can increase yields to 35 mg/g. The most productive strains of *Phycomyces* achieve their full carotenogenic potential on solid substrates or in liquid media without agitation. *Blakeslea trispora* is however more appropriate for production in usual fermentors.

3.1.3   Mucor Circinelloides

Mucor circinelloides wild type is yellow because it accumulates β-carotene as the main carotenoid. The basic features of carotenoid biosynthesis, including photoinduction by blue light [136], are similar in *Phycomyces* and *Mucor. M. circinelloides* is a dimorphic fungus that grows either as yeast cells or in a mycelium form, and research is now focused on yeast-like mutants that could be useful in a biotechnological production.

3.2   Lycopene

Lycopene (Fig. 11) is produced on a large scale by chemical synthesis, and from tomato extracts, in addition to production by fermentation. As with β-carotene, the various preparations differ in the composition of geometric isomers (Table 2). Lycopene is an intermediate in the biosynthesis of all dicyclic carotenoids, including β-carotene. In principle, blocking the cyclization reaction and the cyclase enzyme by

Table 2 Percentage of geometrical isomers in "lycopene" from various sources

Source	(all-*trans*)	(5-*cis*)	(9-*cis*)	(13-*cis*)	Others
Chemical synthesis	>70	<25	<1	<1	<3
Tomato	94–96	3–5	0–1	1	<1
Blakeslea trispora	≥90	(mixed *cis* isomers) 1–5			

mutation or inhibition should lead to the accumulation of lycopene. This strategy is employed for the commercial production of lycopene.

3.2.1 Blakeslea Trispora

A commercial process for lycopene production by *Blakeslea trispora* is now established. Imidazole or pyridine is added to the culture broth to inhibit the enzyme lycopene cyclase. The product, predominantly (all-trans)-lycopene, is formulated into a 20 % or 5 % suspension in sunflower oil, together with α-tocopherol at 1 % of the lycopene level. Also available is an α-tocopherol-containing 10 % or 20 % lycopene cold-water-dispersible (CWD) product. Lycopene oil suspension is intended for use as a food ingredient and in dietary supplements [137]. The proposed level of use for lycopene in food supplements is 20 mg per day.

Approval for the use of lycopene from *B. trispora* was sought under regulation No 258/97/EC of the European Parliament and the Council concerning novel foods and novel food ingredients. The European Food Safety Authority (EFSA) was also asked to evaluate this product for use as a food color. The conclusions were that the lycopene from *B. trispora* is considered to be equivalent to natural dietary lycopene. The toxicity data on lycopene from *B. trispora* and on lycopene from tomatoes do not give indications for concern. In its opinion of January 30, 2008, the EFSA derived a low numerical Acceptable Daily Intake (ADI) of 0.5 mg/kg body weight/day for lycopene from all sources, and the risk assessor also concluded that with the uses and actual use levels presented by the applicants from industry, the intake of lycopene from natural sources and as a food coloring would be expected to remain within this ADI. The main concern is that the proposed use levels of lycopene from *B. trispora* as a food ingredient may result in a substantial increase in the daily intake of lycopene compared to the intakes solely from natural dietary sources. Additionally, the use of lycopene as a health supplement is also becoming very popular. Data from the Framingham Offspring Study – an epidemiological analysis that indicates correlation and not causation – recently reported that increased intakes of lycopene are associated with a reduction in the incidence of cardiovascular disease and coronary heart disease.

3.2.2 Fusarium Sporotrichioides

The fungus *Fusarium sporotrichioides* has been genetically modified to manufacture lycopene from the cheap corn-fiber material, the "leftovers" of making ethanol. By use of sequential, directional cloning of multiple DNA sequences, the isoprenoid pathway of the fungus was redirected toward the synthesis of carotenoids via carotenoid biosynthesis genes introduced from the bacterium *Erwinia uredovora*.

Cultures in laboratory flasks produced 0.5 mg lycopene per g dry mass within 6 days and improvements are predicted.

4 Natural Pigments Produced by Marine-Derived Filamentous Fungi

As their terrestrial counterparts, marine and marine-derived fungi are also able to produce colored compounds. They are therefore able to exhibit bright colors, from yellow to black, mainly belonging to polyketides. Indeed, several papers report that polyketides seem to dominate marine natural products of fungal origin [138]. It is also widespread that the colored molecules identified from terrestrial fungi can often be isolated from the same species living in a marine environment. For instance, catenarin, emodin, erythroglaucin, physcion, questin, and rubrocristin or physcion anthrone are produced by marine derived *Aspergillus* and/or *Eurotium* species, as well as by their terrestrial counterparts. Anyway, fungi from marine ecological niches are today considered as promising novel sources of chemically diverse pigments, and the literature abundantly reports the interest for marine organisms with respect to the production of new molecules and, among them, new pigments [139, 140]. Indeed, many marine ecological niches are still unexplored and it seems plausible that unique features of marine environments can be the inducers of unique substances synthesized by marine or marine-derived microorganisms [141]. The potential of marine derived microorganisms to produce unique and original molecules could therefore come from specific metabolic or genetic adaptation appearing to meet very specific combinations of physico-chemical parameters (high salinity, low O_2 penetration, low temperature, limited light access, and high pressure). Two status lead to particular behaviors and products: either, the challenge of facing unusual living conditions (exogenous fungi), or, the use of specific procedures naturally adapted to the marine niches (for instance marine organisms' fungal endophytes) (i.e., indigenous micromycetes, naturally selected by aquatic environments). For now, the highest diversity of marine fungi seems to be found in tropical regions, mainly in tropical mangroves, which are extensively studied because of their high richness in organic matters [142, 143]. These biotopes seem favorable to the development of a high diversity of heterotrophic microorganisms. Many genera producing pigments have then been isolated either from water, sediments, decaying plants, or from living organisms as invertebrates, plants (endophytes) or algae. Anyway, in unusual biotopes (sometimes extreme), the fungal species with pigmented cell walls (in the spores and/or mycelium), are clearly able to tolerate dehydration-hydration cycles or high solar radiations, better than the moniliaceous fungi, whose cells are devoid of pigments. These aromatic compounds, as melanin, sporopollenin (brown product of oxydative polymerization of β-carotene) or cycloleucomelone (terphenylquinone), often show significant antioxidant activities, and are bound to protect the biological structures, giving them an excellent durability and a high potential for survival in hostile environments [144, 145].

5   The Coding Genes and Biosynthetic Pathways of Pigments in Filamentous Fungi

Biosynthesis of polyketide secondary metabolites has been subjected to more intensive studies among other classes of secondary metabolite pathways in fungi [146]. The majority of genes required for the production of these metabolites are mostly organized in gene clusters, which often are silent or barely expressed under laboratory conditions, making discovery and analysis difficult. Fortunately, the genome sequences of several filamentous fungi are now publicly available and greatly facilitate the establishment of links between genes and metabolites. In the last decade, whole genome sequencing of various fungi has revealed that these microorganisms have immense biosynthetic potential surpassing by far the chemical diversity observed in laboratory culture [147]. For example, the genome of many *Aspergilli* are found to encode for a combined 30 to 80 PKS, non-ribosomal peptide synthetases and polyketide non-ribosomal peptide synthetases hybrids, which far exceed the total number of known polyketides and non-ribosomal peptides [147]. From these, the fungal PKS are of considerable interest due to their interesting enzymology and the polyketide structural diversity [37]. One of the earlier major advances in identification of fungal polyketide secondary metabolite gene clusters is the development of degenerate primed Polymerase Chain Reaction (PCR), based on conserved ketosynthase domain of PKS [148].

5.1   Biosynthesis of Fungal Azaphilone Pigments

Besides the fungal polyketide-derived secondary metabolites, azaphilones are a class of fungal metabolites characterized by a highly oxygenated pyrano-quinone bicyclic core [111, 149–154]. Azaphilone compounds exhibit a wide range of interesting biological activities, such as antimicrobial, antifungal, antiviral, antioxidant, cytotoxic, nematicidal and anti-inflammatory activities [8]. Many of these effects may be explained by the reactions of azaphilones with amino groups, such as those found in amino acids, proteins and nucleic acids. According to Osmanova et al. [155], different azaphilone compounds occur in fungi belonging to 23 genera from 13 families: these azaphilone compounds can be classified into ten different structural groups and the largest group (azaphilones with a lactone ring) includes 68 substances, e.g., the yellow pigment ankaflavin [155]. In total, over 370 fungal azaphilone metabolites have been described in the literature [8]. Considering the variety of azaphilone compounds that occurs, one finds that some fungal species are able to produce a huge variety of diverse types, whereas others are able to produce only one or two different types of azaphilones. An example of the first kind is *Penicillium* spp. of the *Trichocomaceae* family, which produces over 40 azaphilone compounds from five types: azaphilones with a lactone ring; azaphilones with an aliphatic side chain; azaphilones with *o*-orsellinic acid; azaphilones with an ergostane skeleton and *o*-orsellinic acid; and bicyclic spiro-azaphilones. In contrast, *Phomopsis euphorbiae* contains only one type of azaphilone, namely an azaphilone with an aliphatic side

chain. Some of these fungal azaphilone compounds absorb visible light and are colored, namely azaphilone pigments, e.g., ankaflavin (yellow), monascin (yellow), monascorubrin (orange), rubropunctatin (orange), monascorubramine (purple), and rubropunctamine (purple), while others are colorless. Azaphilone pigments are responsible for the bright yellow, red, or green colors of fruiting bodies or mycelia of numerous species of ascomyceteous including genera *Monascus*, *Penicillium*, *Talaromyces* and *Aspergillus*. The color of azaphilone pigments depends on their chemical structure.

Biosynthetically, azaphilone pigments as well as most pigments produced by fungi are polyketide based and involve complex pathways. It is known that the biosynthesis of azaphilone pigments uses both the polyketide pathway and the fatty acid synthesis pathway [8, 156]. In fact, the hexaketide chromophore of azaphilone pigments is derived from the condensation of acetate and malonate by polyketide synthases, while the side chain of these azaphilone pigments arises from a medium-chain fatty acid synthesized via the fatty acid synthetic pathway. The polyketide pathway assembles the main polyketide (hexaketide) chain of the azaphilone pigments from acetic acid (the starter unit) and five malonic acid molecules (the chain extender unit) in a conventional way to generate the hexaketide chromophore structure. The fatty acid synthesis pathway produces a medium-chain fatty acid (octanoic or hexanoic acid) that is then bound to the chromophore by a transesterification reaction in order to form the azaphilone pigment (Fig. 12) [156, 157]. In the literature, biosynthetic pathways are suggested for the following azaphilones: monascorubrin and monascoflavin; mitorubrin and rubropunctatin [149]; ascochitine [150]; ochrephilone [150]; citrinin [150, 156]; monascusones A and B, monascin [158] and sassafrin D [108, 109].

5.2   Biosynthesis of Fungal Hydroxyanthraquinone Pigments

Besides intensively investigated fungal azaphilone pigments, hydroxyanthraquinone compounds have been considered among the most abundant fungal natural products giving color to spores, sclerotia, sexual bodies and other developmental structures [159] as exemplified in case of pigments produced by *Curvularia lunata* [17]. Anthraquinones are a class of chemical compounds of the quinone family that differ in the nature and positions of substituent groups [17]. Anthraquinoid derivatives are derivatives of the basic structure 9,10-anthracenedione or also called 9,10-dioxoanthracene, i.e., a tricyclic aromatic organic compound with formula $C_{14}H_8O_2$ and whose ketone groups are on the central ring. In general, for each anthraquinoid derivative there are eight possible hydrogens that can be substituted. The term "hydroxyanthraquinone" usually refers to derivatives of 9,10-hydroxyanthraquinone, i.e., derivatives of 9,10-anthraquinone where any number n of hydrogen atoms have been replaced by n hydroxyl groups. In this case the number n of hydroxyl group is indicated by a multiplier prefix (mono-, di-, tri-, up to octa-). The hydroxyanthraquinones absorb visible light and are colored, whereas strictly 9,10-anthraquinones are colorless. It appears that the color of the
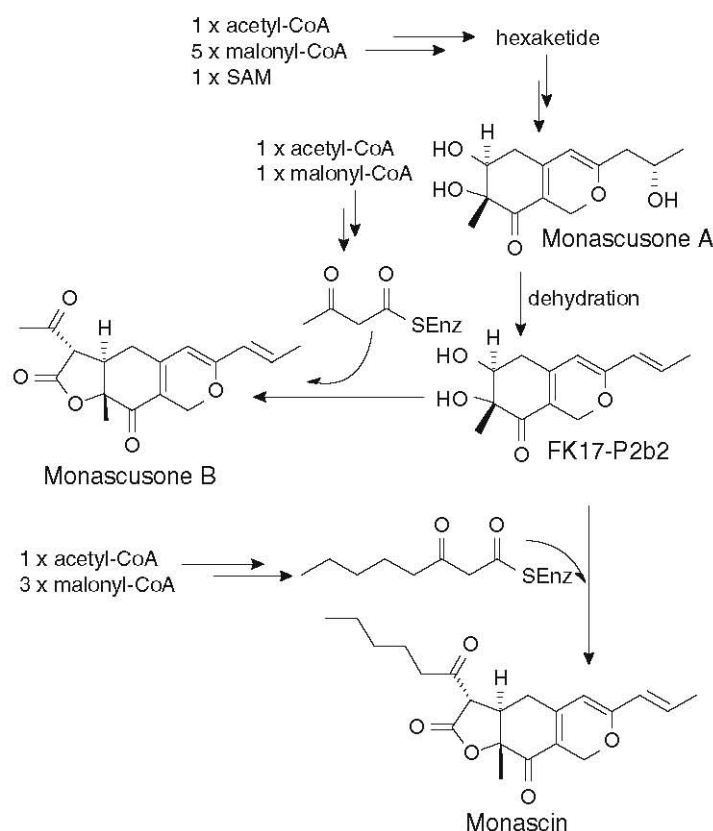
Fig. 12 Possible biosynthetic pathways to azaphilone metabolites, monascusone B and monascin, produced from the fungus *Monascus kaoliang* via fatty acid synthesized pathway proposed by Jongrungruangchok et al. 2004 (Adapted from Gao et al. [8])

hydroxyanthraquinone pigments depends on the position and number of the hydroxyl substituents on the different rings. About 700 anthraquinone derivatives were identified from plants, lichens and fungi; 43 have already been described from fungal cultures [17, 32]. These molecules present a great interest in the field of dyeing molecules: they decline a wide range of nuances in the shades of brown, purple, red, orange to yellow, highly requested in cosmetics, clothes dyeing and foodstuff industries [3, 4, 17, 160]. From their structures, hydroxyanthraquinone pigments, are relatively stable (like the well heat stable hydroxyanthraquinone, carminic acid from insects) and have a superior brightness compared to azo-pigments [3, 4, 17]. Then, they possess good light-fastness properties, which often makes metallization unnecessary, even if hydroxyanthraquinone derivatives can easily form coordination complexes with several cations. In textile industry, hydroxyanthraquinone are, for example, considered as « reactive dyes » as they form a covalent bond with the fibers, usually cotton, although they are used to a small

extent on wool and nylon. Therefore, they made it possible to achieve extremely high washfastness properties by relatively simple dyeing methods. A marked advantage of reactive dyes over direct dyes is that, their chemical structures are often much simpler, their absorption spectra show narrower absorption bands, and the dyeing are brighter [161].

Hydroxyanthraquinone pigments are another interesting set of secondary fungal metabolites and exhibit a wide range of interesting biological activities, such as antioxidant, antimicrobial, antifungal, antiviral, and cytotoxic activities [3, 4, 17, 32, 160]. Numerous hydroxyanthraquinone structures have been described, particularly from members of the *Trichocomaceae* (*Aspergillus* spp., *Emericella* spp., *Paecilomyces* spp., and *Eurotium* spp.), *Pleosporaceae* (Fusarium spp.), and *Nectriaceae* (*Alternaria* spp., *Curvularia* spp., and *Drechslera* spp.) families. Gessler et al. [32] and Hanson et al. [162] explained that anthraquinones are formed via the polyketide pathway and regulated by non reducing polyketide synthases, i.e., multienzymes complexes including acyl carrier protein, transacylase, ketosyntase, malonyl-CoA transacylase, methyltransferases and reductases, ensuring the condensation of acetyl-CoA (starter unit) and malonyl-CoA (extender unit), and producing a unstable β-polyketide chain (containing a free carboxyl group) precursor of different aromatic structures like the hydroxyanthraquinone pigments (Fig. 13).

Fungal hydroxyanthraquinones that are synthesized following this acetate-malonate pathway show a characteristic substitution pattern, i.e., they show substitution on both aromatic rings, and more particularly, at least one hydroxyl group in position R1 and one hydroxyl or methoxyl group in position R8, examples being emodin (yellow), physcion (yellow), dermolutein (yellow), chrysophanol (red), erythroglaucin (red), dermocybin (red), dermorubin (red), tritisporin (reddish brown), cynodontin (bronze) and helminthosporin (maroon), which are produced by *Aspergillus* spp., *Eurotium* spp., *Fusarium* spp., *Trichoderma* spp., *Curvularia lunata* and *Drechslera* spp. According to a practical hydroxyanthraquinone classification [17] based on the position of the functional groups added on the 9,10-anthraquinone backbone, the main hydroxyanthraquinone pigments of fungal origin are classified in the "group A1" (compounds which show substitution on both aromatic rings, and at least two hydroxyl groups in both R1 and R8 positions), or into the "group A2" of hydroxyanthraquinone pigments (compounds which show substitution on both aromatic rings and at least two hydroxyl groups in R1 and R6 positions and one methoxyl group in R8 position). According to this polyketide pathway, the biosynthetic relationships show that the yellow hydroxyanthraquinone pigments (e.g., emodin, physcion and dermolutein) exist in the beginning of the synthesis pathway, whereas the red hydroxyanthraquinone ones, like dermorubin and dermocybin, are more complicated in structure, and occur in the latter part of the biosynthesis pathway [17]. More recently, Bringmann et al. [163] revealed that the pigment chrysophanol is shown to be formed, according to an organism-specific route, by a third folding mode involving a remarkable cyclization of a bicyclic diketo precursor, thus establishing the first example of multiple convergence in polyketide biosynthesis. A complete knowledge about the biosynthetic pathway of hydroxyanthraquinone pigments is not yet available. The genomic approaches of selection of
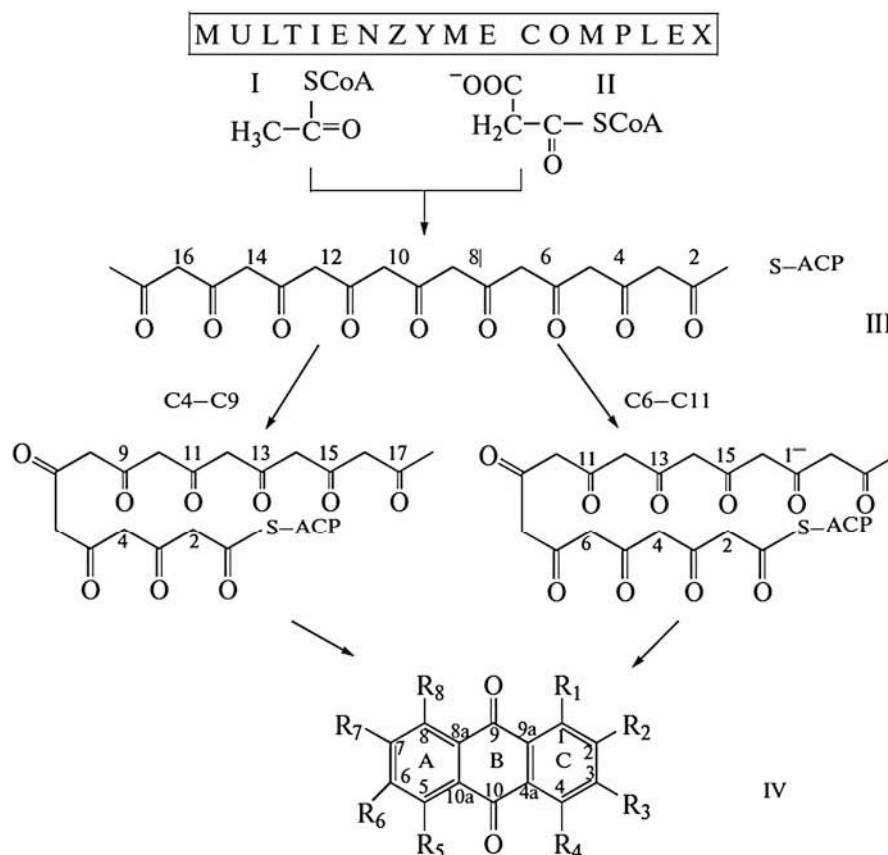
Fig. 13 Scheme of the cyclization of the ß-polyketide chain during the synthesis of anthraquinones in fungi (Based on Gessler et al. [32]). *I, acetyl-CoA; II, malonyl-Coa; III, ß-polyketide chain; IV-anthraquinone (R1-R8 lateral substituents); and ACP, acyl carrier protein*

potential hydroxyanthraquinone pigment producers may not be useful at this point, when none of the fungal hydroxyanthraquinone pigment producers are fully genome sequenced yet. The problem of annoting correct gene sequences should not to be overlooked, especially due to the variation in the domain of the polyketide synthases involved in the biosynthesis of these fungal hydroxyanthraquinone pigments.

5.3   Biosynthesis of Fungal Naphthoquinone Pigments

Naphthoquinone pigments are produced, at least, by a broad range of *Fusarium* species. Few reports have been published on the red naththoquinone, aurofusarin, and bikaverin biosynthetic pathways. The recent sequencing of *Fusarium* genomes has revealed the large number and diversity of secondary metabolic gene clusters

[164, 165]. For example, the genome of *F. fujikuroi* was recently sequenced and partially elucidated, showing that less than half of the putative produced secondary metabolites are known [166]. Examples of discovered secondary metabolites, which could be already linked to the corresponding biosynthetic gene cluster, are the putative carcinogen, fusarin C [167], the histone deacetylase inhibitor, apicidin F [48], the PKS-derived fujikurins [168], the perithecial pigments fusarubins [62], as well as the antiprotozoal mycelial pigment bikaverin [54]. Concerning the bikaverin biosynthesis pathway, the responsible non-reducing PKS-encoding gene PKS4 (FFUJ_06742), later re-named to BIK1 (BIKaverin polyketide synthase), was first described in *F. fujikuroi* by Linnemannstöns et al. [169]. Later, the complete bikaverin gene cluster was characterized by Wiemann et al. [54]. In addition to the PKS-encoding gene, five genes downstream of BIK1 were identified as part of the bikaverin gene cluster. The five genes encode a putative FAD-dependent monooxygenase (BIK2; FFUJ_06743), a putative *O*-methyltransferase (BIK3, FFUJ_06744), a putative NmrA-like transcriptional regulator (BIK4, FFUJ_06745), a putative Zn(II)2Cys6 fungal-type transcription factor (BIK5, FFUJ_06746) and a putative major facilitator superfamily (MFS) transporter (BIK6, FFUJ_06747) [54, 165, 170]. Except for BIK4, gene expression of all BIK genes in *F. fujikuroi* is strictly regulated by nitrogen availability and pH [54–56, 169]. In *F. verticillioides*, the same conditions are conducive for BIK gene cluster expression [171]. Pre-bikaverin (red) has been recognized as the first pathway intermediate and product of the biosynthetic gene BIK1. This intermediate, first described by Ma et al. [172], was identified in *F. fujikuroi* by constitutive overexpression of BIK1 in a DDBIK2/BIK3 double mutant background [54]. The condensation of 8 malonyl-CoA molecules and one acetyl-CoA molecule, catalyzed by the biosynthetic gene BIK1, resulted in the formation of the pre-bikaverin in *F. fujikuroi* (Fig. 14). More recently, the putative biosynthetic pathway for bikaverin synthesis in *F. fujikuroi* was confirmed by Arndt et al. [170]. The structure of a new bikaverin intermediate, oxo-pre-bikaverin (red), was identified by NMR and on the basis of HPLC–HRMS and HPLC–UV measurements. The downregulation of the involved cluster genes was identified by overexpression of BIK2 and BIK3 in the DDBIK2/BIK3 + OE:BIK1 mutant [170]. Neither bikaverin nor the new interme-diate oxo-pre-bikaverin showed cytotoxic effects in Hep G2-Cells [170].

The aurofusarin biosynthetic pathway starts with the condensation of one acetyl-CoA molecule with six malonyl-CoA molecules, which is catalyzed by the aurofusarin polyketide synthase, AUR1 (= PKS12), resulting in the formation of the naphthopyrone YWA1 in *F. graminearum*. The tailoring enzymes modify this compound and the pathway ends with the formation of aurofusarin via the interme-diate red compounds, nor-rubrofusarin and rubrofusarin (Fig. 15) [57, 58]. Moreover, it has been shown that aurofusarin production was the lowest under acidic conditions at pH 4 and 5, while better yields were obtained at pH 6-8 [71].

The recent study by Brown et al. [75] showed that the gene cluster associated with the PKS gene, PGL1, required for the violet perithecial pigment, that accumulates in the walls of sexual fruiting bodies in both *F. verticillioides* and *F. graminearum* [60, 73], consists of the same 6 PKS genes (i.e., PGL1 and the adjacent genes
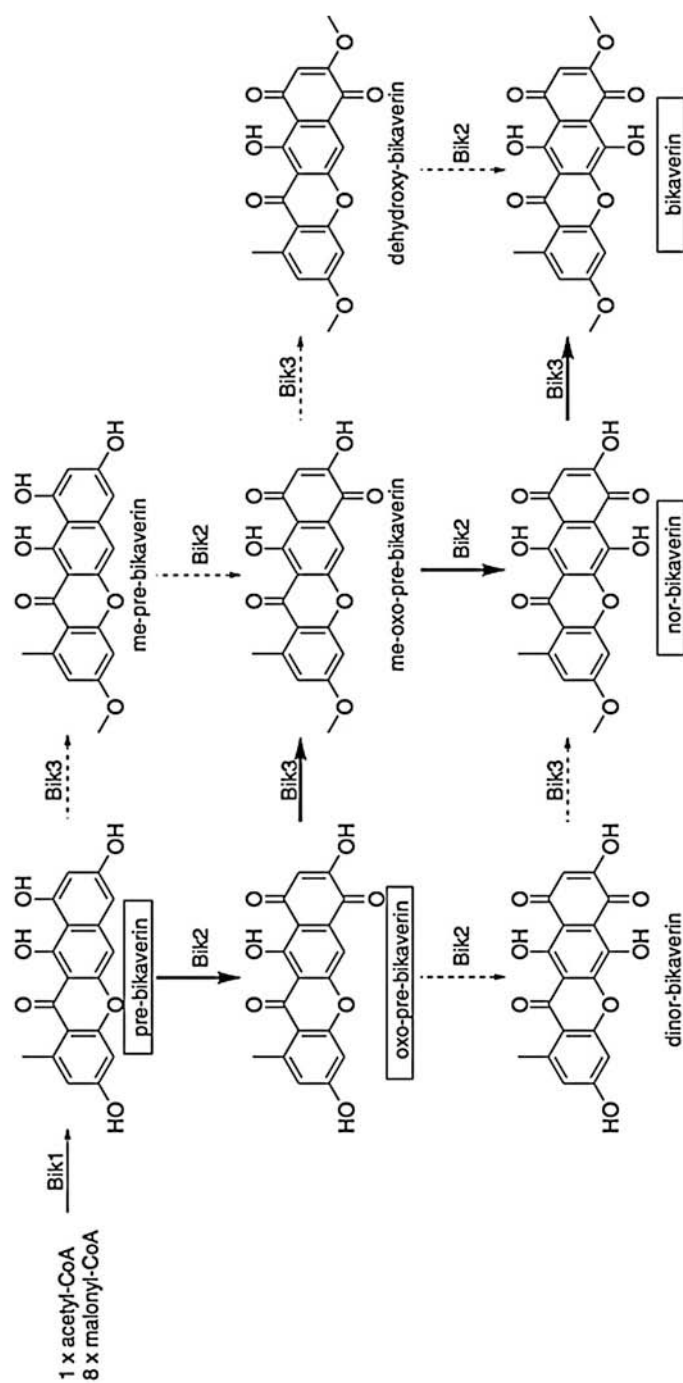
Fig. 14 Models for biosynthesis of naphthoquinone pigment, bikaverin, in *Fusarium fujikuroi* (Based on Arndt et al. [170])
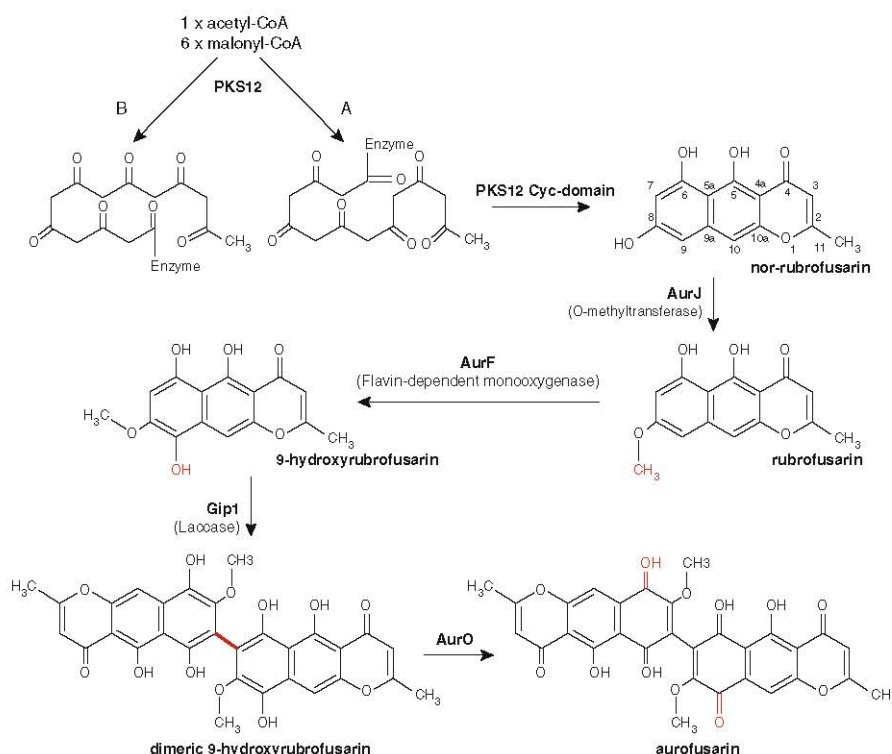
Fig. 15 The biosynthetic pathway for the dimeric naphthoquinone pigment, aurofusarin, in *Fusarium graminearum* reveals a close link between the naphthoquinones and naphthopyrones (Based on Frandsen et al. [57])

PGL2–PGL6). In contrast, the putative gene cluster includes only 3 PKS genes in *F. solani*, and interestingly, the pigment accumulating in the perithecial walls of *F. solani* is red and results from the activity of a different PKS [59]. A homolog of the PGL cluster was also identified in *F. fujikuroi*, and this fungus has been reported to produce particular red fusarubins pigments like 8-*O*-methylfusarubin as the main product [62]. Naphthoquinone pigment related to fusarubin was initially isolated from the fungus *F. solani* [74]. Functional analysis of genes in the cluster demonstrated that the *F. fujikuroi* homologs of PGL1, PGL2 and PGL3 are required for the production of fusarubin pigments, and that the PGL1 homolog is required for perithecial pigmentation [62] (Fig. 16). Although the functional characterization of most of the cluster in *F. fujikuroi* provides strong evidence that fusarubins (red pigment) are precursors of the perithecial pigment (violet hue), the structure of the violet perithecial pigment has yet to be determined [62, 75]. Thus, studies are in progress to determine the relationship between fusarubin pigments and the violet perithecial one. A comparative genomic study of *F. oxysporum*, *F. graminearum* and *F. verticillioides* identified 46 potential secondary metabolite biosynthesis gene clusters, of which 87 % include a polyketide synthase gene [164].
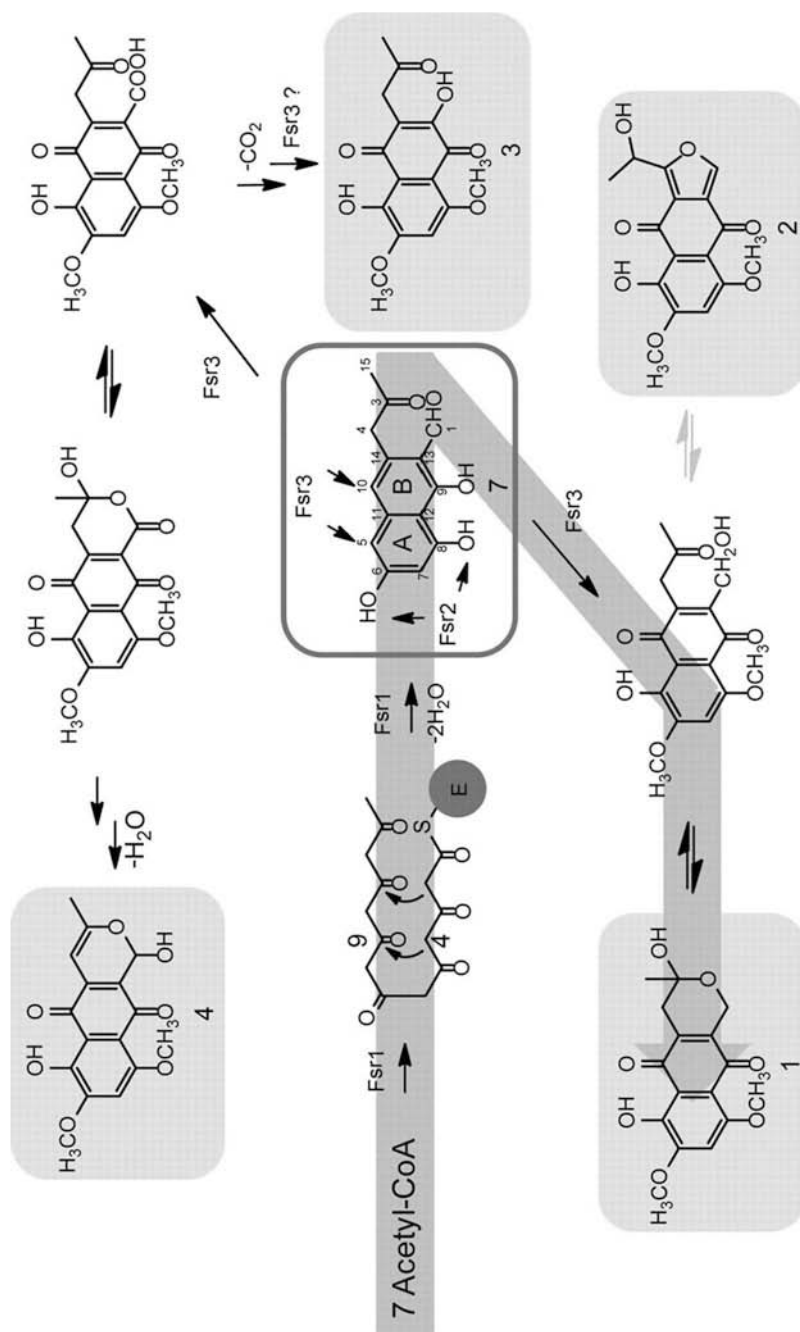
Fig. 16 Biosynthetic pathway of fusarubins in *F. fujikuroi* (Based on Studt et al. [62]). Structures highlighted in *gray* were identified by NMR, mass spectrometry, and UV data in the liquid culture of wild-type *F. fujikuroi*. The aldehyde identified as the first intermediate in this biosynthetic pathway is *boxed*. The *solid dark gray arrow* indicates the main route of naphthoquinone formation. 6-*O*-demethylfusarubinaldehyde (compound 7); 8-*O*-methylfusarubin (compound 1); 8-*O*-methylnectriafurone (compound 2); 8-*O*-methyl-13-hydroxynorjavanicin (compound 3); 8-*O*-methylanhydrofusarubinlactol (compound 4)

The genomic studies by Brown et al. [75], Ma et al. [164], and Hansen et al. [165] shown that the *F. oxysporum* genome contains the BIK gene cluster (i.e., PKS16 (BIK1), PKS17, 18, 19, 20 and 21) involved in the red mycelial bikaverin pigment synthesis, which is consistent with the bikaverin production, reported previously in this fungus [69, 70] and the PGL1 (= PKS3) gene involved in the synthesis of the uncharacterized violet perithecial pigment, although no report on a violet perithecial pigment production by *F. oxysporum* has yet been described in the literature.

5.4   Biosynthesis of Fungal Carotenoids

Carotenoids are the most diverse and widespread pigments found in nature. They are a wide group of isoprenoids synthesized by all photosynthetic organisms and also by some non-photosynthetic bacteria, yeasts, and fungi. The central C40 backbone, made up of eight isoprene units, forms a polyene chain of conjugated double bonds and establishes an extended *pi*-electron system that accounts for its ability to absorb both ultraviolet (UV) radiation and visible light. The number of conjugated double bonds within this basic backbone, as well as cyclic and oxygenic modifications, yields a variety of carotenoids whose colors range from yellow to reddish brown. More than 800 carotenoid structures have been isolated from different natural sources [173].

The biosynthesis of carotenoids has been studied for many years in many organisms including fungi. Hundred of genes involved in the pathway have now been isolated from bacteria, plants, algae, and fungi; and some of these have recently been used in biotechnological research. The productivity of carotenoid can be improved by designing an efficient pathway by selecting genes from different organisms, however neither industry nor consumers did observe a surge or even the appearance of "engineered" carotenoids on the market, thus being a quite disappointing situation after so many millions euros invested, and decades after the beginning of research in this field (i.e., carotenogenic gene cluster from the bacteria *Erwinia herbicola* was described in 1991). This situation seems to change now with the appearance of carotenoids from Genetic Modified Organism (GMO) yeasts such as *Yarrowia lipolytica* (developed by Microbia Inc., now DSM Nutritional Products) or *Kluyveromyces marxianus*, with the incorporation of an algal gene in the latter [174], new strains that try to compete with highly effective production of β-carotene or lycopene using the fungus *Blakeslea trispora*.

Although not essential, carotenoids play significant roles in some groups of fungi, particularly the zygomycetes. β-Carotene is the precursor of trisporoids, a group of compounds involved in the sexual regulation of Mortierellales and Mucorales [175]. Carotenoids are formed in fungi via the mevalonate pathway, which starts at acetyl-CoA and proceeds through mevalonate to isopentenyl-pyrophosphate (IPP), the general precursor of all isoprenoids. Eight molecules of IPP are subsequently condensed to form colorless carotenoids via several dehydrogenation reactions (Fig. 17). Additional modifications bringing color to the molecules then occur through cyclization, hydroxylation, cleavage, etc.
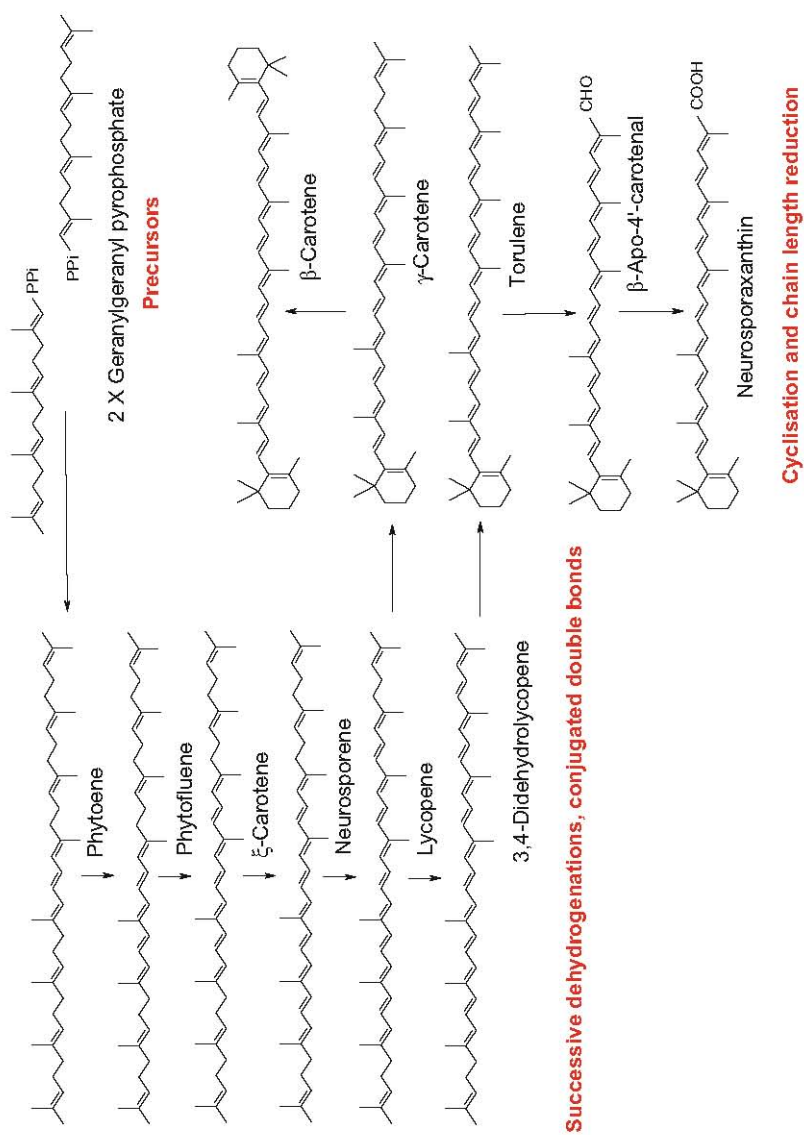
Fig. 17 Biosynthesis of carotenoids in the ascomycete *Gibberella zeae* – anamorph *Fusarium graminearum* (Adapted from Jin et al. [173]))

6   Biotechnological Approaches to Improve Fungal Pigment Production

The past decade was a period of great improvement for pigment productions and the knowledge about the different ways to increase the yields have been greatly extended.

The five major fronts currently ongoing are

- Overall analysis of gene expression i.e., genomics, proteomics, metabolomics. This is to better understand the production pathways and general metabolisms as well as the genes and the molecules involved. More than 100 fungal genomes have already been sequenced, among them several *Aspergillii*, (*A. fumigatus, A. nidulans, A. oryzae, A. niger, A. terreus, A. clavatus*), *Penicillii* (*P. chrysogenum, P. digitatum, Talaromyces marneiffei*) and *Saccharomycetes* (*Saccharomyces cerevisiae, Debaromyces hansenii, Kluyveromyces lactis, Candida albicans...*). Many others are in progress, considering humans, animals or plants pathogens, or strains useful in an industrial context.
- Development of alternative hosts that have already been given GRAS (Generally Recognized as Safe) status by the Food and Drug Agency (FDA) in the USA to be used in food industry (*P. roquefortii, Aspergillus oryzae, A. sojae, A. japonicus, Mortierella vinaceae, M. alpina, Fusarium monoliforme, F. veneratum...*) [176–178].
- Molecular techniques to improve expression and secretion of non-fungal proteins for the biosynthesis of unusual metabolites in filamentous fungi
- Molecular techniques to carry out metabolic engineering to modify and improve particular biosynthetic pathways. Further metabolic engineering to optimize already existing or exogenous biosynthetic pathways.
- Extensive use of Design Of Experiment (DOE) to improve the conditions of pigments production, combining the main physicochemical parameters: temperature, oxygen, carbon, nitrogen and other nutrient sources, pH regulation, light exposure and physiological stage of the fungi.

Today the high productivity of petrochemical-based industry make it difficult for the biotechnologies using microorganisms as platform cell factories to spread. The most important reasons are the higher production costs for the process themselves as well as some drastical changes in basic equipments of technical platforms. Some long ago studied bacteria as *Escherichia coli, Corynebacterium glutamicum, Bacillus subtilis*, or fungi as *Aspergillus niger, Aspergillus oryzae, Penicillium chrysogenum, Saccharomyces cerevisiae* are already operated in industry for enzymes, nutraceuticals or pharmaceuticals.

Indeed, the numerous years of research done on the selected strains led to high robustness and remarkable tolerances against various stresses under industrial conditions, which is the guarantee of stable and efficient productions.

6.1 Genetically Manipulating the Future

Chen and Nielsen [179] published recently a highly interesting paper reviewing the potential future developments for bio-based productions using whole-cell factories. With concrete examples, they reviewed the main tools for metabolic pathways control and strain engineering acceleration and then focused on the development of powerful computational algorithms, omic-based techniques (metabolomic, proteomic, fluxomic, transcriptomic, genomic) combined with modeling refinement, to enable the reduction in development time and, thus, become attractive to industry leaders. Specifically for metabolic optimization and control, a range of computational pathway prediction algorithms has already been generated. Some of them provide a systematic framework for metabolic pathways (re)design by changing existing pathways through introduction of gene knockouts or overexpressions (OptKnock, OptGene, OptForce or FSEOF). Some others can aid in identifying possible pathways from first principles, based on known enzyme reactions (DESHARKY) or based on possible biotransformations of functional groups by known chemistry (BNICE). As an example, their use allowed to identify more than 10,000 possible pathways for the synthesis of 1,4-butane-diol from common central metabolites. Besides predicting a wide range of possible routes, a prioritization scoring algorithm based on binding covalence, chemical similarity, hermos-dynamic favourability and pathway distance, is actually developed to rank the possibilities based on discriminative criteria [180]. Moreover, the recently published web server RetroPath [181] offers a way to retrieve reactions varying in number from the large numbers of reactions found using BNICE to the small numbers of reactions that are presented in the KEGG database. This fully integrative approach will furnish a fast and global analysis contributing to a rapid optimization of the production.

In this field, *Saccharomyces cerevisiae* is one of the most studied strain and it can be considered as an example for production improvement [182–184]. Many of them come from genetic manipulations (insertions, deletions, mutations) directly repressing or promoting the molecules involved in the producing pathways. Unfortunately, the negative aspect of GMOs can be easily concealed, thinking about the actual progress that is changing industrial waste into useful compounds. The use of renewable biomass and processing of industrial waste are becoming more important due to the increasing pollution of the natural environment and growing concerns about climate change. Many efforts have been exerted to extend the carbon substrate range and to improve carbon utilization efficiency or to extend the physicochemical conditions of production by microorganisms. This is clearly illustrated with the example of xylose isomerase overexpression through the engineering of the pentose phosphate pathway [183] or the improvement of succinate production in *S. cerevisiae* [185].

Cost-effective production of dyestuffs could, for instance, come from complete and fast utilization of lignocellulosic biomass. Xylose is the main pentose and second most abundant sugar in lignocellulosic feedstocks. The engineering of the pentose-phosphate pathway enables a rapid xylose utilization and ethanol production. Based on the fact that succinic acid is an important precursor for the synthesis

of high-value-added products, *S. cerevisiae*, as many acidophile fungi, is a valuable platform for acidic productions.

Through a regulation of biotin and urea levels and under optimal supplemental CO_2 conditions in a bioreactor, coupled with engineering strategy, the succinate titer was successfully improved from 6.17 ± 0.34 g/L to 12.97 ± 0.42 g/L at low pH value. Directed mutagenesis has also been developed in the study of Çakar et al. [186], where different culture batches were submitted to oxidative, freezing–thawing, high-temperature, and ethanol stresses before selecting the most tolerant mutants to the environmental conditions generated.

6.2   Cost-Effective Process

As the medium components can represent up to 73 % of the total production cost, by-products of agroindustrial origin have been proposed as low-cost alternative substrates for microbial metabolite production [187]. A wide range of industrial waste, such as fruit pulp, pea pod powder, whey, molasses, corn steep liquor, bran, straw, stem, stalk, leaves, husk, peel, legumes, bagasse, spent grains, mainly composed of cellulose, lignin and residual sugars, can be considered as potential carbon, nitrogen and mineral sources for the microbial production of pigments [188]. One goal is to supply low cost raw material, coupled with the objective of controlling environment pollution. The environmental concern is due to the presence of phenolic and other toxic compounds in these residues (refractory compounds), which may cause deterioration (pollution, saturation) of the environment, when the waste is discarded. These refractory compounds are very difficult to deteriorate in waters and soils, even by microorganisms, and their nitrogen and carbon contents are considered as immobilized. Fungi, as well as actinomycetes, belong to the class of microorganism having the widest enzymatic potential due to a great variety of constitutive and adaptative enzymes, able to recycle the immobilized elements, as key factors of depollution.

6.3   Clean Opportunities for the Future

Clean label is a first response of insistent demand from the consumers for products certified as "natural." Indeed, their use in industrial processes adds more value to the product. However, taking plant culture as an example, the genetic engineering of producing strains does not seem to be the most suitable way to progress towards natural products synthesized from sustainable process. Some European certification organizations as Ecocert, QualiFrance, Certipaq-Aclave, SGS-ICS, and Agrocert Certisud (for organic certifications), have now a strong impact on the market. This gives the possibility to manufacturers to sell the dyes at a higher price compared to their conventional counterparts. These labels provide a composition based on at least 95 % of natural or organic ingredients and they influence the production process that should be more respectful towards the environment.

Thus, many studies deal with searching the paths to naturally foster the productions of suitable compounds, acting on physicochemical parameters of the growths with wild strains. This needs, in the following years, to increase knowledge about the microbial and metabolic biodiversity naturally occurring in our environments. Reducing or suppressing heavy extraction process would also be a beneficial way for several reasons: first, it alleviates the need to use large amount of solvents, whilst from an economic point of view, it reduces production time and eliminates the cost of an extraction process, thus making dye production more economically and friendly viable.

7   Methods for Extraction and Purification of the Colored Compounds

Filamentous fungi produce a mixture of various metabolites such as pigments, fatty acids, proteins, and other cellular metabolites. Thus, the extraction and isolation of the pigmented molecules of interest are necessary steps before proceeding to any further utilization of these metabolites in commercial products. Pigments can be store within the biomass, excreted in the fermentation broth or both, suggesting that extraction methods need to be developed specifically regarding where the pigments are located.

7.1   Pretreatments of Biomass Before Extraction

Fungal pigments can be extracted from both biomass and fermentative broth. Proceeding to pretreatments of the fungal material before applying the extraction protocol itself can improve the overall extraction effectiveness. The biomass is generally separated from the broth either by centrifugation or Büchner filtration [42]. The biomass and/or the broth are then frozen-dried. It renders the biomass easier to grind and allows longer conservation without losing bioactivity of the pigments. Grinding is a common pre-treatment in extraction techniques and results in smaller biomass particles. The smaller the size of every particles, the better the diffusion of the solvent. Consequently, the overall extraction efficiency increases [189]. However, extended pre-treatment of the biomass, could result in denaturation and/or degradation of the pigmented molecules. Shearing forces, high temperatures and pressures when proceeding to grinding or drying processes, can drastically affect the final chemical profile of the pigments. Oxidation and enzyme browning are the main risks encountered in physical pre-treatment of biomass due to prolonged contact with oxygen, and the release of hydrolases from the cells after crushing and grinding [190]. Moreover, during physical treatment, coloured molecules can also react with other cell components, resulting in reducing their final extractability and bioactivity. Thus, the conditions of the pre-treatment of biomass are rather crucial and should remain relatively quick and gentle to ensure the efficiency of the further chemical extraction, as well as the maintenance of the bioactivity of the biomolecules.

7.2 Conventional Extraction Methods Using Organic Solvents

Despite progress done in extraction methods, fungal pigmented molecules are generally extracted by extended contact with one or a mixture of organic solvents, such as in maceration or Soxhlet techniques. The main organic solvents in use are ethanol, methanol, acetone, ethyl acetate, and hexane [42, 191–193]. The efficiency of such process relies on the polarity of the compound to extract. Velmurugan et al. [42] have used a mixture of ethanol and water (1:1, v/v) on the unfiltered fermentation broth of *Isaria farinosa* (formerly *Paecilomyces farinosa*) to extract extracellular red pigments, before separating the filtrate and processing to further analysis. Similarly, extracellular pigments from *Trichoderma harzianium* were extracted with ethyl acetate [194]. These methods can be optimized by adapting the side parameters, i.e., the extraction time, the pre-treatment applied, the pH, the temperature or pressure used, as well as the presence of other salts (NaCl, etc). The afore mentioned organic solvents are still widely used for pigments extraction due to their relative efficiency, ease of use, and their easy application at industrial scale. Only one industrial process using organic solvent extraction of fungal hydroxyan-thraquinones has been reported and patented by the company, ASCOLOR BIO-TECH (now NATURAL RED™), for the production of the pigment Arpink Red™. The process involves a filtration and centrifugation pre-treatment for the removal of the biomass. The pH of the supernatant is then dropped to 2.5–3.0 to precipitate the hydroxyanthraquinones. The dissolution of the precipitate in ethanol is performed, followed by the evaporation of the alcoholic solvent. The fungal pigments are then recovered as a deep-red powder, ready to be used for further application [16]. However, the numerous extraction cycles, the relatively low extraction efficiencies and selectivity, as well as the large volumes of solvent and water used, generally render such methods unsustainable and costly for industrial scale up.

7.3 Alternative Greener Extraction Processes

Over the last two decades and with the emergence of the concept of green chemistry, tremendous progress has been made regarding extraction and separation technolo-gies. The trend is to develop extraction techniques involving cheap and environmental-friendly solvents that can be used under milder conditions with good final efficiency in quicker processes (ideally automatized). Most of them have been tested on fungal material for the extraction of all sorts of biomolecules, such as fatty acids, polyphenols and carotenoids [185, 195–198].

7.3.1 Microwave-Assisted Extraction

Microwave-assisted extractions (MAE) rely on the use of microwave energy to enhance the partitioning of compounds of interest from the biomass matrix into the solvent. The applications of microwaves allow a synergetic effect of both heat and mass transfers occurring in the same direction (from the inside to the outside), while in conventional extraction systems, they are happening in opposite directions

[199]. The efficiency of MAE for extraction of molecules is directly linked to the polarity of the solvent, its ionic conduction and its ability to absorb microwave energy [200]. Thus, this method is more suitable for the extraction of compounds showing medium to high polarity [201]. This explains why MAE has been successfully applied on polyphenolic compound extractions such as phenolic acids [202], curcumin [203], and saffron polyphenols [204]. Hemwimol et al. [205] concluded that coupling MAE with appropriate solvent increased the yield of anthraquinones extracted from *Morinda citrifolia* as well as reduced significantly the extraction time, when compared to Soxhlet extraction or maceration [205]. In Hemwimol work, MAE took 15 min to be completed, while 4 h and 3 days were needed with Soxhlet and maceration techniques, respectively, to reach the same efficiency [205]. Thus, MAE is a very promising technique that has been further optimized for limiting side oxidations by applying nitrogen-controlled atmosphere instead of air, or using MAE under vacuum conditions to protect heat sensitive molecules. The major advantages of MAE are the significantly reduced extraction time, and solvent volumes requirements, as well as the improved extraction efficiency, making this technique interesting for biotechnological applications. However, its possible usages at industrial scales remain limited due to heterogeneous heat propagation at bigger scale, along with cost and maintenance of the material.

7.3.2   Ultrasound-Assisted Extraction

Ultrasound-assisted extraction (UAE) is one of the more straightforward method to use as it requires very limited apparatus and is low-cost [201]. UAE involves the application of acoustic waves in the KHz range (>20 kHz) propagated in the heated extraction solvent [189]. Propagation of the sound waves induces successive expansion and compression cycles of air bubbles within the solvent, resulting in their collapsing. This physical phenomenon disrupts cells membrane, facilitating both the release of molecules of interest and diffusion of the solvent within the sample matrix. UAE has been widely used for enhancing the extraction of bioactive pigmented phenolic molecules from various parts of plants, and was also showed to induce less degradation on phenolic molecules compared to other more intrusive processes such as solid-liquid solvent extraction or MAE. Barreara-Vasquez et al. [206] observed better and quicker extraction of anthraquinones from plant material when performing UAE compared to the conventional Soxhelt method. However, the recurrent issue encountered in UAE is the nonuniformity of the propagation of both the soundwaves and the heat [199]. This heterogeneity is due to the different phases present in the system, which have different heat and mass transfer capacities as well as different compressibilities. These variations can negatively impact on the overall effectiveness of this extraction technique. Nevertheless, UAE has showed good potential for extraction application and is widely used as paired step with MAE and solvent extraction [205, 206].

7.3.3   Pressurized Fluid Extraction

Pressurized fluid extractions (PLE) involve the use of both high pressure and elevated temperatures on the system sample/solvent, where the solvent is near to

its critical stage under the conditions of extraction. The high pressures (100–150 bars) allow the solvent to remain liquid despite the elevation of the temperatures [201]. Additionally, higher temperatures (in the range of 100 °C) enhance the extraction capacities and diffusion of the solvent. Thus, the main advantage of PLE is a significant reduction of solvent volumes used and a shortening of extraction cycles. A wide variety of techniques have been derived from PLE and are still based on the same principles of reaching solvent near critical stage. PLE, also going by its trade name, ASE (accelerated solvent extraction) patented by DIONEX, is a relatively recent method and have promising potentialities. Water becomes a very valuable solvent under high pressure and temperature, and the technique is then called subcritical water extraction (SWE). The water is heated up to 200 °C where it chemical properties change and become similar to those characteristic of organic solvents. CO_2 can also be used as an efficient extraction solvent when compressed to its supercritical state, and is classified amongst the super- and subcritical fluid extraction (SFE) techniques. The advantages of this latter are the absence of light and oxygen during the extraction cycles, which prevent any oxidation and other physical degradation due to the elevated pressure and temperatures. Such process showed great potential regarding preservation of bioactivities of oxygen sensitive chemicals like polyphenols and other pigmented molecules. Borges et al. [207] have concluded that the use of supercritical CO_2 for the extraction of red carminic acid from cochineal insects showed improved yields compared to conventional methods. Moreover, it was said to increase the extraction selectivity for the pigmented hydroxyanthraquinones, and reduce the side extraction of proteins [207]. Such selectivity for pigmented hydroxyanthraquinone is highly desirable as some proteins are of concern regarding allergenic reactions in carminic acid containing products [17]. Nevertheless, few studies have been done on using this technique on fungal biomass for pigments extraction, despite the promising results observed on polyphenols from plants and microalgae [208], as well as hydroxyanthraquinones from insects. PLE allow working quicker, under milder extraction conditions, using more environmental friendly solvent (water, ethanol, methanol or mixtures) at relatively low volumes. However, the complexity of the apparatus needed, as well as the utilisation of high pressure and temperature, remain difficult and costly for any industrial applications yet.

7.3.4 Enzyme-Assisted Extraction

Enzyme-assisted extraction (EAE) is a relatively recent method that was shown as a promising new extraction technique, using enzymatic specific catalytic activities in aqueous solutions [209]. The main types of enzymes used are pectinases, cellulases and hemicellulases, which when applied to the sample matrix, act on the cells membranes components and hydrolyze them. This results in increasing the membrane permeability to solvents and thus, in improving yields of extraction of metabolites. Enzyme catalytic activity can also be used for improving solubility of metabolites in the extraction solvent. Hynninen et al. [191] have used glucosidase for the isolation of hydroxyanthraquinone aglycones from the fungus *Dermocybe sanguinea*. Such enzyme hydrolizes *O*-glycosyl linkages of glucoside and aglycone

anthraquinones (mainly emodin- and dermocybin-1-β-ᴅ-glucopyranoside), enhancing their solubility in organic solvent (acetone). Indeed, after enzymatic treatment, 94 % of the total pigments were yielded in the organic fraction. The different enzymes can be obtained from bacteria, fungi, yeasts or plants, and most of them are commercially available. The major advantages of EAE are the reduction in solvent volumes as well as the shortening of extraction times and a better preservation of the bioactivity of the product due to the usage of milder conditions. The next trend regarding EAE is the engineering of the enzymes for developing tailor-made extraction methods. However, the cost of the enzyme, the difficulty to recycle them, and the loss of catalytic efficiency with larger raw material volumes currently limit industrial application of such method.

7.3.5 Ionic Liquid–Assisted Extraction

Ionic liquids (IL) have been described as novel type of solvents with promising potential for the development of new extraction methods offering milder, greener, and more efficient processes. Over the last decade, they have been widely applied in various fields of chemistry, and they showed great potential in separation technologies. IL are organic molten salts, showing boiling points below 100°C. They are generally made of a bulky organic cation, such as alkyl- ammonium or phosphonium, dialkylimidazolium, N-alkyl-pyrrolidinium or pyridinium, paired with an organic or inorganic anion, such as bromide, chloride, tetrafluoroborate or hexafluorophosphate for the main in use [198, 210]. Low vapour pressure and non-volatile nature, non-flammability, thermal and chemical stability, high solubility, as well as recyclability are some of the unique properties of IL, which classify them as green solvents. Moreover, their properties are tuneable and depend on the couple cation/anion, and can be then optimized accordingly to the molecule to extract. Various types of natural compounds were successfully extracted with IL such as polyphenols [196] or dyes from chilli powder [211]. More recently, focus have been done on using IL coupled with other techniques such as aqueous two-phases system (ATPS), microwave assisted extraction and ultrasound assisted extraction, which was shown to improve the extraction capacities of the IL themselves. Yan et al. [133] concluded that the extraction of hydroxyanthraquinone pigments (e.g., aloe-emodin, rhein, emodin, chrysophanol and physcion) from plant materials yielded better results when performing an ultrasound emulsification of IL for microextraction. Indeed, the dispersion of water insoluble droplets within the sample mixture increases the surface contact of the IL with the hydroxyanthraquinones, which are trapped in the IL- droplets and, later on, separated from the aqueous phase by centrifugation. Similarly, Tan et al. [212] have successfully isolated aloe anthraquinones using microemulsion of IL. Recently, similar techniques coupling IL with ultrasound, microemulsion or ATPS were performed with good results on filamentous fungi biomass and/or culture broth. Ventura et al. [213] succeeded in isolated anthraquinones from 14 days old fermentation broth of *Penicillium purpurogenum*. Similarly, Shen et al. [214] obtained good results when using hydrophobic IL microemulsion extraction of red *Monascus*-pigments from 7 days old fermentative broth.

7.4   Purification Methods

The different extraction methods previously described present more or less selectivity for the pigmented molecules of interest. The desire to use these pigments for cosmetic and food applications requires further purification steps in order for them to be allowed by the different food and drugs regulation agencies, such as the FDA (USA) or the EFSA (Europe). Some pigments – producing species are known to have a paired biosynthesis pathways of pigments and mycotoxins, such as in *Monascus* species where the hepato-nephrotoxic compounds, citrinin, is produced along with the azaphilone pigments [215]. The more commonly used purification techniques are based on solid phase extraction such as column chromatography, cation exchange, and thin layer chromatography (TLC). Column chromatography was used for the generation of sequential elution of pigments that were previously extracted, and is usually followed by a TLC to separate the different compounds present in the pigmented extract. Red pigments from the yeast *Rhodotorula glutinis* were efficiently fractionated on a magnesium oxide-Hyflo Super cell using acetic acid:ethyl ether (1:2, w/w) as elution solvent mixture [216]. The following TLC used petroleum ether:acetone (80:20, v/v) as the mobile phase for the separation of the different chemical species. Three types of β-carotenoid compounds, β-carotene, torulene and torularhodin, respectively, were purified. A sequential silica gel column chromatography using a gradient elution of methylene chloride:methanol (100:0 to 50:50, v/v), followed by TCL using petroleum ether:acetone (3:2, v/v) was performed on pigmented fractions from the ascomycete fungus *Shiraia bambusicola* and yielded 15.5 mg, 42.3 mg, 21.5 mg and 19.6 mg of perylenequinone derivatives hypocrellin A, B, C and D respectively, which are toxic dark red pigments showing interesting anti-cancer activity [217]. Adsorption column chromatography using neutral alumina washed with hexane allowed the elution of β-carotene from the filamentous fungus, *Mucor azygosporus* [218]. The pigment was recovered at 94 % in the first fraction. Silica column showed promising potential as it can perform high purification but need to be paired with another separating steps such as TLC. The main disadvantages of this method are the large volumes of solvents required and the time it requires [219]. TLC show limitations as an effective technique due to the large number of plates that are needed for obtaining enough purified pigment in the end. Moreover, TLC imposes an additional step: scrapping the silica gel to recover the purified pigment, which tend to reduce the overall pigment extraction yield. Cation exchange column is another method, which showed good yields for the purification of pigments from *Fusarium graminearum*, with methanol and methylene chloride (50:50, v/v) used as the mobile phase. Styrene-based strong sulfonic acid columns showed the best purification yields. Then, the main advantages of resins chromatography are the use of reduced amounts of solvent, the quick purification cycles (app. 30 min), and the general good efficiency of the process. However, due to the nature of the resin, isomeric transformation of the pigments may occur.

Thus, there are several options available for developing efficient and selective extraction and purification processes of fungal pigments. However, the limiting steps remain linked to the scale-up to industrial applications, associated with the desire of

using greener methods. Although great efforts have been done to develop more sustainable extraction protocols (PFE, MAE, UAE, etc), the purification steps still involve chlorine-based solvent (methylene chloride) and other solvents (acetonitrile, acetic acid, petroleum ether, ethyl ether and hexane), classified as "usable" or "undesirable" according to the guide for solvent selection, produced by Ghandi [210], for the design of green extraction method. Then, improvements of these techniques are highly desirable to keep developing more environmental-friendly processes with economic potentialities.

8 Industrial-Scale Applications of Fungal Pigments and Perspectives

8.1 Applications as Natural Food Colorants and Dietary Supplements

Since the food company DSM^{TM} has gained the EU approval for food use of fungal originated β-carotene, produced from the fermentation of *Blakeslea trispora* in 2000, industrial interest on fungal metabolites has been revived, and new investigations have been ongoing to develop cost effective fungal colorants ever since [3, 215]. Moreover, the conclusions of the Southampton study, leading to the obligation for food companies to apply a label mentioning that "*azo-dyes (i.e., synthetic dyes) may have an advert effect on activity and attention in children*" have driven all the interests towards bacterial and fungal based pigments for food use. Moreover, the value of international coloring market was estimated to $1.5 billion in 2007 according to Leatherhead Food International (LFI), with the natural colorant (from all natural sources, plants and microbial origins) representing 31 % of the global market, and the synthetic and nature-identical pigments (i. e. chemically similar to natural pigment but synthetically produced) encountering for 40 % and 29 %, respectively [220]. The growth of the natural pigment market has been blooming from 2007 to 2011, and is now overtaking the synthetical color market for the first time, with 39 % of the global food color market against 37 % for the synthetic food dyes [221]. Such market evolutions render fungal pigments an economically valuable niche with great potentialities, where further investigations are needed.

Nowadays, four fungal strains are used for the production of yellow to red pigments at industrial scales: *Blakeslea trispora*, *Penicillium oxalicum*, *Monascus* sp., and *Ashbya gossypi* (Table 3). The β-carotene from *Blakeslea trispora* has been authorized in the food market and classified under the same E number that of plants originated β-carotene, i.e., E160a(ii) [222]. Industries based in Russia, Ukraine and Spain are now producing this pigment with a yield of up to 17 g per liter of culture [223]. Similarly, lycopene from *Blakeslea trispora*, produced by the company Vitatene S.A., has been approved as food colorant by the European Commission in 2006, and labelled with the E-number 160d(iii) [224]. It is used to color non-alcoholic flavored drinks, fine bakeries, dairy-based product, mustards and condiments, as well as soups and sauces. The recommended use level for lycopene

Table 3 Fungal production of pigments (already in use as natural food colorants or with high potential in this field)

Molecule	Colour	Microorganism	Status[a]
Ankaflavin (azaphilone)	Yellow	*Monascus* sp. (fungus)	IP
Anthraquinones	Red and other hues	*Penicillium oxalicum* (+ other fungi)	IP
Azaphilones	Red	*Talaromyces atroroseus* (fungus)	DS
Azaphilones	Red	*Penicillium purpurogenum* (fungus)	DS
Azulenes	Blue	*Lactarius* sp. (fungus)	RP
β-carotene	Yellow-orange	*Blakeslea trispora* (fungus)	IP
β-carotene	Yellow-orange	*Fusarium sporotrichioides* (fungus)	RP
β-carotene	Yellow-orange	*Mucor circinelloides* (fungus)	DS
β-carotene	Yellow-orange	*Neurospora crassa* (fungus)	RP
β-carotene	Yellow-orange	*Phycomyces blakesleeanus* (fungus)	RP
Lycopene	Red	*Blakeslea trispora* (fungus)	IP
Lycopene	Red	*Fusarium sporotrichioides* (fungus)	RP
Monascorubramin (azaphilone)	Red	*Monascus* sp. (fungus)	IP
Naphthoquinones	Deep blood-red	*Cordyceps unilateralis* (fungus)	RP
Riboflavin	Yellow	*Ashbya gossypi* (fungus)	IP
Rubropunctatin (azaphilone)	Orange	*Monascus* sp. (fungus)	IP
Unknown	Red	*Paecilomyces sinclairii* (fungus)	RP

[a]Industrial production (IP), development stage (DS), research project (RP)

from *B. trispora* is 15 to 30 mg/kg depending on the food matrix. Additionally, fungal lycopene has been recognized to be nutritionally equivalent to plant-based lycopene (tomatoes, watermelons, etc) according to an EFSA reports in 2008 [225]. Interestingly, it also has been decided that both β-carotene and lycopene from the fungus *B. trispora* should be only labelled as "β-carotene" and "lycopene," respectively, with no further details on the plant, fungal or synthetic origin.

Riboflavin (vitamin B2 – E101) is a water-soluble vitamin, also commonly used as a yellow pigment, industrially produced by fermentation of a fungal strain, *Ashbya gossypi* [215]. Another strain, *Eremothecium ashbyii*, showed industrial potentialities regarding yields; however, its genetic instability renders it not suitable for cost effective industrial production [226]. Riboflavin is commonly used in cereal-based products, juices and yogurts; however the concentration to be used is limited due to bitterness aftertaste [227]. In the course of the 51st meeting of the Joint FAO/WHO Expert Committee on Food Additive (JECFA) in 1999 (Geneva), ribo-flavin produced by genetically modified bacterial strain of *Bacillus subtilis*, built to overexpress the riboflavin biosynthetic pathway, has been concluded as safe and

nutritionally equivalent as synthetic vitamin B2, and was included in the same ADI group of 0–0.5 mg/kg of body weight for synthetic riboflavin.

Monascus-like pigments, in particular ankaflavin, have been used to color meat, salami, sausages [228, 229], and fish (e.g., to enhance pink coloration in salmon), as well as coloring sauces (ketchup). Food applications of *Monascus*-like pigments have been widely ongoing for centuries in Asia, but are still not allowed in the EU and the United States [215]. Numerous companies, such as Nestlé (Switzerland) (Patent US4145254 A) and The Quaker Oats (USA) (Patent US4418080 and US4418081) have already filled patents for the production and use of *Monascus* red pigments in food applications, and they have been accepted in 1979 and 1983, respectively.

Similarly, red anthraquinones produced by *Penicillium oxalicum* and sold by the Czech company, ASCOLOR BIOTECH (now NATURAL RED™), have been used in meat, dairy and confectionery products, as well as in alcoholic and non-alcoholic drinks, with recommended dosage levels of 100 mg/kg, 150 mg/kg, 300 mg/kg, 200 mg and 100 mg/kg respectively.

Interestingly, the abovementioned fungal pigments do not only display coloring properties, but demonstrate a wide palette of bioactivities from carcinogenesis-preventing roles due to antioxidative, anti-free radical and apoptosis inducing activities, especially regarding lycopene and β-carotene, to antimicrobial and anti-fungal activities. Similarly, *Monascus*-like red pigments (monascorubramin and rubropunctatin) were demonstrated to enhance the organoleptic properties of food (taste and consistency), as well as lowering low density lipoproteins (LDL) choles-terol and increasing high density lipoproteins (HDL) cholesterol due to the presence of a statin-like molecule (monacoline K), reducing salt intake, and preventing gastric and digestive disorder [227, 230]. Furthermore, food supplements of Red Yeast Rice, i.e., *Monascus* sp. pigments, like "SuperSmart" food supplement are sold specifi-cally for the benefits of monacoline K on cholesterol. Interestingly, EU regulations forbid any usage of *Monascus* sp. pigments in food or textile industries, however, these food supplements can be relatively easily found in parapharmacies.

In cheesemaking industries, *Fusarium domesticum* is used as the inoculating strain for the production of Saint Nectaire and Reblochon cheeses. The organoleptic enhancing abilities of this fungus have been known for a long time, as it was called "Anticollanti" before its clear characterization, and was used for its ability to favorize the drying of the cheese surface, and consequently reducing its stickiness [231]. Thus, fungal pigments are unique by showing very interesting and complex profiles: along with being used for their coloring properties, they also could be used as organoleptic properties enhancer, as well as physiologically and health valuable diet supplements.

Numerous other pigments from various fungal strains are currently under inves-tigation: three other pigments (two red azaphilones from *Talaromyces atroroseus* and *Penicillium purpurogenum*, and one β-carotene from *Mucor circinelloides*) are at development stages (Table 3). The next trend now for research projects are focused on developing mycotoxin-free deep red hues producers. Some species such as

Fusarium, *Cordyceps* and *Paecilomyces* sp. are more specifically considered as promising strains (Table 3).

8.2 Applications in Pharmaceutical and Cosmetic Products

Natural bioactive molecules with clinical properties have been widely investigated by pharmaceutical companies, and many microbial secondary metabolites have been used over since as antibiotics, diuretic, anticancer, estrogenic or immune-modulatory compounds. Some examples of pigmented metabolites from fungal strains are under further clinical studies at laboratory scale, such as norsolorinic acid produced from *Aspergillus nidulans*, which is characterised by its anti-proliferative activity in human breast adenocarcinoma MCF-7 cells [232]. Similarly, red anthraquinones molecules, shiraiarin and hypocrellin D, synthesized by the fungal strain *Shriaia bambusicola*, were found to significantly inhibit the growth of tumor cell lines [217, 233], and thus, are potential candidate for future antitumor and anticancer drugs. However, further clinical and toxicity tests are required before considering any commercialisation of these fungal pigments as drugs.

Cosmetic industries are highly interested in inserting biomolecules and natural ingredients for their products. Indeed, the public demands of "*green label*," and organic cosmetic counts for a growing part of the global cosmetic market, and companies are really looking for new type of natural ingredient to use as alternative to conventional synthetic compounds. Some firms are already producing and selling fungal pigments as natural colorant for cosmetic, such as NINGXIA R.D. (natural pigment co. of ningxia light industry design institute), which is manufacturing red pigments from *Monascus* sp. specifically for cosmetic purposes. Another Asian company, KANEBO Ltd (Japan), has been granted a patent for using *Monascus*-like pigments in lipsticks. LEVER HINDUSTAN Ltd (Indian branch of Unilever), UNILEVER and l'OREAL S.A had their patents accepted in 2001, 2002 and 2004 for using *Monascus*-like pigments in skin conditioning and in skincare products giving tan coloration. It is worth noticing that many companies are selling those pigments on the Asian market, as the spread of fungal pigments containing cosmetic products is still limited in Europe and the United State. Despite the facts that numerous patents have been filled and accepted, the actual launches on the market remain highly restricted by allowances from Institutes for Public Health Surveillance (leads by the ANSES in Europe). Indeed, before authorizing any products, clinical tests looking at allergic, dermatologic and endocrine responses are required. Moreover, in the case of cosmetic products, both the ingredient to be added –even more if used for specific bioactivity-, i.e., fungal pigment, and the final cosmetic product would have to be authorized by the appropriate authority. These regulations elements are one of the major points slowing down the use of fungal pigments. Thus, so far, only *Monascus*-like pigments have found actual economic applications; however it does pave the way of dyes and bioingredients market to new types of fungal strains and pigments, such as colorants extracted from *Penicillium*, *Fusarium*, *Emericella*, and *Cordyceps* for instance.

8.3   Applications in Textiles and Painting Industries

As regulations on textiles and material colorants are not as strict as in foodstuff, many more microorganism-originated pigments are in use in textiles, clothing, paints, and polymers. Bacterial pigments, prodigiosin (red) and violacein (purple) from *Serratia marcescens* and *Chromobacterium violaceum*, respectively, were used to dye various types of fabrics like acrylic fiber, silk, cotton, polyester, and polyester microfiber [234]. Similarly, fungal colorants are promising, as these pigments display high colorfastness as well as high staining capability, suggesting that a minimal amount of pigment is required for proper standard staining results. Such properties underpin interesting cost-effectiveness. Moreover, synthetic textile colorants are reported as potential carcinogen due to their content in dioxins, such as polychlorinated dibenzo-p-dioxins (PCCD) and polychlorinated dibenzofurans (PCDF) [235], which strengthen the trend to develop eco-friendly and non-toxic colorant, especially regarding infant and children clothes and toys. Then, economically speaking, in 2008, Mapari et al. [19] have patented the used of *Monascus*-like pigments from various strains of nonproducing mycotoxins *Penicillium* (19 *Penicillium* strains) on textile, cotton, wool, silk, leather, paper, paint, polymer, plastic, inks, and tablet. Additionally, red anthraquinones from *Fusarium oxysporum* and from *Dermocybe sanguinea*, as well as yellow pigments from *Trichoderma virens*, bright olive pigments from *Alternaria alternate*, and melanin pigments from *Curvularia lunata* were demonstrated as potential dye on cellulosic matrix, such as wool and silk with good colorfastness and rub fastness [236–238]. Red dyeing capabilities of other fungal strains, such as *Isaria* spp., *Emericella* spp., *Penicillium* spp., *Monascus* spp., and *Fusarium* spp. were concluded as good natural alternate for leather dyeing, and would be less polluting compared to conventional dyes [42]. Moreover, staining fabrics (silk) with yellow pigments from *Thermocymes* sp. and *Trichoderma* sp. were shown to have antibacterial and/or antifungal activities, and were concluded to be good candidates for producing valuable textiles for hospital/medical uses, such as bed linens, bandages or suture threads [238, 239]. Thus, even when applied for textile dyeing, fungal pigments can enrich the properties of the fabrics with biological activities.

8.4   Limits and Further Opportunities for Industrial Use of Fungal Pigments

The feasibility of developing a wide range of fungal pigments at industrial scale should be considered through the six following main elements: the biological aspect of the strains metabolite production, the health and safety concerns about the putative mycotoxins coproduction, all the legislation and regulations process to do before any actual launch on the market, the trends of the global colorant market along with the cost-effectiveness of such fungal pigments production, and in the end, all the technical and logistical hurdles when designing and running an industrial plant.

Regarding the biological aspect, the main issues could come from the stability of the strains and their pigment production over time, especially if the strains have been previously genetically engineered. However, the use of microorganisms show significant advantages such as fast growth compared to plants, with a complete independency from seasons or weather. Indeed, even cochineal farms production is climate-dependent due to the close interdependency between the cochineal insect and the plants it lives on (Barbary fig), rendering final prices of plants and cochineal-based pigments relatively versatile.

Fungal secondary metabolite commercialization is limited, yet due to health and safety concerns about mycotoxins coproduction with the pigments, or the pigments being toxic themselves. However, similar toxic worries on synthetic dyes and their potential harm on human health are more and more arising. Recently, several research studies, such as the one from Southampton University (2010), show that azo-dyes in food and other synthetic dyes in fabrics should be avoided. Such observations make the public more aware on what sort of additives are added to their food, clothes and cosmetics and drive the demands for "organic" and greener ingredients. To address the health concerns, pigments have to go through a long allowance process in order to be attributed an European number (E number) with an acceptable dietary intake (ADI) before being sold in Europe, for instance. Toxicological and clinical tests are long and require different levels of tests depending on the final application (food, textiles, cosmetics). However, it is worth noticing that numerous patents for the use of fungal pigments (*Monascus*-like pigments) in food and cosmetics have been filled by companies such as Nestlé, Unilever, The Quaker Oat, and l'Oréal, and have all been accepted. Moreover, these pigments have been used for centuries in Asia, which gives good hopes they will be allowed in Europe and in the United States at some point. Then, fungal pigments of *P. oxalicum* (Natural Red™) and β-carotene and lycopene from *Blakesla trispora* have been authorized in Europe. So despites a long process, examples attest that positive progresses are made towards the acceptance of fungal colorant on the EU and US markets.

Concerning the global colorant market, all the last elements are encouraging the development of natural dyes from fungal origins. Such market evolution is coherent with the public demands of more eco-friendly products. Economically speaking, the use of microorganisms has an enormous advantage, which is the complete independence toward the variability of prices of petroleum-based raw materials, rendering the price of the feedstock less variable, reducing significantly its impact on the final pigment price. Moreover, the use of agroindustrial wastes instead is a cheaper and more economically stable feedstocks. When considering the development of biorefineries for fungal pigments biosynthesis, two main hurdles arise: first, the initial investment to design and size the plant, and secondly the impossibility, yet, to set up a continuous fermentation process (the bioreactor would need to be drained, cleaned and refilled for every fermentation batch). Additionally, time and carbon source need to be "wasted" to initiate the strain growth and start the secondary metabolism. However, such fermentation process are little energy demanding and, thus, represent a promising alternative.

9   Conclusion

As an example within the whole industry, the trends in the food and beverage markets as well as in the cosmetic and textile markets push for more natural, organic, and clean label products, so the need for more and more natural ingredients is increasing. The formulation of recipes containing natural colors has steadily increased over recent years. Fungal colorants are constituents of commercial products available for the industry as a natural choice among many natural sources. They are either based on alternative production techniques of well-known pigments (e.g., β-carotene, lycopene, riboflavin) or specific molecules so far not biosynthesized by other organisms such as higher plants (e.g., *Monascus* and *Monascus*-like azaphilones).

The use of natural colors in functional, pharmaceutical, cosmetic, textile, beverage, food, and crossover applications requires an understanding of a variety of attributes and concepts, including heat stability, light stability, in addition to being able to provide exciting color hues. Fungal colorants already proved to exert such properties, and novel compounds from fungal biomass produced by applying biotechnological processes should render possible applicable and optimized solutions to the whole industry.

It can be concluded that despite some safety and regulation difficulties, fungal pigmented secondary metabolites stand for promising colorant alternatives with real cost-effective potentialities in the end. However, it would be advisable to bear in mind that an industrial biorefinery cannot be built similarly to conventional chemical process-based plants. Indeed, new management techniques would have to be developed in order to render such industries competitive.

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2.3. *Penicillium*/ *Talaromyces* Pigments

2.3.1. Overview on the Genera

Penicillium (anamorphic) and *Talaromyces* (teleomorphic) are ascomycetes fungi, ubiquitous in nature, mostly found in soil, rotten fruits, vegetables, meat, many other moist and dead organic matters. The genus *Penicillium* and *Talaromyces* belongs phylogenetically to *Trichocomaceae* [96, 97]. The *Trichocomaceae* comprise a relatively large family of fungi having both positive and negative impacts on human health. Some species of *Trichomaceae* are associated with food spoilage and mycotoxin production while other species are opportunistic pathogens exploited in biotechnology for the production of enzymes, antibiotics and other bioactive metabolites [98]. Amongst all the extrolites produced by *Penicillium* (1338) and *Talaromyces* (316), it has been mainly noted for its potentially of producing natural pigments, which could find extensive use in industrial applications [99]. These genera produce different shades of pigments ranging from yellow, red, orange to reddish-brown. The hues produced by the fungi predominately depend on environmental variables mainly pH, temperature and also regulated by nutrient sources of the culture medium such as carbon, nitrogen sources and addition of trace elements [100].

Strains such as *P. simplicissimum* DPUA 1379, *P. melinii* DPUA-1391, and *P. atrovenetum* isolated from Amazon forest produced yellow pigment with antibiotic activity and it was found that yellow is the chief most pigment produced by most of the *Penicillium* strains [101]. Several species of *Penicillium* such as *P. purpurogenum, P. aculeatum, P. marneffei* and *P. funiculosum* have been reported as novel producers of Monascus-like azaphilone pigments [102]. Monascus pigments belong to polyketide class of azaphilone group produce three types of color components: yellow monascin and ankaflavin; the orange monascorubrin and rubropunctatin and the red monascorubramine and rubropunctamine [103, 104]. So far, the isolated pigments of *Monascus* exhibits potential anti-tumor [105, 106] anti-diabetic, anti-oxidative stress [107], anti-inflammatory [106, 108] anti-cancer activities [109]but the disadvantage being the production of unwanted mycotoxin named citrinin presents a disadvantage of using *Monascus* mycelia and pigments as food colorant [110]. In contrast to *Monascus* species, some *Penicillium* strains such as *P. purpurogenum, P. aculeatum, P. funiculosum,* and *P. pinophilum* were identified *as* potential pigment producers that produced *Monascus*-like pigments but no known mycotoxins [111].

