Chapter 4
Microbial Pigments

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4.1 Introduction

There are three main sources of color additives for foods, drugs, and cosmetics: (1) synthetic colors, (2) plant-derived pigments, and (3) microbial pigments. In chemical terms, soluble colored substances are colorants and insoluble are pigments; however, in biological context, the colored substances are called pigments irrespective of its solubility. Although the term "biopigment" has a bit of redundancy, it is used to refer pigments of natural origin.

Microbial pigments or biopigments are multitude of chemical structures capable of absorbing light in the visible range (400–700 nm). There is an ever-growing number of biopigments. These molecules may possess other properties, which may or may not be compatible with the industrial use: vitamins riboflavin or β-carotene, antioxidants as most carotenoids and xanthophylls, and antimicrobial activity of some fungal polyketides. This chapter presents an overview of microbial pigments as potential food and drug color additives, presenting a brief description of the origin of their color and the physiological role of pigments in microorganisms, followed by a prospect of their use and a final section with representative classes of biopigments which may be produced using agro-industrial wastes.

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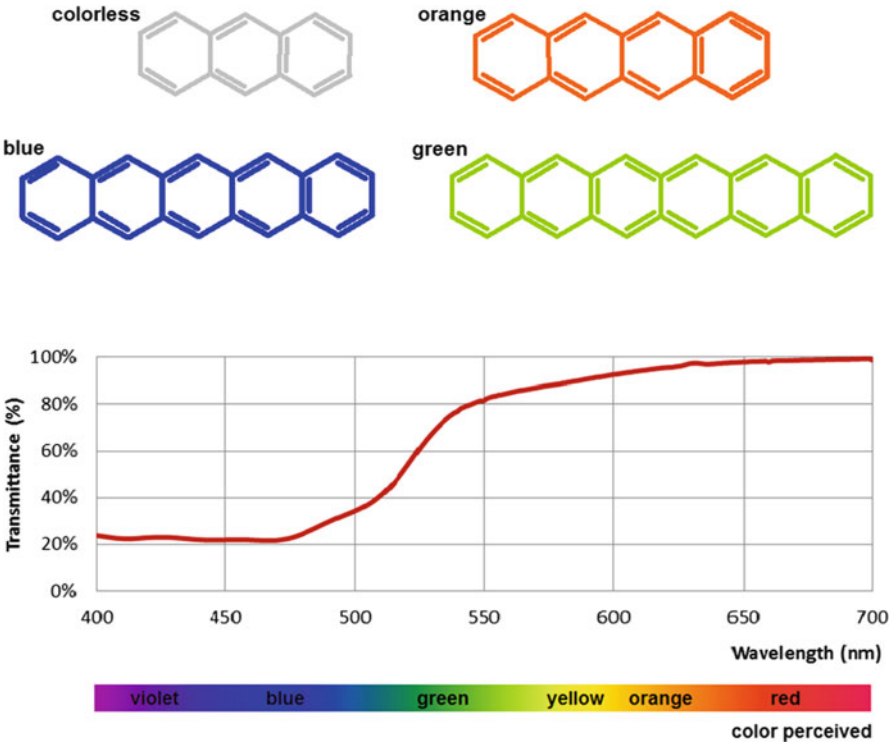

Fig. 4.1 The number of conjugated bonds (molecular structures on *top*) affects the absorption band and the color of the pigments; the *graphic* shows the transmittance spectrum for *Monascus* pigments, which appear *red*. *Sources*: Chemical structures from The Merck index, 2006; transmittance curve obtained at laboratory

4.1.1 The Origin of Color

When light interacts with matter, there may be absorption, reflection, refraction, and even reemission depending on the wavelength of incident light, chemical composition, and physical structure of the material giving rise to the multitude of colors that we see. A material may absorb the incident light unspecifically or selectively: if the absorption is unspecific, we perceive the color of the material as white, gray, or black, while if the absorption of one or more wavelengths is more pronounced, we perceive the material as having the complimentary color of that absorbed. Figure 4.1 illustrates the relationship between structure and color: molecular orbitals absorb and reemit light, and substances with multiple conjugated double bonds (a common trait in organic colored substances) tend to do so in the visible range (Nassau 2003; Meléndez-Martínez et al. 2007). Color may also arise or be modified on interaction of transition metal ions in complexes, as in porphyrin rings in hemoglobin and chlorophyll.

