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Pigments, Microbial[☆]

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Abbreviations

ACNFP Advisory Committee on Novel Foods and Processes
ADI Acceptable daily intake
CWD Cold water dispersible
DDGS Distiller's dry grains with solubles
DXP Deoxyxylulose phosphate
EFSA European Food Safety Authority

GMP Good manufacturing practices
GRAS Generally recognized as safe
HDL High-density lipoprotein
JECFA Joint FAO/WHO Expert Committee on Food Additives
LDL Low-density lipoprotein
OTC Over-the-counter
SCF Scientific Committee on Food

Glossary

Astaxanthin A reddish carotenoid pigment found in salmon, crustacean shells, and bird feathers (e.g., pink flamingos).
Bacteriochlorophyll A form of chlorophyll present in photosynthetic bacteria.
Carotenoids A group of yellow, orange, or red polyene pigments produced in plants and certain microorganisms. Divided into carotenes (hydrocarbons) and xanthophylls (oxygenated carotenes).

Color The sensation resulting from stimulation of the retina of the eye by light waves of certain lengths.
Melanins High molecular weight polymeric indole quinine pigments having brown or black colors.
Phenazines Compounds based on the tricyclic phenazine ring system, some of which are colored, and are secreted by some species of bacteria.
Pigment Any coloring matter in microbial, plant, or animal cells.

Defining Statement

Microorganisms have been used for a long time for production of molecules as diverse as antibiotics, enzymes, vitamins, texturizing agents, and so on. In nature, pigmented bacteria, yeasts, or fungi are quite common, and the main functions of pigments in these living organisms are related to light. The industry is now able to produce some microbial pigments for applications in food, cosmetics, or textile.

Introduction

Nature is rich in colors (minerals, plants, microalgae, etc.), and pigment-producing microorganisms (fungi, yeasts, and bacteria) are quite common (Fig. 1). Among the molecules produced by microorganisms are carotenoids, melanins (Plonka and Grabacka, 2006), flavins, phenazines (Kerr, 2000), quinones, bacteriochlorophylls, and more specifically monascins, violacein, or indigo (Fig. 2; Dufossé, 2004). The success of any pigment produced by fermentation depends on its acceptability in the market, regulatory approval, and the size of the capital investment required to bring the product to market. A few years ago, some expressed doubts about the successful commercialization of fermentation-derived food grade or cosmetic grade pigments because of the high capital investment requirements for fermentation facilities and the extensive and lengthy toxicity studies required by regulatory agencies. Public perception of biotechnology-derived products also had to be taken into account. Nowadays some fermentative food grade pigments are on the market: *Monascus* pigments, astaxanthin from *Xanthophyllomyces dendrorhous* or *Paracoccus carotinifaciens*, Arpink Red (or Natural Red) from *Penicillium oxalicum*, riboflavin from *Ashbya gossypii*, and β -carotene or lycopene from *Blakeslea trispora*. The successful marketing of pigments derived from algae or extracted from plants, both as a food color and a nutritional supplement, reflects the presence and importance of niche markets in which consumers are willing to pay a premium for "all natural ingredients."

Color plays a special role in the food we eat. For example, when confronted with an unattractive color, the consumer assumes that the food is poor or spoiled. On the other hand, products with atypical color – for example, green cheese or blue drink – in most cases, are rejected by the consumers. Typically, one associates colors with food items, such as cherry with red, lemon with yellow, or carrot with orange. Therefore, colors can serve as the primary identification of food and are also a protective measure

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Fig. 1 Spontaneous development of pigmented microorganisms at the surface of nutritive agar Petri dish.

against the consumption of spoiled food. Colors of foods create physiological and psychological expectations and attitudes that are developed by experience, tradition, education, and environment: "We inevitably eat with our eyes."

The controversial topic of "synthetic dyes in food" has been discussed for many years (Mapari *et al.*, 2005). The scrutiny and negative assessment of synthetic food dyes by the modern consumer have given rise to a strong interest in natural coloring alternatives. Some companies decided to "color food with food," using mainly plant extracts or pigments from plants, for example, red from paprika, beetroots, berries, or tomato; yellow from saffron or marigold; orange from annatto; green from leafy vegetables.

Penetration of the fermentation-derived ingredients into the food and cosmetic industries is increasing year after year. Examples could be taken from the following fields: thickening or gelling agents (xanthan, curdlan, and gellan), flavor enhancers (yeast hydrolysate and monosodium glutamate), flavor compounds (gamma-decalactone and diacetyl, methyl-ketones), and acidulants (lactic acid and citric acid). Efforts have been made in order to reduce the production costs of fermentation pigments compared to those of synthetic pigments or pigments extracted from natural sources. Innovations will improve the economy of pigment production by isolating new or creating better microorganisms, by improving the processes. This article focuses on research works related to this field published over the past 10 years by private companies or academic laboratories, with an emphasis on pigments for food use. As recently described by our group, there is "a long way from the Petri dish to the market place," and thus to the product on store shelves.

***Monascus* Pigments – An Old Story for Asians**

***Monascus* Pigment**

Monascus is cultivated on solid medium in Asian countries to produce a red colorant named "Anka" used as a food ingredient. In a Chinese medical book on herbs published in the 1st century, this term "ang-kak" or "red mold rice" was first mentioned. Red mold rice has been used as a food colorant or spice in cooking. In 1884, a purple mold was isolated on potato and linseed cakes and was named *Monascus ruber*. This ascomycete was so named as it has only one polyspored ascus. Then in 1895 another strain was isolated from the red mold rice obtained from the market in Java, Indonesia. This fungus was named *Monascus purpureus*. Later, several other species were isolated around the world. *Monascus* is often encountered in oriental foods, especially in southern China, Japan, and Southeastern Asia. Currently, more than 50 patents have been issued in Japan, the United States, France, and Germany, concerning the use of *Monascus* pigments for food. Annual consumption of *Monascus* pigments in Japan moved from 100 t in 1981 to 600 t at the end of the 1990s and was valued at \$1.5 million. New food applications, like the coloration of processed meats (sausage and hams) and marine products like fish paste, surimi, and tomato ketchup, were described.

The Fungus *Monascus*

The genus *Monascus* was divided into three species: *pilosus*, *purpureus*, and *ruber*, which comprise the majority of the strains mainly isolated in oriental food.

The Fungal Metabolites

The main metabolites produced by *Monascus* are polyketides, which are formed by the condensation of one acetylcoA with one or more malonylcoA with a simultaneous decarboxylation as in the case of lipidic synthesis. They consist of the pigments, monacolins, and, under certain conditions, a mycotoxin.

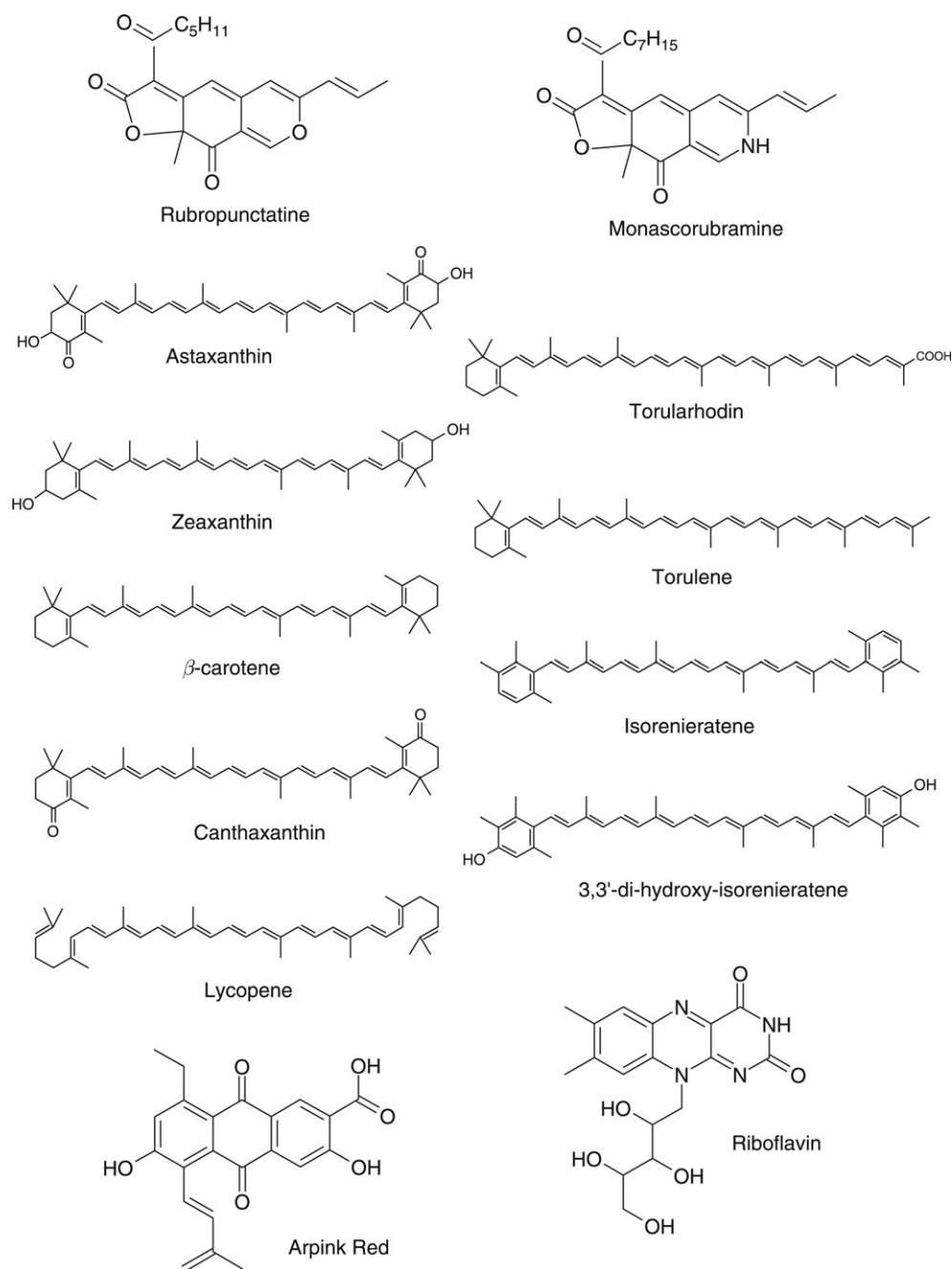


Fig. 2 Chemical formulae of some microbial food-grade pigments.