The *Penicillium* strains may also be pigmented by anthraquinones. Two bioactive bis-anthraquinones (rugulosin and skyrin) were identified as the main products in a strain of *Penicillium chrysogenum* isolated from a saline lake [112]. Similarly, seven pigmented anthraquinones were isolated from the

marine derived fungus *P. oxalicum* 2-HL-M-6 as reported by Wang et al, 2014. **[113]**. Commercially, Arpink red™ (now Natural Red™) was the first fungal red pigment manufactured by ASCOLOR BIOTECH s.r.o., received temporary approval in 2004 to use as food additive, exclusively in the Czech Republic **[29]**. This anthraquinoid type red colorant is an extracellular metabolite obtained by fermentation along with bioprocessing approach from the strain *Penicillium oxalicum* var. *Armeniaca* CCM 8242, obtained from soil as source. The fungus produces pigment upto 2g/L in the culture medium (MW: 550, $C_{25}H_{26}O_{14}$) providing raspberry red color in an aqueous solution **[114]**

2.3.2. *Talaromyces albobiverticillius*: Red Pigment Producer

Talaromyces is the teleomorph genera historically associated with *Penicillium sensu lato* **[98]**. Long ago in 1949, Raper & Thom recognised that there was considerable evidence that *Penicillium* subgenus *Biverticillium* constituted a natural and homogenous group. To delimit phylogenetic species in a complex, RPB1, RPB2, β-tubulin and calmodulin sequences were used whereas ITS barcodes were used to show the relationships among the species **[115]**. Some species of *Talaromyces* secrete large amounts of red pigments, notably species such as *Talaromyces purpurogenus*, *T. albobiverticillius*, *T. marneffei*, and *T. minioluteus* often under earlier *Penicillium* names. Isolates identified as *T. purpurogenus* have been reported to be interesting industrially and they can produce extracellular enzymes and red pigments, but they can also produce mycotoxins such as rubratoxin a and b and luteoskyrin. In a study by Frisvad *et al.* in 2013, they isolated a novel type strain *Talaromyces atroroseus* CBS 133442 which produces diffusible red pigments without any mycotoxin production. Certain strains of *Talaromyces albobiverticillius* were studied and proposed to produce large amount of red pigments thus can be used for coloring foods (Table 2)**[30]**.

Talaromyces albobiverticillius		
Collection No	Source of isolation	Extrolites found
CBS 113168	Sputum of patient male, Copenhagen	mitorubrin, mitorubrinic acid, monascorubramine, PP-R, rubropunctatin,vermicellin
CBS 313.63	Vitis vinifera fruit, South Africa	mitorubrin, monascorubramine, monascorubrin, rubropunctatin
IBT 4466	Punica granata, imported to Denmark	mitorubrinic acid, monascorubramine, a purpactin
CBS 113167	Unknown	mitorubrin, mitorubrinic acid, monascorubrin, a purpactin
CBS 133444	Punica granata, Unknown	mitorubrin, mitorubrinic acid, mitorubrinol
CBS 133452	Cotton duck, Panama	mitorubrin, mitorubrinic acid, monascorubramine, rubropunctatin
CBS 133441	Decaying leaves of a broad leaved tree, Taiwan	mitorubrin, mitorubrinic acid, monascin, monascorubramine, rubropunctatin, vermicellin

Table 2: Extrolites produced by different strains of Talaromyces albobiverticillius detected by HPLC and/or UHPLC-HRMS

Table Source: [30]

2.4.   FUNGAL POLYKETIDE PIGMENTS:

Polyketides are naturally occurring compounds, formed from the condensation of monomers acyl CoA (acetyl-CoA or propionyl-CoA, for example) with malonyl-CoA and methylmalonyl-CoA units, decarboxylation is carried out in parallel to this reaction. Polyketides owe their name to the condensation of primary products bearing β-keto functional groups. They are classified based on the number of carbon units that contribute to the polyketide chain and the type suffered by the cyclization precursor. Certain pigments are part of the family of polyketides, particularly pigments of fungal origin [116]. These pigments consist of 4 to 8 dicarbon units contributing to the polyketide chain. The classes of the most representative polyketide pigments are: anthraquinone, hydroxyanthraquinones, naphthoquinones and azaphilone polyketides. Each pigment class presents a range of different colors (Figure 1).
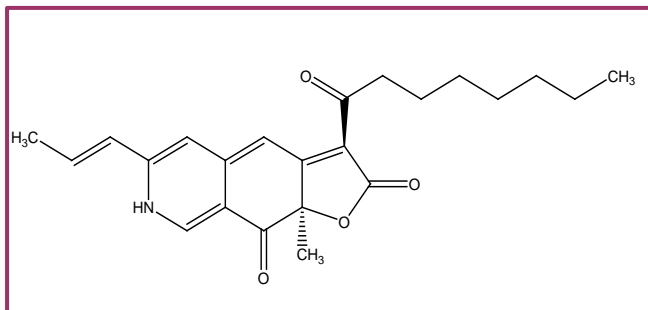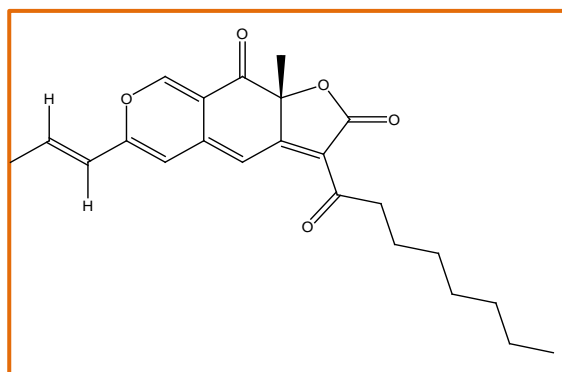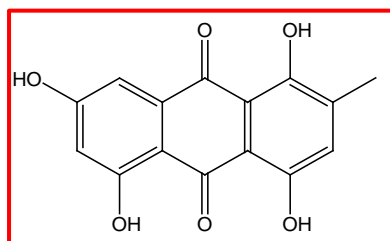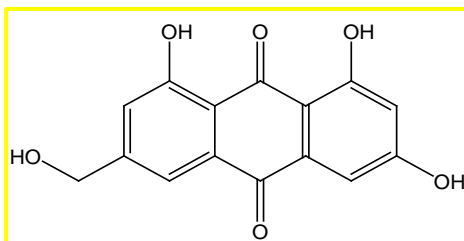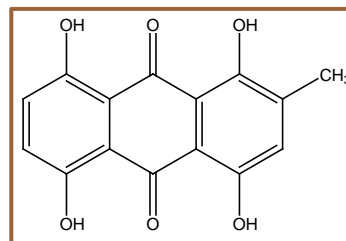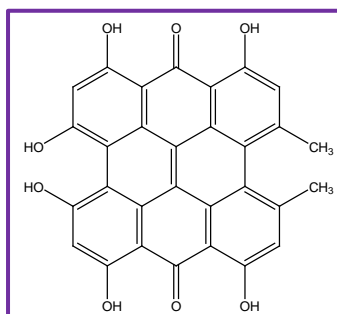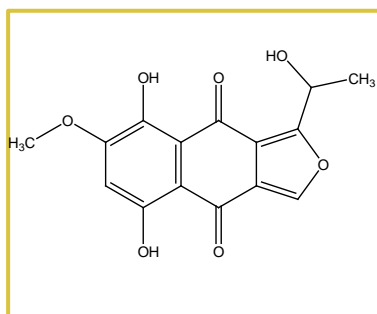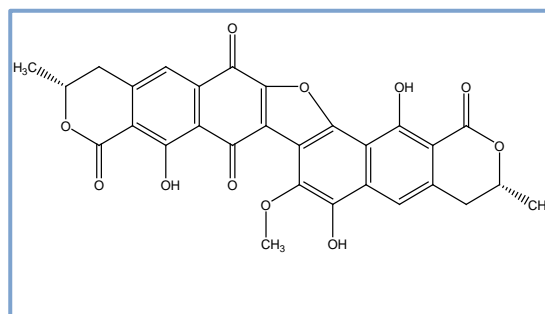
Figure 1: Some exemplary structures of fungal polyketide pigments and their color ranges

2.4.1. Azaphilones

Azaphilones can be defined as a structurally diverse class of fungal secondary metabolites (polyketide derivatives), namely pigments with pyrone-quinone structures containing a highly oxygenated bicyclic core and a chiral quaternary center [2, 3, 4]. Note that both a pyronequinone structure and a chiral quaternary centre are essential for structures to be classified as azaphilones. This class of molecules is characterized by a wide range of biosynthetic modifications of the bicyclic ring system, including oxidation of the *4H*-pyran ring (*cf.* **2** and **3**), annulation, (*cf.* **4**), and halogenation. These molecules exhibit a wide range of biological activities, including sphingosine kinase, fatty acid synthase, gp120-CD4, Grb2-SH2, telomerase, p53-MDM2 interaction, and HIV REV/RRE binding inhibition. The biosynthesis of azaphilones uses both the polyketide pathway and the fatty acid synthesis pathway.

2.4.2. Anthraquinones

Anthraquinones also called anthracenedione or dioxoanthracene, an important members of quinone family constitute a large structural variety of compounds that belongs to the polyketide group [117]. Anthraquinones are structurally built from an anthracene ring with a keto group on position 9 ,10 as basic core and different functional groups such as -OH, -CH$_3$, -OCH$_3$, -CH$_2$OH, -CHO, -COOH, etc. substituted at various positions represented in figure 1 [118]. Anthraquinones and their derivatives, produced as secondary metabolites in plants, lichens, insects and higher filamentous fungi which occur either in a free form or as glycosides, at times as other complexes linked by C- or O- in the side chain [114, 118]. The electronic absorption spectra is a characteristic feature of the parent compound 9, 10-anthraquinone, its dihydroxy- and diamino-derivatives that permits to understand the effect of hydrogen bond, solvent polarity and nature of substituents on the spectral shift. This detailed study is of great importance owing to the wide ranging applications of anthraquinones in many fields [119].

2.4.3. Naphthoquinones

Naphthoquinones are natural polyketide pigments derived from naphthalene. The occurrence of these compounds is widespread in fungi and actinomycetes. Interest in this class of compounds is related to the broad spectrum of their biological activity such as phytotoxic, insecticidal, antibacterial, and fungicidal properties [120]. To determine their structural activity, most of the studies have been directed towards isolation of pigments in sufficient quantities needed for the analysis. More than 100 naphthoquinone metabolites produced by 63 species of fungi which has been studied [121]. Amongst

the fungal species, many studies have been performed specifically on *Fusarium spp* and it has been found to produce naphthoquinones of different shades.

2.5.   References

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2.6. REVIEW ARTICLE

Anthraquinones and Derivatives from Marine-Derived Fungi: Structural Diversity and Selected Biological Activities

This review article focus on anthraquinones and its derivatives listed to date from marine-derived filamentous fungi and its associated biological activities. In addition, it describes the use of certain compounds in industrial applications. This review article forms another part of this chapter.

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Anthraquinones and Derivatives from Marine-Derived Fungi: Structural Diversity and Selected Biological Activities

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Abstract: Anthraquinones and their derivatives constitute a large group of quinoid compounds with about 700 molecules described. They are widespread in fungi and their chemical diversity and biological activities recently attracted attention of industries in such fields as pharmaceuticals, clothes dyeing, and food colorants. Their positive and/or negative effect(s) due to the 9,10-anthracenedione structure and its substituents are still not clearly understood and their potential roles or effects on human health are today strongly discussed among scientists. As marine microorganisms recently appeared as producers of an astonishing variety of structurally unique secondary metabolites, they may represent a promising resource for identifying new candidates for therapeutic drugs or daily additives. Within this review, we investigate the present knowledge about the anthraquinones and derivatives listed to date from marine-derived filamentous fungi's productions. This overview highlights the molecules which have been identified in microorganisms for the first time. The structures and colors of the anthraquinoid compounds come along with the known roles of some molecules in the life of the organisms. Some specific biological activities are also described. This may help to open doors towards innovative natural substances.

Keywords: anthraquinone; marine; fungi; pigment; biological activity; antitumor; antibiotic; cytotoxicity

1. Introduction

In recent decades, marine organisms focused the attention of researchers for their huge potential in producing bioactive compounds [1–4]. Among them, the microorganisms gradually took on an important role because they appear to be prolific producers of a wide diversity of secondary metabolites [5,6]. Cultivated in bioreactors, they also represent a sustainable and easily "upscalable" resource, therefore not endangering fragile marine ecosystems. Among these microorganisms, fungi, which had their terrestrial glory days after the discovery of penicillin, are again at the forefront in the search for new marine molecules in view of their rich biodiversity, even in deep-sea niches [7]. Around 70,000 fungal species have already been described worldwide, and among them about 1500 species of marine-derived fungi were mentioned, primarily from coastal ecosystems [8,9]. As 70% of the earth is submerged, Gareth Jones (1998) [10] estimated the total number of marine and marine-derived fungal species to be a minimum of 72,000, indicating that the inherent discovery of new compounds is still in its infancy. Several fungal metabolites from marine origin have already demonstrated their originality

and efficacy in different domains. As an example, in the field of therapeuthics, we can mention the two chemically unusual cyclodepsipeptides patented in 2008—scopularides A and B—from the marine-derived *Scopulariopsis brevicaulis*, demonstrating anticancer activities [11,12], as well as the halimide (plinabuline) from an endophytic *Aspergillus* sp. *CNC-139* isolated from the green algae *Halimeda lacrymosa*, already achieving phase II clinical tests [13].

Biosynthetically, many extrolites produced by filamentous fungi are polyketides, and several papers report that polyketides seem to dominate marine natural products of fungal origin [14,15]. Polyketides represent an array of often structurally complex natural products including such classes as anthraquinones, hydroxyanthraquinones, naphthalenes, naphthoquinones, flavonoids, macrolides, polyenes, tetracyclines, and tropolones. Many of them have already exhibited either positive or negative effects, as wide as those that are antimicrobial, anticancer, antioxidant, immunomodulatory, cytotoxic, or carcinogenic. This closely concerns the class of anthraquinones whose effects, depending on the nature and amount of compound, can either be beneficial or noxious towards living organisms. These compounds, little studied because of their bad reputation, mainly arising from their benzenic patterns, are, however, worthy of the same attention as other families of fungal compounds, whose members have become pillars of the global pharmacopeia (antibiotics) and are widely used in food or staining industries (azaphilone colorants from *Monascus* spp. in Asia). Within this review, we investigate the present knowledge of the anthraquinonoid compounds listed to date from marine-derived filamentous fungi's productions. This overview highlights the molecules identified for the first time and comes along with interesting characteristics: the panel of colors, their known roles in the biology of the organisms, and some specific *in vitro* biological activities. As the natural products constitute the dynamic element of the present global market, we hope this review can help broadening the horizon towards innovative substances.

2. Anthraquinones from Marine-Derived Fungi

About 700 anthraquinone derivatives were identified in plants, lichens, and fungi; 43 have already been described from fungal cultures [16,17]. Due to their structure, they exhibit interesting chromatic properties and decline a wide range of nuances in colors. Thus, they first presented a great interest in the field of dyeing molecules, highly requested in cosmetics, clothes dyeing and foodstuff industries. From their structures, hydroxyanthraquinone pigments have a relative stability. They also possess good light-fastness properties, which often makes metallization unnecessary. Nevertheless, they can easily form complexes with several metal salts (or cations in general) (aluminium, barium, calcium, copper, palladium, iron) [18–22] and exhibit superior brightness compared to azo-pigments [23,24]. This capacity to form metallic complexes is of a great interest in an industrial context: The complex forms often reduce the soublity in water, enhancing the solvent solubility, without loosing the brightness [20]. In the textile industry, hydroxyanthraquinone are, moreover, considered "reactive dyes," as they form a covalent bond with the fibers, usually cotton, although they are used to a small extent on wool and nylon. Therefore, they have it it possible to achieve extremely high wash fastness properties by relatively simple dyeing methods. Thereby, the literature abundantly reports the interest for marine organisms with respect to the production of new molecules and, among them, new pigments [25,26]. Besides their coloring properties, anthraquinoid compounds exhibit a wide range of diverse biological activities, sparsely mentioned in the literature. Regarding this aspect, their "Dr Jekyll and Mr Hyde" physiognomy [27] needs to be carefully elucidated in order to examine their potential use in pharmaceutical or alimentary fields, with maximum objectivity.

2.1. Anthraquinone's Basic Structure

Anthraquinones represent a class of molecules of the quinone family, based on a structure composed of three benzene rings. The basic structure 9,10-anthracenedione, also called 9,10-dioxoanthracene (formula $C_{14}H_8O_2$), includes two ketone groups on the central ring (Figure 1). The diversity of the anthraquinoid compounds relies on the nature and the position of the substituents,

replacing the H atoms on the basic structure (R1 to R8), as diverse as: –OH, –CH$_3$, –OCH$_3$, –CH$_2$OH, –CHO, –COOH, or more complex groups. When *n* hydrogen atoms are replaced by hydroxyl groups, the molecule is called hydroxyanthraquinone (HAQN). From their structure, HAQN derivatives absorb visible light and are colored.
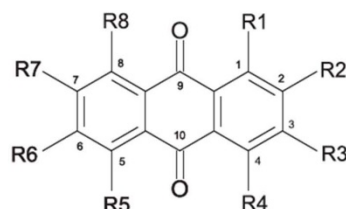
Figure 1. Anthraquinone general structure (R1–R8: lateral substituents).

An important characteristic of the anthraquinone compound is their electronic absorption spectra. The strong absorption in the ultraviolet region is due to the presence of chromophore formed by the system of conjugated double bonds. The spectra of anthraquinone are highly complex because of the presence of absorption bands due to the benzenoid transitions, in addition to quinonoid absorptions. The benzenoid bands appear fairly regularly within the range 240–260, with intense absorption at 250 nm and in 320–330 nm, and with medium absorption at 322 nm, whereas the quinonoid bands absorb at 260–290 nm. These areas of selective absorption are characteristic, and the pattern in the ultraviolet region is not seriously affected by substitution. In addition, hydroxyl anthraquinones show an absorption band(s) at 220–240 nm, not shown by the parent compound. In the visible area, an unsubstituted anthraquinone has a weak yellow color, and its electronic absorption spectrum contains a small peak at 405 nm. The presence of substituents in position 1 and 4 induces a significant bathochromic shift, intensifying the color more significantly than the substituents in the 1,5 and 1,8 positions. Thus, with an alcoholic solution of magnesium acetate, 1,2-dioxyderivative is colored in violet; 1,4-dioxyderivative in purple; and 1,8-dioxyderivative in red-orange [28–30]. Therefore, fungal anthraquinones range from pale yellow to dark red or brown colors, through to violet.

2.2. Ecology of Marine-Derived Fungal Anthraquinones Producers

Marine ecosystems host a wide biodiversity of filamentous fungi found in free waters, inert organic or inorganic matter. They can also be included as endophytes or pathogens in marine plants, planktons, vertebrates and invertebrates [31]. Their different roles in these environments are still poorly known, although their implications in lignocellulolytic compounds degradation and mineralization of organic matter has been repeatedly demonstrated [7,32,33]. Yet the notion of "marine fungus" is still under debate in the world of mycologists. Fungi are usually recognized as ubiquitous because they inhabit a plethora of ecosystems, from terrestrial milieus to aquatic environments (Figure 2). Marine and marine-derived fungi therefore form an ecological, not a taxonomic, group [34]. From the widely adopted definition of Kohlmeyer *et al.* (1979) [8], they are divided into two ecotypes:

- obligate marine fungi (true ones) that grow and sporulate only in seawater. Their spores are able to germinate and form new thalli in salted environment.
- transitional marine fungi (marine-derived fungi) that come from terrestrial or freshwater media and have undergone physiological adaptation to survive, grow, or reproduce in the marine environment.

Figure 2. Marine habitats hosting fungal anthraquinones producers. (**a**) Tree and marine plants growing in a submerged area (**b**) *Aplysina aerophoba* (mediterranean sponge), usual host of endophytic filamentous fungi; (**c**) *Aspergillus versicolor*, exhibiting a pink pigment.

In fungi, anthraquinones are produced from different steps or branches of the polyketides pathway. Today, it is clear that, as far as secondary metabolites and *a priori* anthraquinoid productions are concerned, a great variability appears among species of the same genus, even among strains in the same species. This could undoubtly be related to the capacities a fungus has to develop, in order to face some specific conditions in specific ecosystems. As an illustration, the composition of the quinoid pigment complexes of *P. funiculosum* strains isolated from various types of soils are quite different when cultivated in the same artificial culture media [35]. That is why, even if the polyketides pathway is mentioned in a strain, not all strains inside the species are anthraquinones producers. In the same way, if the fungal metabolism is able to express anthraquinones and (simultaneously) to excrete toxins, the presence of these secondary products are highly dependent on external physico-chemical conditions [36].

Thus, a high diversity of molecules is now expected from unexplored marine-derived fungi, which are considered promising novel sources of chemical diversity. The potential of marine-derived microorganisms to produce unique and original molecules could therefore come from specific metabolic or genetic adaptations appearing to meet very specific combinations of physico-chemical parameters (high osmotic pressure, low O_2 penetration, low temperature, limited light access, high pressure, or regular tidal ebbs and flows) [37]. Indeed, the two marine ecotypes lead to particular behaviors and consecutively to specific products, compared to the terrestrial congeners: either the challenge of facing unusual living conditions (exogenous fungi) or the use of specific procedures naturally adapted to the marine niches (*i.e.*, indigenous micromycetes, naturally selected for aquatic environments). This skill is, for instance, exemplified by marine macroorganisms' fungal endophytes as corals or sponges. For now, the highest diversity of marine-derived fungi seems to be found in tropical regions, mainly in tropical mangroves, which are extensively studied because of their high richness in organic matters. Obviously, these biotopes seem favorable to the development of a high diversity of heterotrophic microorganisms based on the diversity of organic and inorganic substrates [8,38].

The questions on the effect of interactions between organisms on microbes extrolites is amply fueled in the case of a very producive lichen's symbioses. A lichen is a composite organism that emerges from algae or cyanobacteria (or both) living with filaments of a fungus in a mutually

beneficial (symbiotic) relationship. About 20,000 lichen species are known in the world, and there are approximately 700 species known from coastal rocks and urbanized shores [39]. Most work on aquatic lichens was done in temperate areas, as, in the tropics, lichens are less developed on costal rocks. One interesting skill is that lichen associations are primarily terrestrial but require alternate wetting and drying regimes for their survival. In marine environments, these circumstances occur principally in tidal zones on coastal rocks, subject to varying water levels and different degrees of inundation. Another feature is that the whole combined life form has properties that are very different from properties of its component organisms alone. Thus, tropical stream margins are promising biota for species and therefore compounds that are new to science.

2.3. Structural Diversity and Colors of Anthraquinoid Extrolites from Marine-Derived Fungi

2.3.1. Present Knowledge about Anthraquinonoid Compounds from Fungi

Today's knowledge indicates that a large part of compounds identified in terrestrial fungi can often be isolated from the same species living in marine environments. For instance, catenarin, emodin, erythroglaucin, physcion, questin, and rubrocristin or physcion anthrone are produced by marine-derived *Aspergillus* and/or *Eurotium* species, as well as by their terrestrial counterparts. According to Bick *et al.* and Fain *et al.* [40,41], the most widespread anthraquinones in fungi are 1,8-dihydroxy and 1,5,8 or 1,6,8-trihydroxy anthraquinone derivatives. They appear either as simple forms, as glycosides, or other complexes attached through an O- or C-bond in the side chain, which can enhance the water solubility. Some dimeric structures (formed through C–C bonds) are also produced from fungi, (e.g., alterporriols, skyrin, rubroskyrin, luteoskyrin, icterinoidin, rubellin, rufoolivacin, *etc.*). Some dimers may contain not only monomeric anthraquinones but also naphthoquinones and other products of polyketide synthesis. According to Fujitake *et al.* [42,43] and Suzuki *et al.* [44], the dimeric anthraquinone 5,5′-biphyscion (named hinakurin), chrysotalunin, (−)-7,7′-biphyscion, microcarpin, chrysophanol, and physcion are predominant in soil, but they seem rare in organisms. If hinakurin, chrysotalunin, and (−)-7,7′-biphyscion have not been found in fungi yet, it is now clear that chrysophanol and physcion are frequent fungal productions, and that fungi are able to synthesize dimers. These organisms, widely represented in soil, may have a transient appearance of monomeric and dimeric anthraquinones in telluric biotopes, certainly evolving to complex (humic) polymers. However, these statements rely on decades of terrestrial studies. The increase in knowledge on marine and marine-derived anthraquinones from fungi will certainly elucidate this aspect.

2.3.2. Nature and Colors of Compounds from Marine-Derived Fungi

Most anthraquinoid compounds of natural origin have complex structures with several functional substituent groups. In nature and/or cultures, a wide range of hues appears, from pale yellow to dark brown, through to orange, red, or violet pigmentations. Anthraquinoid compounds generaly color sexual stages or resistance forms (ascomata, spores, conidia, *etc.*) but sometimes also impregnate mycelium or are excreted in the growth environment. Thus, the structural localization and the colors of these secondary metabolites seem to highly depend on the fungal species and may vary with the amount of compound produced in relation with the environmental conditions.

Genera and Species

Many genera producing anthraquinones have been isolated from marine environments, either from water, sediments, or decaying plants or from living organisms such as invertebrates, plants (endophytes), and algae. To date, strains in the genera—*Alternaria, Aspergillus, Eurotium, Fusarium, Halorosellinia, Microsphaeropsis, Monodictys, Nigrospora, Paecilomyces, Penicillium, Phomopsis*, and *Stemphylium*—have been clearly mentioned as marine-derived anthraquinones producers.

Some of the common terrestrial genera—*Aspergillus, Eurotium, Alternaria, Penicillium and Fusarium*—have been extensively investigated concerning their secondary metabolite's productions,

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and the anthraquinoid molecules produced were reviewed by Velmurugan *et al.* [45], Caro *et al.* [16], and Gessler *et al.* [17]. They have revealed a large taxonomic distribution, confirming that the biosynthesis of anthraquinones is widespread in the fungal world, from macroscopic fungi to molds, as well as in lichens (symbiosis between algae and fungi). An extended list of the marine-derived fungal species producing anthraquinoid compounds with related differences is presented in Table 1. It indicates that, among the widespread compounds identified in marine-derived isolates, physcion, emodin, and chrysophanol and subsequently catenarin, erythroglaucin, macrosporin, and questin are frequently detected. The stuctural diversity of the compounds identified, along with the colors—(mentioned as a tag accompanying the molecule formula) are described in Table 2.

Ubiquitous Fungi

The Aspergillus glaucus group, A. variecolor, A. versicolor as well as Eurotium cristatum, *E. repens* and *E. rubrum*, very rife on earth, were also identified in marine niches.

To date, *Aspergillus glaucus* is the best anthraquinones producer from this *Aspergillus / Eurotium* cluster, according to the diversity of anthraquinoids molecules listed (11 different molecules from personal data [110]). Nine (9) isolates originate from marine or salted environments (sea, saltern, mangrove), suggesting a developed capacity to face high NaCl concentrations. These marine-derived isolates produce the main part of the new compounds identified in this genus up to now, besides endolichenic *Aspergilli*.

Catenarin, emodin, erythroglaucin, physcion, questin, and rubrocristin or physcion anthrone are commonly produced by marinederived *Aspergillus* and/or *Eurotium* species, as well as by their terrestrial counterparts. Moreover, Variecolorquinone A, which seems specific to the *Aspergillus* family, is synthesized by *Aspergillus glaucus* and *A. variecolor* B-17 from salted environments.

10,10′-dimer of emodin and physcion along with cynodontin, helminthosporin, tritisporin, unusual in this genus are excreted by the mangrove strain *A. glaucus* HB1-19. Two new hexahydroanthrones—tetrahydrobostrycin and 1-deoxytetrahydrobostrycin—are produced by *Aspergillus* sp. 05F16, a strain isolated from an unidentified alga collected in a coral reef (Indonesia).

The new methyl-emodin and 7-hydroxyemodin 6,8-methyl ether, along with emodin, were identified in the *A. versicolor* anendophytic strain isolated from the Red Sea green alga *Halimeda opuntia*. The new 6,8-di-*O*-methyl averantin, along with six known congeners are also synthesized by *A. versicolor* EN-7 (Genbank noEU042148), an endophytic fungus of *Sargassum thumbergii* (brown algae).

The common genus *Eurotium* consists in teleomorphic, often xerophilic, species, usually related to *Aspergillus* anamorphs, especially from the *A. glaucus group*. Anke *et al.* [36] reported that, inside the *Eurotium* genus, *E. rubrum*, and *E. cristatum* produce the highest diversity of compounds; regarding anthraquinones, physcion, physcion anthrone, erythroglaucyn, catenarin, rubrocristin and emodin have been identified in their cultures. They also demonstrated that, inside a species, there was a great variability towards anthraquinones production, as some strains of *Eurotium* (among them *E. rubrum and E. herbariorum)* behaved differently in the same culture conditions. Moreover, some of the strains studied did not produce any anthraquinone, in the conditions of the experiment. Butinar *et al.* [111], noticed that, in Slovenian solar salterns, *E. amstellodami*, *E. herbariorum* and *E. repens* contributed to indigenous fungal community in hypersaline water environments, while *E. rubrum* and *E. chevaliery* were only temporal inhabitants of brine at lower salinities. In contrast, they stated that, for the six *Eurotium* strains isolated from these salterns, the qualitative secondary metabolites profiles were not different from those of strains isolated from foods or other habitats. However, the new 6,3-*O*-(α-ᴅ-ribofuranosyl) questin (anthraquinone gylycoside) is a new questin derivative from the marine-derived *E. rubrum* QEN-0407-G2, isolated from the marine mangrove plant *Hibiscus tiliaceus*. This compound, produced along with questin, seems unusual in the genus [63].

Table 1. Marine-derived fungi producing anthraquinones and some derivatives.

Genus	Species/Strain No	Name of Compounds Produced	Source of Isolation	Refs.
Alternaria	*Al. eichhorniae*	4-deoxyBostrycin, Bostrycin	Mar. Plant pathogen	[46]
	Al. (SK11)	(+) α S-alterporriol C, 6-methylquinizarin, Alterporriol S, Austrocortinin	Mangrove Plant end.	[47]
	Al. sp. ZJ-2008003	Alterporriol C, K–R, Altersolanol B and C, Macrosporin	Mar. Org end.	[48]
	Al. sp. ZJ9-6B	Alterporriols C–M, Altersolanol A, Dactylariol, Macrosporin, Physcion, TetrahydroAltersolanol B	Mar. Plant end.	[49]
Aspergillus	*A. glaucus*	10,10'-dimer of Emodin and Physcion, Catenarin, Cynodontin, Emodin, Erythroglaucin, Helminthosporin, Physcion, Questin, Rubrocristin, Tritisporin, Variecolorquinone A	Mangrove sed.	[50–53]
	A. sp. 05F16	1-deoxytetrahydrobostrycin, Tetrahydrobostrycin	Algal end.	[54]
	A. sp. SCSIOF063	(1'S)-7-chloroaverantin, 1'-O-methylaverantin 1'-O-methyl-7-chloroaverantin, 6-O-methyl-7-chloroaverantin, 6-O-methyl-7-chloroaverythrin, 6-O-methyl-7-bromoaverantin, 6,1'-O,O-dimethyl-7-chloroaverantin, 6,1'-O,O-dimethyl-7-bromoaverantin, 6,1'-O,O-dimethylaverantin, 7-chloroaverantin-1'-butyl ether, 7-chloroaverythrin	Sed.	[55]
	A. variecolor B-17	(2S)-2,3-dihydroxypropyl1,6,8-trihydroxy-3-methyl-9,10-dioxoanthracene-2carboxylate, Catenarin, Emodin, Fallacinol, Physcion, Erythroglaucin, Questin, Questinol, Rubrocristin, Variecolorquinone A,	Sed.	[56]
	A. versicolor	7-hydroxyemodin 6,8-methyl ether, Emodin, Isorhodoptilometrin-methyl ether, Methyl emodin	Algal end.	[57]
	A. versicolor EN-7 (Genbank no EU042148)	6,8-di-O-methylversiconol 6,8-di-O-methylnidurufin 6,8-di-O-methylaverantin 6,8-di-O-methylversicolorin A, Aversin: (−)-isomer	Algal end.	[58]
Curvularia	*C. lunata*	Cytoskyrin A, Lunatin	Mar. Org end.	[50,51,59,60]
Eurotium	*E. cristatum* (ECE)	Catenarin, Emodin, Erythroglaucyn, Physcion, Physcion anthrone, Questin, Rubrocristin	Mar. Org end.	[36,61]
	E. repens	Catenarin, Erythroglaucyn, Physcion, Physcion anthrone	Mar. Org end.	[36,62]
	E. rubrum	6,3-O-(α-D-ribofuranosyl)-questin, Questin	Mar. Plant end.	[63]
Unidentified	Fungus Isolate 1850 and 2526	Averufin, Nidurufin, versicolorin C	Mar. Plant end.	[64]
	Fungus ZSUH-36	1'-O-methyl averantin, 6,8-di-O-methyl averufanin, 6,8-di-O-methyl averufin, 6,8,1'-tri-O-methyl averantin, Versicolorin C	Mar. Plant end.	[65]
Fusarium	*F.* sp. No. B77	5-acetyl-2-methoxy-1,4,6-trihydroxy-anthraquinone	Mangrove Plant end.	[66]
	F. sp. ZZF60	6,8-dimethoxy-1-methyl-2-(3-oxobutyl) anthraquinone	Mangrove Plant end.	[67]
	F. sp. No. ZH-210	Fusaquinon A,B,C	Mangrove sed.	[68]
	F. sp. PSU-F14, *F.* sp. PSU-F135	Austrocortirubin, Bostrycin	Mar. Org end.	[69]

Table 1. *Cont.*

Genus	Species/Strain No	Name of Compounds Produced	Source of Isolation	Refs.
Halorosellinia	*H.* sp. (No. 1403)	1,4,5,6,7,9-hexahydroxy-2-methoxy-7-methyl-5β,9β, 8aβ,6α,10aα—hexahydroanthracene10(10aH)-one, Austrocortirubin, Demethoxyaustrocortirubin, Hydroxy-9,10-anthraquinone, SZ-685C	Mar. Plant-derived	[70,71]
Lichens	Arthonia elegans, Biatorella conspersa, B. ochrophora, Pyrenula cerina, Sphaerophorus fragilis, Stereocaulon corticatulum,v. procerum, Trypethelium aeneum, T. aureomaculata, etc.	Physcion	Lichens	[72]
	Caloplaca sp.	Phallacinol (=Teloschistin=Fallacinol))	Lichen	[73,74]
	Caloplaca ehrenbergii, C. schaereri, C. spitsbergensis, etc.	1-O-methyl-7-chloroemodin, 7-chloro-1,6,8-trihydroxy-3-methyl-10-anthrone, 7-chlorocitreorosein, 7-chloroemodic acid, 7-chloroemodin, 7-chloroemodinal, Emodin, Phallacinol, Physcion	Lichens	[75–77]
	Gliocladium sp. T 31	Citreorosein, Emodin, Isorhodoptilometrin	Lichen	[78]
	Letrouitia hafellneri, L. leprolytoides	7-chloroemodinal, 7-chloroemodin, Fragilin, Physcion	Lichens	[79,80]
Microsphaeropsis	*M.* sp.	1,3,6,8-tetrahydroxyanthraquinone, 1,3,6,8-tetrahydroxy-2-(1-hydroxyethyl)anthraquinone 1,3,6,8-tetrahydroxy-2-(1-methoxyethyl)anthraquinone 1,2,3,6,8-pentahydroxy-7-(1-methoxyethyl)anthraquinone	Mar. Org end.	[81]
Monodictys	*M.* sp.	Chrysophanol, Emodin, Monodictyquinone A, Pachybasin	Mar. Org end.	[14,82]
Nigrospora	*N.* spp.	1-deoxytetrahydrobostrycin, 4-deoxybostrycin, Bostrycin, 4a-epi-9α-methoxydihydrodeoxybostrycin, 10-deoxybostrycin	Mar. Plant/Org end.	[68,83,84]
	N. sp. MA75	4-deoxybostrycin, Bostrycin	Marine	[85]
	N. sp. 1403	4-deoxybostrycin, Bostrycin	Mangrove	[86]
Paecilomyces	*P.* sp. (Tree 1-7)	Chrysophanol, Emodin	Mangrove	[87]
Penicillium	*P. citrinum* PSU-F51 (Accession no JQ66600)	Chrysophanol, Citreorosein, Emodin, Penicillanthranins A and B	Mar. Org end.	[88]
	P. chrysogenum	Skyrin	Salt lake	[89]
	P. flavidorsum SHK1-27	6,8-O-dimethylaverufin, 8-O-methylaverufin, Averufin, Averantin, Versiconol, Versicolorin A&B, Nidurufin	Marine	[90]
	P. oxalicum 2-HL-M-6	Aloe emodin, Chrysophanol, Citreorosein, Citreorosein-3-O-sulfate, Emodin, Emodin-3-O-sulfate, Isorhodoptilometrin	Mangrove sed.	[91]
Phomopsis	*P.* sp. PSU-MA214	Phomopsanthraquinone, 1-hydroxy-3-methoxy-6-methylanthraquinone, Ampelanol, Macrosporin	Mangrove Plant endo	[92]

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Table 1. *Cont.*

Genus	Species/Strain No	Name of Compounds Produced	Source of Isolation	Refs.
Stemphylium	S. sp. 33231	2-*O*-acetylaltersolanol B, Alterporriol T–W, Altersolanol B&C, Auxarthrol C, Macrosporin	Mangrove Plant end.	[93]
	S. globuliferum	6-*O*-methylalaternin, Acetylalterporriol D and E, Alterporriol D and E, Altersolanol A,B and C, Dihydroaltersolanol B and C, Macrosporin, Stemphylanthranol A and B	Salt lake Plant end.	[94]
Trichoderma	*T. aureoviride* PSU-F95	Coniothranthraquinone 1, Trichodermaquinone	Mar. Org end.	[88]
Xylaria	*X.* sp. 2508	Altersolanol A, Bostrycin, Deoxybostrycin, Xylanthraquinone	Marine	[95]

Abreviations: Mar. Plant end.: marine plant endophyte; Mar. Org. end.: marine organism endophyte other than plant; Sed.: sediment.

Table 2. Structural diversity of anthraquinoid compounds identified in marine-derived fungi (increasing mol. mass.).

Mol. Formula/*Mol. mass*	Trivial name	Structure	IUPAC NAME	Source	Refs
$C_{14}H_8O_3$ / 224	Hydroxy-9,10-anthraquinone		1-hydroxy-3-methylanthraquinone	*Halorosellinia* sp. No. 1403	[70]
$C_{15}H_{10}O_3$ / 238	Pachybasin		1-hydroxy-3-methylanthraquinone	*Monodictys* sp.	[14,82]
$C_{15}H_{10}O_4$ / 254	Chrysophanol		1,8-dihydroxy-3-methylanthraquinone	*Monodictys* sp. *Paecilomyces* sp. *P. citrinum* PSU-F51 *P. oxalicum* 2-HL-M-6	[14,82] [87] [88] [91]
$C_{15}H_{10}O_4$ / 254	6-Methylquinizarin		1,4-dihydroxy-methylanthraquinone	*Al.* sp. (SK11)	[47]
$C_{16}H_{12}O_4$ / 268	1-Hydroxy-3-methoxy-6-methyl anthraquinone		—	*Phomopsis* sp. PSU-MA214	[92]
$C_{15}H_{10}O_5$ / 270	Aloe emodin		1,8-dihydroxy-3-(hydroxymethyl)anthraquinone	*P. oxalicum* 2-HL-M-6	[91]
$C_{15}H_{10}O_5$ / 270	Emodin		1,3,8-trihydroxy-6-methylanthraquinone	*A. glaucus A. variecolor* B-17 *A. versicolor Caloplaca* spp. (e.g., *C. ehrenbergii, C. schaereri, C. spitsbergensis, etc.) Eurotium Cristatum Gliocladium* sp. T31 *Monodictys* sp. *Paecilomyces* sp. *P. citrinum* PSU-F51 *P. oxalicum* 2-HL-M-6	[91] [56] [61] [75–77,79,96] [36] [78] [14,82] [87] [88] [57]
$C_{15}H_{10}O_5$ / 270	Helminthosporin		1,5,8-trihydroxy-3-methylanthraquinone	*A. glaucus*	[50–53]
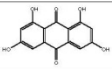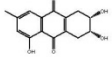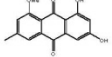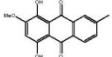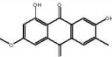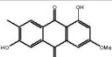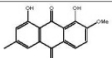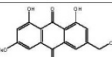
119

Table 2. *Cont.*

Mol. Formula/Mol. mass	Trivial name	Structure	IUPAC NAME	Source	Refs
$C_{14}H_8O_4$ /272	1,3,6,8-Tetrahydroxyanthraquinone		—	*A.versicolor Microsphaeropsis*	[97] [81]
$C_{15}H_{14}O_5$ /274	Coniothranthmaquinone 1		(2S,3R)-2,3,5-trihydroxy-7-methyl-1,2,3,4-tetrahydroanthraquinone	*Trichoderma aureoviride* (PSU-F95)	[88,98]
$C_{16}H_{12}O_5$ /284	1-Methylemodin		1,3-dihydroxy-8-methoxy-6-methylanthraquinone	*A. versicolor*	[57]
$C_{16}H_{12}O_5$ /284	Austrocortinin		1,4-dihydroxy-2-methoxy-7-methylanthraquinone	*Al.* sp. (SK11)	[47]
$C_{16}H_{12}O_5$ /284	Macrosporin		1,7-dihydroxy-3-methoxy-6-methylanthraquinone	*Al.* sp. ZJ9-6B *Al.* sp. ZJ-2008003 *Phomopsis* sp. PSU-MA214 *Stemphylium globuliferum Stemphylium* sp. 33231	[49] [48] [92] [94] [93]
$C_{16}H_{12}O_5$ /284	Marcrospin		1,6-dihydroxy-3-methoxy-7-methylanthraquinone	*Al.* sp. ZJ9-6B	[49]
$C_{16}H_{12}O_5$ /284	Monodictyquinone A		1,8-dihydroxy-2-methoxy-6-methylanthraquinone	*Monodictys* sp.	[14,82]
$C_{16}H_{12}O_5$ /284	Phallacinol/Fallacinol		1,8-dihydroxy-3-(hydroxy-methyl)-6-methoxyanthraquinone	*A. variecolor* B-17 *Caloplaca* spp. (e.g., *C. ehrenbergii, C. schaereri, C. spitsbergensis, etc.*)	[99] [73,74,76,77,79,96]

Table 2. *Cont.*
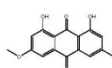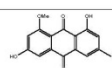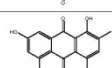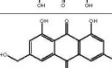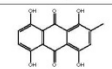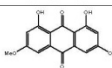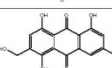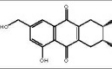
Mol. Formula/Mol. mass	Trivial name	Structure	IUPAC NAME	Source	Refs
$C_{16}H_{12}O_5$ /284	Physcion		1,8-dihydroxy-3-methoxy-6-methylanthraquinone	*Al.* sp. ZJ9-6B *A. glaucus A. variecolor* B-17 *Eurotium repens Eurotium cristatum Caloplaca* spp. (e.g., *C. ehrenbergii, C. schaereri, C. spitsbergensis, etc.*) *Letrouitia hafellneri, L. leprolytoides, Arthonia elegans, Biatorella conspersa, B. ochrophora, Pyrenula cerina, Sphaerophorus fragilis, Stereocaulon corticatulum, v. procerum, Trypethelium aeneum, T. aureomaculata*	[49] [50–53] [56] [62] [36,61] [75–77,79,96] [72,80]
$C_{16}H_{12}O_5$ /284	Questin		1,6-dihydroxy-8-methoxy-3-methylanthraquinone	*A. glaucus A. variecolor* B-17 *Eurotium cristatum* (ECE) *Eurotium rubrum*	[50],[51] [56] [52,53] [36,61,63]
$C_{15}H_{10}O_6$ /286	Catenarin		1,4,5,7-tetrahydroxy-2-methylanthraquinone	*A. glaucus A. variecolor* B-17 *Eurotium cristatum* (ECE) *Eurotium repens*	[50,51] [56] [52,53] [36,62]
$C_{15}H_{10}O_6$ /286	Citreorosein		ω-hydroxyemodin (OHM) or 1,3,8-trihydroxy-6-(hydroxymethyl) anthraquinone	*Gliocladium*. sp. T31 *P. citrinum* PSU-F51 *P. oxalicum* 2-HL-M-6	[78] [88] [91]
$C_{15}H_{10}O_6$ /286	Cynodontin		1,4,5,8-tetrahydroxy-2-methylanthraquinone	*A. glaucus*	[50–53]
$C_{15}H_{10}O_6$ /286	Lunatin		1,3,8-trihydroxy-6-methoxyanthraquinone	*Curvularia lunata*	[50,51,59,60]
$C_{15}H_{10}O_7$ /286	Tritisporin		1,4,5,7-tetrahydroxy-2-(hydroxylmethyl) anthraquinone	*A. glaucus*	[50–53]
$C_{15}H_{14}O_6$ /290	Trichodermaquinone		(2S,3R)-2,3,5-trihydroxy-7-(hydroxylmethyl)-1,2,3,4-tetrahydroanthraquinone	*Trichoderma aureoviride* (PSU-F95)	[88,98]

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Table 2. *Cont.*
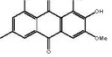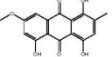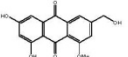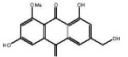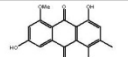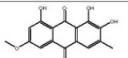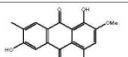
Mol. Formula/*Mol. mass*	Trivial name	Structure	IUPAC NAME	Source	Refs
$C_{15}H_{14}O_6$ /290	Demethoxyaustrocortirubin		1,4-dihydroxy-6-methylanthraquinone	*Halorosellinia* sp. No. 1403	[70,71]
$C_{15}H_{14}O_6$ /290	7-Hydroxyemodin 6,8-methyl ether		2,8-dihydroxy-1,3-dimethoxy-6-methyl anthraquinone	*A. versicolor*	[57]
$C_{16}H_{10}O_4$ /300	Erythroglaucin		1, 4, 5-trihydroxy-7-methoxy-2-methylanthraquinone	*A. glaucus A. variecolor* B-17 *Eurotium cristatum* (ECE) *Eurotium repens*	[50–53] [56] [36,61] [36]
$C_{16}H_{12}O_6$ /300	Carviolin		1,3-dihydroxy-6-(hydroxymethyl)-8-methoxyanthraquinone	*P. dravuni*	[100]
$C_{16}H_{12}O_6$ /300	Questinol		1,6-dihydroxy-3-(hydroxymethyl)-8-methoxyanthraquinone	*A. variecolor* B-17	[56]
$C_{16}H_{12}O_6$ /300	Rubrocristin		1,4,7-trihydroxy-5-methoxy-2-methylanthraquinone	*A. glaucus A. variecolor* B-17 *Eurotium cristatum* (ECE)	[50–53] [56] [36,61]
$C_{16}H_{12}O_6$ /300	6-*O*-methylalaternin		1,2,8-trihydroxy-6-methoxy-3-methylanthraquinone	*Stemphylium globuliferum*	[94]
$C_{16}H_{12}O_6$ /300	1,4,6-Trihydroxy-2-methoxy-7-methyl-anthraquinone		3,5,8-trihydroxy-7-methoxy-2-methylanthraquinone	*Halorosellinia* sp. No. 1403	[70]
$C_{15}H_9O_5Cl$ /304	7-Chloroemodin		—	*Caloplaca* spp. (e.g., *C. ehrenbergii, C. schaereri, C. spitsbergensis, etc.*)	[75–77,79,96]

Table 2. *Cont.*
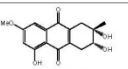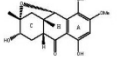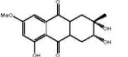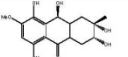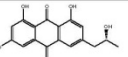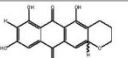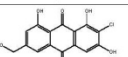
Mol. Formula/*Mol. mass*	Trivial name	Structure	IUPAC NAME	Source	Refs
$C_{16}H_{16}O_6$ /304	Altersolanol B		(2S,3R)-2,3,5-trihydroxy-7-methoxy-2-methyl-1,2,3,4-tetrahydroanthraquinone	*Al.* sp. ZJ-2008003 *Stemphylium* sp. 33231	[48] [93]
$C_{16}H_{18}O_6$ /306	Fusaquinon A		(2R,3S,4aR,9S,9aS)-3,5,8-trihydroxy-7-methoxy-2-methyl-2,3,4,4a,9,9a-hexahydro-2,9-epoxyanthracen-10(1H)-one	*Fusarium* sp. No. ZH-210	[68]
$C_{16}H_{18}O_6$ /307	Dihydroaltersolanol B		(2S,3R)-2,3,5-trihydroxy-7-methoxy-2-methyl-1,2,3,4,4a,9a-hexahydroanthraquinone	*Stemphylium globuliferum*	[94]
$C_{16}H_{19}O_7$ /311	Xylanthraquinone		—	*Xylaria* sp. 2508	[95]
$C_{17}H_{14}$ O_6 /314	Isorhodoptilometrin		(R)-1,3,8-trihydroxy-6-(2-hydroxypropyl)anthraquinone	*Gliocladium* sp. T31 *P. oxalicum* 2-HL-M-6	[78] [91]
$C_{16}H_{12}O_7$ /317	1,3,6,8-Tetrahydroxy-2-(1-hydroxyethyl) anthraquinone		1,3,6,8-tetrahydroxy-2-(1-hydroxyethyl) anthracene-9,10-dione	*Microsphaeropsis*	[81]
$C_{14}H_{11}O_5Cl$ /318	1-*O*-Methyl-7-chloroemodin		2-chloro-1,6-dihydroxy-8-methoxy-3-methylanthraquinone	*Caloplaca* spp. (e.g., *C. ehrenbergii, C. schaereri, C. spitsbergensis, etc.*)	[75–77,79,96]
$C_{15}H_9O_6Cl$ /320	7-Chlorocitreorosein		—	*Caloplaca* spp. (e.g., *C. ehrenbergii, C. schaereri, C. spitsbergensis, etc.*)	[75–77,79,96]

Table 2. *Cont.*

Mol. Formula/*Mol. mass*	Trivial name	Structure	IUPAC NAME	Source	Refs
C₁₆H₁₆O₇ /320	Austrocortirubin		1,4-dihydroxy-2-methoxy-7-methylanthraquinone	*Fusarium* spp. PSU-F14 and PSU-F135 *Halorosellinia* sp. No. 1403 *Nigrospora* sp. ZJ-2010006	[69] [70,71] [84]
C₁₆H₁₆O₇ /320	Altersolanol C		(1R,2R,3R)-1,2,3,5-tetra-hydroxy-7-methoxy-2-methyl-1,2,3,4-tetra-hydroanthraquinone	*Al.* sp. ZJ9-6B *Al.* sp. ZJ-2008003 *Stemphylium* sp. 33231	[49] [48] [93]
C₁₆H₁₆O₇ /320	4-Deoxybostrycin		(2R,3S,4aS,9aS,10R)-2,3,5,8,10-pentahydroxy-6-methoxy-3-methyl-1,3,4,4a,9a,10-hexahydroanthracen-9(2H)-one	*Nigrospora* sp. 1403 *Nigrospora* sp. MA75	[68,83,84,86,101] [85]
C₁₆H₁₆O₇ /320	2,3,5,8-Tetrahydroxy-7-methoxy-2-methyl-1,2,3,4-tetrahydroanthraquinone	—	2,3,5,8-tetrahydroxy-7-methoxy-2-methyl-1,2,3,4-tetrahydroanthracene-9,10-dione	*Al. eichorniae*	[46]
C₁₆H₁₆O₇ /321	10-Deoxybostrycin		—	*Nigrospora* sp.	[84]
C₁₆H₁₈O₇ /323	Dihydroaltersolanol C		(1R,2R,3R)-1,2,3,5-tetra-hydroxy-7-methoxy-2-methyl-1,2,3,4,4a,9a-hexahydroanthraquinone	*Stemphylium globuliferum*	[94]
C₁₆H₂₀O₇ /324	Fusaquinon C		(2S,3R,4aR,9aR,10S)-2,3,5,8,10-pentahydroxy-6-methoxy-3-methyl-1,3,4,4a,9a,10-hexahydroanthracen-9(2H)-one	*Fusarium* sp. No. ZH-210	[68]
C₁₆H₂₁O₇ /325	1-Deoxytetrahydrobostrycin		(2R,3S)-2,3,5,8,10-pentahydroxy-6-methoxy-3-methyl-1,3,4,4a,9a,10-hexahydroanthracen-9(2H)-one	*A.* sp. 05F16 *Nigrospora* sp.	[54] [83]

Table 2. *Cont.*

Mol. Formula/*Mol. mass*	Trivial name	Structure	IUPAC NAME	Source	Refs
C₁₅H₂₀O₈ /328	Fragilin		2-chloro-1,8-dihydroxy-3-methoxy-6-methylanthraquinone	*Letrouitia hafellneri L. leprolytoides*	[79,80]
C₁₇H₁₂O₇ /328	5-Acetyl-2-methoxy-1,4,6-trihydroxyanthraquinone		—	*Fusarium* sp. B77	[66]
C₁₉H₁₆O₆ /328	Isorhodoptilometrin-1-methylether		1,3-dihydroxy-6-2-hydroxypropyl-8-methoxyanthraquinone	*A. versicolor*	[57]
C₁₇H₁₄O₇ /329	1,3,6,8-Tetrahydroxy-2-(1-methoxyethyl)anthraquinone		—	*Microsphaeropsis*	[81]
C₁₈H₂₀O₆ /332	Phomopsanthraquinone		(2R,3S)-7-ethyl-1,2,3,4-tetrahydro-2,3,8-trihydroxy-6-methoxy-3-methylanthraquinone	*Phomopsis* sp. PSU-MA214	[92]
C₁₆H₁₈O₈ /336	Altersolanol A		(1R,2S,3R,4S)-1,2,3,4,5-pentahydroxy-7-methoxy-2-methyl-1,2,3,4-tetrahydroanthraquinone	*Stemphylium globuliferum Xylaria* sp. 2508	[94] [95]
C₁₆H₁₆O₈ /336	Bostrycin		(5S,6R,7S)-5,6,7,9,10-pentahydroxy-2-methoxy-7-methyl-5,6,7,8-tetrahydroanthracene-1,4-dione	*A.* sp. strain 05F16 *Al. eichorniae Fusarium* spp. PSU-F14/PSU-F135 *Halorosellinia* sp. No. 1403 *Nigrospora* sp. *Xylaria* sp. 2508	[46,102] [69] [68,86,101,103] [84] [54] [95]
C₁₆H₁₈O₈ /338	SZ-685C		1,2,3,5,8-pentahydroxy-6-methoxy-3-methyl-1,2,3,4-tetrahydroanthraquinone	*Halorosellinia* sp. No. 1403	[70,101,104–107]

Table 2. *Cont.*
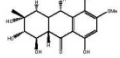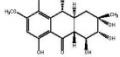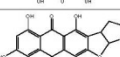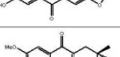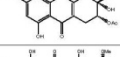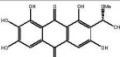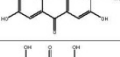
Mol. Formula/*Mol. mass*	Trivial name	Structure	IUPAC NAME	Source	Refs
$C_{16}H_{20}O_9$ /340	Fusaquinon B		(1*R*,2*S*,3*R*,4a*R*,9a*S*,10*S*)-1,2,3,5,8,10-hexahydroxy-6-methoxy-3-methyl-1,3,4,4a,9a,10-hexahydroanthracen-9(2*H*)-one	*Fusarium* sp. No. ZH-210	[68]
$C_{16}H_{21}O_9$ /340	Tetrahydroxybostrycin		1,2,3,5,8,10-hexahydroxy-6-methoxy-3-methyl-1,3,4,4a,9a,10-hexahydroanthracen-9(2*H*)-one	*A.* sp. 05F16 *Nigrospora* sp. MA75	[54] [85]
$C_{18}H_{12}O_7$ /340	Versicolorin C		4,6,8-trihydroxy-3,3a-dihydroanthra[2,3-b]furo[3,2-d]furan-5,10(2*H*,12a*H*)-dione	Fungus ZSUH-36 Fungus Isolate 1850 and isolate 2526	[65] [64]
$C_{18}H_{18}O_7$ /346	2-*O*-Acetylaltersolanol B		(2*R*,3*S*)-3,8-dihydroxy-6-methoxy-3-methyl-9,10-dioxo-1,2,3,4,9,10-hexahydroanthracen-2-yl acetate	*Stemphylium* sp. 33231	[93]
$C_{17}H_{14}O_9$ /347	1,2,3,6,8-Pentahydroxy-7-(1-methoxyethyl)anthraquinone		1,2,3,6,8-pentahydroxy-7-(1-methoxyethyl)anthracene-9,10-dione	*Microsphaeropsis*	[81]
$C_{15}H_{10}O_8S$ /350	Emodin-3-*O* Sulfate		4,5-dihydroxy-7-methyl-9,10-dioxo-9,10-dihydroanthracen-2-yl hydrogen sulfate	*P. oxalicum* 2-HL-M-6	[91]
$C_{16}H_{15}O_9$ /351	Auxarthrol C		(1*S*,2*R*,3*R*,4*R*,4a*R*,9a*S*)-1,2,3,4,5-pentahydroxy-7-methoxy-2-methyl-1,2,3,4-tetrahydro-4a,9a-epoxyanthraquinone	*Stemphylium* sp. 33231	[93]
$C_{19}H_{11}O_7$ /351	8-*O*-Methylversicolorin B		4,8-dihydroxy-6-methoxy-3,3a-dihydroanthra[2,3-b]furo[3,2-d]furan-5,10(2*H*,12a*H*)-dione	*A. versicolor* endolichenic	[108]

Table 2. *Cont.*
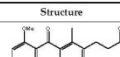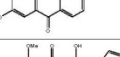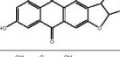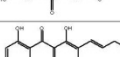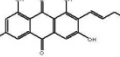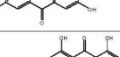
Mol. Formula/*Mol. mass*	Trivial name	Structure	IUPAC NAME	Source	Refs
$C_{21}H_{20}O_5$ /352	6,8-Dimethoxy-1-methyl-2-(3-oxobutyl)anthraquinone		—	*Fusarium* sp. ZZF60	[67]
$C_{19}H_{13}O_7$ /353	8-*O*-Methylversicolorin A		4,8-dihydroxy-6-methoxyanthra[2,3-b]furo[3,2-d]furan-5,10(3a*H*,12a*H*)-dione	*A. versicolor* endolichenic	[108]
$C_{20}H_{18}O_6$ /354	Averythrin		(*E*)-2-(hex-1-en-1-yl)-1,3,6,8-tetrahydroxyanthraquinone	*A.* sp. SCSIO F063 *A. versicolor* endolichenic	[55] [108]
$C_{30}H_{18}O_{10}$ /358	Skyrin		2,2′,4,4′,5,5′-hexahydroxy-7,7′-dimethyl-[1,1′-bianthracene]-9,9′,10,10′-tetraone	*P. chrysogenum*	[89]
$C_{16}H_{12}O_8S$ /364	Macrosporin-7-*O*-sulfate		Sodium 8-hydroxy-6-methoxy-3-methyl-9,10-dioxo-9,10-dihydroanthracen-2-yl sulfate	*Stemphylium* sp. 33231	[93]
$C_{15}H_9O_9S$ /365	Citreorosein-3-*O*-sulfate		4,5-dihydroxy-7-(hydroxymethyl)-9,10-dioxo-9,10-dihydroanthracen-2-yl hydrogen sulfate	*P. oxalicum* 2-HL-M-6	[91]
$C_{20}H_{14}O_7$ /365	6,8-di-*O*-methylversico-lorinA		4-hydroxy-6,8-dimethoxyanthra[2,3-b]furo[3,2-d]furan-5,10(3a*H*,12a*H*)-dione	*A. versicolor* endolichenic *A. versicolor* EN-7 (Genbank no EU042148)	[108] [58]

Table 2. *Cont.*

Mol. Formula/*Mol. mass*	Trivial name	Structure	IUPAC NAME	Source	Refs
C$_{21}$H$_{19}$O$_6$ /367	8-*O*-Methylaverythrin		(*E*)-2-(hex-1-en-1-yl)-1,3,6-trihydroxy-8-methoxyanthraquinone	*A. versicolor endolichenic*	[108]
C$_{20}$H$_{16}$O$_7$ /368	Aversin		4-hydroxy-6,8-dimethoxy-3, 3a-dihydroanthra[2,3-b]furo[3,2-d]furan-5,10(2*H*,12a*H*)-dione	*A. versicolor endolichenic*	[108]
C$_{20}$H$_{16}$O$_7$ /368	Aversin : (−)-isomer		4-hydroxy-6,8-dimethoxy-3, 3a-dihydroanthra[2,3-b]furo[3,2-d]furan-5,10(2*H*,12a*H*)-dione	*A. versicolor* EN-7 (Genbank no EU042148)	[58]
C$_{20}$H$_{20}$O$_7$ /372	Averantin		(*S*)-1,3,6,8-tetrahydroxy-2-(1-hydroxyhexyl)anthraquinone	*A.* sp. SCSIO F063	[55]
C$_{20}$H$_{20}$O$_7$ /372	Averantin = (*S*)-(−)-averantin		(*S*)-1,3,6,8-tetrahydroxy-2-(1-hydroxyhexyl)anthraquinone	*A.* sp. SCSIO F063	[55]
C$_{21}$H$_{18}$O$_7$ /382	6-*O*-Methylaverufin		7,9-dihydroxy-11-methoxy-2-methyl-3,4,5,6-tetrahydro-2*H*-2,6-epoxyanthra[2,3-b]oxocine-8,13-dione	Fungus ZSUH-36 *A. versicolor* EN-7	[65] [58]
C$_{20}$H$_{16}$O$_8$ /384	Nidurufin		5,7,9,11-tetrahydroxy-2-methyl-3,4,5,6-tetrahydro-2*H*-2,6-epoxyanthra[2,3-b]oxocine-8,13-dione	Fungus Isolate 1850 and isolate 2526	[64]
C$_{20}$H$_{15}$O$_8$ /386	Averufin		7,9,11-trihydroxy-2-methyl-3,4,5,6-tetrahydro-2*H*-2, 6-epoxyanthra[2,3-b]oxocine-8,13-dione	*A. versicolor* Fungus ZSUH-36 Fungus Isolate 1850 and isolate 2526	[109] [65] [64]

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Table 2. *Cont.*
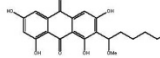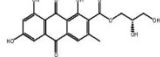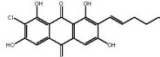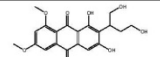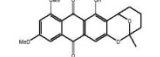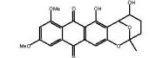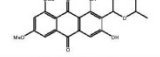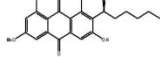
Mol. Formula/*Mol. mass*	Trivial name	Structure	IUPAC NAME	Source	Refs
$C_{21}H_{22}O_7$ / *386*	1'-*O*-Methylaverantin		1,3,6,8-tetrahydroxy-2-(1-methoxyhexyl)anthraquinone	*A*. sp. SCSIO F063 *Fungus* ZSUH-36	[55] [65]
$C_{19}H_{18}O_9$ / *388*	(2*S*)-2,3-Dihydroxy-propyl-1, 6,8-trihydroxy-3-methyl-9, 10-dioxoanthracene-2-carboxylate		(1*S*,5'*S*,6'*R*,7'*S*,8'*R*)-1',2,5',6',7',8,8'-heptahydroxy-3', 6-dimethoxy-3,6'-dimethyl-5',6',7',8',8'a,10' a-hexahydro-[1,2'-bianthracene]-9,9',10,10'-tetraone	*A. variecolor* B-17	[56]
$C_{20}H_{17}ClO_6$ / *388*	7-Chloroaverythrin		(*E*)-2-chloro-7-(hex-1-en-1-yl)-1,3,6, 8-tetrahydroxyanthraquinone	*A*. sp. SCSIO F063	[55]
$C_{20}H_{20}O_6$ / *388*	6,8-Di-*O*-methylversiconol		2-(1,4-dihydroxybutan-2-yl)-1,3-dihydroxy-6, 8-dimethoxyanthraquinone	*A. versicolor* EN-7 (Genbank no EU042148)	[58]
$C_{22}H_{20}O_7$ / *396*	6,8-Di-*O*-methylaverufin		7-hydroxy-9,11-dimetho-xy-2-methyl-3,4,5, 6-tetra-hydro-2*H*-2, 6-epoxyanthra[2,3-b]oxocine-8,13-dione	*A. versicolor endolichenic*	[108]
$C_{22}H_{20}O_7$ / *396*	6,8-Di-*O*-methylnidurufin		5,7-dihydroxy-9,11-dime-thoxy-2-methyl-3,4,5, 6-tetrahydro-2*H*-2, 6-epoxyanthra[2,3-b]oxocine-8,13-dione	*A. versicolor endolichenic A. versicolor* EN-7 (Genbank no EU042148)	[108] [58]
$C_{22}H_{22}O_7$ / *398*	6,8-Di-*O*-methylaverufanin		1,3-dihydroxy-6,8-dimethoxy-2-(6-methyltetra-hydro-2*H*-pyran-2-yl)anthracene-9,10-dione	*Fungus* ZSUH-36	[65]
$C_{22}H_{24}O_7$ / *400*	6,1'-*O*,*O*-Dimethylaverantin		(*S*)-1,3,8-trihydroxy-6-methoxy-2-(1-methoxyhexyl)anthraquinone	*A*. sp. SCSIO F063	[55]
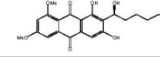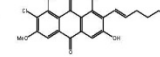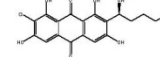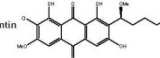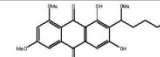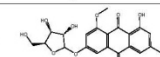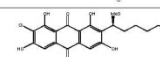
Table 2. *Cont.*

Mol. Formula/*Mol. mass*	Trivial name	Structure	IUPAC NAME	Source	Refs
$C_{20}H_{17}O_9$ / *401*	Variecolorquinone A		(*S*)-2,3-dihydroxypropyl 1,6-dihydroxy-8-methoxy-3-methyl-9,10-dioxo-9, 10-dihydroanthracene-2-carboxylate	*A.glaucus A. variecolor* B-17	[50–53] [56]
$C_{22}H_{24}O_7$ / *401*	6,8-Di-*O*-methylaveran-tin		(*S*)-1,3-dihydroxy-2-(1-hydroxyhexyl)-6, 8-dimetho-xyanthraquinone	*A. versicolor* EN-7 (Genbank no EU042148) *A*. sp. SCSIO F063	[58] [55]
$C_{21}H_{19}ClO_6$ / *402*	6-*O*-Methyl-7-chloroaverythrin		(*E*)-2-chloro-7-(hex-1-en-1-yl)-1,6,8-trihydroxy-3-methoxyanthraquinone	*A*. sp. SCSIO F063	[55]
$C_{20}H_{19}ClO_7$ / *407*	(1'*S*)-7-Chloroaverantin		(*S*)-2-chloro-1,3,6,8-tetrahydroxy-7-(1-hydroxyhexyl)anthraquinone	*A*. sp. SCSIO F063	[55]
$C_{22}H_{23}ClO_7$ / *407*	6,1'-*O*,*O*-Dimethyl-7-chloroaverantin		(*S*)-2-chloro-1,6,8-trihydroxy-3-methoxy-7-(1-methoxyhexyl)anthraquinone	*A*. sp. SCSIO F063	[55]
$C_{23}H_{26}O_7$ / *414*	6,8,1'-Tri-*O*-methyl-averantin		1,3-dihydroxy-6,8-dimethoxy-2-(1-methoxyhexyl)anthraquinone	*A.versicolor endolichenic Fungus* ZSUH-36	[108] [65]
$C_{21}H_{19}O_9$ / *415*	6-3-*O*-(Ribofuranosyl)questin		1,6-dihydroxy-6-*O*-(ribofuranosyl)-8-methoxy-3-methylanthraquinone	*Eurotium rubrum*	[63]
$C_{21}H_{21}ClO_7$ / *420*	1'-*O*-methyl-7-chloro averantin		(*S*)-2-chloro-1,3,6,8-tetrahydroxy-7-(1-methoxy-hexyl)anthraquinone	*A*. sp. SCSIO F063	[55]

Table 2. *Cont.*
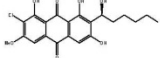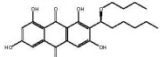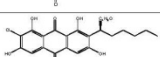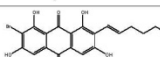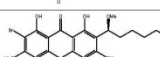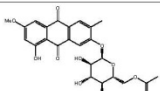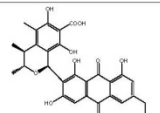
Mol. Formula/*Mol. mass*	Trivial name	Structure	IUPAC NAME	Source	Refs
$C_{21}H_{21}ClO_7$/421	6-O-methyl-7-chloro-averantin		(S)-2-chloro-1,6,8-trihydroxy-7-(1-hydroxyhexyl)-3-methoxyanthraquinone	*A.* sp. SCSIO F063	[55]
$C_{24}H_{28}O_8$/444	Averantin-1′-butyl ether		(S)-2-(1-butoxyhexyl)-1,3,6,8-tetrahydroxy-anthraquinone	*A.* sp. SCSIO F063	[55]
$C_{24}H_{27}ClO_7$/463	7-Chloroaverantin-1′-butyl ether		(S)-2-(1-butoxyhexyl)-7-chloro-1,3,6,8-tetrahydroxyanthraquinone	*A.* sp. SCSIO F063	[55]
$C_{21}H_{21}BrO_7$/465	6-O-Methyl-7-bromoaverantin		(S)-2-bromo-1,6,8-trihydroxy-7-(1-hydroxyhexyl)-3-methoxyanthraquinone	*A.* sp. SCSIO F063	[55]
$C_{22}H_{23}BrO_7$/479	6,1′-O,O-Dimethyl-7-bromoaverantin		(S)-2-bromo-1,6,8-trihydroxy-3-methoxy-7-(1-methoxyhexyl)anthraquinone	*A.* sp. SCSIO F063	[55]
$C_{24}H_{23}O_{11}$/487	Macrosporin2-O-(6′-acetyl)-a-D-glucopymnoside		((2R,3S,4S,5R,6R)-3,4,5-trihydroxy-6-(8-hydroxy-6-methoxy-3-methyl-9,10-dioxo-9,10-dihydroanthracen-2-yl)oxy)tetrahydro-2H-pyran-2-yl)methyl acetate	*Stemphylium* sp. 33231	[93]
$C_{28}H_{24}O_{10}$/520	Penicillanthranin A		(1S,3R,4S)-1-(6-ethyl-1,3,8-trihydroxy-9,10-dioxo-9,10-dihydroanthracen-2-yl)-6,8-dihy-droxy-3,4,5-trimethyliso-chroman-7-carboxylic acid	*P. citrinum* PSU-F51	[88]

Table 2. *Cont.*
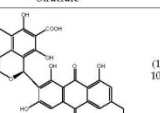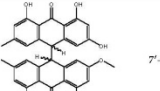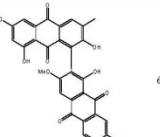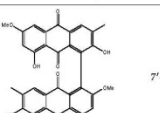
Mol. Formula/*Mol. mass*	Trivial name	Structure	IUPAC NAME	Source	Refs
$C_{28}H_{24}O_{11}$/536	Penicillanthranin B		(1S,3R,4S)-6,8-dihydroxy-3,4,5-trimethyl-1-(1,3,8-trihydroxy-6-(hydroxy-methyl)-9,10-dioxo-9,10-dihydroanthracen-2-yl)isochroman-7-carboxylic acid	*P. citrinum* PSU-F51	[88]
$C_{32}H_{24}O_8$/536	(trans)-R (cis)-Emodin-Physcion bianthrone		2,4,4′,5,5′-pentahydroxy-2′-methoxy-7,7′-dimethyl-[9,9′-bianthracene]-10,10′(9H,9′H)-dione	*A. glaucus*	[50–53]
$C_{32}H_{21}O_{10}$/565	Alterporriol Q		1′,2,7′,8-tetrahydroxy-3′,6-dimethoxy-3,6′-dimethyl-[1,2′-bianthracene]-9,9′,10,10′-tetraone	*Al.* sp. ZJ-2008003	[48]
$C_{32}H_{21}O_{10}$/565	Alterporriol R		2,4′,6′,8-tetrahydroxy-2′,6-dimethoxy-3,7′-dimethyl-[1,1′-bianthracene]-9,9′,10,10′-tetraone	*Al.* sp. ZJ-2008003	[48]
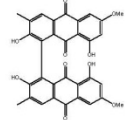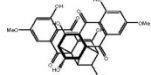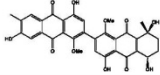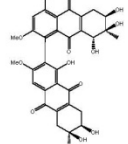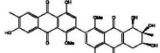
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Table 2. *Cont.*

Mol. Formula/*Mol. mass*	Trivial name	Structure	IUPAC NAME	Source	Refs
C$_{32}$H$_{22}$O$_{10}$/566	Alterporriol V		2,2′,8,8′-tetrahydroxy-6,6′-dimethoxy-3,3′-dimethyl-[1,1′-bianthracene]-9,9′,10,10′-tetraone	*Stemphylium* sp. 33231	[93]
C$_{30}$H$_{22}$O$_{12}$/574	Cytoskyrin A		(6R,14R,17S,18R,19R,20S)-1,7,9,15,17,20-hexahydroxy-3,11-dimethoxy-6,13a,5a,14-(epibutane[1–4]tetrayl)cycloocta[1,2-b:5,6-b′]dinaphtha-lene-5,8,13,16(6H,14H)-tetrone	*Curvularia lunata*	[50,51,59,60]
C$_{32}$H$_{24}$O$_{10}$/586	Alterporriol K		(5S,8R)-4,4′,5,7′,8-penta-hydroxy-1,1′-dimethoxy-6′,8-dimethyl-5,6,7,8-tetrahydro-[2,2′-bianthracene]-9,9′,10,10′-tetraone	*Al.* sp. ZJ9-6B *Al.* sp. ZJ-2008003	[49] [48]
C$_{32}$H$_{30}$O$_{12}$/590	Alterporriol T		(6R,6′S,7R,7′R,8R)-1′,4,6,6′,7,7′,8-heptahydroxy-2,3′-dimethoxy-6′,7-dime-thyl-5,5′,6,6′,7,7′,8,8′-octahydro-[1,2′-bianthracene]-9,9′,10,10′-tetraone	*Stemphylium* sp. 33231	[93]
C$_{32}$H$_{28}$O$_{12}$/601	Alterporriol L		(6S,7R,8R)-4,4′,6,7,7′,8-hexahydroxy-1,1′-dimethoxy-6′,7-dimethyl-5,6,7,8-tetrahydro-[2,2′-bianthracene]-9,9′,10,10′-tetraone	*Al.* sp. ZJ9-6B *Al.* sp. ZJ-2008003	[49] [48]

Table 2. *Cont.*
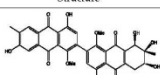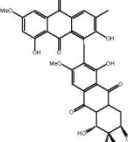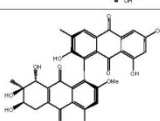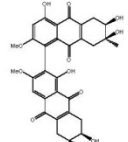
Mol. Formula/*Mol. mass*	Trivial name	Structure	IUPAC NAME	Source	Refs
C$_{32}$H$_{28}$O$_{12}$/601	Alterporriol M		(6S,7S,8R)-4,4′,6,7,7′,8-hexahydroxy-1,1′-dimethoxy-6′,7-dimethyl-5,6,7,8-tetrahydro-[2,2′-bianthracene]-9,9′,10,10′-tetranone	*Al.* sp. ZJ9-6B *Al.* sp. ZJ-2008003	[49] [48]
C$_{32}$H$_{28}$O$_{12}$/601	Alterporriol P		(5′R,6′R,7′R)-1′,2,5′,6′,7′,8-hexahydroxy-3′,6-dimethoxy-3,6′-dimethyl-5′,6′,7′,8′,8′a,10′a-hexahydro-[1,2′-bianthracene]-9,9′,10,10′-tetranone	*Al.* sp. ZJ-2008003	[48]
C$_{32}$H$_{26}$O$_{12}$/602	Alterporriol W		(1′R,6R,7R,8R)-2′,4,6,7,8,8′-hexahydroxy-2,6′-dimethoxy-3′,7-dimethyl-5,6,7,8-tetrahydro-[1,1′-bianthracene]-9,9′,10,10′-tetranone	*Stemphylium* sp. 33231	[93]
C$_{32}$H$_{30}$O$_{12}$/606	Alterporriol U		(6R,6′S,7S,7′R)-1′,4,6,6′,7,7′-hexahydroxy-2,3′-di-methoxy-6′,7-dimethyl-5,5′,6,6′,7,7′,8,8′-octahy-dro-[1,2′-bianthracene]-9,9′,10,10′-tetraone	*Stemphylium* sp. 33231	[93]

Table 2. *Cont.*
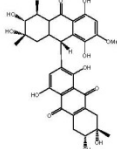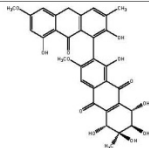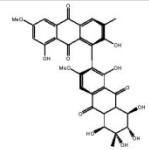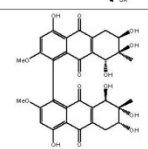
Mol. Formula/*Mol. mass*	Trivial name	Structure	IUPAC NAME	Source	Refs
$C_{31}H_{32}O_{13}/612$	Alterporriol S		(2′S,3′R,4′S,6R,7S,9′R)-1,2′,3′,4,5′,6,7,8′-octahy-droxy-7′-methoxy-2′,4′,7-trimethyl-2′,3′,4′,4′a,5,6,7,8,9′,9′a-decahydro-[2,9′-bianthracene]-9,10,10′(1′H)-trione	*Al.* sp. (SK11)	[47]
$C_{32}H_{25}O_{13}/617$	(+)-aS-alterporriol C		(1S,5′S,6′R,7′S,8′R)-1′,2,5′,6′,7′,8,8′-heptahydroxy-3′,6-dimethoxy-3,6′-dime-thyl-5′,6′,7′,8′,8′a,10′a-hexahydro-[1,2′-bianthracene]-9,9′,10,10′-tetraone	*Al.* sp. (SK11)	[47]
$C_{32}H_{25}O_{13}/617$	Alterporriol C		(1S,5′R,6′S,7′R,8′S)-1′,2,5′,6′,7′,8,8′-heptahydroxy-3′,6-dimethoxy-3,6′-dime-thyl-5′,6′,7′,8′,8′a,10′a-hexahydro-[1,2′-bianthracene]-9,9′,10,10′-tetraone	*Al.* sp. (SK11) *Al.* sp. ZJ-2008003	[47] [48]
$C_{32}H_{29}O_{14}/637$	Alterporriol N		(6R,6′R,7R,7′R,8R,8′R)-4,4′,6,6′,7,7′,8,8′-octahy-droxy-2,2′-dimethoxy-7,7′-dimethyl-5,5′,6,6′,7,7′,8,8′-octahydro-[1,1′-bianthracene]-9,9′,10,10′-tetraone	*Al.* sp. ZJ-2008003	[48]

Table 2. *Cont.*
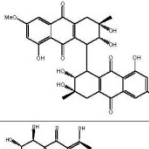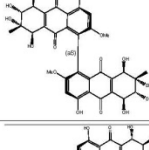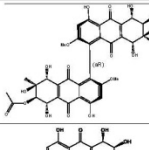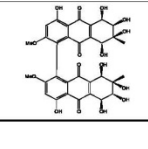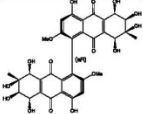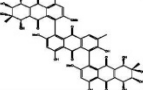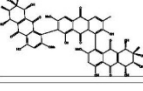
Mol. Formula/*Mol. mass*	Trivial name	Structure	IUPAC NAME	Source	Refs
$C_{32}H_{29}O_{14}/637$	Alterporriol O		(2R,2′R,3S,3′S)-2,2′,3,3′,8,8′-hexahydroxy-6,6′-dimethoxy-3,3′-dimethyl-1,1′,2,2′,3,3′,4,4′-octahy-dro-[1,1′-bianthracene]-9,9′,10,10′-tetraone	*Al.* sp. ZJ-2008003	[48]
$C_{34}H_{33}O_{17}/713$	Acetylalterporriol D		(1′S,5S,5′S,6R,6′R,7S,7′S,8R,8′R)-4,4′,5,5′,6′,7,7′,8,8′-nonahydroxy-2,2′-dimethoxy-7,7′-dimethyl-9,9′,10,10′-tetraoxo-5,5′,6,6′,7,7′,8,8′,9,9′,10,10′-dodecahydro-[1,1′-bianthracen]-6-yl acetate	*Stemphylium globuliferum*	[94]
$C_{34}H_{33}O_{17}/713$	Acetylalterporriol E		(1′R,5S,5′S,6R,6′R,7S,7′S,8R,8′R)-4,4′,5,5′,6′,7,7′,8,8′-nonahydroxy-2,2′-dimethoxy-7,7′-dimethyl-9,9′,10,10′-tetraoxo-5,5′,6,6′,7,7′,8,8′,9,9′,10,10′-dodecahydro-[1,1′-bianthracen]-6-yl acetate	*Stemphylium globuliferum*	[94]
$C_{34}H_{33}O_{17}/713$	Alterporriol D		(1S,5S,5′S,6R,6′R,7S,7′S,8R,8′R)-4,4′,5,5′,6,6′,7,7′,8,8′-decahydroxy-2,2′-dimethoxy-7,7′-dimethyl-5,5′,6,6′,7,7′,8,8′-octahy-dro-[1,1′-bianthracene]-9,9′,10,10′-tetraone	*Stemphylium globuliferum*	[94]

Table 2. *Cont.*

Mol. Formula/*Mol. mass*	Trivial name	Structure	IUPAC NAME	Source	Refs
$C_{24}H_{22}O_{17}$ /713	Alterporriol E		(1R,5S,5'S,6R,6'R,7S,7'S,8R,8'R)-4,4',5,5',6,6',7,7',8,8'-decahydroxy-2,2'-dimethoxy-7,7'-dimethyl-5,5',6,6',7,7',8,8'-octahydro-dro-[1,1'-bianthracene]-9,9',10,10'-tetraone	*Stemphylium globuliferum*	[94]
$C_{48}H_{40}O_{21}$ /952	Stemphylanthranol A		(5S,5''S,6R,6''R,7S,7''S,8R,8''R)-2',4,4'',5,5'',6,6'',7,7'',8,8',8''-dodecahydroxy-2,2'',6'-trimethoxy-3',7,7''-trimethyl-5,5'',6,6',7,7'',8,8''-octahydro-[1,1'5',1''-teranthracene]-9,9',9'',10,10',10''-hexaone	*Stemphylium globuliferum*	[94]
$C_{48}H_{40}O_{21}$ /952	Stemphylanthranol B		(5S,5''R,6R,6''R,7S,7''R,8R,8''R)-2',4,4'',5,5'',6,7,7'',8,8',8''-undecahydroxy-2,2'',6'-trimethoxy-3',6'',7,7''-tetramethyl-5,5'',6,6'',7,7'',8,8''-octahydro-[1,1'2',1''-tetranthracene]-9,9',9'',10,10',10''-hexaone	*Stemphylium globuliferum*	[94]
—	7-Chloroemodic acid	—	—	*Caloplaca* spp. (e.g., *C. ehrenbergii, C. schaereri, C. spitsbergensis, etc.*)	[75–77,79,96]
—	7-Chloroemodinal	—	—	*Caloplaca* spp. (e.g., *C. ehrenbergii, C. schaereri, C. spitsbergensis, etc.*)*, L. hafellneri L. leprolytoides*	[75–77,79,96] [80]
—	7-Chloro-1,6,8-trihydroxy-3-methyl-10-anthrone	—	—	*Caloplaca* spp. (e.g., *C. ehrenbergii, C. schaereri, C. spitsbergensis, etc.*)	[75–77,79,96]

Abbreviations: *A.*: *Aspergillus*; *Al.*: *Alternaria*; *P.*: *Penicillium*. Orange brown: ▬ ; Orange: ▬ ; Yellow: ▬ ; Red: ▬ ; Bronze: ▬ .

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The unidentified fungus ZSUH-36 (isolated from the Shenzhen mangrove plant *Acanthus ilicifolius Linn.*), the isolate 1850 (from a leaf of *Kandelia candel* from an estuarine mangrove in Hong Kong), and the mangrove endophytic strain 2526 produce versicolorin C, although the A enantiomer seems more common from the terrestrial producers *Aspergillus versicolor* and *A. parasiticus*. The two marine-derived strains, isolate 1850 and isolate 2526, also excrete the new nidurufin, along with the known averufin, also found in emerged *Aspergillus parasiticus* and *A. versicolor*. Fungus strain ZSUH-36 produces several new compounds, namely, 6,8-di-*O*-methyl averufin, 6,8-di-*O*-methyl averufanin, 1′-*O*-methyl averantin, and 6,8,1′-tri-*O*-methyl averantin.