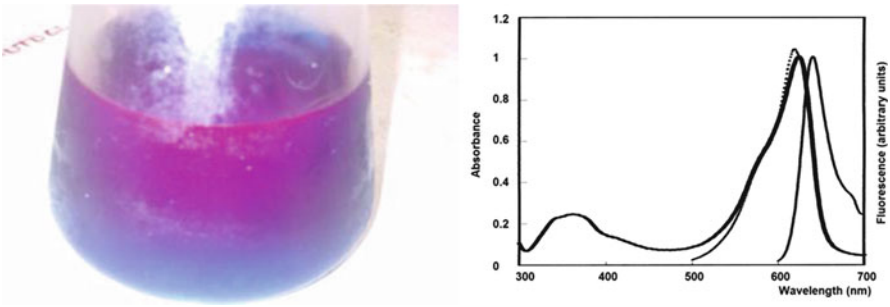

Fig. 4.2 Aqueous solution of phycocyanin from cyanobacteria and its absorption and emission spectra (*Sources*: photograph from Walter et al. 2011; spectra from Tooley et al. 2001)

Electrons in molecular orbital's absorb photons, which leap to higher energetic states and revert towards its fundamental state by releasing the energy, perhaps with several smaller leaps and radiations of different wavelengths. The wavelengths reemitted are usually outside the visible range, the effect being the net and selective absorption of visible light. However, if the reemission occurs in the visible range, we have a fluorescent pigment: for example, phycocyanin absorb mostly green, yellow, and orange light and reemit a bit of red light as can be seen in Fig. 4.2.

The part of a molecule responsible for light absorption is called a chromophore. Despite the enormous variety of biopigments, some recurring structures appear in nature and are illustrated in Fig. 4.3. There are a number of biological roles for these molecules, the most important ones being (a) their antioxidant nature, (b) their use as antennae for energy absorption, and (c) reserve substances.

Several microbial pigments are powerful antioxidants because their conjugated systems are susceptible to electrophilic attack. That is the case for carotenoids and xanthophylls, which are generally several times more efficient than ascorbic acid or butyl hydroxyl toluene (BHT) as antioxidants. Colored substances may also act as a sunscreen, protecting the cell by absorbing UV radiation and thus reducing the formation of DNA-damaging free radicals. Photosynthetic microorganisms such as cyanobacteria and microalgae rely on pigments such as chlorophylls and phycobilins for transferring light energy to electrons which will be used for carbon reduction in photosynthesis—a mechanism which produced the oxygen in our atmosphere and is in the base of virtually all food chains. Some biopigments such as phycobilins, chlorophyll, and prodigiosin may also act as nitrogen reserve in microorganisms. Besides these functions, there are several other cases in which light-absorbing molecules play an important role, such as in eyespots of microorganisms and in light-activated response mechanisms, such as the circadian rhythms of *Neurospora* sp. or as UV-induced damage correction mechanisms. Whenever these light-absorbing molecules selectively absorb in visible range, the result is a colored substance.

