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Red colorants from filamentous fungi: Are they ready for the food industry?

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Abstract

Food components of microbial-origin have a long history in food science and the food industry. Thickening and gelling agents, flavour enhancers, polyunsaturated fatty acids, flavour compounds, vitamins, essential amino acids, and acidulants are some examples of such ingredients. This paper will provide an update on the current worldwide situation for four different fungal reds: (i) carotenoid lycopene (simple compound, complex current status); (ii) molecular biology on *Monascus* to avoid mycotoxin and cholesterol-lowering substance in pigmented extracts; (iii) newcomers with azaphilone-producing fungi such as *Talaromyces atrovirens*, *Penicillium purpurogenum*, and *Talaromyces albobiverticillius*; and (iv) anthraquinones as a possible alternative to the insect-sourced carmine. The future of

Monascus in Europe and the USA is just around the corner, and markets will appear as soon as the citrinin issue has been solved, with the help of the current better knowledge of full genomes from industrial strains. Fungi bring a new class of pigments to the food industry, as azaphilones are not present in plants. These azaphilone-producing strains should now be thoroughly studied through liquid fermentation of *Penicillium*/*Talaromyces* strains, with optimized scale-up. A fungal alternative to carmine insect anthraquinone is further away from the market, however, due to the particular stability of this vibrant red in foods; research efforts should be intensified.

Keywords: Red pigments; Food ingredient; Colorant; Carotenoid; Lycopene; Azaphilone; Anthraquinone; *Blakeslea*; *Monascus*; *Talaromyces*

1 You're already eating microbial metabolites all day long

Ingredients derived from microbial fermentation are steadily gaining ground in the food industries. Thickening or gelling agents (e.g. polysaccharides such as xanthan, curdlan, gellan), flavour enhancers (yeast hydrolysate, monosodium glutamate), polyunsaturated fatty acids (PUFAs), flavour compounds (gamma-decalactone, diacetyl, methyl-ketones), vitamins, essential amino acids, and acidulants (lactic acid, citric acid) are some examples illustrating this trend (Mc Neil et al., 2013). Efforts have been made, and are continuing, to reduce the production costs of pigments produced by microbial fermentation, since synthetic pigments or those extracted from natural plant sources can often be produced more economically (Dufossé, 2008; Dufossé, 2017a). The successful marketing of natural pigments such as β -carotene, lutein, and astaxanthin derived from microalgae (i.e. non-conventional sources) or extracted from 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 non-conventional sources, filamentous fungi are known to produce an extraordinary range of pigments that include several chemical classes such as carotenoids, melanins, azaphilones, anthraquinones, flavins, phenazines, quinones, and more specifically, violacein and indigo (Dufossé, 2008). The success of any class of pigment produced by fermentation depends on its acceptance by the consumers, regulatory approval, and the capital investment required bringing the product onto the market. Twenty years ago, influential representatives from the food industry expressed doubts about the successful commercialization of algae-derived and fermented food grade pigments due to the high investment required for open ponds, photo-bioreactors and fermentation facilities, and the extensive and lengthy toxicity studies required by the regulatory authorities. Poor public perception of fungal-derived products for food use also had to be taken into account (Milićević et al., 2010). Nowadays, some fungal food grade pigments obtained by fermentation already exist on the market worldwide. Among them, fungal *Monascus* pigments, Arpink red™ (now Natural Red™) produced by *Penicillium oxalicum*, riboflavin from the mould fungus *Ashbya gossypii*, lycopene and β -carotene from the tropical mold *Blakeslea trispora*. As an example, the production yield of β -carotene may be as high as 17g/L of the *Blakeslea trispora* culture medium (Dufossé, 2016; Torres et al., 2016).

The present opinion paper gives an update about the worldwide current situation for some fungal reds chosen for the history of their long use in Asia or Europe, the diversity of their chemical structures, and the strategy of the development from lab research to the market (see Fig. 1):

- Carotenoid lycopene (simple compound, long-standing history, complex current status)
- Molecular biology on *Monascus* to avoid mycotoxin and cholesterol-lowering substance in pigmented azaphilone extracts,

- Newcomers with azaphilone-producing fungi such as *Talaromyces atrovirens*, *Penicillium purpurogenum*, *Penicillium marneffei*, *Talaromyces albobiverticillius*
- Focus on anthraquinones.

insert Figure 1

To sum up, those who are still afraid of fungal products in food and feed, please have a look inside your pigmented plant extracts! Are you sure they are mycotoxin free (Solfrizzo et al., 2015)?