Penicillium is a very widespread genus on earth and in marine biotopes. It seems to adjust easily to multiples conditions and to be a source of original compounds. The most commonly represented molecules in the terrestrial strains are emodin (and derivatives), rugulosin, and skyrin (or luteoskyrin), and then carviolin and chrysophanol (personal data, [110]). *Penicillium citrinum* PSU-F51 isolated from the gorgonian sea fan *Annella* sp. and *P. oxalicum* 2-HL-M-6 (from the sea mud sample) synthesize the common chrysophanol and emodin, but also citreorosein (ω-hydroxyemodin), a compound that, to date, is only isolated from the coprophilous ascomycete *Zopfiella longicaudata* IFM4630 [88,91]. Penicillanthranins A and B (two new anthraquinone-citrinin derivatives) are also excreted by *Penicillium citrinum* PSU-F51. Two new molecules—citreorosein-3-*O*-sulfate and emodin-3-*O*-sulfate as well as 1,8-dihydroxy-3-(hydroxymethyl) anthracene-9,10-dione (aloe emodin)—are engendered by *P. oxalicum* 2-HL-M-6. This strain also produces isorhodoptilometrin, which is uncommon, as it is only known from one plant-endophytic *Aspergillus* sp. (strain YL-6) [91,112]. *Penicillium chrysogenum* (from a saline lake in Antarctica) is the only aquatic fungus known for the production of skyrin, similarly to its terrestrial counterpart and four other terrestrial strains (*P. islandicum*, *Talaromyces wortmanii*, and *Dermocybe* spp.) [89]. In a related genera, *Paecilomyces* sp. (Tree 1-7), a mangrove-derived fungus from a Taiwan strait, also produces chrysophanol and emodin [87].

Endophytes and/or Pathogens

Trichoderma is a frequent genus among vegetal root-associated fungi (endophytes and symbionts). Few marine-derived strains have been identified, to date, as anthraquinoid producers. However, the frequent emodin, ω-hydroxyemodin, pachybasin, ω-hydroxypachybasin, 1-hydroxy-3-methoxyanthraquinone, 2-methylquinizarin, and chrysophanol were detected in *Trichoderma aureoviride* PSU-F95, cultured from a gorgonian sea fan *Annella* sp. [88]. This strain also produces the new trichodermaquinone (tetrahydroanthraquinone), the rare coniothranthraquinone, or isorhodoptilometrin. *Trichoderma* strains from terrestrial origin rather produce chrysophanol and pachybasin (personal data, [110]).

Several species of *Alternaria* are terrestrial plants pathogens. Terrestrial *Alternaria* anthraquinones' producers are found in many species, including *A. eichhorniae,* also isolated from marine environments. The main represented compounds in this genus are macrosporin, altersolanol A, and 6-methylxanthopurpurin-3-*O*-methyl ether. However, original compounds were mainly isolated from marine-derived isolates. Indeed, alterporriols (A, B, C), altersolanol A, austrocortinin, bostrycin, physcion, and macrosporin are excreted by *Alternaria* species, originating from marine niches. Alterporriols C is commonly found in terrestrial and aquatic strains, but, until now, alterporriol A and B are only ones known from *Alternaria*'s marine-derived strains or from the terrestrial brother-group *Stemphylium* (*S. globuliferum*). This similarity in extrolite production with this genus also concerns altersolanol A, produced by several *Stemphylium* strains, from terrestrial or salted environments (*S. botryosum v. lactucum*, *S. globuliferum*).

Concerning original compounds, *Alternaria eichhorniae*, a pathogen of the water hyacinth *Eichhornia crassipes*, produces 4-deoxybostrycin, a rare compound only identified in a marine-derived *Nigrospora* sp. [46]. *Alternaria* sp. ZJ9-6B, a mangrove strain from the South China Sea, synthesizes seven new compounds: alterporriol K, L and M, dactylariol, alternariol (AOH), alternariol methyl ether (AME),

and tetrahydroaltersolanol B [49]. The mangrove endophytic strain *Alternaria* sp. SK11, from the root of *Excoecaria agallocha* collected in the South China Sea, produces alterporriol S, (+)-aS-alterporriol C, 6-methylquinizarin along with the known austrocortinin [47]. *Alternaria* sp. ZJ-2008003, a fungus obtained from a *Sarcophyton* sp. soft coral from the South China Sea, is the only known strain to produce alterporriols N–R (five new alterporriol-type anthranoid dimers) [48].

Stemphylium globuliferum (from *Juncus acutus* collected from an hypersaline lake in Egypt) generates the common compounds alterporriol D and E, altersolanol A and B, and macrosporin, as well as the genus specific 6-*O*-methylalaternin [94]. Seven new compounds can also be found in this strain: altersolanol C, dihydroaltersolanol B and C, acetylalterporriols D and E (atropisomers), and stemphylanthranols A and B (the first naturally occurring trimeric anthraquinone derivatives). This endophytic/pathogenic fungus seems very productive in new compounds in marine environments. Indeed, the strain *Stemphylium* sp. 33231, obtained from the mangrove plant *Bruguiera sexangula var. rhynchopetala*, excreted four new alterporriol-type anthranoid dimers, along with 17 analogues [93]. In terrestrial habitats, the genus *Stemphylium* (anamorph of *Pleospora*) consists of plants pathogens/endophytes. The number of original molecules found in its productions is feeding the idea of originality in compounds coming from plant/microbe associations (alterporriol G and H (atropisomers), altersolanols K and L, as well as the new stemphypyrone) [113].

Fusarium is a widespread plant pathogen. The marine-derived *Fusarium* strains, mainly associated with marine organisms, produce four completely new anthraquinoid compounds. *Fusarium* sp. No. B77 (a mangrove endophytic strain from the south China sea) produces the new 5-acetyl-2-methoxy-1,4,6-trihydroxy-anthraquinone, and *F.* sp. ZZF60, another mangrove endophytic fungus from the same area, synthesizes the new 6,8-dimethoxy-1-methyl-2-(3-oxobutyl)-anthraquinone. The strain *F.* sp. No. ZH-210 coming from mangrove sediments of Zhuhai (China) produces the new fusaquinon B and C (red anthraquinone derivatives) along with the new fusaquinon A (colorless) [68]. *Fusarium* spp. PSU-F14 and PSU-F135 (endophytes from the gorgonian sea fan *Annella* sp., collected in Thailand) excrete the known bostrycin but also austrocortirubin, mainly known from the terrestrial macromycete *Cortinarius* spp.

Microsphaeropsis sp. (associated with the mediterranean sponge *Aplysina aerophoba*) produces 1,3,6,8-tetrahydroxyanthraquinone, also extracted from terrestrial *Geosmithia*, *Trichoderma*, and *Verticicladiella*. Three new C2-derivatives of 1,3,6,8-tetrahydroxyanthraquinone were also isolated from this marine derive fungi for the first time.

Monodictys (from a Japanese sea urchin *Anthocidaris crassipina*) is producing the common pachybasin, which was found for the first time from this species, along with emodin and chrysophanol, and also the new monodictyquinone A.

Halorosellinia sp. No. 1403 isolated from *Kandelia* sp. decayed woody tissue in Mai Po (Hong Kong, South China Sea) from a salt lake in the Bahamas excretes austrocortirubin, demethoxyaustrocortirubin, hydroxy-9,10-anthraquinone and two new compounds: 1,4,6-trihydroxy-2-methoxy-7-methylanthracene-9,10-dione and the patented 2,3,4,5,8,10-hexahydroxy-7-methoxy-3-methyl-1,3,4,10-tetrahydro-9(2*H*)-anthracenone (patented compound SZ-685C, [104]).

The endophytic *Nigrospora* sp. isolated from the mangrove plant *Bruguiera sexangula* is able to synthesize the new 1-deoxytetrahydrobostrycin (synonym: 8-hydroxytetrahydroaltersolanol B) along with bostrycin and 4-deoxybostrycin, depending on the culture media [83,114]. *Nigrospora* sp. 1403, endophytic from *Kandelia candel* in a marine mangrove (South China Sea), also produces bostrycin as two terrestrial strains [114,115] along with deoxybostrycin [86]. *Nigrospora* sp. isolated from an unidentified sea anemone excretes the new compounds 4a-*epi*-9α-methoxydihydrodeoxybostrycin and 10-deoxybostrycin along with seven known anthraquinone derivatives [84].

The new (2*R*,3*S*)-7-ethyl-1,2,3,4-tetrahydro-2,3,8-trihydroxy-6-methoxy-3-methyl-9,10-anthracenedione (new tetrahydroanthraquinone derivative) and the compound 1-hydroxy-3-methoxy-6-methylanthraquinone (first time isolation from fungi), along with macrosporin and tetrahydroaltersolanol B&C, were extracted from a culture of *Phomopsis* sp. strain (PSU-MA214)

isolated from a leaf of a mangrove plant *Rhizophora apiculata*. This is different from a terrestrial strain of *P. juniperovora* (PM0409092) producing the common altersolanol A.

Curvularia lunata (anamorphic stage of *Cochliobolus lunatus*), isolated from the marine sponge *Niphates olemda* in Indonesia, excretes the new lunatin and cytoskyrin A, a molecule only known from one another species *Cytospora* sp. CR 200 (endophyte from the buttonwood tree *Conocarpus erecta* in Costa Rica) [60].

Reviewed by Crous *et al.* [116], many species of *Arthinium* are associated with plants as endophytes or parasites, even in marine conditions. Somes strains of *Athrinium phaeospermum* cause cutaneous infections of humans. Others are involved in endophytic plant relationships, producing growth promoting substances, e.g., in *Carex kobomugi*. The endophytic *Arthrinium phaeospermum* CBS 142.55 (type strain of *Botryoconis sanguinea*) produced bostrycin with some minor unidentified red and yellow pigments when cultured on malt extract agar [117]. Bostrycin is produced by *Bostrychonema alpestre*, the causal agent of water hyacinth blight disease, as well as by other plant pathogenic fungi such as *Alternaria eichorniae*, *Nigrospora oryzae*, *Arthrinium phaeospermum* and *Fusarium* spp. *Bostrychonema alpestre* (terrestrial strains) also excretes austrocortilutein and torosachrysone mainly found in macrofungi as *Dermocybe splendida* [118].

As far as bostrycin is concerned, the originally proposed structure [119–121] was revised by Kelly and his co-workers in 1985 [122] on the basis of the total synthesis of (+/−)-bostrycin and an X-ray crystal structure of the *O*-isopropylidene derivative. This revision also concerns its derivative 4-deoxybostrycin found in serveral marine isolates [68,84,86,101,123].

A marine-derived strain of *Xylaria* sp. 2508 produces a new compound, xylanthraquinone, along with three known anthraquinones, altersolanol A, deoxybostrycin, and bostrycin [95]. The *Xylariaceae* is one of the largest families of endophytic filamentous fungi isolated from plants material in terrestrial biotopes. There, they essentially grow under the form of mycelial structure, and their fruiting bodies (stromata) seem to form only when their host is stressed or diseased. However, the pigments seem to be mainly extracted from their fruiting stages [124]. Under marine conditions the morphological structures of fungi are not yet precisely described, and researchers suppose that fungi are mainly growing under mycelial structures. Nevertheless, this does not prohibit the synthesis of these anthraquinoid molecules.

To date, over 100 different anthraquinoid metabolites have been identified in around 27 marine-derived fungal isolates, belonging to at least 22 identified species.

Lichens

Caloplaca, Collema, Collemopsidium, Lichina, Ochrolechia, Ramalina, Tephromela, Verrucaria, and *Xanthoria* species are frequent among lichenic maritime populations [125]. They are known, in marine as in terrestrial biota, to be frequent producers of the common physcion (parietin). This anthraquinoid compound is excreted by *Xanthoria aureola* and *X. parietina* collected from exposed maritime rocks (South Norway) [126,127]. A high diversity of specific compounds can, however, be obtained from lichens, particularly in species producing chlorinated anthraquinones [76,96,128]. The production of special anthraquinones is a major characteristic of most species in the family *Teloschistaceae* (*Caloplaca, Xanthoria, etc.*) [72,77]. In the genus *Xanthoria*, and in closely related species of *Caloplaca*, antraquinoid molecules seem to be the only lichenic secondary compounds present [129,130]). The majority of species produce the widespread parietin and its chemical relatives, but also fragilin, emodin, and chloroemodin, accompanied by varying amounts of their oxidation products [128,129,131]. *Caloplaca* spp. isolated from calciferous rocks in Central Asia (*C. schaereri; C. spitsbergensis, C. ehrenbergii,* and other species) effectively produce 7-chlorocitreorosein and 7-chloro-1,6,8-trihydroxy-3-methyl-10-anthrone, as well as 7-chloroemodic acid, 7-chloroemodinal, and 1-*O*-methyl-7-chloroemodin, but also fallacinol (teloschistin) and fallacinal. Many of these compounds, widespread among lichens, are not found outside from the lichen's group.

Some fungal mycobiontes involved in lichen associations also proved their capacity to produce several original anthraquinoid molecules in separate cultures. For instance, *Aspergillus versicolor*, a mycobionte in *Lobaria retigera* excretes averythin, 8-*O*-methylaverythin, 8-*O*-methylversicolorin A and B, 6,8-di-*O*-methylversicolorin A, 6,8-di-*O*-methylaverufin, and 6,8,1'-tri-*O*-methylaverantin [108].

3. Biosynthesis and Known Roles for Anthraquinones in Fungi

The metabolism is the sum of all the biochemical reactions carried out by an organism. Opposite to the primary metabolism, converging to few products common to many organisms, the secondary metabolic pathways diverge to a great diversity of molecules produced from a few key intermediates of primary metabolism. While endometabolites can be found in almost all species of fungi, exometabolites seem taxonomically shared by species–specific profiles [132,133]. Some authors then assert that the nature of the extrolites can act as signatures, specific to the organisms, coming from biogenetics patterns. Thereby, in *Dermocybe* spp., it was possible to group some subsections with regards to biosynthetis of particular anthraquinoid compounds (skyrin, icterinoidin or hypericin) [134]. Considering the significant shortcomings in these research fields, future studies will gain knowledge about the truth of these claims. Undoubtedly subservient to the genetic skills, the biosynthesis of metabolites such as anthraquinone derivatives is clearly influenced and regulated by a complex set of factors including biotic and abiotic dimensions. Such parameters as temperature, pH, $[O_2]$ availability, light exposure, nature, and abundance of nutritive sources, as well as age and specialization of the fungal structures, have already been proven to have a strong influence on pigments production [135–139].

3.1. Biosynthetic Route and Genes Involved

As polyketides seem to be the most abundant extrolites in fungi, the pathways for their biosynthesis have been widely explored [140]. However, the formation of anthraquinones in fungi is not the most developed area in the literature.

In fungi polyketide compounds are primarily synthesized by the acetate-malonate pathway (Figure 3), which is different from plants using shikimate pathways as well as acetate-malonate metabolism. Depending on the presence of acetate, malonate, or both components, according to the growth conditions, and to the strains involved, the numbers of each residue incorporated differ, and the final secondary metabolites as well. The involvement of each type of molecule can be studied through experiments using $[^{13-14}C$-acetate] and $[^{13-14}C$-malonate], ([141] and other authors such as Gessler *et al.* [17]. In relation to this acetate-malonate pathway, the biosynthetic relationships seem to form yellow hydroxyanthraquinones (e.g., emodin, physcion, and dermolutein) at the beginning of the pathway (simple structures), whereas the red ones, such as dermorubin or dermocybin, with more complex structures, certainly occur in the latter part of the biosynthesis pathway [16].

Hanson *et al.* [15] and Gessler *et al.* [17] summarized that anthraquinones synthesis are regulated by non-reducing polyketide synthases (NR-PKS's). These multidomains enzymes mediate the regioselective cyclization of polyketides, clearly dominating the final structures. NR-PKS's form polyketides in which carbonyl groups are not reduced, whereas reducing polyketide synthases partially or fully reduces the carbonyl groups [142,143]. These multifunction complexes, including acyl carrier protein (ACP), transacylase (STA), ketosynthase (KS), malonyl-CoA transacylase (MTA), thioesterase (TE), product template (PT) domain, methyltransferases, and reductases, first ensure the condensation of acetyl-CoA (starter unit) and malonyl-CoA (extender unit). This produces an instable β-polyketide chain (containing a free carboxylate group) precursor of different aromatic structures. There are several types of PKSs found in different organisms (groups I-VIII), but the principles of constructing the poly-β-keto chain are the same with all PKSs.

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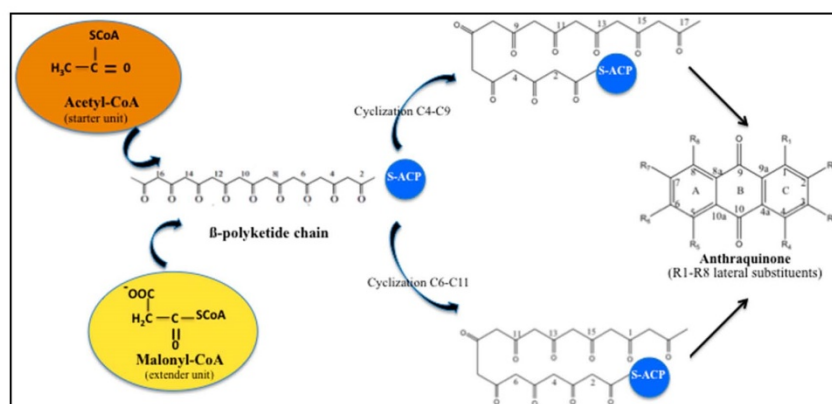
(**a**)
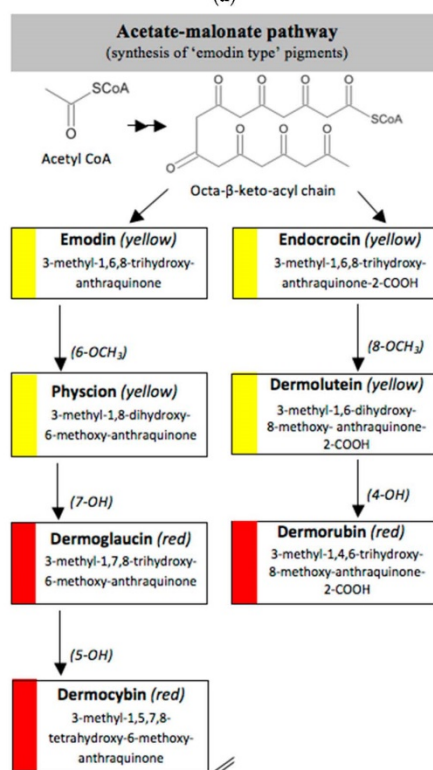
(**b**)

Figure 3. Anthraquinones biosynthetic pathway in fungi. (**a**) Regioselectivity in the formation of the β-polyketide chain during the synthesis of antraquinones in fungi (adapted from Gessler *et al.* [17]). ACP: Acyl Carrier Protein; (**b**) Anthraquinones acetate-malonate pathway in fungi: synthesis of emodin type pigments (from Caro *et al.* [16]).

From these, the fungal PKS are of considerable interest due to their interesting enzymology and the final polyketide structural diversity [144]. One of the earlier major advances in identification of fungal polyketide secondary metabolite gene clusters is the development of a degenerate-primed polymerase chain reaction (PCR), based on the conserved ketosynthase domain of PKS [145]. Javidpour *et al.* [146] asserted that the specific enzyme product template (PT) domain, determined the regioselectivity of the cyclization of the polyketide chain, and then the final structure of the products. Thus, they appeared as key factors in the biodiversity of these secondary metabolites. In fungi PT, domains are found in all 8 groups, but anthraquinoid compounds seem to be mainly produced from PT IV and V groups, indicating C4-C9 or C6-C11 cyclizations.

Based on a bioinformatical analysis, Liu *et al.* [147] interestingly clarified the relationships between enzyme sequences, structures, and functions in fungal PKS PT domains. Besides the basic PKS domain, additional functional domains, including the SAT (starter unit-ACP transacylase) domain, the PT (product template) domain, and the TE (thioesterase) releasing domain, are unique to the NR-PKSs. The PT domains have been demonstrated to be involved in controlling specific aldol cyclization and aromatization of the polyketide precursors. For the first cyclization, three commonly cyclizing patterns appear (C2-C7, C4-C9, and C6-C11). The comparison of 661 NR-PKS sequences belonging to ascomycota and basidimycota revealed that the PT domains can be classified into prominent eight groups (I–VIII) corresponding with the representative compounds and the cyclization regioselectivity. Most of the cavity lining residue (CLR) sites were common in all groups, while the regional CLR site mutations resulted in the appearance of finger-like regions with different orientation. The conservative residues in PT sequences were responsible for the cyclization functions and the evolution of the key residues resulted in the differentiations of cyclization functions. Thus, the cavity volumes and shapes, even the catalytic dyad positions of PT domains in different groups, corresponded to characteristic cyclization regioselectivity and compound sizes.

These authors also noticed that the cyclization route of the polyketide chains even differ between actinomycetes and fungi, certainly due to the involvment of different groups of PT enzymes [148]). Moreover, the late steps of the biosynthesis are responsible for the additions and deletions of lateral substituent groups, generating a great diversity of compounds. For instance, they assert that methyl groups of anthraquinones come from methionine residues via S-adenosylmethionine [149–153].

Miethbauer *et al.* [141] tried to elucidate the biosynthetic pathway of a complex anthraquinone via the synthesis of rubellins. Nineteen species of *Ramullaria* coming from different regions were tested for the production of rubellins A, B, C, D, E, and F. Seventeen biosynthesized the entire panel of rubellins, more or less intensively, except two of them: *R. pratensis* and *R. inaequalis*. They stated that, using [^{13}C-acetate], rubellins are naturally synthesized through the polyketide pathway. They demonstrated that the methyl group of acetate must be converted in part to a carboxyl one via protein turnover, or more specifically by biosynthesis, and the subsequent degradation of lysine in fungi [154]. They suggested that rubellin A and B are certainly originating from a dimeric anthraquinone and that helminthosporin (1,5,8-trihydroxy-3-methylanthraquinone) can be considered the primordial monomer. The conversion of the keto group into a lactone is supposed to be carried out by a Baeyer-Villiger monooxygenase [155]. Thus, they proved that, if the majority of the strains can use these rubellins as nonspecific toxins against plants, some of the species are, surprisingly, completely unable to produce such molecules.

The recent whole-genome sequencing of various fungi revealed that these microorganisms have immense biosynthetic potential, surpassing, by far, the chemical diversity observed in laboratory cultures. For example, the genome of many *Aspergilli* encodes a combined 30 to 80 PKS—non-ribosomal peptide synthetases and polyketide non-ribosomal peptide synthetases hybrids—which far exceed the total number of known polyketides and non-ribosomal peptides [156].

Recently, Bringmann *et al.* [157] revealed that the pigment chrysophanol can come from an organism-specific route, through a third folding mode involving a remarkable cyclization of a bicyclic diketo precursor. This establishes the first example of multiple convergences in polyketide biosynthesis.

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The programming of the fungal PKS seems quite complex and suitable to form sophisticated products. An additional level of complexity can be imagined combinatorializing PKS-based pathways with other metabolic routes.

Kakule *et al.* [158] realized a gene fusions with the idea of testing the connection and compatibility of the PKS and NRPS (nonribosomal peptide synthetase) modules, mediated by the ACP, condensation (C) and ketoreductase (KR) domains. The resulting recombinant gene fusions availed six new compounds. They obtained the first successful fusion between a PKS and NRPS that make highly divergent products as well as previously reported molecules. Thus, they demonstrated that, within the highly reducing (hr) PKS class, noncognate ACPs of closely related members can complement PKS function.

Today, it is rather clear that many of the genes involved in the polyketide pathway are organized in gene clusters, which are often silent or barely expressed under laboratory conditions. This makes their study more difficult. Fortunately, the genome sequences of several filamentous fungi are now publicly available, greatly facilitating the establishment of links between genes and metabolites. To date, complete knowledge about the biosynthetic pathway of hydroxyanthraquinonoid compounds is not yet available, but this knowledge is increasing daily.

3.2. Roles in the Biology of Fungi

Anthraquinones belong to secondary metabolites. The secondary metabolic pathways are today considered as diversions from the primary metabolism, leading to a great diversity of compounds, arising from a few key intermediates. Secondary metabolites, also known as exometabolites, are produced during morphological and chemical differentiation, either accumulated or excreted. In contrast, endometabolites (primary metabolites) fluctuate in concentrations and either transform into other endometabolites or feed into exometabolites. Secondary metabolites are bound to appear when the environmental conditions become unfavorable, in particular when a substrate other than carbon becomes limited [159]. This process of derivation is important in itself. Indeed it is supposed to provide a route for the removal of intermediates, which would otherwise accumulate. This accumulation could probably enable the primary processes leading to these intermediates to remain operational during the time of stress [160]. For researchers, this formerly implied two main features: (1) Cells that are no longer undergoing balanced growth synthesize them for a finite period; and (2) no obvious functions were clearly demonstrated in cell growth for these compounds. Thus, they appeared to researchers to be not vital to the cell life itself. Nowadays, the trend is, through their direct or indirect actions, secondary metabolites are important for the entire organism's survival [161–163]. Due to their chemical and biological properties they are today considered as first-plan actors to help in protection, competition, symbiosis, metal transport, differentiation, *etc.* The biological significance of anthraquinoids pigments, as others such as carotenoids, involves resistance to a variety of adverse environmental factors (dessication, exposure at extreme temperatures, irradiations), to antimicrobial activity (antibacterial, antiviral). Many observations have now been published with regard to the antioxidant activity of carotenoids pigments, protective action against lethal photooxydation, inhibition of mutagenesis, enhancement of the immune response, and inhibition of tumor development [164]. Melanin was also suggested to act as a shield against immunologically active cells [165].

Besides the intensively investigated fungal azaphilone pigments, anthraquinone compounds have been considered among the most abundant fungal natural products, giving color to spores, sclerotia, sexual bodies, and other developmental structures [139]. Present research suggests that, in specific cases, it is doubtful that pigments are really secondary metabolites [164]. It is now clear that anthraquinones should play a role in the cell life.

3.2.1. Anti-Oxidant/Pro-oxidant Activities

Solar radiations contain stress factors for living organisms, especially UV-B. Thus, the biological role of pigments, as the dark brown to black melanin and melanin-like pigments, often restricted

to certain developmental stages or special structures (chlamydospores or microsclerotia), is clearly dedicated to growth, dissemination, and survival under unfavorable conditions. The accumulation of pigment acts as a radiation-screening system that prevents the occurence of damages. It is clear that, in unusual biotopes (sometimes extreme), the fungal species with pigmented cell walls (in the sexual stages, conidia, and/or mycelium) are able to tolerate dehydration-hydration cycles or high solar radiations better than the moniliaceous fungi, whose cells are devoid of pigments. These aromatic compounds, such as melanin, sporopollenin (brown product of oxidative polymerization of β-carotene) and cycloleucomelone (terphenylquinone) [18], often show significant antioxidant activities and are bound to protect the biological structures, giving them an excellent durability and a high potential for survival in hostile environments. However, at higher doses or under certain conditions, antioxidant-type functional compounds may exert toxic pro-oxidant activities, as was demonstrated *in vitro* for vitamin C, E, carotenoids or flavonoids [166]. This is not a new idea, as Paracelsus (1493–1541) already reported toxicity to be a matter of dose. Thus, toxicological risks may arise when daily doses of a compound rise above a certain threshold limit.

Things are not so clear about the relationships between carcinogenic effect and pro-oxidant properties. Indeed, pro-oxidant natural products inducing ROS (reactive oxygen species) were proved to contribute to anticancer effects as for campothecin derivatives [167]. Pro-oxidant natural products then become an attractive anticancer strategy.

Identically, quinonoid structures allow anthraquinones to participate in redox reactions, exhibiting antioxidant or pro-oxidant properties. Emodin and physcion have clearly demonstrated antioxidant and antimicrobial activities [168]. According to Yen *et al.* [169], the basic anthrone chemical structure exhibited the role of electron acceptor, and the ortho-dihydroxy substituent in alizarin, the polyhydroxyl group at position C1, C6 and C8 with methylation at position C3 (emodin), and the polyhydroxyl group at position C1 and C8 with hydroxylmethylation at position C3 (aloe-emodin), are multifunctional antioxidants, combining both chain-breaking and metal-chelating properties. Moreover, they stated that the greater reducing power and the metal chelating activity of alizarin may relate to its marked antioxidant activity. On the other hand, the significant scavenging effect of emodin and aloe-emodin on hydroxyl radicals may contribute to their antioxidant activity. Alaternin also inhibited the peroxidation of linoleic acid by the thiocyanate method in a dose-dependent manner, and showed inhibitory activities in reactive oxygen- and nitrogen-mediated reactions, indicating that it is a potentially effective and versatile antioxidant useful, for protecting biological systems and functions against various oxidative stresses [170]. On the other hand, chrysophanol accelerated the peroxidation of linoleic acid.

Another illustration is the symbiotic foliose lichen *Xanthoria parietina*, which is able to colonize very extreme environments. The cortical anthraquinoid pigment parietin (physcion) is mainly synthesized by the mycobionte and absorbs UV-B and part of PAR (photosynthetically active radiations). Its production depends on several factors. The UV-B induction (280–320nm) and the photosynthetic assimilation of carbon in the photobionte, the green algae *Trebouxia arboricola*, increases the production of parietin; the lack of PAR, dessication, or substancial depression decreases the production [171,172]. Conversely, in artificial culture conditions, it seems that light in the visible part of the spectrum significantly decreases the pigment production in *Isaria farinosa*, *E. nidulans*, *F. verticilloides*, and *P. purpurogenum* [173].

There is therefore a strong correlation between the anthraquinoid contents and site factors, including the openness of the habitat, suggesting that solar radiation plays a crucial role.

3.2.2. Competition and Symbiosis

Endophytic/symbiotic associations may clearly involve some complex regulation mechanisms. Cases of interspecific regulations are already recorded concerning anthraquinones. *In vitro*, anthraquinones production in *Aspergillus kanagawaensis* is, for instance, stimulated by co-cultures with different species [174]. The study also proved that a hydroxyanthraquinone molecule was able

to stimulate the synthesis of exoproteases, in *Aspergillus kanagawaensis* cultures alone and during co-culture with *A. wentii*.

Trichoderma is a frequent genus among vegetal roots associated fungi (endophytes, symbionts) [175]. By colonizing the surface of pinaceae roots, *Trichoderma viride*, for instance, is supposed to confer physiological benefits to the seedlings, enhancing the plant-soil exchanges and probably exerting a photoprotective activity [176]. From this review, this genus is also mentioned to suppress fungal disease in plants and soils, and also to promote plant growth [177]. The frequent antimicrobial activity of anthraquinones clearly enhances the host plant resistance against pathogens (bacteria, fungi). This led, for instance, to a patent describing a plant protection product, using a formulated Polygonaceae extract (*Reynoutria sachalinensis*) marketed as REGALIA® SC by Marrone Bio Innovations, Inc. (Davis, CA, USA) [178]. The MBI-106 active principle contains at least physcion, emodin, chrysophanol, ventiloquinone, emodin glycoside, chrysophanol glycoside, physcion-glycoside, 3,4-dihydroxy-1-methoxyanthraquinone-2-corboxaldehyde, and damnacanthal. The results support a general hypothesis that the presence of the functional metabolites of endophytes enhances the host plant fitness and competitivness in stressful environments [179]. Moreover, they certainly play an important part in inter- or intra-specific competions, combating other microbes in natural micro-environments.

From a functional point of view, in the review of Gessler [17], catenarin and emodin were able to inhibit the DNA-dependant RNA-polymerase of *E. coli*. In *Trichoderma*, the increase in excreted emodin or pachybasin concentrations increases the concentration of cAMP, indicating that these compounds are key substances in the regulation of this secondary messenger and thereby in intracellular signaling. Alterporriol-type dimers from *S. globuliferum* and altersolanol from the endophytic *Ampelomyces* sp., as well as many other anthraquinones, are able to inhibit several protein kinases, playing significant roles in host cells proliferation (PKC-e, CDK4, EGF, *etc.*). The anticancer properties of some anthraquinones can also be partially explained through the inhibition of the Cdc25 phosphatase, demonstrated for emodin, questin, and physcion, as well as through the capacity to activate the capsase's cascade, inducing apoptosis of tumor cells.

The vast array of metabolites produced by microbes in their growth environments undoubtedly has an ecological role in regulating the interactions between plants, microorganisms, insects, and animals. Highlighting these facts, we should ask ourselves about the high capacity of endophytes or symbionts to synthesize original molecules arising from the interactions with their host organisms. Many questions then arise about the contribution of partners in such associations, especially concerning anthraquinoid compounds.

3.2.3. Chelating Properties

Greenaway *et al.* [180] proved that the red anthraquinoid pigment from *Pyrenophora avenae* (1,4,5,8-tetrahydroxyanthraquinone) removed phenyl-Hg^+ ions (Phenyl Mercuric Acetate (PMA)) from an aqueous solution. They stated that strains secreting a visible amount of the pigment were invariably resistant to PMA, and those lacking the pigmentation were susceptible. They noticed that resistance to PMA and pigment production were linked in the segregation of resistant and susceptible progeny of conidiospore from a resistant colony. They concluded that the chelation of the Hg ions by the anthraquinone molecule produces a stable and non-toxigenic complex, protecting the cells proteins from binding with toxic mercury through their sulfhydryl groups. This demonstrated the detoxification potential of anthraquinoid pigments in strain metabolism. However, they demonstrated that pigment-producing strains grew more slowly than unpigmented ones in media lacking PMA. They suggested that an unsaturated chelator produced by resistant strains could act to their detriment by chelating substances required for growth. Several articles refer to the metal-chelating properties of anthraquinoid compounds *in vitro* (such as copper and palladium [181]). These favorable chelating properties towards toxic ions such as aluminium were also demonstrated in plants by studying the impact of soil anthraquinones on the growth of *Alfalfa* and lettuces [182]. However, they stated that

the chelating properties were depending on the structure: Some anthraquinones presented an inactive form (glycoside form), while others demonstrated effective chelating properties (mainly monomeric: physcion, emodin, and chrysotalunin).

4. *In Vitro* Biological Activities of Natural Anthraquinones from Marine-Derived Filamentous Fungi

In recent years, marine microorganisms are increasingly attracting attention of the pharmaceutical community as they produce a wide variety of secondary metabolites that are structurally unique and pharmacologically active [104]. Naturally occurring anthraquinones have been recognized to possess anti-inflammatory, anti-fibrotic, and antitumor properties and thus are applied in human and veterinary therapeutics as active substances of medicinal products. Huang *et al.* [183] demonstrated that anthraquinones isolated from plants, such as emodin, aloe-emodin, and rhein, inhibit the growth and proliferation of various cancer cells, such as lung adenocarcinoma, myelogenous leukemia, neuroblastoma, hepatocellular carcinoma, bladder cancer, and others. The chemical diversity and biological activities of fungal anthraquinone and derivatives from marine origin are considerable; thus, they may represent a promising resource for identifying new therapeutic drugs candidates [184].

4.1. Antitumor Activity and Cytotoxicity

Cancer has become an increasing public health problem due to its high rates of morbidity and mortality. Conventional cancer chemotherapy has the limitation of multidrug resistance caused by an overexpression of integral membrane transporters, which can efflux intracellular anticancer drugs, thus decreasing drug accumulation. Clinical multidrug-resistant (MDR) cells are resistant to cytotoxic effects of various structurally and mechanistically unrelated chemotherapeutic agents. Developing new anticancer drugs that are efficient to MDR cells is a feasible strategy to overcome MDR. In the discovery of new fungal anthraquinone-derivative therapeutic molecules, cytotoxic activity is one of the prominent assays to develop novel anticancer drug candidates [184].

Cancer development largely results from uncontrolled growth of malignant cells in which cell proliferation surpasses cell death. Deregulation of apoptosis, occurring in a majority of cancer types, has since become a non-negligible target for anticancer strategies. Pro-apoptotic compounds derived from marine sources, are thus under active investigation for their therapeutic effects, and for their mode of actions against various cancers as well [104].

Apoptotic cells are usually characterized by several distinctive morphological and biochemical changes, including cell shrinkage, chromatin condensation, presence of phosphatidylserine on cell membrane surface, DNA fragmentation, protein cleavage at specific locations, and increased mitochondrial membrane permeability. A cascade of caspases activation in an ordered and regulated manner is also known to be involved in apotosis as a suicidal process [104].

4.1.1. Breaking the Cell Cycle/Apoptosis Hallmarks

Norsolorinic acid, a fungal anthraquinone derivative originally isolated from the fungus *Aspergillus nidulans*, was investigated for its anti-proliferative activity in human breast adenocarcinoma MCF-7 cells by Wang *et al.* [185]. The results showed that it induced apoptosis of MCF-7 cells.

Using the yeast *Saccharomyces cerevisiae* as a model, Xu *et al.* [103] showed that bostrycin inhibits cell proliferation by blocking the cell cycle at G1 phase. Bostrycin-induced lethal cytotoxicity is actually accompanied with increased levels of intracellular reactive oxygen species and hallmarks of apoptosis such as chromatin condensation, DNA fragmentation, and externalization of phosphatidylserine. This compound also decreases mitochondrial membrane electric potential and causes mitochondrial destruction during the progression of cell death. In this case, apoptosis appears as a mitochondria-mediated but caspase-independent pathway.

In 2011, Ren *et al.* [186] reported the isolation of nidurufin, a hydroxy derivative of averufin, from the marine-derived fungus *Penicillium flavidorsum* SHK1-27. An evaluation of the antitumor activity

indicated that nidurufin induced *in vitro* cell cycle arrest at G2/M transition in the leukemia K562 cell line, in a concentration and time-dependent manner, with an IC_{50} value of 12.6 μM.

Research findings on emodin-induced cytotoxicity and its protective effects against cancer in different body systems, as well as the antitumor mechanisms involved, are well documented in the literature, though still in progress. This compound (1,3,8-trihydroxy-6-methyl-anthraquinone) is reported to have multiple biological activities including antimicrobial, antiviral, anti-inflammatory, anti-ulcerogenic, immunosuppressive, chemo-preventive, and antitumor functions on digestive, respiratory, reproductive, and blood system cancers. It was first assigned to be a specific inhibitor of the protein tyrosine kinase p65lck. Its inhibitory effect on mammalian cell cycle modulation studied in specific oncogene overexpressed cells formed the basis of using this compound as an anticancer agent [187].

Recently, quinofuracins A–E, novel anthraquinone derivatives containing β-D-galactofuranose that were isolated from the fungus *Staphylotrichum boninense* PF1444, also induced p53-dependent cell death in human glioblastoma LNZTA3 cells [188].

4.1.2. Deregulation of ALAS2/c-KIT/miR-221, miR-222, miR-200c, miR-205/Akt

Other pharmaceutical studies have shown that emodin may induce apoptosis but also reverse multidrug resistance in HL-60 and HL-60/ADR cells. It may improve the expression of globin genes in leukemia K562 cells. It also induces K562 cells to erythroid differentiation, possibly *via* upregulating ALAS2 and c-KIT, and downregulating miR-221 and miR-222 [189]. Furthermore, Chen *et al.* [190] reported that emodin exhibited significant anti-leukemic effects *in vitro*. Increasing a dose of emodin could effectively induce growth inhibition and apoptotic effects in NB4 and MR2 cell lines, as well as in primary leukemic cells from acute myeloid leukemia (AML) patients in a dose-dependent manner. This clonal hematopoietic stem cell disorder is characterized by differentiation arrest, inappropriate proliferation, and survival of immature myeloid progenitors. Importantly, emodin was demonstrated as a new inhibitor of phosphatidylinositol 3-kinase (PI3K)/Akt in AML cells. Indeed, cell death and survival are under modulation of a network of transmembrane and/or intracellular signals in which the PI3K/Akt pathway is a prominent mediator of the survival and the proliferation. PI3K/Akt signaling pathway is frequently activated in AML cells and in other malignant phenotypes in a wide variety of cancers. Akt's phosphorylation occurs in response to PI3K activation. Thus, the interruption of the PI3K/Akt signaling pathway should be considered when designing anti-AML therapeutic strategies [190]. Emodin inhibited Akt phosphorylation (p-Akt) at Ser473 as efficiently as mTOR at Ser2448. Consistently, it exerted suppression effects on the phosphorylation of mTOR downstream targets, *i.e.*, 4E-BP1 and p70S6K. Therefore, they provided a demonstration that nontoxic dose of emodin inhibited growth and induced differentiation to a low degree in NB4 and MR2 cells. Moreover, the apoptotic induction in AML cells was associated with the activation of caspase cascades, involving caspase-9, caspase-3, and poly(ADP-ribose) polymerase cleavage. Taken together, these findings indicate that emodin might be considered a promising anti-leukemic agent to overcome all-*trans* retinoic acid-resistance and to improve the patient outcome in AML.

In this topic, previous studies reported the isolation of five anthraquinone derivatives from the marine endophytic fungus *Halorosellinia* sp. No. 1403 [70]. One of these compounds, e.g., the marine anthraquinone called SZ-685C showed strong cytotoxicity toward KB and KBv200 cancer cell lines, with IC_{50} values of 1.40 and 2.58 μg/mL, respectively [70]. This compound inhibits the growth of six tumor cell lines, including human glioma, hepatoma, prostate cancer, MCF-7, and MDA-MB-435 breast cancer cell lines, with IC_{50} < 10 μM [104]. Furthermore, *in vivo* experiments showed that SZ-685C inhibits the tumor growth in nude mice by inducing apoptosis via the Akt/forkhead box protein pathway [104]. Subsequently, Zhu *et al.* [106] found that this marine anthraquinone derivative causes apoptosis in adriamycin-resistant human breast cancer cells, both *in vitro* and *in vivo*. It exerts these antitumor effects through multiple mechanisms mainly involving the suppression of Akt signaling. More recently, Chen et al. [191] reported that SZ-685C significantly inhibited the proliferation of MMQ

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pituitary adenoma cells and induced apoptosis by downregulation of miR-200c. In addition, this compound showed potent anticancer activity in radiosensitive and radioresistant nasopharyngeal carcinoma cells, and the miR-205-PTEN-Akt pathway is the mechanism underlying the anticancer activity [192].

4.1.3. Capsase Dependant Pathway Disturbance/Topoisomerase Inhibition

In 2013, Teiten *et al.* [193] reported that altersolanol A, a natural anthraquinone derivative originally isolated from the endophytic fungus *Stemphylium globuliferum*, showed cytotoxic, cytostatic, anti-inflammatory, and anti-migrative activity against human cancer cell lines (chronic myeloid K562 leukemia and A549 lung cancer cells) in a dose-dependent manner. Interestingly, this compound did not affect the viability of non-cancerous cells. Results clearly demonstrated that altersolanol A induces cell death by apoptosis through the cleavage of caspase-3 and -9, and through the decrease of antiapoptotic protein expression. Acetylation of altersolanol A did not improve activity, whereas other altersolanol derivatives such as tetrahydroaltersolanol B and ampelanol (one of the carbonyl group reduced and some hydroxyl substituents removed) were inactive in comparison.

More recently, a novel anthraquinone derivative with a complex skeleton, naphtho[1,2,3-*de*]chromene-2,7-dione skeleton, identified as aspergiolide A, was isolated from the marine-derived fungus *Aspergillus glaucus* [194]. The compound exhibited distinct cytotoxicities against cancer cell lines A-549, HL-60, BEL-7402, and P388. It was demonstrated that aspergiolide A had anticancer activity targeting topoisomerase II. It clearly decreased the growth of various human cancer cells *in vitro* and induced apoptosis in BEL-7402 cells via a caspase-dependent pathway. *In vivo*, aspergiolide A exhibited significant anticancer activity on the growth of hepatocellular carcinoma xenografts. The maximal tolerable dose of aspergiolide A was more than 400 mg/kg, and it was not considered to be potentially genotoxic or cardiotoxic.

Finally, the marine anthraquinone compound called G503 was isolated in 2014 from the secondary metabolites of the mangrove endophytic fungus *Halorosellinia* sp. No. 1403. This anthraquinone derivative was reported to have antitumor activity [195]. The experiments suggested that the intrinsic mitochondrial apoptosis pathway was also involved in G503-induced apoptosis. The endoplasmic reticulum apoptosis pathway might also be activated by G503 by inducing capase-4 cleavage. In consideration of this inhibition effect on gastric cancer cells, the marine anthraquinone derivative G503 may serve as a promising candidate for gastric cancer chemotherapy.

4.1.4. Cytosolic free Calcium Flux Modification/Reactive Oxygen (ROS) Formation/Mitochondria Dependant Apoptosis

Huang *et al.* [49] isolated three new bianthraquinone derivatives, alterporriol K, L, and M, from the endophytic mangrove fungus *Alternaria* sp. ZJ9-6B. Of these three derivatives, alterporriol K and L were moderately active against MDA-MB-435 and MCF-7 human breast cancer cell lines (IC_{50} values of 13.1–29.1 µM) [49]. Moreover, alterporriol L could induce cancer cell apoptosis or necrosis. Furthermore, the reactive oxygen species production, mitochondrial membrane potential, and cytosolic free calcium level were changed after treatment with alterporriol L, suggesting that alterporriol L played vital roles in breast cancer cells, through destroying the mitochondria [196]. The bianthraquinone derivative alterporriol F was previously isolated from the pathogenic fungus *Alternaria porri*. This compound was found to be highly cytotoxic towards HeLa and KB cells, with IC_{50} values of 6.5 and 7.0 µM [197].

Recently, Hu *et al.* [198] reported that rhein could inhibit the purinergic $P2X_7$ receptor-mediated rat peritoneal macrophages responses, such as increases in the intracellular cytosolic calcium concentration, pore formation, reactive oxygen species production, attenuation of phagocytosis, and cell apoptosis. $P2X_7$ receptor plays important roles in inflammation and immunity. Thus, rhein can be considered as a potential antagonist of the purinergic $P2X_7$ receptor, which is a potential therapeutic target for inflammatory diseases.

4.1.5. Transport Inhibition/Synergic Effects

In 1994, the compound 3,9-dihydroxy-1-methoxy-7-methyl-anthraquinone, identified as the antibiotic C3368-B produced by the fungus strain, *Chrysosporium verrucosum* Tubaki, was found to be a highly-active nucleoside transport inhibitor. This fungal anthraquinone derivative was shown to markedly inhibit thymidine and uridine transport in Ehrlich carcinoma cells, with the half-maximal inhibitory concentration (IC_{50}) values of 7.5 and 9.6 μM, respectively. It showed fairly low cytotoxicity towards tumor cells. The IC_{50} values for epidermoid cancer KB cell lines and hepatoma BEL-7402 cells in clonogenic assays were 77 and 69 μM, respectively. At relatively non-cytotoxic concentrations, it markedly enhanced the cytotoxicity of methotrexate, 5-fluorouracil and mitomycin C against KB cells and BEL-7402 cells. It was also found to partly reverse the multi-drug resistance to vincristine and actinomycin D in mouse leukemia L1210/MDR cells. The IC_{50} values were reduced by 4.9-fold (1.75 to 0.36 μM) for vincristine and 3.3-fold (0.39 to 0.12 μM) for actinomycin D. The results suggest that this anthraquinone derivative may be potentially useful in cancer chemotherapy [199].

At present, the role of emodin in combination chemotherapy with standard drugs, in order to reduce toxicity and to enhance efficacy, is pursued vigorously [200]. For example, the synergic effect of arsenic trioxide (currently used to treat acute promyelocytic leukemia), with emodin in combination with clinically achievable doses of docosahexaenoic acid, reduced arsenic concentrations by 100-fold, while still remaining highly toxic to tumor cells [201]. It was also displayed that emodin enhanced the activity of gemcitabine against pancreatic cancer in mice by promoting the mitochondrial-dependent apoptotic pathway. Gemcitabine is currently the standard first-line chemotherapeutic agent for pancreatic cancer [202].

4.1.6. Regulation of Fibrotic and Tumorigenic Mediators

In a way similar to emodin, clinical studies and experiments with animal disease models or different functional cells demonstrated that the anthraquinone rhein (4,5-dihydroxyanthraquinone-2-carboxylic acid) exerted multiple functions including anti-carcinogenesis, antioxidant, anti-inflammation and immunosuppression [198]. For instance, its application notably suppressed the mRNA and protein levels of various fibrotic and tumorigenic mediators, including alpha-smooth muscle actin, type I collagen, fibronectin, N-cadherin, and matrix metalloproteinases in several mammalian cells (rat pancreatic stellate cells, human pancreatic ductal adenocarcinoma cells, and human colon carcinoma cells SW480 and SW620) [203].

4.1.7. Limitation of Vascularization

Recently, the effect of emodin on the growth of transplanted U14 cervical cancer cells in mice and its antitumor mechanism were reported by Zhang *et al.* [204]. Emodin might suppress the growth of cervical cancer by reducing tumor neovascularization, decreasing macrophage's migration inhibitory factor expression and promoting tumor cell apoptosis. The tumor inhibition rates were 15.83%, 46.92%, and 51.22% in the low-dose emodin group (20 mg/kg), high-dose emodin group (40 mg/kg), and cisplatin group (3 mg/kg), respectively. The tumor inhibition rates were higher in the latter two groups than that in low-dose emodin group. It was also revealed that emodin attenuated tumor cell-induced metastasis and angiogenesis, both *in vitro* and *in vivo* [205]. Finally, emodin has been reported to inhibit the growth of pancreatic cancer PANC-1 cells, which may be related to the demethylation of tumor suppressor genes. The related mechanism may be through the inhibition of methyltransferase expression [206]. Consequently, these results provide important insights into emodin as an anti-invasive agent, for the therapy of human pancreatic cancer at least.

4.1.8. Induction of DNA Damages

Two furofuran precursors of sterigmatocystin, versicolorin A and versicolorin B (*i.e.*, two anthraquinone derivatives), were identified in the culture of the fungus *Aspergillus versicolor* [207]. In a

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cytotoxicity study of these molecules on human adenocarcinoma lung cells A549, the IC_{50} values in the MTT assay (3-(4,5-dimethyl-2-thiazolyl)2,5-diphenyl-2*H*-tetrazolium bromide cell proliferation assay) were as follows: 109 μM for versicolorin A and 172 μM for versicolorin B. The two compounds were found to exert significant DNA damages, compared to the control in the comet assay. Versicolorin B produced the highest DNA damages [207].

4.1.9. Hydroxy Groups and Hydrogen Bonding between Biomacromolecules

In 2010, Zhang *et al.* [184] reported the isolation of fourteen hydroxyanthraquinone derivatives from the mangrove fungi *Guignardia* sp. No. 4382 and *Halorosellinia* sp. No. 1403. Some of these hydroxyanthraquinones showed potent cytotoxicity to drug-sensitive parental KB and KBv200 cancer cell lines. Among them, one monohydroxyanthraquinone— 1-hydroxy-3-methyl-anthraquinone—displayed strong cytotoxicity with IC_{50} values of 3.17 and 3.21 μM to KB and KBv200 cancer cell lines, respectively (MTT assay). The authors suggested that various substituting groups modulated the different contributions of these natural hydroxyanthraquinones towards the anticancer activity. It is believed that hydroxy groups perform important roles by offering hydrogen bonding with biomacromolecules such as proteins. At the same time, the quantity and location of hydroxy groups are fundamental to the activities of the anthraquinone compounds [208]. The compound 1-hydroxy-3-methylanthraquinone, containing only one hydroxy group on position R1, showed the most potent inhibition of growth of KB cells and KBv200 cells. Interestingly, dihydroxyanthraquinones with 1-hydroxy and another hydroxy on another carbon led to the decrease of anticancer activity (*i.e.* 1,8-dihydroxyanthraquinone, 1,8-dihydroxy-3-methylanthraquinone or 1,3-dihydroxy-6-methoxy-8-methylanthraquinone). Hydroxyanthraquinones containing three hydroxy groups at different carbons showed their cytotoxicity IC_{50} values more than 500.0 μM, (*i.e.*, 1,4,7-trihydroxy-2-methoxy-6-methylanthraquinone and 1,3,8-trihydroxy-6-methylanthraquinone). It seems that multi-hydroxy-substitution was unfavorable to the cytotoxic activity. The compound 1-hydroxy-3-methylanthraquinone also induced apoptosis, probably related to mitochondrial dysfunction. Indeed, this compound did not intercalate into DNA according to a DNA binding assay and implied that apoptosis induced by this fungal monohydroxyanthraquinone might not involve DNA intercalation. The apoptosis rates of drug-treated cells with 12.0 μM of this monohydroxyanthraquinone for 48 h were 25.3 and 26.4 for KB and KBv200 cancer cell lines, respectively [184].

4.1.10. Other Compounds

Nemoto *et al.*, in 1996 [209], investigated the antimicrobial and antitumor activities of three benz[a]anthraquinone derivatives, *i.e.*, brasiliquinones A, B, and C (isolated from the pathogenic strain *Nocardia brasiliensis*), against LI210, P388, and drug resistant P388/ADR tumor cells. The benz[a]anthraquinone derivatives brasiliquinones B and C were more effective against L1210 tumor cells than brasiliquinone A. The IC_{50} values of the brasiliquinones B and C against L1210 and P388 tumor cells ranged from 2.9 to 7.0 μg/mL. The 3 compounds were active against Gram-positive bacteria including *Mycobacterium* sp.

According to the study by Ge *et al.* [210], three anthraquinone derivatives isolated from the culture of the endophytic fungus *Pleospora* sp. IFB-E006, *i.e.*, altersolanol B, deoxybostrycin, and dactylariol, exhibited significant cytotoxic activity against human colon cancer (SW1116) and leukemia (K562) cell lines, while physcion, 7-methoxy-2-methyl-3,4,5-trihydroxyanthraquinone, and pleospdione (hexahydroanthraquinone) were only weakly or moderately active.

The marine anthraquinone derivative, anhydrofusarubin isolated from the marine endophytic fungus *Fusarium* sp. No. b77, showed a significant inhibition of the growth of HEp2 and HepG2 cells, with IC_{50} values of 8.67 and 3.47 μM, respectively [66,211].

Metabolomic investigations focusing on the marine-derived fungus *Aspergillus* sp. SCSIO F063 have unveiled seven new chlorinated anthraquinones related to averantin, a well-known fungal

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anthraquinone derivate. One of them, 6-*O*-methyl-7-chloroaverantin, displayed significant inhibition activity against three human tumor cell lines (SF-268, MCF-7, and NCI-H460) with IC_{50} values of 7.11, 6.64, and 7.42 μM, respectively [55].

In a recent review, Wang *et al.* [101] have summarized the sources and structures of 110 natural compounds including marine anthraquinone derivatives and other marine anthracene-9,10-diones mainly extracted from mangrove-derived fungi. They focused on their bioactivities reported between 2008 and mid-2013 and mentioned several moderate to significant anticancer activities.

4.1.11. Carcinogenic Effects

On the other hand, Ueno *et al.* [212] reported that the anthraquinone derivative rugulosin produced by fungi such as *Penicillium rugulosum* exhibited hepatocarcinogenic effects on male mice.

Moreover, the safety and effectiveness of emodin in naturopathic treatment have not been approved by the U.S. Food and Drug Administration (FDA). Side effects of emodin actually include potential carcinogenesis, nausea, diarrhea, and renal failure. Li *et al.* [213] reported that emodin showed no mutagenic activity in the *Salmonella* mutation assay, but caused genotoxicity in the thymidine kinase gene mutation assay in TK6 cells and in the micronucleus test. Results were in accordance with a previous study which indicated that emodin was genotoxic in several assays (comet assay, micronucleus test, and mutation assay in mouse lymphoma L5178Y $tk^{+/-}$ cells), whereas chrysophanol and physcion (two other anthraquinones often isolated from fungal cultures) showed no effects [214].

Two anthraquinone-type agents—danthron, a drug for constipation, and diacerein, an anti-inflammatory drug for osteoarthritis—were developed and approved by the U.S. FDA. However, danthron was withdrawn by the FDA in 1999 due to the risk of carcinogenesis. Therefore, clinical use of the naturally occurring anthraquinones should be considered cautiously [215].

Finally, all these findings clearly indicate that anthraquinone derivatives from marine and marine-derived fungi might be considered as potent sources of novel anticancer drugs and, at least, promising anti-leukemic agents, anti-invasive agents for human pancreatic and gastric cancers chemotherapy, and antitumor agents for hepatocellular carcinoma, bladder cancer, and others. However, the cytotoxicity caused by quinones is very complex and seems to occur through several mechanisms. Thus, due to differences in structures and characteristics among quinones, and to the dose-dependant responses observed, the molecular mechanism of the toxicity of each compound remains to be fully elucidated.

4.2. Special Focus on Protein Kinase Inhibition

The protein kinases are a large family of enzymes that transfer phosphate from adenosine triphosphate (ATP) to proteins as a means of regulating their activity and conformational state. The crucial role of protein kinases in cell signaling, gene expression, and metabolic regulation is highlighted by the fact that nowadays this family of enzymes is the second most important drug target. Actually, abnormal activity of individual protein kinases is often associated with human diseases, especially tumors whose treatment has been so far restricted to cytotoxic and hormonal agents. Many kinase inhibitors are currently in clinical trials, mostly as antitumor drugs, and two of them, Gleevec (STI-571) and rapamycin, are in clinical use for the treatment of a form of leukemia and for the prevention of tissue rejection after organ transplantation, respectively. One major problem with kinase inhibitors is that the human genome encodes 500 different protein kinases; therefore, inhibitors designed to target specifically an individual kinase are likely to bind to closely related kinases as well, thus interfering with other cell functions [216].

Many of the chemical scaffolds or building blocks studied as ATP site-directed kinase inhibitors are based on more or less complex heterocyclic molecules (mainly with nitrogen and oxygen as heteroatoms). The most common scaffolds are derivatives of the following: quinazolines; phenylamino-pyrimidines, pyrido-pyrimidines, pyrrolo-pyrimidines, pyrimido-pyrimidines, or

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pyrazolo-pyrimidines; pyrrolo-pyridines; indolin-2-ones; purines; pyridinyl-imidazoles or pyrimidinyl-imidazoles; and phthalazines [216].

The interest of the scientific community has been also focused on the anthraquinone family as anthraquinones have been used for the purification of proteins by affinity techniques taking advantage of their nucleotide specific ligand capability. This enables them to interact with ATP, ADP, and NAD binding sites of enzymes such as dehydrogenases, kinases, and ATPases. A potential drawback of these compounds is that their cyclic planar structure confers them the feature of DNA-intercalators with expectable cytotoxic effects. Even with this limit, the optimization of highly specific and selective inhibitors of this category should be exploited [216].

Emodin is a biologically active natural compound extracted from the biomass of many marine or marine-derived fungi that can be chemically classified as an anthraquinone derivative (1,3,8-trihydroxy-6-methylanthraquinone). Several scientific studies have been performed that indicate the vast variety of effects mediated by this compound. Emodin is known to have anti-microbial, immunosuppressive, and anti-inflammatory activities. It exerts anti-proliferative effects in a vast array of cancer cell lines, often enhancing the sensitivity of cancer cells to chemotherapeutic drugs. The efficacy of emodin in inhibiting tumorigenesis is due, at least in part, to its ability to induce apoptosis [217].

Although the exact mechanism(s) of apoptosis induction by emodin remain unclear, several studies have indicated that this compound is an effective inhibitor of protein kinases that are known to regulate a wide range of cellular processes, including apoptosis. Emodin is a cell permeable inhibitor of protein kinase CK2, a constitutively active Ser/Thr kinase that is highly conserved and ubiquitously expressed in eukaryotic cells [217]. CK2 (an acronym derived from the misnomer "casein kinase 2") is probably the most pleiotropic protein kinase known with more than 300 protein substrates identified. At variance with the great majority of protein kinases which are normally inactive and are turned on only in response to specific stimuli, the enzyme is ubiquitously expressed in all eukaryotic cells. Abnormally high levels of CK2 have been observed in various types of cancer cells as compared to normal tissues, and CK2 is in fact invariably elevated in a wide variety of tumors [218]. Following this pharmaceutical hit with emodin, studies about anthraquinones were expanded with the addition of bromo (brominated anthraquinones exist in some marine fungal biomasses), nitro, amino or bromoacetamido groups, and many compounds were also active (Table 3). Citreorosein (ω-hydroxyemodin) demonstrated similar kinase-inhibiting properties [219], and this kinase inhibiting action of anthraquinones was further linked to an anti-hypertensive potential as therapeutic agents [220]. Thereby, it would be very interesting to investigate the properties of the very large list of natural anthraquinones (over 100 compounds) produced by marine and marine-derived fungi, gathered for the first time in this review (Table 2).

Table 3. Effective anthraquinones with a protein kinase CK2 inhibitor activity (adapted from Meggio *et al.* [218]).

Name	Structure	IC$_{50}$ (μM)—K$_i$ (μM)
Emodin (3-methyl-1,6,8-trihydroxyanthraquinone)		K$_i$ = 1.85
1,8-dihydroxy-4-nitro-anthracene-9,10-dione		IC$_{50}$ = 0.30; K$_i$ = 0.78
1,8-dihydroxy-3-methyl-4-nitro-anthracene-9,10-dione		IC$_{50}$ = 0.30; K$_i$ = 0.95
1,8-dihydroxy-4,5-dinitro-anthracene-9,10-dione		IC$_{50}$ = 0.30
1,4-dihydroxy-5,8-diamino-anthracene-9,10-dione		IC$_{50}$ = 0.30; K$_i$ = 0.42
1-bromo-4,5-dihydroxy-8-nitro-anthracene-9,10-dione		IC$_{50}$ = 0.40
1,4,5-trihydroxy-8-(2-bromoacetamido)-anthracene-9,10-dione		IC$_{50}$ = 0.70

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4.3. Immunomodulatory Activity

The term "immunomodulation" means the alteration of immune response which may increase or decrease. Enhancement in the immune responsiveness is called immunostimulation and reduction in the immune responsiveness is called immunosuppression. An immunomodulator may be defined as a substance, biological of synthetic, which can stimulate, suppress or modulate any of the components of the immune system including both innate and adaptive arms of the immune response [221]. The essence of immunomodulation is that a pharmacological agent acting under various dose and time regimens displays an immunomodulating effect.

The extreme manifestations of immunomodulating action of biologically active substances are immunosuppression and immunostimulation; hence, both immunostimulating agents and immunosuppressing agents have their own standing, and the search for better agents exerting these activities is becoming a field of major interest all over the world. Natural adjuvants, synthetic agents, and antibody reagents are used as immunosuppressive and immunostimulative agents. However, there are major limitations to the general use of these agents, such as increased risk of infection and generalized effect throughout the immune system. To overcome these problems, a number of drugs from natural source either plant or fungi have been used to alter the human immune system [221], with cyclosporine A, a fungal metabolite, being a successful immunosuppressive agent.

Among anthraquinones from marine-derived and non-marine-derived fungi, carviolin (roseo-purpurin), 1-*O*-methylemodin, and ω-hydroxy-emodin (citreorosein) were found to have moderate immunosuppressive activities. The immunosuppressive activities (IC50 values) of carviolin (roseo-purpurin), 1-*O*-methylemodin, and ω-hydroxyemodin (citreorosein) were calculated against concanavalin A-induced (T cell) and LPS (lipopolysaccharides)-induced (B cell) proliferation of mouse splenic lymphocytes [222].

As immunosuppressive anthraquinones, emodin (1,6,8-trihydroxy-3-methylanthraquinone) [223], aloe-emodin [224], questin (1,6-dihydroxy-8-methoxy-3-methylanthraquinone), and rubrocristin (1,4,6-trihydroxy-8-methoxy-3-methylanthraquinone) were already known [222,225], it was estimated that the immunosuppressive activity (e.g., inhibition of phytohemagglutinin-induced lymphoproliferative responses) of emodin and similar anthraquinones might be partly mediated through H_2O_2 generated from its semiquinone form, and the free OH group at the β-position of the anthraquinone nucleus seems to play an important role in its immunosuppressive effect.

4.4. Antimicrobial, Antiviral, Antiparasitic Activities

4.4.1. Antimicrobial Activities

The search for components with antimicrobial activity has gained increasing importance in recent times due to growing worldwide concern about the alarming increase in the rate of infection by antibiotic-resistant microorganisms. There has also been a rising interest in research for natural products from marine-microorganisms for the discovery of new antimicrobial agents in the last three decades [2,226]).

The two new hexahydroanthrones tetrahydrobostrycin and 1-deoxytetrahydrobostrycin isolated from the marine-derived fungus *Aspergillus* sp. strain *05F16* collected at a coral reef (Indonesia) demonstrated antibacterial activities. Tetrahydrobostrycin inhibited the growth of *Staphylococcus aureus* and *Escherichia coli*, at 100 mg/disc, with the inhibition zones of 15 and 9.2 mm in diameter, respectively. Another compound, 1-deoxytetrahydrobostrycin from the same strain, was active against *S. aureus* (12 mm at 100 mg/disc). The growth of *Saccharomyces cerevisiae* and *Mucor hiemalis* were not affected by these compounds at 100 mg/disc. It is interesting that the presence of an OH group at C-1 is important for the antibacterial activity of tetrahydrobostrycin against *E. coli*, although the antibacterial activities of these compounds are very weak [54].

Monodictyquinone A (1,8-dihydroxy-2-methoxy-6-methylanthraquinone) was produced from a culture of a marine-derived fungus of the genus *Monodictys*. Its antimicrobial activity was determined

against *Bacillus subtilis*, *Escherichia coli*, and *Candida albicans*. This compound showed antibacterial activities against the three species with 2.5 µg/disk [82].

(2*R*,3*S*)-7-ethyl-1,2,3,4-tetrahydro-2,3,8-trihydroxy-6-methoxy-3-methyl-9,10-anthracenedione, a tetrahydroanthraquinone derivative, showed modest antibacterial activity against standard *Staphylococcus aureus* ATCC25923 and methicillin-resistant strains *S. aureus* SK1 with the MIC (Minimum Inhibitory Concentration) values of 128 and 64 µg/mL respectively. The compound was obtained from the EtOAc extract of the culture broth of the mangrove-derived fungus *Phomopsis* sp. PSU-MA214 [92].

Catenarin and 1,4,6,8 tetrahydroxyanthraquinone, products of the *Aspergillus glaucus* group exhibited the highest antibacterial activity against exponentialy growing cells of *Bacillus brevis*. The concentration at 1 µg/mL of catenarin and tetrahydroxyanthraquinone resulted in a complete inhibition of the incorporation of uracil and leucine [36].

Trichodermaquinone and Conioanthraquinone were isolated from *T. aureoviride* PSU-F95 and both exhibited antibacterial activity against MRSA (MIC, 200 µg/mL and 8 µg/mL respectively) [88].

Isorhodoptilometrin-1-methyl ether was isolated from *A. versicolor* and exhibited antibacterial activity against the three Gram-positive bacterial strains *Bacillus cereus*, *B. subtilis* and *S. aureus* (inhibition zones 2, 3, and 5 mm at 50 µg/disk, respectively), and the C-6 propanol group is important for its activity, as determined by a comparison with inactive compound 1-methyl emodin [57].

As mentioned in Khamthong *et al.* 2012, anthraquinone-citrinin derivatives are rare natural products. Penicillanthranin A, an anthraquinone-citrinin derivative was isolated from the sea fan-derived fungus *Penicillium citrinium* PSU-F51. This molecule displayed a moderate antimicrobial activity against *Staphylococcus aureus* ATCC25923 with equal MIC values of 16 µg/mL. The same inhibitory effect was observed against *S. aureus* SK1 for penicillanthranin A, which was four fold more active than chrysophanol [88].

Two other natural anthraquinone derivatives, *i.e.* 4-deoxybostrycin and nigrosporin, obtained from the strain *Nigrospora* sp. showed inhibitory effects against mycobacteria. 4-deoxybostrycin also showed significant inhibition of some clinical multidrug-resistant *Mycobacterium tuberculosis* strains (maximal inhibitory concentration <15.7 µM) [123].

To date, most of the anthraquinones studied, isolated from various sources (plants, microbes) exhibited more antibacterial than antifungal activities.

The production of a virulence factor, essential for causing SSTIs (skin and soft tissue infections) by *Staphylococcus aureus*, is controlled by quorum sensing (QS), mediated by the accessory gene regulator (agr). From Daly *et al.* [227], ω-hydroxyemodin (OHM), a polyhydroxyanthraquinone isolated from solid-phase cultures of *Penicillium restrictum*, was identified as a suppressor of QS and a compound sought for the further characterization of the mechanism of action in mouse model. At concentrations that are nontoxic to eukaryotic cells and subinhibitory to bacterial growth, OHM prevented agr signaling by all four *S. aureus* agr alleles. OHM inhibited QS by direct binding to AgrA, the response regulator encoded by the agr operon, preventing the interaction of AgrA with the agr P2 promoter. Decreased dermonecrosis with OHM treatment was associated with enhanced bacterial clearance, and reductions in inflammatory cytokine transcription and expression at the site of infection. Furthermore, OHM treatment enhanced the immune cell killing of *S. aureus in vitro* in an agr-dependent manner. These data suggest that bacterial disarmament through the suppression of *S. aureus* QS may bolster the host innate immune response and limit inflammation.

4.4.2. Antiviral Activity

The search for compounds useful in combating viral infections has resulted in relatively few successes. Most of the clinically useful compounds discovered so far have been nucleoside analogues, the usefulness of which has often been limited by the development of toxic side effects and the emergence of drug-resistant viruses. Consequently, the discovery of new non-nucleoside compounds,

which are less toxic to host cells and have different mechanisms of action than nucleoside analogues, would be of great value [228].

Some naturally occurring anthraquinones and their derivatives have been studied for antiviral activity against human immunodeficiency virus (HIV) and other retroviruses [229–232]. Some of these compounds have also been reported to possess *in vitro* antiviral activity against the herpes simplex viruses 1 and 2 (HSV-1, HSV-2), the influenza virus, the vesicular stomatitis virus [233], and the Epstein-Barr virus [234]. Murine Friend leukemia virus infections in mice have also been reported to be inhibited by polycyclic anthraquinone hypericin [235]. However, other investigators have seen no effect of hypericin on this virus infection in mice [232]. Other anthraquinones have been tested against HIV, but very few have been found to be active against this virus [231]. Tang *et al.* [232] have found that some anthraquinones also have virucidal activity against RNA and DNA viruses.

Alterporriol Q and tetrahydroaltersolanol C, isolated from the culture broth and mycelia of *Alternaria* sp. ZJ-2008003, which was collected from a soft coral (*Sarcophyton* sp.) from the South China Sea, expressed antiviral activity against the porcine reproductive virus and respiratory syndrome virus, with IC50 values of 39 and 65 μM, respectively [48].

Alizarin, emodin, emodin anthrone, emodin bianthrone, but also other anthraquinones such as quinalizarin, rhein, hypericin, and protohypericin, showed activity against HCMV strain AD-169 (human cytomegalovirus), distinguishable from cytotoxic effects on cells. Of these, quinalizarin had the highest therapeutic index at 3.4, and emodin anthrone and emodin bianthrone the lowest, probably due to their low solubility indices. The EC_{50} values (effective concentrations giving half-maximal response) of alizarin, quinalizarin, and rhein were lower in experiments with the ganciclovir-resistant strain than with the AD-169 virus strain [236].

Aloe-emodin, a bioactive anthraquinone possesses antiviral and anticancer potential [237] reportedly inhibiting replication of varicella-zoster, herpes simplex Types 1 and 2, pseudorabies, influenza, human cytomegalovirus, and/or Japanese encephalitis virus [236,238,239]. Other anthraquinone derivatives like emodin, chrysophanic acid, and hypericin have demonstrated antiviral activity against hepatitis B/C, poliovirus, and HIV [240–242]. Anthraquinones directly kill enveloped viruses [238]. Aloe-emodin also inhibits replication of the un-enveloped enterovirus *71 in vitro*, showing Types I and II interferon (IFN) signaling inductions in mammalian cells [239].

Aloe-emodin showed the strongest inhibition of virus yield among emodin and chrysophanol towards MDCK cells. Aloe-emodin treatment caused more than 1-log reduction (equal to 90% effective concentration [EC90]) in virus RNA loads. Subsequent plaque assay determined half maximal inhibitory concentration (IC50) value of aloe-emodin on virus yield. Aloe-emodin showed dose-dependent inhibition of virus-induced cytopathic effect. Infected cells showed about 50% cytopathic effect, those treated with aloe-emodin at concentrations of 1 and 2.5 μg/mL were less than 10% [237].

4.4.3. Antiparasitic Activity

The biological activities of fungal hydroxyanthraquinone derivatives, *i.e.*, 1,3,8-trihydroxy-6-methyl-anthraquinone, aloe-emodin 8-*O*-glucopyranoside, 1,8-dihydroxy-3-methoxy-6-methyl-anthraquinone, and 1,4,5-trihydroxy-7-ethoxy-2-methyl-anthraquinone, isolated from fungal extract of *Drechslera rostrata* and *Eurotium tonpholium* were studied by Awaad *et al.* [243]. Of these fungal anthraquinones, the 1,8-dihydroxy-3-methoxy-6-methyl-anthraquinone from *E. tonpholium* showed a significant anti-leishmanial activity against *Leishmania major* (with an IC_{50} value of 10.38 μg/mL). On the other hand, oral administration of this compound (50 mg/kg) showed very good anti-leishmanial activity.

4.5. Other Identified Biological Activities

In a review published in 2015, Chien *et al.* [215] outlined the chemical structure and biological properties of the naturally occurring anthraquinones and their derivatives with an emphasis on recent

findings about their therapeutic potential in autoimmune diabetes. So far, 79 naturally occurring anthraquinones have been highlighted, which include emodin, physcion, cascarin, catenarin, and rhein. A large body of literature has demonstrated that the naturally occurring anthraquinones possess a broad spectrum of bioactivities, such as anticancer, antimicrobial, antiinflammatory, but also cathartic, diuretic, vasorelaxing, and phytoestrogenic. This suggests their possible clinical application in many diseases. Despite the advances that have been made in understanding the chemistry and biology of the anthraquinones in recent years, research into their mechanisms of action and therapeutic potential in autoimmune disorders is still at an early stage [244–250].

4.5.1. Antioxidant Activities

Oxidative stress contributes to free radical-mediated diseases such as aging, atherosclerosis, cancer, ischemic heart disease, diabetes, hyperlipidaemia, hepatotoxicity, and neurodegenerative diseases. There is considerable interest in the isolation of potent radical scavenging compounds from natural resources to treat these pathologies. Natural and synthetic anthraquinones and their derivatives clearly demonstrated their antioxidants potential [63,246,251–254].

3-*O*-(α-D-Ribofuranosyl)-questin was isolated and identified in *Eurotium rubrum*, an endophytic fungal strain that was isolated from the inner tissue of the stem of the marine mangrove plant *Hibiscus tiliaceus*. The compound was evaluated for its radical scavenging activities by using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay and showed weak-moderate activity [63].

From Nemeikaite-Ceniene *et al.* [255] there is a correlation between the cytotoxicity of natural hydroxyanthraquinones (e.g., emodin and chrysophanol) or model quinones on FLK cells (bovine leukemia virus transformed lamb kidney) and HL-60 cells (human promyelocytic leukemia), and the redox cycling reactions inducing the formation of superoxide. This ability can then be determined by the E1 7values (values of single-electron reduction midpoint potential at pH 7.0). They concluded that the rate constants of the single-electron enzymatic reduction of natural hydroxyanthraquinones may serve as a useful tool for the quantitative description of their cytotoxicity with the involvement of oxidative stress.

4.5.2. Excretion Functions

Diuretic Activity

The diuretic action of emodin and aloe-emodin is probably due to their strong competitive inhibition on Na$^+$-K$^+$-ATPase activity. From a study of Zhou *et al.* [247], these compounds demonstrated IC$_{50}$ values of 9.8 μg/mL and 19.3 μg/mL and Ki values of 1.33×10^{-6} and 7.41×10^{-6}, respectively.

Laxative Activity

Anthranoid laxatives of natural origin, mainly extracted from plants are widely used [256,257]. The basic structure for all anthranoid laxatives is an anthracene ring, to which a hydroxyl or carbonyl function is substitued at C-9, and hydroxy groups at C-8.

Aloe-emodin and chrysophanol are among the most common anthraquinone laxatives [258]. Emodin also forms the basis of a range of purgative anthraquinone derivatives and, from ancient times, has been widely used as a laxative compound. It is believed that the presence of hydroxyl groups in position 1 and 8 of the aromatic ring system is essential for the purgative action of the compound [259].

Because of its chemical structure, emodin glycosides (and other anthraquinones) are carried unabsorbed to the large intestine in mammals, where metabolism to the active aglycones takes place by intestinal bacterial flora. The aglycone exerts its laxative effect by damaging epithelial cells, which leads directly and indirectly to changes in absorption, secretion and motility [258,260]. Emodin also inhibits the ion transport (Cl$^-$-channels) across colon cells, contributing to the laxative effect [249,261].

There is good evidence that anthrones/anthraquinones, known as active metabolites of emodin-type O- and C-glycosyl compounds, influence the ion transport across colon cells, although the target transport systems have not yet been elucidated. To solve the problem of contradictory explanations about the laxative action of these drugs, a study tested 25 different anthrone/anthraquinone metabolites of plant drugs. Their influence was assayed on different ion transport systems in Ehrlich cells as a model system. Comparing the laxative potency of these substances, with their influence on the different ion transport systems involved in trans-epithelial ion transport, made it possible to exclude some transport processes as primary targets of the drugs. The results showed that Na^+-K^+-2Cl- cotransport was not inhibited by any of the substances tested and that Na^+/K^+-ATPase (pump) was inhibited by those 1,8-dihydroxyanthrones/anthraquinones that bear an additional phenolic hydroxyl group. This inhibition is indirect by interference with oxidative ATP production. However, there is no direct correlation to laxative action. In addition, cation channels were not influenced by these drugs, and Cl^--channels were inhibited significantly by those drugs that also showed a laxative action. These results make it very likely that inhibition of Cl^--channels is the primary action responsible for the laxative action. Interference with oxidative ATP production as an additional effect may explain the known synergistic action described for the combination of different anthrones/anthraquinones or anthranoid drugs, respectively [261].

In addition, 1,3,6,8-trihydroxymethylanthraquinone was used in a patented laxative preparation for intravascular injection, active by stimulating the neuromuscular junction of the bowel wall [262].

Studies in humans have also suggested tumor promoting activities for these laxatives. Although the short-term use of these substances is generally safe, long-term utilization cannot be recommended.

4.5.3. Vasorelaxant or Contractile Effects

In a first study of Huang *et al.* [263], the vasorelaxant effect of emodin was assayed as the ability to relax rat thoracic aortic rings precontracted with phenylephrine. The vasorelaxant activity of emodin was expressed as a percentage of relaxation of the maximal tension increase produced by phenylephrine. The concentration evoking 50% relaxation (IC_{50} value) showed that emodin exhibits vasorelaxant effect, and dose-dependently relaxed the contractile responses of rat aortic rings. In the same study, emodin also dose-dependently suppressed the responses of human mononuclear cells to phytohemagglutininand mixed lymphocytereaction. Thus, this compound may be useful as a new template for the development of better immunosuppressive agents, with vasorelaxant actions useful against transplantation rejection and autoimmune disease.

The possible mechanism underlying the vasorelaxant effect of emodin was investigated in a second study by Huang *et al.* [264]. Emodin dose-dependently relaxed isolated vascular rings of several vessels in animals and human, in case of induced contraction. The study also investigated the inhibition, attenuation, or potentiation of relaxation response to emodin by various compounds. It suggested that the vasorelaxant effect of emodin might be mainly due to cGMP accumulation, as a result of guanylate cyclase activation by free radicals and/or hydrogen peroxide generated from semiquinone.

The effects of emodin on skeletal muscle were studied in a mouse-isolated diaphragm and in sarcoplasmic reticulum (SR) membrane vesicles [265]. Emodin dose-dependently caused muscle contracture, simultaneously depressing twitch amplitude. Neither tubocurarine nor tetrodotoxin (neuromuscular non-depolarizing agents) blocked the contraction, suggesting that it was caused myogenically. These data suggest that muscle contraction induced by emodin is dose-dependent and is due to Ca^{2+} release from the SR of skeletal muscle, as a result of oxidation of the ryanodine receptor and influx of extracellular Ca^{2+} through voltage-dependent Ca^{2+} channels of the plasma membrane.

4.5.4. Effects on Lipid and Glucose Metabolism

Several drawbacks of many pharmaceutic drugs used for the treatment of Diabetes mellitus have contributed to the use of "natural" products. Emodin, which was extensively studied, has

anti-inflammatory, analgesic [266], and antipancreatitic [267] effects. Its anti-diabetic activity was investigated by evaluating its hypoglycaemic and hypolipidaemic effects, together with its potential effects on L-type calcium-channels in dyslipidaemic-diabetic rats. The results demonstrated significant dose-dependent reductions in blood glucose, serum total cholesterol, triglycerides, free fatty acids, and malonaldehyde in dyslipidaemic-diabetic rats. In addition, emodin caused dose-dependent increases in their plasma superoxide dismutase activity. The results suggest that emodin has antidiabetic and lipid-modulating effects that involve, in part, upregulation of L-type calcium channel expression in the pancreas and heart of dyslipidaemic-diabetic rats [268].

The antidyslipidemic effect of several anthraquinone derivatives as chrysophanol and emodin obtained from *Rheum emodi*'s rhizomes was evaluated. These compounds significantly reduced plasma lipid levels. The most active compound emodin showed significant lipid-lowering activity in the HFD-fed model. The effect of emodin on enzymes modulating lipid metabolism confirms and supports its efficiency as a potent antidyslipidemic agent [269].

4.5.5. Estrogenic Activity

Insufficiency of endogenous estrogen secretion is known to cause several physical disorders in postmenopausal women, such as osteoporosis, hypercholesteremia, and symptoms of menopause. Synthetic estrogen-replacement therapy has been reported to be effective for these diseases. Recently, the estroenic activity of phytoestrogens was reported. A study of these compounds, which are distributed in vegetables, fruits, and medicinal plants, evaluated the estrogenic activity of the methanolic extracts from several medicinal herbs. The study was guided by the detection of a proliferative activity of MCF-7,1 (estrogen-sensitive cell line) [244]. A bioassay-guided separation led to emodin, which enhanced proliferation of MCF-7 from 1 to 10 mM in a concentration-dependent manner. Aloe-emodin and chrysophanol showed weak activity. By comparison of the activities, the 6-hydroxyl group seemed to be essential for enhancement of the estrogenic activity.

To clarify the affinity of the active anthraquinones to human estrogen receptors ERα and Erβ, a competitive binding assay was performed using 17β-estradiol. Aloe-emodin and chrysophanol did not compete with ERα and ERβ for 17β-estradiol binding at a high concentration (40mM). Conversely, emodin competed for 17β-estradiol binding with both ERα and ERβ.

Concerning the structure–activity relationships of anthraquinones regarding the oestrogenic activity, it is quite clear that the unchelated hydroxyl group is essential for a strong estrogenic competency. This is the first report for estrogenic activity of anthraquinones. The findings that emodin bonds with human ERα and ERβ may be useful for replacement therapy for human menoxenia and post-menopausal diseases.

5. Conclusions

The recent developments in technology helped in promoting the increased use of natural molecules in industries. Thereby, the microorganisms naturally producing bioactive or useful extrolites recently appeared as a great source of potentially interesting molecules. Opposite to plants and insects, these easily renewable and upscalable resources (short production cycles), independent of seasonality, can give regular productions with potentially higher yields. Unfortunately, far more than the chemical synthetic routes, the biological synthesis requires a deep comprehension of the phenomena involved, and often implies very thin adjustments. This is one of the reasons why some have expressed doubts about the successful production and commercialization of fermentation-derived compounds. Others dealt with the high capital investment requirements for fermentation facilities and the extensive and lengthy toxicity studies required by regulatory agencies. However, several microbial genera are capable of producing highly original compounds in large quantities, and could meet the economic needs of companies, if we are ready to embark on the challenges of the production of natural molecules through biotechnology. For now, the question is: Can the recent and future research efforts on the fungal strains, the productions' methods, and the biosynthetic improvments reveal secure ways to

produce targeted molecules? Thus, it seems that anthraquinoid compounds produced by fungi are quite new tracks for research and applications.

Conflicts of Interest: The authors declare no conflict of interest.

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2.7. CONCLUSION

In the beginning of this chapter, we briefly described the potential of numerous microorganisms focusing on the production of pigments. The literature studies of various authors have suggested that fungal pigments are produced under natural conditions, once the growth of the fungal colony is well established or when the supply of vital nutrients have become depleted. In this condition, parts of the mycelium may switch biochemical activity to pathways of secondary metabolism. Rather than producing new fungal materials, this gives rise to other compounds such as secondary metabolites. Fungal pigments are produced in this way and may contain a mixture of several different pigments [1]. The pigments may provide protection to the host fungi against harmful effect of sunlight and ultraviolet radiation, bacterial attack and attack of insects [2, 3]. In addition, these fungal pigments have huge potential to use in food industries [4, 5] and some are associated with biological effects on humans [6-9].

Considering filamentous fungi, they are known to produce several classes of pigments that include carotenoids (orange red), melanins (dark brown), azaphilones, flavins, phenazines, quinones, and more specifically, monascins, violacein, and indigo [5, 10].