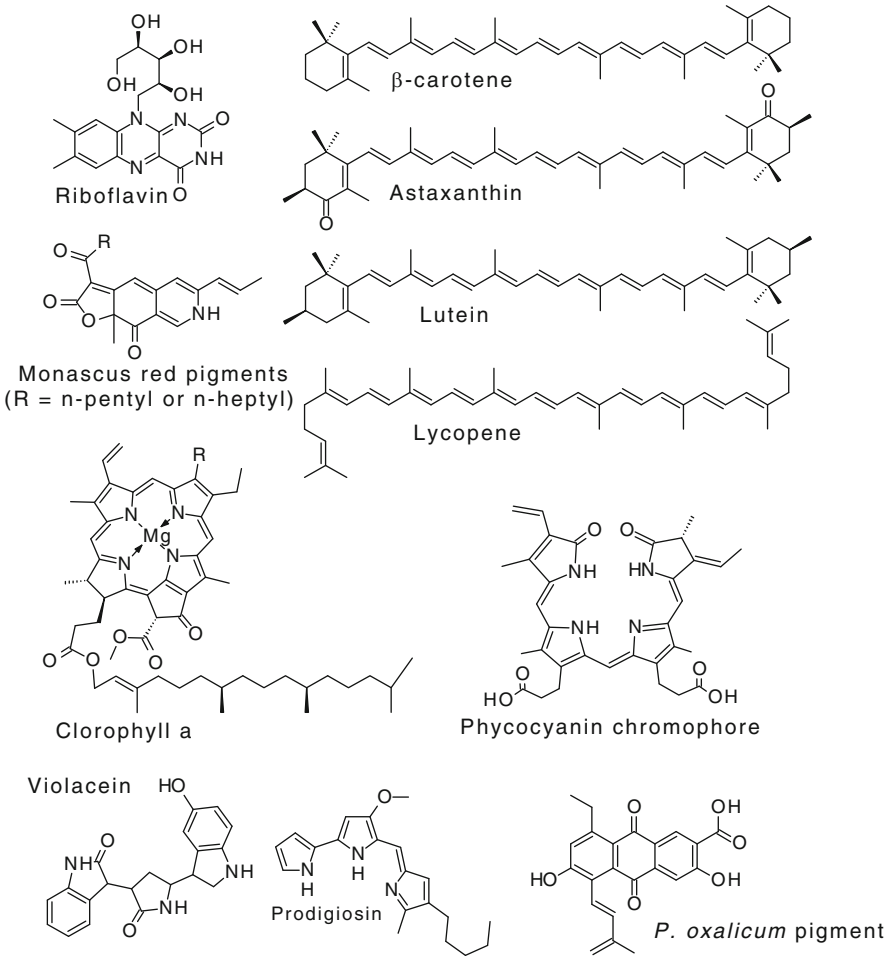

Fig. 4.3 Common microbial pigment core structures

4.1.2 Current Use of Biopigments

Several foods are naturally strongly colored and may be processed into extracts or powders used as food colors. That is the case of tomato-based lycopene, beet powder, paprika, and carrot oil. However, several nonfood plants are used for FD&C (food, drug, and cosmetic) colors, such as alfalfa for chlorophyll, marigold for lutein, annatto for bixin and norbixin, and cochineal (this, an insect) for carmine. Table 4.1 lists the natural color additives approved by the FDA (United States Food and Drug Administration) for food and feed use, excluding the mineral pigments. The pigments in the list are exempt from certification and may be used according to specific legislation for each type of food product (in general, *ad quantum satis* or as much as needed according to current good manufacturing practices).

Table 4.1 Color additives approved by the FDA for use in human food

Straight color	Uses and restrictions
Algae meal, dried	Chicken feed only
Annatto extract	GMP
Astaxanthin	Salmonid fish food only
Beet juice or powder	GMP
Canthaxanthin	Foods, salmonid fish feed, broiler chicken feed
Caramel	GMP
β-Apo-8′-carotenal	Foods and feeds
β-Carotene, natural and synthetic	GMP
Carmine or cochineal extract	GMP
Carrot oil	GMP
Corn endosperm oil	Chicken feed only
Copper chlorophyllin, sodic	Citrus-based dry beverage mixes
Cottonseed flour, toasted partially defatted cooked	GMP
Ferrous gluconate or lactate	Ripe olives
Fruit juice	GMP
Grape color extract	Non-beverage food only
Grape skin extract (enocianina)	Beverages and beverage bases
Haematococcus algae meal	Salmonid fish feed only
Lycopene, tomato extract, or concentrate	GMP
Paprika and paprika oleoresin	GMP
Paracoccus pigment	Salmonid fish feed only
Xanthophyllomyces dendrorhous (*Phaffia*) yeast	Salmonid fish feed only
Riboflavin	GMP
Saffron	GMP
Tagetes (Aztec marigold) meal and extract	Chicken feed only
Turmeric and turmeric oleoresin	GMP
Vegetable juice	GMP

From: FDA (2012). GMP, "good manufacturing practices," vary with the class of food

Biopigments are usually more susceptible to chemical attack than their synthetic counterparts (Aberoumand 2011) and may not resist the processing or the intended shelf life in some formulated products. However, this very limitation may be a reflex of a desirable trait at least in some cases: for example, the molecules would be easily degraded in the body.

There is a high degree of homology among eukaryotic metabolism, and animals are expected to manage diverse metabolites from fungi, yeast, and algae (with some notable exceptions such as mycotoxins). Most of the natural pigments are easily oxidized (e.g., carotenoids), hydrolyzed (e.g., phycobilins), or excreted due to its aqueous solubility (e.g., riboflavin). Table 4.2 shows the most important natural food colors derived from microorganisms, its origin, and possible metabolic roles. While some pigments such as astaxanthin are merely healthy, others such as those with provitamin A activity (β-carotene and β-cryptoxanthin) are actually essential for human nutrition.

Table 4.2 Microbial production of pigments in use as natural food colorants or with technology well developed and possible metabolic roles

Molecule (color)	Microorganism	Metabolic role
Lutein (yellow)	*Spongiococcum excentricum*	Antioxidant, may help slow macular degeneration
Ankaflavin (yellow)	*Monascus* sp.	Antimicrobial
Anthraquinoid (red)	*Penicillium oxalicum*	
Astaxanthin (salmon)	*Haematococcus pluvialis* *Xanthophyllomyces dendrorhous* *Paracoccus carotinifaciens*	Antioxidant
β-carotene (orange)	*Blakeslea trispora* *Dunaliella salina*	*Provitamin-A activity*, *antioxidant*
Monascorubramin (red)	*Monascus* sp.	Antimicrobial
Phycocyanin (blue)	*Arthrospira platensis*	*Antioxidant*
Riboflavin (yellow)	*Ashbya gossypii*	*Vitamin B2*
Rubropunctatin (orange)	*Monascus* sp.	Antimicrobial
Lycopene (red)	*Blakeslea trispora*	Antioxidant