2 Fungal red carotenoid already produced on a large scale: lycopene from *Blakeslea trispora*

In the European Union, the United States of America (USA), Canada, Australia and New Zealand, pioneering work on large-scale production of fungal colorants has been done on carotenoids. Academics knew for a long time that fungi belonging to the order Mucorales were able to produce β -carotene (Ciegler, 1965). The first papers dealing with *Blakeslea trispora* carotenoid production were published in the late 1950s (Ciegler et al., 1959; Ciegler, 1965). It took four decades to move to industrial production, waiting for consumer interest in natural colorants, and to develop biotechnological techniques. The first fungal carotenoid launched in Western Europe was β -carotene between 1995 and 2001 (European Commission directive N° 50/2001), by the Dutch company Gist Brocades (now DSM). Soviet Union companies had already been doing the same thing in Eastern Europe a decade earlier. As lycopene is a metabolic intermediate in the biosynthesis of β -carotene, the use of inhibitors opens the doors for its industrial production, together with the use of lycopene-accumulating and overproducing mutants. Vitatene, a Spanish company, filed a novel foods and novel food ingredients application in 2003, to place lycopene from *Blakeslea trispora* on the European

market (under Regulation EC N° 258/97). A positive response was published on 23 October 2006 (European Commission decision N° 721/2006).

On the market, this biotech colorant has to compete with lycopene extracted from tomato (E160d(ii), listed in European directive 94/36/EC) and with the cost-effective synthetic lycopene (E160d(i)), from chemical synthesis. As a result of the increased demand of consumers for natural and safe food ingredients, the toxicological aspects of biotech lycopene E160d(iii) was investigated within the framework of a ninety-day oral toxicity study in rats (Jonker et al., 2003). The results from this study do not provide any evidence of toxicity for lycopene extracted from the biomass of *Blakeslea trispora* at dietary levels up to 1.0% (w/w, as a suspension in sunflower oil) as demonstrated by the findings of clinical observations, neurobehavioral observations, motor activity assessment, body weight and food consumption measurements, ophthalmoscopic examinations, hematology, clinical chemistry, urinalysis, organ weights, gross pathology, or histopathology. The No-Observed-Effect Level (NOEL) was 1.0% (w/w) in the diet, the highest dietary concentration tested (Jonker et al., 2003).

Lycopene has received particular attention in recent years as a result of studies that have reported that it is a highly efficient antioxidant and has a high singlet-oxygen and free-radical scavenging capacity. Many researchers have shown that lycopene is detected in the plasma and tissues of humans following incorporation into the daily diet. Such studies indicate that lycopene is absorbed and subsequently distributed to the tissues. The natural lycopene sales started on this health market, on functional foods.

Average dietary intakes of lycopene from foods in different populations are, according to dietary surveys, estimated to be between 0.5 and 5 mg/day, with high intakes up to about 8 mg/day. High intakes of fruit and vegetables, especially tomato products, may result in occasional intakes of 20 mg/day or more.

118 In 2005 Vitatene company informed the European Food Safety Authority (EFSA) that use
119 levels of lycopene from *B. trispora* in foodstuffs would lead to an additional intake of up to
120 about 2 mg/day. The proposed use level of lycopene in food supplements would give rise to
121 an additional intake of 20 mg/day. To date, no long-term feeding studies conducted with
122 lycopene extracted from the microorganism *B. trispora* have been performed. The
123 toxicological data on α -tocopherol containing oil suspensions of lycopene from *B. trispora*
124 (90-day oral feeding study) are not sufficient to derive an acceptable daily intake (ADI).
125 EFSA concluded at that time that α -tocopherol-containing an oil suspension of lycopene,
126 obtained from *B. trispora*, for use as a novel food ingredient in foodstuffs leading to an
127 additional intake of up to about 2 mg/day was not of concern from the safety point of view.
128 However, this does not hold for the proposed levels of use of lycopene in foods that would
129 give rise to an additional intake of 20 mg per day.