Monascus pigments are a group of fungal polyketide metabolites called azaphilones, which have similar molecular structures as well as similar chemical properties. Two molecular structures of the *Monascus* pigments are shown on Fig. 2. Ankaflavine and monascine are yellow pigments, rubropunctatine and monascorubrine are orange, and rubropunctamine and monascorubramine are purple. The same color exists in two molecular structures differing in the length of the aliphatic chain. These pigments are produced mainly in the cell-bound state.

They have low water solubility, are sensitive to heat, unstable in the pH range of 2–10, and fade with light. A number of methods have been patented in order to make water-soluble pigments. The principle is the substitution of the replaceable oxygen in monascorubrine or rubropunctatine by nitrogen of the amino group of various compounds such as amino acids, peptides, and proteins, changing the color from orange to purple. *Monascus* pigments can be reduced, oxidized, and react with other products, especially amino acids, to form various derivative products sometimes called the complexed pigments. Glutamyl-monascorubrine and glutamyl-rubropunctatine were isolated from the broth of a submerged culture.

Stability of the pigments is affected by acidity, temperature, light, oxygen, water activity, and time. It was shown that these pigments added to sausages or canned pâté remained stable for 3 months' storage at 4°C, while their stability ranged from 92 to 98%. Thus, the main patents have focused on the solubilization, the stability and the extraction in solution of pigments. The pigments can easily react with amino group-containing compounds in the medium, such as proteins, amino acids, and nucleic acids, to form water-soluble pigments.

A series of hypocholesteremic agents has been isolated from *Monascus* and named monacolin J, K, and L. These polyketides were first isolated from cultures of *Penicillium citrinum* and they can inhibit specifically the enzyme controlling the rate of cholesterol biosynthesis. They are currently used in China in traditional and modern medicine.

Antibacterial properties of *Monascus* were first mentioned in 1977. The so-called monascidin A was effective against *Bacillus*, *Streptococcus*, and *Pseudomonas*. It was shown that this molecule was citrinin and its production by various *Monascus* species was studied using different culture media and conditions.

The Production in Various Modes of Cultures

Submerged cultures

Considerable contradiction exists in the published works as to the best carbon source for red pigment production in liquid cultures. Traditionally cultured on breads and rice, *Monascus* grows on every amylaceous substrate. *Monascus* grows quite well on starch, dextrans, glucose, maltose, and fructose. High production of pigments was achieved using glucose and maltose. The nitrogen source seems to have more importance than the carbon source and ammonium, and peptones as nitrogen sources gave superior growth and pigment concentrations compared to nitrate. The best results were obtained using glucose and histidine. The C/N ratio was also shown to be important: at a value close to 50 g g⁻¹ growth would then be favored, while in the region of 7–9 g g⁻¹, pigmentation would be favored.

Solid-state cultures

The classical Chinese method consists in inoculating steamed rice grains spread on big trays with a strain of *Monascus anka* and incubating in an aerated and temperature-controlled room for 20 days.

In these types of cultures, moisture content, oxygen and carbon dioxide levels in the gas environment, as well as cereal medium composition, are the most important parameters to control.

Moisture content is a very important parameter. Red pigments were produced in plastic bags containing rice grains. It was observed that pigmentation occurred only at a relatively low initial moisture level (26–32%). Initial substrate moisture content regulated pigmentation as it was found that glucoamylase activity increased along with a rise in initial substrate moisture content. Therefore, at high moisture content, as high enzyme activity was produced, glucose was rapidly liberated in amounts (120 g L⁻¹), which inhibited pigmentation. The sugar was then transformed into ethanol.

As far as cultures on rice are concerned, an optimal pigmentation was found at an initial moisture content of 56%, while lower moisture contents led to a large decrease in pigment formation. Thus, it was confirmed that solid culture was superior to liquid culture for red pigment production by *M. purpureus*. This result has been attributed to the derepression of pigment synthesis in solid systems due to the diffusion of intracellular pigments into the surrounding solid matrix. In submerged culture, the pigments normally remain in the mycelium due to their low solubility in the usually acidic medium.

Levels of oxygen and carbon dioxide in the gas environment influence pigment production significantly while affecting growth to a lesser extent in solid state culture. With *M. purpureus* on rice, maximum pigment yields were observed at 0.5 × 10⁵ Pa of oxygen partial pressure in closed pressure vessels. However, high carbon dioxide partial pressures progressively inhibited pigment production, with complete inhibition at 105 Pa. In a closed aeration system with a packed-bed fermentor, oxygen partial pressures ranging from 0.05 to 0.5 × 10⁵ Pa at constant carbon dioxide partial pressures of 0.02 × 10⁵ Pa gave high pigment yields with a maximum at 0.5 × 10⁵ Pa of oxygen, whereas lower carbon dioxide partial pressures at constant oxygen partial pressures of 0.21 × 10⁵ Pa gave higher pigment yields. Maximum oxygen uptake and carbon dioxide production rates were observed at 70–90 and 60–80 h respectively, depending on the gas environment. Respiratory quotients were close to 1.0 except at 0.05 × 10⁵ Pa of oxygen and 0.02 × 10⁵ Pa of carbon dioxide partial pressures.

When studying various cereal media, it was shown that the best results were obtained using “mantou” meal (yeast-fermented wheat meal).

Methods Developed to Avoid Mycotoxin Production

In order to identify chemically the so-called monascidin A discussed by some Chinese scientists in their papers as a component suitable for the preservation of food, it was isolated and chemical investigations using mass spectrometry and NMR were undertaken. Monascidin A was characterized as citrinin. Thus, in order to avoid the production of this toxin, various strains were screened in order to see if all were toxinogenic, and it was shown that in the species of *Monascus* available in public collections, nontoxinogenic strains were obtainable.

Another way to avoid the production of citrinin can be through controlling the biosynthesis of the metabolite. To control the biosynthesis, the metabolic pathway has to be investigated. The metabolic pathway is the same for citrinin and the pigment: the

polyketide pathway in which condensation of acetates and malonates occurred. In the case of the pigment, there is at the end of the pathway an esterification of a fatty acid on the chromophore to obtain the colored molecules. Several modifications of the culture conditions are possible in order to increase the pigment production or reduce the citrinin one: addition of fatty acids and change of the nitrogen source. Adding fatty acids to the medium was effective in favoring the synthesis of pigment, but the citrinin production remained unchanged.

The final modification of the culture conditions was the replacement of glutamic acid by other amino acids. *M. ruber* was cultivated in a liquid medium containing glucose and various amino acids, and histidine was found to be the most effective nitrogen source regarding citrinin production inhibition. When the pathway of histidine assimilation was investigated, it was shown that during its catabolism, one molecule of hydrogen peroxide was produced per molecule of consumed histidine, and it is known that peroxidases can destroy citrinin in the presence of hydrogen peroxide. So, the production of citrinin can be avoided by control of the medium especially by the selection of a suitable amino acid, usually histidine.

Despite the enormous economic potential of *Monascus* pigment, it does not lead to a commercial exploitation in the Western world, mainly because of ignorance and also reluctance to change from food public agencies. Indeed these agencies do not approve *Monascus* pigments for use in the food industry, although they do appear to be non-toxic if correctly used. Thus, even though species of *Monascus* have been consumed in the Far East for many years, this does not help the pigment to gain approval in the European Union or the United States.

Using next-generation sequencing and optical mapping approaches, a 24.1-Mb complete genome of a *M. 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 (Yang *et al.*, 2015). It consists of eight 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, 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 it seems 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.

Monascus-Like Pigments From Non-Toxicogenic Fungal Strains

Some species of *Talaromyces* secrete large amounts of red pigments. In the literature, this biosynthetic potential has been linked to species such as *Talaromyces purpurigenus*, *Talaromyces marneffei*, and *Talaromyces minioluteus* often known under their previous *Penicillium* names. However, since some of them do not exert enough stability for pigment production, then such species should be avoided for scale-up production.