Sources: adapted from Nelis and De Leenheer (1989); Margalith (1992); Soni (2007)

For application in processed products, a biopigment must be formulated (which will be discussed at the end of the chapter) to be stable enough in its life cycle. For products with low water activity such as liquors, drugs, and several cosmetics, stability is hardly an issue, however in moist formulations two problems arise: first, the pigment must be adequately dispersed—and lipophilic pigments require a suitable vehicle for dispersion—and, second, the chromophores may behave differently in different pHs, just as happens with fruit anthocyanins. Finally, some pigments may require the addition of an antioxidant in the formulation, or a protective barrier (such as specific packaging) in the processed food, but this is an issue already addressed because of the other sensitive chemical components of foods and cosmetics.

The market for food biopigments grows steadily despite of economic turmoil. Estimated as a 35 million USD market in the late 1980s, 250 million in 2000, and 600 million in 2011 (Yarnell 2012), biopigments grew from an 11 % niche of food colors in 1987 to a 27 % share in 2000. When dried foods (vegetables and microalgae) and nature-identical carotenoids used for fish and poultry feeds are included, the market surpasses 1.2 billion, with an annual growth of 2.3 % (BBC Research 2010). The steep rise in the biopigment market may be attributed to a mix of more stringent regulations regarding synthetic colors, the development of new pigment formulations, and the increase in fish farming. Considering the industrialization and consumption of processed food by countries like China, India, and Brazil, it would not be an over estimation that the biopigment market will grow to over 1.5 billion USD by 2020.

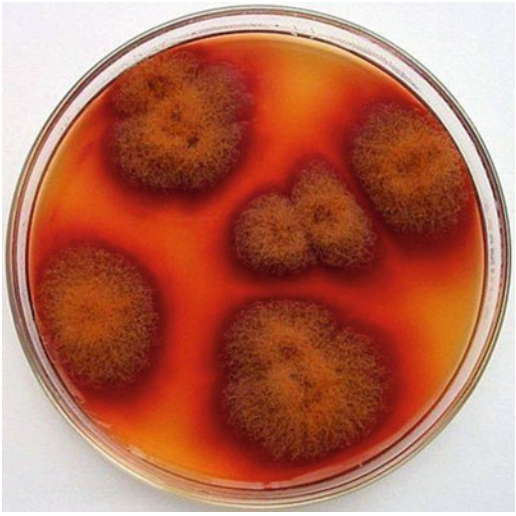

Fig. 4.4 Monascus colonies in an YM agar plate. Mycelium is colored and pigments diffuse through the medium

4.1.3 Why Microbial Pigments?

There are several natural pigments derived from vegetables and animals, in some cases from agro-industrial wastes such as tomato (lycopene), grape (anthocyanins), and palm (carotenoids) processing residues. However, source variability and presence in low concentration of pigment in those target fruits require processing of large amounts of agro-industrial waste. On the other hand, the use of selected microorganisms which are able to synthesize specific pigments has the advantages of consistent batches and high concentration and quality. Besides these advantages, microorganisms may be selected or modified in search of suitable color additives.

The selection of color-producing microorganisms is straightforward: the observation of colored colonies in agar plates. A diffusion halo is formed if the pigment is liberated to the medium and is at least partially water soluble (Fig. 4.4). The isolation of new microorganisms may be directed towards an acid stable pigment by controlling the pH of the isolation medium.

Further mutagenesis may lead to enhanced production. After initial isolation, the microorganism must be cultivated in a suitable medium so that the pigment may subsequently be isolated in order to determine eventual biological activity and finally be identified by LC-MS. In order to enhance the production of pigments, the careful evaluation of the effect of several substrates, macro- and micronutrients, as well as pH and temperature is done. The comprehension of the metabolic pathways leading to the target molecule is also important, for these pathways may be manipulated either by using inductors or repressors in the culture media or by gene knockout or promotion.

Despite of possible usefulness as bioactive compounds, the antibiotic pigments have much restricted use, and the route to market will be far more complicated. For example, *Monascus* pigments have been used for centuries in the Western countries but are still not regulated (therefore not permitted) in the USA and Europe (Puttananjaiah et al. 2011). Antioxidant pigments, at the other side, are generally welcome and even if the molecule is new, there is the possibility of its use as a nutraceutical ingredient, although petitions to regulatory agencies still will have to be done for FD&C compliance. This is easier with pigments derived from GRAS microorganisms, such as phycocyanin from *Spirulina* sp. Table 4.2 shows the most important microbial biopigment either in production or in late development state.