The fungal biodiversity from terrestrial and marine origins and the pigments produced by these diverse groups of fungi, biosynthetic pathways for pigment production (carotenoids and polyketide derivatives), biotechnological approaches to improve pigment production, prevailing extraction process and alternative eco-friendly/green extraction techniques, current industrial applications of pigments along with their limitations and future opportunities of pigment usage in other industries such as beverage, food, pharmaceutical, cosmetic, textile, and painting areas have been presented in detail under the section 2.2 as book chapter (page no:)[10].

Besides, there are many other extrolites produces by filamentous fungi which belong to polyketide family including classes such as anthraquinones, hydroxyanthraquinones, naphthalenes, naphthoquinones, flavonoids, macrolides, polyenes, tetracyclines, and tropolones. The chemical structures of the compounds produced by these classes, source of fungi producing the particular compounds, their biological activities have been described in an elaborate way under the section 2.4 as review article (page no) [6].

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CHAPTER 3

SCREENING OF FUNGI FROM MARINE ENVIRONMENT IN LA RÉUNION ISLAND, INDIAN OCEAN FOR THE PRODUCTION OF PIGMENTS: IMMENSE ESSENCE OF EXCELLENCE

3.1   BACKGROUND

3.1.1.   Marine environment, a treasure hunt for secondary metabolites

Filamentous fungi are ubiquitous organisms, whose role is often overlooked in marine ecosystems. Marine environment is extremely complex; however reveal a great diversity of fungi that could have significant impacts on the biogeochemical evolution of habitats [1]. Recently, marine derived fungi have been recognized as one of the last barely tapped sources for new biologically active secondary metabolites such as enzymes, pigments and other bioactive compounds [2]. These secondary metabolites are considered as natural products find its importance in food, cosmetic, medical fields. Overall, research on marine-derived fungi has led to the discovery of 272 new natural products including many that have novel carbon skeletons, thus, providing evidence that marine-derived fungi have the potential to be a rich source of pharmaceutical leads [3]. Among the production of profusion of secondary metabolites, pigments has gained scientific and industrial interest due to their taxonomic importance and its attractive wide array of colors respectively.

The objective of this study was to search filamentous fungi in some marine biotopes of La Réunion Island's reef flat (sediments, open water, coral reef). The main focus was on isolating fungal populations that require minimum conditions (aerobic, basic nutrient sources) which would facilitate to grow the strains in laboratory and industrial context, especially with regard to biomass or pigments production. Different approaches have been performed to screen as well as to select pigment producing fungi among all the 47 isolates. Having their morphological features (Phenotypic or Phenetic) from microscopic observations and identifying the ideal differences among others, fungal species were described. Even though it was the classical approach used by mycologists, at times distinction between a population and an individual is not always easy. The genetic concepts of some species will be unknown and different mycologists consider different concepts while delineating them. This has been improved by phylogenetic concept, particularly

the analysis of DNA nucleotide sequences using base pairs [4] For all the 47isolates, molecular identification was carried out based on selective gene sequence (18S, ITS region, 5S,β tubulin, Calmodulin, etc) to obtain the relative information about the species [5-7].

Among the recovered fungal species, *Aspergillus* and *Penicillium* genera seems to be much represented. Nearly twenty seven isolates synthesized pigments ranging from pale yellow, dark brown, red, green according to the media used for growth. Some species such as *Aspergillus versicolor, A. glaucus, Penicillium purpogenum, P. citrinum, P. herquei, P. rubrum* produces pigments of anthraquinone and azaphilone families. To identify its pigment nature, the pigmented extract was measured for its optical density from 200 to 700 nm and the absorption maxima were noted. Among these pigments, according to the strains, it seems that carotenoids (λ_{max} visible 440-500) and/or anthraquinones (λ_{max} UV 200-280, Visible 420-550nm) were produced [8].

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3.3   RESEARCH ARTICLE

Biodiversity of Pigmented Fungi Isolated from Marine Environment in La Réunion Island, Indian Ocean: New Resources for Colored Metabolites

This research article reveals the diversity of pigment producing fungi isolated from different samples in the marine environment of Reunion Island. The color hues and its absorption in the visible area, amount of pigments produced are presented. This research article forms the basis of this chapter.

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Article

Biodiversity of Pigmented Fungi Isolated from Marine Environment in La Réunion Island, Indian Ocean: New Resources for Colored Metabolites

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Abstract: Marine ecosystems cover about 70% of the planet surface and are still an underexploited source of useful metabolites. Among microbes, filamentous fungi are captivating organisms used for the production of many chemical classes of secondary metabolites bound to be used in various fields of industrial application. The present study was focused on the collection, isolation, screening and genotyping of pigmented filamentous fungi isolated from tropical marine environments around La Réunion Island, Indian Ocean. About 150 micromycetes were revived and isolated from 14 marine samples (sediments, living corals, coral rubble, sea water and hard substrates) collected in four different locations. Forty-two colored fungal isolates belonging to 16 families, 25 genera and 31 species were further studied depending on their ability to produce pigments and thus subjected to molecular identification. From gene sequence analysis, the most frequently identified colored fungi belong to the widespread *Penicillium, Talaromyces* and *Aspergillus* genera in the family Trichocomaceae (11 species), then followed by the family Hypocreaceae (three species). This study demonstrates that marine biotopes in La Réunion Island, Indian Ocean, from coral reefs to underwater slopes of this volcanic island, shelter numerous species of micromycetes, from common or uncommon genera. This unstudied biodiversity comes along with the ability for some fungal marine inhabitants, to produce a range of pigments and hues.

Keywords: fungi; biodiversity; Indian Ocean; Marine; coral reef; genotyping; pigment production

1. Introduction

With the growing demand for natural compounds in the industrial sector, marine derived fungi appear to present many interests. Filamentous fungi are ubiquitous in nature due to their huge capacity of adaptation and their ability to produce an assortment of new secondary metabolites. Literature now abundantly reports the significant involvement of fungi in the industry, through the production of various useful substances, such as antibiotics, immunosuppressants, anti-cancer drugs, plant hormones, enzymes, acids and also natural pigments [1–5]. Both the pigments and enzymes equally find their usages in food and beverages, animal feeds, pharmaceuticals, cosmetics, textile, leather, pulp and paper industries, biofuel production, and environment bioremediation [6].

Nevertheless, the distribution of the marine-derived fungal species and their contribution to marine biotopes are still in infancy, and more has to be explored [7–11]. The highest diversity of marine fungi seems to appear in tropical regions, mainly in tropical mangroves, which are extensively studied because of their high richness in organic matters and especially lignocellulosic materials, favorable to the development of a wide range of heterotrophic microorganisms [11–14]. Anyway, many marine ecological niches are still unexplored and it seems plausible that unique features of marine environments can be the inducers of unique substances, biosynthesized by marine or marine-derived microorganisms [15,16].

Considering the immense genetic and biochemical diversity of these fungi, partially derived from the specificity of the biotopes they are facing, marine-derived fungi are regarded as a potential bright source of new molecules with likely application in pigment production [17,18]. Many genera producing pigments have then been isolated either from water, sediments, and decaying organic residues, or from living organisms such as invertebrates, plants or algae. Fungi belonging to genera such as *Aspergillus, Penicillium, Paecilomyces, Eurotium, Alternaria, Fusarium, Halorosellinia, Monodictys* and *Microsphaerospsis* have already been identified from marine biotopes [19–21]. They are therefore able to exhibit bright colors, from yellow to black, mainly belonging to polyketides. Indeed, polyketides pigments and particularly azaphilones and anthraquinones seem to dominate marine natural products of fungal origin [22]. Colored compounds, usually described as secondary metabolites, do not seem to be directly involved in the primary growth of the fungus in which they occur [23]. However they may play some important roles in the resistance to a variety of adverse environmental factors (desiccation, exposure at extreme temperatures, irradiations and photo-oxidation) or in ecological interactions with other organisms (macroorganisms such as sponges, corals or other microbial communities) [24]. For this reason, many fungal secondary metabolites exhibit useful biological activities and are of interest to the pharmaceutical, food, and agrochemical industries [16,25].

This study initiated the search for filamentous fungi in some tropical marine biotopes of coral reefs and underwater slopes of the volcano from La Réunion Island. Fungal isolates from samples of sediments, seawater, hard substrates, coral rubbles or living coral individuals (*Pocillopora* sp.) were characterized both by phenotypic and molecular ways. The production of pigments of quinoid-type produced from the mycelia cultured in liquid media was used as a first approach to screen for the pigment production. This work reveals a part of the mycofloral biodiversity in La Réunion Island tropical marine environment and its potentiality to propose new pigment sources to expand in an industrial setting.

2. Materials and Methods

2.1. Samples Collection

La Réunion Island lies in the Indian Ocean and is located 800 km east of Madagascar ($21°06'54.5''$ S and $55°32'11.0''$ E) (Figure 1a). This tropical island arose two million years ago from a volcanic hot spot (Piton de La Fournaise) and is known for its rainforested interior and its fringing reefs holding most of the marine wealth.

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Figure 1. (**a**) La Réunion island location (Indian Ocean, 21°06′54.5″ S and 55°32′11.0″ E); (**b**) geolocation of sampling sites around La Réunion Island (West: La Saline; and East: Sainte Rose and Tremblet); and (**c**) geolocation of the three sampling spots at La Saline fringing reef: Trou d'eau (TDE inner reef and TDE outer slope) and Planch'Alizé (PA) (back arrow represents the main water flow).

A first set of samples was collected on the fringing reef from La Saline, which lies on the dry west coast of the island. It is more than 9 km long and ranges in width from 50 m in its northern part to 600 m in the south [26]. For the purpose of research, samples were collected from three sampling spots on the west coast that cover the sites of Trou d'Eau (TDE) (inner reef flat at −1 m depth and outer slope at −17 m) and Planch' Alizé (PA) (inner reef flat, −1 m) (Figure 1b,c). Planch' Alizé is considered as a sheltered site, located downstream of seawater flowing over the Trou d'Eau (Figure 1c). The outer slope is found at the outer edge of the reef, closest to the open ocean, and is characterized by spurs and grooves extending downward to the sand bottom, while the inner reef flat displays wide transversal strips of branched coral colonies alternating with narrow detrital channels perpendicular to the reef flat [27–31]. Low water flow and high solar radiation contribute to heating the reef water during the day, inducing important daily sea surface temperature variations. This area is also heavily laden with organic and mineral matter coming from nearby human activity (seaside area).

A second set of samples was collected in Sainte Rose area (south-east) on the submerged lava flows (Figure 1b). Indeed, the Piton de la Fournaise is one of the most active effusive volcanoes in the world with 27 eruptions between 1998 and 2007 and a mean frequency, over a century, of an eruptive phase every nine months. Submerged lava flows appear on the south-east part of the island when, during eruptions, the pool of lava overflows the active volcano mouth and pours down on the slopes

of the volcano, to the sea. These costal marine ecosystems facing the deep ocean, are then regularly subjected to natural hazards such as being covered by incandescent lava flows, temporary changes in physicochemical conditions of water bodies and exceptional rises of temperature. Besides, this area is poorly inhabited and urbanized and, as a consequence, the amount of organic matter poured in the sea is reduced compared to other coastal ecosystems. It provides a natural laboratory to study the colonization of a blank substrate and the evolution of the biodiversity all around, during the following years. Samples were then obtained from sediments extracted from 1977 lava flow (−25 m depth) and 2004 lava flow (−70 m), as well as from surrounding free water at −70 m.

Seawater, sediments, parts of living corals and hard substrates (volcanic rocks or coral rubbles) were collected in sterile bottles, during the months of April and May 2012, stored in a cooling box (4 °C), brought to the laboratory, and treated immediately for the fungal isolation.

2.2. Culture and Purification of Fungi

To cultivate the revivable fungi from the collected seawater, 100 mL of water was filtered using a 0.45 μm sterile cellulose-nitrate filter (Sartorius Stedim, Göttingen, Germany). The filters were then placed in Petri plates containing malt extract agar (MEA) and Sabouraud agar (BD Difco, Franklin Lakes, NJ, USA) prepared with natural seawater collected near La Saline, and beforehand sterilized at 121 °C, 15 min.

The other samples such as sediments, hard substrates and parts of living/dead coral were treated separately. The samples were first washed with 70% alcohol and rinsed in sterile seawater. Then they were ground using sterile pestle and mortar. Ground material (5 g) was taken from each sample and added to 15 mL of sterile diluent (1.6 g of tryptone (Sigma- Aldrich, T-9410, Saint Louis, MO, USA), 0.05 g of tween 20, 1 L of sterile seawater of pH = 7.5). After stirring for 20 min at 150 rpm on a shaking table (Edmunt Bühler GmbH, VKS 75 Control, Hechningen, Germany), the suspension was diluted by employing serial decimal dilution method up to 10^{-3} [32]. Each diluted sample (1 mL) was poured on Petri plates containing MEA and Sabouraud agar prepared with natural seawater.

All the platings were performed in triplicates and incubated at 25 °C for 21 days. During this period, the plates were checked each day for the appearance of new colonies. Each new colony was individually isolated and cultured on new MEA solid medium. During the growth period, the production of colors was observed.

All the isolated fungi were cultured using monospore technique for future experiments and long-term storage. The fungi grown after 5 days were scraped and transferred into a sterile vial containing a cryoprotectant medium composed of 15% skimmed milk and 2% glycerol for long term storage at −80 °C [33,34]. In total, 42 fungal isolates were then selected for pigment production based on the visual appearance of the thalli grown on solid culture media.

2.3. Fungal Identification

2.3.1. Fungal DNA Extraction

To extract DNA from the 42 purified isolates, a small amount of mycelium along with spores was cultivated on potato dextrose agar (PDA) at 25 °C under day light exposure. After 5 days of growth, the fungal mycelium was scraped and DNA was extracted using DNeasy Blood and Tissue kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. DNA amount and purity contained in each extract were evaluated by measuring the absorbances at 230, 260 and 280 nm (Nanodrop 2000, Thermo Scientific, Waltham, MA, USA) and calculating the ratio A_{260}/A_{280} and A_{260}/A_{230}. DNAs were stored at −20 °C prior to use for amplification studies [35].

2.3.2. Primers, PCR Amplification and Sequencing

The choice of PCR primers was made based on observed phenotypic characteristics for molecular identification. *Aspergillus* species were amplified for calmodulin gene using primers Cmd5/Cmd6

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and *Penicillium* species for β-tubulin using primers T10/Bt2b [36]. To amplify and sequence the DNA from *Trichoderma* and *Hypocreales* species, EF-1H/EF-2T primer pair was used to amplify a fragment of the translation elongation factor 1 alpha gene (*Tef1*) [37]. For uncharacterized fungi, the fragments containing ITS region were amplified using ITS1-F_KYO2/ITS2 or ITS3_KYO2/ITS4, and, when necessary, the large subunit rDNA was also amplified using V9G/LR3 primer pair (Table 1) [36–38].

Table 1. PCR amplification and the sequencing primers used for the identification of fungal isolates.

Primers	Direction	Sequences (5′→ 3′)	Note	Hybrid. T °C	Refs.
ITS1-F_KYO2	Forward	TAGAGGAAGTAAAAGTCGTAA		56	
ITS2_KYO2	Reverse	TTYRCTRCGTTCTTCATC		47	[36]
ITS3_KYO2	Forward	GATGAAGAACGYAGYRAA	Small sub-unit, ITS 1, 5.8S, ITS 2, Largest sub unit rDNA	47	
ITS 1	Forward	TCCGTAGGTGAACCTGCGG		55	
ITS 2	Reverse	GCTGCGTTCTTCATCGATGC		55	[39]
ITS 3	Forward	GCATCGATGAAGAACGCAGC		55	
ITS 4	Reverse	TCCTCCGCTTATTGATATGC		55	
V9G	Forward	TTACGTCCCTGCCCTTTGTA	Large sub unit D1/D2 for basidiomycetous yeast	55	
LR3	Reverse	TGACCATTACGCCAGCATCC		57	[38]
Cmd 5	Forward	CCGAGTACAAGGARGCCTTC	Calmodulin, specific for *Aspergillus*	52	
Cmd 6	Reverse	CCGATRGAGGTCATRACGTGG		52	
T 10	Forward	ACGATAGGTTCACCTCCAGAC	β- tubulin, specific for *Penicillium*	55	[38]
Bt2b	Reverse	ACCCTCAGTGTAGTGACCCTTGGC		55	
EF1-728F	Forward	CATCGAGAAGTTCGAGAAGG	Elongation factor 1 for *Trichoderma*	55	[38]
TEF1-LLErev	Reverse	AACTTGCAGGCAATGTGG		55	

PCR reactions were carried out in a total volume of 30 μL: 1× of MasterMix (Applied Biosystems, Foster city, CA, USA), 0.5 μM of forward and reverse primers and at least 1.3 ng/μL of genomic DNA. Amplifications were carried out on a thermal cycler GeneAmp® PCR System 9700 (Applied Biosystems) according to the following program: 94 °C for 5 min + 35 × (94 °C for 30 s, 55 °C (or 52 °C for the primers of calmodulin: Cmd5/Cmd6) for 60 s, 72 °C for 60 s) + 72 °C for 5 min for final elongation step.

2.3.3. Sequence Analysis

Amplicons were sequenced in both directions (GENOSCREEN, Lille, France). The obtained electropherograms were read and corrected with Chromas software (version 2.13, Technelysium pty Ltd., South Brisbane, Australia). The extracted sequences for each gene were separately used to perform nucleotide searches using online BLAST algorithm, provided by NCBI (http://www.ncbi.nlm.nih.gov/BLAST/). BLAST results were sorted based on the maximum identity to the query sequence and considered as the best hit. Sequence-based identities with a cutoff of 97% or above and query coverage >90% were considered as significant [40,41]. Because of low recovery rates and concordance values, some isolates were amplified and sequenced a second time, with additional sets of primers, mainly among the isolates of the genera *Penicillium* and *Trichoderma*.

2.4. Culture Conditions for Pigment Production and Separation of Biomass from Liquid Medium

2.4.1. Culture Conditions

Erlenmeyer flasks (250 mL) containing 80 mL of potato dextrose broth (PDB) medium were autoclaved at 121 °C for 15 min. Then, 120 mg of mycelia from interesting fungal species grown on PDA Petri plates were transferred into the sterile flasks and incubated at 25 °C under daylight exposure, with an agitation of 150 rpm for 10 days (Multitron Pro, Infors HT, Bottmingen, Switzerland).

2.4.2. Separation of Biomass from Culture Liquid

After the end of the fermentation period, the culture medium containing extracellular pigments was separated from mycelia by vacuum filtration using Whatman filter paper No. 2 (Merck, Darmstadt, Germany). Thus, liquid medium and biomass were treated separately. The wet mycelium was further used for the extraction of pigment content.

2.5. Production of Pigments

2.5.1. Determination of Pigments Production in Liquid Cultures

Chromophore is a chemical group that absorbs light of specific frequency and confers color to a molecule. Widespread polyketide pigments such as anthraquinones or azaphilones are often highly substituted aromatic molecules, with fused benzene rings [42]. Thus the majority of the common chromophores from fungi absorb in the UV region (one or several peaks between 200–300 nm), whereas absorbance in the visible region (400–700 nm) highly depends on the nature and the number of substituted groups.

To compare the pigment production of all isolates cultured in PDB medium, the amount of pigments produced in liquids was expressed as mg equivalent (mg eq.) of a chosen commercial standard per liter of culture medium (mg eq. purpurin L^{-1}). Purpurin was chosen as a polyketide pigment in orange-red hue, which absorbs in the UV area (250–270 nm) as many polyketides [43], and also in the visible range 458–520 nm [44]. Thus, the absorbance of an authentic colored standard purpurin (Sigma-Aldrich) was estimated at different concentrations using an UV-visible spectrophotometer (Shimadzu UV-1800 Spectrophotometer). Then, in regard with the diversity of pigments content in the fungal cultures and as a preliminary approach, the absorbance of each sample was measured at 254 nm and the amount of pigments produced was expressed in "mg equivalent purpurin L^{-1}" (Figure S1). In addition, for each isolate, the intracellular (IC) pigments (extracted from the biomass) and extracellular (EC) contents (liquid from culture, separated from the biomass) were scanned between 200 and 600 nm with a UV-1800 spectrophotometer (Shimadzu UV spectrophotometer, Shimadzu Corporation, Kyoto, Japan) in a quartz cell of 10 mm path length.

2.5.2. Extraction of Pigments

IC pigments contained in the wet mycelium were extracted using a methanol: water combination (1:1 v/v) as conventional extraction method. The mixture was immersed in an ultrasonic bath at 45 °C for 30 min. The suspension was allowed to stir overnight at room temperature on a shaking table (VKS 75 Control, Edmunt Bühler GmbH). On the following day, it was filtered through Whatman filter paper No. 2 to recover the solvent containing the pigments extracted from biomass.

To compare the amount of pigments produced within the cells with the one diffused into the extracellular medium, we performed the nonparametric Mann–Whitney–Wilcoxon test as our data did not follow the normal distribution using the R software (R Development Core Team 2016) [45].

3. Results

3.1. Diversity of Isolated Fungi

More than 150 isolates were first recovered from the 14 samples collected among four locations. Among them, 42 were selected for identification, according to their capacity to develop colored mycelia or to secrete colored compounds in the media.

After sequencing, the 42 colored isolates were assigned to 16 families, 25 genera and 31 species (accession numbers mentioned in Table 2). The vast majority of the isolates have been identified with more than 98% concordance rate and recovered with high precision at the species level. However, few fungi (*Acremonium* sp., *Periconia* spp. and *Biscogniauxia* sp.) were identified to the genus level only, according to the gene chosen. The genetic characterization with these primers partially failed for two

isolates (*Whalleya microplaca B* and *Wallemia sebi*). *Wallemia sebi* was only characterized according to morphological criteria.

Table 2. Fungal isolates from La Réunion Island marine biotopes from different sample types and sampling sites: Trou d'Eau (TDE); Planch' Alizé (PA); Lava flow corresponds to 1977 lava flow in Sainte Rose/Tremblet area.

Family	Fungal Species	Sampling Site	Gene Accession Number
Water Bodies			
Davidiellaceae	*Cladosporium Cladosporioides*	Lava flow (−70 m)	JF949719.1
Didymellaceae	*Peyronellaea glomerata* (syn: *Phoma glomerata*)	Lava flow (−70 m)	JQ936163.1
Nectriaceae	*Nectria haematococca* A	Lava flow (−70 m)	XM_003053163.1
Pleosporales Incertae Sedis	*Periconia* sp. A	Lava flow (−70 m)	HQ608027.1
	Periconia sp. B	Lava flow (−70 m)	HQ608027.1
Sporidiobolaceae	*Rhodosporidium paludigenum*	Lava flow (−70 m)	AF444493.1
Stachybotryaceae	*Myrothecium atroviride*	Lava flow (−70 m)	AJ302002.1
Teratospheriaceae	*Hortaea werneckii* (syn: *Cladosporium werneckii*)	Lava flow (−70 m)	JN997372.1
Trichocomaceae	*Aspergillus sydowii* B	Lava flow (−70 m)	KC253961.1
	Emericella qinqixianii	TDE outer slope	AB249008.1
	Penicillium broae NRRL 32599	TDE outer slope	DQ123642.1
	Penicillium viticola B	TDE inner reef	AB606414.1
	Talaromyces rotundus	TDE inner reef	EU497950.1
	Talaromyces verruculosus	PA inner reef	KC416631.1
Wallemiaceae	*Wallemia sebi*	Lava flow (−70 m)	Morphological Identification
Living Coral Pocillopora sp.			
Hypocreaceae	*Acremonium* sp.	PA inner reef	FJ770373.1
	Hypocrea koningii	TDE inner reef	JX174420.1
Trichocomaceae	*Aspergillus creber* A	TDE inner reef	JN854049.1
	Aspergillus creber B	TDE inner reef	JN854049.1
	Aspergillus sydowii A	PA inner reef	JN854052.1
	Eurotium amstelodami	TDE outer slope	FR727111.1
	Penicillium viticola C	PA inner reef	AB606414.1
Coral Rubbles			
Chaetomiaceae	*Chaetomium globosum or Chaetomium murorum*	TDE outer slope	JN209898.1
Trichocomaceae	*Penicillium herquei*	TDE outer slope	JN246042.1
	Talaromyces albobiverticillius B	TDE outer slope	JN899313.1
	Talaromyces albobiverticillius C	TDE outer slope	JN899313.1
Hard Substrate/Rock Substrate			
Nectriaceae	*Fusarium equiseti* A	Lava flow (−25 m)	JQ936153.1
	Fusarium equiseti B	Lava flow (−25 m)	JF311925.1
	Fusarium equiseti C	Lava flow (−25 m)	JQ936153.1
	Nectria haematococca B	Lava flow (−25 m)	XM_003053163.1
Pleosporaceae	*Epicoccum sorghi* (syn: *Phoma sorghina*; *Peyronellaea stemphylioides*)	Lava flow (−25 m)	KC106717.1
Sordariomycetes	*Nigrospora sphaerica* (or Env. sample from marine air)	TDE outer slope	KC505176.1
Sporidiobolaceae	*Rhodotorula mucilaginosa*	TDE outer slope	KC515367.1
Trichocomaceae	*Penicillium citrinum*	Lava flow (−25 m)	EU030332.1
	Penicillium viticola A	Lava flow (−25 m)	AB606414.1
Ustilaginaceae	*Sporisorium exsertum*	TDE outer slope	JN367293.1
Xylariaceae	*Biscogniauxia* sp.	PA inner reef	FJ884075.1
	Whalleya microplaca A	TDE outer slope	JQ760548.1
	Sordariomycete (or *Whalleya microplaca* B)	TDE outer slope	FJ416301.1
Sediments			
Didymosphaeriaceae	*Paraconiothyrium variabile*	TDE outer slope	JQ936271.1
Hypocreaceae	*Trichoderma atroviride*	TDE outer slope	KC008065.1
Trichocomaceae	*Talaromyces albobiverticillius* A	TDE outer slope	JN899313.1

Pigment producing fungi (42) were isolated from all types of samples: sediments (3), living coral *Pocillopora* sp. (7), unidentified coral rubbles (4), hard substrates (reef basis or volcanic rocks) (13) and seawater (15) (Table 2).

The most represented fungi, in the selection of colored micromycetes, belonged to the family Trichocomaceae with *Penicillium, Talaromyces* and *Aspergillus* genera (11 species); and then came the Hypocreaceae with *Trichoderma, Hypocrea* and *Acremonium.*

A high diversity of pigmented isolates was observed from the so-called "hard substrates" (rocky basis on which the coral colonies recruit, or submerged lava flows). Some *Nigrospora, Sporisorium, Whalleya,* and *Rhodotorula* isolates were collected from the outer slope at Trou d'Eau (TDE), although they are rather rarely isolated from marine environment. In Sainte Rose, *Penicillium* species (*P. citrinum, P. viticola*) as well as *Fusarium equiseti, Epicoccum sorghi, Nectria haematococca,* were successfully revived from lava flow, sampled at −25 m.

From our study, the coral rubbles (dead parts of corals) contained colored *Penicillium* or related species: *P. herquei* and two isolates of *Talaromyces albobiverticillius* (B and C), as well as an isolate of *Chaetomium globosum.* Coral rubbles or hard substrates naturally appear diversely colored underwater. Indeed, they support the colonization by multiple organisms (colored algae or other aquatic organisms), visually detectable when sampling.

The revivable colored fungi sheltered by the living coral *Pocillopora* sp. belonged to the genera *Aspergillii* (*A. creber, A. sydowii* and *Eurotium amstellodami* (the teleomorphic form of *A. amstellodami*), as well as to *Penicillium* (*P. viticola*), *Hypocrea* (*H. koningii*) and *Acremonium.*

Some fungal species were identified from different types of samples in the same area. At TDE outer slope, *Talaromyces albobiverticillius* A came from sediment and *T. albobiverticillius* B and C were revived from coral rubbles. *Nectria haematococca* was found in lava substrate (−25 m) (isolate B) as well as seawater (−70 m near lava flow) in the same area (isolate A).

Some similar species also appeared in separate locations: *Aspergillus sydowii* was found near lava flow on the east coast (seawater, −70 m) (isolate B) and also in living *Pocillopora* colonies (isolate A), from the west coast back reef (PA site). *Penicillium viticola* was isolated from the west coast on living *Pocillopora* sp. coral in PA (isolate C), from seawater in TDE back reef (isolate B), as well as from lava flow hard substrate (−25 m), on the east coast (isolate A).

These fungi found in several samples and/or in different locations may be considered as frequent in this marine environment.

3.2. Pigment Production

3.2.1. In Culture Broth

The majority of the isolates produced pigments after four days of fermentation in PDB. The colors of the broth (biomass plus liquid culture medium) always darkened over time, which indicated their potential for pigment production (Figure 2).
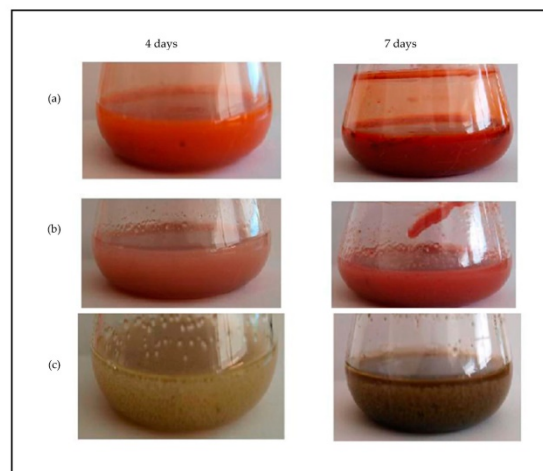
Figure 2. Colors observed in potato dextrose broth cultures from (a) *Talaromyces albobiverticillius* B, (b) *T. albobiverticillius* C, and (c) *Aspergillus creber* B, after four and seven days.

Overall, it was observed that the coloring trend was not directly related to the genus. Even if dominant colors such as yellow, red, brown, purple, orange, pink and green were observed in flasks, the hues were extremely diverse according to the species, even to the isolates (Table 3).

Table 3. Dominant colors of culture broth [1], extracellular (EC) [2] and intracellular (IC) [3] pigments from fungal isolates.

Fungal Isolates	Approximate Hues			Fungal Isolates	Approximate Hues		
Isolates *with Intense Hues (Purple/Red/Maroon)*				*Isolates with Orange Hues*			
	Broth	EC	IC		Broth	EC	IC
Acremonium sp.				*Penicillium viticola* A			
Talaromyces albobiverticillius A				*Penicillium viticola* B			
Talaromyces albobiverticillius B				*Epicoccum sorghi*			
Talaromyces albobiverticillius C				*Penicillium brocae* NRRL 32599			
Aspergillus sydowii A				*Penicillium herquei*			
Aspergillus creber A				*Aspergillus sydowii* B			
Aspergillus creber B				*Chaetomium globosum* or *C. murorum*			
Emericella qinqixianii				*Penicillium viticola* C			
Trichoderma atroviride				*Penicillium citrinum*			
Biscogniauxia sp.				*Hypocrea koningii*			
Paraconiothyrium variabile							
Myrothecium atroviride							
Isolates with Yellow Hues				**Isolates with Green/Brown Hues**			
	Broth	EC	IC		Broth	EC	IC
Peyronellaea glomerata				*Talaromyces verruculosus*			
Eurotium amstelodami				*Talaromyces rotundus*			
Rhodosporidium paludigenum				*Wallemia sebi*			
Periconia sp. A				*Sporisorium exertum*			
Periconia sp. B				*Hortea werneckii*			
Rhodotorula mucilaginosa				*Whalleya microplaca* A			
Fusarium equiseti A				*Whalleya microplaca* B			
Fusarium equiseti B				*Nigrospora sphaerica* or *Env.* sample			
Fusarium equiseti C				*from marine air*			
Nectria haematococca A				*Cladosporium cladosporioïdes*			
Nectria haematococca B							

[1] Culture broth: mycelium + liquid medium; [2] EC: filtrate from liquid culture medium; [3] IC: intracellular extract of fungal pigments.

Indeed, some similar-looking fungi, identified under a unique accession number (i.e., sharing the same sequence for the considered gene), nevertheless developed different color-phenotypes, while cultured under the same culture conditions. As an example *A. creber* A developed a red hue, clearly different from the green-like color *of A. creber* B ("broth" column in Table 3). Moreover, if the same coloring trend was applicable to all the three isolates of *T. albobiverticillius* (A–C) or *P. viticola* (A–C), different shades of red or yellow-orange hues, respectively, were noticed (Figure 3).

Figure 3. Colors observed from culture filtrates from three isolates *of Penicillium viticola* (**A–C**) (seven-day cultures in potato dextrose broth).

Oppositely, no clear difference could be visually established among the pale pink shades of the three isolates of *F. equisetti* (A–C) or the two *N. haematococca* isolates (A and B).

3.2.2. Pigmented Contents from Mycelium

For all pigment-producing isolates, the intracellular pigments (from mycelium) were extracted from the biomass. The approximate colors visualized after extraction are presented in Table 3. The pigments from most of the extracellular fungal culture filtrates were of dominating red, orange, yellow, green, brown, pink and violet. However, after extraction from biomass, many intracellular samples were uncolored, especially for isolates producing extracellular culture filtrates of pink, yellow and green color. This is probably characteristic of isolates essentially secreting water-soluble colored molecules in the culture media.

Instead, many dark colored cultures, mainly in the shades of red or maroon extracellular pigments, gave dark pigmented intracellular extracts from the biomass, indicating that the pigment was also highly concentrated inside the mycelium. These mainly concerned the isolates included in the group "isolates with intense hues", and in the group "isolates with orange hues" to a lesser extent (Table 3). Thus, isolates appeared with different status and varying capacities, towards pigment production.

3.3. Spectrophotometric Characterization of Pigments

As shown in Figure 4, the absorbance spectra of intra- and extracellular solutions from a single isolate revealed quite similar profiles characterized by a strong absorbance in the UV region and also an area of absorbance in the visible range of wavelengths. The values were principally located in the 400–480 nm area for pale yellow to yellow-orange pigments. The maximal absorbances spread in the 500–550 nm region for red colors.

(a)

(b)

Figure 4. *Cont.*

(c)
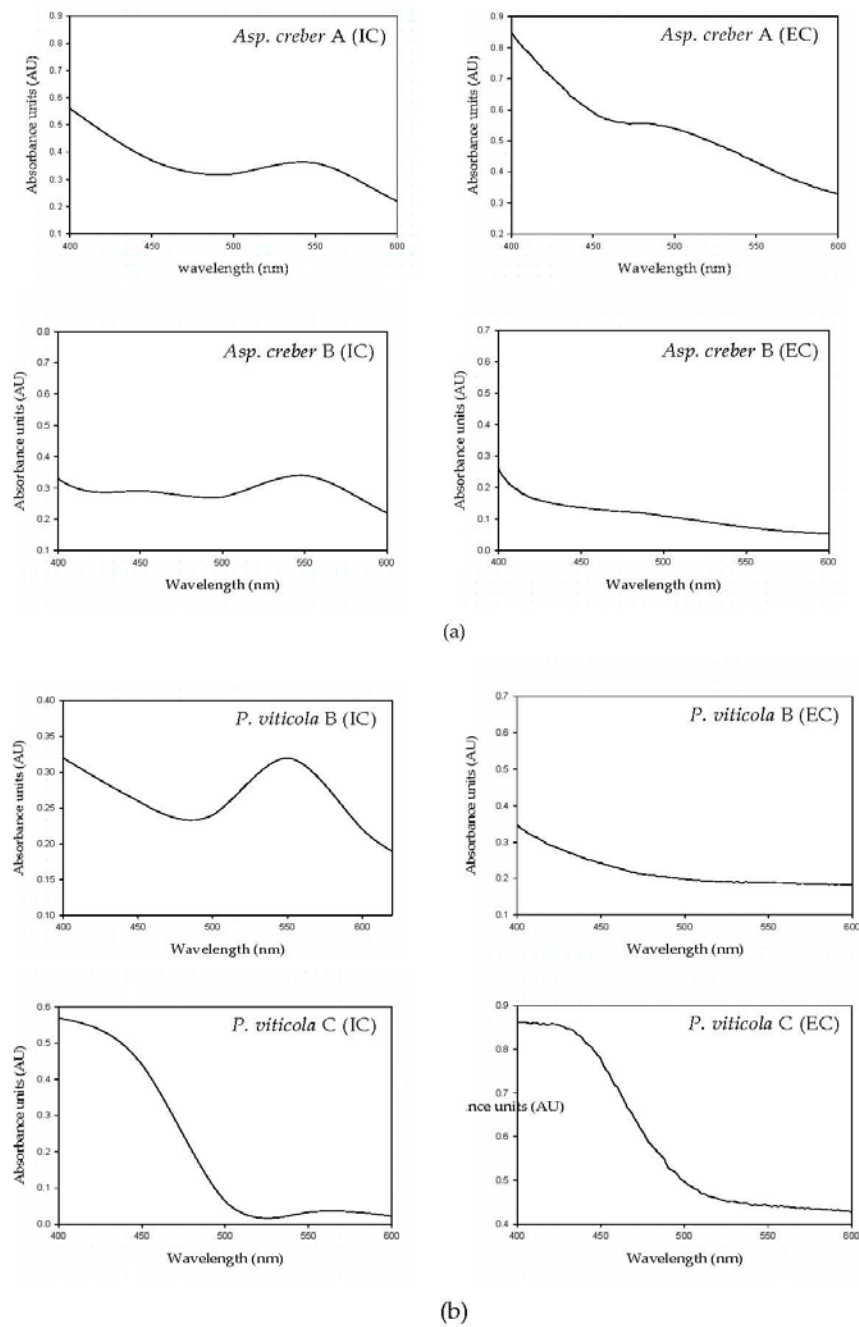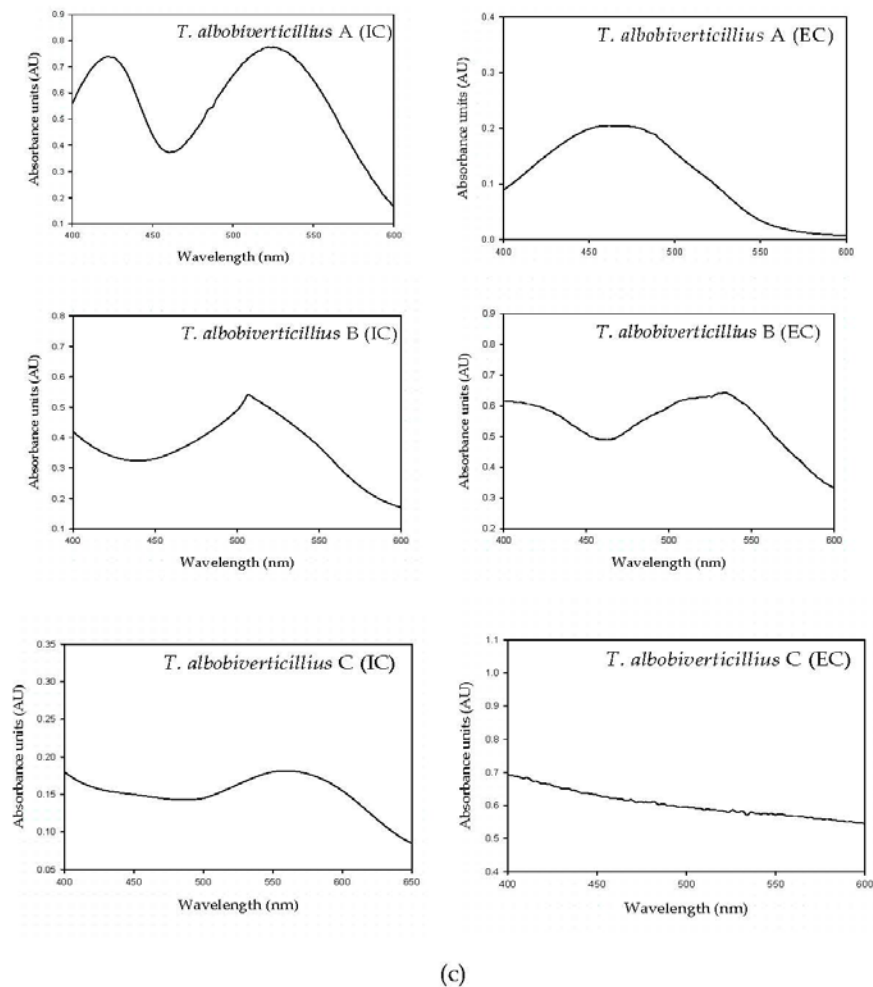
Figure 4. Intracellular (IC) and extracellular (EC) UV-visible spectra of: (**a**) *Aspergillus creber* A and B; (**b**) *Penicillium viticola* B and C; and (**c**) *Talaromyces albobiverticillius* A–C cultures in potato dextrose broth (7 days).

However, slight variations were noticed between extra- and intracellular liquids: in *A. creber* A as an example, extracellular maximum absorbance was around 470 nm (yellow-orange hue) instead of 550 nm (red shade) for intracellular liquid (Figure 4a). These slight variations however indicate that intra- and extracellular solutions may contain different assortments of colored compounds, in different proportions, resulting in different hues (Figure 5).
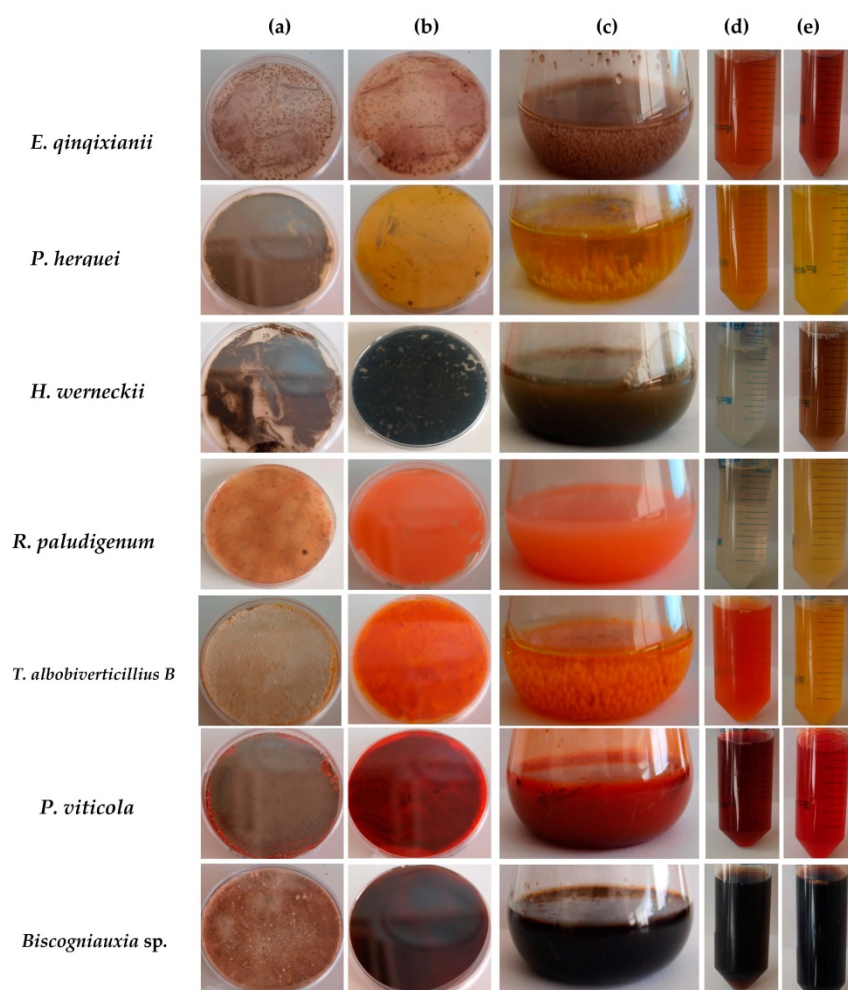
Figure 5. Colors observed in different fungal species: (**a**) obverse face on PDA; (**b**) reverse face on PDA; (**c**) culture in PDB (seven days); (**d**) extract of intracellular pigments (Ethanol/water 50/50) (IC); and (**e**) filtrate from liquid culture (EC).

Differences were also observed among the spectral profiles of different isolates belonging to the same species. As shown from the intracellular profiles of *A. creber* A and B, *P. viticola* B and C, and *T. albobiverticillius* A–C (Figure 4a–c, respectively, and Table 4), maximal absorbance areas differed in the visible region (510–560 nm for *P. viticola* B and 420–450 nm for *P. viticola* C; and 422–525 nm for *T. albobiverticillius* A, 500 nm for *T. albobiverticillius* B and 520–580 nm for *T. albobiverticillius* C), but, for *A. creber* A and B, the spectra looked similar (Figure 4a,b). Similar variation was stated between the extracellular profiles.

These results clearly imply that isolates from a same species produce and secrete different pigments and therefore have different behavior towards colored compound production.

Table 4. Summary of main peaks (λ_{max}) noticed in 10-days old culture of *Talaromyces albobiverticillius* isolates A–C cultivated in liquid medium (potato dextrose broth).

T. albobiverticillius	Sample	Peaks in the UV Region (nm)		Peaks in the Visible Region (nm)	
		200–250	**250–300**	**300–400**	**>400**
A	IC	235	286	362	422, 425, 511, 525
	EC		265	365	458, 469.8, 480
B	IC	232	268, 292		410, 440, 460, 500
	EC		288		412, 524, 532
C	IC	222	283	385	520–580
	EC		283	370, 385	436

3.4. Evaluation of Intracellular and Extracellular Contents in Pigments

The amount of pigments in IC and EC solutions, expressed in mg eq. Purpurin L^{-1}, are presented in Figure 6.
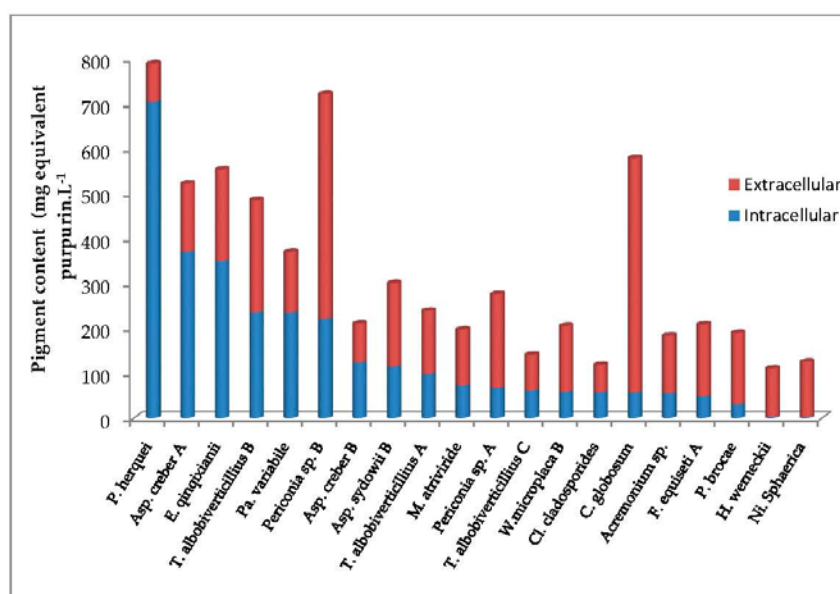
Figure 6. Colored compounds in biomass (intracellular, IC) and culture filtrate (extracellular, EC) for 20 isolates of marine derived fungi isolated around La Réunion Island, in mg eq. purpurin L^{-1} of culture medium (potato dextrose broth, absorbance at 254 nm).

In regard with the diversity of isolates and colored compounds involved in this study, instead of the intensity of the color, the values express the global amount of polyketides compounds produced by each isolate, hues ranging from pale green, light yellow to dark red or maroon.

In the extracellular samples, the maximum amount was produced by *Chaetomium globosum* (521.44 mg equivalent purpurin L^{-1}), followed by *Periconia* sp. B (498.39 mg equivalent purpurin L^{-1}). For intracellular samples, the maximum levels were measured for *P. herquei, A. creber* A, *E. qinqixianii* with 704.55, 371.18 and 350.93 mg equivalent purpurin L^{-1}, respectively.

The amount of intracellular content was significantly lower than the one of the extracellular content in this population ($n = (20,20)$, $V = 10$, $P = 6 \times 10^{-4}$). However, looking at each isolate separately, the amount of intracellular pigments was significantly higher than the extracellular one for *P. herquei* (704.55 vs. 84 mg equivalent purpurin L^{-1}), and *A. creber* A (371.18 vs. 151.11) and B (125.25 vs. 86.35), and *E. qinqixianii* (350.93 vs. 202.74).

4. Discussion

4.1. Biodiversity of Marine-Derived Fungi around La Réunion Island

From the sampling in La Réunion Island marine biotopes, 31 different species distributed in 25 genera were identified as pigment producers. The identification of isolates collected in coral reefs and lava flows of La Réunion Island coincides with identifications conducted from various marine environments. Indeed, the majority of the studied fungi, such as those sampled from north of the Indian Ocean, belong to the phylum Ascomycetes. The fungi of the genus *Aspergillus*, particularly *A. sydowii*, are also found in the Caribbean corals (*Porites lobata*), Polynesia, and in sediments off the coast of India [46,47]. *Penicillium citrinum* was isolated from the red algae *Actinotrichia fragilis*, from sponges, and the species was also found on other substrates such as hard substrate or water [48–51]. The genera *Penicillium*, *Cladosporium*, *Chaetomium*, and *Fusarium*, and species *Nigrospora oryzae* and *Hortea werneckii*, have been identified in marine sediments collected at different depths in the central basin of the Indian Ocean and considered to be coral pathogens [8,52]. Fungi, namely *Alternaria* sp., *Acremonium* sp. and *Rhodotorula mucilaginosa*, were isolated from salt lakes in Antarctica, as were *P. chrysogenum* and *P. crustosum* [53]. *Rhodotorula mucilaginosa* was also found in the sediments of central Indian basin [52].

The diversity within the isolated fungal population was crucial while comparing the ability of pigment production [54]. However, in our samples, the highest diversity of pigmented fungi was revealed from the water column (13 species) and from hard substrates (limestone or lava flow) (11 species). If the water column can be suspected of carrying a multitude of fungal propagules originating from terrestrial environments, hard substrates are probably more representative of marine and marine-derived biodiversity.

Our study demonstrates that the living coral *Pocillopora* sp. shelters fungi from the genera *Aspergillus* (*A. creber*, *A. sydowii* and *Eurotium amstellodami*) and *Penicillium* (*P. viticola*), as well as *Hypocrea koningii* and *Acremonium* sp. Widely disseminated on land, this mainly saprophytic genus *Acremonium* sp., has already been isolated from marine environments (sea fans, sea water, sea cucumbers, and intertidal sediment samples) [55–57]. These fungi, were extracted from the inner parts of the coral structure. They are then supposed to be at least endophytic species for this coral genus.

The coral rubbles (dead part of corals) from our samples contained colored *Penicillium* (*P. herquei*) or related species *T. albobiverticillius* as well as an isolate of *Chaetomium globosum*. *Chaetomium globosum* is a common fungal species from soil and environment.

Most of the fungi we identified can also be found on land, in soil, on plants or insects, but some of them have rarely been isolated from marine environments such as *Whalleya microplaca*, *Biscogniauxia* sp., *Paraconiothyrium variabile*, *Myrothecium atroviride*, *Nectria haematococca*, *Peyronellaea glomerata*, *Epicoccum sorghi*, *Sporisorium exsertum* and *Periconia* sp.. From our study, the genera *Aspergillus* and *Penicillium* or the close ones such as *Talaromyces*, *Emericella* or *Eurotium* (from the Trichocomaceae family) are much more diverse than others in these tropical marine biotopes (12 different species), and are represented in several types of samples and locations. These aerobic and xerophilic species are well-known for populating dry and/or salty biotopes. However, their ability to subsist or develop underwater, with widely varying oxygenation conditions is less known. These cosmopolitan fungi are well-known to produce a wide range of secondary metabolites such as polyketide-based pigments in solid and liquid cultures. Overall, in our study, some fungal species (*T. albobiverticillius* or *N. haematococca*) were identified from different types of samples in the same area. Some others (*A. sydowii*, *P. viticola*)

appeared in separate locations. These fungi found in several sample types and/or in different locations may be considered as frequent in marine environment around La Réunion Island.

4.2. Qualitative Aspect of the Pigment Production

For marine-derived isolates, two statuses lead to particular behaviors and products: the challenge of facing unusual living conditions (exogenous fungi) and the use of specific procedures naturally adapted to the marine niches (for instance fungal endophytes of marine microorganisms, i.e., indigenous micromycetes, naturally selected by aquatic environments).

Overall, in unusual biotopes (sometimes extreme), the fungal species with pigmented cell walls (in the spores and/or mycelium), are clearly able to tolerate dehydration-hydration cycles or high solar radiations, better than the moniliaceous fungi, whose cells are devoid of pigments. These aromatic compounds, as melanin, sporopollenin (brown product of oxydative polymerization of β-carotene) or cycloleucomelone (terphenylquinone), often show significant antioxidant activities, and are bound to protect the biological structures, giving them an excellent durability and a high potential for survival in hostile environments [58,59].

From the available literature, the microorganisms of the genus *Trichoderma* are frequent in marine environments and some terrestrial strains are able to produce anthraquinone-like compounds [60]. Indeed, isolates of the family Hypocreaceae (*Trichoderma, Hypocrea* and *Acremonium*) are also represented in our study and exhibit orange to purple hues. Some strains of the common soil fungus *Cladosporium cladosporioides*, also isolated from our samples with green shades, have already been studied for their production of intracellular melanin [61].

The most important colored compounds produced by *Aspergillus* and *Emericella* species are respectively, hydroxyanthraquinones and azaphilone pigments, exhibiting a very wide range of hues. Furthermore, *A. sydowii* and *Eurotium amstelodami* isolated from La Réunion Island showed red and yellow colors respectively, as produced by their terrestrial counterparts [62].

Penicillium species and related ones seem to adjust easily to multiple conditions and to be a source of original compounds as they appear among the most chemically inventive fungi. In *Penicillium* and *Talaromyces* species, polyketide-based pigments are also very common, and, particularly, the azaphilones, such as the derivatives of monascorubrin and rubropunctatin [63]. *Monascus*-like azaphilone pigments such as *N*-glutarylmonascorubramine, *N*-glutarylrubropunctamine, monascorubramine homologues PP-V [(10Z)-12-carboxyl-monascorubramine] and PP-R [(10Z)-7-(2-hydroxyethyl)-monascorubramine] are frequently identified in their cultures [64,65]. However the commercial production of red anthraquinoid pigments (Arpink Red™, Natural Red™) has already been carried out with *P. oxalicum var. armeniaca* [1]. The most common hues produced by both genera include yellow, red, orange and reddish-brown. Nevertheless, it was found that the yellow pigments seem predominant in most of the *Penicillium* species, while *Talaromyces* species mainly produce red pigments with few synthesizing yellow compounds of azaphilone series [66]. The colored molecules sometimes demonstrate mycotoxic activities such as rubratoxins A and B, rugulovasins and luteoskyrin [67].

Some strains of the widespread *Acremonium* sp. produce the yellow oosporein (chaetomidin) (biquinone, benzoquinone) and also some toxic compounds as diterpene glycosides [68].

Chaetomium globosum, isolated from the coral rubbles biosynthesizes maroon pigments in the culture conditions of our experiment. Many members of the family produce metabolites with antifungal properties. *C. globosum* is already known to biosynthesize yellow azaphilones named chaetoviridins (A–D), antifungal compounds involved in the induction of chlamydospores-like cells [69]. It also produces nitrogenous azaphilones (4′-epi-*N*-2-hydroxyethyl-azachaetoviridin A, and *N*-2-butyric-azochaetoviridin E) and isochromophilone XIII, with orange to red hues. Some strains generate pigmented chaetoglobins, chaetoglobosins, chaetomugilins, and seco-chaetomugilins, while others can secrete a purple pigment called cochliodinol [70–73].

Associated with lava flows, *Fusarium equiseti* belongs to a group of widespread plant pathogens, but marine-derived *Fusarium* strains are also frequent in mangroves or associated with marine organisms. These are already known to produce original colored anthraquinoid compounds (5-acetyl-2-methoxy-1,4,6-trihydroxy-anthraquinone;6,8-dimethoxy-1-methyl-2-(3-oxobutyl)-anthraquinone and fusaquinones) [19]. Among the *Fusarium* secondary metabolites, numerous polyketide pigments have already been identified, such as naphthoquinone pigments which are the most abundant (bikaverin, nor-bikaverin, javanicin, anhydrojavanicin, fusarubin, anhydrofusarubin, bostrycoidin, and novarubin) and the hydroxyanthraquinones emodin, physcion, dermolutein, chrysophanol, erythroglaucin, dermocybin, dermorubin, tritisporin, cynodontin, helminthosporin or aurofusarin (review in [19,21]). All these molecules develop a palette of colors, ranging from yellow to purple or brown. Some species are also able to produce orange carotenoids (neurosporaxanthin by *F. fujikuroi*) [74]. The putative carcinogen, fusarin C, apicidin F, fujikurins, the perithecal pigments fusarubins as well as the mycelial pigment bikaverin are also produced in the family.

From our work, *Periconia* sp. A isolate produced an impressive violet hue in PDB culture. *Periconia* is a cosmopolitan genus, often found in soil, and decaying herbs and forages. Some *Periconia* strains were nevertheless identified from marine environments (*P. abyssa* (deep sea), *P. byssoides* (sea slug *Aplysia kurodai*)) [75–77]. They attract interest because of the production of promising anti-cancer drugs, such as the carbosugar pericosine A. Some strains may produce an unidentified hepatoxin.

4.3. Quantitative Aspect of Pigment Production

As a promising factor, several of the marine-derived fungi isolated in this study had the ability to grow and biosynthesize pigments in unsalted synthetic conditions (e.g., Czapek Dox medium, PDB). During the period of fermentation, the pigment production started between Day 1 and Day 4 for the majority of isolates such as *Aspergillus, Eurotium, Fusarium, Nigrospora, Pencillium* and *Talaromyces*. For some fungi, the detection of the pigment production was notably delayed (e.g., *Acremonium, Epicoccum*, or *Myrothecium*). This might be due to the low level of pigment producing ability of the fungi or due to unfavorable environmental conditions for pigment production such as pH, temperature, nutrient sources, osmolarity and illumination conditions [78].

Considering the visual observation of pigment color in flasks and the respective UV-visible spectra, fungi belonging to the same species may produce different colored mixtures (e.g., *Aspergillus creber* A and B or *Talaromyces alboverticillius* A–C). They may then belong to different varieties and thus produce pigments of distinct natures. The slight variations observed between intra- and extra-cellular solutions also indicate that the solutions may contain different assortments of colored compounds, in different proportions, resulting in different hues.

From these findings, it is understood that a higher quantity of pigments has been mainly purified from extracellular filtrates in a significant manner (11/21 isolates). In our experimental conditions, the maximum pigment production was obtained in the extracellular samples for *C. globosum* and *Periconia* sp. B. On average, the values measured in the cells were significantly lower; indicating that pigments secretion in the liquid medium seems a widespread behavior in the conditions of the experiment. Only the isolate *P. herquei* had a very high level of intracellular pigment biosynthesis (704.55 mg equivalent purpurin L^{-1}). Nevertheless, for high intracellular pigment production from biomass, *A. creber* A and *E. qinqixianii* present a true production potential. On the other hand, the extraction of intracellular colored compounds appeared sometimes not completely effective. The fungal biomass was still colored even after extraction. The efficiency of the extraction process could probably be improved to recover higher pigment quantities from intracellular samples [79].

This work highlights different behaviors of fungal isolates towards the secretion of colored molecules compared to internal storage. Anyway, the production of secondary metabolites often occurs after fungal growth has ceased, as a result of nutrient limitation coupled with excess carbon availability. This makes it possible to manipulate their formation [80,81].

5. Conclusions

Marine and marine-derived fungi are promising resources for the production of new metabolites of interest, and, among them, pigments are attractive [82–84]. The potential of marine-derived microorganisms to produce unique and original molecules may come from specific metabolic or genetic adaptation appearing to meet very specific combinations of physical and chemical parameters (high salinity, low O_2 penetration, low temperature, limited light access and high pressure). Based on this statement, our study explores, for the first time, the biodiversity of fungi from marine environments around La Réunion Island, Indian Ocean, along with the ability of the isolates to produce pigments. The potentiality of these marine derived isolates to secrete pigments or to concentrate colored compounds inside the cells was highlighted. Several isolates collected from lava flows, hard substrates sediments and corals (living or dead) turned out to be the interesting producers of intense colors on PDA culture medium. The main types identified, *Aspergillus*, *Penicillium* and related genera, are also found in other marine regions (such as Polynesia or along the coast of India). However, a great biodiversity (31 species) emphasizes the range of possible hues and molecules susceptible to be isolated. The majority of the isolates, probably marine optional, may also be able to grow in synthetic media, devoid of sea salts and may show the competence of producing pigments in an industrial scale. The most promising pigmented products, probably of intense red or purple hues, which seem to consist in mixtures, will be subjected to purification and further analyses by analytical techniques such as liquid chromatography–mass spectrometry/time-of-flight (LC-MS/TOF) and Nuclear Magnetic Resonance (NMR). The interesting isolates will also be subjected to further analyses to determine their ability as antibiotics or for enzyme production.

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3.4 CONCLUSION

In this segment, major groups of fungi are discussed briefly to highlight the extent of biodiversity and nature of pigment production followed by the examples that deserve great attention. Nearly 150 micromycetes were reviewed from the several samples collected in four different sites of marine environments. Forty-seven colored fungal strains were identified through genomic sequencing and the most predominant fungal genus includes *Penicillium, Talaromyces* and *Aspergillus*. These widespread fungi produce a large number of interesting secondary metabolites, which often show pharmaceutically relevant bioactivities and may be candidates for the development of natural colorants. By and large, the world of fungi provides a fascinating and almost endless source of biological diversity, which is a rich source for exploitation.

CHAPTER 4

GENERAL CHARACTERIZATION OF FUNGAL GROWTH AND ITS PIGMENTS PRODUCTION IN SUBMERGED FERMENTATION OF THE MARINE-DERIVED *Talaromyces albobiverticillius* 30548: A DOE BASED APPROACH

4.1 BACKGROUND

Due to the extensive interest in process development for pigment production from the isolated fungi, there must be given an emphasis towards optimizing culture conditions for maximum productivity. Media composition and growth conditions influences culture growth and thus in turn affect pigment production. Traditional optimization techniques such as "One factor at a time" fails to identify the variables that gives rise to maximum response, also consumes labor and time. Statistical methods are an alternate to traditional methods, used to optimize a process by considering the mutual interactions among the variables and its response. Design of Experiments (DoE) is one of the most valuable techniques for organized and efficient planning, execution and statistical evaluation of experiments [1, 2]. The concept of DoE is to vary process parameters simultaneously over a set of planned experiments and then to interpret the results with the proven mathematical model that allows greater understanding of the process.
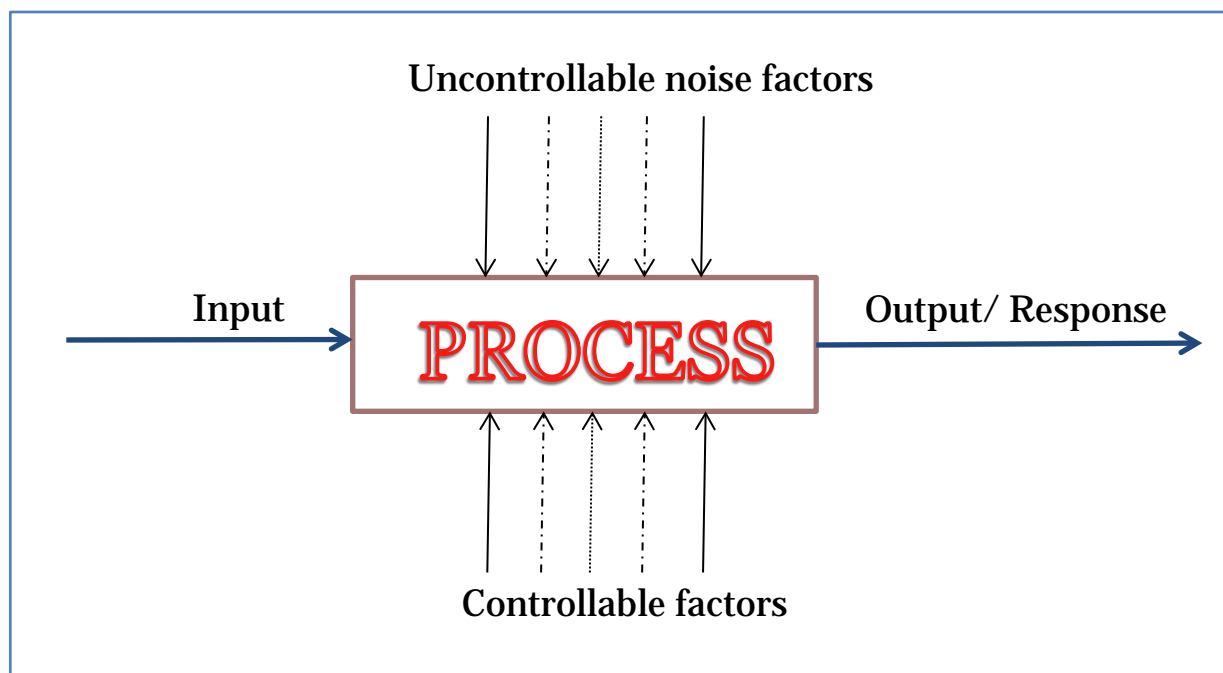
Figure 4.1 The Parameter diagram or P-diagram used during DoE

4.1.1 Basic process parameters

Initially, the key target is the optimization of basic process parameters such as temperature, pH, agitation speed, fermentation time, dissolved oxygen, illumination conditions, and inoculum age. Amongst all, the main factors dominating in submerged state fermentation are temperature and pH. Both the parameters affects the rate of biochemical reactions therefore affects the rate of biomass growth and pigment formation. However, by changing the levels of temperature and pH, the biomass and pigment production varies. For example, from numerous studies of pigment production on *Penicillium* and *Monascus sp.,* the ideal pH range for pigment production lies between pH 4 – 7[3, 4]. In *Monascus sp,* usually at low pH values (pH 4), there was the domination of yellow pigments and once the pH reaches above 6, red pigment production exceeds than the yellow. In *Talaromyces albobiverticillius* 30548, similar pattern of pigment production was observed and thus the levels of pH were varied from acidic to alkaline levels (pH 4-9). Similarly, change in other process parameters influences the final desired output of the pigment yield.

With a view to gather information on the effect of various factors on the response variables (pigment yield and biomass growth) and also to determine the optimal settings of those factors (initial pH, temperature, agitation speed and fermentation period), experimental designs were implemented. Using Response Surface Methodology (RSM), a three level four factors box behnken design was used to identify the effect of process variables such as initial pH, temperature, agitation rate and fermentation period on pigment production and biomass growth.

4.1.2 Optimization of culture media and components:

With the optimized environmental/process conditions, further study was carried out to formulate the suitable media for enhanced pigment productivity and fungal growth in *Talaromyces albobiverticillius* 30548. Formulating liquid medium for lab scale experiments includes the addition of water, carbon, nitrogen, minerals and other supplements in pure form. In general, the color of the pigments produced by the fungus varies depending on the culture conditions but mostly influenced by the nutrient sources [5, 6]. In fungal growth, composition of the nutrient medium (type of carbon and nitrogen sources and C/N ratio), have been shown to influence the biomass growth and type, and the yield production coefficient of secondary metabolites [5, 7-9]. The carbon source generally provides energy for growth and secondary metabolism. It also provides the carbon for making various cell structures, organic chemicals and metabolites. For instance, in *Monascus sp.,* it was noticed that the type and concentration of the carbon source affected its growth and most commonly used sources are glucose, maltose and starch [10]. Certain other studies have been performed using other sources such

as lactose, fructose, xylose, sucrose and presented different results which found to be inferior for growth and pigment production [5].

Similarly, a source of nitrogen is essential for microbial growth and it comprises approximately 10% of dry weight of fungi [11]. The type of nitrogen source influences growth, sporulation and type of pigments production. Besides, the consumption of different nitrogen sources produces different pH profiles in uncontrollable fermentations and this in turn affects the growth pattern and pigment production [12, 13]. Organic sources of nitrogen such as peptone, monosodium glutamate (MSG) and inorganic ammonium and nitrates have been reported to stimulate cell growth and promote pigment production in *Monascus sp., Penicillium sp., Fusarium sp., Phoma sp.* [7, 14-16]. Further, it was reported that optimal carbon and nitrogen sources for production of pigments by fungi are strain dependent [17]. In addition, trace elements such as zinc, manganese, iron, magnesium and phosphorus have been reported to profoundly influence the secondary metabolism of fungi. The composition of these trace elements was chosen from literature studies and fixed throughout the experiment only by changing carbon and nitrogen sources for each experiment [4].

After reviewing a number of available literatures, different carbon and nitrogen sources have been chosen to perform the experiments to optimize better culture media for biomass and pigment production of *Talaromyces albobiverticillius* 30548. Screening of different carbon and nitrogen sources influencing better pigment production was carried out Plackett-Burman design and method of steepest ascent was used to find out the process variables range. The details and soundness of Plackett – Burman design in screening the variables for media optimization, results from the experiments and interpretation of those results were summarized in manuscript 4.3

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4.2 RESEARCH ARTICLE

Production of pigments from the tropical marine-derived *Talaromyces albobiverticillius*: new resources for red natural colored metabolites

This research manuscript emphasize the morphology, genotyping, pigment production, color characteristics, spectral data of pigmented extracts of *Talaromyces albobiverticillius* 30548. This article is the outset of this chapter.

1 # Production of pigments from the tropical

2 # marine-derived *Talaromyces albobiverticillius*:

3 # new resources for red natural colored

4 # metabolites

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14 **Abstract**

15 Filamentous fungi are ubiquitous organisms that produce many chemical classes of secondary

16 metabolites bound to be used in various fields of industrial applications such as cosmetics,

17 food, paper, textiles, medicine, biodegradation, waste remediation, etc. The present study is

18 focused on pigment production from *Talaromyces albobiverticillius*, fungi isolated from

19 marine environments around Reunion Island, Indian Ocean (21°06'54.5" S, 55°32'11.0" E).

20 This study demonstrates their ability to produce a wide range of colored compounds

21 exhibiting a specific orange/red hue under submerged fermentation in potato dextrose broth

22 (PDB), both seen in the mycelia and liquid medium. For the most promising strain, maximum

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23 levels of extracellular orange-yellow pigments (22.39 UA at 470 nm) and red pigments

24 (18.67 UA at 500 nm) were attained in 8 days with an initial pH of 5.0 at 24°C. Instead, the

25 maximum biomass (5.48 g/L) was noticed in 7 days under the same culture conditions. After

26 8 days of culture in PDB, two compounds were produced in larger amounts in comparison

27 with the total pigmented extracts. The compound I, red colored, presents two absorbance

28 maxima ($\lambda_1 = 422$ nm, $\lambda_2 = 511$ nm); the compound II, orange colored, absorbs at $\lambda_1 = 469.8$ nm

29 as a maximum. Both compounds belong to the family of polyketides. The color

30 characteristics of the extracellular fungal products were measured by LAB quantitative

31 colorimetric system and compared to commercially available *Monascus* colorant. The values

32 of a* and b* were found to be positive and hue values (h) ranged from 36.05 to 72.05

33 confirming the existence of red-orange colored compounds in the fungal pigment production.

34 A first approach of the pigment production in relation with the growth phases is presented

35 here as a main objective. In addition, this article provides information about the future

36 directions towards large scale cultivation of these fungi which may support the industrial

37 applications of these red pigments.

38 Keywords: *Talaromyces albobiverticillius*; marine-derived fungi; Reunion island;

39 genotyping; red pigments production; biomass; intracellular; extracellular

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Highlights

- Filamentous fungi started to be investigated as readily available sources of chemically diverse pigments and colorants
- Literature had revealed some species of *Talaromyces* secrete large amount of red pigments
- *Talaromyces albobiverticillius* produces *Monascus*-like red pigments without co-production of mycotoxins
- The main target is to characterize and optimize the production of toxin free red pigments in a large scale from the marine-derived *T. albobiverticillius*

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1. INTRODUCTION

65 **1. INTRODUCTION**

66 While extremely complex, marine environment reveals a great diversity of fungi that could

67 present significant impacts on the biogeochemical evolution of habitats (Newell, 1996).

68 Recently, marine-derived fungi have been recognized as one of the last barely tapped sources

69 for new original secondary metabolites such as enzymes, pigments and other bioactive

70 compounds (Vita-Marques et al., 2008). These secondary metabolites considered as natural

71 products, find their importance in food, cosmetic and medical fields. Overall, research on

72 marine-derived fungi has led to the discovery of 272 new natural products until 2002 and

73 currently it exceeds 1,000 compounds. Many of these have novel carbon skeletons, thus,

74 providing evidence that marine-derived fungi have the potential to be a rich source of

75 pharmaceutical leads and to provide original compounds (Bugni and Ireland, 2004; Imhoff,

76 2016a; Rateb and Ebel, 2011; Saleem et al., 2007). Among the profusion of secondary

77 metabolites, production of pigments has gained scientific and industrial interest due to their

78 economic importance and the attractive wide array of possible natural colors respectively.

79 *Penicillium* and the related genus *Talaromyces* are ubiquitous fungi that include many species

80 requiring minimum conditions (oxygen requirements, basic nutrient sources) to grow. This

81 makes these genera interesting candidates to facilitate the growth of the strains in laboratory

82 and industrial context, especially with regard to biomass or pigment production. Many

83 secondary metabolites commonly produced by *Penicillium* and *Talaromyces* species, and

84 including similarly colored compounds belong to the family of anthraquinones and

85 azaphilones. As a matter of facts, *Penicillium* species including *P. brunneum*, *P. frequentans*,

86 *P. islandicum*, *P. rugulosum* produce diverse anthraquinones and related metabolites showing

87 antitumor properties (Bouhet et al., 1976; Howard, 1947; Howard and Raistrick, 1950;

88 Kiyoshi et al., 1984; Nicoletti et al., 2008; Sankawa et al., 1973). According to recent

89 literature studies, *Penicillium* strains namely *P. purpurogenum*, *P. aculeatum*,

4

90   *P. funiculosum,* and *P. pinophilum,* are novel producers of *Monascus*-like natural colorants

91   such as mitorubrin, monascorubrin, PP-V, PP-O, PP-R, xanthomonasin A, threonine

92   derivatives of rubropunctatin without coproduction of mycotoxins (Frisvad, 2014; Hailei et

93   al., 2011; Mapari et al., 2009; Méndez et al., 2011a).

94   *Talaromyces* is the fifth most productive genus of exometabolites, and those are unique to

95   that genus or only shared with few other species (Samson et al., 2011a). For instance, colored

96   extrolites such as mitorubrins, certain bisanthraquinones (rugulosin, skyrin), duclauxin and

97   glauconic acid were detected in *Talaromyces,* but never found in *Penicillium* sensu stricto

98   (Frisvad et al., 1998). In total, at least 316 exometabolites are produced by different species

99   of *Talaromyces* and the average number of exometabolites per species is evaluated as 3.58

100   **(Frisvad, 2015).** Such species namely *T. albobiverticillius, T. atroroseus, T. marneffei,*

101   *T. minioluteus* and *T. purpurogenus* produce polyketide red pigments in different proportions

102   depending on the species and even on the isolates (Frisvad et al., 1990a; Frisvad et al., 1990b;

103   Mapari et al., 2009; Samson et al., 2011a; van Reenen-Hoekstra et al., 1990). Generally,

104   *Talaromyces spp.* produce pigments in various colors mainly in yellow, orange and red hues

105   seen either in the mycelium or diffusing into the culture medium (Yilmaz et al., 2014).

106   These pigmented secondary metabolites do not seem to be directly involved in the initial

107   mycelial growth of the fungus in which they occur (Calvo et al., 2002; Singh et al., 2012b).

108   However they may play an important role in cell protection or ecological interactions with

109   other organisms (Frey-Klett et al., 2011; Pagano and Dhar, 2015). For these reasons, many

110   fungal secondary metabolites exhibit useful biological activities and are of interest to the

111   pharmaceutical, food and agrochemical industries (Demain and Fang, 2000; Imhoff, 2016b;

112   Imhoff, 2016a).

5

113 The present study focuses on *Talaromyces albobiverticillius* 30548 and two other *T.*
114 *albobiverticillius* (30570 & 30570_3) isolated from marine biotope of outer slopes, from the
115 West Coast of Reunion Island (Southwestern Indian Ocean). These strains were purified,
116 identified and studied for their potential to produce water-soluble pigments in synthetic
117 culture media, under laboratory conditions. The fungi were characterized both by phenotypic
118 and molecular ways for accurate identification. The pigment productions were analyzed
119 through spectrophotometry and color values through spectrocolorimetry, with pigments
120 coming either from the mycelium or from the culture filtrate.

121 **2. MATERIALS AND METHODS**

122

123 **2.1.   Samples collection: origin of the fungal strains**
124 Reunion Island is located in the Southwestern Indian Ocean (21°06'54.5" S and
125 55°32'11.0" E), 800 km east of Madagascar. This tropical island arose 2 million years ago
126 from a volcanic hot spot, and a coral reef covers a concentrated part of the West Coast.
127 Situated at La Saline reef, Trou d'Eau is a 500 m-wide sheltered reef flat located downstream
128 of seawater flowing from the ocean through a narrow path, in the compact reef (Conand et
129 al., 1997; Montaggioni and Faure, 1980; Naim et al., 2000; Peyrot-Clausade et al., 1999;
130 Turner and Klaus, 2005).

131 For the purpose of research, samples of dead coral, living coral, hard substrates, sediments
132 and open water were collected from different marine locations that cover both inner reef flat
133 and outer slope of the fringing reef of Trou d'Eau (Fig. 1a). The samples were collected using
134 sterile bottles during May 2012. The collected samples were stored in a cooling box (4°C)
135 during the transport to laboratory and were treated immediately to isolate the fungi under
136 sterile laboratory conditions.

137

6

138 <mark>insert Fig.1a</mark>

2.2.   Culture and purification of the fungi

140 The collected hard substrates and parts of living/dead coral were washed with 70° alcohol

141 and rinsed in sterile sea water. Then they were ground separately using sterile pestle and

142 mortar. Five grams of ground material was taken from each sample and added to 15 mL of

143 sterile diluent [1.6 g of tryptone (Sigma, T-9410), 0.05 g of Tween 20, 1 L of sterile sea water

144 pH= 7.5]. After stirring for 20 min at 150 rpm on a shaking table (Edmunt Bühler GmbH,

145 VKS 75 Control), the suspension was diluted by employing serial decimal dilution method.

146 For each diluted sample, 1 mL was poured on Petri plates containing malt extract agar (MEA)

147 and Sabouraud agar prepared with seawater. All the platings were performed as triplicates

148 and incubated at 25°C for 21 days. During this period, the plates were checked each day for

149 the appearance of new colonies. Each new colony was individually isolated and cultured on

150 new MEA medium.

151 The studied fungi were then cultured using monospore technique to isolate single pure fungal

152 thalli. For long-term storage, the fungi grown on potato dextrose agar (PDA) for 5 days were

153 scraped and transferred into a sterile vial containing a cryoprotectant composed of 15%

154 skimmed milk and 2% glycerol. The vials were stored at -80°C. In addition, the stock

155 cultures were also maintained on PDA plates at 4°C and sub-cultured at regular intervals.

156 Subject to the pigment production characteristics, three fungal strains producing red pigments

157 were initially selected for experiments. Those strains were collected from the external slope

158 of Trou d'Eau, with collection numbers viz. 30548 (refereed hereafter to strain A) isolated

159 from sediment, 30570 and 30570_3 (refereed as B and C, respectively), both isolated from

160 dead coral. Strain A was further selected for detailed studies because it exhibited deep red

161 color pigment production.

7

162 **2.3. Determination of morphological features**

163 For phenotypic characterization, strain A was cultivated as triplicates and studied on five

164 different agar media such as Czapek yeast autolysate agar (CYA), dichloran glycerol

165 chloramphenicol agar (DG-18), oatmeal agar (OA), MEA and PDA (Difco) under a constant

166 culture condition at 25°C, under day light exposure. During the growth period, colony

167 characteristics such as shape/size of the colonies, mycelial textures and colors, production of

168 pigments on both obverse and reverse surfaces were reported on each day. Radial growth rate

169 was determined by measuring the colony diameter which is frequently used as parameters of

170 fungal characterization. In addition to the visual observation, morphological characteristics

171 such as conidia, conidiophores, phialides and branching patterns were examined under a

172 photonic microscope (Olympus CX41, Japan) after staining using the protocol of James and

173 Natalie (2001) (James and Natalie, 2001).

174 **2.4. Fungal DNA extraction**

175 To extract DNA from the purified strains, a small amount of mycelium along with spores was

176 taken and cultivated on PDA at 25°C under day light exposure. After five days of growth, the

177 fungal mycelium was scraped and DNA was extracted using DNeasy Blood & Tissue kit

178 (Qiagen™, France). The amount and purity of the DNA contained in each extract was

179 evaluated by measuring the absorbances at 230, 260 and 280 nm (Nanodrop 2000, Thermo

180 Scientific) and calculating the ratio A_{260}/A_{280} and A_{260}/A_{230}. The DNA was stored at 4°C

181 prior to amplification (Knebelsberger and Stoger, 2012).

182 **2.5. Primers selection, PCR amplification and sequencing**

183 On the basis of observed phenotypic characteristics, the PCR primers were chosen

184 accordingly for molecular identification. The β-tubulin and ITS regions were amplified using

8

185   T10/Bt2b and V9G/LS266 primer pairs (for large subunit rDNA), respectively <mark>(Table 1)</mark>

186   (Glass and Donaldson, 1995; Samson et al., 2014; Samson et al., 2011b; Toju et al., 2012).

187   The fragments of fungal DNA were amplified using PCR thermal cycler (GeneAmp® PCR

188   System 9700, Applied Biosystems™). PCR reactions were carried out in a total volume of

189   30 µL: 1X of MasterMix (Applied Biosystems, Foster city, CA, USA), 0.5 µM of forward

190   and reverse primers and at least 1.3 ng/µL of genomic DNA. Amplifications were carried out

191   according to the following program: 94°C for 5 min + 35 × (94°C for 30 s, 55°C for 60 s,

192   72°C for 60 s) + 72°C for 5 min for final elongation step. Amplification products were

193   subjected to electrophoresis on a 1.5% agarose gel to check the size, concentration and

194   quality of amplified DNA (Johansson, 1972).

195   <mark>insert Table 1</mark>

196   **2.6.   Sequences Analysis**

197   Amplicons were sent for sequencing in both directions to Genoscreen (Lille, France) on a

198   capillary sequencer ABI 3730XL (Applied Biosystems). The obtained electrophoregrams

199   were read and corrected with the software Chromas v.2.13. The extracted sequences for each

200   primer pairs were separately used to perform nucleotide searches using online BLAST

201   algorithm provided by NCBI (**http://www.ncbi.nlm.nih.gov/BLAST/**). Following to that,

202   the outcomes from the BLAST searches were sorted based on the maximum identity to the

203   query sequence and considered as the best hit. Sequence-based identities with a cutoff of 97%

204   or above and query coverage > 90% were considered as significant (Romanelli et al., 2010;

205   Sutton et al., 2013).

206   **2.7.   Phylogenetic construction and data analysis**

207 ITS and β-tubulin gene sequences for representative species of *T. albobiverticillius* obtained

208 from GenBank, were aligned using CLUSTAL W in MEGA v.6. Phylogenetic analyses of

209 these two regions were performed using maximum likelihood (ML) method using MEGA

210 v.6. The robustness of the phylogeny was tested using 1000 iterations of bootstrap analysis

211 and these values underpin the percentage of iterations supporting the constructing topology.

212 *Talaromyces purpurogenus* was selected as a suitable outgroup in all the phylogenies.

213 **2.8. Determination of dry biomass weight in liquid cultures**

214 **2.8.1 Culture conditions**

215 Erlenmeyer flasks (250 mL) containing 50 mL of PDB were autoclaved at 121°C for

216 15 minutes. Mycelium of strain A grown on PDA Petri plates (120 mg) was taken and

217 transferred into the sterile flasks and incubated at 25°C under day light exposure, with a

218 continuous agitation of 150 rpm for 10 days (Infors Multitron Pro).