Isolates identified as *T. purpurigenus* have been reported to be of industrial interest. They can produce extracellular enzymes and red pigments, but may also produce mycotoxins such as rubratoxin A and B and luteoskyrin in addition to extrolites that may be toxic following intraperitoneal (spiculisporic acid) and intravenous (rugulovasine A and B) injections in cats. Consequently, mycotoxin production may limit the use of isolates of a particular species in biotechnology, and some authors concluded that *T. purpurigenus* may thus not be recommended for industrial production of red pigments. *Talaromyces atrovirens* sp. nov. produces the azaphilone biosynthetic families mitorubins (Frisvad *et al.*, 2013) and *Monascus* like-pigments without being accompanied by mycotoxin synthesis (patent applications WO2012022765, US 20110250656). As it has been found for *Monascus*, these azaphilone pigments may react with amino groups containing compounds, to which reaction they owe their name, providing intense dark red colors. *Talaromyces albiverticillius* isolated from Réunion island lagoon, Indian Ocean, is also under investigation.

Arpink Red From *Penicillium oxalicum* – Still a Strange and Unclear Case

Many patents from Ascolor s.r.o. (Czech Republic) relate to a new fungus strain having the properties to produce a red colorant that can be applied in the food and cosmetic industries (WO9950434, CZ285721, EP1070136, US6340586). The strain *P. oxalicum* var. *Armeniaca* CCM 8242, obtained from soil, produces a chromophore of the anthraquinone type (Figs. 1 and 3). Some strains of the same species are effective as biological control agents, for example, reduction of the incidence of *Fusarium* wilt of tomato under glasshouse and field conditions. Others have been described for production of a milk-clotting enzyme.

The cultivation of the fungus in liquid broth requires carbohydrates (such as sucrose and molasses), nitrogen (corn extract and yeast autolysate or extract), zinc sulfate, and magnesium sulfate. The optimum conditions for performing the microbiological synthesis are pH value 5.6–6.2 and temperature 27–29°C.

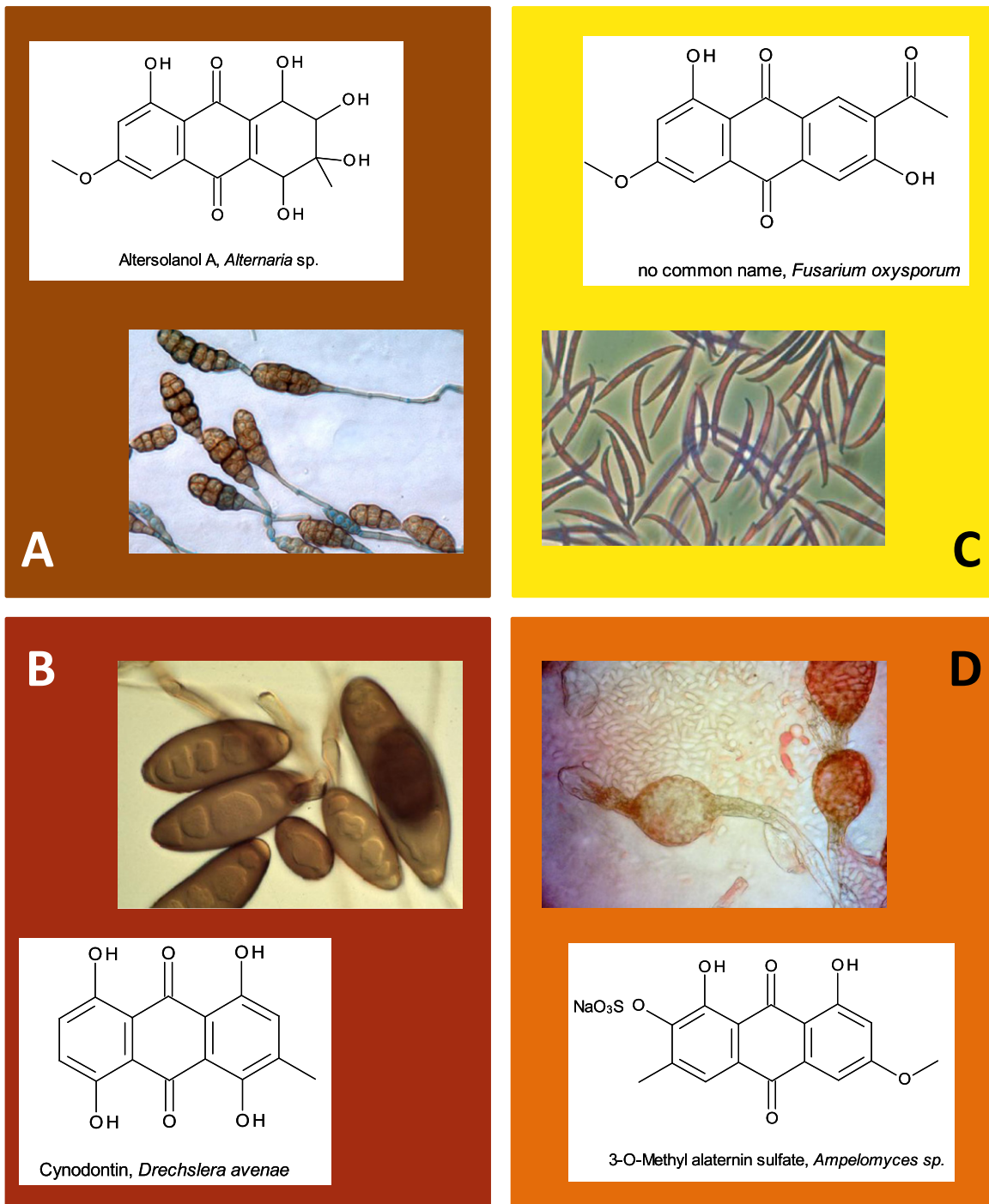


Fig. 3 Some anthraquinones from fungal origin (A) maroon, (B) bronze, (C) yellow, (D) red-orange (color of the box reflects color of the main pigment produced by the fungus).

On the second day of incubation a red colorant is released into the broth, increasing up to 1.5–2.0 g L⁻¹ of broth after 3–4 days. After biosynthesis of the red colorant is completed, the liquid from the broth is filtered or centrifuged for being separated from the biomass. The liquid is then acidified to pH 3.0–2.5 to precipitate the colorant. The precipitate is dissolved in ethyl alcohol and filtered. Following removal of alcohol, the colorant in the crystalline form is obtained, that is, dark red powder.

The colorant gives a raspberry red color in aqueous solution, stable at pH over 3.5. Neutral solutions are stable even after 30 min of boiling and color shade does not change in relation with pH.

Many toxicological data are available on this red pigment: acute oral toxicity in mice 90-day subchronical toxicological study, acute dermal irritation/corrosion, acute eye irritation/corrosion, antitumor effectiveness, micronucleus test in mice, AMES test (*Salmonella typhimurium* reverse mutation assay), estimation of antibiotic activity, and results of estimation of five mycotoxins. A new patent on Arpink Red was filed in 2001 with claims of anticancer effects of the anthraquinone derivatives and applications within the food and pharmaceutical fields.

After evaluating all the materials provided by the company Ascolor s.r.o., the Codex Alimentarius Commission (Rotterdam meeting, 11–15 March 2002) made the following statement: “there will not be any objections to use the red coloring matter Arpink Red” in:

- meat products in the amount up to 100 mg kg⁻¹,
- meat and meat product analogs in the amount up to 100 mg kg⁻¹,
- nonalcoholic drinks in the amount up to 100 mg kg⁻¹,
- alcoholic drinks in the amount up to 200 mg kg⁻¹,
- milk products in the amount up to 150 mg kg⁻¹,
- ice creams in the amount up to 150 mg kg⁻¹,
- confectionery in the amount up to 300 mg kg⁻¹.

JECFA evaluation process is in progress and Arpink Red situation was discussed during the 63rd meeting of Joint FAO/WHO Expert Committee on Food Additives in Geneva, 8–17 June 2004. File is also under progress at the European Food Safety Authority (EFSA) under the reference EFSA-Q-2007-021.

Other Fungal Anthraquinones

Anthraquinones are widely spread in the kingdom of fungi (Fouillaud *et al.*, 2016), and thus, the latter might serve as alternative sources being independent of agro-climatic conditions in contrast to plant and animal derived sources. For example, anthraquinones were found in *Aspergillus* sp., *Eurotium* sp., *Fusarium* sp., *Dreschlera* sp., *Penicillium* sp., *Emericella purpurea*, *Curvularia lunata*, *Mycosphaerella rubella*, *Microsporium* sp., etc.

Anthraquinones exhibit a broad range of biological activities, including bacteriostatic, fungicidal, antiviral, herbicidal, and insecticidal effects. Presumably, in fungi, these compounds are involved in interspecific interactions. For example, anthraquinones synthesized by endophytic fungi protect the host plant from insects or other microorganisms.

The present picture of fungal anthraquinones is quite complex, with a great variety of chemical structures, a huge number of factors or parameters which may have impact on the composition of quinoidal pigments biosynthesized by a particular species. Among them, for example, habitat, light, pH, temperature, O₂ transfer, liquid/solid media, culture medium, C and N sources, C:N ratio, presence of organic acids, mineral salts, and inoculum have been considered.