4.2 Biopigment Production Cases

With hundreds of microbial genera capable of producing biopigments, there are only a handful of substances which are industrially produced, as shown in Table 4.2. For these biopigments, technological and regulatory barriers have been transposed, which does not mean that there is no space for further development. Actually, these are the most likely platforms to be used at agro-industrial valorization initiatives, from which new or improved strains and products may progressively be developed. This section describes some cases selected by microorganism type, i.e., the production using yeasts, microalgae and cyanobacteria, filamentous fungi, and bacteria, for selected pigments.

4.2.1 Carotenoids from Yeast and Fungi

The color and nutraceutical properties of carotenoids have attracted the attention of food, cosmetic, and pharmaceutical industries. The food industry uses this kind of molecule as natural food colorants, as dietary supplements, and fortified foods (Vílchez et al. 2011). For example, β-carotene, the carotenoid with the largest market share can be found in margarine, cheeses, and fruit juices. The carotenoids are also used in pharmaceutical and cosmetic due to their nutraceutical properties.

Carotenoids are naturally found in bacteria and fungi (Table 4.3) and microalgae (Table 4.4). These important natural pigments have colors ranging from yellow to red (Perez-Fons et al. 2011). More than 600 different structures of these biomolecules, synthesized in vegetables and microorganisms, have been characterized.

It is clear, from Table 4.3, that the productivity of biopigments is quite diverse. For comparison, the vegetative cycle of carrots is around 100 days, with 70 mg/kg of β-carotene and for watercress 50–70 days, with 60 mg/kg of β-carotene. The slowest growing microorganism from Table 4.3 has a 5-day cycle (considering 10 % inoculum), with a final carotenoid concentration of 250 mg β-carotene/kg of biomass, on average.

Table 4.3 Comparison of microbial carotenoid production using different waste substrates as carbon source

Microorganism	Molecule	Culture medium	X_{max} (g/L)	P_{max} (mg/L)	Conc. (mg/g)	$\sim\mu_x$ (h^{-1})	References
Blakeslea trispora (*fungus*)	β-carotene	Corn steep liquor	20	800	40	0.022	Papaioannou and Liakopoulou-Kyriakides (2010)
Blakeslea trispora (*fungus*)	β-carotene	Whey	8	1,360	170	0.023	Varzakakou et al. (2010)
Sporobolomyces roseus (yeast)	β-carotene	Reconstituted whey	4.71	2.58	0.55	–	Marova et al. (2012)
Rhodopseudomonas palustris (bacterium)	β-carotene	Sodium succinate	2.58	1.78	0.69	–	Kuo et al. (2012)
Rhodotorula glutinis (yeast)	β-carotene	Potato extract	5.70	1.08	0.19	–	Marova et al. (2012)
Dietzia natronolimnaea (bacteria)	Canthaxanthin	Whey	3.29	2.87	0.87	0.020	Khodaiyan et al. (2008)
Phaffia rhodozyma (*yeast*)	Astaxanthin	Cassava residues	8.6	2.98	0.35	0.060	Yang et al. (2011)
Sporobolomyces ruberrimus (yeast)	Torularhodine	Technical glycerol	30	3.7	0.12	0.040	Razavi and Marc (2006)

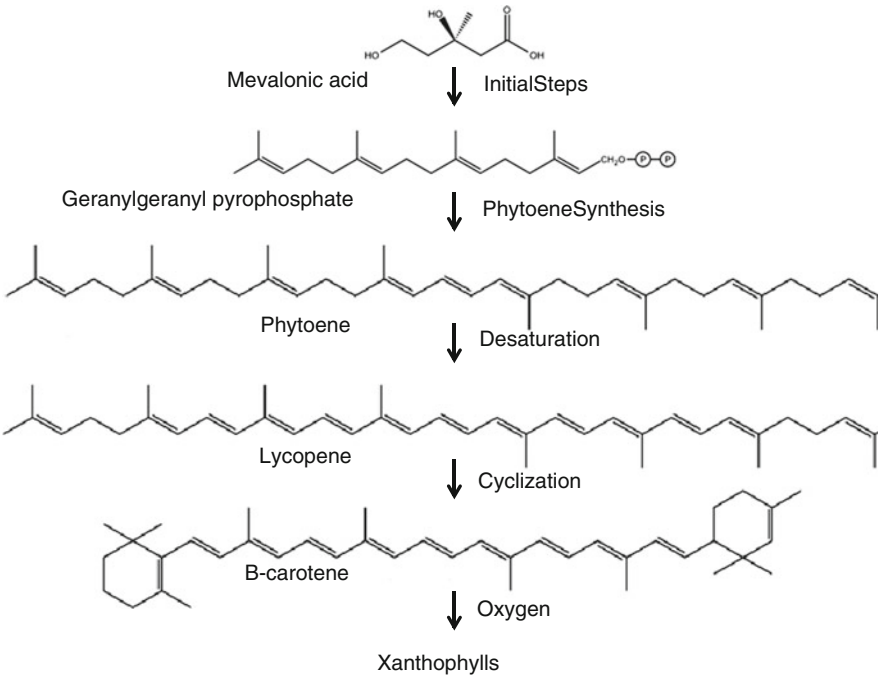

Fig. 4.5 Carotenoid biosynthesis pathway (Silva 2004)