219 **2.8.2 Determination of fungal dry biomass**

220 Separation of biomass and culture filtrate was made through a 48 μm Nitex filter cloth

221 (SEFAR AG, Switzerland), the mycelial biomass was dried in a thermostatic oven (model

222 100, Memmert) at 105°C until the weight becomes constant. The dried samples were allowed

223 to cool by placing in a desiccator for a minimum of 30 min. The total dry biomass weight was

224 determined by gravimetric analysis and calculated using the following formula:

225 $$Dry\ biomass = \frac{(w - w_f)}{v_s} \quad (g/L) \quad ......(1)$$

226 Where:

227 W = weight of filter with dried biomass (g)

228 W_f = weight of empty filter (g)

10

229   V_s = Sample volume (L)

230   **2.9.   Production of pigments**

231   **2.9.1   Daily monitoring of pigment yield**

232   The production of pigments was evaluated from both biomass and culture liquid, incubated

233   using the same condition as described under 2.8.1. The kinetics of growth and pigment

234   production was monitored once in every 24 hours as triplicates. For daily monitoring, 5 mL

235   of liquid culture were sampled from the fermented broth under sterile conditions. The liquid

236   culture medium containing extracellular pigments (EC) was separated from mycelia

237   (containing the intracellular pigments, IC) by centrifugation at 8000 rpm for 6 minutes and

238   followed by filtration through nylon filter of 48 μm pore size (Nitex, SEFAR AG,

239   Switzerland). The filtration, as a second step after centrifugation, removes the cells and

240   mycelial fragments coming along with supernatant, minimizing the analytical error in

241   spectrophotometer during absorbance measurement. The absorbance of the culture filtrate

242   containing EC was immediately scanned by spectrophotometer in the wavelength range

243   between 230 and 700 nm. After the end of the fermentation period, *i.e.* 10 days, the

244   remaining culture medium was separated from mycelia by vacuum filtration using Whatman

245   N°2 filter paper (Merck). The separated contents were frozen at -80°C, lyophilized and then

246   the pigments were extracted from both lyophilized biomass and culture filtrate using organic

247   solvents.

248   **2.9.2   Extraction of pigments**

249   Intracellular pigments (IC) contained in the wet mycelium were extracted using solvents of

250   methanol: water combination (1: 1 v/v) as conventional extraction method. The solvent

251   mixture was immersed in an ultrasonic bath at 45°C for 30 minutes. The suspension was

252   allowed to stir overnight at room temperature on a shaking table (Edmunt Bühler GmbH,

11

253 VKS 75 Control). On the following day, it was filtered through Whatman N°2 filter paper to

254 separate the solvent containing the pigments extracted from biomass. Similarly, the

255 extracellular pigments (EC) in the culture filtrate were extracted using the same solvent

256 combination.

2.9.3 Spectrophotometric characterization of pigments

258 To analyze the extracellular pigment contents, the absorbance of the crude colored solutions

259 were measured using a microplate reader (Tecan Infinite® 200 PRO series) (Kim et al., 2002).

260 The colored solution was adjusted using purified water as a diluent obtained from Milli-Q

261 system (Merck Millipore). The absorption maxima were determined by scanning the colored

262 solutions over the range of 230-700 nm. The chromophore is a chemical group that absorbs

263 light of specific frequency and imparts color to a molecule. Common fungal polyketide

264 pigments, such as anthraquinones or azaphilones, are often highly substituted aromatic

265 molecules with fused benzene rings (Zhou et al., 2010). Thus the majority of the common

266 chromophores from fungi absorb in the UV region (one or several peaks between 200-

267 300 nm), whereas absorbance in the visible region (from 400-700) highly depends on the

268 nature and the number of substituted groups.

269 The quantification of pigments was estimated by measuring the absorbance of filtrates at

270 254 nm, at which several polyketide pigments show strong absorbance (Caro et al., 2012).

271 Absorbance was also measured at 470 nm, 500 nm representing the region of absorbance for

272 yellow and red colors, respectively. Even though, the maximum absorbance varies depending

273 on the growth phase; daily monitoring is necessary due to logging of fungal growth with

274 change in color of the media correlating to pigment production. The pigment yields of strain

275 A were expressed in absorbance units (UA) at its maximum wavelength region i.e. 470 and

276 500 nm (General et al., 2014).

277

278 **2.9.4 Colorimetric characterization of pigments**

279 The extracellular colored solution after separation from biomass was used to obtain

280 quantitative descriptions of the color. The L*a*b* c h° color coordinates were measured in

281 the CIE L*a*b* color space using spectrocolorimeter (Minolta CM-5000 d, Konica Minolta,

282 Mahwah, NJ). The CIE L*a*b* colorimetric system was interpreted as follows: $L*$ indicates

283 lightness read from 0 (black) to 100 (white). The positive $a*$ value indicates the red color

284 while the negative a* value represents the green color. Similarly, positive and negative $b*$

285 indicate the yellow colors and blue colors. Chroma values ($c*$) denote the saturation or

286 intensity of color. In the color wheel, values close to the center, at the same L* value, indicate

287 dull or gray colors, whereas values near the circumference represent vivid or bright colors.

288 Hue angle values ($h*$) represent the degree of redness, yellowness, greenness, and blueness,

289 the maximum is at 0, 90, 180 and 270 degrees, respectively (Mapari et al., 2006).

290

291 **3. RESULTS**

292 **3.1. Ecology of the habitat**

293 The fungal strain A used in this study was isolated from a sediment sample collected from the

294 outer slope of the fringing reef (Trou d'Eau, Reunion Island, Southwestern Indian Ocean;

295 22°5'23.99" S, 55°14'7.03" E). Two other colored strains belonging to the genus

296 *Talaromyces* (B, C) were isolated from dead coral, also collected in the outer slope from the

297 same zone (Fig. 1b).

298 Insert Fig. 1b

299 The studied strain (A) was isolated at a depth of 17 m from the sea surface and this zone is

300 recorded with a temperature of 26-27°C during the sampling period in May. Most fungi are

301 mesophilic and some fungi adapt to grow in cold environments with a stable temperature of

302 few degree Celsius (Kohlmeyer and Kohlmeyer, 1979; Robinson, 2001). Herein, the isolated

303 strains produce pigments even when cultured using varying laboratory conditions pointing to

304 pH 4.0-9.0, temperature of 21-27°C, conditions of dark and light exposure (data not shown).

305 This indicates that *T. albobiverticillius* is capable of living or surviving underwater, in this

306 tropical euphotic area, under different physicochemical conditions.

307 **3.2.   Morphological features of Strain A**

308 Phenotypic features of strain A were studied on five different agar media under a constant

309 culture condition (static incubation under day light illumination at 25°C). In general, the

310 mycelium appeared as white and yellow, sometimes red that grew rapidly by dense to

311 moderately dense sporulation, according to the culture media. The colonies became mature

312 within 120 h and red metabolites were released during this stage. After the maturation period,

313 further incubation of the culture led the pigments to leach off which drastically changed the

314 red pigments to brown compounds. All the tested culture media supported the growth of the

315 fungus to varying degrees and the growth of colonies mentioned in the following were

316 measured on day 7 (Fig. 2).

317 On PDA, the fungus showed rapid growth (21-24 mm) by producing green mycelia along

318 with diffusing water-soluble red pigments to the exterior surface of the medium. After

319 maturation, the texture of the fungus became powdery.

320 In MEA, this strain exhibited faster growth (24-28 mm) by producing white and yellow

321 mycelia with an orange velvety texture in the center. Soluble pigment was absent on the agar

322 surface but visible red droplets emerged on top of the thallus.

323 Whereas on CYA, white and yellow mycelia were observed with floccose to velvety texture

324 at the center (18-20 mm). The colony was slightly raised at the center, possessing red

325 droplets, also diffusing soluble red pigments on the entire surface of culture medium and

326 notable dark red color on the reverse side (Fig. 2).

14

327   <mark>Insert Fig. 2</mark>

328   When grown on OA (23-28 mm), mycelia appeared as white and yellow with velvety texture

329   wearing orange droplets. Soluble pigments were absent in the agar while the back side was

330   dark pink to red in color. Albeit grown on DG-18, the strain showed rapid growth (24-

331   36 mm) with green mycelia, in addition producing soluble red pigments on the agar surface.

332   The reverse side of the colony appeared as dark brown after 7 days of maturation.

333   Mentioning the micro-morphological structures, when cultivated on MEA, conidiophores are

334   strictly biverticillate; stipes are smooth walled and 2.1-2.9 μm wide; metulae (1.3 x 4 μm) are

335   10 to 18 per stipe; phialides are acerose (1.5 x 3 μm and 3-5 per metulae). While grown on

336   PDA and OA, conidia are smooth to finely rough walled, globose to subglobose, (1.2-2 μm

337   wide) <mark>(Fig. 3).</mark>

338   <mark>Insert Fig. 3</mark>

339   **3.3.   Molecular characterization of fungi**

340   From taxonomic identification, the three fungal strains (A, B, C) isolated from the marine

341   environment exhibited exactly the same sequence for both genes (ITS and β-tubulin). Both

342   alignments were 659 and 660 bp long, respectively. The phylogenetic reconstructions

343   allowed identifying them as *T. albobiverticillius*. Strain A was moreover double characterized

344   by CBS-KNAW (The Netherlands) due to its interesting nature of dark red pigment

345   production. This teleomorphic species belongs to the family *Trichocomaceae* (Ascomycota)

346   and the genus is holophyletic in a cladistics sense and polythetic class in an anagenetic or

347   functional sense (Frisvad, 2015). The fungal ITS ribosomal and β-tubulin gene sequences

348   were submitted to the Genbank and the accession numbers of each identified fungal taxon

349   were mentioned in <mark>Table 2.</mark>

15

350 <mark>Insert Table.2</mark>

351

352 These three new strains resolved in a distinct clade, separated from all the other species in

353 both phylogenies but remained closely related to other red pigment producing species such as

354 from *T. albobiverticillius*, *T. purpurogenus* and *T. atroroseus* group <mark>(Fig. 4, 5)</mark>.

355 <mark>Insert Fig. 4</mark>

356 <mark>Insert Fig. 5</mark>

357 **3.4.   Pigment and biomass production**

358 **3.4.1   Pigment profile based on absorbance spectrum**

359 Overall, it was observed that cultures of all three *T. albobiverticillius* strains (A, B, C)

360 produced red pigments with varying intensities in their color range, becoming more intense

361 with time (Fig. 6)

362 <mark>Insert Fig. 6</mark>

363 Pigmented solutions (IC and EC) prepared from the three strains A, B, C after 10 days of

364 culture, were orange to red in color giving light to dark hues. The electronic absorption

365 spectra of the colored extracts (from the lyophilized biomass and liquid culture) were

366 measured using microtiter plates at 230-700 nm to determine the maximum absorbance

367 wavelength of its pigment components in the UV and visible areas. They showed that the

368 pigment profiles were not identical among the three isolates: A was different from B and C

369 <mark>(see Table 3, 4)</mark>. Strains B and C demonstrated peaks mainly in the UV (260 – 385 nm) and

370 also in the visible region (410-470 nm) indicating yellow or orange hues, whereas A

371 exhibited an additional peak in the visible region, at 511 nm as absorption maximum (red

372 color). Moreover intracellular and extracellular extracts also presented different profiles,

373 particularly for strain A. For intracellular pigmented extract of A, the first absorption peak

374 was in the range of 410-428 nm and the second peak has a maximum at around 490-525 nm,

375 which indicated the presence of red colored compounds in the whole crude pigments (Fig. 7

376 a). For extracellular pigments, the UV-visible spectrum showed maximum peak at 469.8 nm,

377 implying that the compounds exhibited an orange hue and the profile of IC pigments bound

378 to the mycelium diverged from the EC ones (Fig. 7 b).

379 Insert Fig. 7 a, b

380 All three strains supported growth in PDB in conjunction with pigment production but in

381 comparison strain A developed an intense dark red color. Thereupon, the rate of pigment

382 production was further monitored merely for strain A on a daily basis.

383

384 **3.4.2   Colorimetric characterization**

385 Commercially available *Monascus* red yeast rice (Wuhan Jiacheng Biotechnology Co., Ltd)

386 used as a reference to compare the color characteristics of red pigments produced by three

387 strains (A, B, C) (Table 4 a). Lightness values (L*) of the colored solutions obtained from the

388 three strains extended from 57.12 to 75.32 representing the intensity range of light absorption

389 and thus in comparison with *Monascus* standard (54.02), the L* value of A (57.12) was close

390 rather than B (62.12) and C (75.32). In fact, the average observer will see hue differences

391 first, chroma differences second and lightness differences last.

392 The a* values of the pigmented solutions (A, B, C) extended from 28.66 to 56.73 which

393 appeared to be positive. While the b* values were also positive and ranging from 22.69 to

394 42.94. The experimental pairs (a*, b*) of the three strains showed different pigmentations

395 that displayed diverse hues (red, orange) and thus in turn supported the interpretation that hue

396 variation existed among the three isolated strains (A, B, C).

17

397   The hue angles (h°) of the extracellular colored solutions varied from 36.05 to 50.18, this

398   range signifying orange-red colored pigments in the extract. As represented in color wheel

399   (Fig. 8), the color coordinates of the three strains exhibiting dark red color. The commercially

400   available reference standard *Monascus* red (R), indicated h° of 47.19 which is nearly close to

401   the value of fungal pigments representing orange red color. Ultimately, the control red

402   pigment, *Monascus* red had maximum wavelengths of absorption at 416 and 526 nm, similar

403   to the absorption range of the three fungal strains indicating almost similar redness (Table

404   4 a).

405   Insert Table 4 a

406   The intensity or saturation level of a particular hue was illustrated here in terms of Chroma,

407   as a most descriptive factor. Color differences of the three strains were best separated by their

408   purity. For the three fungal strains, C* responses widely varied as 44.68 (for C), 63.36 (for

409   B), 71.14 (for A). Although, a pale color was noticed for the pigments from strain C, strain B

410   displayed a moderate red color and strain A exhibited a significantly brighter red color. But,

411   none of these three strains showed any evidence of having purity level to orange color. In this

412   case, the colors from strains A and B appeared brighter like *Monascus* red (C*: 84.48)

413   certainly due to its mixed nature of different compounds.

414   Assessment of daily color changes were recorded for strain A in terms of La*b* color values,

415   during the entire fermentation period (from day 1 to 10) and the values are reported in (Table

416   4 b).

417   Insert Fig 8

418   Insert Table 4 b

419   **3.4.3   Characterization of growth and pigments production**

18

420 As shown by Fig. 9, strain A produced red pigments from the 3rd day of fermentation and the

421 color became more intense with time. Observing the wavelength data from day 1 (after 24

422 hours of fermentation) to day 10 of the pigments from fermented culture broth, strain A

423 displayed a wide range of absorption from 400-600 nm (Fig. 9).

424 Maximum productions of orange pigments (22.39 UA) at 470 nm and red pigments

425 (18.67 UA) at 500 nm were noticed on 8th day of culture (Fig. 10). The dry biomass weight

426 was found to be a maximum on 7th day of fermentation, which increased to 5.24 g/L;

427 thereafter the biomass production was limited.

428 Insert Fig. 9

429 Insert Fig. 10

430 **4. DISCUSSION**

431 After a decisive approach towards investigating the marine microbial biodiversity at Reunion

432 Island (Southwestern Indian Ocean), three interesting fungal isolates were found to produce

433 water-soluble red pigments. Normally in the marine habitat, the fungi appear to be low in

434 number when compared to bacteria. Thereupon a special effort is necessary to isolate a large

435 number of fungi from particular samples. However, studies up-to-date conducted on the

436 marine fungi reported that the majority of isolates were from the water and sediments

437 sources. These are supposed to be very closely associated to the land suggesting that

438 environmental influences such as floods and winds carry terrestrial fungi toward the marine

439 environment and thought that the produced compounds and morphological characters were

440 similar to that of terrestrial counterparts (Arumugam et al., 2015; Méjanelle et al., 2000;

441 Morrison-Gardiner, 2002). Indeed, sediments on the seafloor originate from a variety of

442 sources including biota from the overlying ocean water, eroded material from land

443 transported to the ocean by rivers or wind, mechanical fracture of surrounding rubbles, ash

444 from volcanoes, and chemical precipitates derived directly from seawater. This results in a

19

445 heterogeneous mixture of small grains of material ranging in size from micrometers to 1 or 2

446 millimeters. The richness in the composition of these seawater sediments allows the

447 development of a high diversity of micro-habitats sheltering an unstudied diversity of

448 microbes, adapted to these specific physicochemical parameters.

449 In this present work, molecular identification of the isolated fungal strains was performed

450 using phylogenetic analyses based on ITS and β-tubulin gene sequences due to their adequate

451 discriminating potential to distinguish the novel taxa from other recognized taxa (Schoch et

452 al., 2012; Tsui et al., 2011). The three identified strains were grouped in their own clade and

453 related to other strains of *T. albobiverticillius* (Fig. 4, 5). They are brand new identified for

454 the first time from the marine environment and produce intense water soluble red pigments.

455 Past identifications of red pigment production from some species of *Talaromyces*, especially

456 *T. purpurogenus, T. ruber, Penicillium sanguineum* and *P. crateriforme* have generated lot of

457 confusions in identification and resulted in misconception of those species. Relating to

458 *P. rubrum,* hence the unavailability of type material, Raper and Thom (1949) used two strains

459 to describe *P. rubrum*, NRRL 1062 (= CBS 370.48) and NRRL 2120 (= CBS 133452) (Raper

460 and Thom, 1949). Later, a detailed taxonomic study by Yilmaz et al. (2012) described NRRL

461 1062 remained as *T. ruber* (teleomorphic form of *Penicillium*) but NRRL 2120 (= CBS

462 133452) was identified as *T. albobiverticillius* but not *T. ruber* (Yilmaz et al., 2012). Under

463 these circumstances, the isolated fungal strains from the marine environment of Reunion

464 Island were identified and recognized as *T. albobiverticillius* from the phylogenetic analyses.

465 Interestingly, this is the first ever report discussing marine-derived strain of

466 *T. albobiverticillius* among all other available strains which were isolated from terrestrial

467 environments (Frisvad et al., 2013).

468 The strain A demonstrated its ability to produce more intense red pigments on PDB than the

469 other two strains (B and C) (see Fig. 3). There upon, it seems that the metabolites produced

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470   are different, even though the stains belong to the same taxon. This infers that the strains may

471   slightly differ in their behavior (and maybe genetically), depending on the environmental

472   conditions of the isolation areas (Jensen et al., 2007). Similarly, some strains of

473   *T. albobiverticillius* (CBS 113168, CBS 133440[T], CBS 133441, CBS 133452, CBS 313.63)

474   produced red soluble azaphilone pigments on various culture media and others such as CBS

475   133444 and CBS 133448 did not produce any soluble pigments (Frisvad et al., 2013). Though

476   the cell bound and hydrophobic nature of the six prime *Monascus* pigments, the exogenous

477   release of *Monascus*-like pigments was mostly seen in the fermented culture broths of

478   *T. albobiverticillius*. It has been proposed that the release of water-soluble pigments into the

479   culture medium is due to a reaction of aminophilic moieties of the fungi with amino group

480   containing compounds in the medium (Shah et al., 2014a).

481   A report by Frisvad et al (2013) examined the extrolites produced by different strains of

482   *T. albobiverticillius* through HPLC-DAD and/or UHPLC- HRMS and found the presence of

483   *Monascus*-like pigments including yellow (monascin), orange (monascorubrin and

484   rubropunctatin), red (monascorubramine), other series of yellow mitorubrins (mitorubin,

485   mitorubrinol, mitorubrinic acid). In addition to the above compounds, other minor

486   compounds like vermicellin, a purpactin, have been identified from few strains of this taxon

487   (CBS 113167, CBS 113168, CBS 133441, IBT 4466) (Frisvad et al., 2013). As a note to

488   that, research on subsequent color shift of *Monascus* pigments (yellow to orange) could be

489   due to the oxidation of water insoluble yellow pigments which forms orange pigments (Blanc

490   et al., 1994; Lin, 1991). Then the oxygen moiety of the orange pigments is replaced by the

491   nitrogen moiety of a nitrogenous compound to form red compounds (Lin and Demain, 1991).

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494   Considering biomass production, our results indicated that there was an increasing trend up to

495   day 8 until the nutrient medium was used up for biomass synthesis. Many researchers have

496   claimed that different morphology of fungal mycelium under different initial pH levels acts

497   as a crucial factor in biomass formation and metabolite production in submerged fermentation

498   (Kim et al., 2005; Shu and Lung, 2004; Wang and McNeil, 1995). A study by Arumugam et

499   al (2014) on *Nigrospora* sp.(piezotolerant deep sea fungus) observed that biomass and

500   pigment production were influenced by initial pH of the medium and that the biomass was

501   higher when the initial culture medium was set at pH 6.0 (Arumugam et al., 2015). Likewise

502   for *P. purpurogenum* GH2 strain, the maximum biomass concentration (6.045 g/L) was

503   obtained at pH 7.0 and 34°C but pigment production was not favorable in this condition. This

504   indicates that no direct relationship exists between biomass accumulation and pigment

505   production (Méndez et al., 2011b). In relation to the above, dry biomass ranged between 6.02

506   and 11.3 g/L for *Monascus ruber* 62478 and *M. purpureus* 288.34, respectively in a defined

507   medium with an initial pH of 6.5 but produced very low amount of pigments (ratio

508   500/400 nm: 0.69 for *M. ruber* 62478, 0.71 for *M. purpureus* 288.34) compared to other

509   strains with low biomass, indicating no relation between biomass and pigment production

510   (Pisareva et al., 2005).Relating to the red pigment production by strain A in terms of

511   *Monascus* equivalents (500 nm) it displayed maximum production (1.92 g/L) compared with

512   *Monascus purpureus* AKI & AKII (0.92 g/L), *M. purpureus* 915 (1.03 g/L) and not quite less

513   compared to *M. ruber* (2.07 g/L). However, some experiments using different culture media

514   can still improve the pigment production to higher amounts in strain A. (Danuri, 2008;

515   Hamano and Kilikian, 2006).

516   With reference to color measurements observed under D_{65} light, three pigmented solutions of

517   *T. albobiverticillius* exhibited strong differences in their colors and calculated as ΔE^*_{ab}

518   The total difference among each pigmented solution of the three strains exceeded by a

519 margin of visual perceptible threshold, reaching $\Delta E^* = 45$ units for strain C. The differences

520 in ΔL, Δa, Δb with *Monascus* red standard were found negative, indicating that all the three

521 strains had less red color compared to *Monascus* red yeast rice. Jung et al (2003) studied

522 color characteristics of amino acid derivative pigments of *Monascus* and underpinned the fact

523 that various pigments are in the range of orangish red to deep red. Most of the derivative red

524 pigments had two λ_{max} values of 417-427 nm and 498-525 nm and this is in accordance with

525 the absorption range of pigments produced by strain A (410-428 nm and 490-525 nm) (Jung

526 et al., 2003).

527 From the marine sampling, several different species were identified as pigments producers

528 and the diversity within the fungal population was crucial while comparing the ability of

529 pigment production (Yahr et al., 2016). Strains belonging to *Penicillium* and *Aspergillus*

530 genera are the most frequently found fungi in marine environment as well as on earth and it

531 extents somewhat to *Fusarium* and *Cladosporium* genera when given special focus to

532 pigments. Indeed, the genus *Penicillium* has already been identified in marine sediments

533 collected at different depths in the central basin of the Indian Ocean and some of them are

534 considered to be coral pathogens (Cathrine and Raghukumar, 2009; Singh et al., 2012a).

535 Different species of *Penicillium* strains such as *P. marneffei*, *P. purpurogenum*, *P. aculeatum*,

536 *P. pinophilum* have been reported to produce various *Monascus*-like pigments namely

537 monascorubramine and monascin (Büchi et al., 1965; Frisvad, 1989; van Reenen-Hoekstra et

538 al., 1990). *Penicillium verruculosum* SG, a newly isolated fungus, produces water soluble

539 glutamyl monascorubrine in addition to monascorubramine and monascin (Shah et al.,

540 2014b). Other than the uses of pigments in food industries, many reported the anti-

541 inflammatory (Hsu et al., 2012; Yasukawa et al., 1994) and antibiotic activity of the reddish

542 orange pigment monascorubramine (Martinkova and Veselý, 1995).

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543   Regardless of other factors, the degree and intensity of pigment production mainly depends

544   on the composition of the culture medium used for fermentation. For example,

545   *P. funiculosum* IBT 3954, a process patented strain, produces both red and yellow *Monascus-*

546   like pigments under specific media or culture conditions: With the condition of glucose as a

547   carbon source (40 g/L), MSG as nitrogen source (2 g/L), pH of 8.0, C/N ratio of 96.39

548   yielded high amount of red pigments (0.138 g/L) in terms of *Monascus* equivalents but not

549   yellow pigments and the values were estimated at the end of 7 days culture (Shamsuddin et

550   al., 2012). Production of secondary metabolites often occurs after fungal growth has ceased

551   as a result of nutrient limitations but with an excess carbon source available, making it

552   possible to manipulate their formation (Debbab et al., 2011; Gunatilaka and Wijeratne). No

553   doubt that the amount of pigments produced in one condition can be improved, by increasing

554   the knowledge about the particular strain and its cultivation conditions.

555   **5. CONCLUSION**

556   In conclusion, this study confirms that Reunion Island marine environment shelters revivable

557   filamentous fungi with the ability to produce water soluble pigments. This study is a first

558   approach to characterize the pigments produced mainly by *Talaromyces albobiverticillius,*

559   isolated from a sediment sample extracted at -17 m on the outer reef slope. It had been

560   showed that strain A consistently produced dark red *Monascus*-like azaphilone pigments in

561   potato dextrose broth medium in 10 days of fermentation at 24°C and initial pH of 5.0.

562   Some questions arise about the *in vitro* behavior of this strain towards the pigment production

563   in salty environment, pressure, illumination conditions, varying pH ranges which strongly

564   differ from their native biotopes. The behavior of the fungus, its morphology and pigment

565   producing ability using different carbon and nitrogen sources will be studied further under

566   different experimental conditions.

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567   Existing literature works on *T. albobiverticillius* provides an insight about the safety of using

568   this strain for pigment production and specified as highly captivating for industrial scale up

569   without co-production of mycotoxins. Future investigations on this strain should be more

570   focused on pigment production from shake flasks to fermenter by understanding the fungal

571   morphology, growth pattern, influence of oxygen and limiting factors in the fermentation

572   process.

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805

806 **Table 1**

807 PCR amplification and the sequencing primers used for the identification of fungal strains

Primers	Direction	Sequences	Amplification region	References
V9G	Forward	TTACGTCCCTGCCCTTTGTA	ITS	(Hoog and Ende, 1998)
LS266	Reverse	GCATTCCCAAACAACTCGACTC	ITS	(Masclaux et al., 1995)
T10	Forward	ACGATAGGTTCACCTCCAGAC	β-tubulin	(Glass and Donaldson, 1995)
Bt2b	Reverse	ACCCTCAGTGTAGTGACCCTTGGC	β-tubulin	(Glass and Donaldson, 1995)

808

809 **Table 2**

810 Identification of three fungal strains of *Talaromyces albobiverticillius* sourced from marine

811 environments in the reef of Trou d'Eau (Reunion Island) and their color production

812

Talaromyces alboverticillius strains	Identification number	Sampling source	Color intensity	GenBank Accession N°
A	30548	Sediment	Dark red	XXXX
B	30570	Dead coral	Dark red	XXXX
C	30570_3	Dead coral	Red	XXXX

813

814 **Table 3**

815 Summary of the peaks (λ_{max}) noticed in 10-days old culture of *Talaromyces albobiverticillius*

816 strains A, B, C cultivated in liquid medium (PDB)

817

Talaromyces alboverticillius strains	Sample	Peaks in the UV region			Peaks in the visible region
		200-250	250-300	300-400	>400 nm
A	Intracellular	235	286	362	422, 425, 511, 525
	Extracellular		265	365	458, 469.8, 480
B	Intracellular	232	268, 292		410, 440, 460
	Extracellular		288		412

35

233

C	Intracellular	222		283	385	
	Extracellular			283	370, 385	436

818

819 **Table 4**

820   CIELAB color coordinates of (a) Reference standard and extracellular coloured samples of

821   strains A, B and C (10-days old culture) (b) Extracellular coloured samples of strain A for a

822   period of 10 days culture (PDB, initial pH 5.0, 25°C, day light exposure)

823   **(a)**

Sample/ fungal name	Ref code	L	a*	b*	h°a	Chromab	Absorption (nm) $\lambda_{max\,1}$	spectrum λ_{max2}
Monascus red	R	54.02	57.41	61.97	47.19	84.48	420	498
30548	A	57.12	56.73	42.94	37.08	71.14	410-428	490-525
30570	B	62.12	51,22	37.31 67,3	36.05	84,57 53.36	52,75 450-470	
30570_3	C	75.32	28.66	34.28	50.18	44.68	410-440	

824   [a]Hue angle (h_{ab}) = tan[-1] (b */ a *); [b]Chroma (C) = [(a *)2 + (b *)2]$^{1/2}$

825   **(b)**

Day	L	a*	b*	h°a	Chromab	Absorption spectrum λ_{max} (nm)
1	98,54	-0,45	5,38	5,40	95,18	---
2	93,67	9,18	5,93	10,93	32,96	---
3	84,52	19,76	24,34	31,54	50,03	486
4	82,63	24,87	31,39	51,61	40,01	481
5	78,68	30,42	38,56	38,23	49,11	482
6	75,55	35,98	47,53	37.08	59,67	482
7	67,72	49,32	53,07	47.09	72,73	482
8	62,42	45,92	39,80	40,89	62,92	481
9	58,85	54,32	40,79	36,89	58,28	482
10	57,12	56,73	42,94	37.08	71,14	482

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LIST OF CAPTIONS

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831 **Fig. 1. (a)** Location of Reunion Island in the Southwestern Indian Ocean (21°06'54.5" S and

832 55°32'11.0" E) and sampling area **(b)** The two sampling sites on the Western coast (back reef

833 and outer slope in Trou d'Eau, La Saline).

834

835 **Fig. 2.** Macroscopic observations of strain A on five different culture media **(a)** Obverse face

836 of 5-days old colony cultured using three-points fashion; **(b)** Front view (3-days old colony);

837 **(c)** Back view (3-days old colony); **(d)** 7-days old colony

838

839 **Fig. 3.** Strain A: Microscopic structures (25°C, 7 days culture, 100 x magnification, stained

840 with lacto phenol blue) **(a)** mono- and bi-verticillate conidiophores on MEA; **(b)** Conidia

841 on PDA; **(c)** Red colored hyphae on CYA; **(d)** Globose ascomata on OA (a-d: scale bar

842 5 μm).

843

844 **Fig. 4.** Maximum likelihood tree comparing the ITS region of *Talaromyces* species based on

845 the Tamura-Nei model (bootstrap values above branches). *Talaromyces purpurogenus* was

846 used as an outgroup. GenBank accession numbers inside parentheses. Asterisk indicates the

847 three studied strains of *T. albobiverticillius* (A, B, C)

848

849 **Fig. 5.** Maximum likelihood tree comparing the β–tubulin gene region of *Talaromyces*

850 species based on the Tamura-Nei model (bootstrap values above branches). *Talaromyces*

37

851   *purpurogenus* was used as an outgroup. GenBank accession numbers inside parentheses.

852   Asterisk indicates the three studied strains of *T. albobiverticillius* (A, B, C)

853

854   **Fig. 6.** Production of pigments by Strain A, B, C: From left to right: (a) observe and reverse

855   face of culture on PDA; (b) PDB (initial pH 5.0, 25°C); (c) intra (IC) and extra-cellular (EC)

856   crude extracts after methanol/ water extraction (1:1V/V)

857

858   **Fig. 7.** Absorbance scanning of strain A cultured on PDB for 10 days: (a) UV-Visible

859   absorption spectrum of intracellular pigments (IC, extracted from dried biomass); (b) UV-

860   Visible absorption spectrum of extracellular pigments (EC, from culture medium)

861

862   **Fig. 8.** (a) Color wheel with the correspondence between the color of the radiation absorbed

863   by the compounds (wavelength in nm) and the complementary color (color of the

864   object/compound); (b) Scatter plot showing the color values of extracellular pigments of

865   strain A in the CIELAB colorimetric system (day 10)

866   R - *Monascus* red, A – 30548, B – 30570, C- 30570_3

867

868   **Fig. 9.** UV-Visible absorbance spectrum of extracellular pigments (EC) for strain A during

869   the culture period of 10 days (D1 to D10) (200 ml shake flasks, PDB, pH 5.0, 24°C)

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38

871   **Fig. 10.** Absorbance of extracellular pigments (EC) in UA and dry biomass weight (in g/L) of

872   strain A (200 mL shake flasks, PDB, pH 5.0, 24°C). The error bars indicate the standard error

873   from three independent samples.

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4.3 MANUSCRIPT

OPTIMIZATION OF PIGMENTS AND BIOMASS PRODUCTION FROM *Talaromyces albobiverticillius* **30548 UNDER SUBMERGED FERMENTATION CONDITIONS USING RESPONSE SURFACE METHODOLOGY**

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Abstract

Talaromyces albobiverticillius 30548, a marine-derived pigment producing filamentous fungi isolated from La Réunion Island, Indian Ocean has the competence to produce water soluble pigments. Hence, the objective of this study was to examine and optimize the effect of process parameters such as initial pH (4-9), temperature (21 – 27 °C), agitation speed (100 – 200 rpm) and fermentation time (0 – 336 h) on maximal pigments (orange and red) and biomass production. The bio-process chosen was submerged fermentation in 250 mL shake flasks using 100 mL potato dextrose broth (PDB) as working volume and the experiments were carried out employing Box-Behnken response surface statistical experimental design (BBD). From the experimental data, mathematical models were developed to predict the pigment and biomass yields. Response surface methodology (RSM) was adopted to investigate the individual and interactive effects between variables, also used to determine the optimal condition for maximum pigments and biomass production. Evaluation of the experimental results signified that the optimum conditions for maximum production of

pigments and biomass were as follows: initial pH of 6.4, temperature of 24 °C, agitation speed of 164 rpm and fermentation time of 149 h respectively. This is the first report optimizing the process parameters for maximal pigments (orange and red) and biomass production for *T. albobiverticillius* 30548 using PDB by Box-Behnken response surface statistical experimental design (BBRD) under submerged fermentation.

Keywords: Optimization, Pigments, Biomass, *Talaromyces albobiverticillius,* Box-Behnken Design, Response Surface Methodology

1. Introduction

In recent years, development of alternate sources for the production of natural pigments has been focused to overcome the unlimited usage of synthetic pigments, which found to be hazardous to human health and environment [1]. Natural colorants can be obtained from plants and microbial sources which are found to be alternatives for synthetic pigments [2, 3]. Plant derived pigments are highly produced but has limited usage in certain food formulations because of their low water solubility, instability against light and heat [4]. Nowadays, pigments from microbial origin is of great interest worldwide and gaining more importance in food industry applications. Microorganisms including bacteria, algae, yeasts and filamentous fungi from marine or terrestrial origins are capable of producing natural dyes, which paves way to increase the production of natural compounds [5-7]. The use of fungi for the production of commercially important products has increased rapidly over the past half century [5, 8]. Taxonomically, it represents a large group, often prevailing in seawater, corals, sponges, sediments, rocks, sand and others [6]. In general, the colorants produced from fungi are associated with increased yields, and huge array of potential compounds due to their vast diversity in the environment [9]. The majority of the pigments produced by fungi are quinones, flavonoids, melanins and azaphilones, which belong to the aromatic polyketide chemical group [10-12] and have been widely described for medicinal uses and potential use

as dyes [13, 14]. More specifically, filamentous fungi are considered as a promising source of natural pigments because they do not only promote color, but also often possess bioactive properties like anti-cancer, immunomodulatory, anti-proliferative, antibiotics, and so on [15]. Especially fungi isolated from the marine environment reveals a plethora of known or new compounds with still relatively unexplored bioactivities [16-18]. *Talaromyces albobiverticillius* 30548, marine-derived filamentous fungi was isolated from a sediment sample of La Réunion Island, Indian Ocean. Some species of *Talaromyces* such as *T. purpurogenus, T. albobiverticillius, T. marneffei, T. minioluteus* secrete large amount of pigments, thus reported to produce diffusible azaphilone-like red compounds [19-22]. These pigments are homologues of *Monascus* pigments with similar chromophore structures.

Considering fungal pigment production, submerged fermentation appears to be a convenient and economical way. Indeed it provides the space to produce the pigments, owing to their deep commitment to sophisticated control devices and easy monitoring methods during experiments [23-25]. Nevertheless, the culture conditions play a major role in the production of these secondary metabolites and influence the fungal growth to the maximum. To produce pigments in large scale, it is very important to determine and control the factors that have a strong impact on the desired product. Apart from the composition of the culture medium, various abiotic factors affect the submerged fermentation process, such as pH of the medium, temperature of growth,agitation rate (gaz diffusion), fermentation time, and impose a strong pressure on the pattern that brings out the interesting compounds such as pigments [26-29]. The complexity of the scientific experimental design is to evaluate the impact of each factor, but also and above all, their correlated effect. In this context, with the aim of increasing the production capacity of natural pigments by *T. albobiverticillius* 30548, submerged fermentation was carried out in this study.

Box-behnken response surface statistical experimental design was employed in this-work to investigate and optimize the effect of combined process parameters such as initial pH, temperature, agitation speed and fermentation time on the two main colored components yields (orange and red) along with biomass production, through submerged fermentation in potato dextrose broth (PDB). Box-Behnken response surface statistical experimental design is a spherical, revolving response surface methodology (RSM) design that consists of defining a central point and the middle points of the edges of the cube circumscribed on the sphere. Thus it consists of three interlocking 2^2 factorial designs with points lying on the surface of a sphere surrounding the center of the design. Several studies have successfully employed RSM to model and optimize the biochemical and biotechnological processes related to food systems **[30-35].**

2.   Materials and Methods

2.1   Microorganism and maintenance

The filamentous fungus used in this study was isolated from the sediments collected from the marine environment of La Réunion Island, Indian Ocean (GPS coordinates: 21° 06' 22,11" S, 55° 14' 15,78"E). From taxonomic identification of the fungal biodiversity center (CBS Knaw, The Netherlands), the fungus was identified as *Talaromyces albobiverticillius* and named *T. albobiverticillius* 30548. The stock culture was maintained on PDA agar plates at 4 °C (Difco) and sub-cultured at regular intervals for the experiments.

2.2   Preparation of Seed culture

Laboratory grade potato dextrose broth (PDB) (Difco, France) was used to prepare the seed medium. For inoculum preparation, 0.100 g of mycelia with spores was taken from a seven days old pre-culture grown on Petri plate and transferred into 250 mL Erlenmeyer flask containing 80 mL of sterilized PDB medium. The flasks were incubated at 25 °C at 200 rpm

of agitation speed for 48 hours before being transferred to main fermentation medium, and thus considered as pre-culture.

2.3   Fermentation conditions

After 48 hours growth, the pre-culture broth was centrifuged at 8000 rpm for 6 minutes at 24 °C to separate the mycelia and the culture filtrate. In order to optimize as per the defined experimental design (Design-Expert® Software (version **9)**, Stat-Ease Inc.), Minneapolis, MN, U.S.), 200 mg of mycelia was transferred as inoculum into 200 mL of fresh media (PDB) at three different levels of initial pH (4, 6.5 and 9) contained in 500 mL Erlenmeyer flasks, and then incubated at three different temperatures (21, 24, 27 °C) with three different speeds of agitation (100, 150, 200 rpm) for 336 hours. At predefined regular intervals, the fermented broth was sampled to measure the pigments concentration and biomass yield.

2.4   Spectrophotometric quantification of extracellular pigments

About 5 mL of the fermented culture broth was drawn at regular intervals (**Table 2**) and filtered using nylon mesh of 48 µm pore size (SCRYNEL, NY, 48 HC) to separate supernatant and mycelia from the culture broth. The obtained colored supernatant contained two major sets of pigments: orange-yellow and red [36], whose concentrations were determined by measuring optical density through UV-visible spectrophotometer (UV-VIS spectrophotometer UV-1800, Shimadzu, Tokyo, Japan) at 470 and 500 nm respectively. The optical density (AU) at these two absorbance maxima was expressed as the concentration of orange and red pigments produced by *T. albobiverticillius* 30548. The concentration of pigments in the extracts from optical density units was converted in terms of mg/L.

2.5   Dry biomass yield

The biomass obtained through filtration was dried at 80 °C in a hot air oven until it attained a constant weight and the values were represented using the formula as follows (**Eq.1**):

$$Dry\ biomass: \frac{(W-W_f)}{Vs} \qquad (1)$$

W= Weight of the filter with biomass obtained after drying (g)

W_f = Weight of the corresponding empty filter (g)

V_s = Volume of sample (L)

2.6   Experimental design

Box-Behnken design with 4 factors at 3 levels was employed to study the effect of process parameters on the pigments (orange and red) production and biomass yield for the studied strain, according to the defined experimental design (Design-Expert® Software (version **9),** Stat-Ease Inc.). Based on several literature studies and results from experiments conducted in laboratory, the process parameters and their ranges were chosen for this study (Table 1). Each independent design variables was coded at three levels as -1 (low), 0 (central point) and +1 (high) which were: temperature (21 – 27 °C), pH (4 - 9), Fermentation time (24 – 336 h) and agitation speed (100 – 200 rpm). The process variables were respectively denoted as X_1, X_2, X_3 and X_4 in uncoded forms and these variables were converted to coded forms: x_1, x_2, x_3 and x_4 using the following equation (**Eq. 2**) **[37].**

$$x = \frac{X - ((X_{max} + X_{min})/2)}{(X_{max} - X_{min})/2} \qquad (2)$$

Table 1: Coded and actual values of the variables for four factor Box-Behnken design

Variables	Symbol	Coded and actual values		
		-1	0	+1
pH	X_1	4	6.5	9
Temperature (°C)	X_2	21	24	27
Agitation (rpm)	X_3	100	150	200
Fermentation period (h)	X_4	24	168	336

In Box-Behnken method, a total number of 29 experiments including five center points were carried out and the experimental conditions, the corresponding results (responses), are presented in **Table 2**. The performance of the process was evaluated by analyzing the responses (Y), which depend on the input factors $x_1, x_2..., x_k$, and the relationship between the response and the input process parameters is described by **Eq. 3**:

$$Y = f(x_1, x_2...x_k) + e \tag{3}$$

Where "f" is the real response function, the format of which is unknown and "e" is the error which describes the differentiation. In order to analyze the curvature in response, second-order polynomial model must be employed. The second-order polynomial equation depicts the interaction between the factors and the response. For determining the critical points (maximum, minimum and saddle point), the quadratic terms must be embodied in the polynomial model, thus the following second-order quadratic polynomial equation was employed to fit the experimental data. The relevant model terms were obtained using the statistical software, Design Expert 9.0.3.1. A quadratic model, which also includes the linear model, can be described as **Eq. 4**:

$$Y = \beta_0 + \sum_{j=1}^{k} \beta_j x_j + \sum_{j=1}^{k} \beta_{jj} x_j^2 + \sum_{i}^{k} \sum_{<j=2} \beta_{ij} x_i x_j + e_i \tag{4}$$

Where Y is the response; x_i and x_j are variables (*i and j range from* 1 to *k*); β_0 is the model intercept coefficient; β_j, β_{jj} and β_{ij} are interaction coefficients of linear, quadratic and the second-order terms, respectively; *k* is the number of independent parameters (k= 4 in this study); and e_i is the error **[38, 39].**

2.7 Statistical analysis

Experimental design data were analyzed using multiple regressions through the least square method. Statistical testing of the model for each response was done using Fisher's statistical

test for analysis of variance (ANOVA). The fitted polynomial equation was expressed as three-dimensional surface plots to visualize the relationship between the responses and the experimental levels of each factor used in the design. These graphs were drawn by maintaining two factors constant (in turn at its central level) and varying the other two factors in order to understand their main and interactive effects on the dependent variables. The goodness of fit of the model can be checked by the determination coefficient (R^2) and by adjusted R^2 and predicted R^2. R^2 represents the proportion of variation in the response data that can be explained by the fitted model. High R^2 is considered as confirmation for the applicability of the model in the range of variables included. It should be noted that a R^2 value greater than 0.75 indicates the suitability of the model.

Adjusted R^2 is the value of R^2, adjusted down for a higher number of variables in the model which makes it much more useful than the regular value of R^2 and it is suggested to use adjusted R^2 rather than the regular R^2 to assess the fit of a multiple regression model. Predicted R^2 is used in regression analysis to indicate how well the model predicts responses for new observations, predicted R^2 can prevent overfitting. Larger values of predicted R^2 suggest models of greater predictive ability. The coefficient of variation (CV%) indicates the degree of precision with which the treatments are compared. Usually, higher values of CV% indicate lower reliability of the experiment. PRESS means the Predicted REsidual Sum of Squares for the model, a measure of how well a particular model fits each point in the design. Adequate precision (Adeq. Pre.) measures the signal-to-noise ratio and generally, a ratio > 4 is desirable.

2.8 Optimization

After the surface-response results, optimization of the process conditions was carried out by multi-response analysis **[40]** using Derringer's desired function methodology. The general

approach of desirability function is to first transform the response into a dimensionless individual desirability function (g_i) that varies from 0 to 1 (lowest to highest desirability). The overall desirability function (G) was obtained (**Eq. 5**) from the geometric means of individual desires. The Design expert 9.0.3.1 was used to maximize the G.

$$G = (d_1^{n1} \times d_2^{n2} \times d_3^{n3} \times .... \times d_k^{nk})^{1/k} \tag{5}$$

Where d_i is the individual desirability ranged from 0 to 1, k is the number of considered responses, and n_i is the weight of each response.

The following equation (**Eq. 6**) was used to maximize and transform the response into dimensionless desirability (g_i).

$$g_i = \frac{Y_i - Y_{min}}{Y_{max} - Y_{min}} \tag{6}$$

Where, Y_{min} is the response minimum value and Y_{max} is the response maximum value.

3. Results

3.1 Box-Behnken design analysis

Experimental design is widely used for understanding the effect of parameters in a system or a process, so as to decrease the number of experiments, time, and material resources. Furthermore, the analysis performed on the results can be easily realized, and experimental errors are minimized. Statistical methods measure the effects of change in operating variables and their mutual interactions on a system or a process through experimental design [41]. In this study, four factors at three level Box-Behnken design was employed to investigate and optimize the influence of process variables on the pigments and biomass yield by the fungus *T. albobiverticillius* 30548 and the results were listed in **Table 2**.

Table 2: Box Behnken design matrix with the experimental responses

Exp. Run	pH	Temperature (C)	Agitation speed (rpm)	Fermentation time (h)	Orange pigment yield (mg/L)	Red pigment yield (mg/L)	Biomass yield (g/L)
1	0	0	0	0	47.15	192.75	12.19
2	0	0	1	-1	34.02	137.41	11.57
3	0	1	0	1	24.01	97.68	8.33
4	-1	0	0	1	20.01	85.23	10.23
5	0	-1	0	-1	29.01	118.52	10.62
6	0	1	1	0	36.02	139.58	10.74
7	-1	0	0	-1	25.01	94.70	8.74
8	0	0	0	0	46.28	192.05	12.10
9	0	0	0	0	47.08	192.86	12.05
10	0	1	-1	0	10.01	43.17	5.61
11	1	1	0	0	26.01	107.83	8.46
12	0	0	0	0	46.02	191.98	12.07
13	0	0	0	0	47.34	192.83	12.57
14	0	-1	-1	0	32.02	130.76	8.74
15	-1	0	1	0	36.02	150.51	10.01
16	-1	-1	0	0	18.01	83.44	8.75
17	0	1	0	-1	26.01	112.12	7.50
18	1	0	-1	0	36.02	147.90	9.68
19	0	-1	1	0	25.01	103.06	9.15
20	1	0	1	0	43.02	172.83	11.55
21	1	0	0	-1	36.02	147.61	11.45
22	0	-1	0	1	27.01	111.63	9.27
23	0	0	-1	-1	30.02	122.89	9.17
24	1	-1	0	0	35.02	143.78	10.78
25	-1	1	0	0	18.01	82.25	7.37
26	1	0	0	1	40.02	168.34	10.50
27	-1	0	-1	0	14.01	56.88	8.06

| 28 | 0 | 0 | 1 | 1 | 37.02 | 147.49 | 10.87 |
| 29 | 0 | 0 | -1 | 1 | 25.01 | 104.49 | 7.79 |

Model adequacy checking was performed on the experimental data to determine whether the approximating model would give poor or misleading results. Four high degree polynomial models viz., linear, interactive (2 factor interaction), quadratic and cubic models were fitted to the experimental data. Two different tests namely the sequential model sum of squares and model summary statistics were carried out in this study to conclude the adequacy of models among various models to represent the responses. The adequacy of model summary output indicates that, the quadratic model is statistically highly significant for this present work. Quadratic model was found to have maximum R^2, adjusted R^2 (0.94), predicted R^2 (0.84) and also exhibited low *p*-values (0.0001). Hence, quadratic model was selected in this study to investigate the effect of process variables on the pigment and biomass production using *T. albobiverticillius* 30548 strain.

3.2   Development of second order polynomial mathematical models

Mathematical models were developed to obtain a better understanding of the nature of the true relationship between the input variables and the output variables of the system under study. This approximate formula could be used as a proxy for the full-blown simulation itself in order to get at least a rough idea of what would happen for a large number of input-parameter combinations. An empirical relationship expressed by a second-order polynomial equation with interaction terms was fitted between the experimental results obtained on the basis of Box–Behnken experimental design and the input variables. The final equations obtained in terms of coded factors are given below:

For orange pigment yield (OPY) (**Eq. 7**)

$$OPY = 46.77 + 7.09X_1 - 2.17X_2 + 5.34X_3 - 0.58X_4 - 2.25X_1X_2 - 3.75X_1X_3 + 2.25X_1X_4 + 8.25$$
$$X_2X_3 + 0.001X_2X_4 + 2X_3X_4 - 8.42X_1^2 - 13.55X_2^2 - 7.05X_3^2 - 7.67X_4^2 \qquad (7)$$

For red pigment yield (RPY) (**Eq. 8**)

$$RPY = 192.49 + 27.94X_1 - 9.05X_2 + 20.4X_3 - 1.53X_4 - 8.69X_1X_2 - 17.18X_1X_3 + 7.55X_1X_4 + 31.03$$
$$X_2X_3 - 1.89X_2X_4 + 7.12X_3X_4 - 33.17X_1^2 - 54.11X_2^2 - 31.21X_3^2 - 32.32X_4^2 \qquad (8)$$

For biomass yield (BMY) (**Eq. 9**)

$$BMY = 12.2 + 0.77X_1 - 0.77X_2 + 1.24X_3 - 0.17X_4 - 0.24X_1X_2 - 0.021X_1X_3 - 0.61X_1X_4 + 1.18$$
$$X_2X_3 + 055X_2X_4 + 0.17X_3X_4 - 1.02X_1^2 - 2.31X_2^2 - 1.35X_3^2 - 0.97X_4^2 \qquad (9)$$

3.3 Statistical analysis

The adequacy and fitness of the models were tested by multiple regression analysis through the least square method. Significance of the developed models can be determined through ANOVA and the results were shown in **Table 3**. The results of ANOVA indicated that, the developed models adequately represented the actual relationship between the independent variables and responses (**Table 3**). Analysis of variance followed by Fisher's statistical test (F-test) was applied to evaluate the significance of each variable. The large F- values (67.05 for orange pigment yield (OPY) at 470nm, 36.61 for red pigment yield (RPY) at 500nm and 34.02 for biomass yield (BMY)) indicate that most of the variation in the response can be explained by the developed regression equation. The associated p-value were used to estimate whether F is large enough to indicate statistical significance and p-values lower than 0.05 indicate that the developed model and the terms were statistically significant. In our study, the p- values were lower than 0.0001 for all the responses and it exhibits the precise and accuracy of the developed models.

Determination coefficient (R^2), Adjusted- R^2 (R^2adj), predicted- R^2 (R^2pre) and coefficient of variation (CV%) were calculated to check the adequacy and accuracy of the developed

models. The R^2 gives the proportion of the total variation in the responses predicted by the models and the values of R^2 (0.985 for OPY at 470 nm, 0.973 for RPY at 500 nm and 0.971 for BMY) ensures a satisfactory fit of the quadratic model to the experimental data. The values of R^2adj (0.971 for OPY at 470 nm, 0.946 for RPY at 500 nm and 0.942 for BMY) were also high and advocates a high correlation between the observed and the predicted values. R^2pre is a measure of how good the model predicts a response value. The R^2adj and R^2pre should be within approximately 0.20 of each other to be in reasonable agreement. If they are not, there may be a problem with either the data or the model. In our case, the R^2pre of (0.917 for OPY at 470 nm, 0.847 for RPY at 500 nm and 0.845 for BMY), is in reasonable agreement with the R^2adj. The CV%, indicating the relative dispersion of the experimental points from the predictions of the second-order polynomial (SOP) models, were found to be (5.65 for OPY at 470 nm, 7.41 for RPY at 500 nm and 4.26 for BMY) respectively. The very low values of CV % clearly indicated a very high degree of precision and a good reliability of the experimental values. The high R^2 value and a small CV% value indicate that the model developed will be able to give a reasonably good estimate of response of the system over the ranges studied. Adequate precision (Adeq. Pre.) measures the signal to noise ratio and compares the range of the predicted values at the design points to the average prediction error. This ratio, greater than 4, was desirable and indicated adequate model discrimination. In this work, the ratio was found to be > 21 for all the responses, which indicates an adequate signal.

Table 3. Regression coefficient (RC) of the models and their statistical parameters on the responses

Source	DF	Orange pigment Yield		Red pigment yield		Biomass yield	
		RC	p value	RC	p value	RC	p value
Model	14	46.77	< 0.0001	192.49	< 0.0001	12.20	< 0.0001
X_1	1	7.09	< 0.0001	27.94	< 0.0001	0.77	< 0.0001
X_2	1	-2.17	0.0009	-9.05	0.0058	-0.77	< 0.0001

X_3	1	5.34	< 0.0001	20.40	< 0.0001	1.24	< 0.0001
X_4	1	-0.58	0.2767	-1.53	0.5901	-0.17	0.1837
X_{12}	1	-2.25	0.0245	-8.69	0.0927	-0.24	0.2788
X_{13}	1	-3.75	0.0009	-17.18	0.0031	-0.02	0.9224
X_{14}	1	2.25	0.0245	7.55	0.1394	-0.61	0.0117
X_{23}	1	8.25	< 0.0001	31.03	< 0.0001	1.18	< 0.0001
X_{24}	1	0.001	0.0985	-1.89	0.7011	0.55	0.0210
X_{34}	1	2.00	0.0418	7.12	0.1615	0.17	0.4359
X_1^2	1	-8.42	< 0.0001	-33.17	< 0.0001	-1.02	< 0.0001
X_2^2	1	-13.55	< 0.0001	-54.11	< 0.0001	-2.31	< 0.0001
X_3^2	1	-7.05	< 0.0001	-31.21	< 0.0001	-1.35	< 0.0001
X_4^2	1	-7.67	< 0.0001	-32.32	< 0.0001	-0.97	< 0.0001
R^2		0.985		0.973		0.971	
Adj-R^2		0.971		0.947		0.943	
Pre-R^2		0.917		0.847		0.845	
CV%		5.65		7.41		4.26	
Adeq. Pre.		28.3		21.05		22.71	

3.4 Effect of process variables on pigment yield

Growth phase is a key parameter in the production of secondary metabolites. In the first stage of fermentation, fungi utilize carbon and nitrogen from the fermentation medium for the primary metabolites synthesis, and then the secondary metabolites are produced at the end of the fungal growth [42]. In our study, the pigments often considered as secondary metabolites, begin to be produced only a few hours after the start of the fermentation, and the synthesis roughly follows the growth profile of the fungus. The working strain of *T. albobiverticillius* 30548 is able to produce a range of colored molecules such as orange and red compounds. During the growth of *T. albobiverticillius* 30548, a process of granular liquid extrusion occurres through the point of hyphae growth in PDB medium and this granular liquid, initially

white, gradually develops from a pinkish color to orange and red hues, depending on the culture media. The red pigments with relatively high maximum absorption,along with orange compounds of lower maximum absorption spectra were observed [43]. These pigments excreted into the fermentation broth (extracellular) were submitted to various solvent systems for their extraction and purification. UV–Vis spectral analysis of the red components showed maximum absorbance at 500 nm and the orange compounds at 470 nm respectively.

3.4.1   Optimization of orange pigment yield (OPY)

The effect of interaction of variables on orange pigments yield was studied by changing levels of any two independent variables while keeping the other two independent variables at their constant level. The response surface plots or contour plots can be used to predict the optimal values for different test variables. Therefore, three response surface plots were obtained by considering all the possible combinations. **Fig. 1a** shows the effect of interaction between pH and temperature on orange pigment production (maximum absorbance at 470nm). It revealed that both the components at their lower levels have relatively small effect on the pigment production. Increase in pH and temperature leads to gradual increase in the pigment production up to pH of 7.5 and temperature of 24 °C. Increasing the value of both independent variables (pH > 7.75 and T > 27 °C) showed negative effect on pigment production. As shown in **Fig. 1b**, increasing agitation speed (up to 155 rpm) and increasing pH (from 5.5 – 7.5) lead to high pigment production (47.99 mg/L of orange pigments and 198.67 mg/L red pigments) while the agitation speed above 155 rpm and alkaline pH above 7.7 showed negative effect on orange pigment production. Thus, both the parameters at their maximum level were found to have negative effect for pigment production.

The interaction between fermentation time and pH also play an important role in pigment production (Fig. 1c); pH in the range up to 8.1 (alkaline pH) at higher level of fermentation time was found to be significant for orange pigment production. It was noticed that

fermentation time up to certain level (234 h) as well as pH (< 8.1) supports orange pigment production but both at higher level negatively affects the pigment production. Temperature and agitation speed showed significant influence on each other and also on orange pigment yield. Both the parameters showed the linear and quadratic response for pigment yield (**Fig. 1d**). Maximum yield was observed in above mid values of both the parameters (temperature of 25 °C and agitation speed of 165 rpm) and above that, yields of pigments were decreased.

The interactive effects of temperature and fermentation time against orange pigment yield were depicted in **Fig. 1e**. When temperature was increased from 21 to 24 °C and fermentation time between 24 and 259 h, it resulted in a gradual enhancement in pigment production up to a maximum level. Further increase in temperature (above 24 °C) and fermentation time (above 259 h) leads to decrease in pigment yield. The three dimensional (3D) response interactive plot of agitation speed and fermentation time illustrated that, orange pigment yield was increased with the increase in agitation speed and fermentation time up to around 180 rpm and 24 h, respectively **(Fig. 1f).** The yield of orange pigment was decreased with the increase in the agitation speed and fermentation time above 180 rpm and 247 h, respectively.

According to the response surface point prediction analysis, combining the effects of all the process variables studied, initial pH of 6.5, temperature of 23.91 °C, agitation speed of 154.89 rpm and fermentation time of 229.08 h could give maximum orange pigment yield up to 47.99 mg/L.
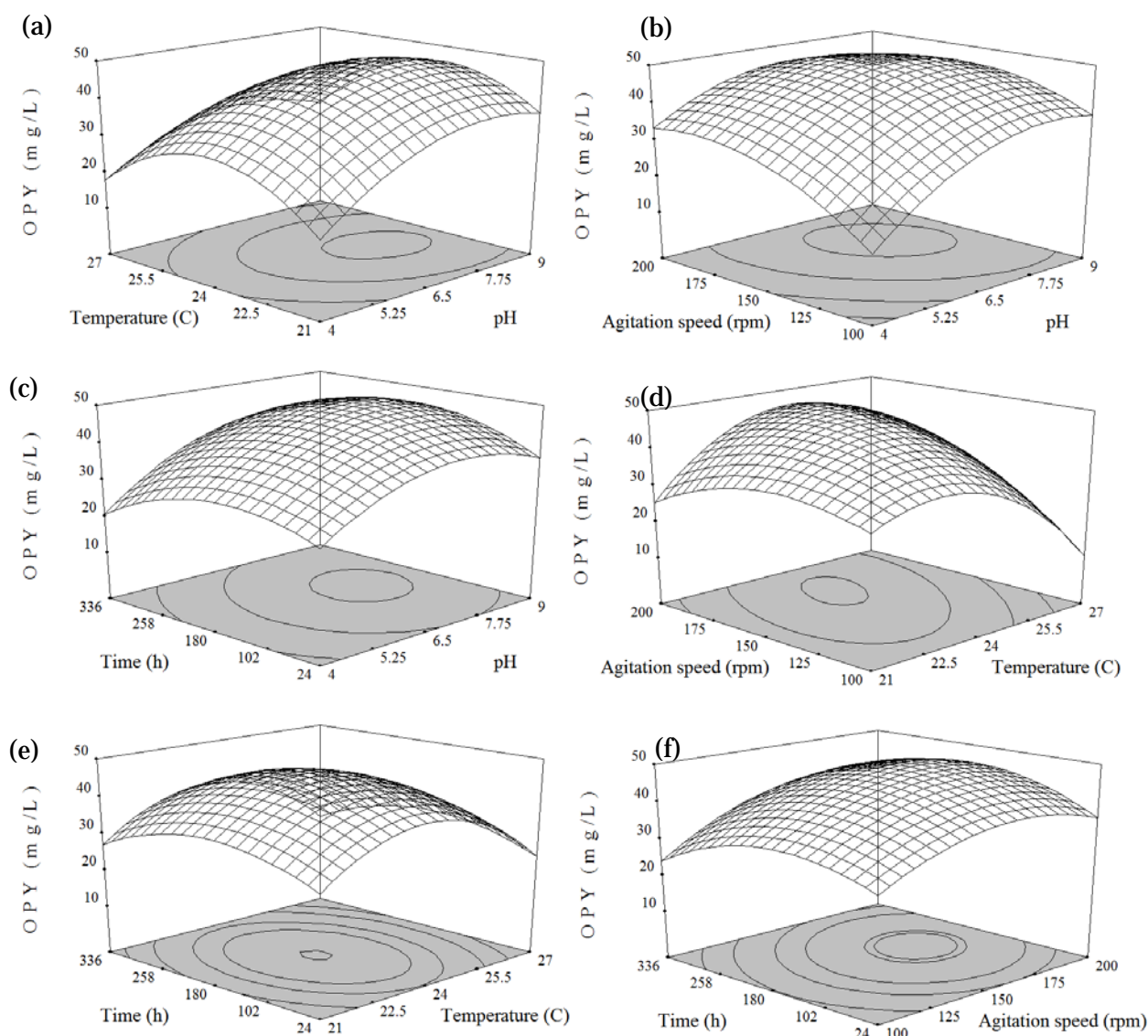
Fig.1. Response surface plots showing the influence of proces0s variables on orange pigment yield (OPY) a) Temperature vs pH; b) Agitation speed vs pH; c) Time vs pH; d) Agitation speed vs temperature; e) Time vs temperature; f) Time vs agitation speed

3.4.2 Optimization of red pigment yield (RPY)

Data obtained from the experiments were used to study the effect of process variables on red pigment production. From the results, it was confirmed that increasing pH level from 4.0 – 6.5 enhanced the red pigment production and it decreased if the pH has been increased to alkaline level **(Fig. 2a and 2b).** Intensity of pigment production by *T. albobiverticillius* 30548 was found to be increased from acidic to neutral pH. When pH is coupled with temperature as

shown in **Fig. 2a,** the maximum production was noticed from 22 – 25 ℃ together with pH in the range of 4.0 – 6.5. Increasing agitation speed from 100 – 158 rpm and pH value of 4.0 - 6.5 was expected to maximize the red pigment production **(Fig. 2b).**

Fermentative production of any substance depends on the incubation time. So the synthesis of the pigments was monitored at regular intervals of time. The maximum production of red pigments was attained at 120 - 168 hrs of fermentation and further increase in incubation time lead to decrease in the production (**Figs. 2 c, e, f**). The mycelium of the species grows rapidly in the beginning of fermentation (12 – 48 hrs) and upon maturation stage (48- 96 hrs); more extracellular red metabolites were released (up to 168 hrs). Further increasing incubation time leads to bleaching of the extracellular red pigments and then decreased the pigments yield.

To examine the influence of agitation speed on red pigment production, experiments were carried out in various agitation speeds (100 – 200 rpm). The results showed that, red pigment production was increased from 100 -158 rpm and rapidly it decreases (Fig. 2d, f).

The application of response surface methodology resulted in empirical relationship between response and independent variables in terms of individual and interactive effects. All the responses were illustrated in **Figs. 2a-f** and observed that, the pigment yield was increased from low to middle level and further decreases. The linear and quadratic relationships of all process variables revealed most significant influence on the red pigment production based on the low p-value (< 0.05). The interactive effect of pH and agitation speed, temperature and fermentation time had significant effect on the red pigment production yield (**Table 3**). Maximum pigment production was expected at the middle level of the medium variables and strong interaction exists between the variables since the shape of the response surface and contour plot was elliptical in nature. By applying numerical optimization method to the 4 process variables, the optimal condition was attained as follows: initial pH of 6.45,

temperature of 24.01 °C, agitation speed of 158.37 rpm and fermentation time of 198.62 h
with maximal red pigment yield of 198.67 mg/L respectively.
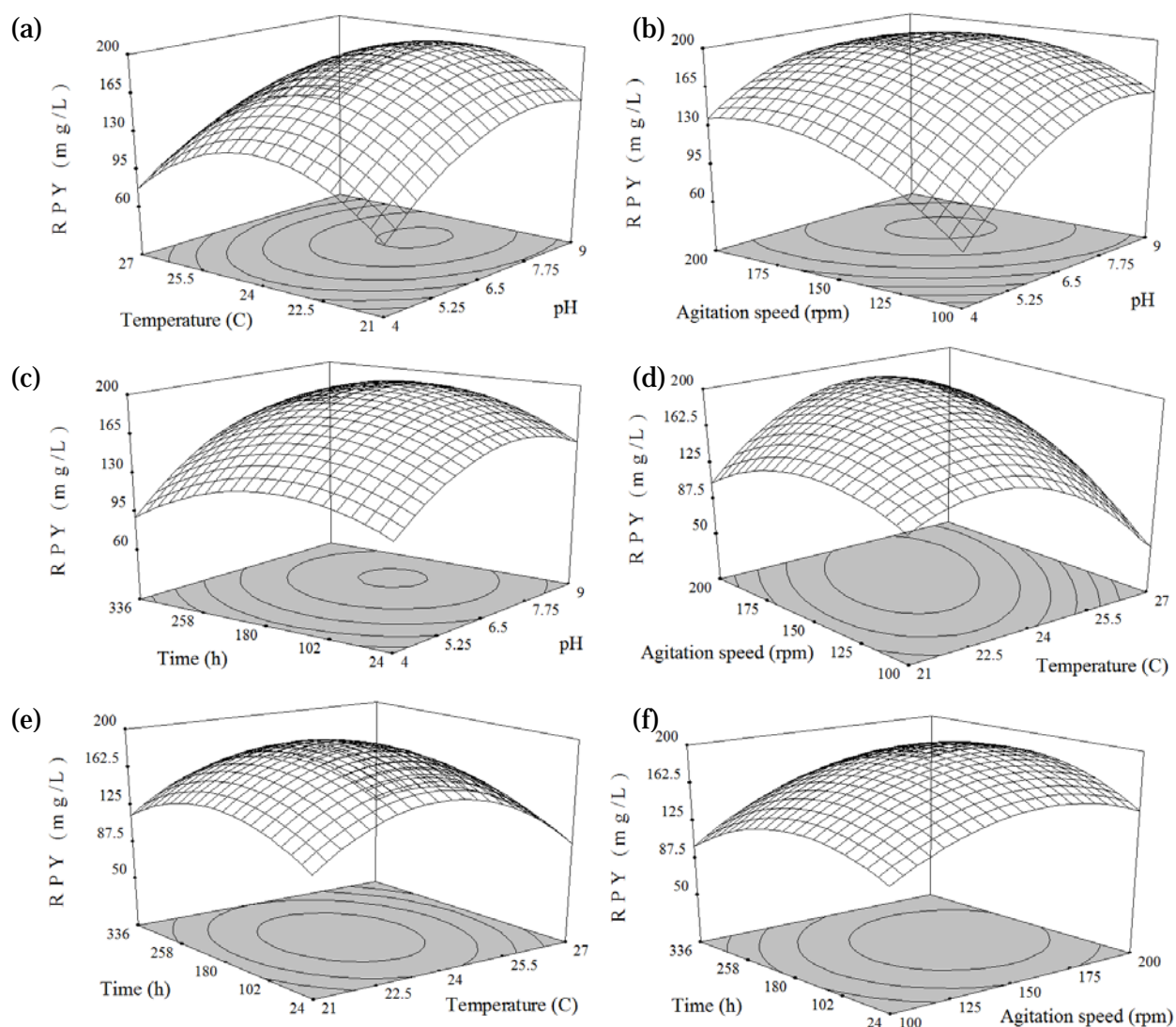
Fig.2. Response surface plots showing the influence of process variables on red pigment yield (RPY) a) Temperature vs pH; b) Agitation speed vs pH; c) Time vs pH; (d) Agitation speed vs temperature; (e) Time vs pH; (f) Time vs agitation speed

3.5   Effect of process variables on biomass yield (BMY)

Fig. 3 shows the results obtained from Box -Benhken design regarding the studied variables: temperature (21-27 °C), pH (4-9), agitation speed (100-200 rpm) and fermentation time (24 – 336 h). As seen in each **fig. 3 (a, b, c, d, e, f)**, a strong interaction was exhibited between any

two factors on the biomass yield while the other ones were kept at constant level. The graphs demonstrated the biomass yield was linearly increased with increasing levels of pH up to7.8 and also temperature up to 25 °C and then the yields decreased gradually. **Fig. 3b** shows the effect of interaction between pH and agitation speed on biomass production, which revealed that both the components at their lower level have no significant effect on the biomass production but increase in pH and agitation speed leads to gradual increase in the biomass production up to pH of 7.7 and agitation speed of 180 rpm, while increasing the value of both independent variables beyond, showed negative effect on biomass production.

From the **fig. 3c**, it can be seen that the biomass yield increased with increasing of pH and fermentation time up to its middle level. But further increasing of pH and fermentation time would decrease the yield of biomass. The 3D response surface/ contour plots based on independent variables such as temperature and agitation speed were developed, while other variables were kept at middle levels **(Fig. 3d).** And it is evident that the biomass yield was enhanced with increasing temperature (24.5 °C) and agitation speed (185 rpm) and the yield decreased beyond the limits. Biomass production increased with increase in both the variables (temperature and fermentation time) **(Fig. 3e),** above the constant level up to temperature of 25 °C and fermentation time of 245 h. However there was a sharp convergence of the curve near the boundary, explaining that in the presence of temperature and fermentation time above certain limit (above temperature of 25 °C and fermentation time of 245 h) it would not contribute for increasing biomass production.

The interaction between agitation speed and fermentation time also play an important role in biomass production **(Fig. 3f).** Agitation speed and fermentation time at above middle level was found to be significant for biomass production. It was noticed that agitation speed up to certain level (190 rpm) as well as fermentation time (245 h) supports biomass production but both at higher level negatively affects the biomass production. The analysis of response

surface was performed in order to determine the optimal condition to produce maximal biomass yield. The optimal condition was initial pH of 6.6, temperature of 23.86 °C, agitation speed of 168.88 rpm and fermentation time of 145.17 h, respectively. The maximum predicted yield of biomass was 12.58 g/L.
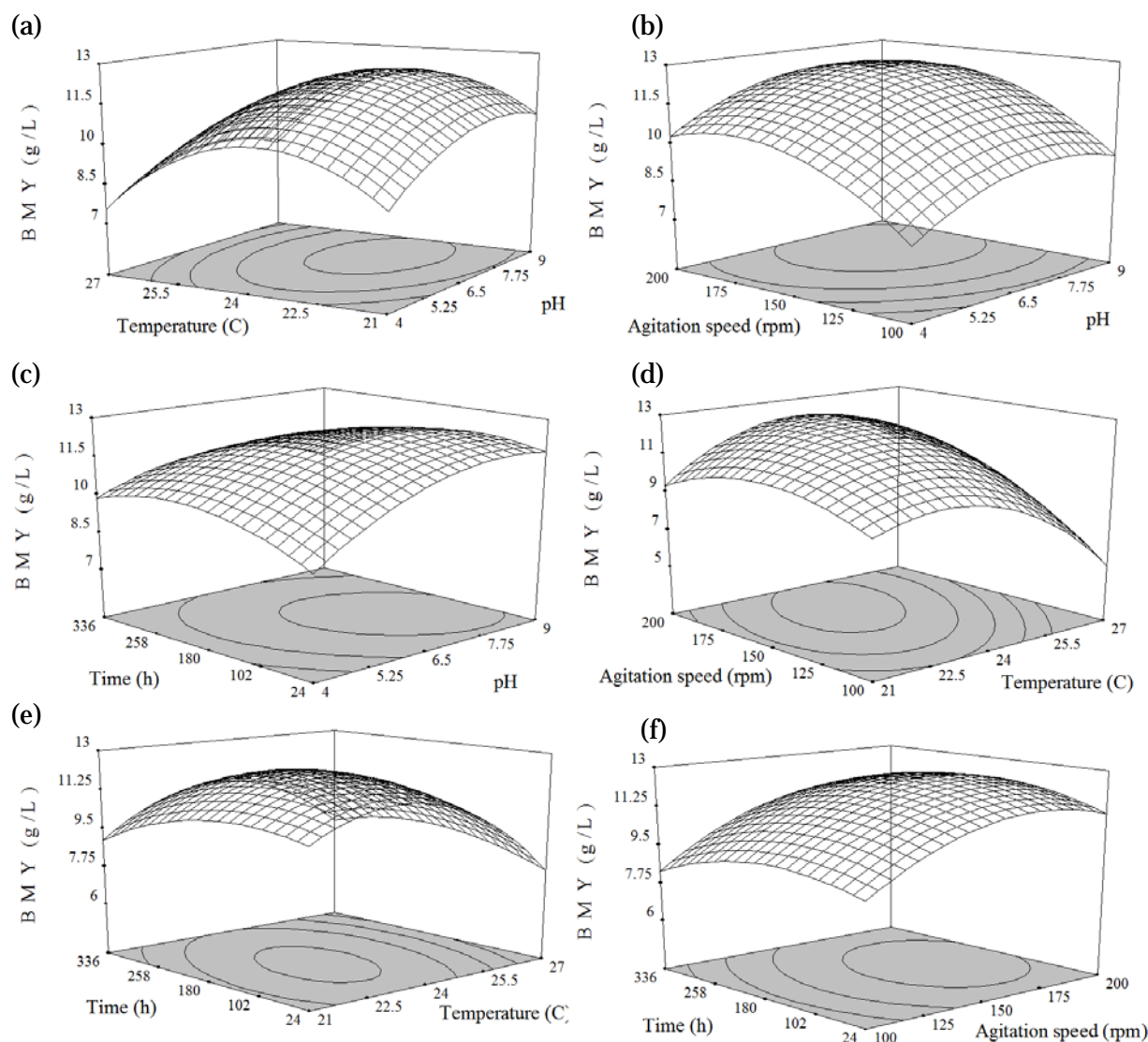

Fig.3. Response surface plots showing the influence of process variables on biomass yield (BMY) a) Temperature vs pH; b) Agitation speed vs pH; c) Time vs pH; d) Agitation speed vs temperature; e) Time vs temperature; f) Time vs agitation speed

3.6 Multi response optimization and validation of optimized condition

An optimum condition for the maximal production of pigments (orange and red) and biomass yield by *T. albobiverticillius* 30548 under submerged fermentation was derived by Derringer's desired function methodology. Second order polynomial models developed in this study were utilized for each response in order to obtain specified optimum conditions. Simultaneous optimizations of the multiple responses were then carried out using Derringer's desirability function method. This function searches for a combination of factor levels that simultaneously satisfies the requirements for each response in the design. This numerical optimization evaluates a point that maximizes the desirability function. The desired goal was selected by adjusting the weight (or importance) that might alter the characteristics of a goal. A weight factor, which defines the shape of the desirability function for each response, was then assigned. The main objective of optimization was to maximize the final pigments yield and biomass production with recalculating all responsible independent factors by using desirability functions, therefore, the goal for pH, temperature, agitation speed and fermentation time was assigned as in range.

The goal for orange pigment yield, red pigment yield and biomass production was assigned to get maximum. A weight factor of 1 was chosen for all individual desirability in this work. The "importance" of a goal can be changed in relation to the other goals. It can range from 1 (least importance) to 5 (most important). The default is for all goals to be equally important in a setting of 3. The optimization procedure was conducted under these settings and boundaries. Under the optimal conditions, the predicted orange pigment yield of 47.88 mg/L, red pigment yield of 196.15 mg/L and biomass production of 12.63 g/L with a desirability value of 0.983. The maximized overall desirability (G = 0.983) was calculated from geometric means of the individual desirability functions (g_i) of the each response.

Due to applicability of optimal extraction condition in practical manner, the attained optimal condition was altered as follows: initial pH of 6.4, temperature of 24 °C, agitation speed of 164 rpm and fermentation time of 149 h respectively. Triplicate experiments were carried out under the modified optimized conditions and mean values of experimental results were compared with the predicted values. The experimental efficiency of the production of pigments and biomass under the optimum condition was found to be 47.93 ± 0.5 mg/L of orange pigment, 196.28 ± 0.76 mg/L of red pigment and 12.58 ± 0.41 g/L of biomass respectively.

4.   Discussion and conclusion

RSM involving BBD was employed in this present research work minimizes time, also cost effective which has been applied to optimize the effects of independent variables such as pH, temperature, agitation speed and fermentation time on the maximal production of pigments and biomass under submerged fermentation using PDB. The actual values obtained through the experimental studies were used to construct second order quadratic model for the responses to predict the observed data and the 3 D Surface/ contour plots are generally used to visualize the interactions between the variables graphically [37]. From the results, the regression analysis of the experimental data showed that linear and quadratic effects of independent process variables have significant influence on the responses.

Studies by many researchers have revealed that among the numerous environmental factors, medium pH and source of nitrogen determines pigment prodution in submerged culture [44-49]. Indeed, acidic pH may enhanced the hydrolysis of the substrates and subsequently favored the generation of the metabolite production [48]. It is supposed that the compounds produced in 3- 4 days may react with ammonium ion or free amino groups to transform to red amine derivatives towards neutral pH and beyond [50]. These results were consistent with Babitha et al. (2007), who reported maximum production of pigments were noticed in *M.*

purpureus at pH 4.5 to 7.5 **[51]**.These results showed that the color of the pigment remained stable over a wide range of pH, which could open as an avenue for industrial application of the pigments produced by *T. albobiverticillius* 30548.

To find the optimal temperature for mycelial growth and pigment production, *T. albobiverticillius* 30548 was cultivated under various temperatures (21-27 °C). Consequently, the optimal temperature for both mycelial growth and pigment production was found to be 24 °C. Taking into account that higher fungi usually require long periods for submerged culture, exposing them to contamination risk, this optimal temperature was regarded as a favorable physiological property of *T. albobiverticillius* 30548 **[26, 52].**

Pigment production, as measured by absorbance lagged growth and was in peak at 149 hours (day 6) of fermentation time. Thereafter, a decrease in pigment production was absorbed after the mentioned fermentation time. A hypothesis could be stated here, the decrease of red pigment production during fermentation was caused by decomposition of pigment or due to change in pigment structure. Also, the decreased pigment absorption might be due to the degradation of chromophore pigment group. Almost identical behavior was observed in the pigment production of *Monascus purpureus* described by Chen *et al.* 1993 **[49]**.

Basically, in liquid fermentation agitation speed increased the amount of dissolved oxygen and makes the oxygen more accessible to cells, leads to greater growth **[53].** For *Penicillium aculeatum* ATCC 10409, several experiments were conducted at agitation speeds of 100 and 150 rpm yielded highest level of yellow pigments (1.38 g/L) in combination with pH value of 6.5, a temperature of 30 °C **[54]**. Similarly for *T. albobiverticillius* 30548, increasing agitation from 100-158 rpm enhanced the supply of oxygen in the growth phase and thus enhanced the biomass and pigment yield. However, above 158 rpm of agitation speed damage the formation of mycelia growth by high shear force at high agitation speed and leads to decrease

the pigment yield. Mohamed *et al.* (2012) reported the similar result for highest pigment production obtained at agitation speed of 150 rpm using *Monascus purpureus* [55].

The results obtained through confirmation experiments indicate the suitability of the developed quadratic models and it may be noted that these optimal values are valid within the specified range of process parameters. The elliptical nature of the response surface and contour plot illustrated the applicability of maximization function in order to attain an optimal condition to produce maximal amount of red pigment yield. Muralidhar *et al.* (2001) reported previously the existence of a perfect interaction between the independent variables if the contour plots are elliptical in shape [56]. The results were closely related with the data obtained from optimization analysis using desirability functions. Also indicating Box–Behnken design in corporate with desirability functions could be effectively used to optimize the design for experiments on the maximal production of pigments (orange and red) and biomass yield for the strain *T. albobiverticillius* 30548 using potato dextrose broth under submerged fermentation.

Applying the methodology of desired function, the optimum level of various process parameters were obtained as follows: initial pH of 6.4, temperature of 24 °C, agitation speed of 164 rpm and fermentation time of 149 h. Among the evaluated conditions, the maximum experimental efficiency of the production of orange pigments (47.93 ± 0.58 mg/L) under the optimal conditions of pH value of 5.5, a temperature of 24 °C and an agitation speed of 150 rpm, and fermentation time of 120 h. Maximum red pigments (196.28 ± 0.76 mg/L) with pH value of 6.2, a temperature of 24 °C, agitation speed of 150 rpm and fermentation time of 180 h. For maximum biomass (12.58 ± 0.41 g/L), the best condition was found to be initial pH of 6.4, temperature of 26 °C, agitation speed of 164 rpm and fermentation time of 149 h. respectively. This model could be useful for the production of pigments at large scale and given these findings, further studies are required for characterization of the produced

pigments and its use in food applications. As a next step, more detailed experiments using different carbon and nitrogen sources must be performed to demonstrate a direct relationship between biomass growth and pigment production.

ACKNOWLEDGEMENT

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4.4 MANUSCRIPT

Culture media optimization for enhanced pigment production and biomass growth by *Talaromyces albobiverticillius* 30548 using one-variable-at-a-time and response surface methodology

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Abstract:

Species of *Talaromyces* produces *Monascus-like* azaphilone pigments have potential applications as natural colorants in food applications. Enhancement of pigment production using various carbon and nitrogen sources from various fungal strains using statistical methods is customary now-a-days. The present work is the first attempt for the production optimization of pigments and biomass in *Talaromyces albobiverticillius* 30548. Different nutrient combination media was screened using one-variable-at-a-time (OVAT) analysis and found sucrose combined with yeast extract was chosen as the best media. It provided maximum yield of orange (695.17 mg/L RYR equivalents) and red pigments (738.58 mg/L RYR equivalents) and also higher dry cell weight (6.60 g/L). Significant medium components (yeast extract, K_2HPO_4 and $MgSO_4 \cdot 7H_2O$) were identified form OVAT analysis for pigment and biomass production. A five-level central composite design of the response surface method was applied to evaluate the optimal concentration and the interaction effects between the selected components. Predicted maximum response of OPY (70.87 mg/L), RPY (262.34 mg/L), DBW (15.98 g/L) was obtained at the optimal level of medium variables of yeast extract, K_2HPO_4 and $MgSO_4 \cdot 7H_2O$ at 3 g/L, 1 g/L and 0.2 g/L, respectively. Model verification was performed at the predicted optimal level and the model was well fitted with the experimental results.

Keywords: Optimization, Pigments, Biomass, one-variable-at-a-time, Central composite design, Talaromyces albobiverticillius 30548

1. INTRODUCTION

As a number of food additives perform certain technological functions in improving food quality, color is often the first element noticed in the appearance of a food product. Synthetic or artificial colors remain the most popular as compared to natural colorants, as they are brighter, more uniform, better characterized, and of higher tinctorial strength, encompass a wider range of hues, and are less expensive [1, 2]. Though some artificial colorants approved for food use, organizations like the U.S. Food and Drug Administration (FDA), the European Food Safety Standards Authority (EFSA) have instituted warnings on consumption and have recommended the safe dosage in foods, cosmetics and drugs [3-5]. Nonetheless, many colors have been banned due to its potential downside to consumers as it's linked to cause hyperactivity disorders, cancer, allergies and toxicological problems [6].

As a replacement to synthetic dyes, there has been increasing interest towards the search for natural pigment producing agents. Among all the natural sources, microorganisms are getting more interest owing to their easy cultivation, massive production in cheaper nutrient medium, high pigment stability [7-9]. Fungal pigments are extensively studied for their potential as a promising pigment source and produces astonishing variety of pigments including carotenoids, melanins, flavins, phenazines, quinones, monascins, violacein or indigo [10-12]. Fungal pigments are considered as secondary metabolites usually begin after active growth of cells. Furthermore due to insufficient nutrient supply, pigments are produced by fungal mycelia to survive in the adverse situation [13-15]. Besides, fungal pigments have been reported to have bioactive properties that are of considerable interest for new drug development in pharmaceuticals [16, 17].

Biosynthetically, most pigments produced by fungi are polyketide-based and involve complex pathways catalyzed by frequent type of polyketide synthases. The biosynthesis of azaphilones uses both the polyketide pathway and the fatty acid synthesis pathway. Biosynthetic pathways are suggested for the following azaphilones: monascorubrin and monascoflavin [18], mitorubrin and rubropunctatin [19], citrinin [20, 21]. However, some researchers are of the opinion that biosynthetic mechanisms of these pigments are poorly understood including the extensively studied Monascus pigments [22].