Today, research priority is laid on a small number of fungal anthraquinone-producing species meeting the following profile of requirements established during the identification of potentially safe fungal cell factories for the production of polyketide natural food colorants using chemotaxonomic rationale:

- fungus shall be non-pathogenic to humans,
- fungus shall be non-toxic under a broad range of production conditions,
- fungus shall be able to produce in liquid media.

Riboflavin – The Vitamin B2 But Also a Yellow Food Colorant

Riboflavin (vitamin B2) has a variety of applications as a yellow food colorant. Its use is permitted in most countries. Applications include dressings, sherbet, beverages, instant desserts, ice creams, tablets, and other products. Riboflavin has a special affinity for cereal-based products, but its use in these applications is somewhat limited due to its slight odor and naturally bitter taste. There are numerous microorganisms that produce riboflavin fermentatively. Riboflavin fermentation could be classified into three categories: weak overproducers (100 mg L⁻¹ or less, e.g., *Clostridium acetobutylicum*), moderate overproducers (up to 600 mg L⁻¹, e.g., yeasts such as *Candida guilliermondii* or *Debaryomyces subglobosus*), and strong overproducers (over 1 g L⁻¹, e.g., the fungi *Eremothecium ashbyii* and *A. gossypii*).

Current Carotenoid Production Using Microorganisms

Commercial processes are already in operation or under development for the production of carotenoids by molds, yeasts, and bacteria. The production of β-carotene by microorganisms, as well as by chemical synthesis or from plant extracts is well developed (Table 1; Britton *et al.*, 2004), and several other carotenoids, notably lycopene, astaxanthin, zeaxanthin, and canthaxanthin are also of interest.

Table 1 Microorganisms, a carotenoid source among many others. β -Carotene and β -carotene-containing preparations from various sources

| Trademark | Company | Origin |
|-------------------------------|---------------------------|---|
| AL CARC 9004 | Diana naturals | Carrot |
| Altratene | Allied Industrial Corp. | Chemical synthesis |
| Betanat | Vitatene | <i>Blakeslea trispora</i> |
| Betatene | Cognis nutrition & health | <i>Dunaliella salina</i> |
| CaroPure | DSM | Chemical synthesis |
| CaroPure | DSM | <i>B. trispora</i> |
| Caroxan | Pot au Pin | Carrot |
| Lucarotin | BASF | Chemical synthesis |
| Mixed carotenoids | Global palm products | Palm oil (<i>Elaeis guineensis</i>) |
| Panaferd | JX Nippon Oil & Energy | <i>Paracoccus carotinifaciens</i> |
| Astaxanthin under development | Ajinomoto | <i>Xanthophyllomyces dendrorhous</i> (formerly <i>Phaffia rhodozyma</i>) |

β -Carotene

β -Carotene from *Blakeslea trispora*

The source organism, *B. trispora*, is a commensal mold associated with tropical plants. The fungus exists in (+) and (–) mating types, of which the (+) type synthesizes trisporic acid, a metabolite of β -carotene. On mating the two types in a specific ratio, the (–) is stimulated by trisporic acid to synthesize large amounts of β -carotene. The mold has shown no pathogenicity or toxicity, in standard pathogenicity tests in mice, by analyses of extracts of several fermentation mashes for fungal toxins and by enzyme immunoassays of the final product, the β -carotene crystals, for four mycotoxins.

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 extracted with ethyl acetate, suitably purified and concentrated, and the β -carotene 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 EU specifications as set out in Directive 95/45/EC for coloring materials in food.

The first β -carotene product from *B. trispora* was launched in 1995 at the Food Ingredients Europe business meeting in London. Following the optimization of the fermentation process, many aspects had to be addressed many aspects before the product could be marketed:

- Presentation of the microorganism: A fungus isolated from a natural environment, not genetically modified; yield improvement achieved by classical genetics.
- Guidelines for labeling: Natural β -carotene; natural β -carotene from *B. trispora*; fermentative, natural β -carotene; natural β -carotene from a fermentative source.
- Lobbying from other β -carotene producers (nature-identical, mixed carotenoids from palm oil, β -carotene from the microalgae *Dunaliella*): The EU Health and Consumer Protection Directorate General was asked to give an opinion on the safety of β -carotene from a dried biomass source, obtained from a fermentation process with *B. trispora*, for use as a coloring matter for foodstuffs.
- Safety of the fermentation-produced β -carotene: HPLC analysis, stability tests, and microbiological tests have shown that the β -carotene obtained by cofermentation of *B. trispora* complies with the EC specification for E 160 a(ii), also including the proportions of cis and trans isomers, and is free of mycotoxins or other toxic metabolites. Tests in vitro for gene mutations and chromosomal aberrations with the β -carotene produced by the manufacturer in the European Union showed it to be free of genotoxic activity. In a 28-day feeding study in rats with the β -carotene manufactured in the European Union, no adverse findings were noted at a dose of 5% in the diet, the highest dose level used. In conclusion, evaluation of the source organism and the production process yielded no grounds to suppose that the final crystalline product, β -carotene, differs from the chemically synthesized β -carotene used as a food colorant. The final crystalline fermentation product has been shown to comply with the specification for β -carotene E 160 a(ii) listed in Directive 95/45/EC. The committee considers that " β -carotene produced by co-fermentation of *B. trispora* is equivalent to the chemically synthesized material used as food colorant and is therefore acceptable for use as a coloring agent for foodstuffs" (Table 2).

Today there are other industrial productions of β -carotene from *B. trispora* in Russia and Ukraine, and in León (Spain). The process has been developed to yield up to 30 mg of β -carotene per gram dry mass or about 17 g L⁻¹. *B. trispora* is now also used for the production of lycopene.

Table 2 Isomers described in “ β -carotene” from various sources

| Source | Carotenoids | | | |
|--------------------------------------|-----------------------------|------------------------|--------------------|-------|
| | All-trans β -carotene | Cis- β -carotene | α -Carotene | Other |
| Fungus (<i>Blakeslea trispora</i>) | 94 | 3.5 | 0 | 2.5 |
| Chemical synthesis | 98 | 2 | 0 | 0 |
| Algae (<i>Dunaliella salina</i>) | 67.4 | 32.6 | 0 | 0 |
| Palm oil | 34 | 27 | 30 | 9 |

β -Carotene from *Phycomyces blakesleeanus*

Another mold, *P. blakesleeanus*, is a potential source for various chemicals including β -carotene. The carotene content of the wild-type grown under standard conditions is modest, about 0.05 mg per gram dry mass, but some mutants accumulate up to 10 mg g⁻¹. As for *B. trispora*, sexual stimulation of carotene biosynthesis is essential and can increase yields up to 35 mg g⁻¹. The most productive strains of *Phycomyces* achieve their full carotenogenic potential on solid substrates or in liquid media without agitation. *B. trispora* is more appropriate for production in usual fermenters.

β -Carotene from *Mucor circinelloides*

M. circinelloides wild type is yellow because it accumulates β -carotene as the main carotenoid. The basic features of carotenoid biosynthesis, including photoinduction, are similar in *Phycomyces* and *Mucor*. *M. circinelloides* responds to blue light by activating biosynthesis. Wild-type strains grown in darkness contain minimal amounts of β -carotene because of the low levels of transcription of the structural genes for carotenogenesis. When exposed to a light pulse, the level of transcription of these genes increases strongly, leading to high pigment concentrations. *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.

Applications of β -carotene

Research reports indicate that natural β -carotene possesses numerous benefits for the human body and consistently support the use of β -carotene as part of the human diet. The human body converts β -carotene to vitamin A via body tissues as opposed to the liver, hence avoiding a buildup of toxins in the liver. Vitamin A is essential for the human body in that it assists the body's immune system and helps battle eye diseases, such as cataracts and night blindness, various skin ailments such as acne, signs of aging, and various forms of cancer. β -Carotene has antioxidant qualities. Antioxidants help mediate the harmful effects of free radicals, which are implied in over 60 life-threatening diseases including various forms of cancer, coronary heart disease, premature aging, and arthritis. Additionally, the antioxidant qualities of β -carotene assist the body in suppressing the effects of premature aging caused by UV rays. β -Carotene is also added to numerous cosmetic and body-care products as a nonharmful colorant to improve the attractiveness of the product.

β -Carotene is one of the leading food colorants in the world. β -Carotene has been applied to a range of food and beverage products to improve their appearance to customers, including items such as margarine, cheese, fruit juices, baked goods, dairy products, canned goods, confectionary, and health condiments, to name just a few. β -Carotene is used in various pet foods as both a colorant and a precursor to vitamin A. β -Carotene can be applied to an array of animal foods designed for pets, including dogs, cats, fish, and birds. The antioxidant and precursory vitamin A properties increase the appeal and application of β -carotene in pet foods. Additionally, β -carotene is an important carotenoid that may assist in improving the color of birds, fish, and crustaceans, as well as improving the appearance of the pet food.

Lycopene

Lycopene is an intermediate in the biosynthesis of all dicyclic carotenoids, including β -carotene. In principle, therefore, blocking the cyclization reaction and the cyclase enzyme by mutation or inhibition will lead to the accumulation of lycopene. This strategy is employed for the commercial production of lycopene.