The sequence of carotenoid biosynthesis in microorganisms is summarized in Fig. 4.5. The biosynthesis starts with the mevalonic acid that by different reactions (initial steps) produces the geranylgeranyl pyrophosphate (GGPP). Two molecules of GGPP are condensed to synthesize phytoene, which is transformed into lycopene through few steps of desaturation. β-carotene is finally formed from lycopene through cyclization. Oxygenation of carotenoids gives the xanthophylls such as astaxanthin (Wang et al. 2007).

There are several ways to improve carotenoid synthesis in fungi and yeasts, such as addition of inducing substances and light stimulation. But since these organisms are heterotrophs, culture media optimization is by far the most important aspect for a production system. Nowadays, the challenge is to reduce the production costs of carotenoids from bioprocesses. The use of cheap industrial by-products as nutrient sources and use of a microorganism with high carotenoid yield can contribute to the minimization of production cost (Tinoi et al. 2005). As showed on Table 4.3, carotenoids can be produced by microorganisms able to use different kinds of waste substrates as carbon sources. Grape must, glucose syrup, beet molasses, sugar cane molasses, soybean flour extract, technical glycerol, and whey are examples of by-products that have been reported in the literature as low-cost substrates for carotenoid production (Buzzini and Martini 1999; Razavi and Marc 2006; Khodaiyan et al. 2008; Papaioannou and Liakopoulou-Kyriakides 2010; Yang et al. 2011).

Marova et al. (2012) have shown in their work the ability of the yeast *Rhodotorula glutinis* to use industrial waste (whey) for high-value β-carotene production.

Table 4.4 Selected carotenoid-producing algae

Microalga	Carotenoid	Medium[a]	*X* (g/L)	Conc. (mg/g)	Maximum specific growth rate	References
Chlorella zofingiensis	Astaxanthin	BBM with glucose	10.2	1	0.031 h^{-1}	Ip and Chen (2005)
Coelastrella striolata	Canthaxanthin Astaxanthin *β*-carotene	BBM	2.7	47.5 1.5 7	0.30 day^{-1}	Abe et al. (2007)
Coccomyxa onubensis	*β*-carotene Lutein	K9	1.6	2.88 6.48	0.50 day^{-1}	Vaquero et al. (2012)
Haematococcus pluvialis	Astaxanthin	BBM	2.2	13.5	–	Harker et al. (1996)
Chlorella zofingiensis	Astaxanthin	Bristol, modified	10	1.25	0.043 h^{-1}	Ip et al. (2004)
Dunaliella salina	*β*-carotene	f2	–	14[b]	0.55 day^{-1}	Kleinegris et al. (2011)
Haematococcus pluvialis	Astaxanthin	Standard	3	12–15	0.56 day^{-1}	Garcıa-Malea et al. (2005)
Muriellopsis sp.	Lutein	Arnon, modified	5.37	6.51	0.17–0.23 h^{-1}	Del Campo et al. (2000)
H. pluvialis (wild type)	Astaxanthin	NIES medium	1.6	47.62	0.07	Hong et al. (2012)
H. pluvialis (mutant)			2.25	54.78	0.08 h^{-1}	

[a]Except where specified, these are mineral-based media. Recipes may be found at UTEX, SAG, or CCMP collections web sites
[b]Estimated. The original reference reports 28.1 mg/L carotenoids

Another source of carotenoids from yeast may be as by-products of nutraceutical oil production, although the fractionation of the mixture may be challenging.

The production of β-carotene by *Blakeslea trispora*, which is one of the best known processes today, was at a time elusive: carotenoids after conversion into pheromones are used as basis for communication in this fungus, and the use of two mating types is the key for the production of the large amounts of carotenoids (Papaioannou and Liakopoulou-Kyriakides 2010). However, microalgal carotenoid productivity may surpass that of fungi, as is discussed in the next section.

4.2.2 Carotenoids from Microalgae

Carotenoids are synthesized by microalgae for photoprotection, oxidation-protective agent, and as part of the light-harvesting complexes for photosynthesis. Being ubiquitous in microalgae, carotenoids are even used as a primary classification key for genera. Table 4.4 presents cases of carotenoid-producing microalgal cultures.