130 The true use of lycopene as a food colorant is a more complex situation. In Europe, EFSA
131 currently allows this use within the framework of an Acceptable Daily Intake (ADI) of 0.5
132 mg/kg body weight (bw)/day based on No-Observed-Adverse-Effect Level (NOAEL)
133 published data. In 2009 the FAO/WHO Joint Expert Committee on Food Additives (JECFA)
134 replaced the group ADI of 0-0.5 mg/kg bw with a group ADI 'not specified' for lycopene
135 from all sources, creating a divergence (EFSA, 2010). Then, in the USA the Food and Drug
136 Administration approved a petition from LycoRed company seeking the green light to use
137 higher levels of tomato lycopene to restore colour to processed meats, giving manufacturers
138 of sausages, deli meats and jerky an alternative to synthetic FD&C Red #40 (Allura Red AC,
139 EC 129) and 'bug-derived' carmine (Watson, 2014). The present situation will continue to
140 evolve and it is possible that biotech lycopene will be clearly allowed in the next
141 months/years as a true colour in the food industry.

To conclude this overview, it is noteworthy to mention that the development of biotech lycopene took decades (Dufossé, 2017b; Mantzouridou and Tsimidou, 2008) and regulatory aspects continue to affect its use. Such an impressive complexity with this well-known carotenoid is just a small ‘sneak peek’ at the huge efforts yet to be accomplished with other pigmented molecules such as azaphilones and anthraquinones. However, as for some suppliers, lycopene red is ‘more of an orangish-red, and not a true, vibrant red shade’, and is ‘also one of the more expensive natural colour options to use’, the scientific community must continue to investigate for natural reds.

3 Towards a safe use of *Monascus*

Monascus has been used to produce natural colorants and food supplements for more than one thousand years in Asia, and 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 to Qing Yilu in CE 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 (Yang *et al.*, 2015), because azaphilones easily combine with nitrogen-containing compounds. 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 (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 (Yang *et al.*, 2015), 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.* (2007), who have 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.

4 *Monascus*-like pigments (MLPs) produced by *Penicillium*/*Talaromyces* species

Some species of *Talaromyces* (the teleomorphic (sexual) stage of *Penicillium*) secrete large amounts of red pigments. In the literature, this biosynthetic potential has been linked to species such as *Talaromyces purpureogenus*, *T. albobiverticillius*, *T. marneffei*, and *T. minioluteus*, often known under their previous *Penicillium* names (e.g. *Penicillium* sp. from

Japan, Ogihara *et al.*, 2000). However, some of them do not exert enough stability for pigment production, and should then be avoided for scaled-up production (Figure 2).

Woo *et al.* (2014) from Hong Kong investigated another filamentous fungus, *Penicillium (Talaromyces) marneffei*, for 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 (Woo *et al.*, 2014). 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 (Woo *et al.*, 2014).

The aforementioned polyketide gene cluster and pathway have also been shown to be responsible for the biosynthesis of ankaflavin and citrinin, the latter being a mycotoxin exerting nephrotoxic activity in mammals (Kumar *et al.*, 2014). Twenty-three putative PKS genes and two putative PKS-non-ribosomal peptide synthase hybrid genes were identified in the *P. marneffei* genome (Woo *et al.*, 2014). Woo *et al.* (2014) 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 characterized 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 undesirable in normal foods) and citrinin-free red pigments from *P. marneffei*, as the latter, a mycotoxin, appears to be an early byproduct of the biosynthetic pathway.

Isolates identified as *T. purpurogenus* have been reported to be of industrial interest (some of which are investigated in Mexico, Morales-Oyervides *et al.*, 2015). 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 (spiculisporeic acid) and intravenous (rugulovasine A and B) injections in cats (Frisvad *et al.*, 2004). Consequently, mycotoxin production may limit the use of isolates of a particular species in biotechnology, and Frisvad *et al.* (2013) concluded that *Talaromyces purpurogenus* may thus not be recommended for industrial production of red pigments. *Talaromyces atrovirens* sp. nov., described by the same group from Denmark, produces the azaphilone biosynthetic families mitorubins and *Monascus*-like pigments without being accompanied by mycotoxin synthesis (patent applications WO2012022765 Mapari *et al.*, 2012, US20110250656 Mapari *et al.*, 2011). 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 colours (Mapari *et al.*, 2010; Gao *et al.*, 2013).

A strain of *Talaromyces albobiverticillius* isolated in the lagoon from Réunion Island, Indian Ocean, is currently being developed and optimized for red azaphilones production (please refer to the two papers from this Pigments in Food congress, *Journal of Food Composition and Analysis*, current special issue: Fouillaud M. et al., Production of pigments from the tropical marine-derived fungus *Talaromyces albobiverticillius*: new resources for red natural coloured metabolites; Venkatachalam M., Partial characterization of the pigments produced by the marine-derived fungus *Talaromyces albobiverticillius* 30548. Towards a new fungal red colorant for the food industry).

insert Figure 2

5 The anthraquinone quest

Anthraquinones are widely spread throughout the kingdom of fungi (Caro *et al.*, 2016), and thus, the latter might serve as alternative sources since they are 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*, *Microsporum* sp., etc. (Caro *et al.*, 2012; Gessler *et al.*, 2013).