Lycopene from *Blakeslea trispora*

A process for lycopene production by *B. trispora* is now established, with the aim of marketing this product in Europe for use as a nutritional food ingredient and dietary supplement or a food color. Lycopene is an intermediate in the β -carotene biosynthetic pathway and microbial strains that accumulate lycopene are easy to obtain by mutagenesis, molecular biology, or use of inhibitors. In the commercial process imidazole or pyridine is added to the culture broth to inhibit the enzyme lycopene cyclase. The product, predominantly (all-trans)-lycopene (Table 3), is formulated into a 20 or 5% suspension in sunflower oil with α -tocopherol at 1% of the lycopene level. Also available is an α -tocopherol-containing 10 and 20% lycopene cold water dispersible (CWD) product. Lycopene oil suspension is intended for use as a food ingredient and in dietary supplements. The proposed level of use for lycopene in food supplements is 20 mg day⁻¹.

Table 3 Comparison of the chemical compositions of synthetic lycopene, lycopene from tomatoes and lycopene from *Blakeslea trispora*

| Lycopene | Chemical synthesis (%) | From tomatoes (%) | From <i>Blakeslea</i> |
|-------------------|------------------------|-------------------|------------------------|
| All-trans isomer | > 70 | 94–96 | ≥ 90% |
| 5-Cis isomer | < 25 | 3–5 | |
| 9-Cis isomer | < 1 | 0–1 | 1–5% |
| 13-Cis isomer | < 1 | 1 | (Total of cis isomers) |
| Other cis isomers | < 3 | < 1 | |

Approval for the use of lycopene from *B. trispora* was sought under regulation (EC) No 258/97 of the European Parliament and the Council concerning novel foods and novel food ingredients. The European Food Safety Authority was also asked to evaluate this product for use as a food color. It was stated in the application that it is likely that lycopene from *B. trispora* in food supplements would simply replace those supplements containing lycopene from other sources that are already being marketed so that overall consumption levels would not increase. Incorporation of lycopene into foods would result in additional intake.

The dossier first satisfied the United Kingdom Advisory Committee on Novel Foods and Processes (ACNFP) in 2004, but the panel from the European Food Safety Authority, in opinions adopted on 21 April and 5 October 2005, was unable to conclude whether the proposed use levels of lycopene from *B. trispora* would be safe.

Existing authorizations and evaluations on lycopene from various sources are quite numerous. Lycopene, extracted from tomatoes, is authorized as food coloring agent within the European Union (E160d) (Directive 94/36/EC) and the United States (CDR 21 73.295). This restricts the amount that can be added to foods. Lycopene was evaluated by the Scientific Committee on Food (SCF) in 1975 when it was unable to allocate an acceptable daily intake (ADI), but felt able to accept the use of lycopene prepared from natural foods by physical processes, without further investigations, as a coloring matter in food, provided that the amount consumed does not differ significantly from the amount consumed through the relevant foodstuffs. This opinion was reiterated by SCF in 1989. When JECFA evaluated lycopene from natural sources in 1977, they postponed a decision because of lack of data. In 1999 the SCF evaluated synthetic lycopene, but the available data were not sufficient to allow for an acceptance. The SCF concluded "The Committee is not able to allocate an ADI and considers its use in food is unacceptable at present." Synthetic lycopene is currently used as food ingredient but is not approved for coloring matters within the European Union and it is considered generally recognized as safe (GRAS) in the United States (GRAS notice No GRN 000119). In Australia and New Zealand, lycopene is permitted for use as a food color in processed foods in accordance with GMP under Schedule 3 of Standard 1.3.1 in the Food Standards Code. In Japan, tomato color, defined as "a substance composed mainly of lycopene obtained from tomato fruits," is permitted for use as a food additive under the Food Sanitation Law.

To summarize, the lycopene from *B. trispora* is considered by the EFSA Panel to be nutritionally equivalent to natural dietary lycopene, but further safety trials are necessary. While the toxicity data on lycopene from *B. trispora* and on lycopene from tomatoes do not give indications for concern, nevertheless these data are limited and do not allow an ADI to be established. 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.

Lycopene from *Fusarium sporotrichioides*

The fungus *F. sporotrichioides* has been genetically modified to manufacture lycopene from the cheap corn fiber material, the "leftovers" of making ethanol. Corn fiber is abundant (the US ethanol industry generates 4 million tons annually) and costs about five cents a pound. Distiller's dry grains with solubles (DDGS) could also be used as a substrate. Using a novel, general method for the sequential, directional cloning of multiple DNA sequences, the isoprenoid pathway of the fungus was redirected toward the synthesis of carotenoids. Strong promoter and terminator sequences from the fungus were added to carotenoid biosynthetic genes from the bacterium *Erwinia uredovora*, and the chimeric genes were assembled, introduced in the fungus, and expressed at levels comparable to those observed for endogenous biosynthetic genes. Cultures in laboratory flasks produced 0.5 mg lycopene per gram dry mass within 6 days and this is predicted to increase in the next few years.

Astaxanthin

Astaxanthin (3,3'-dihydroxy- β,β -carotene-4,4'-dione) is widely distributed in nature and is the principal pigment in crustaceans and salmonid fish. The carotenoid imparts distinctive orange-red coloration to the animals and contributes to consumer appeal in the market place. Since animals cannot synthesize carotenoids, the pigments must be supplemented in the feeds of farmed species. Salmon and trout farming is now a huge business and feeding studies have shown that astaxanthin is very effective as a flesh pigmenter. There are also reports of beneficial actions of astaxanthin for human health, so its use in supplements is of interest.

Astaxanthin from *Xanthophyllomyces dendrorhous*, formerly *Phaffia rhodozyma*

Among the few astaxanthin-producing microorganisms, *X. dendrorhous* is one of the best candidates for commercial production of (3R, 3'R)-astaxanthin. Many academic laboratories and several companies have developed processes suitable for industrial production.

Several reports have shown that constituents in the medium, among other environmental factors, affect astaxanthin production in this yeast. The effects of different nutrients on *X. dendrorhous* have generally been studied in media containing complex sources of nutrients such as peptone, malt, and yeast extracts. By-products from agriculture were also tested, such as molasses, enzymic wood hydrolysates, corn wet-milling coproducts, bagasse or raw sugarcane juice, date juice, and grape juice. Although such media are often convenient because they contain all nutrients, they suffer from the disadvantage of being undefined and sometimes variable in composition; this may mask important nutritional effects. For this reason, several studies have yielded results that are difficult to interpret in detail because of inadequate characterization of growth-limiting factors in the media. Thus, in order to elucidate the nature of nutritional effects as far as possible, chemically defined or synthetic media were used by some authors. In one study, 11 strains were assayed for their ability to utilize 99 different compounds as single carbon source. In a second study, carotenoid biosynthesis was increased at low ammonium or phosphate levels and stimulated by citrate. Factorial design and response surface methodology could be used to optimize the astaxanthin production. The optimal conditions stimulating the highest astaxanthin were 19.7°C temperature, 11.25 g L⁻¹ carbon concentration, 6.0 pH, 5% inoculum, and 0.5 g L⁻¹ nitrogen concentration. Under these conditions the astaxanthin content was 8.1 mg L⁻¹. Fermentation strategy also has an impact on growth and carotenoid production of *X. dendrorhous*, as shown with fed-batch (e.g., limiting substrate is fed without diluting the culture) or pH-stat (i.e., a system in which the feed is provided depending on the pH) cultures. The highest biomass obtained was 17.4 g L⁻¹.

A major drawback in the use of *X. dendrorhous* is that disruption of the cell wall of yeast biomass is required before addition to animal diet, to allow intestinal absorption of the pigment. Several chemical, physical, autolytic, and enzymic methods for cell wall disruption have been described, including a two-stage batch fermentation technique. The first stage was for "red yeast" cultivation. The second stage was the mixed fermentation of the yeast and *Bacillus circulans*, a bacterium with a high cell wall lytic activity.

Another starting point in optimization experiments is the generation of mutants, but metabolic engineering of the astaxanthin biosynthetic pathway is now attractive. It should be possible to manage carbon fluxes within the cell and resolve competition between enzymes such as phytoene desaturase and lycopene cyclase.

The case of *X. dendrorhous* (*P. rhodozyma*) is peculiar as hundreds of scientific papers and patents deal with astaxanthin production by this yeast but the process has not been economically efficient up to now. New patents are filed almost each year, with improvement in astaxanthin yield, for example, 3 mg g⁻¹ dry matter in a US Patent. European Union recently decided to invest again in this yeast, with research programs led by Goethe University in Germany or the Royal University of London, United Kingdom (PROCAR, ERA-IB-14-073 project).

Astaxanthin from Agrobacterium aurantiacum and other bacteria

Compared to the huge research effort devoted to *X. dendrorhous*, astaxanthin production by *A. aurantiacum* has been investigated to a lesser extent. The first description of astaxanthin biosynthesis in this bacterium was published in 1994. Astaxanthin is one of 10 carotenoids present. The biosynthetic pathway, the influence of growth conditions on carotenoid production, and the occurrence of astaxanthin glucoside were described in two subsequent papers, but commercial processes have not yet been developed.

Numerous screenings have been conducted with the aim of characterizing new biological sources of astaxanthin, and positive targets were isolated such as *P. carotinifaciens* or *Halobacterium salinarium*. The latter is particularly interesting because (1) the extreme NaCl concentrations (about 20%) used in the growth medium prevent contamination with other organisms, so no particular care has to be taken with sterilization; (2) NaCl concentrations under 15% induce bacterial lysis, so that no special cell breakage technique is necessary; and (3) pigments may be extracted directly with sunflower oil instead of organic solvents. This eliminates possible toxicity problems due to trace amounts of acetone or hexane and facilitates pigment assimilation by animals.