Although β-carotene holds the largest market for carotenoids, astaxanthin—a high-value keto-carotenoid pigment—is increasingly being used as feed additive in aquaculture. Farmed salmonid production was almost nonexistent in 1980 but reached 2.41 million tons in 2010 and had an average increment of 3.8 % per year in the previous 8 years (data from FAO 2012). This represents a huge market for fish feed, in which astaxanthin is included in order to confer the attractive coloration of these fish, and helps maintain their normal growth and survival (Shen et al. 2009). In addition, the strong antioxidative activity of astaxanthin over other carotenoids such as β-carotene, zeaxanthin, and lutein has attracted tremendous commercial interest for medicinal and nutraceutical uses (Miki 1991).

The occurrence of astaxanthin in the freshwater microalga *Haematococcus pluvialis* led to the development of two-stage cultures of this alga: the production intracellular pigment involves changes in the metabolism associated with a morphological transformation from green vegetative cells to deep-red, astaxanthin-rich, immobile aplanospores (Elliot 1934) that take place when the culture is subjected to stress conditions such as high irradiances (Kobayashi et al. 1992) usually in combination with nutrient deprivation (Boussiba et al. 1992; Margalith 1999; Orosa et al. 2001).

The incorporation of well-developed *Haematococcus* and *Dunaliella* production systems into conventional (agro industrial) wastewater treatment reduces production costs of algal biomass, which in turn can be applied to production of bioactive substances, bioenergy, or valuable chemicals (Hoffmann 1998). Municipal wastewaters and piggery wastes are very rich in nutrients but must not be used to feed microalgae until effluent preprocessing and biomass post-processing guarantee pathogen destruction. But agro-industrial wastes such as *manipueira* (cassava processing wastewater) and vinasse (ethanol production wastewater) may be conveniently used as culture media for mixotrophic growth of microalgae (Soccol et al. 2012). Table 4.5 shows the main components of autotrophic of culture media and of two residues.

In a conventional biological wastewater treatment process, external carbon sources such as methanol or acetate are usually needed to convert nitrate into nitrogen gas (Tchobanoglous et al. 2003), and excess biomass generated needs to be treated and disposed of in a safe and cost-effective way—which leads to high operating costs (Yang et al. 2003). However, the assimilation of nitrate by microalgae has two advantages over conventional biological nutrient removal: (1) nitrate can be converted into biomass without any external carbon source, and (2) high-value products such as astaxanthin can be extracted from excess biomass. Because of the low rates of growth and nitrate uptake in microalgae, it may be difficult for this microalgal treatment process to be used in a mainstream treatment process, but it may have potential application as a subsidiary process in biological nutrient removal (Kang et al. 2006). Actually, the final step in wastewater treatment (stabilization) usually has a healthy population of microalgae.

At the other side, *direct* (i.e., untreated) use of agro-industrial wastewaters, even with a high organic load is possible, for suitably adapted species. Direct cultivation in these residues may even work as a selective trait: extreme pH or high osmolality, coupled to cultivation in shallow ponds, and high inoculum concentration may be

Table 4.5 Main components of culture media (in mg/L) for microalgae with potential for production of value-added biomass and production (in g/L) for *Chlorella* and *Spirulina*

	Seawater	*Vinasse*	*Manipueira*	*Spirulina*	*Chlorella* and *Scenedesmus*	*Porphyridium*	*Dunaliella*
HCO_3^-	145			12,200		28.6	1,235
Na^+	10,768	51.6		5,670	300	10,626	46,465
K^+	399	1,689	1,863	672	313	406.1	199
Ca^{2+}	412	368	227.5	10.9	4.2	408	11.9
Mg^{2+}	1,292	135	405	19.5	24.4	1,306	131.4
Fe^{2+}	0.002	17.6	15.4	2.0	0.9	4. 4	0.1
Cl^-	19,353	1,219		626		19,068	71,050
Br^-	66						
$H_2BO_3^-$	27		26.8		2.5	0.6	6
HPO_4^{3-}		92	498	276	385	49.9	10.0
SO_4^{2-}	2,712	1,538		633	98	2,576	468.3
NO_3^-	0.3			5,670	993	614	310
NH_4^+	0.03	13.2					
EDTA				0.2	3.1	15.6	0.5
Organic matter		9,300	55,000				
BOD		16,950	55,000				
COD		28,450	85,400				
Organic N		343.4	4,900				
Soluble carbohydrates			5,100				
Biomass production, g/L		Residue diluted to 30 %	Residue diluted to 30 %				
Chlorella	–	1.6	3.1	–	5–10	–	–
Spirulina	–	4.47	2.6	1–10	–	–	–

Source: adapted from Soccol et al. (2012), with permission. Empty cells indicate low or zero concentration, for media and seawater and low or unknown, for residues

enough to guarantee the predominance of one algal species in open systems. In closed systems, the problem is reduced to a question of sterilization—to which bioindustries are well acquainted.