Anthraquinones exhibit a broad range of biological activities, including bacteriostatic, fungicidal, antiviral, herbicidal, and insecticidal effects (Gessler *et al.*, 2013). 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 (Gessler *et al.*, 2013).

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 an effect on the composition of quinoidal pigments biosynthesized by a particular species (Fouillaud *et al.*, 2016). Among them, e.g. 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 (Caro *et al.*, 2012).

Today, research places the priority on a small number of fungal anthraquinone-producing species meeting the following profile of requirements established by Mapari *et al.* (2009) during the identification of potentially safe fungal cell factories for the production of polyketide natural food colorants using chemotaxonomic rationale:

- the fungus shall be non-pathogenic to humans;
- the fungus shall be non-toxigenic under a broad range of production conditions; and
- the fungus shall be able to be produced in liquid media.

6 Plant-pigment preparations may contain fungal metabolites

For most people, plants for food use appear safe, and they appear as sources of pristine ingredients. This is not always true, and we mention here a grape byproduct, namely grape pomaces, as an example. Grape pomaces are used as plant food supplements and food colourings (anthocyanins). Unfortunately, these grape pomaces can be contaminated by ochratoxin A (OTA), a mycotoxin produced mainly by *Aspergillus carbonarius* on grape berries on the vine. OTA possesses nephrotoxic, immunosuppressive, teratogenic, and carcinogenic properties.

Levels of contamination are presented in a nice paper from Solfrizzo et al. (2015). OTA was found in 69% of food colouring samples at levels between 1.16 and 32.00 µg/kg. The situation appear so bad (high incidence of OTA contamination) that the authors recommend to established maximum permitted levels for this mycotoxin in food colouring agents derived from *Vitis vinifera*.

There is also much awareness in the wine industry where OTA decontamination of musts and wines is under study, using inactivated yeasts or yeast cell walls (Petruzzi et al., 2015). Since vintage year 2006, with the adoption of Regulation CE123/05, the level of OTA in commercial wines cannot exceed 2µg/L, but many trade agreements usually require lower limits (e.g. 0.5µg/L).

7 Conclusion: Simple messages I wanted to deliver

These four examples of fungal reds for the food industry describe diverse situations, from products already on the market (lycopene from *Blakeslea*, red rice from *Monascus*) to products still under development (azaphilones from *Penicillium/Talaromyces*, anthraquinones). The future of *Monascus* in Europe and the USA exists, and markets will

appear when the citrinin issue has been resolved with the help of the current better knowledge of full genomes from industrial strains. Fungi bring a new class of pigments to the food industry, because azaphilones are not present in plants. These azaphilone-producing strains must now be better studied through liquid fermentation of *Penicillium/Talaromyces* strains, with optimized scale-up. A true fungal anthraquinone alternative to the insect-based carmine is further away from the market, however, due to the nice stability of this vibrant red in foods, and research efforts should be continued and intensified.

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387 **List of captions**

388 **Figure 1. Fungal reds *i*) already in use in the food industry (carotenoid lycopene from**
389 ***Blakeslea*, polyketides from *Monascus*), *ii*) at development stage (*Monascus*-like pigments from**
390 ***Talaromyces* species, hydroxy-anthraquinones from many fungi).**

391 **TOP LEFT: mycelium (morphological detail) of *Blakeslea trispora*; chemical formula of the**
392 **carotenoid lycopene; red-colored foods using fungal lycopene.**

393 **TOP RIGHT: *Monascus purpureus* growing on a Petri dish; chemical formula of the polyketide**
394 **monascorubrin.**

395 **BOTTOM LEFT: microscopic view (morphological detail) of *Talaromyces* sp.; chemical formula**
396 **of N-threonine monascorubramine.**

397 **BOTTOM RIGHT: mycelium (morphological detail) of *Eurotium cristatum*; chemical formula of**
398 **erythroglauzin; foods which could be colored by the red hydroxy-anthraquinones.**

399 **(for color view, please refer to the online version of this article).**

400 **Figure 2. Food-oriented fungal reds that emerged independently at a world level (initial**
401 **genus/species^a and geographical location).**

402 ^a Taxonomic rearrangements have occurred since the first publications about some of the microorganisms. (Please refer to the
403 main text for references of the researches conducted in each country).