Microbial pigments are better produced in submerged fermentation compared to solid substrate fermentation to get homogenous growth of cells when supported with proper agitation under aerobic conditions [2, 23]. Various ascomycetes fungi including *Drechslera*

sp. [24, 25], *Fusarium* sp. [26-28], *Monascus* sp.[29-32], *Paecilomyce*s sp. [33, 34], *Penicillium* sp. [35-40], *Talaromyces* sp. [41, 42] , *Cordyceps unilateralis* [43], *Curvularia lunata, Herpotrichia rhodosticta* [44] were described to produce pigments by fermentative technology. Especially for *Monascus spp.,* several studies by many researchers have revealed that numerous environmental factors regulate the ability of pigment production particularly the medium pH and nitrogen source [45-52].

In fermentation, optimization of medium components is one of the tedious processes due to the involvement of numerous factors influencing the process. The formulation of culture media containing complex nutrients is generally preferred in order to reduce the production cost so as to give maximum product yield [53-55]. In the classical optimization, only one factor or variable is varied at a time while keeping other variables constant in one-variable –at – a – time – analysis (OVAT). Because of its ease and convenience, the OVAT has been the most preferred choice among the researchers for designing the medium composition and used in the initial stages in diverse fields [56]. This methodology is still in use even today, during the initial stages of medium formulation for the production of new metabolite or known compound from new source.

After finding the critical factors through one- variable- at- a- time analysis, the next step is to optimize the actual values of these process factors. Statistical design of experiments is a powerful approach for media optimization which offers a systematic way of simultaneously evaluating multiple parameters and analyzing the resulting process outputs. To achieve this purpose, central composite response surface design was used and this empirical technique enables to evaluate the relationship between independent variables to predict the response [57, 58]. For *Monascus spp*., several experiments have been continuously made to find better carbon and nitrogen sources for the higher microbial production of pigments [59-63]. With this backdrop on mind, a study was undertaken to focus inexpensive and most suitable nutrient medium for pigment production.

2. MATERIALS & METHODS

2.1 Microorganism and cultivation

The studied strain, *Talaromyces albobiverticillius* 30548 was isolated from the marine sediment source of Réunion Island, Indian Ocean. The fungus was grown on potato dextrose agar (PDA) at 25 °C for 7 days and after, the cultures were stocked by maintaining at 4 °C.

2.2　Primary screening of medium components by OVAT analysis

In earlier studies, the major influencing factors for fungal growth and pigment production was screened and optimized by culturing the fungi only in potato dextrose broth (PDB). The concentration ranges of the variables were chosen based on the existing literature data and available reports [64, 65]. To find the other best nutrient sources for pigment production, different carbon and nitrogen sources were chosen for this study by 'one-variable-at- a-time method'. Preliminary experiments were carried out using 5 different carbon (glucose, sucrose, fructose, soluble starch and malt extract), 4 nitrogen sources (sodium nitrate, peptone, tryptone and yeast extract) and inorganic salts (K_2HPO_4, $MgSO_4.7H_2O$, $FeSO_4.7H_2O$, KCl) to evaluate their suitability for fungal growth and pigment production by the strain *Talaromyces albobiverticillius* 30548. The chemical composition of the medium is (g/l): 15; carbon source, 3; nitrogen source, 1; K_2HPO_4, 0.2; $MgSO_4.7H_2O$, 0.2; $FeSO_4.7H_2O$, 0.25; KCl. The working stocks of culture media components such as carbon, nitrogen and trace elements were prepared and sterilized separately in an autoclave (121 °C, at 15 psi for 15 mins). Upon cooling down to room temperature, the sterile components were mixed aseptically at right proportions.

2.3　Submerged fermentation

A small loop of fungal mycelia grown on PDA was taken and transferred into 80 mL of sterile PDB culture media. The flasks were incubated on a rotary shaker at 150 rpm at 24 °C for 48 hours and considered as pre-inoculum. After 48 hours of inoculum, the culture broth was allowed to centrifuge at 8000 rpm for 6 minutes at room temperature to separate mycelia. 100 mg of mycelia was added to the fermentation media containing 100 mL of working media, and then incubated at 200 rpm at 24 °C for 10 days. All the experiments were performed as triplicates and pigment production was monitored on an everyday basis.

2.4　Quantification of pigments

Throughout the period of fermentation, 5 ml of sample was withdrawn from the flasks to measure the optical density of the solution. The culture broth was centrifuged at 8000 rpm of 6 min at room temperature to separate the supernatant and mycelia. The color-rich supernatant which contains the extracellular pigment was quantified using UV-vis spectrophotometer. The pigment analysis was done by measuring the solution absorbance at 470, 500 and 520 nm representing the regions for orange, red and reddish brown colors respectively [66, 67].

Pigment yield was observed as OD units at its maximum wavelength and the values were converted into pigment units using the following equation

$$color\ value = \frac{OD*DF*V}{Vt}$$ …………………. (1)

Where

OD = Optical density at 500nm; DF = Dilution factor; V = Volume of extract; V_t = Total volume

2.5   Dry biomass estimation

Each day, the fermentation broth and cells were separated using 0.48 µm filters (SEFAR, Nitex, Switzerland). The mycelia mat was dried at 105 °C in the hot air oven to the constant weight. The dry weight of the fungus was calculated using the following formula

$$Dry\ weight = (Weight\ of\ filter\ paper + weight\ of\ mycelia) - weight\ of\ filter\ paper……… (2)$$

2.6   Optimization using central composite response surface design

To find out the optimum concentration of the most effective variables (yeast extract [X1], KH_2PO_4 [X2], and $MgSO_4.7H_2O$ [X3]) identified by OVAT analysis and to study their interactions, RSM using CCD was applied. Each variable in the design was studied at five different levels, with all variables taken at a central coded value of zero (Table 1).

Table 1: Experimental range and the levels of three independent variables for central composite design (CCD) matrix in terms of actual and coded factors

Factor code	Factors (in g/L)	Levels of the factors				
		$-\alpha$	-1	0	+1	$+\alpha$
A	Yeast extract	1.32	2	3	4	4.68
B	KH_2PO_4	0.16	0.5	1	1.5	1.84
C	$MgSO_4.7H_2O$	0.03	0.1	0.2	0.3	0.37

Accordingly, a factorial experimental design, with an axial point (α=1.68) and six replicates at the center point, with a total number of 20 experiments, was employed (Table 2). Orange pigment yield, red pigment yield and dry biomass weight were taken as responses Y1, Y2, Y3 respectively, and a multiple regression analysis of the data was carried out for obtaining an

empirical model that relates the response measured to the independent variables. The relationship of the independent variables and the response was calculated by the second-order polynomial equation

$$Y = \beta_0 + \beta_1 . X_1 + \beta_2 . X_2 + \beta_3 . X_3 + \beta_{11} . X_1^2 + \beta_{22} . X_2^2 + \beta_{33} . X_3^2 +$$
$$\beta_{12} . X_1 . X_2 + \beta_{13} . X_1 . X_3 + \beta_{23} . X_2 . X_3 + \beta_{123} . X_1 . X_2 . X_3 \qquad \ldots\ldots\ldots\ldots\ldots.. \qquad (3)$$

where Y represents the response value, β_0 is a constant which shows where the line intersects the y-axis and allows the average impact of the factors, β_1, β_2 and β_3 are regression coefficients, β_{12}, β_{13}, β_{23} and β_{123} are the regression coefficients for the interaction of variables [68].

2.7   Statistical analysis and data validation

Using Design Expert Statistical Software package 9.0.3.1 (Stat Ease Inc., Minneapolis, USA), the response surface models were obtained for CCD. For CCD, ANOVA through Fisher's test was used to evaluate the effect of independent variables on the response and significant results were identified by a p-value of < 0.05. Multiple correlation coefficient (R^2) and adjusted R^2 were used as quality indicators to evaluate the fitness of the second order polynomial equation. Contour and three dimensional surface plots were employed to demonstrate the relationship and interaction between the coded variables and the response. The optimal points were determined by solving the equation derived from the final quadratic model and grid search in RSM plots.

3.   Results

3.1.   Primary screening of media components

Among the tested carbon and nitrogen sources, some sources showed positive and some showed less effect on pigment production. The experimental combinations for OVAT are shown in Table 1. The results of Fig. 1(a), (b) & (c) indicated that when sucrose and yeast extract was used as a combined source, it provided maximum yield of orange (695.17 mg/L RYR equivalents) and red pigments (738.58 mg/L RYR equivalents) and also higher dry cell weight (6.60 g/L) then followed by fructose with yeast extract (623.09 mg/L of orange pigments 635.35 mg/L of red pigments , 5.96 g/L of dry cell weight). Evidently, sodium nitrate used as an inorganic nitrogen source gave very low yields of all the nitrogen sources tested (Fig. 1(a), (b), (c)). Nitrogen sources, peptone and tryptone individually combined with any of the carbon sources provided quite nearby yields (see fig. 1(a), (b) & (c)). In the context of above observations, sucrose and yeast extract was shown to improve the yield of responses.
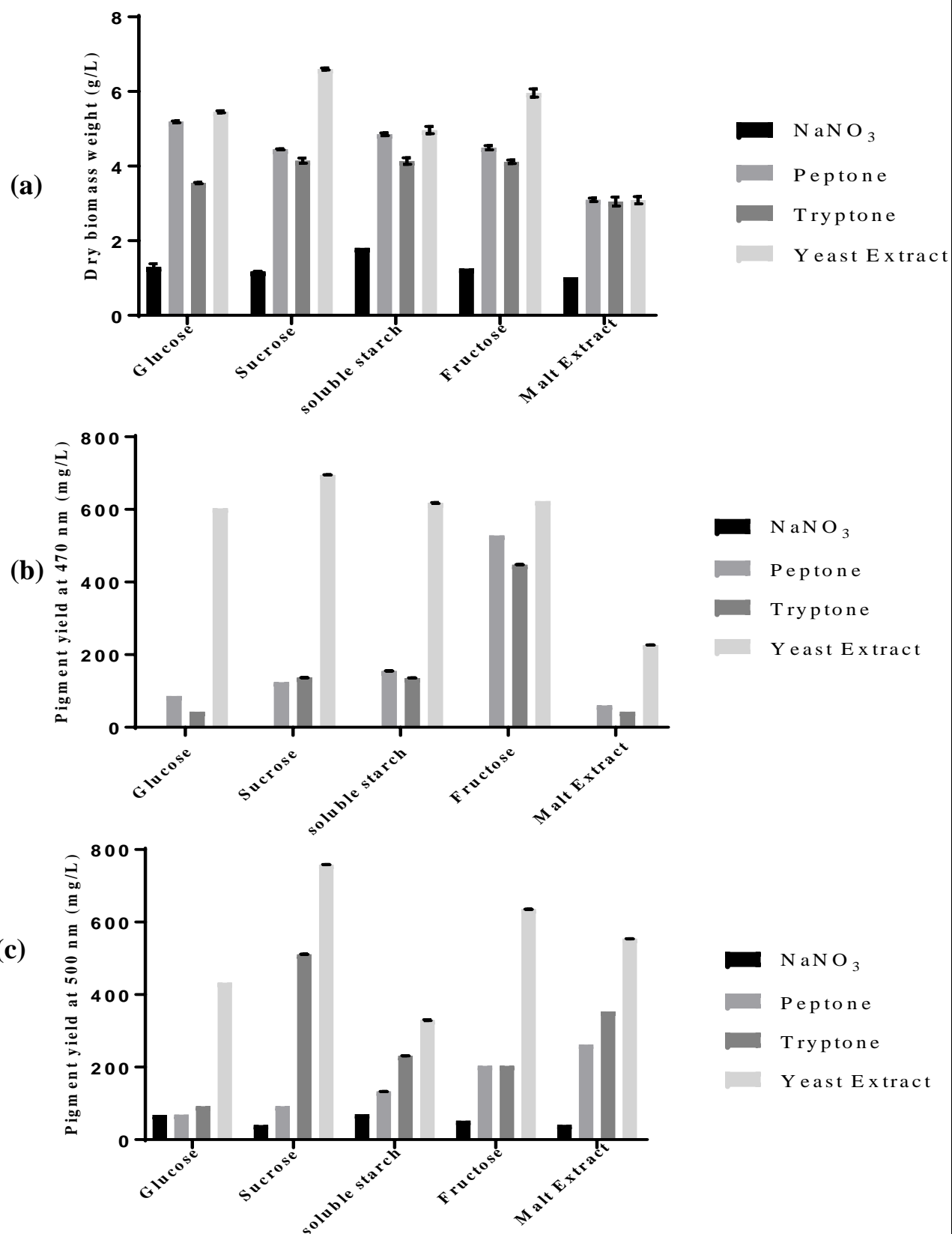
Figure 1: The main effects of combined different carbon and nitrogen sources on (a) Dry biomass weight of *T. albobiverticillius* 30548, (b) Pigment yield at 470 nm in terms of red yeast rice equivalents (mg/L), (c) Pigment yield at 500 nm in terms of red yeast rice equivalents (mg/L). Data plotted are means ± SD of three replicate growths per culture medium.

3.2. Central composite design and Response surface methodology

Twenty experiments were carried out at different combinations of variables and the responses were tabulated in Table 2. The coefficients of the response surface model were evaluated by regression analysis and tested for their significance. A second-order polynomial equation for pigment production (orange and red) and dry biomass weight was obtained using the regression co-efficient. This equation asserts the relationship between the process factors and the responses. After neglecting the insignificant factors, this can be presented in terms of coded factors as:

Orange pigment yield (OPY) Y_1 = + 65.45 - 4.47 * A - 2.73 * B + 5.16 * C + 0.67 * AB + 0.098 * AC - 2.35 * BC -15.51 * A^2 – 8.15 * B^2 – 6.16 * C^2 ………………….. (4)

Red pigment yield (RPY) Y_2 = + 259.45 + 4.07 * A - 0.30 * B + 3.04 * C – 0.44 * AB + 0.26 * AC + 0.65 * BC – 43.80 * A^2 – 42.83 * B^2 - 21.89 * C^2 …………………. (5)

Dry biomass weight (DBW) Y_3 = + 14.47 – 0.76 * A + 0.089 * B + 1.23 * C - 0. 011 * AB + 0.28 * AC – 0.044 * BC – 3.10 * A^2 – 1.43 * B^2 – 0.99 C^2 ………………….. (6)

Where, A represents yeast extract, B is KH_2PO_4 and C is $MgSO_4.7H_2O$.

Table 2: Central composite experimental design matrix with observed results by using three independent variables

Std order	Run order	Yeast extract (g/L)	KH_2PO_4 (g/L)	$MgSO_4.7H_2O$ (g/L)	OPY (mg/L)	RPY (mg/L)	DBW (g/L)
20	1	3	1	0.2	62.98	260.12	14.23
19	2	3	1	0.2	64.9956	262.78	14.98
8	3	4	1.5	0.3	31.25	163.21	8.95
6	4	4	0.5	0.3	37.14	158.32	9.03
1	5	2	0.5	0.1	38.21	137.25	8.89
4	6	4	1.5	0.1	26.11	156.8	6.81
5	7	2	0.5	0.3	52.36	140.02	10.08
7	8	2	1.5	0.3	44.03	139.96	10.27
10	9	4.68179	1	0.2	19.35	129.65	5.03
2	10	4	0.5	0.1	22.37	161.22	6.49
11	11	3	0.159104	0.2	49.65	142.51	10.57
15	12	3	1	0.2	65.99	247.2	15.98
18	13	3	1	0.2	63.98	261.39	13.12
13	14	3	1	0.0318207	37.73	188.32	9.12
9	15	1.31821	1	0.2	21.87	144.81	7.05
3	16	2	1.5	0.1	39.05	141.28	9.03
17	17	3	1	0.2	70.869	262.27	13.63
12	18	3	1.8409	0.2	33.23	137.43	10.95
16	19	3	1	0.2	64.22	262.34	14.77
14	20	3	1	0.368179	56.41	210.08	14.89

From the developed models (Eqs. 4, 5, 6), three dimensional response plots were plotted to visualize the relationship between factors and responses. Response surface curves for the variation in pigment production (orange and red) and dry biomass weight were constructed, depicted in figure 2 (a), (b) & (c). In each set, responses were plotted on Z axis against two factors while maintaining other two factors at constant at their middle level [69, 70].

Fig. 2 (a) depicts the production of orange pigments with respect to yeast extract versus KH_2PO_4. From the interaction response of yeast extract with KH_2PO_4, orange pigment yields increased with increasing yeast extract and KH_2PO_4 concentration up to 2.9 g/L and 0.9 g/L, respectively. Fig. 2 (b) represents the interaction effect of yeast extract and $MgSO_4.7H_2O$ on production of orange pigment. With an increase in yeast extract (2.0 - 2.9 g/L) and $MgSO_4.7H_2O$ (0.1 - 0.2 g/L) concentration, OPY increased. Thereafter, an increase in MgSO4 concentration up to 0.3% (w/v) resulted in decreased orange pigment production. Fig. 2 (c) reveals the orange pigment production with KH_2PO_4 versus $MgSO_4.7H_2O$ and the maximum range was found to be 0.2 g/L $MgSO_4.7H_2O$ and 0.9 g/L KH_2PO_4. However, the response curve did not show curvature, rather it was flattened. This suggested a demand for yeast extract rather the influence of trace elements ($MgSO_4.7H_2O$ and KH_2PO_4) used here.

Fig. 3 (a, b, c) illustrates the interaction effect of individual factors on red pigment production. In all the three graphs, interaction of yeast extract with KH_2PO_4 (Fig. 3(a)), yeast extract versus $MgSO_4.7H_2O$ (Fig. 3(b)), KH_2PO_4 versus $MgSO_4.7H_2O$ (Fig. 3(c)), there was an increased pigment production in the range of 2.9 g/L yeast extract, 0.2 g/L $MgSO_4.7H_2O$ and 0.9 g/L KH_2PO_4. In these concentrations, the red pigment production was maximum with a value of 262.78 mg/L.

Fig. 4 (a, b) exemplifies the interaction effect of yeast extract with KH_2PO_4 (fig. 4 (a)), yeast extract with $MgSO_4.7H_2O$ (fig. 4 (b)) on dry biomass weight. It represents an increase of biomass until 2.9 g/L yeast extract, and then started to decrease in their amount. Moreover, the influence of KH_2PO_4 and $MgSO_4.7H_2O$ was not very high on biomass production as it is shown on graphs. Fig. 4 (c) depicts the interaction of KH_2PO_4 versus $MgSO_4.7H_2O$ and whilst, increasing the phosphate to 0.9 g/L and sulphate to 0.2 g/L concentration has just slightly increased the biomass content toward the end (14.98 g/L).
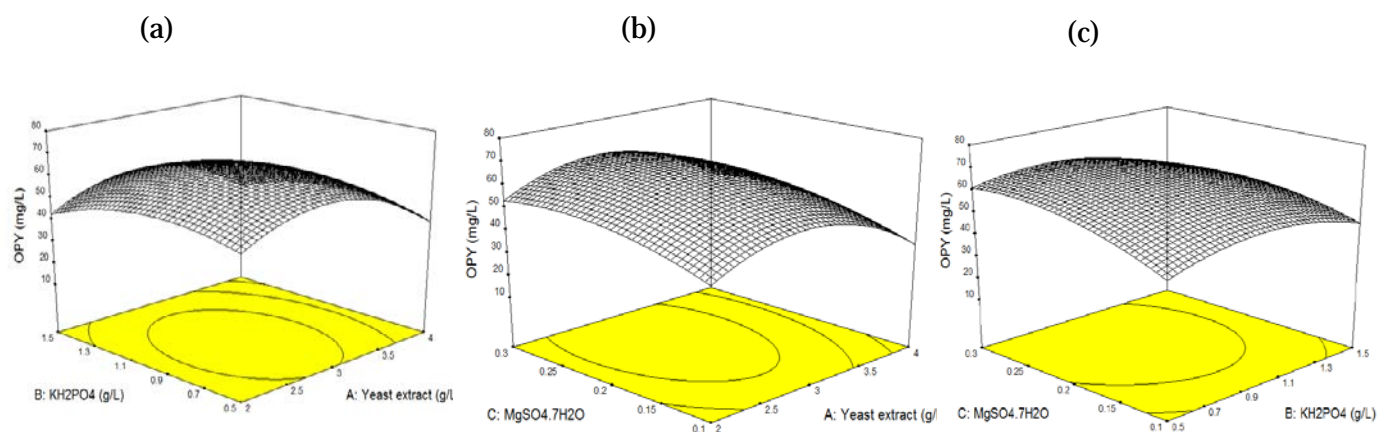
Fig. 2: 3D surface plots showing the interactive effects of factors on orange pigment yield (OPY)
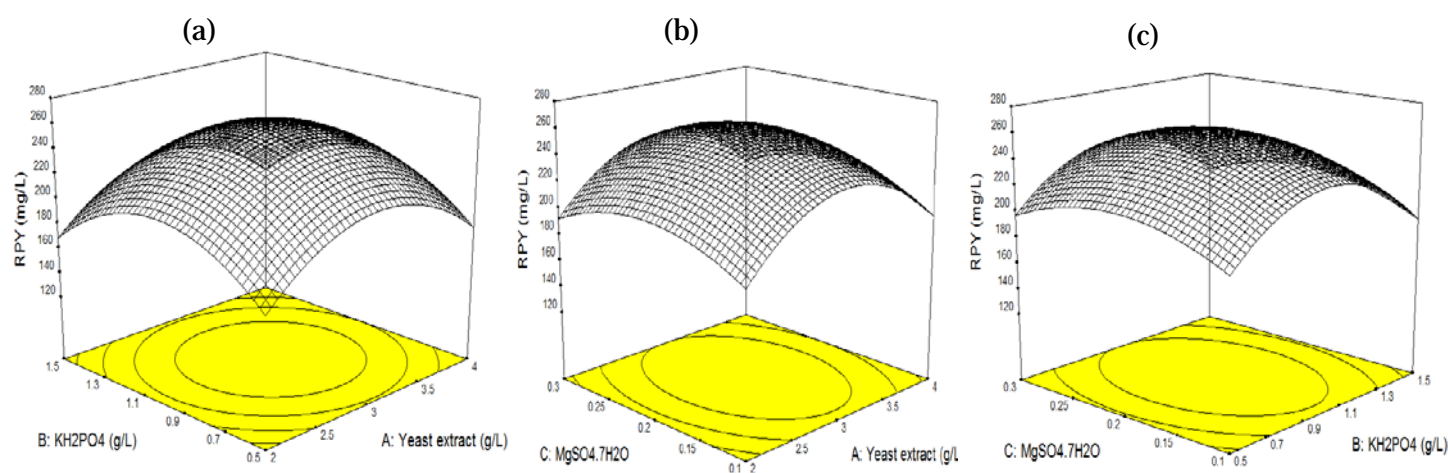
Fig. 3: 3D surface plots showing the interactive effects of factors on red pigment yield (RPY)
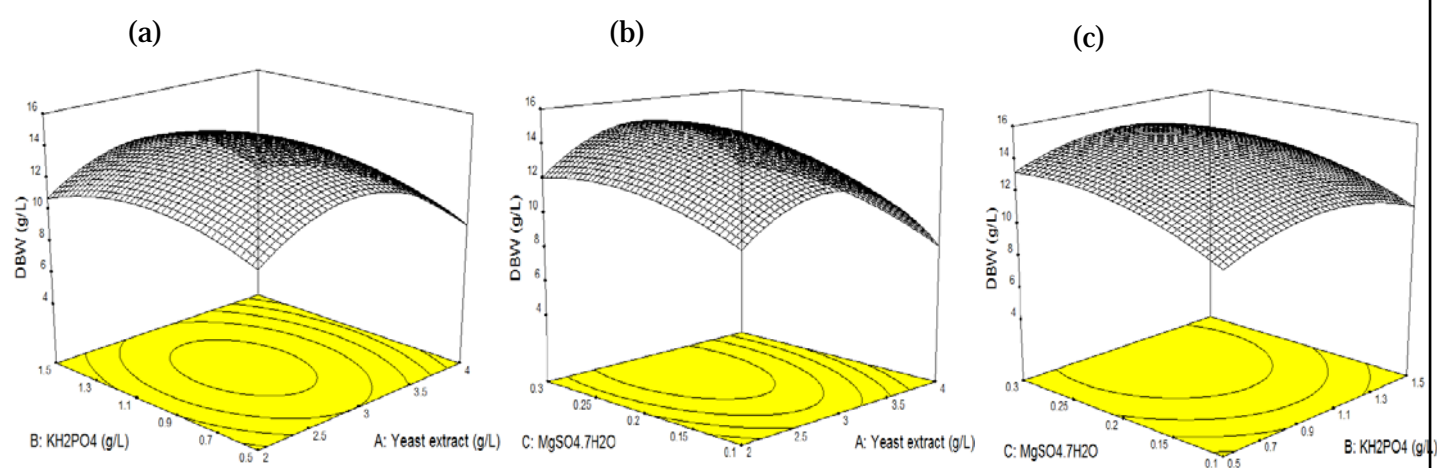
Fig. 4: 3D surface plots showing the interactive effects of factors on dry biomass weight (DBW)

3.3. Analytical validation

ANOVA statistics for the response surface was shown in Table 3, 4, 5. Unexplained deviations of the model are indicated through determination co-efficient and in this experiment, ANOVA of regression model demonstrated the determination coefficient ($R^2=$ 0.9573 for OPY, 0.9800 for RPY and 0.9553 for DBW) which discloses the deviations were 0.042, 0.02 and 0.044 of the deviations were unexplained [71]. With the values of coefficient (CV) it is possible to determine the degree of precision and reliability of the conducted experiments. A minimum value of CV% (10.61% for OPY, 5.58% for RPY and 8.85% for DBW) interpret the low deviation between the experimental and predicted value that has good degree of precision. Signal to noise ratio is referred as adequate precision and a value greater than 4 is desirable [72]. In this study, the adequate precision was found to be >15 for three responses, also proves the model can be used to navigate the design space.

Table 3: Analysis of variance (ANOVA) for the effect of the independent variables on the dependent variables and the regression coefficients, of the fitted quadratic equations on orange pigment yield (OPY)

Source	DF	Orange pigment yield (mg/L)			
		Sum of squares	Mean square	F-value	P-value
Model	9	5126.09	569.57	24.90	< 0.0001
A-Yeast extract	1	272.63	272.63	11.92	0.0062
B-KH_2PO_4	1	101.63	101.63	4.44	0.0613
C-$MgSO_4.7H_2O$	1	363.48	363.48	15.89	0.0026
AB	1	3.56	3.56	0.16	0.7013
AC	1	0.076	0.076	3.325E-003	0.9552
BC	1	44.18	44.18	1.93	0.1948
A^2	1	3467.87	3467.87	151.60	< 0.0001
B^2	1	956.75	956.75	41.82	< 0.0001
C^2	1	546.39	546.39	23.89	0.0006
Residual	10	228.75	22.88		
Lack of Fit	5	189.20	37.84	4.78	0.0555
Pure Error	5	39.56		7.91	
Cor. Total	19	5354.84	19		
SD		4.78			
Mean		45.09			
CV %		10.61			
PRESS		1493.79			
R^2		0.9573			
R^2_{adj}		0.9188			
R^2_{pred}		0.7210			
Adeq. precision		15.195			

Table 4: Analysis of variance (ANOVA) for the effect of the independent variables on the dependent variables and the regression coefficients, of the fitted quadratic equations on red pigment yield (RPY)

Source	DF	Red pigment yield (mg/L)			
		Sum of squares	Mean square	F-value	P-value
Model	9	52455.40	5828.38	54.51	< 0.0001
A-Yeast extract	*1*	*225.90*	*225.90*	*2.11*	*0.1767*
B-KH$_2$PO$_4$	*1*	*1.23*	*1.23*	*0.012*	*0.9166*
C-MgSO$_4$.7H$_2$O	*1*	*126.45*	*126.45*	*1.18*	*0.3023*
AB	*1*	*1.53*	*1.53*	*0.014*	*0.9071*
AC	*1*	*0.53*	*0.53*	*4.961E-003*	*0.9452*
BC	*1*	*3.41*	*3.41*	*0.032*	*0.8619*
A^2	*1*	*27643.42*	*27643.42*	*258.55*	*< 0.0001*
B^2	*1*	*26434.07*	*26434.07*	*247.24*	*< 0.0001*
C^2	*1*	*6903.79*	*6903.79*	*64.57*	*< 0.0001*
Residual	10	1069.17	106.92		
Lack of Fit	*5*	*887.56*	*177.51*	*4.89*	*0.0532*
Pure Error	*5*	*181.61*	*36.32*		
Cor. Total	19	53524.57			
SD		10.34			
Mean		185.35			
CV %		5.58			
PRESS		7037.93			
R^2		0.9800			
R$^2_{adj}$		0.9620			
R$^2_{pred}$		0.8685			
Adeq. precision		17.878			

Table 5: Analysis of variance (ANOVA) for the effect of the independent variables on the dependent variables and the regression coefficients, of the fitted quadratic equations on dry biomass weight (DBW)

Source	DF	Dry biomass weight (g/L)			
		Sum of squares	Mean square	F-value	P-value
Model	9	191.65	21.29	23.77	< 0.0001
A-Yeast extract	*1*	*7.90*	*7.90*	*8.82*	*0.0141*
B-KH$_2$PO$_4$	*1*	*0.11*	*0.11*	*0.12*	*0.7368*
C-MgSO$_4$.7H$_2$O	*1*	*20.70*	*20.70*	*23.10*	*0.0007*
AB	*1*	*1.013E-003*	*1.013E-003*	*1.130E-003*	*0.9738*
AC	*1*	*0.63*	*0.63*	*0.71*	*0.4203*
BC	*1*	*0.015*	*0.015*	*0.017*	*0.8986*
A^2	*1*	*138.80*	*138.80*	*154.92*	*< 0.0001*
B^2	*1*	*29.66*	*29.66*	*33.11*	*0.0002*
C^2	*1*	*14.25*	*14.25*	*15.91*	*0.0026*
Residual	10	8.96	0.90		
Lack of Fit	*5*	*3.75*	*0.75*	*0.72*	*0.6372*
Pure Error	*5*	*5.21*	*1.04*		
Cor. Total	19	200.61			
SD		0.95			
Mean		10.69			
CV %		8.85			
PRESS		35.94			
R^2		0.9553			
R$^2_{adj}$		0.9151			
R$^2_{pred}$		0.8209			
Adeq. precision		15.026			

Table 3, 4, 5 interprets that the significance of the developed model is sharp with a probability value less than 0.0001. In this study, the F values are found to be 24.90 for OPY, 54.51 for RPY and 23.77 for DBW with a corresponding very low probability value <0.0001. The high F-test value indicates that the model is suitable and can adequately explain the real relationship among the factors used. These results show that the model chosen can satisfactorily explain the square of parameters ($p<0.05$ for A^2, B^2, C^2) and interaction ($p<0.05$ for A.B, A.C, B.C) effects of selected medium components on pigment production using yeast extract in shake flask cultures.

3.4. Model validation

The validation was carried out in shake flasks under optimum conditions of the media predicted by the model. The predicted maximum response of OPY (70.87 mg/L), RPY (262.34 mg/L), DBW (15.98 g/L) was obtained at the optimal level of medium variables of yeast extract, K_2HPO_4 and $MgSO_4 \cdot 7H_2O$ of 3 g/L, 1 g/L and 0.2 g/L, respectively. To verify the accuracy of the model, validation experiment was carried out at the predicted optimal levels of the medium components. In the validation experiment we obtained maximum orange and red pigment yields of 70.22 mg/L and 263.11 mg/L. The obtained amount of dry biomass weight under optimized conditions was 16.11 g/L showing a good coincidence with the predicted value. The accuracy of the fitted model was found to be 0.9827. Results of validation explained that the predicted model for the red pigment production was well fitted with the experimental results.

4. DISCUSSION AND CONCLUSION

It is well known that the secondary metabolite production of microorganisms is largely influenced by carbon and nitrogen sources in addition to trace elements [66, 73, 74]. In addition, it has been shown that the media conditions have varying effects on the production of fungal secondary metabolites [75, 76]. Carbon and nitrogen sources constitute the major cost of the fermentation medium and several studies on alternate sources reported economical production of red pigment using various agro products and byproducts [14–19]. This requirement necessitated the present study to optimize carbon and nitrogen sources and growth factors for *Talaromyces albobiverticillius* 30548 so that a commercial production process could be evolved.

One-variable- at-a-time using different carbon sources presented varying levels of pigment production and among all; sucrose was the best for *Talaromyces albobiverticillius* 30548. Previous reports have also indicated that pigment production is more in the presence of glucose in case of *Monascus* species [62, 77-81] . There was an increase in the biomass as well as pigment production with increasing glucose concentration. Some workers reported that a high glucose concentration (50 g/L) leads to low growth rates and pigment synthesis (Chen and Johns, 1994). Results of Barun *et al.* (2016) suggested that the maximum production of pigments in *Pezicula sp.* BDF 9/1 was obtained with glucose as a carbon source [82]. Earlier Cho *et al.* (2002) reported maximum pigment production with soluble starch medium from *Paecilomyces sinclairii* [83] whereas Tseng *et al.* (2000) reported fructose as a suitable carbon source for maximum pigment production from *Monascus purpureus* [84]. In a study by Kim *et al.,* 1998, sucrose gave a maximum yield of yellow pigment and it may be due to the fact that sucrose can be easily assimilated in the metabolic pathway for biosynthesis of pigment production [85].

A stimulatory effect of nitrogen source on pigment formation has been reported that utilization of different nitrogen sources in fermentation had effects on microorganism growth and pigment production [23]. In *Thermomyces sp.*, ammonium and peptone serves as a good nitrogen source for pigment production that yielded better growth [86]. Inorganic nitrogen does not influence pigment formation but organic nitrogen such as malt extract, yeast extract, peptone and soybean flour or their combination promotes growth as well as pigmentation [87, 88]. The effect of different nitrogen sources on pigment production by a number of *Monascus* species concluded that when the source of nitrogen is yeast extract or nitrate, red pigments are formed, whereas with ammonium or ammonium nitrate, orange pigments are formed [11, 89]. As well as for biomass, yeast extract stimulates conidiation, represses the sexual cycle and increases biomass production [90]. This is in agreement with the pigment production and biomass growth for *Talaromyces albobiverticillius* 30548 using yeast extract.

Although trace elements are considered as a highly important parameter to reach higher growth rates and biomass production but, its different amount did not significantly change the pigment production in our studied fungi. In this case, the red pigment production had an impact of three to four times higher than orange pigment yield and dry biomass weight when using trace elements (Fig. 4, 5, 6). In a study for *Monascus anka* mycelia, the dry cell weight was three to four times higher than conventional batch when trace elements were fed in the fed-batch culture. While, the total pigment production decreased by 9.0 to 14.6 %, which may

be partly due to the strong negative-regulating effect of high concentration of $MgSO_4$ on the action of pigments synthase(s). These findings suggested that feeding carbon and nitrogen sources only played a role in facilitating cell growth, while the trace elements were key factors to improve the cell growth and control pigment synthesis in the high density culture [65]. On contrary, the only trace element reported to support growth and pigment production by *Monascus* species is zinc [91-93]. This effect could be due to the participation of zinc in the uptake and utilization of the carbon source. According to Lin *et al*. (1991), high concentrations of phosphate and magnesium sulfate ($MgSO_4$) inhibit pigment production and the growth is a crescent linear function of $MgSO_4$ concentration, in the range from 0.5 to 16 mM [46]. This is not in agreement with the results obtained in the experiments conducted using *Talaromyces albobiverticillius* 30548.

The orange pigment yield (OPY) with media optimized using central composite design was found to be 70.87 mg/L, which is about nine times less than that obtained in media optimized by conventional one-variable-at-time method (623.09 g/L). Similarly for red pigment yield (RPY), it was about 262.34 mg/L using response surface analysis which is threefold less than obtained using OVAT analysis (635.35 g/L). On the contrary, biomass yield was higher in central composite design experiments (15.98 g/L) but using OVAT analysis, the biomass weight was very low (5.96 g/L). This change in cultivation time and decrease in pigments production is probably due to change in concentrations of media constituents obtained by CCD.

In the present study, optimum media composition was determined for the production of pigments and biomass using *T. albobiverticillius* 30548 by means of the one-factor-at-a-time method, and CCD. The classical one-factor-at-a-time method indicated sucrose, fructose, yeast extract to be high-pigment-yielding media components. Dihydrogen potassium phosphate (KH_2PO_4) and magnesium sulfate ($MgSO_4.7H_2O$) were other significant factors after yeast extract. The CCD design was used to check interactions and concentration of significant media components. About a fivefold increase in biomass production within 148 h cultivation time was found, after using statistical techniques for media optimization. A higher yield of pigment production in comparison with the conventional method of optimization and a decrease in cultivation time using a lower concentration of components (yeast extract— 2.9 g/L, KH_2PO_4—0.9 g/L, and $MgSO_4.7H_2O$ —0.2 g/L) is the major outcome of this work. The yield of pigments can also be increased by using fed-batch fermentation, which overcomes the problem of substrate inhibition.

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4.5   CONCLUSION

Monascus and *Monascus-like* azaphilone pigments are high molecular weight pigments which has huge application value and development potential in food industry. Various microorganisms have been reported to produce an array of primary and secondary metabolites, but in a very low quantity. Medium optimization is still one of the most critically investigated phenomenon that is carried out before any large scale metabolite production, and possess many challenges too. Optimization of production medium is required to maximize the metabolite yield and this can be achieved by using a wide range of techniques from classical "one-factor-at-a-time" to modern statistical design of experiments.

An initial study was conducted to identify the optimum process factors and environmental conditions for pigment production using potato dextrose broth as a fermentation media. Using Box-Behnken Design, optimum conditions for maximum production of pigments was found to be initial pH of 6.4, temperature of 24 °C, agitation speed of 164 rpm and fermentation time of 149 h respectively. The biomass was produced maximum with the above conditions but gave better yield at 26 °C.

The selection of carbon and nitrogen source, its concentration in the media also plays a crucial role in pigment production as well as fungal biomass synthesis. Even though, experimental designs offer considerable advantage of reliable shortlisting of medium components, one-variable- at-a-time approach determines the influence of each variable on responses. With this experimental set up, sucrose and yeast extract gave maximum yield of orange (623.09 mg/L) and red pigments (635.35 mg/L) as well as biomass production of 5.96 g/L. The significant factors identified through OVAT analysis were considered for the next stage in media optimization by using response surface central composite design. Predicted maximum response of orange pigment yield (70.87 mg/L), red pigment yield (262.34 mg/L), dry biomass weight (15.98 g/L) was obtained at the optimal level of medium variables of yeast extract, K_2HPO_4 and $MgSO_4 \cdot 7H_2O$ at 3 g/L, 1 g/L and 0.2 g/L, respectively. In addition to trace elements, use of specific amino acids can increase the productivity in some cases but appropriate concentration is necessary for the production of desired metabolite at large scale. In order to standardize the production medium, the concept of medium optimization has emerged. However, the medium formulated after employing various designs still needs further evaluation under realistic production conditions and lastly with full scale models that reflect the production environment.

CHAPTER 5

EFFECT OF SEA SALTS ON FUNGAL GROWTH AND PIGMENT PRODUCTION IN MARINE-DERIVED FUNGI, *Talaromyces albobiverticillius* 30548

This chapter provides brief information about pigment production in marine derived fungus, *Talaromyces albobiverticillius* 30548 and its growth based on the effect of changing environmental conditions particularly sea salts concentration in the culture medium.

5.1 BACKGROUND

Oceans and Seas have abound of microorganisms such as bacteria, fungi, viruses, protists and they are considered as a unique living environments of high salinity, pressure, temperature and nutrition. In the past two decades, nearly 690 new compounds have been found from marine fungi showing that marine microorganisms are potent source of natural compounds [1-3]. Following to that, much research has been focused on marine and marine derived fungi to study their capability to produce secondary metabolites such as enzymes, pigments, antibiotics, etc. The microorganisms isolated from the marine sources exhibit distinct mechanism than the terrestrial ones in order to adapt to the living environments [4]. There are number of factors which influences the occurrence and metabolism of marine fungi such as nature of habitats, geographical distribution and temperature, salinity, inhibition competition, dissolved organic nutrients, hydrogen ion concentration, osmotic effects, oxygen availability, pollutants, abundance of propagules in the water, ability to impact on to and attach to suitable substrata, hydrostatic pressure, substrate specificity, temperature and tidal amplitude and perhaps even light [5]. Though the presence of many chemicals makes the ocean water salty, the outweighing compound is sodium chloride or simply salt. The salinity in most of the marine areas is usually 35 parts per thousand. i.e.; 35 g of salt in every 1000 g of water.

Marine fungi have the ability to grow and produce secondary metabolites by altering their morphological characters according to the changing environment. This competence pinpoints the ecological advantage of marine fungi over their terrestrial counterparts. However, some studies have shown that the secondary fungal metabolite production is influenced by the culture conditions which might be regarded as the environmental changes when growing at laboratory conditions [6]. Only limited publications dealt with the effects of salinity on growth and secondary metabolites of marine fungi. The non-marine red pigment producing fungal

species namely *Monascus sanguineus* and *Monascus purpureus,* when exposed to salt stress above 3 %, decrease in the yield of pigment was noted also the strains lost their viability above 12% of salt in the culture media.

Marine-derived *Pencillium dravuni* F01V25 produces the anthraquinone carviolin and the polyketide dictyosphaeric acids A and B. As a note that it was sourced from marine environment, a study has been attempted to determine its growth on salt Malt Extract Agar (MAE) supplemented with 3% sodium chloride (NaCl) w/v and 1% other salts (potassium chloride [KCl], magnesium chloride [MgCl$_2$], calcium chloride [CaCl$_2$]) w/v. The fungi grew at these concentrations but a decrease in growth was observed beyond increasing the concentration to 10%. Moreover, two different secondary metabolites were produced in 10% concentration when compared to salts level at 3% [7]. As a part of our research, a study was carried out by varying the concentrations of sea salts at different levels from 3.65-9% in the culture media to understand the influence of sea salts on the growth and pigment production as well as to compare the response with control (0% salt concentration) in *Talaromyces albobiverticillius* 30548.

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5.3 MANUSCRIPT (RESEARCH)

This chapter is based on the manuscript "Effect of sea salts on fungal growth and pigmented metabolites production in marine-derived fungal strain Talaromyces albobiverticillius" presenting the effects of sea salts concentrated media on fungal growth and pigment production of *Talaromyces albobiverticillius*

Journal: Marine drugs

Special issue entitled: "Bioactive compounds from Marine-derived *Aspergillus, Penicillium, Talaromyces* and *Trichoderma* species"

Guest Editors: Dr. Rosario Nicoletti & Dr. Francesco Vinale

Submission deadline: October 2017

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Effect of sea salts on fungal growth and pigmented metabolites production in marine-derived fungal strain *Talaromyces albobiverticillius*

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Abstract: Marine-derived fungi have gained increasing interest by producing structurally unique natural products that inhabit severe changing environments. *Talaromyces* strains as well as the close *Penicillium* genera are among the most innovative towards bioactive compounds even amongst colored metabolites. Coupling pigment producing capability with bioactive effectiveness would be a valuable challenge in some specific industries as dyeing, cosmeceutical or food industries. In this sense, a red pigment producing strain *Talaromyces albobiverticillius* has been isolated from the marine environment of Réunion Island, Indian Ocean. The marine origin of this strain may impact its potential to synthesize metabolites, for instance the intense red pigment which is one of its characteristics. In this research we analyzed the effect of different temperatures and salinity levels on biomass growth and pigment production. This study emphasizes the impact of salted / unsalted media on the production of metabolites of interest in marine-derived fungal strains.

1. Introduction

The use of natural colorants is gaining interest due to the increasing demand for natural and healthy products among consumers. In that quest for new molecules and efficient pigment producers, filamentous fungi are found to produce a diverse range of bioactive secondary metabolites of interest such as antibiotics, alkaloids, enzymes, organic acids, carotenoids, toxins and pigments since long [1-3]. These compounds have been apparent to have potential interest in agrochemical, biotechnological, food and pharmaceutical industries [4, 5]. Filamentous fungi dwell in various ecological habitats including marine and terrestrial environments, also thrive on a various range of substrates either in solid or liquid base [6]. Many of the fungal secondary metabolites are not directly involved in the growth of organism instead it will be produced after the active growth, which may play a pivotal role in sporulation, stress tolerance, protection against UV, nutrition and interactions with other organisms [7, 8].

Considering pigments, divergent sources of filamentous fungi produces different chemical classes of pigments such as carotenoids, melanins, flavins, phenazines, quinones and more specifically monascins, violacein, and indigo [9-11]. For instance, carotenoids or anthraquinones play an important role for the adaptation of fungi to their environment, by protecting their cells against oxidative stress or by promoting various synthesis of physiologically active by products [12-15]. However, the underlying mechanisms of secondary metabolites biosynthetic gene cluster regulation are emerging but the factors and pathway involved in the regulation of these secondary metabolites are poorly understood till now [16, 17]. Furthermore, pigments from marine-derived ascomycetes fungi considered as prolific source for their production of structurally and biologically unique compounds with pharmaceutical potential. This has increased the attention of natural products chemists and researchers to scrutinize the diversity, chemistry and biology of marine fungi [18-20].

Exposure to both biotic and abiotic stress causes variety of responses in microbes including direct nutritional effects, toxic effects, biochemical and morphological changes [21]. Despite their native habitat, number of evidences proved that under marine environmental conditions, particularly in the presence of sea salts, unique secondary metabolites have been found to be produced as compared to the related strains of terrestrial / fresh water fungi [22-24]. Novel classes of secondary metabolites were identified from the salted-water cultures including antiviral cum neuroprotective sorbicillactone A and 2′,3′-dihydroanalog sorbicillactone B from *Penicillium chrysogenum* [25], antibacterial terremides A and B from *Aspergillus terreus* PT06-2 [26], asperic acid

from the saltwater culture of sponge derived *Aspergillus niger* [27], the polyketide, trichoharzin was isolated from the salty medium of *Trichoderma harzianum* [28].

Therefore, a special emphasis has been assigned to collect samples from different habitats of marine environment and to analyze the pigment producing strains among the diverse selection of fungal community. The samples were collected in the marine environment of La Réunion Island, located 800 km east of Madagascar. The study was conducted in order to support future research towards identification of pigments from marine-derived fungi and eventual secondary metabolites with potential bioactivities. Among the screened microorganisms collected from the marine environment of La Réunion Island (Indian Ocean), a species of *Talaromyces* (*T. albobiverticillius* 30548), was found to originally synthesize large amount of red pigments. It has been reported that some strains of *T. albobiverticillius* does not produce any toxins [29] and thus considered as a promising source for industrial production of red pigments.

Attributable to various reports, an intuitive study was carried out to investigate the behaviour of this strain and its ability of pigment production under submerged fermentation, using potato dextrose broth (PDB) supplemented with artificial sea salts. However, only a few published research articles deals with the effects of salts (mostly NaCl) on growth, extrolites and pigments production by marine-derived filamentous fungi [30, 31]. Huang et al (2011) studied the effects of salinity on growth, secondary metabolites production and biological activities of several marine-derived strains of *Penicillium* [32]. In his report, it was found that NaCl promoted growth in 91.5% of their 47 strains, and antimicrobial activity in 14.5%. These results confirm that salinity is a crucial factor influencing growth and secondary metabolites production [33]. Based on these findings, this work was designed to investigate the effects of salinity on fungal growth and behaviour, intensity and amount of pigment production, ability of stress tolerance level of marine-derived *T. albobiverticillius* 30548 towards this particular factor.

2. Results

2.1 Effect of temperature on fungal growth and biomass

For a better understanding of temperature effect on fungal growth rate and biomass production, fermentation was carried out at three different temperatures (21, 24 and 27 °C). With dry weight of fungal biomass used as a criterion, the biomass production rates have been calculated during the maximal growth phases (2 to 7 days for 21 °C, 2 to 8 days for 24 °C and 1 to 5 days for 27 °C). The weight of final dry biomass was given for day 10 and amount was examined once the growth had stopped completely in the stationary phase, after 9 days **(Table 1).**

Table 1. Biomass growth rate and final dry biomass weights based on the temperature in *Talaromyces albobiverticillius* 30548 cultured in PDB with an initial pH of 4.0 under the agitation speed of 200 rpm

Temperature	Exponential phase	Fungal growth rate (g/L/day)	SD	R^2	Maximum dry biomass weight (g/L)	SD
21 °C	Days 2 to 7	0.51	0.03	0.9696	6.22	0.02
24 °C	Days 2 to 8	0.48	0.03	0.9311	5.48	0.02
27 °C	Days 1 to 5	1.11	0.03	0.9893	5.66	0.02

The growth rate values were found to be 0.51±0.03, 0.48±0.03 and 1.11±0.03 mg/L/day implying the temperature and time factor had an influence on fungal growth. In the experiments conducted at 3 different temperatures, the final dry biomass weights were 6.22±0.02, 5.48±0.02 and 5.66±0.02 g/L at temperatures 21, 24 and 27 °C respectively. These results significantly showed that the fungi grown at the temperature of 21 °C exhibited maximum growth rate in 7 days, and the biomass produced was higher compared to the growth at 24 & 27 °C **(Figure 1)**.
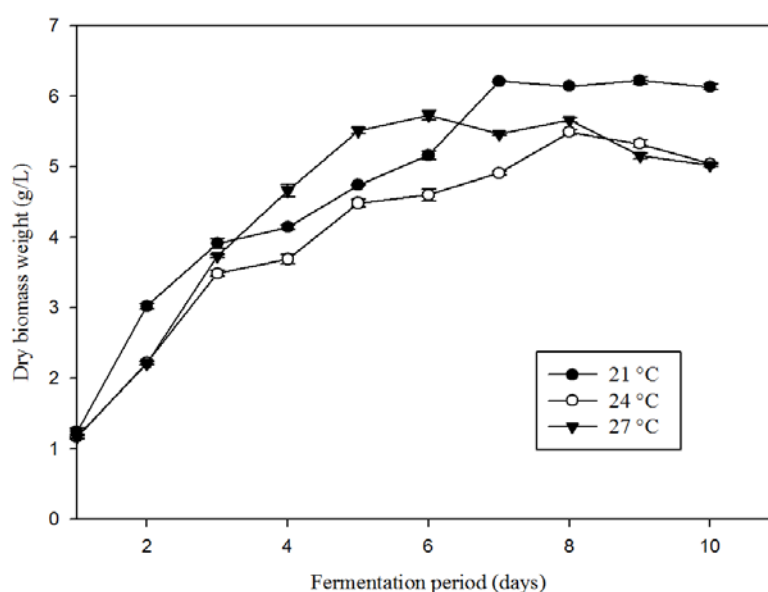
**Figure 1.** Compared effects of culture at three different temperatures (21 °C, 24 °C & 27 °C) on biomass growth for *Talaromyces albobiverticillius* 30548 cultured in PDB with an initial pH of 4.0 under the agitation speed of 200 rpm

Considering the maximum dry biomass at three different temperatures, incubation at 21 °C gave maximum biomass yield on day 6 but culturing at this temperature made the condition

unfavourable to produce high amount of pigments compared to 24 °C & 27 °C (see paragraph 2.2). And also, at 27 °C, the growth rate is maximum (1.1 mg/L) but the exponential phase is shorter and the final biomass is lower than 21 & 27 °C.

One way ANOVA was performed and the difference in the mean values of the three groups is not great enough to reject the possibility that the difference is due to random sampling variability. There exists no statistical significance between the temperature conditions ($P = 0.359 > 0.05$).

2.2 Effect of temperature on pigment production

The studied fungal strain produces red pigments at three different temperatures and the highest production of pigments (expressed in red rice equivalents mg/L) was observed on day 9 at 24 °C. In all the treatments (21, 24 & 27 °C), pigment production was initiated on day 3 and a large deviation among the triplicates was seen in the beginning of exponential phase, which is clearly indicated in **Figure 2**. This demonstrates the asynchronous nature of the culture. The maximum pigment production was attained on day 8 for all the three sets and then followed by the stationary phase from day 8 until day 10. Whereas at 24 °C, pigment production was more effective and reached nearly maximum on day 6 compared to the production at 21 & 27 °C.
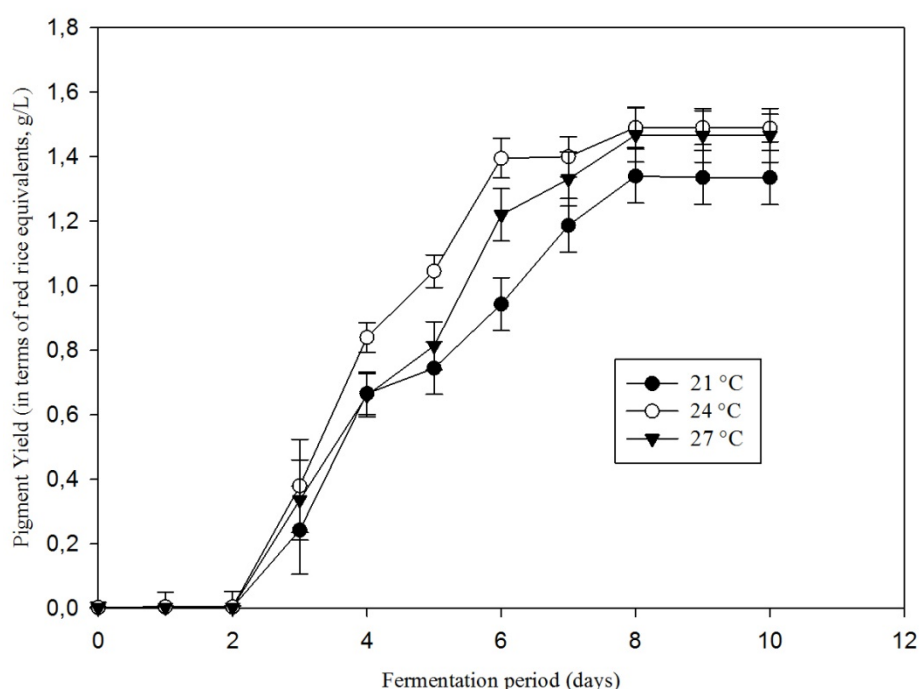

Figure 2: Compared effects of culture at three different temperatures (21 °C, 24 °C & 27 °C) on red pigment yield (at 500 nm) (expressed in red rice equivalent g/L) for *Talaromyces albobiverticillius* 30548 cultured in PDB with an initial pH of 4.0 under the agitation speed of 200 rpm

In addition, rate of pigment production has been calculated during exponential growth of the fungi and the yields were 0.2204±0.01g/L/day at 21 °C, 0.3446±0.02g/L/day at 24 °C and 0.2910±0.02 g/L/day at 27 °C **(Table 2).** Hence, it can be concluded that temperature had a significant impact on pigment yield and obtained values were significantly different (p<0.05), even though the final pigment yields were nearly the same.

Table 2. Pigment production rate (in equivalents Red Rice) in *Talaromyces albobiverticillius* 30548 depending on temperature cultured in PDB with an initial pH of 4.0 under the agitation speed of 200 rpm

Temperature	Exponential phase	Production rate (mg/L/day)	SD	R^2	Maximum pigment yield (g/L)	SD
21°C	Days 2 to 8	220	0.01	0.97	1.34	0.08
24°C	Days 2 to 6	344	0.02	0.98	1.49	0.06
27°C	Days 2 to 7	291	0.02	0.98	1.46	0.08

2.3 Effect of sea salts on fungal growth rate and biomass

Based on the biomass criterion, the implemented linear model was found to be significant for two sea salt concentrations (0% and 9% sea salts) and moderately adequate for 6% **(Table 3).** From the obtained slopes for all sea salts concentrations, it could be noted that the biomass production tends to be greater with lower sea salts concentrations. Lag phase was observed before the beginning of growth for lower sea salts concentrations (3.65%) as the culture for fermentation was taken from seed culture (same media without added salts) while it was in its exponential phase. However, a small lag phase (one day, pigment production started on day 2) was observed for higher sea salts concentrations (6% and 9%). This could be explained by the fact that a short time was required by the fungi for its adaption to grow in higher sea salts concentration.

Table 3: Rate of fungal growth in *Talaromyces albobiverticillius* 30548 based on salinity in the fermentation media cultured in PDB with an initial pH of 4.0 under the agitation speed of 200 rpm

Sea salts concentration	Reference code	Exponential phase	Fungal growth rate (mg/L/day)	SD	R^2
0%	T1	Days 2 to 6	291***	0.02	0.986
3.65%	T2	Days 3 to 9	212*	0.03	0.904
6%	T3	Days 2 to 7	122**	0.005	0.988

| 9% | T4 | Days 2 to 8 | 39*** | 0.004 | 0.950 |

The significance of sea salts concentration during exponential phase was statistically analysed using Tukey's test. The results indicated that there was no significant difference between the groups 0% & 3.65%, 3.65% & 6% (p>0.05) whereas there exists a difference between 0% & 6%, 0% & 9% (p<0.05).

2.4 Effect of sea salts on dry biomass weight

From the results **(Figure 3),** it was clear that dry biomass yield was influenced by sea salts concentration from day 1 **[34]**. It can be observed that culture media with 9% salinity (T4) exhibited linear increase from day 1 until maximum biomass yield obtained on day 9. Media with sea salt concentrations (0%, 3.65% and 6%) obtained maximum biomass yield on day 8 (T1), 7 (T2) and 8 (T4) respectively. From the results, it was inferred that the amount of final dry biomass weight and the time required in obtaining maximum dry biomass increase with an increase in sea salts concentration and this was in agreement with the work done by Masuma et al, 2001 **[35].** There is a clear difference in the final dry biomass weight of salted media which was higher for T3 (8.28 g/L) and notably higher for T4 (9.22). From fig. 3, it is highlighted that the final dry biomass was nearly the same for T1 (4.88 g/L) and T2 (5.01 g/L) which was lower than obtained for salted media.
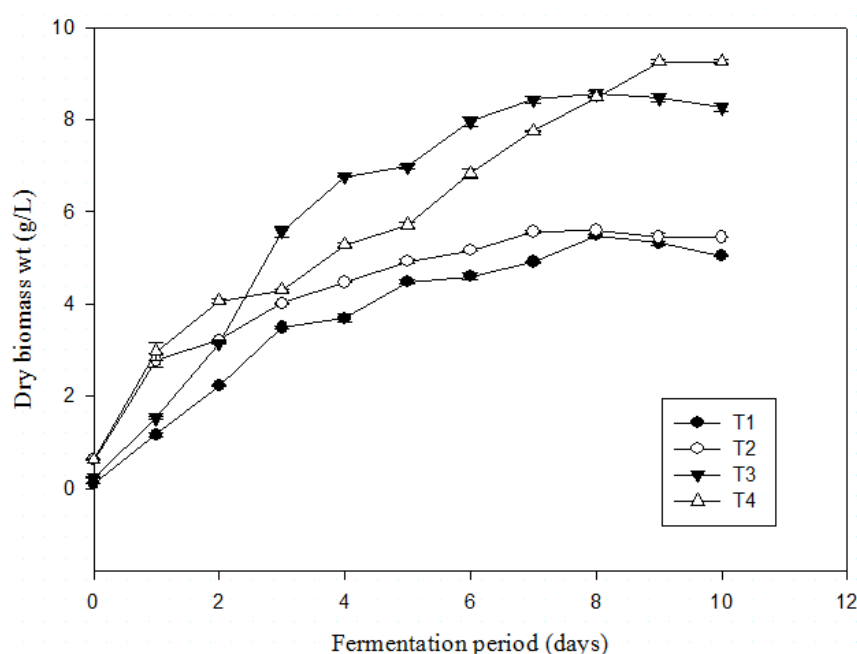
Figure 3: Effect of different levels of sea salts concentration (0% (T1), 3.65% (T2), 6% (T3), 9% (T4)) on dry biomass weight at 27 °C cultured in PDB with an initial pH of 4.0 under the agitation speed of 200 rpm

The addition of sea salts was found to significantly increase the final dry biomass weight in *Talaromyces albobiverticillius* 30548 which was confirmed by ANOVA. **Table 4** shows the length of exponential phase, fungal growth rate and final biomass for all the treatments applied.

Table 4: Rate of fungal growth and final biomass weight in *Talaromyces albobiverticillius* 30548 based on salinity in the fermentation media cultured in PDB with an initial pH of 4.0 under the agitation speed of 200 rpm

Sea salts concentration	Reference code	Exponential phase	Fungal growth rate (mg/L/day)	Final biomass (g/L)	SD	R^2
0%	T1	Days 2 to 8	484	4.88	0.03	0.9312
3.65%	T2	Days 2 to 7	446	5.01	0.03	0.9652
6%	T3	Days 2 to 7	965	8.28	0.06	0.8944
9%	T4	Days 2 to 9	784	9.22	0.09	0.9884

2.5   Effects of sea salts on pigment production

The rate of pigments production for different levels of salinity is presented in **Figure 4**. Enhanced pigment production was noticed in the culture media without the addition of sea salts (T1) then followed by minimum salinity percentage (T2). The time required for initiation of pigments production in sea salts added media was found to be higher when compared to 0% salt concentration media; this could be explained by an initial adaptation time which might have been required for the fungi in sea salts concentrated media **[36]**. Indeed, the seed culture was grown in PDB without sea salts added in the culture media. From the results it was noticed that media with 0% salt concentration (T1) exhibited prominent pigment production from day 2 (0.0167 g/L) to day 7 (maximum pigment yield 1.36 g/L). Though media with 3.65% (T2) sea salt concentration exhibited a short delay in pigment production until day 5 (0.23 g/L), it exhibited a drastic change in pigment production after day 6 (0.92 g/L) until day 9 (maximum pigment yield 1.46 g/L).
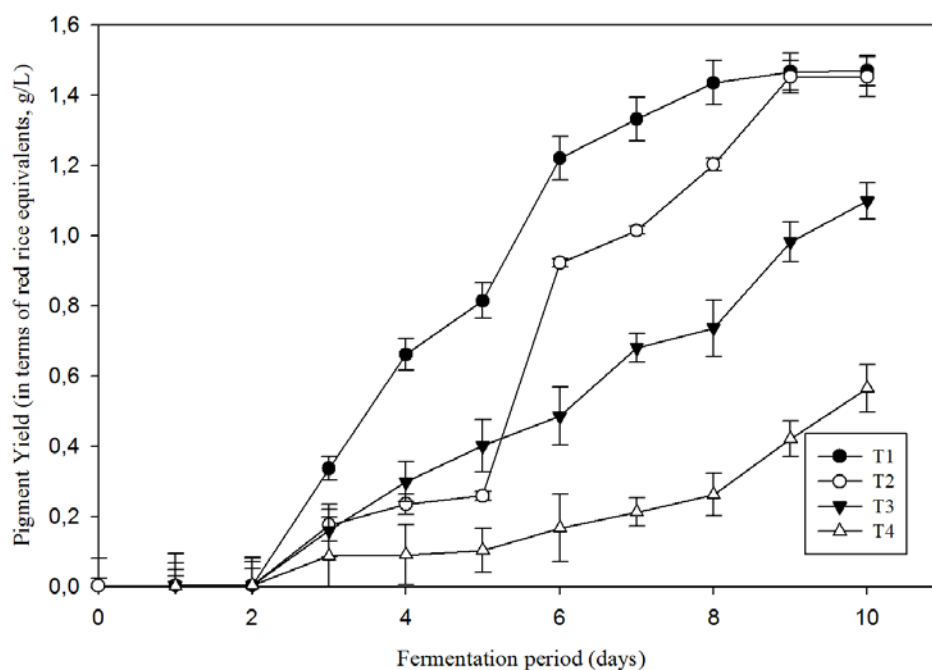
Figure 4: Effect of sea salts concentration on pigment yield expressed in red rice equivalent g/L (500nm) at 27 °C in *Talaromyces albobiverticillius* 30548 cultured in PDB with agitation speed of 200 rpm ; 0% (T1), 3.65% (T2), 6% (T3), 9% (T4).

Salt contents in the medium had a significant influence on the pigment yield and its production corresponded to the change in color values. The final pigment yield was higher for treatment T1, 1.46 g/L and for T2 (1.45 g/L) which is nearly the same as T1. For treatment T3, the final pigment yield was 1.09 g/L and for T4, 0.56 g/L which is very low compared to all other treatments **(Table 5).**

Table 5: Rate of pigment production and final pigment yield in *Talaromyces albobiverticillius* 30548 based on salinity in the fermentation media cultured in PDB with an initial pH of 4.0 under the agitation speed of 200 rpm

Sea salts concentration	Reference code	Exponential phase	Production rate (mg/L/day)	SD	Final pigment yield (g/L)	SD
0%	T1	Days 3 to 8	225	0.01	1.46	0.08

Sea Salts concentration	Reference code	Days			
3.65%	T2	Days 3 to 9	232	0.02	1.45	0.06
6%	T3	Days 2 to 9	130	0.02	1.09	0.08
9%	T4	Days 3 to 10	65	0.05	0.56	0.07

Pigments production rate was statistically analysed through R^2 value. The R^2 value of 0% and 3.65% salt concentration was found to be around 0.95 and 0.98 which indicates the linear increase of pigment concentration. To test the statistical significance, t- test was performed between the treatments. Between treatment T1 and T2, the t-test was successful and the P- value was 0.45. There exists no significant difference between the treatments as the P-value 0.45 > 0.05. But among the treatments T1, T3 and T4 there exists a significant difference which is given by the P-value 0.002 < 0.05.

2.6 Effect of sea salts on color hues

The commercially available *Monascus* red (R) and orange quinizarin (O) were used as reference colorants (1 g/L) to compare La*b*c h° color values of fungal culture filtrates. The a* values of all the cultured filtrates were found to be positive ranging from 6.87 to 81.25 (pale pink to red) and similarly for b*, only positive values exists from 6.87 to 67.12 (grey to yellow) **(Table 6).**

Table 6: L, a*, b*, c, h° values of *Talaromyces albobiverticillius* 30548 fungal extracts at different sea salts concentrations on day 10 cultured in PDB with an initial pH of 4.0 under the agitation speed of 200 rpm and standards (Monascus red and Quinizarin)

Sea Salts concentration	Reference code	L	a*	b*	h°_{ab}	Chroma
0%	T1	57.12	56.73	42.94	37.08	76.14
3.65%	T2	48.16	61.38	46.08	36.89	71.75
6%	T3	75.25	49.87	21.25	23.07	54.20
9%	T4	81.25	6.87	29.87	77.04	30.62
Red yeast rice	R	54.02	57.41	61.97	47.19	84.48
Quinizarin	O	91.24	42.18	65.12	57.06	35.17

The colored extracts produced in control media (0%) and (3.65%) sea salts concentrated media represented red color when compared to media with higher sea salts concentrations (6% & 9%). The hue angles of the culture filtrates (0, 3.65 and 6%) ranged from 23 to 37 signifying orange –red color. While, the hue angle of 9% sea salts concentration was 76.8 which was very near to yellow region (Figure 5) and implies that addition of Sea salts to the culture medium modified the color of pigments produced in *Talaromyces albobiverticillius* 30548. In the culture media without the addition of sea salts (at 0%), "dark red" hue was observed instead, salt concentrated media altered the color from dark red to brown indicating a clear change in the pigment content and structure. However, T1, T2, T3 exhibit close hue angles, compared to T4 which appears really different. Comparing the hue angles of T1 and T2, it seems closer to the reference colorant, Red Rice (R). Whereas the hue angle of T4 is on the angle of quinizarin (O) which is orange in color but exhibited very low lightness. The saturation, C* responses of the 4 different treatments (T1, T2, T3, T4) widely ranged from 30.62 to 76. 14. Among T2 then T1 exhibited significantly high concentration of pigments presented by high saturation values. Comparatively T3, then T4 are positioned lower. The saturation of the color is thus clearly modified by the variations in salt concentrations in the culture media.
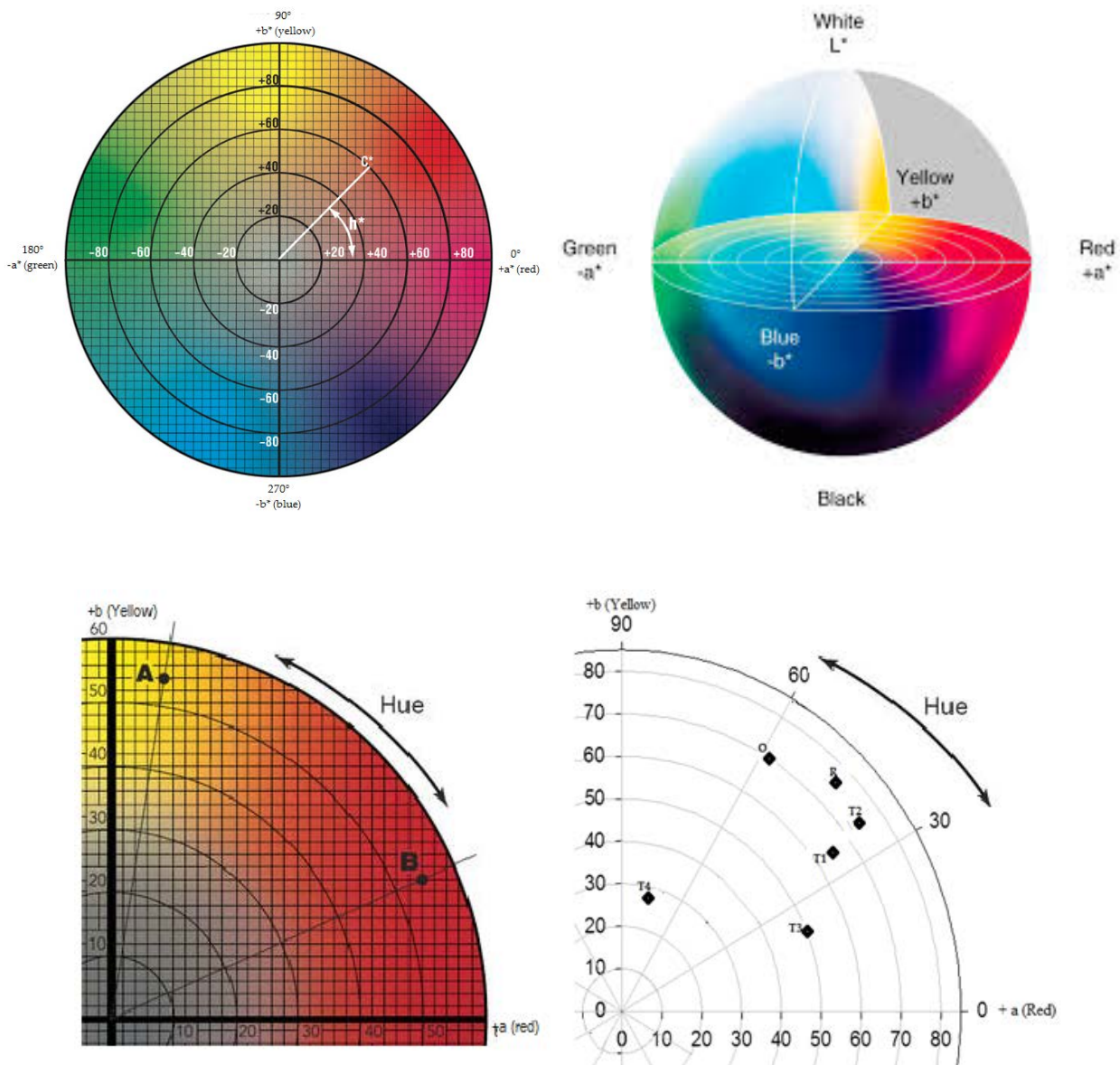
Figure 5: Polar scatter plot showing the positions of *Talaromyces albobiverticillius* 30548 cultured in 4 different sea salts concentrated media T1 (0%), T2 (3.65%), T3 (6%) & T4 (9 %) including reference standards Quinizarin (O); Red yeast rice (R)

3. Discussion

Since our interest was to test the tolerance of marine derived fungi to high salt stress and the reaction towards pigments production, we have used artificial sea salts to study their behaviour in liquid media. Indeed literature already mention that in the presence of marine salts, fungi produces different metabolic profiles **[18].** Numerous studies on various fungal species have reported that temperature is an important physiochemical factor that has a great impact on cell

growth **[37]**. Nonetheless, incubation at low temperature (21 °C) increases broth viscosity that can result in increased biomass but had negatives effect on pigment production in the studied range (220 mg/L/day Red Rice equivalents, compared to 344mg/L/day at 24°C). From the negligible starting viscosity, immediately after the inoculation, viscosity in the fermentation medium increases due to the increased cell concentration at low temperature and release of any metabolites into the medium **[38, 39].** High viscosity of the media may result in reduced oxygen transfer and increased fraction of oxygen limited-cells. Thus the associated oxygen limited environment may yield low pigment production. Ahn *et* al. (2006) observed a similar pattern of decreased *Monascus* pigment production, linked to the increased viscosity which was due to the highly entangled mycelial clumps **[40]**.

Several studies have reported that changes in temperature whether increase or decrease; induce changes in the production of pigments. In this regard, Babitha *et al.* (2007) described increasing the temperature to 30 °C resulted in higher red pigment production in *Monascus purpureus* LPB 97 at 500 nm, beyond increment of temperature to 40 °C produced more yellow pigments absorbing at 400 nm **[41]**. Considerably, high viscosity of the broth was observed in a culture at 21 °C which apparently decreases the availability of dissolved oxygen, thus in turn affected the higher production of pigments absorbing at 500 nm. Similar behaviour was remarked in the submerged fermentation of *Monascus sp.* J101 for red pigment production **[40].**

Next to temperature, it is well known that salinity is an important abiotic factor affecting the distribution of at least some marine derived fungi. Sea water normally holds salt content around 3.5 % and there seems to be continuous alteration in the salinity based on intertidal amplitude, which considerably affects the fungal biodiversity **[42].** Tolerance to high salt stress is a property of marine-derived fungi, predominantly some species of *Aspergillus* and *Penicillium* **[43]**. The present study shows a remarkable influence of sea salts on the growth of the isolated marine-derived fungi. The growth was enhanced when the concentration was increased to 9% at 27 °C. In actuality, as long ago in 1957 and 1959 Ritchie had found a fact that the growth of various tropical marine isolates can be faster at higher salinities as long as higher temperatures are maintained **[44, 45]**. Extending this evidence to some strains, similar pattern was observed in *Aspergillus nidulans, Curvularia sp., Dendryphiella salina, Penicillium chrysogenum, P. citrinum, P. corylophilum P. dravuni, Pestalotia sp., Phoma sp.* **[46-49]**.

Most fungi disposed to be more halotolerant than halophilic and do not require salt concentrations for their viability **[50]**. The microbial growth in highly saline environments requires numerous adaptations more specifically at cell wall level since it protects them against mechanical

damages, as well as from high concentrations of salts by maintaining osmotic homeostasis of cells **[51-54].** The influence of high salinities on biomass growth of *Talaromyces albobiverticillius* 30548 is depicted in figure 3. Most studies revealed that fungi cultured in highly saline medium undergo decreased growth when compared to control. The salinity tolerance level in terms of NaCl concentrations towards growth was discussed for *Aspergillus spp.*(4%), *Cladosporium* (4%), *Pestalotiopsis* (4%) [35]; *Penicillium sp* Ty01b-8 (3-6%) [32]; *Microporus xanthopus* (3.5%), *Pycnoporus sanguineus* (6%), *Schizophyllum commune* (7%) **[55]** and furthermore growth was suppressed if the concentration increases above the mentioned tolerance level. Under high saline conditions (6% & 9%) in the culture media, the marine isolate *T. albobiverticillius* 30548 exhibited higher dry biomass weight indicating this species is well adapted to salt stress.

Our results on salinity effects towards biomass growth showed stimulatory effects whereas the production of pigments was inhibited with increasing salt concentrations. Present findings were in agreement with Chintapenta *et al.* (2014), who noticed a decreased production of red pigments in *Penicillium sp* when the salt concentration was increased to 2% (w/v) in the culture media. Additionally, it was reported most of the pigments were adhered to the mycelia instead soluble into the media in the increased salt concentration from 8 to 10% NaCl **[56]**. However, in our study there was diffusion of red pigments but the concentration of excreted pigments was low partly due to the change in pH level (data not shown). Indeed, the electrolytes present in the salt might have altered the osmolarity and also the pH. The occurrence of color change may be due to the production of other molecules or modifications of the parent compound in the media of increasing salt concentrations. Also, it could be explained that the increasing salt concentration might have altered the pH of the media and prevented diffusion of pigments or modified the metabolism. On the contrary, pigments were diffused into the media without containing any salts (0%) and hence the water soluble pigments gave a dark red hue compared to highly saline medium **[57].** However, in highly saline medium, if pigments do not diffuse into the culture media, it might have been adhered to biomass either didn't produce pigments at all.

Further study of this strain exposing to different pH ranges in saline medium as well as evaluation of intracellular pigments content may provide insight in the physiological behaviour and fungal growth. As a contrary, Babitha et al (2007) proposed significantly larger amount of red pigments production in high salinity at 10% NaCl on *Monascus purpureus* LPB97 cultured on solid state fermentation for 12 days period **[41]**. Besides, in *Talaromyces verruculosus*, the pigment production was hampered due to stress induced by NaCl that influences optical density measurement based on dose dependent manner **[58]**. In an opinion, salt stress highly enhanced

primary metabolism covering increased biomass growth but showed little influence on secondary metabolism in terms of pigment production with regards to quantity[59]. Fungal pigments are special natural products considered as secondary metabolites which are usually produced after the active growth of the organism. In this experiment, the pigments started to produce in the middle of the log phase and reached maximum at the end of log phase, which is usual for secondary metabolites [8].

Obvious color diversity of extracellular pigments in the culture filtrates of *T. albobiverticillius* 30548 was located through color analysis using spectrocolorimetric system. Alignments of hue values ranged from 23.07 to 77.04 exhibited the shades from pink to yellow depending on salt levels in the medium. Similarly for *P. chrysogenum* IFL₂ hue angles of all of the fungal extracts ranged from 1.57 to 90.41. Thereby, indicated the color variation from pink to red to orange and yellow to light green-yellow obtained in PDB [60]. To our knowledge, this is the first report to deal with the color characteristics of red pigments produced from marine-derived *T. albobiverticillius* cultured in sea salts enriched medium.

To sum up, marine-derived fungi seem quite distinct from terrestrial that discrete a specialized ecological niche and also possess differences in some aspects of metabolism [61, 62]. Among the different salinity levels, pigment production and biomass are quite similar in evolution up to 3.5%, there after exhibited an unequal relationship in 6% and 9% sea salts added media. Ultimately, the resistance to salinity is partially controlled by several genes, which have been found in various marine-derived fungi. Abiotic stress affects the expression of several genes in cellular mechanism that could raise the genes to be up- or down regulated [63, 64]. By studying the gene behaviour, it may shed light on underlying mechanisms of salt tolerance.

4. Materials and Methods

4.1 Microorganism and preparation of seed media

The studied fungus was isolated from sediment source in the external slope of the site Trou d'Eau (22°5'23.99" S, 55°14'7.03" E) around Réunion Island (Indian Ocean, France). This fungal isolate is identified as *Talaromyces albobiverticillius* through genomic sequencing (CBS knaw, The Netherlands) and stored in the laboratory collection of LCSNSA (Laboratoire de Chimie des Substances Naturelles et des Sciences des Aliments, Université de La Réunion) under the reference code 30548 at -80 °C for long term storage. The culture was also maintained on PDA slants at 4 °C and sub cultured at regular intervals.

Seed media was prepared by taking 80 mg of mycelia from Petri Plates of 7 days old culture grown on PDA in a sterile eppendorf tube and vortexed with 1 ml of distilled water. Then, it was transferred into 250 ml Erlenmeyer flask containing a volume of 80 ml sterile potato dextrose broth (Difco Ref.254920) and incubated at 24 °C, under 200 rpm agitation for 2 days (Infors Multitron Pro).

4.2   Preparation of culture media and submerged fermentation

The culture media was prepared using PDB supplemented with artificial sea salts (Sigma Sea Salts S-9883). The percentage of salinity was varied at four different levels (0%, 3.65%, 6% and 9%). A volume of 80 mL media was dispensed into 250 mL Erlenmeyer flasks and the flasks were autoclaved at 121 °C for 15 min and then left to cool at room temperature. The initial pH was adjusted to 4.0 using 2M HCl solution prior to sterilisation in the autoclave. Indeed, earlier laboratory experiments demonstrated that pH 4.0 seems to be favourable for the fungal growth and pigment production in *T. albobiverticillius* 30548. Moreover, some species of *Penicillium* and *Talaromyces* reported in literature studies produced highest amount of red pigments at acidic levels of pH [65].

To study the relationship between the temperature and fungal growth as well as pigment production, flask culture experiments were initially performed at three different temperatures (21, 24 & 27 °C) using sterile PDB without the addition of sea salts (0%). For inoculation, the homogenous 2 days old liquid pre-culture was allowed to centrifuge at 8000 rpm for 6 min (Centrifuge SIGMA 3K 3OH and 19776-H rotor). The supernatant was discarded and 80 mg of mycelia was added to 1 mL sterile distilled water in sterile Eppendorf tubes and vortexed. Further, the content was inoculated to each flask under sterile conditions and incubated at 21, 24 & 27 °C for 10 days with an agitation of 200 rpm. After temperature optimization, fermentation using different sea salt concentrations was performed at 27 °C.

4.3   Monitoring methods

To monitor the fungal growth and pigment production throughout the entire fermentation period, 5 ml of fermented broth from each flask was sampled once every 24 hours. The pH was measured and recorded for each flask using a pH meter (pH 1500, Eutech Instruments, with a Bioblock Scientific probe). The samples were then filtered using labelled nylon cloth of pore size 48 μm (Nitex, Sefar) to separate the biomass and supernatant.

4.4   Determination of dry biomass

The amount of wet biomass obtained after filtration was noted using a precise analytical weighing balance (Adventurer Pro AS214, d=0.0001g). The wet filters were dried in a hot air oven

(Memmert SNB 100, Germany) at 105 °C for 17 hours and weighed after keeping in desiccator for 30 minutes to get it at room temperature **[66].**

The dry biomass weight was calculated using the following formula and presented in grams per litre.

$$Dry\ biomass: \frac{(W - W_f)}{Vs} \quad (g/L) \quad\quad\quad (1)$$

W = Weight of the filter with biomass obtained after drying, in g

W_f = Weight of the empty filter, in g

V_s = Volume of the sample, in L

4.5 Estimation of pigment absorbance and standard curve

The absorbance measurement of pigments in the extracellular culture filtrate was carried out using UV-1800 Spectrophotometer (Shimadzu). The colored culture filtrates were scanned at 230-700 nm for maximum wavelength absorbance the pigments. Absorbance of extracellular pigments from fungal liquid culture was read at 500 nm, which represent a widespread maximum for orange and red pigments respectively **[67]**. The pigment yield was expressed in terms of g/L Red rice equivalents. This was done by extrapolating the absorbance vs. concentration (g/L) calibration curve of commercially available red yeast rice (Wuhan Jiacheng Biotechnology Co., Ltd).

Red yeast rice is a product obtained by fermentation using *Monascus purpureus* and considered as one of the food supplements by having active main constituents **[68].** It was used as a reference standard, since the pigments produced by *T. albobiverticillius* 30548 was found to be *Monascus-* like pigments, reported by HPLC-MS and NMR studies **[69].**

The concentration of the pigments were calculated using the following formula

$$C = \frac{Abs_{500} - 0.0097}{1.6925} \quad\quad\quad (2)$$

Where,

C = Concentration of pigment in g/L

Abs_{500} = Absorbance at 500 nm wavelength

The gradient (0.0097) and intercept (1.6925) represent the values obtained from the standard curve of *Monascus* red yeast rice.

4.6 Color analysis of pigments

The extracts used for absorption scanning were again used to determine CIELAB color coordinates. The color measurements were performed using Spectrocolorimeter (Minolta CM-3500d Spectrocolorimeter) and the values of L*, a*, b*, c and h° were obtained automatically with the help of SpectraMagic™NX which is a color data software (Minolta Co., Osaka, Japan, ver 1.9). Color measurements were performed on an alternate days with a 30 mm filter (Konica Minolta, Osaka, Japan). The standard illuminant D65 was used throughout the colorimetric measurements

intended to represent average daylight. The colorimetric system defines the characters, L for brightness ranging from 0 (black) to 100 (white), where a* represents the change from green (negative values) to red (positive values); b* represents the change from blue (negative values) to yellow (positive values). Chroma is the color strength of the object, denoted by C and hue is the saturation or purity of a color, represented as h°. Values close to the centre at the same L* values express dull or grey color, though values near circumference indicates bright or vivid colors [70]. Chroma and hue angle are calculated from the a* and b* coordinates in L*a*b*.

$$\text{Chroma C*} = \sqrt{(a^*)^2 + (b^*)^2}$$

$$\text{Hue angle h°} = \tan^{-1}\left(\frac{b^*}{a^*}\right)$$

4.7   Quality control

Random samples were taken from several flasks during the early fermentation period to examine the phenotypic characters under a light microscope as well as to check the purity of the culture (Olympus CX41, Japan).