Advantages of astaxanthin over other carotenoids

Some of the advantages of astaxanthin over other carotenoids are

- more stable compared to other carotenoids,
- high antioxidant potential (10 times more than β -carotene and 500 times more than α -tocopherol),
- easily cross the blood-brain barrier,
- high tinctorial property.

Astaxanthin is a powerful bioactive antioxidant and has demonstrated efficacy in animal or human models of macular degeneration, a cause of blindness in a large population. It is also helpful in treating Alzheimer's and Parkinson's diseases, and is known to offer protection against cancer. Astaxanthin has been shown to be a powerful quencher of singlet oxygen as evident from in vitro studies. Astaxanthin has stronger antioxidant activity, 10 times higher than β -carotene and more than 500 times more effective than α -tocopherol; astaxanthin has been proposed to be the super vitamin E. The antioxidant property has been demonstrated in a number of biological membranes, and astaxanthin has preventive effects against aflatoxin B1 carcinogenicity. Astaxanthin also has strong activity as an inhibitor of lipid peroxidation mediated by active forms of oxygen. The strong antioxidative activities of astaxanthin suggest its potential as photoprotectant against UV irradiation, and astaxanthin-containing preparations for prevention of light-induced aging of skin have been developed.

Mammals lack the ability to synthesize astaxanthin, or to convert dietary astaxanthin into vitamin A. Unlike β -carotene, astaxanthin has no provitamin activity in these animals. Astaxanthin has been shown in both in vitro and in a study with human

subjects to be effective for the prevention of the oxidation of low-density protein, suggesting that it can be used to prevent arteriosclerosis, coronary artery disease, and ischemic brain development. A number of astaxanthin health products are under study. Studies on rats had shown no toxicity of astaxanthin preparations. Dietary administration of astaxanthin has proved to significantly inhibit carcinogenesis in the mouse urinary bladder, rat oral cavity, and rat colon. In addition it is reported to induce xenobiotic-metabolizing enzymes in rat liver. Astaxanthin has been shown to enhance in vitro antibody production by mouse spleen cells stimulated with sheep red blood cells and in human blood cells in vitro. Furthermore it has not exhibited any mutagenicity in in vitro study at doses up to 14.4 mg day⁻¹ for 2 weeks. There was strong evidence to suggest that astaxanthin was shown to modulate the humoral and nonhumoral immune systems. It enhances the release of interleukin-1 and the tumor necrosis factor α in mice, to a greater extent than canthaxanthin or β -carotene and has the highest cytokinin-inducing activity. Astaxanthin has a significant enhancing action on the production of immunoglobulin A, M, and G and on T-helper cell antibody production, even when suboptimal amounts of antigen are present.

Astaxanthin for salmon and trout feeds

The predominant source of carotenoids for salmonids has been synthetic astaxanthin, which has been used for pigmentation for the last 20 years with FDA approval in 1996. Natural sources of astaxanthin for commercially raised salmonids have been utilized including processed crustacean waste from the krill, shrimp, crab, and crawfish. However, crustacean waste products contain high amounts of moisture, ash, and chitin. Another natural source, *P. rhodozyma* (*X. dendrorhous*) requires a large amount of feed for sufficient pigmentation leading to higher ash contents. The efficiency of utilization of dietary astaxanthin using microalgae for flesh pigmentation of Atlantic salmon and rainbow trout has been demonstrated. For salmon, astaxanthin is even considered as a vitamin essential for the proper development and survival of juveniles. In rainbow trout fed with algae up to 6% of the diet, no major effect on growth or mortalities was observed, and it was concluded to be a safe and effective source of pigment. Astaxanthin has been used to enhance the immune response of fish and shrimp for maximum survival and growth. Also, natural microalgal astaxanthin has shown superior bioefficacy over synthetic form. Full approval in Japan has been received as a pigment in feeds and foods; registration for approval is in progress for the United States, the European Community, and Canada. An amount of 25–100 ppm of carotenoids in the final feed has been considered to give desired pigmentation in various salmonid species. However, the livestock feed market for astaxanthin, presently small, will grow to a comparable size as synthetic ones, which is estimated to \$185 million. The largest market for astaxanthin being aquaculture – making up 24% of total global fisheries – production is currently valued at \$35 billion per annum and is expected to grow to \$49 billion by 2010 (the total carotenoid market is planned to reach 1.53 billion USD by 2021).

Astaxanthin for humans

Limited studies have been carried out about dietary astaxanthin intake by humans. In a study, an astaxanthin-containing drink was used to protect low-density lipoprotein (LDL) from oxidation (astaxanthin was administered 3.6–14.4 mg day⁻¹ over a period of 2 weeks). Progressively slowing down LDL oxidation with increasing doses of astaxanthin was observed, and no ill effects were reported. When 100 mg of synthetic astaxanthin in olive oil-containing meal was given to male volunteers, maximum plasma concentration of 1.24 mg L⁻¹ astaxanthin was observed in the first 6 h postprandially. The relative concentration of total astaxanthin in high-density lipoprotein (HDL) decreases compared to the other lipoprotein fractions in the 72-h study. Based on a study conducted with 40 healthy volunteers, the effect of astaxanthin on mammalian muscle function was reported. Volunteers received one capsule of 4 mg astaxanthin each morning along with food. No significant difference was observed between the treatment and placebo group in any physical parameter measured. The effect of dietary astaxanthin on the health of humans as carried out by Aquasearch Inc. on 33 volunteers consuming daily 3.85 mg (low dose) and 19.25 mg (high dose) for a period of 29 days indicated no ill effects or toxicity due to the consumption of astaxanthin as analyzed by medical and clinical parameters. Other scientists suggested that astaxanthin could be useful for prevention and treatment of neuronal damage associated with age-related macular degeneration, and it may also be effective in treating ischemic reperfusion injury, Alzheimer's disease, Parkinson's disease, spinal cord injuries, and other types of central nervous system injuries. Astaxanthin was found to easily cross the blood–brain barrier and did not form crystals in the eye.

Zeaxanthin

Zeaxanthin (β,β -carotene-3,3'-diol) can be used, for example, as an additive in feeds for poultry to intensify the yellow color of the skin or to accentuate the color of the yolk of their eggs. It is also suitable for use as a colorant, for example, in the cosmetics and food industries, and as a health supplement that may help to prevent age-related macular degeneration.

In the mid-1960s, several marine bacteria were isolated that produced zeaxanthin. Cultures of a *Flavobacterium* sp. (ATCC 21588, classified under the accepted taxonomic standards of that time) in a defined nutrient medium containing glucose or sucrose, as carbon source, were able to produce up to 190 mg of zeaxanthin per liter, with a concentration of 16 mg g⁻¹ dried cellular mass. One species, *Flavobacterium multivorum* (ATCC 55238), is currently under investigation in many studies.

Recently, a zeaxanthin-producing "*Flavobacterium*" was reclassified as a new *Paracoccus* species, *Paracoccus zeaxanthinifaciens* and earlier findings that IPP biosynthesis occurs exclusively via the mevalonate pathway were confirmed. A second strain, isolated in a mat from an atoll of French Polynesia, is peculiar as it also produce exopolysaccharides.

Sphingobacterium multivorum, the new name for *F. multivorum*, was recently shown to utilize the alternative deoxyxylulose phosphate (DXP) pathway. A strain was constructed for overproduction of zeaxanthin in industrial quantities.

As more bacteria are examined, the distribution of the mevalonate and DXP pathways will become better defined, thus facilitating the metabolic engineering of microorganisms with improved production of commercially important isoprenoid compounds including carotenoids.

Canthaxanthin

Canthaxanthin (β,β -carotene-4,4'-dione) has been used in aquafeed for many years in order to impart the desired flesh color in farmed salmonids. A *Bradyrhizobium* sp. strain was described as a canthaxanthin producer and the carotenoid gene cluster was fully sequenced. Interest in canthaxanthin is decreasing since the discovery of extreme overdosage, that is, the deposition of minute crystals in the eye, a fact leading to adverse media attention in the past, and some pressure to limit its use in aquafeeds.

A second bacterium under scrutiny for canthaxanthin production is *Haloferax alexandrinus*, which belongs to the extremely halophilic Archaea, chemo-organotrophic organisms that satisfy some of their energy requirements with light. Members of the family Halobacteriaceae are characterized by red-colored cells, the color in most cases being due to the presence of C50-carotenoids (especially bacterioruberins) as the major carotenoids. Some species have been reported to produce C40-carotenoids and ketocarotenoids as minor carotenoids. Recently, the biotechnological potential of these members of the Archaea has increased because of their unique features, which facilitate many industrial procedures. For example, no sterilization is required, because of the extremely high NaCl concentration used in the growth medium (contamination by other organisms is avoided). In addition, no cell-disrupting devices are required, as cells lyse spontaneously in freshwater. A 1 l scale cultivation of the cells in flask cultures (6 days) under nonaseptic conditions produced 3 g dry mass, containing 6 mg total carotenoid, and 2 mg canthaxanthin. Further experiments in a batch fermenter also demonstrated increases in the biomass concentration and carotenoid production.