4.8   Statistical analysis

All the experiments were performed in triplicates to calculate the means and standard deviations. The statistical functions and the corresponding descriptive graphs were created using Sigmaplot software version 11 (Systat Software Inc., USA). To determine the adequacy of fit data and its significance, one-way analysis of variance (ANOVA) was performed to compare the mean values of individual variable between the four conditions followed throughout the experiment at 95% significance level. For all the treatments, the exponential growth phase was modelised through a linear model and the slope was calculated to determine the fungal growth rate.

5. Conclusions

In conclusion, behavior of the marine-derived strain *Talaromyces albobiverticillius* 30548 to various levels of sea salts (0-9%) was studied in order to understand its impact on fungal growth and pigment production. Through this investigation, the isolated marine-derived fungus *T. albobiverticillius* 30548 appeared to be a halo- tolerant by producing higher biomass production at 9% salinity. Subsequently, the pigment production was not effective at higher percentage of saline media (0.56 g/L in terms of red rice equivalents at 9%) instead high level was achieved in control media (1.47 g/L in terms of red rice equivalents at 0%). This finding revealed that broth medium with 6% salinity (T3) presents a quick adaptation to salinity with high biomass production compared to other treatments but treatment T4 gave maximum dry biomass weight on day 9 (9.22 g/L). On the contrary, media without added salts (T1, control media) was the best for maximum pigment production compared to all degrees of salinity used in the experiments.

Further studies on pigment characterization and isolation of pigmented compounds will be needed in order to fully exploit the variation in pigment production by the same fungal isolate to varying salinity concentrations. In this current era towards seeking the microbial sources for natural pigments, extremophilic behaviour of halophilic fungi makes them particularly interesting candidates for biotechnological applications in a very efficient manner of producing different compounds by resting on its physical conditions. Certainly, the total findings may be useful for designing selective experiments to further understand the pathways associated with differences in metabolite formation with salt stress.

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5.4   CONCLUSION

This chapter covers the effects of sea salts on the pigment production of marine-derived fungus Talaromyces albobiverticillius 30548. From this work, it was understood that Secondary metabolite production and its concentration varies depending on the salt concentration. In this fungus, when the concentration of sea salts increased more than 3.65% (w/v), the pigment production was less in amounts. Besides, there was increased biomass growth in high salt concentration (6%) when compared to the control without added any salts. Marine derived fungi which are halotolerant may evolve unique metabolic mechanisms that are responsive to salt concentrations. For fungi to grow in the marine environment, they must have osmoregulatory mechanisms that signal the production of polyols and amino compounds of polyols and amino compounds in conjunction with increasing the concentration of cytoplasmic ions [1]. Since the biosynthesis of these solutes for osmoregulation is energetically costly, fungi may exhibit decreased secondary metabolite production or slower rates of metabolite production in the presence of high salt concentrations. These findings suggest that marine derived *Talaromyces albobiverticillius* 30548 could be sensitive in the media containing seawater salt concentrations and thus have implications on the production of pigments.

CHAPTER 6

DETECTION AND PARTIAL CHARACTERIZATION OF PIGMENTS PRODUCED BY T*alaromyces albobiverticillius* USING HPLC AND NMR TECHNIQUES

6.1   BACKGROUND

There is no surprise that micro fungi are a rich source of chemical diversity and together with the actinomycetes, serves as a source of 50% of metabolites used in the pharmaceutical industry either in the native or derived form [1-3].Though, most fungi produce several unknown metabolites and fungi are the objective in numerous high-throughput screening (HTS) programs targeting new pharmaceutics and other bioactive components [4-6]. Since isolation and characterization of "new" candidates are very time consuming and costly [4] it is important to develop an early and quick dereplication approach to eliminate already known components [7]. The diversity of chemical compounds is very high with the micro- fungi and almost all types of chemical structure including small acids, alcohols, ketones, alkaloids, anthraquinones and cyclic peptides can be expected in the extracts. To cope with this broad range of chemical structures, most methods are based on reverse- phase liquid chromatography combined with diode array detection (DAD) [8, 9] and atmospheric pressure ionization/ electrospray ionisation (ESI) mass spectrometry (MS).

Generally, RP-HPLC with water–acetonitrile gradient elution has been preferred over isocratic for full pigment separation. Empirical correlations from ^{1}H, ^{13}C nuclear magnetic resonance (NMR) aid in the interpretation of molecular spectra for the elucidation and confirmation of the structure of pure pigment fractions.

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Partial characterization of the pigments produced by the marine-derived fungus *Talaromyces albobiverticillius* 30548. Towards a new fungal red colorant for the food industry

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1 **Partial characterization of the pigments produced**

2 **by the marine-derived fungus *Talaromyces***

3 ***albobiverticillius* 30548. Towards a new fungal red**

4 **colorant for the food industry.**

5

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21

22   **ABSTRACT**

23

24   The interest about red color in the food industry has been growing because of its wide

25   application in variety of foods and beverages and also due to the carcinogenic and teratogenic

26   effects of some synthetic colorants. Many ascomycetous fungi naturally synthesize and

27   secrete pigments and thus provide readily available additional and/or alternative sources of

28   natural colorants that are independent of agro-climatic conditions. Some species of

29   *Talaromyces* produce large amounts of *Monascus*-like azaphilone red pigments without any

30   toxins. In this study, *Talaromyces albobiverticillius* 30548 was isolated from the outer slope

31   of the coral reef of the Reunion Island, Indian Ocean. The biosynthesized intracellular and

32   extracellular pigments were extracted by successive cold extractions or by single solvent

33   extraction methods. The pigments were then analyzed by HPLC-PDA-ESI/MS system in

34   positive and negative ionization modes. Twelve different compounds were detected and four

35   were tentatively identified as *Monascus*-type pigments, based on the results obtained and the

36   available   literature.   In   particular,   N-threonine-monascorubramine,   N-glutaryl-

37   rubropunctamine and PP-O were tentatively identified; further, this work reports for the first

38   time on the PDA, MS and NMR characterization of the here named as N-GABA-

39   monascorubramine   derivative   (6-[(*Z*)-2-Carboxyvinyl]-N-GABA-monascorubramine)

40   pigment bearing a *cis* configuration at the C10-C11 double bond, in *Talaromyces*

41   *albobiverticillius* 30548.

42

43   Keywords: Filamentous fungus; *Talaromyces albobiverticillius*; Marine – derived food

44   colorant; Red colored pigments; Azaphilone; HPLC-MS; NMR

45

46

47

1. Introduction

49

50   In the present world, there is growing interest for the use of natural colors mainly from the

51   consumers due to the harmful concerns associated with synthetic dyes and pigments.

52   Currently, natural pigments are derived from various sources, mainly from plants and

53   microalgae and have applications in many foods and beverages. However, they have several

54   drawbacks like instability, seasonal availability and high cost when considering the industrial

55   application (Dufossé et al., 2005; Gunasekaran and Poorniammal, 2008; Jiang et al., 2005).

56   Aside from these sources, microorganisms provide an alternative to synthetic pigments as

57   they are able to grow in different culture systems (Campoy et al., 2003; Yan et al., 2005), are

58   independent of climatic conditions and supply of agricultural raw materials (Mapari et al.,

59   2006). Also, some of the pigments produced by microbes possess a high stability towards

60   light, heat and pH. (Joshi et al., 2003; Malik et al., 2012). With these advantages, special

61   attention has been focused on filamentous fungi which are the potential producers of

62   numerous shades of pigments ranging from yellow, red, reddish brown, bronze and maroon

63   (Caro et al., 2012). In fungi, these pigments have been thought to serve different ecological

64   functions, for example, melanins protect them against environmental stress, carotenoids

65   against lethal photo-oxidations, and flavins act as cofactors in enzyme catalysis (Firn and

66   Jones, 2003; Spiteller, 2015).

67   Fungal colorants can be chemically classified as carotenoids, melanins, polyketides, etc. in

68   which the polyketides constitute the most representative class of pigments. Current industrial

69   fungal productions are running at multi metric tons level with yellow-orange-red food-

70   colorants β-carotene and lycopene, biosynthesized by *Blakeslea trispora* (Finkelstein et al.,

71   1995; López-Nieto et al., 2004; Xu et al., 2007). Polyketide based pigments are structurally

72 complex and involve pathways catalyzed by the enzymes polyketide synthases. The main
73 classes of polyketide pigments include anthraquinones, hydroxyanthraquinones,
74 naphthoquinones, and azaphilone structures, each of which exhibits an array of color hues
75 (Mapari et al., 2010). Since ancient times, azaphilone pigments produced by *Monascus sp*
76 have widely been used in the oriental countries (particularly Japan and China) to color rice
77 wine, koji, soyabean, cheese and meat. However, the use of *Monascus* pigments as food
78 colorants is still forbidden in European countries owing to the time-to-time production of the
79 mycotoxin citrinin (Liu et al., 2005) and also the production of the unwanted cholesterol-
80 lowering drug mevinolin when added to foods (Patakova, 2013). Some species of *Aspergillus*
81 *sp (A. glaucus, A. cristatus,* and *A. repens)* produce hydroxyanthraquinoid (HAQN) pigments
82 like emodin (yellow), physcion (yellow), questin (yellow to orange-brown), erythroglaucin
83 (red), catenarin (red), and rubrocristin (red) along with several mycotoxins such as secalonic
84 acid, oxaline, citrinin, tanzawaic acid A, cyclochlorotine, islanditoxin, luteoskyrin,
85 erythroskyrin, rugulosin or aspergiolide A. Many of these mycotoxins are pigmented and
86 show substitution on both aromatic rings which arise biosynthetically by the polyketide
87 pathway (Caro et al., 2012; Goyal et al., 2016).

88 During the world's scientific quest to identify potential non-toxic pigment producers for
89 industrial application, several species of fungi have been evaluated and identified belonging
90 to the genus *Paecilomyces, Cordyceps, Penicillium, Aspergillus, Epicoccum, Fusarium* (Cho
91 et al., 2002; Pradeep et al., 2013; Suhr et al., 2002; Unagul et al., 2005). On the other side,
92 several non-pathogenic to humans *Talaromyces sp* producing azaphilone series of yellow and
93 red pigments without the production of mycotoxin seem to be an alternative to *Monascus* red
94 pigments (Frisvad et al., 2013). Azaphilones are interesting set of fungal secondary
95 metabolites namely pigments with pyrone – quinone structures containing a highly
96 oxygenated bicyclic core and a chiral quaternary center (Osmanova et al., 2010). Studies have

323

97 shown that some *Talaromyces sp* such as *Talaromyces aculeatus, T. pinophilus, T.*

98 *purpurogenus, T. funiculosus, T. amestolkiae, T. ruber* and *T. stolii* naturally produce

99 polyketide azaphilone *Monascus* red pigments and their amino acid derivatives (Mapari et al.,

100 2008; Mapari et al., 2009b). But, the later three species do not diffuse pigments into the

101 culture medium and also *T. purpurogenus* produces mycotoxins such as rubratoxins A and B,

102 rugulovasins and luteoskyrin which limits the biotechnological production of pigments by

103 using this species (Yilmaz et al., 2012). Such compounds, for example, rubratoxin was

104 produced in a high concentration in a rhubarb-wine contaminated with *T. purpurogenus* and

105 induced an immediate liver transplant when consumed by a teenager (Richer et al., 1997;

106 Sigler et al., 1996).

107 Some other species, specifically *T. atroroseus, T. albobiverticillius, T. minioluteus,* and *T.*

108 *marneffei* produce diffusing strong red pigments and some yellow pigments. One potential

109 pigment producer among them, namely *T. albobiverticillius* collected from different sources

110 produces several purple-red-orange azaphilone pigments such as monascorubramine (red),

111 monascorubrin (orange), rubropunctatin (orange), PP-R (purple-red) (Mapari et al., 2005;

112 Ogihara et al., 2001; Ogihara et al., 2000) and a series of yellow-orange pigments such as

113 monascin (yellow), mitorubrin (orange-yellow), mitorubrinic acid (yellow) or mitorubrinol

114 (yellow) (Frisvad et al., 2013). (see structures in Table 3)

115 The current study describes the pigment production from the marine derived fungus

116 *Talaromyces albobiverticillius* strain 30548 isolated from the outer slope of the Réunion

117 island coral reef (Indian Ocean) and the characterization of those pigments by high-

118 performance liquid chromatography-diode array detection-electrospray ionization mass

119 spectrometry (HPLC-PDA-ESI/MS), followed by the isolation of major compound(s) and the

120 structure elucidation of a novel red azaphilone using NMR analysis.

121

2. Material and Methods

123

2.1. Isolation of fungal strain and identification

125

The fungus used in this study was sampled from the outer slope of the Réunion island coral-reef (Indian Ocean). After sampling, 5 g of sediment was crushed and cultured on Potato Dextrose Agar (PDA) using serial dilution method. During the period of incubation, several isolates producing colored metabolites were observed visually. The red pigment producing strain was isolated, purified by monospore culture technique and stored at -80 °C for long term preservation. To study its pigment production ability, the fungus was grown on potato dextrose agar (PDA) (Samson et al., 2010) (Fig. 1).

133

Insert Fig. 1.

135

The fungal strain was genetically identified as *Talaromyces albobiverticillius* using gene sequencing at molecular level (30548 indicates the Université de La Réunion collection reference number of the newly isolated strain) (Domsch, 1980; Foster et al., 2011).

139

2.2. Submerged fermentation of fungal strain

For submerged fermentation, potato dextrose broth (PDB) was used as a culture medium and prepared using sterile distilled water. The pH of the culture medium was adjusted to 5.5 ± 0.2 using 0.1M HCl prior to sterilization at 121 °C for 15 minutes. Pre culture was prepared by taking a loop of fungus from 7-day old culture grown on PDA Petri plates and transferred into 60 mL sterilized culture medium. The flasks were incubated at 24°C for 72 hours.

146   Cultivations were then carried out in 250 mL Erlenmeyer flasks containing volume of 100

147   mL sterilized culture medium. The flasks were inoculated with 1% (w/v) 72-h-old pre culture

148   and incubated at 24 °C for 8 days with the agitation of 150 rpm using rotary agitator (Infors

149   Multitron HT).

150

151   *2.3. Separation and extraction of fungal pigments*

152

153   After 8 days of fermentation, the culture broth and fungal biomass were separated by

154   centrifugation at 8000 rpm for 6 min (Centrifuge Sigma 3K 3OH and 19776-H rotor). Both

155   samples were immediately frozen (-80 °C) and then lyophilized into a fine powder (Cryotec

156   cosmos, France). The pigments from both the biomass and the culture filtrate were extracted

157   at room temperature successively with solvents of increasing polarity: n- hexane, chloroform,

158   ethyl acetate and ethanol. The crude extracts were dried using rotary evaporator (Büchi,

159   Germany) at 30 °C under reduced pressure yielding red colored dried residues. The residues

160   were then dissolved in 1 mL of methanol (1:1 v/v), filtered through Minisart® syringe filter

161   of 0.20 µm pore size housing with PTFE membrane (Sartorius) . The crude filtrates were

162   stored at 4 °C in an amber vial prior to HPLC analysis.

163

164   *2.4. HPLC-DAD-ESI-MS analysis*

165

166   The analyses were carried out on a Shimadzu Prominence LC-20A system (Shimadzu, Milan,

167   Italy) equipped with a CBM-20A controller, two LC-20AD pumps, a DGU-20A$_3$ degaser, a

168   SIL-20AC autosampler and a SPD-M20A photo diode array detector. The data were

169   processed with the software Shimadzu Labsolution ver. 5.53. For MS analyses a mass

170   spectrometer was used (LCMS-2020, Shimadzu), equipped with an ESI interface, both in

171   positive and negative ionization modes. HPLC separations were performed on a C18 Kinetex

172   (Phenomenex) column (100 x 2.1 mm-1.7 μm); the mobile phases consisted of water (0.1 %

173   formic acid; eluent A) and acetonitrile, (0.1 % formic acid; eluent B), using a gradient

174   program as follows: 0 min, 5% B; 15 min, 95% B; 17 min, 95% B; 18 min, 5% B. The flow

175   rate was 0.2 mL/min and the injection volume was 1 μL. The column oven temperature was

176   30°C. The UV-Vis spectra were acquired in the range of 200-600 nm, while the

177   chromatograms were extracted at 470 nm and 360 nm (sampling frequency: 1,5625 Hz; time

178   constant: 0.64 s). The MS was set as follows: Scan, both ESI positive (+) and negative (-);

179   nebulizing gas flow (N$_2$): 1.5 L/min; Event Time: 0.3 sec; Detector Voltage: 4.5 kV; m/z

180   range: 60-600; Interface Voltage: ± 3.5 kV; Interface Temperature: 350 °C; DL Temperature:

181   250 °C; Heat Block: 400 °C.

182

183   *2.5. Nuclear Magnetic Resonance (NMR) spectroscopy*

184

185   Compound n. 4 in Fig. 2, was collected after LC separation in an almost pure form. The

186   eluent was evaporated and the red residue (around 0.6 mg) was dissolved in 500 μL of

187   CD$_3$OD and analysed by NMR; afterward the solution was freeze dried and dissolved again

188   in 500 μL of CD$_3$COCD$_3$. This strategy was applied in order to rule out possible solvent

189   effects and/or artefacts. ^{1}H and ^{13}C{^{1}H} NMR spectra of compound n. 4 were recorded on an

190   Agilent Propulse 500MHz spectrometer equipped with a OneNMR probe and operating at

191   499.74 and 125.73 MHz respectively. The sample in a 5 mm test-tube was analysed after

192   locking on the deuterated lock signal, search for a good field homogeneity (shimming) and

193   frequency modulation (tuning). The saturation 90° pulse was calculated to be 8us at 59 dB of

194   power level and the protonic spectrum was obtained with 2 s of acquisition time, 2 s of scan

195   delay and 16 scans; all the other techniques were designed starting from this simple

196   experiment. The complete and unambiguous assignment (Table 2 with the numbering scheme

197   in Fig. 4), was confirmed by homo nuclear 2D-COSY, TOCSY and ROESY (Derome, 2013),

198   and heteronuclear (Willker et al., 1993) $^{13}C\{^1H\}$-HSQC and ^{13}C-HMBC experiments.

199   Calibration was attained using as internal standard residual proton signal of the solvent

200   (CD_2HOD quintet: $\delta = 3.31$ ppm; CD_3COCD_2H quintet $\delta = 2.05$ ppm and the ^{13}C solvent

201   septuplets at $\delta = 49.0$ ppm and $\delta = 29.84$ respectively) (Gottlieb et al., 1997) and data were

202   processed by vNMRj software and by the PC software package ACD/Lab, which was also

203   exploited to validate the goodness of the structure elucidation.

204

205   **3. Results**

206

207   *3.1. Behavior of fungal pigments during extraction(s)*

208

209   An ideal solvent for fungal pigment extraction must have low toxicity, and must be able to

210   solubilize a range of target pigment molecules (Robinson et al., 2014). Initial extraction trials

211   were conducted with the commonly used solvents from low to high polarity such as n-

212   hexane, chloroform, ethyl acetate and ethanol successively which yielded differences in

213 amounts of extracted pigments for both biomass and culture filtrate. Simultaneously,

214 extraction was carried out using ethyl acetate and ethanol as single solvent extraction. On the

215 basis of liquid chromatography-diode array detector (LC-DAD) chromatogram, among the

216 used solvents, ethyl acetate as single solvent was found to be the best solvent for extraction of

217 major pigmented compounds followed by ethanol. Indeed, *Monascus*-like polyketide

218 pigments are hydrophilic in nature, slightly polar and so they are easily handled with polar

219 solvents (Padmavathi and Prabhudessai, 2013). In non-polar solvents like n- hexane and

220 chloroform, the extraction and recovery of pigments was very low and chloroform yielded

221 two compounds (peaks 1 and 2) which were unpigmented. The yield from these solvents was

222 very poor compared to ethyl acetate which yielded 12 different compounds and among them

223 10 compounds were pigmented (figure 2).

224

225 *3.2. Characterization of fungal pigments using HPLC-DAD-ESI-MS*

226 Fig. 2 shows a typical representative chromatogram of the detected pigments (compounds n.s

227 3-12) in *Talaromyces albobiverticillius* 30548 obtained from the EtOAc pigment extract and

228 detected at the wavelength of 470 nm; in the same Fig. 2, it is also shown an insert

229 representing the better detection for compound n.1 and n.2 obtained from the $CHCl_3$ extract

230 and recorded at the wavelength of 360 nm. Table 1 presents all of the detected compounds,

231 their corresponding retention times, PDA and MS data, with a relative tentative identification

232 based on the obtained spectroscopic data and the comparison with literature data. Together

233 with PDA, an on line MS detector operating in both ESI positive and ESI negative ionization

234 mode was used in order to have a double confirmation of the mass values.

235 Twelve different compounds were detected and four were tentatively identified as *Monascus*-

236 type pigments (Table 1). The identified compounds 3, 5 and 8 are similar to the already

237 known N-threonine-monascorubramine, N-glutaryl-rubropunctamine and PP-O respectively.

238 Further, one compound was also characterized by NMR analysis and a new structure for this

239 molecule, here named as 6-[(Z)-2-Carboxyvinyl]-N-GABA-monascorubramine, (or as N-

240 GABA- monascorubramine-derivative), is provided for the first time based on PDA, MS and

241 NMR data (see sections 2.5 and 3.3 of this paper).

242

243 insert Fig. 2.

244

245 insert Table 1

246

247 Compound 3: Under the assumption that the α-amino acid threonine was incorporated into

248 pigment, compound n. 3 was tentatively identified as N-threonine-monascorubramine; the

249 corresponding $[M + H]^+$ m/z 484, and $[M - H]^-$ m/z 482, pseudomolecular ions mass values

250 were consistent with the values reported by Jung et al. 2003 (Jung et al., 2003) and the

251 corresponding PDA data were also consistent with the reported values for *Monascus* type

252 pigments (Mapari et al., 2008).

253 Compound 4: Interestingly compound n.4 was here identified as a never previously reported

254 compound. Under the assumption that the γ-amino acid, γ-aminobutyric acid was

255 incorporated into pigment, the name of 6-[(Z)-2-Carboxyvinyl]-N-GABA-monascorubramine

256 or simply as N -GABA-monascorubramine derivative was proposed for compound n.4; the

257 structure was determined on the basis of the obtained PDA and MS data and of a detailed

258 NMR investigation (see sections 2.5 and 3.3 of this paper). The PDA data are in agreement

259   with the reported values for *Monascus* type pigments (Mapari et al., 2008) and the compound

260   showed corresponding $[M + H]^+$ *m/z* 498, and $[M - H]^-$ *m/z* 496, pseudomolecular ions (see

261   Fig. 3) which are consistent with the proposed structure (Fig. 4) for a compound having a

262   molecular formula of $C_{27}H_{31}NO_8$, with a mass value of 497 amu.

263

264   insert Fig. 3.

265

266   The PubChem database (PubChem CID : 44715338) reports the existence of a compound

267   named as *4-{6-[(E)-2-Carboxyvinyl]-9a-methyl-3-octanoyl-2,9-dioxo-9,9a-dihydrofuro[3,2-*

268   *g]isoquinolin-7(2H)-yl}butanoic acid* with a *trans* configuration at the C10-C11 double bond,

269   but no information was available/ reported on the source or in any literatures for this

270   compound. Therefore, this work reports for the first time on the characterization of the

271   pigment N-GABA-monascorubramine derivative bearing a *cis* configuration at the C10-C11

272   double bond, in the investigated *Talaromyces albobiverticillius* species.

273

274   insert Fig. 4.

275

276   Compound 5: Compound n.5 was tentatively identified as N-glutaryl-rubropunctamine; it

277   showed the $[M + H]^+$ *m/z* 484 and $[M - H]^-$ *m/z* 482 pseudomolecular ions, and UV-vis

278   absorbance values in agreement with the literature reported values (Mapari et al., 2009a).

279 Compound 8: Compound n.8 was tentatively identified as PP-O; it showed the corresponding

280 $[M + H]^+$ m/z 413 and $[M - H]^-$ m/z 411 pseudomolecular ions, and UV-vis absorbance

281 values in agreement with the literature reported values (Mapari et al., 2008; Ogihara and

282 Oishi, 2002).

283

284 *3.3. Characterization of fungal pigments using NMR*

285

286 As explained in the experimental part, HOMO and HETERO nuclear 2D techniques allowed

287 the chemical shift (δ) assignment of the proton resonances and of relative ^{13}C parent

288 resonances specifically defined by the HSQC-DEPT spectroscopy. HMBC spectrum,

289 connecting ^2J, ^3J and few ^4J ^1H-^{13}C resonances (last two columns in Table 2), is consistent

290 with the proposed structure and allowed to assign the quaternary ^{13}C resonances.

291

292 insert Table 2

293

294 Homonuclear TOCSY experiments evidenced the nature of the azaphilone side chains: a) the

295 propyl moiety on the N-7 endocyclic atom; b) aliphatic seven-member peripheral chain

296 bound to the C13 carbonyl group; c) two contiguous unsaturated CH (namely C10 and C11),

297 linked to the C6 endocyclic carbon atom (long-range coupling C10-H5 in HMBC spectrum).

298 Terminal CH_2 (3') and CH (11) of the N7 and C6 side chains were connected to carboxylic

299 acid groups because the HMQC spectrum showed correlations between their proton

300 resonances and those of quaternary carbon atoms at 176.4 ppm (C4') and 171.4 ppm (C12)

301 respectively. Chemical nature of these termini was also confirmed by the mass molecular

302 peak of a compound having a molecular formula of $C_{27}H_{31}NO_8$ (see sections 2.4 and 3.2 of

303 this paper). As these molecules are pretty unusual, some calculated ^{13}C chemical shifts

304 (ACD/Lab calculator) deviate significantly from the experimental value; specifically this is

305 the case of C10, C11 and C4. On the other hand, we are confident about the assignment in

306 Table 2 because the well detected HMBC spots are strongly supported by "through the space"

307 ROESY connections (Fig. 5).

308

309 insert Fig. 5.

310

311 These same ROESY connections, joined to the $^3J_{H10\text{-}H11}$ coupling constant (11.7 Hz), allowed

312 to definitely assess the Z configuration at the unsaturated C10-C11 bond. After all some other

313 similar molecules, called monascorubramines, were thoroughly analysed (Ogihara and Oishi,

314 2002), and specifically the compound called PP-R with the same Z configuration and

315 framework has shown compatible NMR constants (Ogihara et al., 2001) thus providing a

316 very sound support to our overall discussion.

317

318 **4. Discussion**

319

320 *Talaromyces* species, the teleomorph (sexual reproductive) stages of the well-known

321 *Penicillium* fungi, have a long common history with foods and beverages consumed by

322 human beings. As examples, in Europe, *Penicillium camemberti* or *Penicillium roqueforti* are

323 used in cheese production, and many other *Talaromyces/Penicillium* strains are of great

324 importance in Asia, for soy products.

325 From a more global point of view, ingredients derived from microbial fermentation are

326 steadily gaining ground in the food industries. Thickening or gelling agents (e.g.

327 polysaccharides such as xanthan, curdlan, gellan), flavour enhancers (yeast hydrolysate,

328 monosodium glutamate), polyunsaturated fatty acids (PUFAs), flavour compounds (gamma-

329 decalactone, diacetyl, methyl-ketones), vitamins, essential amino acids, and acidulants (lactic

330 acid, citric acid) are illustrating this trend. Efforts have been made and continue to be done in

331 order to reduce the production costs of pigments produced by microbial fermentation, since

332 synthetic pigments or those extracted from natural plant sources can often be produced more

333 economically (Dufossé, 2008). The successful marketing of natural pigments such as β-

334 carotene, lutein, and astaxanthin derived from algae (i.e. non-conventional sources) or

335 extracted from plants (conventional sources), both as food colorants and nutritional

336 supplements, reflects the presence and importance of niche markets in which consumers are

337 willing to pay a premium for 'natural healthy ingredients'. Among other non-conventional

338 sources, filamentous fungi are known to produce an extraordinary range of pigments that

339 include several chemical classes such as carotenoids, melanins, azaphilones, anthraquinones,

340 flavins, phenazines, quinones, and more specifically, violacein and indigo (Caro et al., 2016;

341 Dufossé, 2008; Fouillaud et al., 2016). The success of any class of pigment produced by

342 fermentation depends on its acceptance by the consumers, regulatory approval, and the

343 capital investment required in bringing the product onto the market. Twenty years ago,

344 influential representatives from food industry expressed doubts about the successful

345 commercialization of algae-derived and fermented food grade pigments due to the high

346 investment required for open ponds, photo-bioreactors and fermentation facilities, and the

347 extensive and lengthy toxicity studies required by the regulatory authorities. Poor public

348   perception of fungal-derived products for food use had also to be taken into account.

349   Nowadays, some fungal food grade pigments obtained by fermentation already exist on the

350   market worldwide. Among them, fungal *Monascus* pigments, Arpink red™ (now Natural

351   Red™) produced by *Penicillium oxalicum*, riboflavin from the mold fungus *Ashbya gossypii*,

352   lycopene and β-carotene from the tropical mold *Blakeslea trispora*. As an example, the

353   production yield of β-carotene may be as high as 17g/L of the *Blakeslea trispora* culture

354   medium (Dufossé, 2016).

355   In the Western World (the Occident), pioneering work about large scale production of fungal

356   colorants was done on carotenoids. Academics knew for a long time that fungi belonging to

357   the order Mucorales are able to produce β-carotene. First papers dealing with *Blakeslea*

358   *trispora* carotenoid production were published in the late fifties (Ciegler et al., 1959). It took

359   four decades to move to industrial production, waiting for consumer interest about natural

360   colorants, developing biotechnological techniques, and gaining regulatory approval. For this

361   last aspect, Vitatene, a Spanish company, filled a novel foods and novel food ingredients

362   application in 2003, to place lycopene from *Blakeslea trispora* on the European market

363   (under Regulation EC N° 258/97). The positive answer was published on 23 October 2006

364   (European Commission decision N° 721/2006).

365   Red azaphilone pigments are similarly known for a long-time by scientists from Asia and, as

366   explained in the introduction, researchers are trying to find new strains, non-mycotoxigenic,

367   to use as an alternative to the citrinin-producing *Monascus*.

368   Pioneering work started at Denmark Technical University (DTU) during the PhD thesis of

369   Sameer Mapari, with co-workers such as Ulf Thrane, Anne S. Meyer, Jens C. Frisvad and co-

370   funding from the world-leading natural colors Chr. Hansen A/S company, represented by

371   Annette Salskov-Iversen. Many papers were published between 2005 and 2009 (Mapari et al.,

372 2005; Mapari et al. 2006; Mapari et al. 2008, Mapari et al., 2009), setting a general

373 framework in the development of fungal reds. International patents were issued such as

374 EP2262862 (=WO2009026923, priority date August 28, 2007) or EP2011/064152

375 (=WO2012022765, priority date August 19, 2010). Despite the very nice results obtained in

376 these works, no industrial application of red azaphilone (polyketide) *Monascus*-like pigments

377 appears on the market up to now. A few years later, in 2013, Jens C. Frisvad and co-workers

378 from DTU, the CBS–KNAW Fungal Biodiversity Centre (Utrecht, The Netherlands) and the

379 Department of Biology, Utrecht University (Utrecht, The Netherlands), described a new

380 strain of *Talaromyces*, they named *Talaromyces atroroseus* sp. nov., and recommended as an

381 effective producer of the azaphilone biosynthetic families mitorubrins and *Monascus*-like-

382 pigments without any production of mycotoxins.

383 In our work, we isolated in the tropical marine environment of Réunion island, Indian Ocean,

384 a different red pigment producing strain belonging to *Talaromyces albobiverticillius* and this

385 paper brings new information about the pigments produced. The literature (Frisvad et al.

386 2013) lists ten extrolites (excreted metabolites) in seven collection strains (Table 3):

387 mitorubrin $C_{21}H_{18}O_7$ formula weight 382.36 (6 occurrences among 7 strains, *i.e.* 6/7),

388 mitorubrinic acid $C_{21}H_{16}O_9$ formula weight 412.35 (6/7), monascorubramine $C_{23}H_{27}NO_4$

389 formula weight 381.46 (5/7), rubropunctatin $C_{21}H_{22}O_5$ formula weight 354.39 (4/7),

390 monascorubrin $C_{23}H_{26}O_5$ formula weight 382.45 (2/7), a purpactin (2/7), vermicellin (2/7),

391 PP-R = [(10Z)-7-(2-hydroxyethyl)-monascorubramine] $C_{25}H_{31}NO_5$ formula weight 425.52

392 (1/7), mitorubrinol $C_{21}H_{18}O_8$ formula weight 398.36 (1/7) and monascin $C_{21}H_{26}O_5$ formula

393 weight 358.43 (1/7).

394

395 insert Table 3

397   During the research presented here four out of twelve compounds were identified in the

398   investigated pigmented extract from *Talaromyces albobiverticillius* 30548 using HPLC-PDA-

399   ESI/MS and NMR: N-threonine monascorubramine ($C_{27}H_{33}NO_7$ formula weight 483.55), N-

400   glutaryl rubropunctamine ($C_{26}H_{29}NO_8$ formula weight 483.51), PP-O = ((10Z)-12-

401   carboxylmonascorubrin) ($C_{23}H_{24}O_7$ formula weight 412.43) and a new N-monascorubramine

402   compound, a N-GABA-monascorubramine derivative (6-[(Z)-2-Carboxyvinyl]-N-GABA-

403   monascorubramine) ($C_{27}H_{31}NO_8$ formula weight 497.53), pigment bearing a *cis* configuration

404   at the C10-C11 double bond.

405   This new compound will enlarge the list of 63 *Monascus* and *Monascus*-like pigments (24 O-

406   containing compounds and 39 N-containing compounds) recently summarized by (Gao et al.,

407   2013). Azaphilones are a large group of pyrano-quinones structures with a high electron

408   acceptor tension determining sensitivity of oxygen in the primary ring. This yields γ-

409   pyridones, exhibiting chromophore properties in which colors depend on their chemical

410   structure. Their name comes from their ability to react with ammonia. Thus in the media, they

411   readily interact with compounds containing amino groups such as proteins, amino acids, or

412   nucleic acids resulting in water soluble colored products. In microorganisms such as fungi, L-

413   glutamate is the main precursor of 4- amino-butyrate (GABA). The production of GABA is

414   considered as a shunt of the tricarboxylic acid (TCA) cycle which can lead to the production

415   of succinic semi-aldehyde (GABA bypass) (Kumar and Punekar, 1997). The reaction of

416   GABA with O-containing rubropunctatin precursor is mentioned here in *Talaromyces*

417   *albobiverticillius*, in *Talaromyces* genus, in fungi, for the first time. The specific role of this

418   product in the fungal metabolism has to be clarified. Indeed, the azaphilone skeleton is

419 essential for certain biological activities of these metabolites. The differences observed in

420 their activities can however be ascribed to differences in their reactivity with amines.

421

422 The work will continue with large scale cultivation of *Talaromyces albobiverticillius* 30548

423 in fermenter, analysis of pigmented extracts with liquid chromatography-mass spectrometry

424 ion trap time-of-flight (LCMS-IT-TOF) mass spectrometer (MS) through an atmospheric-

425 pressure chemical ionization (APCI) source, operating in both positive and negative mode,

426 and finally isolation of still unknown compounds for additional NMR.

427

428 **5. Conclusion**

429

430 Research efforts on fungal reds will continue in the next years or decades. It is now proven

431 that some *Talaromyces/Penicillium* species are able to produce pigments with no associated

432 mycotoxin(s) (e.g. *Talaromyces atroroseus*; *T. albobiverticillius* – up to now we were unable

433 to detect any mycotoxin(s) in all our extracts, prepared with various solvents, from

434 biomasses produced in many different media). Feeding rats in order to test toxicity on living

435 animals is one of the next steps, as it was done previously with other pigmented extracts or

436 molecules (Jonker et al., 2003; Sanjay et al., 2007).

437 These *Talaromyces atroroseus*, *T. albobiverticillius* fungal reds will also be challenged by

438 new generations of *Monascus* pigments, biosynthesized by new strains unable to produce the

439 mycotoxin citrinin. The very popular and rapid-evolving technique CRISPR/Cas9 allowing

440 fine targeted genome editing sure opens a new era in molecular biology applied to fungal

441 pigments. More data are soon expected about deletion(s) of polyketide synthase(s) involved

442 in mycotoxin(s) biosynthesis, deletion(s) that should maintain pigment(s) production, such

443 providing safe fungi for the production of food colorants.

444

455

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Table 1

Overall compounds detected by HPLC-PDA-ESI/MS in IC and EC extracts* of *Talaromyces albobiverticillius* 30548, with reference to the chromatogram

shown in Figure 2.

Compound N.	R.t.	PDA λ nm	MS/ ESI	Tentative identification
1	4.89	207, 362	255 [M + H]+	n.i.
2	7.35	223, 390	269 [M + H]+ 267 [M - H]-	n.i.
3	10.04	222,273,422, 511	484 [M + H]+ 482 [M - H]-	N-threonine-monascorubramine
4	10.32	221,273,425, 522	498 [M + H]+ 496 [M - H]-	**N-GABA-monascorubramine derivative (see NMR)**
5	10.92	223, 430, 499	484 [M + H]+ 482 [M - H]-	N-glutaryl-rubropunctamine
6	11.57	225, 409	375 [M + H]+	n.i
7	12.6	222, 280, 461	413 [M + H]+ 411 [M - H]-	n.i.
8	12.8	223,286,458,470	413 [M + H]+ 411 [M - H]-	PP-O
9	13.4	224, 287, 458	459 [M + H]+ 457 [M - H]-	n.i.
10	14.4	225, 421	503 [M + H]+ 501 [M - H]-	n.i.
11	15.2	224, 446	445 [M + H]+ 443 [M - H]-	n.i.
12	15.6	225, 458	459 [M + H]+	n.i.

* the analysed samples were extracted with different solvent systems. In Table 1 are reported the compounds detected in all the different samples

and, in particular, the EtOAc extract was the most representative as shown in Figure 3. Only compounds 1, 2 and 6 were detected mainly in the

CHCl₃ extract.

n.i.=not identified

18 **Table 2**

19 ^{1}H and ^{13}C NMR spectroscopic data in CD$_3$OD for compound n. 4 reported in Fig. 4.

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Position	δ_C (ppm)[a]	type	δ_H (ppm)[b]	H Mult.[c] (J (Hz))	COSY[d]	ROESY[e]	HMBC[f]
C2	173.79	C					
C3	102.75	C					
C3a	174.25	C					H-Me9a
C4	99.02	CH	6.663	s	H8	H5	H5
C4a	153.20	C					H4, H8
C5	121.40	CH	6.939	s		H4	H4, H10
C6	151.18	C					H1', H5, H8
C8	143.49	CH	8.324	s	H4	H1'	H1'
C8a	120.05	C					H4, H5
C9	196.25	C					HMeC9a, H8
C9a	87.13	C					HMeC9a, H4
C-Me9a	30.54	CH$_3$	1.656	s			
C10	126.67	CH	6.712	d (11.7)	H11	H1', H11	H5
C11	137.30	CH	6.475	br d (11.7)	H10	H10	
C12	171.15	C					H11
C13	198.71	C					H14
C14	41.34	CH$_2$	2.798	br t (6.6)	H15	H15	
C15	26.44	CH$_2$	1.584	m (6.1)	H16, H14	H14	H14
C16	30.33	CH$_2$	1.324	om	H14		
C17	30.65	CH$_2$	1.285	om			H15, H14
C18	32.99	CH$_2$	1.283	om			H20, H16
C19	23.75	CH$_2$	1.297	om	H20		H20
C20	14.47	CH$_3$	0.887	br t (7.1)	H19		
C1'	56.00	CH$_2$	4.139	t	H2'	H2', H3', H10, H8	H3', H8
C2'	26.51	CH$_2$	2.081	p (6.8)	H3', H1'	H1'	H3'
C3'	31.48	CH$_2$	2.4	br t (6.6)	H3', H1'	H1'	
C4'	176.37	C					H3'

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22   a) Chemical shift of the given C. b) Chemical shift of the attached Hs for the given C position c)

23   proton signal multiplicity with standard labeling: br = broad, o = overlapped, m = undefined multiplet,

24   d, t, q, p = doublet, triplet, quadruplet and quintet respectively. d) H labels of the detected H-H COSY

25   connection toward the given H resonance; e) H labels of the detected H-H ROESY connection toward

26   the given H resonance; f) H labels of the detected C-H HMBC connection toward the given C

27   resonance.

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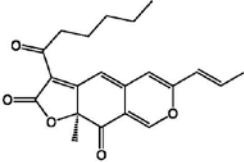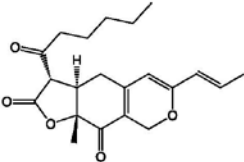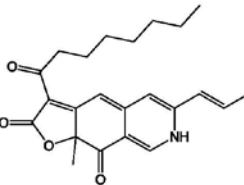
34 Reported structures of pigmented extrolites produced by different collection strains of *Talaromyces albobiverticillius*, mentioned in (Frisvad et

35 al., 2013) and in this study.

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Compound	Chemical structure	Color	Formula	Monoisotopic mass	Average Mass	References
Rubropunctatin		Orange	$C_{23}H_{22}O_5$	354.1467	354.39	(Frisvad et al., 2013)
Monascin		Yellow	$C_{21}H_{26}O_5$	358.1780	358.43	(Frisvad et al., 2013)
Monascorubramine		Red	$C_{23}H_{27}NO_4$	381.1940	381.46	(Frisvad et al., 2013)
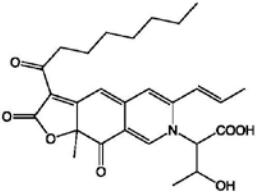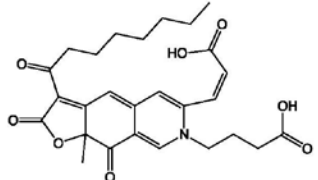
7

Mitorubrin		Orange – yellow	$C_{21}H_{18}O_7$	382.1053	382.36	(Frisvad et al., 2013)
Monascorubrin		Orange	$C_{23}H_{26}O_5$	382.1780	382.45	(Frisvad et al., 2013)
Mitorubrinol		Yellow	$C_{21}H_{18}O_8$	398.1002	398.36	(Frisvad et al., 2013)
Mitorubrinic acid		Yellow	$C_{21}H_{16}O_9$	412.0794	412.35	(Frisvad et al., 2013)

PP-O		Red orange	$C_{23}H_{24}O_7$	412.1522	412.43	This study
PP-R [(10Z)-7-(2-hydroxyethyl)-monascorubramine		Purple red	$C_{25}H_{31}NO_5$	425.2202	425.52	(Frisvad et al., 2013)
N-glutaryl rubropunctamine		Red	$C_{26}H_{29}NO_8$	483.1893	483.51	This study

N-threonine monascorubramine		Purple red	$C_{27}H_{33}NO_7$	483.2265	483.55	This study
6-[(Z)-2-Carboxyvinyl]-N-GABA-monascorubramine		Red	$C_{27}H_{31}NO_8$	497.2050	497.53694	This study

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5   **Fig. 1.** Morphological features of *Talaromyces albobiverticillius* 30548: (a) Obverse face of

6   fungus grown on Potato Dextrose Agar (PDA) media, (b) Reverse face, (c) Red pigment

7   production in Potato Dextrose Broth (PDB) medium incubated for 7 days at 24°C, (d)

8   Conidiophores produced on PDA, stained with lactophenol blue (scale bar 5 μm) (for color

9   view, please refer to the online article).

10

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19   **Fig. 5.** Selected COSY (doubled headed blue arrows), ROESY (doubled headed red arrows)

20   and HMBC (green arrows) for compound n. 4 reported in Fig. 4 (for color view, please refer

21   to the online article).

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Fig. 1.

Fig. 2.

Fig. 3.

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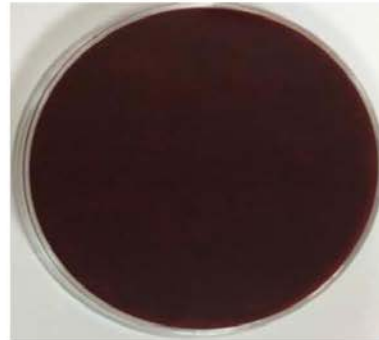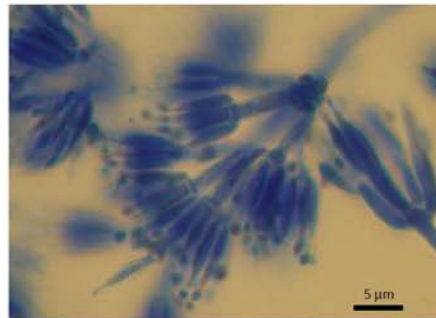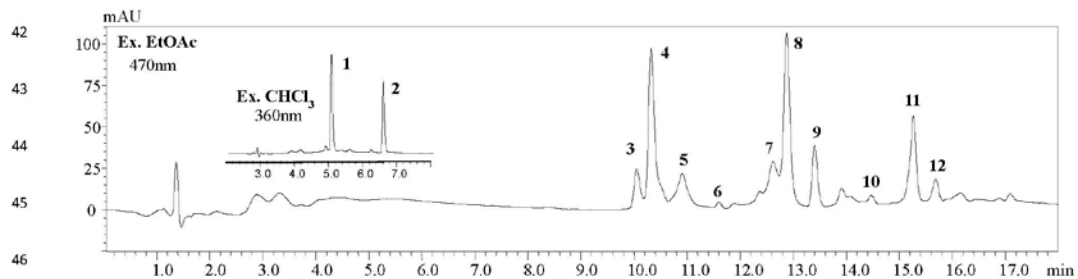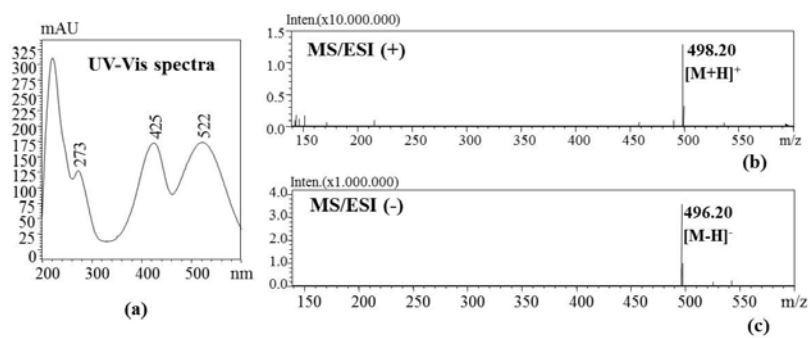
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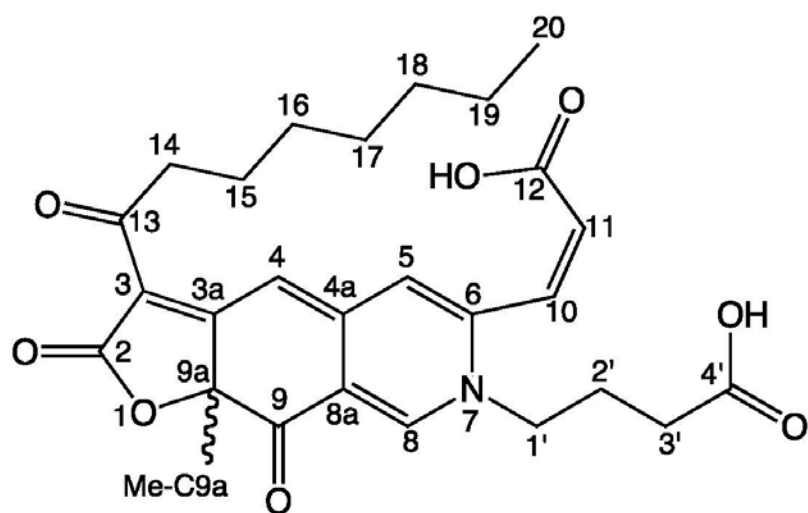
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59 Fig. 4.

60
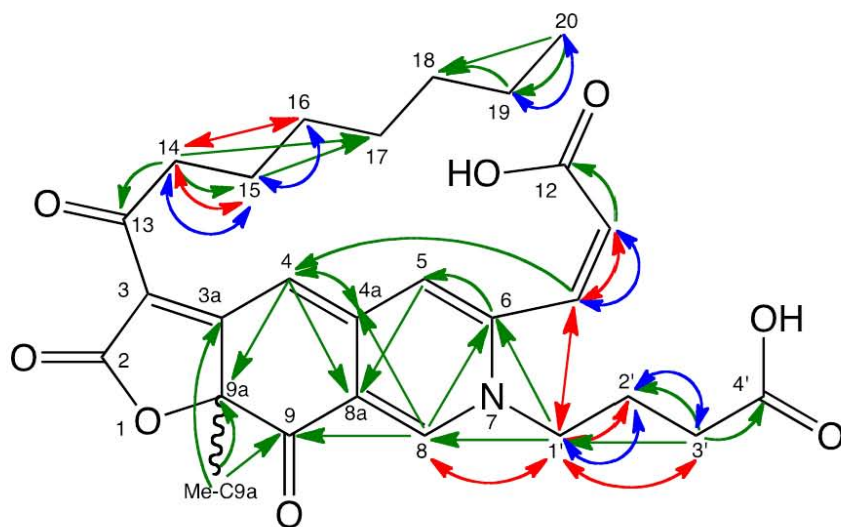
Fig. 5.

Highlights

● Microbial pigments are now well established among the natural colors used in food and beverages, competing with plant and microalgae colorants

● Filamentous fungi appear to be effective producers of many hues

● Studies about many species of *Talaromyces* started in many parts of the world within a few years

● The main target is to find a non-toxigenic fungal cell factory

● *Talaromyces albobiverticillius* is among the most promising sources of fungal reds for food use

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6.3   CONCLUSION

This chapter summarizes the applications of analytical tools and discusses the detection of twelve pigmented compounds from the intracellular and extracellular extracts of the fungus, *T. albobiverticillius*. Analysis by HPLC-DAD-ESI-MS serves as an efficient tool for determining the molecular composition of many target components, and by calculation of accurate masses from several adducts. The compounds 3, 5, 8 among the twelve are similar to the already known N-threonine-monascorubramine (red), N-glutaryl-rubropunctamine (red) and PP-O (orange) respectively based on the obtained spectroscopic data and the comparison with literature data. Further, one red compound with new structure named 6-[(Z)-2-Carboxyvinyl]-N-GABA-monascorubramine, (or as N-GABA- monascorubramine-derivative) was determined using NMR spectroscopy.

The remaining compounds were in few milligrams (**1 -2 mg)** of amounts to make ^{13}CNMR and so it was difficult to make the scan and to obtain useful spectrum. Hence, the amount of material needed should be increased, then 2D NMR experiments such as HSQC and HMBC **will be done to characterize the new compounds as well as to elucidate its molecular structure.** Detailed study of these identified compounds belongs to structurally diverse family of azaphilones, which is a well- known family among fungi and also in *Penicillium /Talaromyces* genera. And the identified new compound detailed in this chapter confirms the high diversity of molecules potentially produced by fungi in general as well as from marine-derived fungi.

CHAPTER 7

AN APPROACH TO DETERMINE THE EFFICIENT TECHNIQUE FOR THE EXTRACTION OF FUNGAL PIGMENTS: COMPARISON OF MECHANICAL AND CONVENTIONAL EXTRACTION METHODS

7.2 BACKGROUND

The preparation of fungal pigments for chemical analysis involves multitude of steps, aiming to improve the separation of interesting compounds while analyzing through analytical systems [1]. In liquid fermentation, most of the pigment producing fungi are able to diffuse water soluble pigments into the culture media and so the fungal pigments can be extracted both from biomass and culture filtrate [2]. Generally the biomass and culture broth are separated either by centrifugation or Buchner filtration. Then the biomass is allowed to freeze and subjected to lyophilization until it becomes dry powder. It offers easy grinding of the dried matter and reduces the particle size which in turn improves the extraction efficiency [3]. To extract fungal pigments, traditional solid liquid extraction (SLE) is normally used to extract soluble pigments from the freeze dried solid matrix. Suitable organic solvents are chosen based on the polarity of the compounds to be extracted. The main organic solvents with increasing polarities used for extraction involve hexane, chloroform, ethyl acetate, acetone, methanol, ethanol [4].

The current traditional SLE used to extract fungal pigments involves maceration and Soxhlet extraction techniques. Although these techniques are still in use, they relatively require large volume of solvents and consume long time to complete the extraction process. Moreover, many natural products including pigments are thermally unstable and can be degraded during extraction at high temperature commonly from 50°C depends on the pigment type [5]. To overcome these flaws, efficient and eco-friendly extraction techniques have been introduced to extract fungal biomolecules in cheap and efficient ways. These novel techniques include Microwave Assisted Extraction (MAE), Ultrasound Assisted Extraction (UAE), Enzyme Assisted Extraction (EAE), Pressurized Solvent Extraction (PSE), Supercritical Fluid Extraction (SFE), Pulsed Electric Field (PEF), extraction with Ionic Liquids (ILs) and with

some switchable solvents [6, 7]. These emerging techniques offer the advantages of shorter extraction time, reduced organic solvent consumption and waste pollution.

Among the novel extraction techniques, MAE and UAE are two efficient and easy to operate SLE methods widely applied in extracting compounds of interest from sample matrices. Recently, the application of ultrasound in pigment extraction has been the topic of many investigations. UAE involves the application of acoustic cavitation, which produces high intensity acoustic waves in a liquid phase. This enhances the heat and mass transfer also solvent penetration into the solvent matrix by disrupting the outer surface of the material [8, 9]. MAE uses non-ionizing microwave energy which gives high yield of extraction and accelerated process by the result of synergistic combination of heat and mass gradient working in the same direction. Indeed, whereas in conventional methods, mass transfer takes place from the inside to outside on the contrary heat transfer in the opposite direction. In the process of MAE, the heat is dissipated volumetrically inside the medium[10].

Though both the techniques offer several advantages over conventional systems, the extraction efficiency depends on a number of factors. That includes solid to solvent ratio, extraction time, temperature, effect of microwave or ultrasound power and nature and choice of solvent used. This chapter investigates and presents the extraction of fungal pigments from *Talaromyces albobiverticillius* 30548 using novel extraction methods such as MAE, UAE by comparing with conventional solvent extraction.

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7.3 MANUSCRIPT

This chapter is based on the extraction methods applied to extract the pigments from *Talaromyces albobiverticillius* and the following script will be submitted to a journal.

Extraction of azaphilone-like red pigments produced by *Talaromyces albobiverticillius* 30548: Comparative study of different extraction methods

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Abstract

Effect of ultrasound assisted extraction (UAE) and microwave assisted extraction (MAE) to extract pigments from the culture of marine derived fungus *Talaromyces albobiverticillius* 30548 were compared with the conventional extraction method using ethyl acetate as extraction solvent. Extracted crude pigments yields of the different methods were measured and quantified by RP-HPLC to assess the extraction performances. Extractions were performed on extracts coming both from filamentous cells (IC) and from liquid from the culture (EC). Comparing the chromatograms and the chemical profile of three different extraction methods, it was found that, MAE exhibited higher crude pigment yield (29.8 mg of intracellular pigments and 29.6 mg of extracellular pigments per gram of lyophilized sample) in 10 minutes under a constant power of 1000W when compared to UAE and conventional methods. Nevertheless, UAE exhibited the extraction of high quantity of interesting pigments in both intra and extracellular when compared to MAE which was portrayed in the chromatograms. Also, higher absorbance signal was noticed in the elution peaks of UAE for the extracts obtained in 10 minutes under 77% amplitude using ethyl acetate as solvent. To achieve the same recovery of pigments as with MAE and UAE, conventional solvent extraction required longer time (24 hrs) and the yield was lower (20.1 mg crude pigment/g of lyophilized biomass and 17.3 mg crude pigment/g of lyophilized culture filtrate). Furthermore, lyophilization of biomass before extraction reduces the particle size of the material. Thus in turn leads to easy diffusion of solvents to the matrix and provides rapid extraction of pigments.

1.   Introduction

There is an increasing hunt for new sources of natural pigments owing to the toxic and adverse effects of synthetic colors used in foods, pharmaceuticals, cosmetics and textiles. Since prehistoric times, natural colorants are extracted from plants, vegetables, fruits, insects, microorganisms, animals and ores. Among the available natural sources, microorganisms offer promising avenues due to their better biodegradability, undemanding production of pigments besides several secondary metabolites [11]. Furthermore, the produced secondary metabolites may demonstrate biological activities such as anticancer, antioxidant, anti-inflammatory which increases its marketing potential [12]. Amongst microbes, filamentous fungi serves as a promising source of producing diverse chemical colorants and it facilitates the scientists and researchers to focus their research on filamentous fungi [13]. Microbial pigments have advantages over higher forms of life like plants and animals in terms of massive controlled production, independence of agro climatic conditions, minimizing batch to batch variations, colors of different shades and growth on inexpensive substrates [14, 15].

Traditionally microbial pigments are extracted using organic solvents based on polarity of the solvents and pigments to be extracted. For example, extracellular pigments from *Trichoderma harzianium* and *Penicillium purpurogenum* IAM15392 were extracted using ethyl acetate. Cho et al. (2002) have used n-butyl acetate in acidified broth to extract red pigment from *Paecilomyces. sinclarii.* [16-18]. However, conventional extraction methods usually involve extended contact with large volumes of solvents or mixture of solvents performed at high temperatures sometimes (>100° C). They often result in relatively low extraction yield, poor selectivity and in the necessities for further purification steps. Thus, both scale-up and cost-effectiveness of the production of natural pigments at industrial scale are yet limited and require optimization [2].

 Alternative and novel extraction methods namely microwave-assisted extraction (MAE), ultrasound-assisted extraction (UAE), supercritical fluid extraction (SFE), pressurized solvent extraction (PSE), pulsed electric field-assisted extraction (PEF), enzyme-assisted extraction (EAE), and extraction with switchable solvents and ionic liquids (ILs) are currently being used to extract pigments from plants, rhizomes, microalgae, bacteria and fungi [1]. Each of these techniques has their own advantages but mostly it reduces the extraction time, volume of the

solvent used, shortens the sample preparation time and result in high efficiency compared to the conventional extraction method.

Microwave-assisted and ultrasound-assisted extractions are known to be novel, simpler and efficient extraction techniques to extract target compounds from the sample matrix. MAE uses microwave energy that disrupts the hydrogen bond promoted by the dipole rotation of the molecule and it facilitates the migration of dissolved ions [19]. Unlike other conductive heating methods, microwaves heat the sample and solvent simultaneously and accelerate the release of compounds into the solvent from the sample [20]. UAE uses acoustic waves in the KHz (>20 kHz) range on liquid medium thereby creating successive compressions and expansions of air bubbles on the molecules of the medium [3]. During the expansion phase, the air bubbles have large surface area, causing the bubble to expand resulting to collapse. This phenomenon extracts compounds from the solid matrices by diffusing solvent into the medium [21, 22]. The extraction efficiency is linked to the polarity of the solvents and both methods have the possibility to use several solvents of varying polarities. UAE has showed better extraction rate when coupled with MAE and both methods allow faster extraction, use low amount of solvents and are cost effective [23, 24].

Emerging green and novel solid liquid extraction methods would support the development of sustainable and cost-effective production of natural colorants at an industrial scale. In summary, this work reveals the effects of microwave and ultrasound assisted extraction techniques on the extraction of intracellular and extracellular pigments from the fungus, *Talaromyces albobiverticillius 30548* by comparing its efficiency on pigment yield with conventional extraction method. The comparison was carried out by injecting the crude pigments into HPLC to analyze and characterize the extracted pigments in the samples.

2. Experimental setup

2.1 Fungal material and culture conditions

The fungus used in this study was isolated from marine sediment source of La Réunion Island, south west of Indian Ocean area and identified as *Talaromyces albobiverticillius* 30548. Throughout the study, the fungus was maintained on potato dextrose agar slants (Sigma Aldrich) stored at 4°C and sub-cultured at monthly intervals. A loop of the fungus along with spores was taken and inoculated into 40 ml of potato dextrose broth (PDB) of initial pH 5.5 (Sigma Aldrich) to make a 72 h old pre-culture by culturing at 24°C, at 150 rpm

(Multitron Pro, Infors HT). After 72 h of growth, 1% (v/v) of pre-culture was transferred into 100 ml of PDB and allowed the fermentation to 8 days under the same conditions used for pre culture. After 8 days of fermentation, fungal biomass and culture filtrate containing pigments were separated using centrifugation at 8000 rpm for 6 minutes (3K3OH-Fisher Bioblock Scientific). The materials were frozen immediately (-80°C), followed by lyophilization (Cryotec) for 48 hours and then stored at room temperature for the extraction of pigments.

2.2   Solvents

Ethyl acetate of purity 99% (Sigma) was used as a solvent to extract the pigments from the lyophilized material. Ethyl acetate (4.4 polarity index) was chosen as the extraction solvent from preliminary results as it effectively extracts the orange and red pigments compared to other solvents tested such as hexane, chloroform and methanol.

2.3   Conventional extraction

1 g of lyophilized fungal biomass and culture filtrate containing pigments were taken and 50 ml of ethyl acetate was added in 150 ml Erlenmeyer flask. The flask was wrapped with aluminum foil and kept in the orbital shaker (VKS_75 Control, EdmuntBühler GmbH) at 150 rpm, allowed for 24 hours. Solvent containing the extracted pigments was filtered using nylon mesh of size 48 μm (Nitex, Sefar) and the solvent was evaporated using Rota vapor (R210, Buchi, Germany) at 30°C under reduced pressure. After rotary evaporation, to the pigmented extract 2 ml of methanol (99% purity) was added and the liquid was filtered using Minisart® syringe filter of 0.20μm pore size housing with PTFE membrane (Sartorius Stedim Biotech). The filtered crude extracts were stored at 4°C in amber vials prior to HPLC injection.

2.4   Microwave Assisted Extraction

MAE experiments were performed using "Milestone DryDist" laboratory microwave oven (Milestone srl, Italy) working at constant atmospheric pressure (1.5 bar) but varying extraction time and microwave power. One gram of freeze dried intracellular (IC) and extracellular (EC) samples were mixed with 50 ml of ethyl acetate and the experiments were carried out under different MAE conditions. In this microwave module, two parameters were made to be changed including extraction time (5, 10, 15 minutes) and power (400, 600, 1000W). A

cooling system outside the microwave cavity continuously condenses the distillates. The condensates and the excess water were refluxed back to the sample in the reactor to bring uniform temperature and humidity for extraction [25]. Once the extraction was completed, the oven was allowed to cool for few minutes before opening. The crude extracts after microwave treatment were centrifuged for 6 minutes at 8000 rpm (3K3OH-Fisher Bioblock Scientific, SIGMA) and the supernatant was transferred to round bottom flask, concentrated under reduced pressure at 30°C using rotary evaporator (R210, Buchi, Germany). Finally, 2 ml of methanol (99% purity) was added to dissolve the residue. The solution was filtered using filter syringe of pore size 0.2μm (Minisart ®, Sartorius Stedim) and then the filtrate was stored at 4°C prior to HPLC analysis.

2.5   Ultrasound Assisted Extraction

The ultrasound experiments were carried out using ultrasonic chamber with probe operating at 40 W power, 80 KHz frequency, 77% amplitude and the system was maintained at the temperature of 40°C. The most powerful ultrasound probe (Vibra Cell 75043, Fisher Bioblock Scientific) was used in this experiment to handle small extraction volume. Ultrasonic probe was directly submerged into the solvent mixture containing 1 g of lyophilized sample and 50 ml of ethyl acetate. Time and desired power or amplitude of the whole apparatus was controlled with the help of amplitude controller. The extracted sample mixtures were allowed to separate which was identical to the procedure mentioned under microwave assisted extraction.

2.6   HPLC analysis

The intra and extracellular pigments extracted by UAE, MAE and conventional techniques were analyzed through RP-HPLC equipped with PDA detector (Ultimate 3000 DAD, Dionex) using C18 hypersil gold column (150 x 4.6mm, 5 μm). The mobile phases consisting of milliQwater and acetonitrile, both solvents were buffered with 0.1% formic acid to stabilize the pH. The gradient conditions used for separation were as follows: 5% solvent B for 0-4 min then increasing to 90% in 20 min, keeping 90% solvent B to 30 min and reduced to 5% from 30-31 min and equilibrated for 12 min before the next injection. An injection volume of 10 μl, column temperature at 25°C, flow rate of 0.4ml/min were used as constant operating conditions throughout the HPLC program. The PDA (Photo Diode Array Detector) acquisition wavelengths were set at 470 nm and 530 nm where the main compounds of EC (orange) and IC (red) samples respectively showed their maximum absorbance.

2.7   Calculation methods:

After evaporating the solvents in the extraction mixture to dryness, the amount of crude pigments extracted from each of the three different methods was calculated based on the dry weight of pigments remained.

$$\text{Crude pigment yield } (\tfrac{mg}{g}) = \frac{\text{weight of pigment in the balloon after RV} - \text{weight of empty balloon}}{\text{initial amount of matrix used for extraction}}$$

(1)

Where RV represents rotary evaporation

The results of HPLC analysis were expressed as a means of yield of extracted pigments in terms of % relative area.

3.   Results & Discussion

3.1   Effect of conventional solvent extraction on pigments yield

Conventional extraction using ethyl acetate for 24 hours was employed to determine the yield of intra and extracellular pigments. The analysis of the results showed that conventional extraction process has a yield of 20.1 mg/g ± 0.23 intracellular pigments (IC) and 17.3 mg/g ±0.59 extracellular pigments (EC). Comparing results of IC and EC pigments, the yield of intracellular pigments is higher than that of extracellular using the same amount of lyophilized biomass and culture filtrate respectively.

3.2   Effect of microwaves on pigment extraction

Effects of microwave power and extraction time over the yield of fungal crude pigments were studied and the values were represented in **Fig. 1**. As these experiments were done only once, this could be considered as preliminary results. The experiments in triplicates will determine the statistical power of this study. From these first results **(Fig. 1)** it can be observed that the crude pigment yield of intracellular and extracellular increases with the increase in microwave power (400 to 1000W) and process time (5 to 15 minutes). Microwave power and process time are interrelated by explaining that when microwave power increases, the time required to extract the pigments out may decreases. Because in theory as microwave power increases, the temperature of extraction solvent increases, which in turn leads to decrease in surface tension and solvent viscosity. This phenomena helps in improving sample wetting and matrix penetration **[26]**. In our study, the extraction rate increased when the power was increasing in

a range up to 1000 W except for 1000 W keeping at 15 minutes. For longer periods of extraction (15 min) at 1000 W power, the difference of the pigment yields appears more significant compared to short irradiation time (10 min). The extraction efficiency was good when the interaction of power and time was set at 1000 W for 10 minutes. Extraction at 1000 W for 10 minutes yielded 7.72% increased IC and 8.05% increase for EC pigments rather gave low yields when the parameters were set at 1000 W for 15 minutes. Hence, the pigment yields were strongly correlated with the time of application, for short times of application, a linear correlation was seen, which is given by the value $R^2 = 0.97$ **(Fig. 1).** This could be explained by the fact that for longer periods above 10 min, a maximum value of pigment yield was reached earlier between 600 and 800 W. This may indicate that all the extractable pigments not too much bounded in the cells might be extracted and a stationary phase for yields was reached between 600 and 800 W and between 10 and 15 minutes of application. For extracellular (EC), the maximum mass yield of pigments was 29.8 mg per 1 g of dried culture filtrate (1000W, 10 min), which was quite similar compared to the pigment obtained from intracellular biomass (29.6 mg/g IC). Extraction at 1000W, 15 min gives yields values of 27.2 and 25.9 mg/g for EC an IC respectively. The repetition of these experiments may demonstrate if these results are significantly different.

In this closed microwave apparatus used in this study, the temperature was uncontrollable and found to increase 6-8°C for each experiment compared to previous set. The temperature of the solvent could be increased in this vessel and as a result, the extraction efficiency increases. This is because the higher temperature opens the cell matrix and effectively extracts the pigments due to decreased intermolecular interactions within the solvent, raising the motion between the molecules [27].
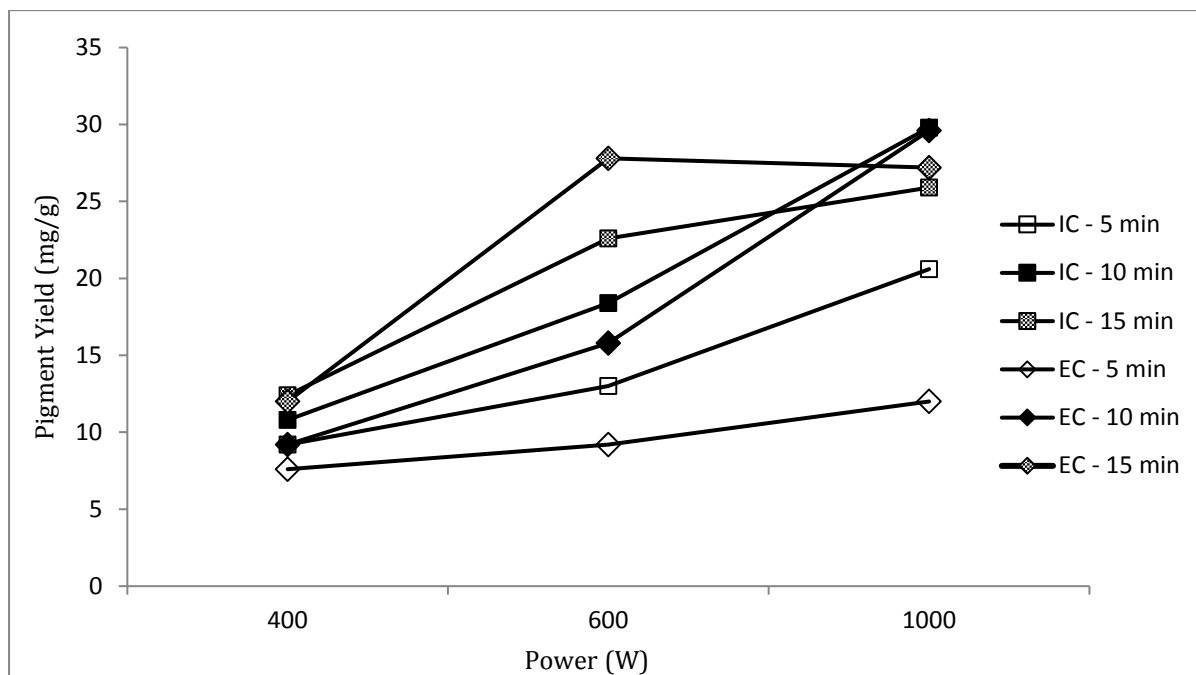
Figure 1: Effect of varying microwave power and extraction time of MAE on crude pigment yield of intra (IC) and extracellular (EC) matrix

3.3 Effect of ultrasound on pigments extraction

In UAE, to extract pigments from both intra and extracellular freeze dried samples (IC, EC), time was varied as 5, 10, and 15 minutes with the following extraction conditions as constant: solvent-ethyl acetate, temperature 40°C, sample-solvent ratio of 1:50 and amplitude of 77%. The results are provided in **Fig. 2.** In both the extracts, there was higher yield of pigments when increasing time duration from 5 to 10 minutes. Increasing the time thereafter to 15 minutes seems to conversely decrease the amount of pigments extracted by keeping solvent volume and amount of the sample matrix constant.

For IC samples, the overall pigment yield (35 mg/ 1 g of lyophilized sample) exhibited an increasing pattern for the 5 and 10 minutes of application, after which the yield was found to decrease. As a result of cavitation and micro-jet formation, solute-solvent interaction was accelerated during the initial stages of extraction. Cavitation occurs in the solvent as a result of the compression and rarefaction cycle of ultrasonic waves. During cavitation micro-bubbles will be formed which will grow and disrupt with the release of enormous amount of energy. This results in the disruption of cell walls and formation of micro-jets which increases the solute-solvent interaction **[28-31].** Fick's second law of diffusion states that the solute concentration in the solid matrix and solvent attains equilibrium after a certain period of time. However, extracted pigment on prolonged exposure to ultrasonic treatments will undergo degradation and in this case, the pigments may lose its color to some extent. These

phenomena may explain the difference in yield of pigment after 10 minutes and it must be proved with repetition of experiments.

Extracellular pigment yield was found to increase linearly with the increase in time for the first 10 minutes (25.4 mg/g EC) and the yield was found decrease while increasing the extraction time to 15 minutes. The similar profile follows for the pigments extracted from dried intracellular biomass but the yield was higher at 10 minutes (33.8 mg/g IC) (Fig. 2). This phenomenon can be explained by the fact that mass transfer of solute occurs from the higher concentration (solid matrix) to lower concentration (solvent) until equilibrium in concentration was attained. Whereas, prolonged exposure of extracted pigments to ultrasound waves may results in the degradation of the pigment molecules due to the shock waves produced by cavitation. The maximum crude pigment yield obtained was 25.4 mg/ 1 g of sample at 10 minutes of extraction time. It can be seen that there is 33.07% increase of IC pigments yield compared to the yield of EC pigments, mentioning 13.01% decrease.
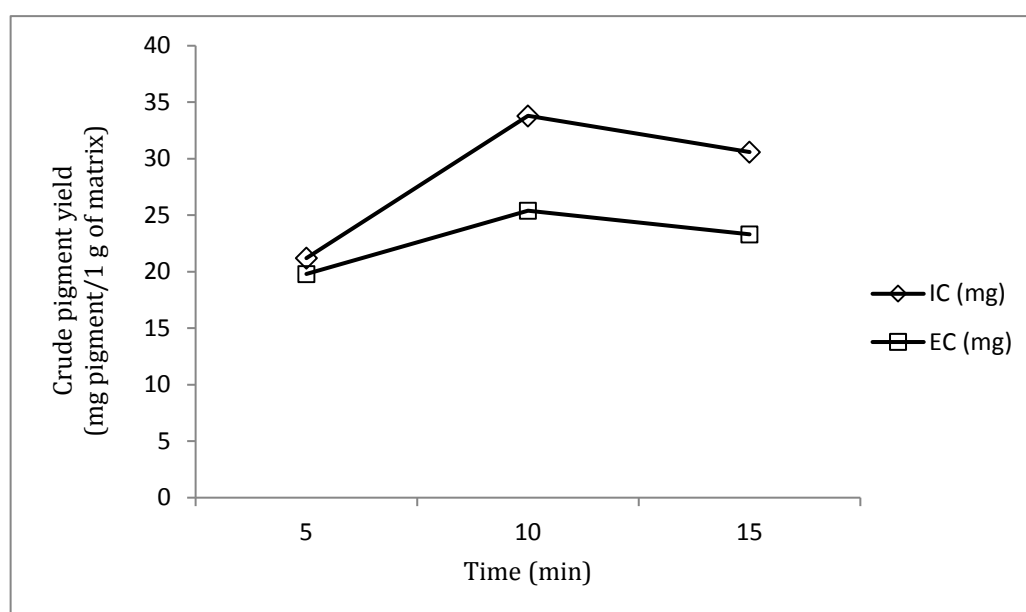
Figure 2: Crude Pigment yield obtained from both intra and extracellular samples (IC, EC) through Ultrasound Assisted Extraction at different time duration

3.4 HPLC analysis of pigments by different extraction methods

Using the optimized HPLC method, the crude extracts of the different methods were subjected to HPLC analysis. The extraction efficiency of different methods was calculated and

the similarity in chromatograms was compared. The relative peak areas of characteristic compounds were calculated for quantitative expression of the HPLC fingerprint. Besides, each characteristic peak was identified by comparing the retention time and their relating UV absorption spectra that unveils chemically related information.

3.3.1 Intracellular pigments

The pigment profile of *Talaromyces albobiverticillius* 30548 under three different extraction methods (conventional, UAE and MAE) changed in terms of the number of compounds extracted as well as the type of colored compounds noticed by the change in absorption maxima (Table 1). Chromatograms of the intracellular pigments (IC) were monitored and extracted at 530 nm as the main compound which belongs to polyketide azaphilone family has its maximum absorbance at 426 and 525 nm. In intracellular pigment profile (IC), there were at least three red pigmented compounds with two maximum absorbance in the region of 423-471 and 523 – 558 but there are different compounds in the whole extract. The main compound is the one absorbing in 426 and 523 denoted as peak 2 in conventional and MAE, peak 1 in UAE. This compound is present in major quantities in all the intracellular crude extracts obtained by the three different extraction methods. The other red compounds could be some intermediates produced during the metabolism of pigment synthesis.

The percentage of individual compounds in the mixture of crude extracts was given by the percentage of relative area. In UAE, the main red compound (peak 1) occupied 85.06 % relative area and few other compounds were extracted in small quantities. Contour to that, the same compound extracted by MAE (48.33 % for peak 2) and conventional method (41.44% for peak 1) occupied very low %relative area compared to UAE. This indicated that the ultrasonic assisted process was many times faster to extract the main compound and the yield was higher than that of conventional and MAE method.

Table 1: Comparative intracellular (IC) pigment profile of *Talaromyces albobiverticillius* 30548 extracted using conventional, UAE and MAE methods

Extraction methods	No of colored peaks	Retention time(min)	Peak no	UV- vis absorption wavelength (λ_{max}) nm	Pigments group	Relative Area (%)
Conventional	Not detectable	15.18	1	196,244,273,**423,526**	R	3.65
		15.71	2	195,245,273,**426,525**	R	41.44
		16.40	3	195,249,287,**426,542**	R	6.53
		18.47	4	198,210,235,**456,464**	O	33.57
		19.26	5	195,235,285,**458,460**	O	11.21

		22.26	6	193,211,242,282,**456,471**	O	2.77
UAE	Not detectable					
		15.35	**1**	**195,245,273,426,523**	**R**	**85.06**
		16.02	2	249,296,427,**471,558**	R	7.59
		18.81	3	239,285,**462**	O	2.74
		20.09	4	195,232,285,**462**	O	3.75
MAE	Not detectable	16.07	1	196,244,273,**422,523**	R	5.77
		16.59	**2**	**195,245,273,426,523**	**R**	**48.33**
		17.25	3	196,219,249,287,**425,547**	R	5.91
		19.37	4	199,203,235,279,**455,466**	O	27.48
		20.04	5	194,233,285,**462**	O	10.61
		23.04	6	196,242,283,**457,471,475**	O	1.90

-R- Red pigments, O- Orange pigments

-Not detectable indicates there are uncountable many small peaks with less than 1% relative peak area.

-Bold fonts represent the maximum absorbance in visible r
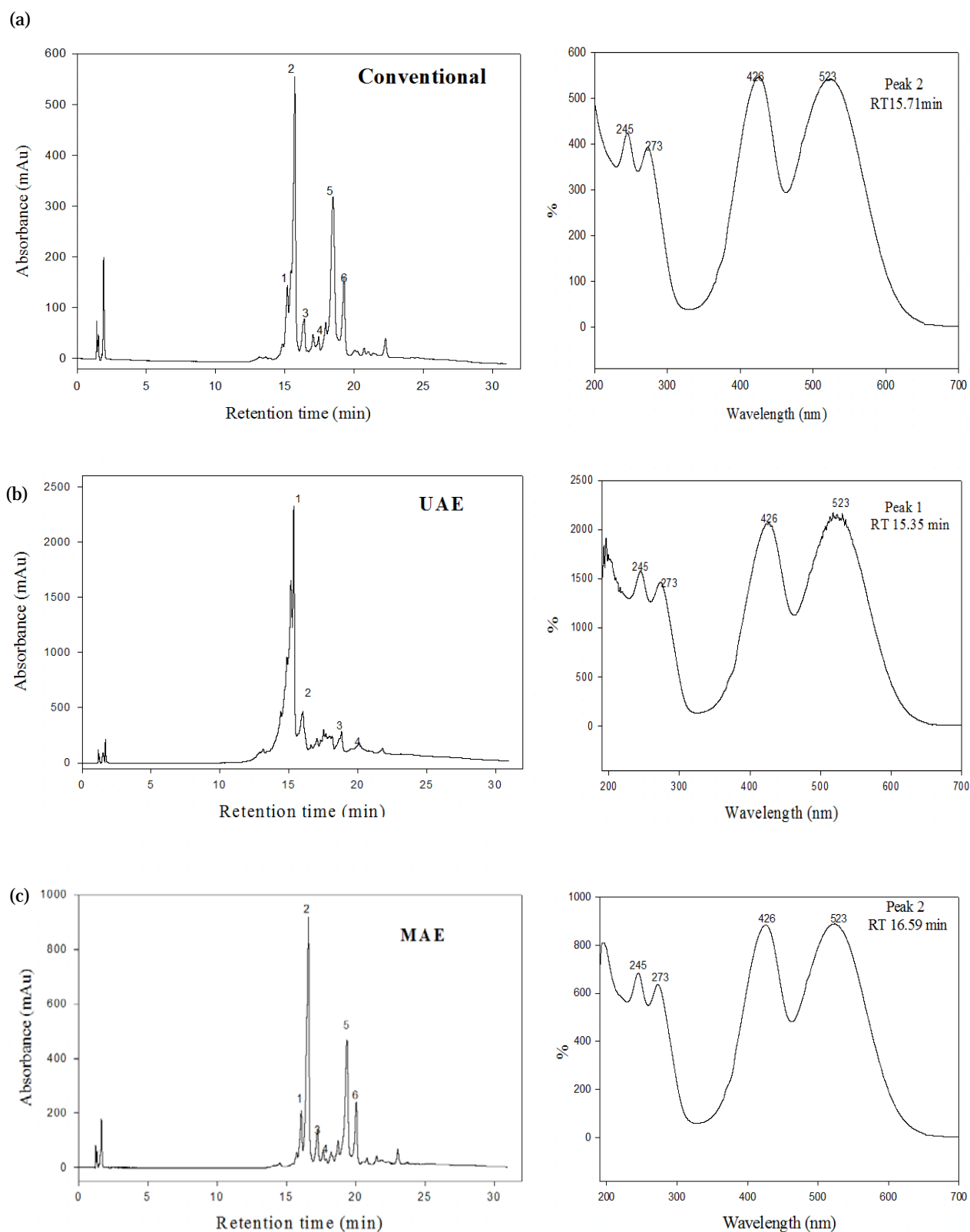
Figure 3: HPLC chromatograms of intracellular pigments (IC) of *Talaromyces albobiverticillius* 30548 extracted using different extraction methods (a) conventional method for 24 hours at 150 rpm (b) UAE of 10 minutes at 40 W power (c) MAE of 10 minutes at 1000 W power

3.3.2   Extracellular pigments

Qualitative HPLC analysis of extracellular pigments (EC) was carried out and the pigment profiles were compared for the three different extraction methods (Table 2). In extracellular culture filtrate, most of the extracted compounds absorbing in the yellow-orange region (458 – 481) but a single compound absorbing in the red region was noticed in both conventional and MAE method (peak 2). The orange pigment (λ_{max} 469.8) was mainly diffused into the extracellular culture medium and the compound was better extracted by UAE (96.53% for peak 2), followed by MAE (90.69% for peak 4) and less amounts by conventional extraction (87.70% for peak 4). Similarly, as noticed in intracellular pigment profiles, UAE shows the advantage of extracting few compounds and to extract the main one in major quantities (Table 2). Considering conventional extraction for 24 hours in ethyl acetate, the chromatogram shows that the yield was lower than UAE and MAE. The reason for this was it requires longer times and large amount of solvents. Considerably, UAE (96.53% for peak 2) and MAE (16.85% for peak 4) gave comparable higher recovery percentage for extracellular pigment yield.

Table 2: Comparative pigment profile of extracellular pigments (EC) extracted using three different methods (conventional, UAE and MAE) and maximum absorbance wavelength of each compound

Extraction method	No of colored peaks	Peak no	Retention time(min)	UV- vis absorption wavelength (λ_{max}) nm	Group	Relative Area (%)
Conventional	Not detectable	1	13.42	209,245,287,**481**	O	1.21
		2	15.03	244,273,**422,520,525**	R	2.40
		3	16.11	204,264,**470**	O	6.27
		4	**16.81**	**197,264,469.8**	**O**	**87.70**
		5	19.11	216,240,285,**406,459,480**	O	1.45
UAE	5	1	13.21	209,245,287, **481**	O	1.79
		2	**15.56**	**197,265,365,469.8**	**O**	**96.53**
		3	18.80	216,219,238,**461,471**	O	1.67
MAE	6	1	14.45	210,245,287,**481**	O	1.77
		2	16.08	197,244,273,**422,521,523**	R	1.57
		3	16.40	210,266,306,**469**	O	2.83
		4	**16.85**	**197,265,365,469.8**	**O**	**90.69**
		5	19.41	240,280,**463,470**	O	1.87
		6	20.05	216,234,285,**458**	O	1.27

R- Red pigments, O- Orange pigments

Not detectable indicates there are uncountable many small peaks with less than 1% relative peak area.
Bold fonts represent the maximum absorbance in visible region
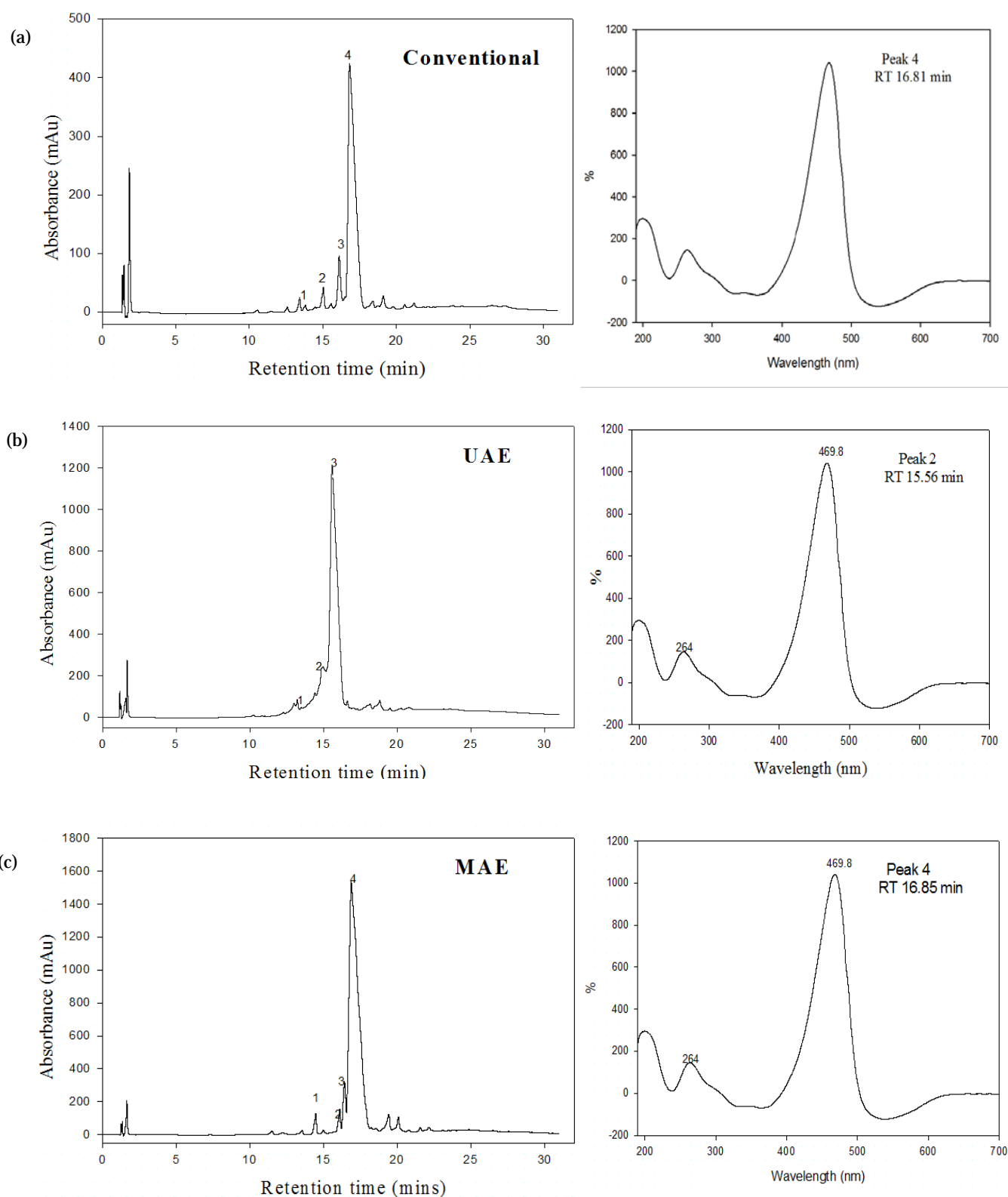
Figure 4: HPLC chromatogram of extracellular pigments (EC) extracted from *Talaromyces albobiverticillius* 30548 recorded at 470 nm (a) conventional extraction for 24 hours at 150 rpm (b) UAE of 10 minutes at 40 W power (c) MAE of 10 minutes at 1000 W power (d) UV-visible spectra of main peaks and a compound with its maximum absorbance at 469.8 nm

3.5   Comparison of MAE, UAE with conventional extraction

The three different extraction methods conventional, UAE and MAE were performed to compare the extraction efficiency of pigments from the lyophilized intracellular biomass (IC) and extracellular culture filtrate (EC) of the fungus *Talaromyces albobiverticillius* 30548 by calculating the yields and HPLC-DAD analysis. In all these experiments, few operating conditions were kept as constant: ethyl acetate as solvent, solid to solvent ratio of 1:50. In comparison, we have shown that there are diverse colored pigments produced by this fungus mainly absorbing in the red, yellow and orange regions and diversely extracted by the three different methods. From HPLC chromatogram, it was detected that among many compounds, there were two main components, red compound in intracellular (λ_{max} 426 and 523) and orange in extracellular (λ_{max} 469.8) appearing in large amounts in all of the three extraction methods.

Thus, the study was mainly focused to concentrate on two main compounds of interest. The intracellular (IC) red pigment (absorption maxima at 426 and 523 nm), is characteristic of the *Monascus*-like azaphilone red pigment [32]. But the elution of the same pigment differs when extracted using different methods and changes in the retention time were observed in the chromatograms of each method **(Fig. 3 & 4)**.

From the results, it was clear that the intracellular biomass (IC) provides higher crude pigment yields through all the three extraction methods (Conventional, UAE and MAE) when compared with extracellular culture filtrate (EC) **(Fig. 1 & 2)**. UAE (34.9 mg in 10 min with 40 W) was found to extract maximum crude pigment yield followed by MAE (29.8 mg in 10 min with 1000 W) and conventional method (20.1 mg in 24 hours), which is shown below in **Table 3**. For intracellular pigments, the extraction yield through UAE was 73.63% higher than conventional and 17.11% higher than MAE. Likewise, MAE gave 48.25% greater yield than conventional method. At the same time, extraction through conventional method yielded 42.4% decreased amount of pigments than UAE and 32.55% decrease compared to MAE. The efficient yield through MAE and UAE is due to direct generation of heat within the matrix, by friction between polar molecules [33]. Thus, in conventional heating, both heat and mass gradients work in opposite directions; also only the surface of the matrix is heated directly, and subsequent heating is by conduction from the surface to the core of the matrix particle [34].

Table 3: Crude pigment yield (mg/g) of intracellular (IC) and extracellular (EC) pigments extracted from *Talaromyces albobiverticillius* using three different extraction procedures

Extraction methods	Time	Power (W)	Temperature (°C)	Crude pigment yield (mg pigment/g sample)			
				IC	% increase compared to conventional	EC	% increase compared to conventional
Conventional	24 h	-----	25	20.1± 0.73	-----	17.3± 0.59	-----
UAE	10 min	40	40	34.9± 0.31	68.15%	25.4± 0.45	46.82%
MAE	10 min	1000	38-62	29.8± 0.27	48.25%	29.6± 0.32	71.0%

4.   Conclusions

In the present study, three different extraction methods such as MAE, UAE and conventional techniques were used in a preliminary study to extract fungal pigments from both intracellular biomass and extracellular culture filtrate. The yield by MAE represents total of 29.8 mg IC pigments and 29.6 mg EC pigments per 1 g of each sample matrix under the following conditions: sample to solvent ratio 1:50 (1g in 50 ml solvent), microwave power of 1000W and extraction time in 10 minutes. This is an expected result; increasing power along with proper extraction time will produce a faster breakdown of the cell walls, providing an easier diffusion of the solvent to extract the pigments. The temperature at the condition of overall maximum pigment yield (IC & EC) was around 60°C in MAE. Considering UAE, 34.9 mg IC and 25.4 mg EC pigments were extracted within 10 minutes using ultrasonic probe of amplitude 77%.

From the HPLC-DAD chromatogram, it was evident that both methods MAE and UAE found to be more efficient in extracting pigments (red in IC, orange in EC) in a short time than conventional technique. Furthermore, the % relative area of main pigments of intra and extracellular matrix extracted by UAE suggested that the possibility to select the ultrasound for extraction of a specific compound than MAE. Obtaining the same results through conventional extraction demonstrates it is more laborious, time consuming and extract components in low amounts compared to other techniques. The same experimental techniques may be reproduced to confirm the preliminary results obtained. Moreover, it is mandatory to investigate some more effective parameters such as different levels of sample to

solvent ratio, temperature to optimize the most influencing factor for pigment extraction in the fungus *Talaromyces albobiverticillius* 30548.

Acknowledgements

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7.4   CONCLUSION

To our knowledge, this study provided information that in comparison with traditional extraction method, using alternative methods such as UAE, MAE are less time consuming and low solvent consumption. The interpretation of the results showed that amount of intracellular pigments was extracted in higher quantities than extracellular pigments in all the three different methods used. It indicates fungal biomass holds much of the pigments than dispersed into the liquid media. Comparison of the results of conventional method with extraction using microwaves and ultrasound indicated that there was a decrease in the pigment yield. This proves the non-conventional methods yielded higher amount of pigments in less time using minimum volume of solvents and its high power combined with temperature.

HPLC characterization performed on the extracted pigments confirmed the extraction of interested compound in higher amounts among others, whatever the methods used. However, the differences in Rt and peak elution was observed for both intracellular and extracellular pigments which is due to the influence of different factors on the extraction. But, to prove the efficiency of these techniques, some more experiments should be carried out. The solid to solvent ratio could also be varied as well as the extraction solvent and temperatures. However, to define a relevant and reproducible pigment yields, the experiments should be conducted in triplicates. For industrial application purposes, further investigations are required to develop mathematical model to control and predict the optimization parameters of the extraction process.

Also, it is highly desirable to conduct experiments in order to extract pigments using other eco-friendly techniques such as Pressurized Fluid Extraction (PFE), Enzyme Assisted Extraction (EAE), using Ionic liquids (IL's) to develop these techniques with economic prospective. In addition, for some bioactive compounds, mutual interplay or combination of different separation techniques are essential for their separation. It is thus of great importance to study the combination ways of different separation techniques in the future.

CHAPTER 8

PILOT SCALE FERMENTATION STUDIES TO IMPROVE RED PIGMENT PRODUCTION FOR INDUSTRIAL SCALE- UP

8.2 BACKGROUND

In the development of producing microbial pigments on a commercial scale, using the results of laboratory and pilot scale studies presents new challenges. Generally, scale-up studies refers to the act of using laboratory results for designing a prototype and a pilot plant process. Pilot scale fermentation allows investigating the production process and level of product yield that could be used to extrapolate and build the large scale industrial fermenters with confidence [1]. Especially, bench scale fermenters operating with volumes of 0.5-2 L allow the control of oxygenation, pH and provide options for easy sampling as well as to control fermentation conditions [2].
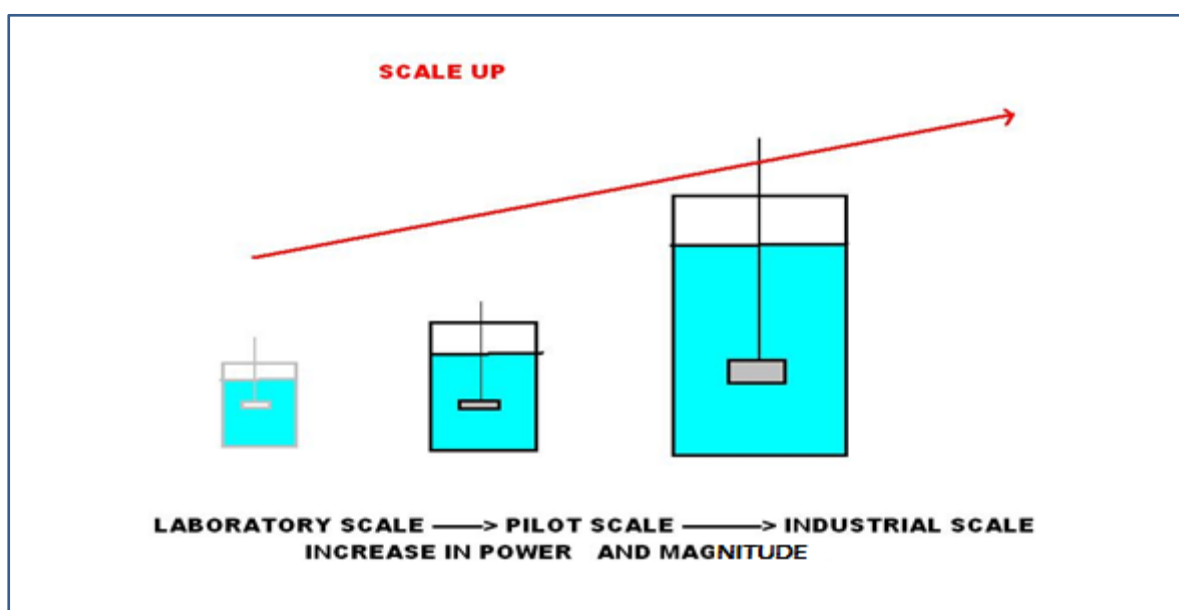
Figure 8.1 Scale up from laboratory to industrial level for commercial production

Image source: reference [3]

During scale up studies, some additional parameters need to be considered, modified and adjusted. This is because, as the size of fermentation increases during scale up, various measured parameters might not show a linear co-relationship. But the ultimate goal is to

obtain high throughput of desired product yield and fermentation efficiency at most economical values [3].

There are few considerations and monitoring to be applied when studying behavior of fungi and pigment production during scale up which are mentioned below:

- Inoculum development and its percentage of inoculation (v/v)
- Proper sterilization establishments (correct temperature cycle at larger loads, proper calibration, pre and post sterilization volumes of growth media)
- Environmental parameters such as nutrient sources, pH of the media, temperature, dissolved O_2 and CO_2
- Foam production and its control
- Type of impeller, agitation speed and shear stress.

The present study was carried out to understand the behavior, biomass growth and pigment production of the fungus *Talaromyces albobiverticillius* 30548 in a bioreactor with a capacity of 2 L. The details of the fermentation, evaluation of results, performance efficiency and future considerations are communicated below in manuscript form.

8.2   References

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8.3   MANUSCRIPT

This chapter is based on the manuscript written about the results obtained from the fermenter studies and its comparison with shake flasks experiments in terms of fungal growth and pigment production

Scale-up of pigment production in the marine-derived filamentous fungus, *Talaromyces albobiverticillius* 30548 from shake flasks to stirred bioreactor-condition optimization and comparison

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Abstract:

Talaromyces albobiverticillius 30548, a marine derived fungus produces *Monascus*- like azaphilone red/orange pigments. Experiments were carried out at laboratory scale in 200 ml Erlenmeyer flasks and others in 1.5 L bioreactor with a capacity of 2 L. The influence of dissolved oxygen, mechanical forces, temperature and pH on pigment production and biomass growth of this fungus in 2 L bioreactor was investigated. The fungal morphology, duration of pigment production, growth kinetics studies in bioreactor were compared with the observations noticed from Erlenmeyer flasks cultured with the identical inoculum. The highest orange and red pigment production in bioreactor was noticed after 160 hours of fermentation (for the experiment at 70% of pO_2) with 25.95 UA_{470} nm and 22.79 UA_{500} nm while maintaining pH of the medium as 5.0 respectively. Whereas, the fermentation in Erlenmeyer flasks of 200 ml effectively producing pigments with 22.39 UA_{470} nm and 14.84 UA_{500} nm releasing yellow and red pigments in the culture medium respectively under the same experimental conditions on day 8 . Relating to the fungal morphology, growth of fungus in the bioreactor was in the form of pellets rather than filaments when the oxygen transfer was very low in the medium. In aerobic fermentations of filamentous fungi, oxygen is a key substrate for their growth, maintenance and production of secondary metabolites. Due to the low solubility of oxygen in liquid broths, continuous supply of oxygen is needed and the rate

of oxygen transfer should be monitored and controlled throughout the entire fermentation period. From the observed difference in Erlenmeyer flasks and closed bioreactor, it is understood that the process was significantly influenced by dissolved oxygen rate. Thus, oxygen transfer appears as the rate limiting factor which highly influences the overall growth and production of pigments in *Talaromyces albobiverticillius*.

1.   INTRODUCTION

With the increase of industrial applications of colors in day to day products, pigments have become a point of interest among manufacturers and researchers. They are especially used in food and cosmetics, also in medicines and dyeing clothes [1]. The harmony between chemical and biological pigment production has become an opposition since the increasing consumers concern about using natural sources of ingredients, to ensure the safety and quality of their products consumption. Indeed, some synthetic colors induce severe allergic reactions or hyperactivity among adults and children [2]. Today, due to the environmental concerns and harmful human-health effects of synthetic colors, a benefic marketing impact has been promoted to use natural pigments instead of synthetic ones [1, 3]. In the case of pigments, natural colors alone do not have the same color intensity as synthetics, and some (not all) are less economical on a dosage basis; however, technological advances have reduced this performance gap. Therefore, natural colors production continue to rise due to its adequacy to meet consumer expectations, despite of its higher cost of production [4, 5]. Moreover, the large diversity of pigments from microorganisms is an additional argument, and its study gives possibilities to identify new sources of valuable compounds.

The field of pigments production by vegetables and animals, especially insects, is attractive sometimes with benefits to human health, because of being natural sources. Nevertheless, it is less competitive towards industrial productivity than microbial production [3, 6, 7]. Indeed it is partly due to lower stability and solubility of pigments due to their strong binding nature to the cells [8]. Furthermore culture conditions can be under control in the case of microbial production compared to vegetal-or-animal productions and this is an efficient way to increase the pigment yields. Thus microorganisms present advantages compared to plant or animal extracts: less space needed, independence towards seasons, rapid growth, fast and unlimited production. Especially, some species of fungi, like *Monascus* and *Talaromyces* are efficient

producers of pigments. However, to be accepted as a pigment producer, fungi must be non-toxic and non-pathogenic in nature [9]. The legislation for food industry is very restrictive and many microorganisms have not been approved due to regulatory compliance.

The culture of the fungi could be carried out in a solid state or submerged fermentation [6]. With submerged fermentation, it can be considered an approximation that all the media are made up essentially of water. In this environment, temperature and pH regulations are trivial and do not pose problems during the scaling-up of a process [10]. As it is the case, the submerged fermentation is more adapted here because the pigments will be diffused into the extracellular culture broth. This provides an improved understanding of the interactions between the variables and attempts have been focused on modeling micromorphology, hyphal fragmentation and rheology of fungi [11-14]. Then, the optimization of pigment production does not directly result from the optimization of cell-growth. Therefore parameters like pH and substrate concentration, can be managed in a submerged fermentation and thus the monitoring of the culture will be easier [15].

Talaromyces albobiverticillius, teleomorphic genera of *Penicillium sp.* produces *Monascus* like azaphilone pigments noticed in the laboratory scale studies cultivated in Erlenmeyer flasks [16]. Literature studies have shown that some species of *Talaromyces* synthesize yellow (monascorubrin and rubropunctatin) and red (monascorubramine and rubropunctamine) colored pigments and among them few species do not produce any mycotoxin along with it [4, 8, 15, 17-19]. This factor allows the biotechnological production of azaphilone pigments by using the strains favorable for large scale production. The pigments produced by *T. atroroseus, T. albobiverticillius, T. purpurogenus, T. aculeatus, T. funiculosus* are diffusing into the culture medium in submerged fermentation. However, the submerged fermentation in small volumes is not suitable for industrial commercialization, in regard with the relatively low production yields.

Scale up fermentation is generally performed to produce molecules in large quantities and if possible to yield desired metabolite at specified level. As a means of increasing pigment yield, also to understand the behavior of fungal growth, fermentation involving bioreactor was carried out The operating condition of bioreactor depends on the amount of working volume, desired product level but mainly on the type of microorganism used [20]. Previous experiments in flasks allowed obtaining optimal conditions of production in 200 ml of liquid culture for temperature, orbital agitation rate, and composition of the culture media. In the

submerged fermentation using 2 L bioreactor, some additional parameters such as pH and dissolved oxygen are monitored by setting the above optimized factors as constant. It is a first step to produce pigments in high amount, and a precursor to a future study in fermenter of ten liters or higher volume. The present work is aimed to understand the influence of oxygen supply, maintenance of constant pH and agitation speed on pigment production and fungal growth by conducting the fermentation in closed bioreactor.

2. MATERIALS AND METHODS
2.1 Organism and conservation

Talaromyces albobiverticillius 30548 has been isolated from marine sediment source in Reunion Island, Indian Ocean sampled from Trou d'Eau, on the external slope of the coral reef, at 17 m of depth (21° 06' 22,11" S, 55° 14' 15,78" E). The fungus was grown on potato dextrose agar in Petri dishes (PDA, Sigma Aldrich) at 24°C for a period of 7 days. After the growth period, the solid culture was maintained at 4°C for the conservation. This cycle was repeated in a monthly interval to maintain the strain.

2.2 Inoculum preparation

To make liquid pre-inoculum, small amount of spores from the solid cultivation were aseptically inoculated into 250 ml Erlenmeyer flasks containing 100 ml of potato dextrose broth (PDB, Sigma Aldrich) which was sterilized for 15 minutes at 121°C. The pre-inoculum was cultivated at 24°C at 200 rpm in an agitating incubator (Multitron Pro, Infors HT) for a period of 72 hours.

2.3 Fermentation in shake flasks

The pigment production and biomass growth was monitored in 500mL shake flasks containing 200 mL of potato dextrose broth. The medium pH was initially adjusted to 5.0 under sterile conditions with 0.1 M HCl after sterilization. 1% (w/v) of the seed culture was used to inoculate the flasks and the culture flasks were incubated on agitating incubator at 24°C under 200 rpm for 10 days. All the experiments were performed as triplicates and statistical analysis such as One-way ANOVA was performed with SigmaPlot ver10 (Systat Software Inc, CA, USA).

2.4   Fermentation in bioreactor

Batch fermenter cultures were performed in a 2L bioreactor (BIOSTAT® A PLUS, Sartorius Stedim Biotech) sealed with a stainless steel head- plate. A working volume of 1.3 liter PDB media was sterilized at 121°C for 15 min and upon cooling; it was inoculated with the whole pre-inoculum (1.3 g/L wet mycelia) with a peristaltic pump. After the inoculation, there was no change in the working volume of the media. The used bioreactor is equipped with temperature control, pH control, oxygen probe and flat bladed impeller for stirring. The temperature (24°C) and pH (5.0) maintained in this study were the same as that of the shake flasks fermentation. The pH was controlled automatically using a pH control module (LH Fermentation Ltd, Stoke Poges, UK) equipped with a steam-sterilisable pH electrode (EF—12/120 K8-HM-UniVessel, Sartorius Stedim Biotech, France) by passing sterile solutions of 0.1 M HCl.

Based on the results of preliminary tests in the bioreactor, agitation speed and air input were fixed at the beginning of the experiment and later, it was changed depending on the objective and the evolution of the culture. During the entire fermentation process, the agitation speed was changed by a flat-bladed impeller (200- 1000 rpm) and sterile air was supplied at 1.3 L/min (100% DO_2, dissolved oxygen) but varied at 50%, 70% & 90% depending on the experiments.

2.5   Biomass estimation

The amount of fungal biomass in the bioreactor was determined by sampling 5 ml of the fermentation broth. The sample was drawn aseptically at intervals of 24 hours to separate the biomass from the liquid medium. The sample was filtered using Nitex filter with 0.48 μm pore size (Nitex 03-48/31, Sefar) and the collected mycelia were dried at hot air oven at 80°C to a constant weight. Then the dried biomass was weighed to calculate the daily dry biomass weight.

2.6   Estimation of pigments concentration

The culture filtrate was separated from the biomass by centrifugation at 8000 rpm for 6 minutes (Centrifuge SIGMA 3K 3OH and 19776-H rotor, Sigma). The absorption maxima of the pigments were determined by scanning the colored extracts for their absorption spectra over the range of 200-700 nm (UV-vis area). The quantification of extracellular pigments was estimated by measuring the absorbance of the filtrates at 470 nm (orange pigments), 500 nm (red pigments) using microplate reader (Infinite® 200 PRO series, Tecan Life Sciences) [21].

The pigments produced in the liquid culture by *Talaromyces albobiverticillius* 30548 were expressed in absorbance units (UA).

2.7   Color characteristics

The same extracts, after determining the pigment concentrations by absorbance, were then used for determining CIElab color coordinates using spectrocolorimeter (Colorimeter CM-5000 d, Konica Minolta, Mahwah, NJ). The CIELab colorimetric system was interpreted as follows: The value L* indicates lightness, covers from 0 (black) to 100 (white). The positive to negative a* value indicates red to green colors whereas positive and negative b* represents yellow or blue colors respectively. Chroma denoted by C gives saturation or purity of color. Hue angles, h° denotes the degree of redness, yellowness, greenness and blueness by locating at 0, 90, 180 and 270° respectively.

3. RESULTS AND DISCUSSION

3.1   Relationship between oxygen transfer and pellets formation

Fermentation was conducted at two different agitation speeds according to the rate of aeration (30 rpm at 100% DO_2, 200 rpm at 50% DO) by maintaining the medium pH as 5.0 during the experiment. From figs. 1 (a), (b) it was observed that pellet formation was mainly influenced by the agitation rate rather than air injection. When the rotational speed of the impeller was maintained at 200 rpm, the growth of the fungus was observed as mycelial forms/filaments (fig. 1 (a)). In this experiment, air was diffused above the surface of the liquid culture so that the fungus was fed with dissolved oxygen throughout the stirring action in the fermenter. We noticed that the medium was homogenously red colored (opposite to pellet culture) proving the efficiency of pigment production in the batch. As another hypothesis, the movement of liduid might be due to the rotational movement of the air bubbles if arising from the bottom induces the formation of pellets. And when stirring at the same time (200 rpm), this movement of pellets in the liquid was broken and allows the generation of filaments. Fig. 1 (b) shows the culture media without pigment production, also exhibiting the growth of fungus as pellets. In this experiment, the oxygen was diffused through the probe (100% DO_2) and a gentle stirring (30 rpm) was applied to mix the liquid culture throughout the fermentation. The oxygen transfer rate decreased dramatically after 16 hours of cultivation and then went down to 1% (fig. 2).

Figure 3 (a): surface of the liquid culture of *T. albobiverticillius* without bubbling (50 % DO$_2$), with an agitation of 200 rpm (day 7)

Figure 3 (b): liquid culture *of T. albobiverticillius* with bubbling (100% DO$_2$) and agitation of 30 rpm (day7)
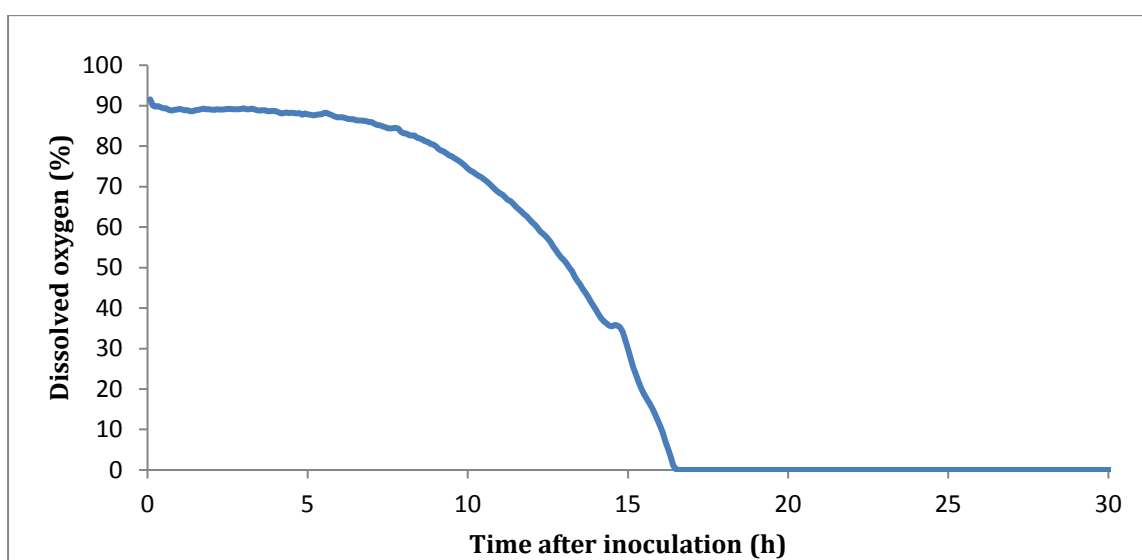
Figure 2: Decreased response of dissolved oxygen (1% DO$_2$) after fungal inoculation at 25 °C when the bubbling was set at 190 ml/min (90% DO$_2$) and stirring at 30 rpm

Other influencing factor is viscosity; it was usually found to increase when the microorganism grew fast with high oxygen consumption in bioreactor, starting from day 2. However, a strong difference appears in the fungal structures when sufficient stirring was applied or not: gentle stirring (30 rpm) does not allow adequate aeration in the whole fermenter and fungal structures seem modulated by bubbling (pellets). On the contrary, efficient stirring (200 rpm) allows the growth under the form of filaments (mechanical effect), increases the penetration of oxygen and therefore the pigment production is favored. In the shake flasks at 200 rpm, higher pigment production and mycelial growth were observed due to the low working

volume, the constant oxygen input through the silicon stopper which provides molecular-scale openings through which oxygen can easily diffuse. Anyways it was very difficult to measure this parameter in shake flasks.

3.2 Oxygen is a necessary parameter and pellet is not the limiting factor for pigment production

In *Talaromyces albobiverticillius* 30548, two main pigments were of interest. Red pigments produced in major quantities which were mainly diffused into the extracellular medium and yellow, main one which was bound to the cells of the fungi **[22]**. Even with changing parameters such as agitation speed, rate of oxygen supply to increase the pigment production, pellet formation was a phenomenon observed in bioreactor but not in flask fermentation. To understand if the formation of pellets inhibits the pigment production, 100 ml of medium containing pellets was drawn from the bioreactor (50 %DO$_2$, 200 rpm) on day 5 and transferred into 250 mL Erlenmeyer flasks and to Eppendorf tubes (full filled, 0%DO$_2$). These cultures were cultivated again for 5 days under shaking at 200 rpm (both flasks and Eppendorf tubes) without adjusting the pH. When starting this experiment the medium pH was 4.8.

As can be observed in fig. 3(b), the effect of oxygen transfer in shake flasks influenced red pigment production by increasing the absorption spectra in the red visible region of 500 nm. On the opposite, in the Eppendorf tubes, which were tightly closed in order to prevent the air passing inside, there was no observation of pigments (UV – visible scanning 200-600 nm) (fig. 3(a)). Hence, it was confirmed that fungi growing as pellets doesn't have a strong influence on pigment production. In contrast, oxygen availability seemed to be the main factor in this experiment.

(a)   (b)
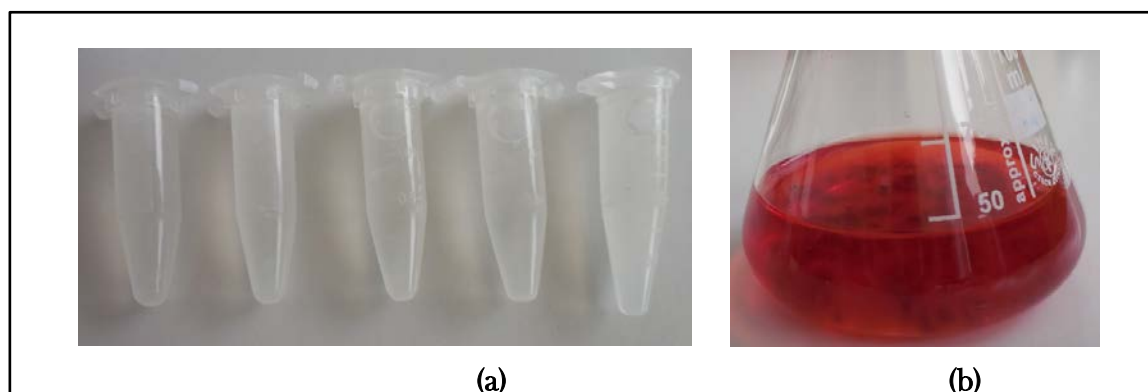
Figure 3: (a) Cultivation of pelleted fungus along with PDB media in full filled Eppendorf tubes by closing lids (day 5); (b) Cultivation of the same pelleted fungus along with PDB media in Erlenmeyer flask with silicon stopper (day 5).

3.3   Importance of pH on pigment production

Normally, pH of the culture medium was found to have an effect on biomass growth and pigment production in most of the filamentous fungi [23]. To fix the pH for fermentation in the bioreactor, several experiments were previously carried out in 250 ml shake flasks with 100 ml volume of culture media by varying the levels of pH (3, 4, 5, 6, 7, and 8) and adjusted each day to maintain the constant pH throughout the fermentation period (Table 1 & 2). Results indicated that growth at pH 5.0 exhibited high production of pigments on day 8. The pigments yield obtained was approximately 22.39 $UA_{470\ nm}$ ± 2.59 and 14.84 $UA_{500\ nm}$ ± 1.93 indicating the orange and red pigments respectively. Pigments excretion into the medium was observed nearly similar rates in both pH 5.0 & 6.0, if considering the standard deviations at both wavelengths of 470 nm and 500 nm (21.7 $UA_{470\ nm}$ ± 1.66; 13.42 $UA_{500\ nm}$± 0.83). For pH 4.0, absorbance at 500nm is also at a similar value (13.29 ± 0.42) compared to pH 5.0 and 6.0, whereas absorbance at 470nm is significantly lower (14.91± 1.03). This shows that the acidic pH favors high pigment production in *T. albobiverticillius* 30548 as noticed in many other strains such as *Penicillium purpurogenum* GH2 [8], *P. aculeatum* ATCC 10409 [24] *Monascus spp.*[25]. However pigments in orange hues (maximum absorbance at 470nm) seem slightly depressed when the pH lowers down to 4.0.

Table 1: Maximum absorbance at 470 nm, representing the region for yellow pigments

pH	3	4	5	6	7	8
Day of maximum absorbance	9	7	8	9	8	9
Maximum absorbance (UA)	6.74	14.91	22.39	21.70	10.14	7.22
± Standard error	± 0.84	± 1.03	± 2.59	± 1.66	± 0.91	± 0.33

Table 2: Maximum absorbance at 500 nm, representing the region for red pigments

pH	3	4	5	6	7	8
Day of maximum absorbance	9	7	8	8	8	9
Maximum absorbance (UA)	4.04	13.29	14.84	13.42	9.94	7.41
± Standard error	± 0.36	± 0.42	± 1.93	± 0.83	± 0.35	± 0.26

The results from shake flasks experiments were used to compare with the values obtained from the batch fermentation in bioreactor (Figs. 4, 5). The pH of value 5.0 was chosen to permit the experiment throughout the entire fermentation period in the bioreactor.

3.4 Effect of aeration rate on pigment production

At pH 5.0 combined with 10% oxygen saturation and at 30 rpm of agitation speed, the mycelia in the bioreactor were noticed as filaments along with the growth of many small

pellets. But we could notice that with a DO_2 of 50% there was no formation of small pellets, neither at 70% nor 90% of dissolved oxygen, with the same stirring speed. This is also influenced by the viscosity of the culture broth and the fluffy pellets clumps makes the air bubbles clumped together rather dispersed even with agitation, resulting lower oxygen transfer rate. So there certainly exists a relation between a supply of O_2 transfer and the fungal cell morphology from filaments to pellets.

At 10% of oxygen supply, the fermentation broth became orange red in color after 4 days of growth and the maximum pigment production was reached on day 8. If the DO_2 increases, the formation of pigments in the media began sooner (day 2 for 50% of DO_2 and less than a day for 70% of DO_2). This is applicable to the fermentation condition up to 70% of DO_2 but in another experiment with DO_2 of 90%, the media allowed the production of red pigments on day 2. In the shake flasks, the broth became red within 3 days of fermentation and the growth was ended after 7 days due to the depletion of nutrients. On day 7, the observed pigment absorbance (4.5 $UA_{470\ nm}$, 5.3 $UA_{500\ nm}$) and biomass (4.88 g/L) was less in bioreactor comparing with shake flasks (20.38 $UA_{470\ nm}$ ±0.43, 18.23 $UA_{500\ nm}$± 0.31, and biomass of 8.10 g/L). The low growth rate in bioreactor might be due to the low transfer rate of oxygen thereby also lowering the pigment production.
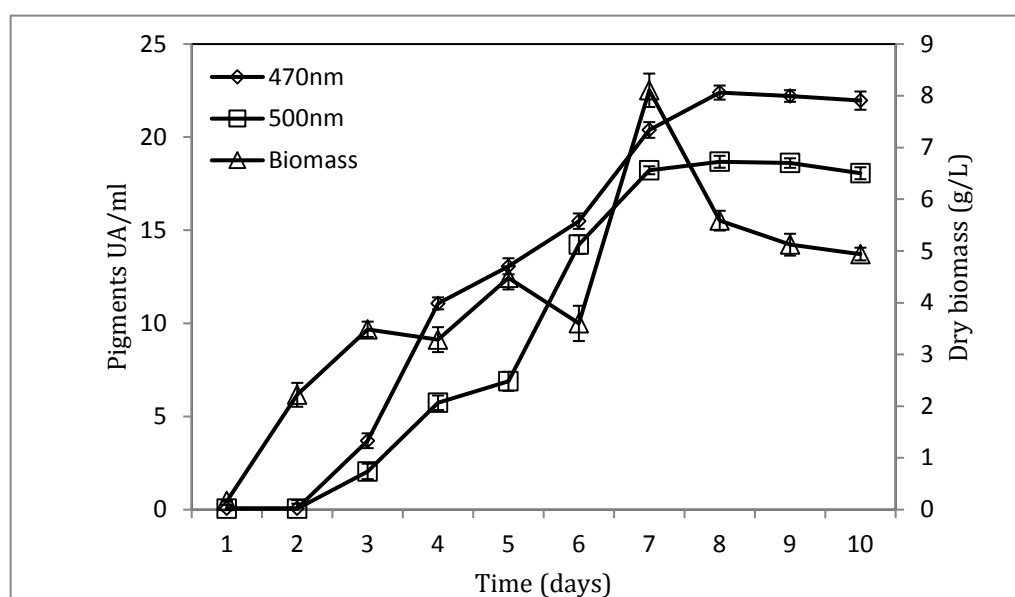
Figure 4: Absorbance of culture liquid (extracellular pigments) and biomass weight of *Talaromyces albobiverticillius* 30548 cultivated in 200 ml working volume in shake flasks with adjusted medium pH 5.0 through the fermentation period. The errors bars in the figure indicates the standard deviation from three independent samples
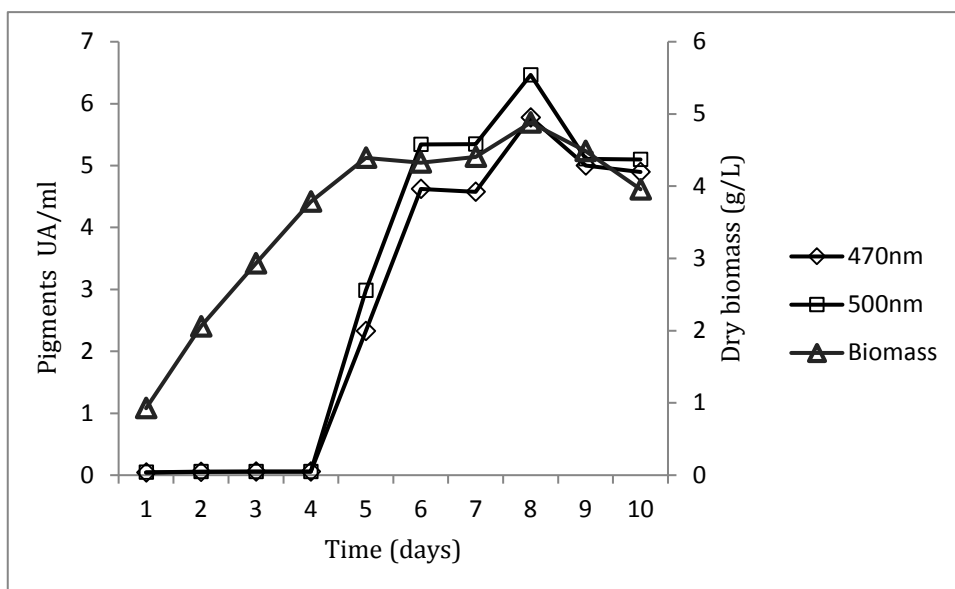
Figure 5: Absorbance of extracellular pigments and dry biomass weight of *Talaromyces albobiverticillius* 30548 in working volume of 1.3 L bioreactor with adjusted medium pH 5.0 throughout the experiment (10% DO₂ and 100 rpm of agitation speed). No standard deviation was calculated from the results as single experiment was made for all the runs performed

3.5 Comparison of color values in fermenter and shake flasks

Absorption spectral graphs for pigments produced in both shake flasks and fermenter has maximum absorption at both 470nm (orange pigments) and 500 nm (red pigments) ; it's absorption values were reported as fig. 4 & 5. The color values of the extracellular fungal extracts of the strain *T. albobiverticillius* 30548 is shown in table 3. For the culture in shake flasks, the chroma values increased to a maximum (40.69) but very low for the extract in the fermenter (1.14). The hue angle observed in the shake flasks was 56.13 which is close to 60 yellowish- red color.

Table 3: Color of the filtrates observed on day 7 using Spectrocolorimeter.

	Coloration	L	a	b	c	h
Colored filtrates without bubbling (pH 5.0 controlled, 0% DO₂ , 200 rpm)		98.5	1.12	-0.21	1.14	349.39
Colored filtrates in shake flasks (initial pH 5.0, 200 rpm)		79	22.68	33.78	40.69	56.13

There is obvious color difference in the extracts obtained from shkae flask and fermenter experiments. The total color difference ΔE in the extracellular pigmented extracts was evaluated and found to be 11.8 which is greater than the value of 6, indicates there exists an

obvious difference in the pigment production. Fig. 6 clearly indicates the difference in the lightness value of colors.
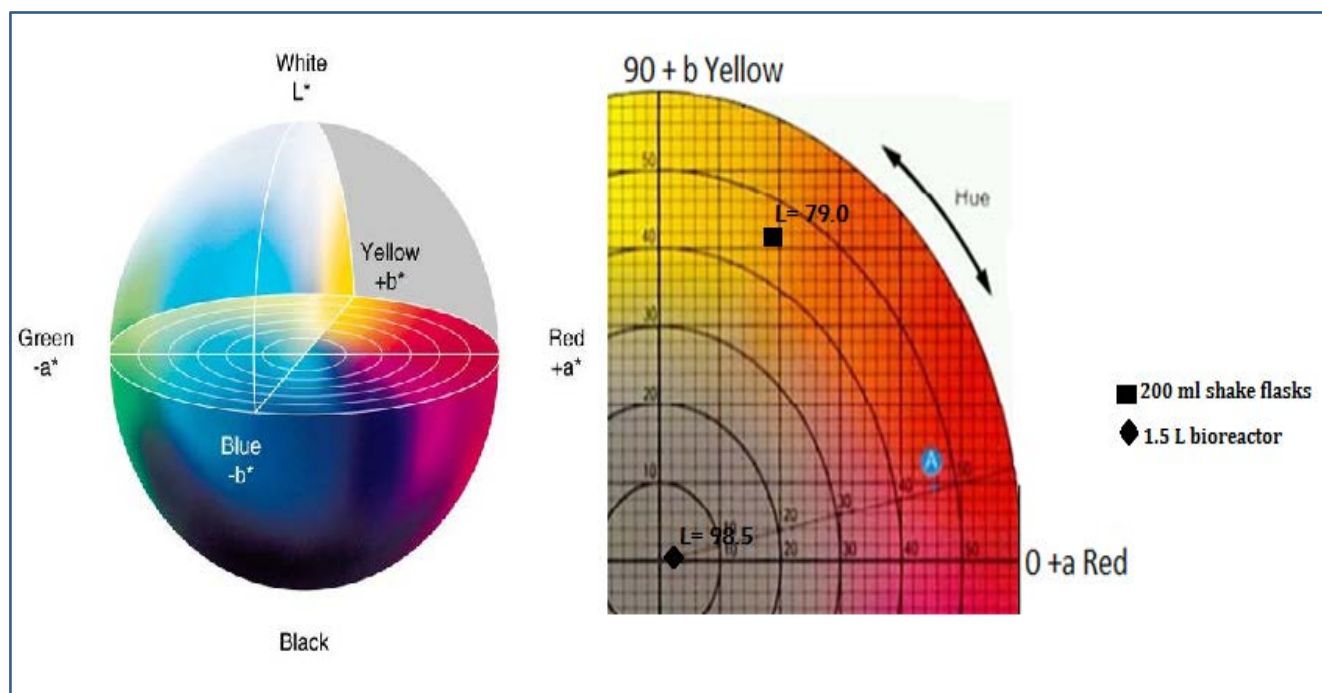
Figure 6: Color wheel showing lightness of colored filtrates in bioreactor (1.5 L working volume) without bubbling (0%DO₂), agitation rate 200 rpm and also in shake flasks (200 mL, 200 rpm) observed on day 7

4.   DISCUSSION

The process of upscaling from laboratory to pilot level then to industrial scale presents several inherent challenges. Changes in fungal morphology such as growing as pellets or mycelia in submerged fermentation has been noticed in many different filamentous fungi such as *Aspergillus*, *Rhizopus* or *Penicillium* strains [26-29]. The formation of pellets due to low agitation speed and the high bubbling may interfere with the O_2 penetration or dissolution, the substrate uptake and thus considerably influences the efficiency of target product formation [30-32]. In this study, the pigment production is thus dramatically depressed. In orbital shake culture flasks using the same PDB as culture media, growth always took place in the form of mycelia, but pellets were observed in slow stirred tank bioreactor under the same basic culture parameters (pH 5, temperature 24° C). This may be partly due to lack of proper oxygen transfer on the culture media in bioreactor, and also to the type of shear forces given by stirrer. This leads to produce growing cells with different morphologies as pellets or filaments

and to decrease the pigments yield in the case of pellets formation. The same behavior was noticed in the *Monascus* fungi in which cell morphology as pellets had an unfavorable effect on final pigment yield, which diminished the pigment production [33]. As a side effect, 2 L bioreactor used has a larger surface area containing liquid media in contact with gas compared to shake flasks, and the fermenter's equipments (agitation impeller, pH & temperature control probes, foam control probe) offer static aerated surfaces. Therefore, the free filamentous fungi seemed to attach and grow on the probes and wall, where P_{O_2} is maximum and feeding medium regularly spilled.

While studying the impact of biomass and pigment production in *Talaromyces albobiverticillius* 30548, it was observed that at low agitation speed (30 rpm) and variable DO_2 rates (10-100 %DO_2) the pigment production was very less and fungus was grown as pellets. Similar observation has been reported for fungal growth as pellets on *Neurospora intermedia* and *Rhizopus oryzae* based on the influence of agitation rates [27, 30]. In addition, agitation speed played a major role in determining the fungal morphology but when considering the pigment production, the effect of dissolved oxygen concentration was greater than that of agitation speed. From observation of current research, at high-speed agitation (>1000 rpm, for 8 days) the structure of fungal mycelia was not damaged.

In shake flasks, the pigment production starts after 48 hours observed as light orange and the fermentation completes within 9 days with dark red pigment production. It has been suggested that red pigments are derived by the chemical reaction from orange precursors [34]. Similar color shift was noticed with the absorbance scan of pigmented extracts of *T. albobiverticillius*, the first one nearby 420 nm and the second one at 500 nm and few days later, the first one moved in the wavelength range from 420 nm to 470 nm.

During scale up, there was a drastic decrease in pigment yield and biomass comparing to shake flask cultures. In the conditions of the experiments, the pigments yield obtained from the bioreactor was considerably low (4.89$UA_{470\ nm}$, 5.09 $UA_{500\ nm}$, in 1,3 PDB working volume, X %DO_2 and Y rpm agitation speed compared to shake flasks (22.21 $UA_{470\ nm}$, 18.61 $UA_{500\ nm}$, 200 ml working volume, at 200 rpm agitation speed). This might be partly due to the low oxygen diffusion which adversely affected the growth (4.88 g/L in fermenter vs 8.10 g/L in shake flasks) thereby the pigment production. Different pH levels influenced the physiology of fungi, conidial development and pigment synthesis. Reducing the pH inhibits the formation of conidia and increases pigment production, suggesting that the pH of the medium might affect

the transport of certain media constituents, such as glucose and nitrogen sources [35]. The pH can affect the activity of enzymes involved in the biosynthesis of pigments [36].

The studied fungus was initially living in a marine environment, at surface of -17m under water. The pressure increases about one atmosphere for every 10 meters of water depth and -17 m was suggested to be between 2 and 3 atm [37].

5.   CONCLUSION

Red and orange pigments, from the azaphilone family were produced with varying concentration, under several sets of conditions tested in bioreactor and flasks.   From this study, it was found that the oxygen supply and stirring mechanical effect may play a vital role on the production of secondary metabolites such as pigments, also on fungal growth. Thus, fungal morphology during cultivation has a major effect on the biomass yield, which again linked to final pigment yield. To study the interaction between the mechanical forces and fungal cells behavior, dissolved oxygen concentration has to be fixed at different levels. Based on the present data, it can be understood that both fungal growth and pigment production are not only affected by medium pH, carbon and nitrogen sources used, but also mainly depends on oxygen transfer and shear forces [38]. In order to obtain a high pigment yield, more experiments should be done to optimize the cultivation conditions specifically improving dissolved oxygen concentration and balancing shear stress. The problem of pH regulation should also be fixed. The obtained results would be helpful to design the oxygen supply strategy in bioreactors for better growth and higher pigment production on large scale.

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8.4   CONCLUSION

The fermentation bioprocess in large scale (1.3 3 L working volume fermenter BIOSTAT®
A PLUS) was carried out under previously optimized conditions (temperature, pH,
concentration of biomass, nutrients, agitation speed). But, the overall mass production in the
set fermenter was not easy to control because different phenomena were simultaneously taking
place. Indeed the relative importance of these parameters changes at least with the scale and
the type of bioreactor. Therefore, oxygen transfer rate appears as a main factor, is influenced
by high number of parameters including physical properties of gas and liquid as well as the
geometrical parameters of the bioreactor. However, in this experiment, there was a "classical"
malfunction particularly with the dissolved oxygen electrode which was regularly invaded by
fungal growth. This should further be anticipated and measures should be taken to provide
consistent air supply despite this major inconvenience. The results of the above work clearly
demonstrates the individual and combined effects of aeration, agitation, gaz pressure and pH
variations on the production of pigments by *Talaromyces albobiverticillius* 30548. Indeed, it
equips an idea for designing an efficient fermentation process in large scale for pigment
production in the future studies.

GENERAL CONCLUSIONS

➤ The research on coastal and marine environment of La Reunion over the years has been conducted by different university laboratories and this study provides an opportunity to explore the effective fungal pigment producers to generate an application of colorants to the food industry. Although, marine-derived strains are promoted as a new and unexplored source of chemistry, few comparative data exist to test the hypothesis that a search of other underexplored ascomycete or basidiomycete fungi would result in an equal, if not better probability of encountering novel biosynthetic gene clusters and associated chemistry. In this study, a variety of marine-derived fungal genera have been isolated from different ecological niches, such as samples collected from dead corals, living corals, hard substrates, sediments and water. Molecular identification of the 47 pigment producing strains included 25 genera and 30 species of which the most predominant ones are *Aspergillus, Emericella, Eupenicillium, Eurotium, Fusarium, Penicillium* producing different hues of pigments.

From the availability of countable pigment producing strains (47 studied strains), a novel red pigment producing potential fungal strain was selected for this complete research work. The chosen marine-derived fungal strain was identified at the species level based on the analysis of ITS, β tubulin and RPB1 gene sequences. It was double characterization by sending the strain to **CBS-KNAW**, Fungal Biodiversity Centre culture collection, Utrecht, the Netherlands. Analysis of the ITS region revealed that the isolated fungi belongs to *Talaromyces albobiverticillius* with 99 % similarity. However, less conservative regions of β-tubulin gene showed the same identification to a level of 99% similarity. Testing evolutionary hypothesis in the form of phylogenetic tree becomes more reliable and it estimates the

relationships among the species represented by those sequences. We generated neighbor-joining analysis with 100 bootstrap pseudo-replicates for each of the data sets using the program MEGA v6. For all analyses, it was identified the species resolved in a clade together with other red pigment producing species such as *T. albobiverticillius, T. atroroseus and T. purpurogenus.*

- *Talaromyces albobiverticillius* produces large amounts of Monascus red pigments and/or their amino acid derivatives as secreted by other species of *Talaromyces* particularly *T. atroroseus, T. aculeatus, T. pinophilus, T. purpurogenus, T. funiculosus, T. amestolkiae, T. ruber and T. stollii.* The red pigment producer *Monascus* species is known to producer six major azaphilone pigments but the production of mycotoxins (citrinin) and unwanted drug (mevinolin) limits the use of *Monascus* for industrial purposes. Some novel producers of *Monascus*-like pigments from *Penicillium* subgenus *Biverticillium, Talaromyces spp* are not reported to produce citrinin or any other known mycotoxins. However, few species produces toxins which are metabolic intermediates not essential to growth or life of the producing organism. Notably, the toxins produced by some fungal species were listed below in the form of a table

Fungal species	Pigments produced	Toxic metabolites	Ref
Monascus spp			
Monascus pilosus, M. ruber, M. purpureus	Monascin, ankaflavin, Rubropunctatin, Rubropunctamine, Monascorubrine, Monascorubramine	Citrinin (Yellow)	
Penicillium spp			
Penicillium citrinum	Anthraquinones (yellow)	Citrinin (yellow)	

P. oxalicum	Arpink red- anthraquinone derivative (red)	Secalonic acid D (yellow)	
P. islandicum	---------	Emodin (yellow) Erythroskyrin (orange-red) Luteoskyrin (yellow) Skyrin (orange) Cyclochlorotine Islanditoxin Rugulosine Rugulovasine A and B	
P. marneffei	Monascorubin (red) Rubropunctatin (orange) Mitorubrinol Monascorubramine (purple red) Purpactin	Secalonic acid D (yellow)	
P. rugulosum	---------	Rugulosin (yellow)	
P. variabile	---------	Rugulosin (yellow)	
P. purpurogenum	Mitorubrin (yellow) Mitorubrinol (orange-red) PP-V (violet) PP-O (orange)	Rubratoxin A (red) Rubratoxin B Luteoskyrin	
P. rubrum		Rubratoxin A (red) Rubratoxin B	
Talaromyces spp			
Talaromyces atroroseus	Gluconic acid Mitorubrin Mitorubrinic acid Mitorubrinol Monascin Monascorubramine Monascorubrin PP-O PP-R Purpuride Purpuroquinone A		

	Rubropunctatin Vermicellin		
T. albobiverticillius	Mitorubrin, Mitorubrinic acid Monascin Monascorubramine Monascorubrin PP-R Rubropunctatin, Vermicellin		
T. purpurogenus		Rubratoxin A Rubratoxin B Rugulovasins Luteoskyrin	

The identified *T. albobiverticillius* 30548 showed rapid growth when cultured using different media (18 – 28 mm) by producing green (on PDA), white and yellow mycelia (MEA, CYA, OA). The fungus diffuses red pigments into the surface media either on agar or into the liquid culture depending on the state of fermentation employed. The culture became mature within 120 h of fermentation and red metabolites will be released during this stage. After the maturation period, further incubation of the culture led the pigments to leach off which drastically changed the red pigments to brown compounds, notably after 192 hours. The morphological features of this strain are strictly biverticillate; stipes are smooth walled and 2.1-2.9 µm wide; metulae (1.3 x 4 µm) are 10 to 18 per stipe; phialides are acerose (1.5 x 3 µm and 3-5 per metulae). While grown on PDA and OA, conidia are smooth to finely rough walled, globose to sub globose, (1.2-2 µm wide).

➢ The extraordinary color range of pigments produced by *T. albobiverticillius* 30548 is in the red and the yellow spectra, although there are definitely more color shades

that need to be explored from the other parts of the visible spectrum. The Commission Internationale de l'Eclairage (CIE) defined a system of describing the colour of an object based on three primary stimuli: red (700 nm), green (546.1 nm), and blue (435.8 nm). Because of the structure of the human eye, all colors appear as different combinations of these. The colorimetric data is measured on the basis of CIELAB system where it uses Cartesian coordinates to calculate a color in a color space. Assessment of the color difference is made using commercially available red, orange and yellow reference standards. The color of the pigments is expressed in terms of hue (color), lightness (brightness) and saturation (vividness). The color value of the extracellular colored solution obtained from the strain is compiled by: L*: 57.12 ±0.06, a*: 56.73±0.12, b*: 42.94±0.19 representing the intensity range of light absorption; hue and chroma values were represented as 37.08±0.23 and 71.14±0.27. Similarly, for the intracellular crude pigmented extract had the colored values with L*: 62.34 ±0.09, a*: 59.28±0.20, b*: 49.33±0.15, h°: 39.8 and C: 73.19. The color variation is influenced by the variation of pigment productions for fermentation; their formation is affected by stage of the fungal strain, substrate composition, environmental factors and length of fermentation. In ethanol, the absorption spectra in the visible region of the whole extracellular colored components showed different patterns of absorption maxima at 427-430, 466-483 nm, 511-522 nm except for a peak which had a single maximum absorbance at 469 nm which is the main compound in the extract; While, the intracellular extract had two absorption maximum of 422 and 511 nm for the red *Monascus*-like pigment and the values are in agreement with the findings for the red N-threonine-monascorubramine.

➢ HPLC-based analyses of crude samples of these pigmented extracts are important because it acts as a powerful tool to access the diversity of pigments production. Reverse phase HPLC with a DAD coupled to a MS, a hyphenated technique in the pigments field unambiguously confirmed the pigment peak and greatly facilitated the recording of MS spectra. Analysis using HPLC-DAD-ESI-MS stated the detection of 12 different compounds in the biosynthesized intracellular and extracellular pigments obtained by submerged fermentation. Four compounds were tentatively identified as *Monascus*-type pigments based on the obtained spectroscopic data and the comparison with literature data. The identified peaks 3, 5 and 8 among the total twelve compounds are similar to the already known N-threonine-monascorubramine, N-glutaryl-rubropunctamine and PP-O respectively.

Compound 3: N-threonine-monascorubramine, under the assumption that the α-amino acid threonine was incorporated into pigment. $[M + H]^+$ m/z 484, and $[M - H]^-$ m/z 482 and the corresponding PDA data were also consistent with the reported values for Monascus type pigments.

Compound 5: N-glutaryl-rubropunctamine, $[M+H]^+$ m/z 484 and $[M - H]^-$ m/z 482 pseudo molecular ions. This compound was discovered for the first time from the extracellular pigment extract obtained from the liquid medium (N1) of *P. purpurogenum* IBT 11181 and also produced by *Monascus ruber*. Having molecular formula $C_{26}H_{29}NO_8$ and mass of 483.20 exhibited the UV-visible spectra (at 430 and 522 nm) very similar to N-glutaryl monascorubramine as the two compounds differed only in their aliphatic side chain.

Compound 8: PP-O, [M + H]$^+$ m/z 413 and [M - H]$^-$ m/z 411 pseudo molecular ions. This compound has also been found to be produced by *Penicillium purpurogenum*IAM15392, which is Monascus azaphilone pigment homologues in culture with a specific medium (20 g soluble starch, 2 g yeast extract per liter of 50mM citric acid/ Na3 citrate buffer, pH 5.0).

The extrolites only identified by HPLC-DAD might in some cases not be the actual metabolite but a derivative with the same chromophore and retention on the column. The tentative identification of the above compounds was only based on the existing literature available data and several reports by various authors. Indeed, for detailed structure analysis and confirmation of the identified compound, the pure fraction was analyzed by ^{1}H NMR and^{13} C NMR.

- Based on the HPLC identifications, the structure of the pigmented compound no: 4 was accomplished using NMR spectroscopy. ^{1}H NMR spectrum to determine bond status, connectivity of protons in a molecule and 2D- NMR spectroscopic methods of correlated homonuclear spectroscopy (COSY) and nuclear overhauser effect spectroscopy (NOESY) was used to obtain even more detailed structural information by analyzing the coupling of protons associated with one or more bonds. NMR analysis was performed for a single compound (compound no: 4) and interestingly, it was identified as a never previously reported compound. A new structure for this molecule, named as 6-[(Z)-2-Carboxyvinyl]-N-GABA-monascorubramine, (or as N-GABA- monascorubramine-derivative) is provided for the first time based on PDA, MS and NMR data by the incorporation of γ-amino acid, γ-aminobutyric acid into the pigment.

 The present NMR data showed the evidence associated with nature of the azaphilone side chains: a) the propyl moiety on the N-7 endocyclic atom; b)

aliphatic seven-member peripheral chain bound to the C13 carbonyl group; c) two contiguous unsaturated CH (namely C10 and C11), linked to the C6 endocyclic carbon atom (long-range coupling C10-H5 in HMBC spectrum). Terminal CH2 (3') and CH (11) of the N7 and C6 side chains were connected to carboxylic acid groups because the HMQC spectrum showed correlations between their proton resonances and those of quaternary carbon atoms at 176.4 ppm (C4') and 171.4 ppm (C12). The compound showed corresponding $[M + H]^+$ m/z 498, and $[M - H]^-$ m/z 496, pseudo molecular ions which are consistent with the proposed structure. The compound has a molecular formula of C27H31NO8, with a mass value of 497 amu.

- This work also focused on the effects of different extraction methods to efficiently extract pigments from the lyophilized fungal biomass (IC) and culture filtrate (EC). Conventionally, the compound is successively extracted with various organic solvents of increasing polarity starting from n-hexane (4 hours), chloroform (15 hours), ethyl acetate (24 hours) to ethanol (24 hours).The extract percentage varied greatly between different solvents, with the highest one obtained for ethyl acetate (96% for IC and 98% for EC) followed by ethanol extract (91% for IC and 93%. for EC). Although this successive solvent extraction method is less laborious and consumes less solvent of other extraction methods (maceration, reflux, decoction, infusion, etc), it presents low selectivity for certain pigmented compounds of interest, mainly in hexane and chloroform extracts. Therefore, it was important to develop a more selective and efficient technique to obtain pigments in larger amounts. In this direction, Microwave-assisted extraction (MAE), Ultrasonic-assisted extraction (UAE), has been used for the extraction of pigments and the yields were compared with conventional extraction.

Effect of various variables such as applied power, volume of solvent, extraction time on the extraction yield and efficiency was investigated. It was found that, MAE exhibited higher crude pigment yield (29.8 mg of intracellular pigments and 29.6 mg of extracellular pigments per gram of sample) in 10 minutes under a constant power of 1000W when compared to UAE and conventional methods. In a study using microwave-assisted extraction process, it offers the advantage of a homogenous thermal regulation of the medium. It is confirmed by obtaining 34.9 mg IC and 25.4 mg EC pigments within 10 minutes using ultrasonic probe of amplitude 77% with a constant temperature of 40°C.

The extracted pigments were quantified by RP-HPLC and extraction performance was assessed in terms of peak reproducibility and extraction yields. The chromatograms were obtained at 470 and 520 nm of ethyl acetate extracts of conventional extraction, MAE and UAE. As expected, in intracellular pigment profile, the main compound is the one absorbing at 426 and 523 nm, identified by a retention time between 15.18 – 16.59 min, denoted as15 peak 2 in conventional and MAE method, peak 1 in UAE 1 This compound is present in major quantities in all the intracellular crude extracts, whatever the extraction process used.

For extracellular pigment profile, an orange pigment (λ_{max} 469.8) was mainly diffused into the extracellular culture medium and the compound was better extracted by UAE (96.53% recovery for peak 2), followed by MAE (90.69% for peak 4) and less amounts by conventional extraction (87.70% for peak 4). But in HPLC chromatogram, the compound had different retention time from 15.56 to 16.85 due to extraction by different techniques. Finally, the extraction techniques can be highly improved and the time of extraction greatly reduced by applying different techniques to disrupt fungal cell wall and membranes.

➢ Some reports have suggested that marine-derived fungi are sensitive to seawater concentration and postulated that the fungi produce decreased amounts of secondary metabolite production. In this study, marine isolate of *Talaromyces albobiverticillius* 30548 was assessed for their tolerance to salinity (0, 3.65, 6, 9% sea salt concentration) towards growth and pigment production. However, the isolated strain can grow very well in sea water/ NaCl free media, the results indicated that the biomass growth of *T. albobiverticillius* 30548 was enhanced in the presence of sea salt to 9.48 g/L at 9% salinity whereas 4.17 g/L is produced under control conditions (0% salinity) grown at 27 °C at 200 rpm for 10 days.

Considering pigment production of this strain at different sea salts concentrated media, the soluble red pigments were diffused into the media at all concentrations of salinity but the concentration of pigments was less (0.46 g/L PY in terms of red rice equivalents at 6% salinity; 1.03 g/L PY in terms of red rice equivalents at 9% salinity) compared to that of control and low sea salt concentration (1.42 g/L PY in terms of red rice equivalents at 0% and 3.65% salinity). This might be due to the electrolytes present in the media altered the pH and prevented the diffusion of pigments. When the concentration of sea salts in the media increased from 3.65 to 9% w/v, pigment solubility decreased and the cells might have encountered stress that inhibit metabolic functions. In addition, high salt concentrations affected the fungal spores which turn the color of spores to brown instead of red. These results suggest that the regulation of secondary metabolite production will be modulated by the sea salt concentration of the culture media.

Discussing the color values of pigments produced in sea salts concentrated media, the color of pigments produced in control media (0%) differs significantly from 6 and 9% salinity. The culture media with 0%, 3.65% and 6% salinity represented red color pigments with hue angles of 23.07 − 37.08 representing purple-red to

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dark red in the color space. While 9% sea salts concentrated media represented a hue angle of 77.08 indicating the dominance of yellow colored pigments in the colored solution. The overall results indicated among the different salinity levels, pigment production and biomass are quite similar in evolution up to 3.5%, there after exhibited an unequal relationship in 6% and 9% sea salts added media.

➢ To more fully realize the biosynthetic potential of the fungi, shaken-submerged and solid-substrate fermentations are often performed in parallel. Scale up of fermentation from laboratory scale to fermenter involves careful attention but designed to address all the limitations coming out from shake-flask experiments. In fermenter systems, careful control of pH, temperature, oxygen supply and gas exchange by the use of sensors. In this study, a small scale fermenter of 2 L is used because it is most similar to in situ conditions for fungal growth because it can be monitored and optimized throughout a run. Among cultivation systems, the pigments yield obtained from the bioreactor was considerably low (4.89UA_{470} nm, 5.09 UA_{500} nm, in 1. 3 PDB working volume, 30 %DO_2 and 100 rpm agitation speed compared to shake flasks (22.21 UA_{470} nm, 18.61 UA_{500} nm, 200 ml working volume, at 200 rpm agitation speed). This could be pointed to some inherent disadvantages, such as poor oxygen dissipation and uncontrolled pH, supply of agitation rate, fungal morphology especially the shape of mycelia in the form of filaments or pellets.

Increasing the agitation speed to 200 rpm and with considerable variation of oxygen transfer rate (70%) resulted in the reduction of pellet formation instead the culture grows rapidly with the production of red pigments (1.25 mg/L pigments on day). The pigment production was enhanced if the pH was maintained at 5.5±0.04 by adding 0.4 N of HCL. It was important to maintain good pH control, because

the activities of the fungal growth as well as diffusion of pigments are known to be pH sensitive.

The results from this study indicate that the mass transfer in the systems was an important process parameter, and that it may have been too high for optimal production of many of the metabolites. It can therefore be inferred that the production of secondary metabolites is influenced not only by factors like carbon or nitrogen limitation but may also be dependent on oxygen limitation and other mass transfer or shear stress parameters.

➢ Optimization of media components and fermentation conditions is becoming acute for large scale production of pigments. Statistical design of experiments (DoE) is a powerful approach to screen the nutrients of fermentation media, has the ability to quickly detect how interactions between factors can affect product yield and quality, outweigh the disadvantages of one factor at a time (OFAT) approach by consuming less time that is achieved by less number of experiments. Initial experiments for optimization of fermentation conditions/ process parameters were carried out based on Box-Behnken design (BBD). The parameters were chosen according to initial experiments carried out in laboratory (pH: 4-9; temperature: 21 – 27 °C; agitation speed: 100 – 200 rpm; fermentation time: 24-336 hours) and also based on the research notes of pigment production in *Penicillium, Talaromyces* and *Monascus spp.*

Box-Behnken design with 4 factors and 3 levels was employed to investigate the best optimized conditions. The data analysis revealed there was an increase in pigment production with an increase in temperature up to 25.5°C beyond this range of temperature, there was a decrease in the pigment production because it affects the rate of biochemical reactions and the enzymatic activity involved in

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biopigment synthesis. Increase in incubation time up to 245 hours (10.2 days) and initial pH ($4 - 7.7$) increased pigment production. The agitation speed up to 180 rpm affected the yield of pigments implying the importance of through mixing for growth and pigment yield.

The adequacy of quadratic models for all responses was on the basis of R^2, F-value and p-level at 1 and 5 per cent level of significance accordingly. The coefficient of determination (R^2) for fungal growth and pigment production was between 0.97-0.98, which is very close to 1 and can explain the fitness of the model. The predicted R2 value of 0.84-0.91 is in reasonable agreement with the adjusted R2 value of 0.82-0.89.

To optimize suitable carbon and nitrogen sources, different nutrient combination media was screened using one-variable-at-a-time (OVAT) analysis and found sucrose combined with yeast extract was chosen as the best media. It provided maximum yield of orange (695.17 mg/L RYR equivalents) and red pigments (738.58 mg/L RYR equivalents) and also higher dry cell weight (6.60 g/L). Significant medium components (yeast extract, K_2HPO_4 and $MgSO_4 \cdot 7H_2O$) were identified form OVAT analysis for pigment and biomass production. A five-level central composite design of the response surface method was applied to evaluate the optimal concentration and the interaction effects between the selected components. Predicted maximum response of OPY (70.87 mg/L), RPY (262.34 mg/L), DBW (15.98 g/L) was obtained at the optimal level of medium variables of yeast extract, K_2HPO_4 and $MgSO_4 \cdot 7H_2O$ at 3 g/L, 1 g/L and 0.2 g/L, respectively. Model verification was performed at the predicted optimal level and the model was well fitted with the experimental results.

FUTURE PERSPECTIVES

➢ Typically, NMR experiments are sensitive both to structural features and to dynamics, and hence the measured data contain information on both. Despite major progress in both experimental approaches and computational methods, obtaining a consistent view of structure and dynamics from experimental NMR data remains a challenge. Herein, due to the insufficient amount of pure compounds, NMR analysis did not reveal the details of all structures for the identified compounds (11 among 12) through HPLC-DAD-ESI-MS. After having higher amounts of pure fractions, the structures of other 11 isolated compounds will be elucidated with the aid of NMR and mass spectroscopic methods.

➢ The bioassays related to the toxicity of red pigments produced by *T. albobiverticillius* were not addressed in this study. Though, *Monascus* red pigments have been exploited as natural food colorants in Western countries; *Monascus spp.* excretes citrinin, a yellow metabolite which possesses nephrotoxic and hepatotoxic properties which is toxic for humans. In addition to citrinin, which has been the major potential threat so far, other potential toxic metabolites of *Monascus* such as monascopyridines have also been reported. Monascopyridines C and D induced metaphase arrest, these effects are indicating an aneuploidic potential which might contribute the formation of tumours. Contamination of these toxic fungal pigments has raised a great concern in the food industry. Similarly, some species of *Penicillium* including *Penicillium citrinum, P.* islandicum, *P. rugulosum, P. tardum*, *P. oxalicum* (secalonic acid, a yellow pigment). If imperative toxicological testing is carried out, fungal pigments

could be accepted by the current consumer. Therefore, studies which address the evaluation of toxicity should be performed in future studies.

- Fungal polyketide azaphilone pigments exhibit a wide range of interesting biological activities, such as anticancerous, antimicrobial, inhibitory effects on HIV-1 replication and gp120-CD4 activity, antifungal, nematicidal and anti-inflammatory activities. Several secondary metabolites from microbial origin inhibit 15-lipoxgyenase (15- LOX). More recently the fungal pigment, (+)-sclerotiorin was found to inhibit lipoxygenase-1, also known as 15-LOX. Geumsanols isolated from *Penicillium sp.* KCB11A109, were evaluated for their anti-cancer, anti-bacterial, anti-malarial activities in zebrafish development. Secondary metabolites of *Colletotrichum sp.* showed anti-bacterial activities against two commonly dispersed environmental strains of *Escherichia coli* and *Bacillus subtilis,* and against two human pathogenic clinical strains of *Staphylococcus aureus* and *Pseudomonas aeruginosa.* The potent nonselective biological activities of azaphilones may be related to their production of vinylogousc-pyridones.

Azaphilones were also found to be a new class of heat shock protein Hsp90 inhibitors. More recently, azaphilones have shown to inhibit tau aggregation and dissolve tau aggregates. *Aspergillus nidulans* secondary metabolites were tested for their ability to inhibit tau aggregation in vitro and it was found that several were active inhibitors at micromolar concentrations, although they did not have tau disaggregation properties. Therefore finding the biological activities of the prodcued secondary metabolites of *Talaromyces albobiverticillius* 30548 enriches the possibilities of new drug discovery towards various applications.

➢ Although many azaphilones have been isolated and identified, their biosynthetic pathways remained unknown for more than half a century. Efficient gene-targeting technology is a powerful tool used to mine the genome for novel secondary metabolites and identify the genes in the biosynthetic pathway. Biosynthetically, most pigments produced by fungi are polyketide-based (some may involve polyketide-amino acid mixed biosynthesis) and involve complex pathways catalyzed by iterative type I polyketide synthases. Generally, the biosynthesis of azaphilones uses both the polyketide pathway and the fatty acid synthesis pathway. There is no current evidence at hand to understand the biosynthetic pathway of pigment production in *T. albobiverticillius* 30548 and so the future study should be focused on the elucidation of biosynthetic pathway of red pigments, which is a proof-of- the-concept study.

➢ At present, the contradiction between the low production and high demands of pigments is acute, giving rise to the need for large-scale production in fermenter. Pigment production process performed in bioreactors outcompete the processes performed in shake flasks in terms of information level due to the higher degree of control. The improved growth and pigment production may be a result of the better hydrodynamics and oxygen transfer. Continuous cultivation and fed batch cultivation offers an opportunity to study the fungal metabolism, characterize the fungal morphology, fine tuning the process and to optimize the production yield under a set of well-defined operating conditions. However, in-depth physiological characterization would be required before continuous process could be designed and implemented for pigment production. Until now, there is no bioreactor data for any species of *Talaromyces* because the cultivation has been made on agar plates or in shake flasks so far.

In addition, extractive fermentation offers a way to extract orange cell-bound *Monascus* pigments into the extracellular broth using a nonionic surfactant micelle solution. This type of solution helps producing higher quantity of red pigments due to the compactable lipophilic nature of orange pigments versus the solubility of hydrophilic amino acids in aqueous solution. Hence, assessing the yield coefficients of the process offers the possibility of quantitatively comparing different process designs. Also, experiments using different parameters help in understanding the response time of biological systems and gives knowledge on the influence of different parameters on pigment production when cultured in large-scale fermenter.

➤ Utilization of low cost substrates is one of the areas under focus on microbial pigment production. There is a growing interest for fungal pigment production, but high cost associated with laboratory nutrient media made the researchers to find the alternative carbon and nitrogen as lost cost substrates. For example, cultivation of *Monascus* in solid-state fermentation (SSF) over steamed rice is very exuberant. There are reports describing some other raw materials used as substrate for Monascus, which include cassava starch, prickly pear juice, and dairy milk. Similarly, an agro-industrial residues such as rice bran, wheat bran, coconut oil cake, sesame oil cake, tamarind seed powder, groundnut oil cake, cassava bagasse, sugarcane bagasse and rice flour (carbon source) were used for carotenoid production in nutrient broth under submerged fermentation to find a cheap nutrient source in order to minimize cultivation cost. Likewise, a systemic evaluation of low cost media components on pigment production in *T. albobiverticillius* 30548 is highly recommended. The utilization of agro-industrial waste as growth substrates may represent an added value to the industry and may

change the production of pigments and color hues giving rise to the possibility of producing various shades.

➢ Although conventional processing technologies, based on solvent extraction, offer a simple approach to isolate microbial pigments, they suffer several inherent limitations including low efficiency (extraction yield), selectivity (purity), high solvent consumption, and long treatment times, which have led to advancements in the search for innovative extraction technologies. In this research work, two different efficient mechanical extraction methods using ultrasound and microwaves were employed and the results stated that both the methods were efficient. Despite these two extraction techniques currently available, there are other different innovative techniques such as pulsed electric fields, liquid pressurization, supercritical fluids, subcritical fluids, and high-pressure homogenization. Focus of the future work is to present an overview of the potentialities of the main emerging extraction methods on fungal matrixes for pigments isolation and recovery. Also to analyse the using technologies, characteristics, advantages, and shortcomings of the different innovative processes, highlighting the differences in terms of yield, selectivity, and economic and environmental sustainability.

➢ The study of colorant stability is an important attribute to predict the quality changes occurring in the food products because their color is easily affected by a number of reactions occurring in the processed foods. In terms of understanding the stability of colorants to pH, heat, light, oxygen, it is mandatory to note the variation of applied polyketide colorants in liquid food model systems like soft

drinks. The quantitative and qualitative color change pattern of the fungal pigment extracts should be compared with the commercially available control colorants like red yeast rice (red), quinizarin (orange), carmine (purple-red to rose).

pH: At certain pH levels, some colorants degrade or shift to a different, less-stable color. Most synthetic dyes have good low-pH stability; while for natural colorants it precipitates or break down at low pH. For example, annatto will precipitate at a pH less than 4.0, and carmine breaks down at a pH less than 3.5; however, these colorants can be used in acidic applications by modifying their form. For instance, an annatto emulsion resists precipitation at low pH. The anthocyanins are most stable and supply red hues at pH less than 3.8, while at higher pH levels, the color is either lost or shifts to unstable blue and/or purplish tones.

Heat: Both synthetic and natural colors generally have good heat stability, in particular Red No. 3, Blue No. 1, Blue No. 2, caramel color, turmeric and carmine. Exceptions include Red No. 40 and some of the anthocyanins, which are not retort-stable. A color's ability to withstand heat is dependent on end-product processing conditions, specifically the temperature, time and point of color addition.

Light: All colors, both synthetic and natural, will eventually fade if exposed to sufficient light. Colorants especially susceptible to light include Red No. 3 and turmeric, while annatto, carmine and some anthocyanins have moderate to good light stability. "Caramel colors have good light stability, plus they absorb UV light, which helps to prevent the degradation of UV-sensitive ingredients in a product, like some nutraceuticals."

Oxygen: Colorants like carmine, carotenoids and paprika can fade in the presence of oxygen. Antioxidants like ascorbic acid or tocopherols can improve the shelf life of the color itself and help maintain the desired shade in the end product. For instance, the ascorbic acid in a beverage protects some colors by scavenging residual oxygen.

Effective colorants not only supply consistent hues, they also disperse evenly in a product without bleeding or precipitating out over time. Color stability can also be affected by the moisture, protein or fat content of the end product at times microbial growth even affects the color stability in moisture containing food products. The color changes in food product across different colorants will be analyzed using the color parameters L*, a* and b* to emphasize the positive or negative effect of colorants on their stability.

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LIST OF PUBLICATIONS & COMMUNICATIONS

<u>RESEARCH ARTICLES</u>

1. Biodiversity of pigmented fungi isolated from marine environment in La Réunion Island, Indian Ocean: New resources for colored metabolites. Mireille Fouillaud, **Mekala Venkatachalam,** Melissa Llorente, Hélène Magalon, Pascale Cuet and Laurent Dufossé. **Journal of Fungi (2017),** 3(3), 36; DOI: 10.3390/jof3030036

2. Production and new extraction method of polyketide red pigments produced by ascomycetes fungi from terrestrial and marine habits. Juliana Lebeau, **Mekala Venkatachalam,** Mireille Fouillaud, Thomas Petit, Francesco Vinale, Laurent Dufossé and Yanis Caro. **Journal of Fungi (2017),** 3(4), 34; DOI: 10.3390/jof3030034

3. Production of pigments from the tropical marine-derived *Talaromyces albobiverticillius*: new resources for red colored metabolites. **Mekala Venkatachalam,** Hélène Magalon, Laurent Dufossé and Mireille Fouillaud. Accepted for publication in **Journal of food composition and analysis,** Manuscript JFCA-D-17-00507, submitted on April 29, 2017.

4. Partial characterization of the pigments produced by the marine-derived fungus *Talaromyces albobiverticillius* 30548. Towards a new fungal red colorant for the food industry. Accepted for publication in **Journal of food composition and analysis**, Manuscript JFCA-D-16-01022, submitted on November 22, 2016.

5. Optimization of pigments and biomass production from *Talaromyces albobiverticillius* 30548 under submerged fermentation conditions using Response Surface Methodology. **Mekala Venkatachalam,** Alain Shum-Chéong-Sing, Prakash Maran Jeganathan, Laurent Dufossé, Mireille Fouillaud. **(Manuscript to be submitted, 2017)**

6. Culture media optimization for enhanced pigment production and biomass growth by *Talaromyces albobiverticillius* 30548 using one-variable-at-a-time and Response Surface Methodology. **Mekala Venkatachalam,** Alain Shum-Chéong-Sing, Laurent Dufossé, Mireille Fouillaud. **(Manuscript to be submitted, 2017)**

7. Effect of sea salts on fungal growth and pigmented metabolites production in marine-derived fungal strain *Talaromyces albobiverticillius*. **Mekala Venkatachalam,** Gérard Lea, Cathie Milhau, Francesco Vinale, Laurent Dufossé and Mireille Fouillaud. **Marine drugs,** under the special issue entitled "Bioactive compounds from marine-derived Aspergillus, Penicillium, Talaromyces and Trichoderma species" **(Manuscript to be submitted before 31st October 2017)**

REVIEW ARTICLES

1. Anthraquinones and derivatives from marine-derived fungi: structural diversity and selected biological activities. Mireille Fouillaud, **Mekala Venkatachalam,** Emmanuelle Girard-Valenciennes, Yanis Caro and Laurent Dufossé. **Marine drugs (2016),** 14(4): 64. DOI: 10.3390/md14040064

BOOK CHAPTERS

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2. Different compounds of phenolic compounds related to foods- Anthraquinones. Mireille Fouillaud, Yanis Caro, **Mekala Venkatachalam,** Alain Shum-Chéong-Sing, Isabelle Grondin and Laurent Dufossé. Chapter 14 in: Phenolic Compounds in Food: Characterization and Analysis, 4th edition, Leo M.L. Nollet & Janet Alejandra Gutiérrez- Uribe (Eds.), CRC Press, Boca Raton, Florida, USA **(in press)**

ORAL COMMUNICATIONS

1. Anthraquinones from marine-derived fungi, Challengers for natural colors in industrial fields?, Mireille Fouillaud, **Mekala Venkatachalam,** Pascale Cuet, Hélène Magalon, Yanis Caro and Laurent Dufossé*. Oral communication, 1st International Conference of the Marine Fungal Natural Products Consortium (MaFNaP 2015), joint with the 14th International Marine and Freshwater Mycology Composium, Nantes, France, July 22-24, 2015 (* presenting author).

2. Isolation and characterization of pigments from *Talaromyces albobiverticillius*: a marine-derived fungus producing polyketide red pigments. **Mekala Venkatachalam*,** Christian Fog Nielsen, Danielle Giuffrida, Yanis Caro, Emmanuelle Girard-Valenciennes, Laurent Dufossé and Mireille Fouillaud. Oral communication, 8th International Conference of Pigments in Food, "Colored foods for health benefits", Cluj-Napoca, Romania, June 28-July 01, 2016 (* presenting author).

POSTER PRESENTATIONS

1. Optimization of fermentation conditions for fungal growth and production of red pigments from *Talaromyces albobiverticillius* using Response Surface Methodology. **Mekala Venkatachalam,** Alain Shum-Chéong-Sing, Yanis Caro, Laurent Dufossé and Mireille Fouillaud. 8th International Conference of Pigments in Food, "Colored foods for health benefits", Cluj-Napoca, Romania, June 28-July 01, 2016

2. Marine-derived fungi producing red anthraquinones: new resources for natural colors? Mirielle Fouillaud, **Mekala Venkatachalam,** Yanis Caro and Laurent Dufossé. 8th International Conference of Pigments in Food, "Colored foods for health benefits", Cluj-Napoca, Romania, June 28-July 01, 2016

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5. Azaphilones pigments from Talaromyces albobiverticillius. Miroslava Zelena, Laurent Dufossé, Daniele Giuffrida, Francesco Cacciola, **Mekala Venkatachalam,** Mireille Fouillaud, Emmanuelle Girard-Valenciennes, Lenka Ceslova, Paola Dugo and Luigi Mondello. 40th International Symposium on Capillary Chromatography and 13th GCxGc Symposium. Palazzo dei Congressi, Riva del Garda, Italy, May 29-June 03, 2016