A third example is *Gordonia jacobea* (CECT 5282), a Gram-positive, catalase-negative, G + C 61% bacterium that was isolated in routine air sampling during screening for microorganisms that produce pink colonies. Analysis of the carotenoid extracts by HPLC-MS revealed that the main pigment in the isolates is canthaxanthin. The low carotenoid content (0.2 mg g⁻¹ dry mass) in the isolate does not support an industrial application, but after several rounds of mutations, a hyperpigmented mutant (MV-26) was isolated, which accumulated six times more canthaxanthin than the wild-type strain. Apart from their high pigment production, the advantages of mutants of this species from the industrial point of view are (1) the optimal temperature for growth and carotenogenesis is 30°C, which is usual in fermentors; (2) the use of glucose, an inexpensive carbon source, for optimal growth and pigmentation; and (3) the fact that >90% of the total pigments can be extracted directly with ethanol, a nontoxic solvent allowed for human and animal feed. Many other culture media were tested, giving canthaxanthin from 1 to 13.4 mg L⁻¹.

Torulene and Torularhodin

Yeasts in the genus *Rhodotorula* synthesize carotenoids, mainly torulene (3',4'-didehydro- β,ψ -carotene) and torularhodin (3',4'-didehydro- β,ψ -caroten-16-oic acid), accompanied by very small amounts of β -carotene. Most of the research has focused on the species *Rhodotorula glutinis*, but some papers deal with other species such as *Rhodotorula gracilis*, *Rhodotorula rubra*, and *Rhodotorula graminis*.

Feed supplemented with a *Rhodotorula* cell mass has been found to be safe and non-toxic in animals. Its use in the nutrition of laying hens has also been documented. As the β -carotene content in wild strains of *R. glutinis* is low, efforts have been made to increase it through strain improvement, mutation, medium optimization and manipulation of culture conditions (temperature, pH, aeration, and C/N ratio). These studies mainly resulted in an increased yield of torulene and torularhodin, which are of minor interest, though some did succeed in increasing the β -carotene content up to about 70 mg L⁻¹.

Prospects for Carotenoid Production by Genetically Modified Microorganisms

Escherichia coli and Other Hosts

Metabolic engineering is, in a narrow sense, defined as the use of recombinant DNA techniques for the deliberate modification of metabolic networks in living cells to produce desirable chemicals with superior yield and productivity. The traditional assumption was that the most productive hosts would be microbes that naturally synthesize the desired chemicals, but microorganisms that have the ability to produce precursors of the desired chemicals with superior yield and productivity are also considered as suitable hosts.

As a starting point a large number (200) of genes and gene clusters coding for the enzymes of carotenoid biosynthesis have been isolated from various carotenogenic microorganisms and the functions of the genes have been elucidated. Then large range whole genome sequencing arrived and this number increased tremendously.

In bacteria such as *E. coli*, which cannot naturally synthesize carotenoids, carotenoid biosynthesis de novo has been achieved by the introduction of carotenogenic genes. *E. coli* does possess the ability for synthesize other isoprenoid compounds such as dolichols (sugar carrier lipids) and the respiratory quinones. It is thus feasible to direct the carbon flux for the biosynthesis of these

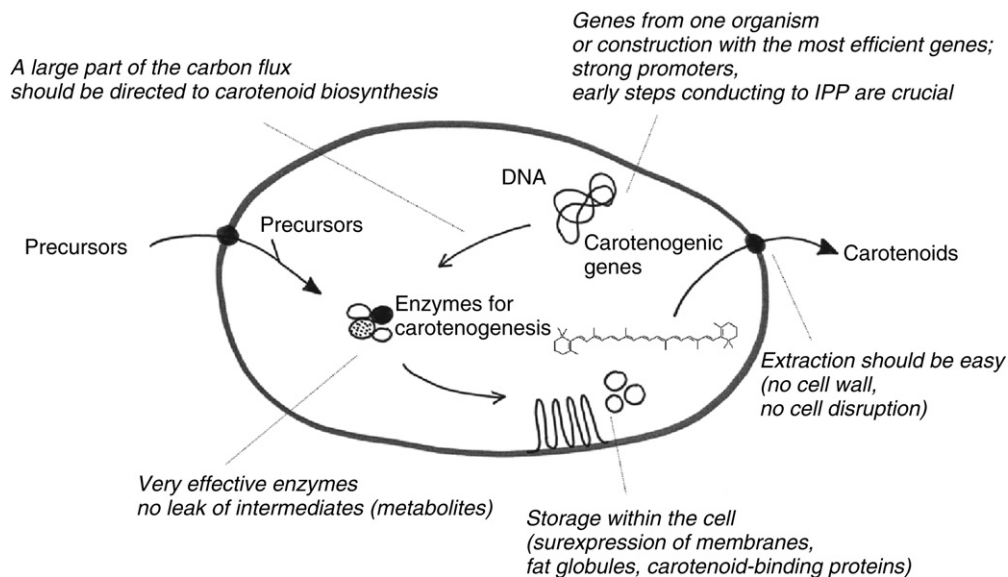


Fig. 4 Metabolic engineering of microbial carotenoid production.

isoprenoid compounds partially to the pathway for carotenoid production by the introduction of the carotenogenic genes. For example, plasmids carrying *crt* genes for the synthesis of lycopene, β -carotene, and zeaxanthin have been constructed and expressed in *E. coli*. Transformants accumulated 0.2–1.3 mg g⁻¹ dry mass of lycopene, β -carotene, and zeaxanthin in the stationary phase. Several intermediates accumulated at the expense of the end product of the pathway. The use of shotgun library clones constructed with *E. coli* chromosomal DNA has revealed that genes not directly involved in the carotenoid biosynthesis pathway are important, such as *appY* that encodes transcriptional regulators related to anaerobic energy metabolism and can increase the lycopene production to 4.7 mg g⁻¹ dry cell mass.

The most important task for biotechnology will be to identify rate-limiting steps or to eliminate regulatory mechanisms in order to enhance further the production of valuable carotenoids. Some stimulating effect has also been reported for *E. coli* strains that overexpress the DXP synthase gene from *Bacillus subtilis* or *Synechococcus leopoliensis*.

Optimization for high-yield carotenoid production should focus on several different aspects (Fig. 4) First, sufficient amounts of endogenous precursors (i.e., substrates for the reactions involved) should be available (e.g., by control of the pyruvate/glyceraldehyde 3-phosphate ratio, a yield of 25 mg lycopene per gram dry mass has been reported). Second, a balanced system of carotenogenic enzymes should be expressed, to enable efficient conversion of precursors without the formation of intermediate metabolite pools. Third, the correct plasmid combination is important to minimize the accumulation of intermediates and to increase the yield of the end product. Last, the host organism should exhibit an active central terpenoid pathway and possess a high storage capacity for carotenoids.

It has also been shown that the edible yeasts *Candida utilis* and *Saccharomyces cerevisiae* acquire the ability to produce carotenoids when the required carotenogenic genes are introduced (same result was observed for the oleaginous yeast *Yarrowia lipolytica*).

Directed Evolution and Combinatorial Biosynthesis

Directed evolution involves the use of rapid molecular manipulations to mutate the target DNA fragment, followed by a selection or screening process to isolate desirable mutants (Schmidt-Dannert *et al.*, 2006). By various directed evolution protocols, several enzymes have been improved or optimized for a specific condition. Directed evolution was applied to geranylgeranyl diphosphate (GGPP) synthase (a rate-controlling enzyme) from *Archaeoglobus fulgidus* to enhance the production of carotenoids in metabolically engineered *E. coli*. The library of mutated genes that was created was transformed into an *E. coli* strain containing the reconstructed isoprenoid pathway and colonies were then color-screened for the increased conversion of glucose into astaxanthin or lycopene. Eight color-enhanced mutants were obtained from over 10,000 colonies. The production of lycopene was increased by about twofold.

A second example deals with the membrane-associated phytoene synthase, which appears to be the major point of control over product diversity. By engineering the phytoene synthase to accept longer diphosphate substrates, variants were produced that can make previously unknown C45 and C50 carotenoid backbones from the appropriate C20 and C25 isoprenyl diphosphate precursors. A C35 carotenoid backbone has also been biosynthesized. Various downstream enzymes (desaturases and cyclases) from the C30 and C40 carotenoid pathways were found to be functional on these unnatural substrates, leading to the production of a series of novel C35, C45, and C50 carotenoids. Thus, it appears that once a carotenoid backbone structure is created, downstream enzymes, either natural or engineered, such as desaturases, cyclases, hydroxylases, and cleavage enzymes, can accept the new substrate, and whole series of novel carotenoids can be produced.

A different approach to expand the recombinant production of known carotenoids and to synthesize new structures is to combine available biosynthetic genes and evolve new enzyme functions through random mutagenesis, recombination (DNA-shuffling), and selection. Prerequisites for this approach are that crt enzymes from different species can function cooperatively in a heterologous host and display enough promiscuity regarding the structure of their substrates. With a few exceptions, such as the zeaxanthin C(5,6) epoxidase gene, almost all cloned carotenoid biosynthetic genes are functionally expressed in *E. coli*. The success of functional color complementation in transgenic *E. coli* for the cloning of a number of carotenoid biosynthesis genes demonstrates that enzymes from phylogenetically distant species can assemble into a functional membrane-bound multienzyme complex through which carotenoid biosynthesis presumably takes place. The enzymes phytoene desaturase (CrtI) and lycopene cyclase (CrtY) have been targeted for evolution in vitro to achieve synthesis of novel carotenoids in *E. coli*. A variant enzyme, a desaturase chimera, efficiently catalyzed the extended desaturation of the linear C40 carotenoid pathway and introduced six rather than four double bonds into phytoene, to favor the production of the fully conjugated carotenoid (3,4,3',4'-tetrahydro- ψ,ψ -carotene).

A related strategy is to combine carotenogenic genes from different bacteria that alone normally produce different end products and to express them in a simple *E. coli* host that carries the biosynthetic machinery for phytoene production. In conjunction with the four-step phytoene desaturase that yields lycopene, a five-step desaturase was used to produce 3,4-didehydro- ψ,ψ -carotene. Further diversification of the C40 skeleton using a 1,2-hydratase and a C-3,4 desaturase yielded a range of carotenoids, including 3,4-didehydro-1,2-dihydro- ψ,ψ -caroten-1-ol, 3,4,3',4'-tetrahydro-1,2,1',2'-tetrahydro- ψ,ψ -carotene-1,1'-diol, and 1',2'-dihydro- β - ψ -caroten-1'-ol.

Concluding Comments

There are several advantages of microorganisms for the study of biosynthesis and function of pigments. Bacteria and fungi offer a tremendous resource in that they produce hundreds to thousands of various pigmented molecules. It is likely that many more natural pigments will be isolated from this biomass (Dufossé, 2016).

Synthetic pigments traditionally used by food or cosmetic processors continue to be utilized with success; however, with the increasing consumer preference for natural food additives, and the surge of the so-called Southampton study which started a worldwide ban on artificial colorants, natural colorants from plants is now a big business and most of the research efforts within the scientific field of colorants are conducted on natural ones. Regarding bacteria, yeast, or fungi (Table 4), despite common belief about the high production cost of fermentation pigments, two initiatives started in Europe these last decades: β -carotene from the

Table 4 Microbial production of pigments (already in use as natural colorants or with high potential in this field)

| Molecule | Color | Microorganism | Status |
|-------------------|----------------|---|--------|
| Ankaflavin | Yellow | <i>Monascus</i> sp. (fungus) | IP |
| Antraquinone | Red | <i>Penicillium oxalicum</i> (fungus) | IP |
| Astaxanthin | Pink-red | <i>Xanthophyllomyces dendrorhous</i> (yeast), formerly <i>Phaffia rhodozyma</i> | DS |
| Astaxanthin | Pink-red | <i>Agrobacterium aurantiacum</i> (bacteria) | RP |
| Astaxanthin | Pink-red | <i>Paracoccus carotinifaciens</i> (bacteria) | RP |
| Canthaxanthin | Dark red | <i>Bradyrhizobium</i> sp. (bacteria) | RP |
| Canthaxanthin | Dark red | <i>Haloferax alexandrinus</i> (bacteria) | RP |
| Canthaxanthin | Dark red | <i>Gordonia jacobea</i> (bacteria) | DS |
| Lycopene | Red | <i>Blakeslea trispora</i> (fungus) | DS |
| Lycopene | Red | <i>Fusarium sporotrichioides</i> (fungus) | RP |
| Melanin | Black | <i>Saccharomyces neoformans</i> var. <i>nigricans</i> (yeast) | RP |
| Monascorubramin | Red | <i>Monascus</i> sp. (fungus) | IP |
| Naphtoquinone | Deep blood-red | <i>Cordyceps unilateralis</i> (fungus) | RP |
| Riboflavin | Yellow | <i>Ashbya gossypii</i> (fungus) | IP |
| Rubrolone | Red | <i>Streptomyces echinoruber</i> (bacteria) | DS |
| Rubropunctatin | Orange | <i>Monascus</i> sp. (fungus) | IP |
| Torularhodin | Orange-red | <i>Rhodotorula</i> sp. (yeast) | DS |
| Zeaxanthin | Yellow | <i>Flavobacterium</i> sp. (bacteria) | DS |
| Zeaxanthin | Yellow | <i>Paracoccus zeaxanthinifaciens</i> (bacteria) | RP |
| Zeaxanthin | Yellow | <i>Sphingobacterium multivorum</i> (bacteria) | RP |
| β -carotene | Yellow-orange | <i>B. trispora</i> (fungus) | IP |
| β -carotene | Yellow-orange | <i>F. sporotrichioides</i> (fungus) | RP |
| β -carotene | Yellow-orange | <i>Mucor circinelloides</i> (fungus) | DS |
| β -carotene | Yellow-orange | <i>Neurospora crassa</i> (fungus) | RP |
| β -carotene | Yellow-orange | <i>Phycomyces blakesleeanus</i> (fungus) | RP |
| Unknown | Red | <i>Penicillium purpurogenum</i> (fungus) | DS |
| Unknown | Red | <i>Paecilomyces sinclairii</i> (fungus) | RP |

Abbreviation: DS, development stage; IP, industrial production; RP, research project.

filamentous fungi, *B. trispora* (produced by Gist-Brocades, now DSM; approved in 2000 by the EU Scientific Committee on Food Safety) and Arpink Red from *P. oxalicum* (manufactured by Ascolor now Natural Red) (Dufossé *et al.*, 2014). These companies invested a lot of money as any combination of new source and/or new pigment drives a lot of experimental work, process optimization, toxicological studies, regulatory issues, and tremendous paper work. Time will tell whether investment was cost-effective. Another development under process is the production of lycopene using *B. trispora* by Vitatene, a subsidiary of Spanish penicillin firm Antibioticos. Exploration of fungal biodiversity is still going on, with special interest in water-soluble pigments. The case of *X. dendrorhous* (*P. rhodozyma*) is very peculiar as hundreds of scientific papers and patents deal with astaxanthin production using this yeast and the process has not been economically efficient up to now. Microorganisms could either be used for the biosynthesis of “niche” pigments not found in plants, such as aryl carotenoids. Carotenoids play an exceptional role in the fast-growing “over-the-counter (OTC) medicine” and “nutraceutical” sector. Among carotenoids under investigation for coloring or for biological properties, a small number are available from natural extracts or chemical synthesis. The list is rather short compared to the long list of 750 entries in the “Carotenoid Handbook.” With imagination, biotechnology could be a solution for providing additional pigments including interesting aryl carotenoids. Isorenieratene (Φ,Φ -carotene), and its monohydroxy and dihydroxy derivatives, can be produced by bacteria, that is, *Brevibacterium linens*, *Streptomyces mediolani*, or *Mycobacterium aurum*.

Research projects mixing molecular biology and pigments were investigated all over the world, and it seems that current productions are not effective in terms of final yield. Nowadays, combinatorial genetic engineering is being addressed, based on an increasing number of known carotenogenic gene sequences. By combining genes, some authors were able to obtain more efficient biosynthesis, or new carotenoids, never described in nature, such as multihydroxylated ones, which could be very efficient as antioxidants.

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Abstract

Nature is rich in colors (minerals, plants, microalgae, etc.), and pigment-producing microorganisms (fungi, yeasts, and bacteria) are quite common. Among the molecules produced by microorganisms are carotenoids, melanins, flavins, phenazines, quinones, bacteriochlorophylls, and more specific monascins, violacein, or indigo. Focus will be first dedicated to *Monascus*, which is cultivated on solid medium in Asian countries to produce a red colorant named "Anka," used as a food ingredient. Despite the enormous economic potential of *Monascus* pigment, it does not lead to a commercial exploitation in the Western world, mainly because of ignorance and also reluctance to change from food public agencies. The second and third cases present, respectively, the production of Arpink Red, a molecule supposed to have a chromophore of the anthraquinone type, and the biosynthesis of riboflavin, the vitamin B2 but also a yellow food colorant. As most industrial applications in the field of microbial pigments deal with carotenoids, examples were selected such as β -carotene, lycopene, astaxanthin, zeaxanthin, and torulene. Applications are numerous in health supplements, animal feed, nutraceuticals, and food colorants. The last part concludes with some prospects for carotenoid production by genetically modified microorganisms, especially directed evolution and combinatorial biosynthesis.

Biographical Sketch



Since 2006 Professor Laurent DUFOSSE has a position at Reunion Island University, located in the Indian Ocean, nearby Madagascar and Mauritius. That volcanic island is one of the French overseas territories with almost one million inhabitants and 15,000 students. Previously, he was researcher and senior lecturer at the University of Western Brittany, Quimper, France, where Pigments in Food International congress occurred in 2004. Laurent DUFOSSE attended the University of Burgundy, Dijon, where he received his PhD in Food Science in 1993. He has been involved in the field of Biotechnology of Food Ingredients for more than 26 years. Before joining the University he was Research Project Leader at private companies such as Sanofi-SKW or Lesaffre in charge of natural aroma production using fermentation. His main research subject is now focused on microbial production of pigments. This activity started 18 years ago and studies were and are mainly devoted to aryl carotenoids, such as isorenieratene, C50 carotenoids and anthraquinones/azaphilones. Links with food science are established within the cheese industry, the sea salt industry, etc.