Comparing media in Table 4.5, one may note that the residues have high potassium and low nitrate concentration but carry organic nitrogen and phosphate, which explain why some algae thrive in these wastewaters. Kang et al. (2006) introduced the cultivation of *H. pluvialis* into secondary treatment of the primary-treated sewage (PTS) and primary-treated piggery wastewater (PTP), containing low and high concentrations of nitrate, respectively. The authors examined the characteristics of algal growth, nitrate assimilation, and astaxanthin biosynthesis by red cyst cells of *H. pluvialis* through subsequent strong photoautotrophic induction. The work showed that the inorganic wastes in the wastewater were removed successfully by *Haematococcus* cultivation, after which green vegetative cells were transformed by photoautotrophic induction to red aplanospores with substantial astaxanthin content of 39.7 mg L^{-1} and 83.9 mg L^{-1} on PTS and PTP-2 cultures, respectively.

Carotenoid bioavailability depend on post-processing of the biomass produced; intact astaxanthin-rich cysts of *Haematococcus* are poorly absorbed in salmonids (Sommer et al. 1991), and the biomass should be processed in order to enhance digestibility, e.g., via high-pressure homogenization.

4.2.3 Other Photosynthetic Pigments from Microalgae

Chlorophyll (which exists in several forms in microalgae and cyanobacteria) is usually produced from alfalfa, through solvent extraction and then purified and converted to cupric complexes. However, microalgae have much higher contents of chlorophylls (for example, 37.1 mg/g biomass in *Chlorella*, against 3.84 mg/g in alfalfa), and the prospective production of large amounts of microalgal biomass for biofuels or protein may provide raw material for microalgae-based chlorophyll.

In order to enhance the absorption of light, besides chlorophyll and carotenoids, several microorganisms have specialized phycobilin proteins. These antennae pigments are present in cyanobacteria and some algae (rhodophyta, cryptophyta, and glaucophyta) and have absorption spectra complementary to that of chlorophylls. Among phycobilins, phycocyanin is one of the most interesting pigments with a distinct blue color. Also, being a water-soluble pigment, it offers a large array of potential applications. Table 4.6 presents the production characteristics for representative photosynthetic microorganisms.

Phycocyanin production is done by cultivating the chosen microorganism and then processing the biomass. Since *Spirulina* supports very high pH for growth, large-scale open cultures of this cyanobacterium have been done even with mixotrophic cultures. However, without the total dependence of light, cells may reduce the concentration of photosynthetic pigments—for example, chlorophyll content drops in mixotrophic cultures, from approximately 23 mg/g in Chlorella to around 4 mg/g

Table 4.6 Selected phycocyanin (PC), phycoerythrin (PE) and chlorophyll (CHL), and allophycocyanin (APC)—producing microorganisms

Microorganism	Molecule	Culture medium	X_{max} (g/L)	P_{max} (mg/g)	μ (day^{-1})	References
S platensis	PC	ZRK + 2.5 g/L glucose, 4klux	2.66	121	0.62	Chen et al. (1996)
S platensis	PC	ZRK		167		Yan et al. (2011)
	APC	ZRK		36.6		Yan et al. (2011)
S platensis		ZRK	10.24	54–125		Chen and Zhang (1997)
A platensis	PC	ZRK, 2.5 g/L glucose, 12 kLux	1.33		0.49	Ben et al. (2010) Better production in high light, low glu; not optimized
Synechocystis sp.	PC	Modified BG-11	0.2	120		
Galdieria sulphuraria	PC	Glucose and ammonia based, fed batch, high concn.	109	27	1.4	Graverholt and Eriksen (2007)
Pseudanabaena sp.	PE	ASN-III	0.89	44	0.1	Mishraa et al. (2012)
S. platensis	CHL	Mineral, nitrate based	1.9	11.6	0.15	Rangel-Yagui et al. (2004)

ZRK Zarrouk medium; *S* (*Spirulina*) and *A* (*Arthrospira*) refer to the same microorganism, but the original denomination given by the reference was maintained

(Cheirsilp and Torpee 2012); light intensity also affects the production, although cell concentration must be taken into account when analyzing irradiance.

While chlorophyll may be extracted from algal biomass just as in plant-based processes, phycocyanin processing requires cell disruption in a buffer, followed by filtration or centrifugation of the debris, concentration, and drying. Broiler additives may be produced by simply drying the biomass. Pure phycobilins may be obtained by fractional precipitation at 25 and 60 % ammonium sulfate, followed by a DEAE-Sepharose chromatography with a gradient from pH 5–3.6, reaching phycocyanin purity of 5.59 (A_{620}/A_{680}) and allophycocyanin purity of 5.19, with recoveries of 67 % and 80 %, respectively (Yan et al. 2011).

As it could be expected with a protein, phycocyanin is stable only in the pH range of 5.5–6, with a temperature below 47 °C (Chaiklahan et al. 2012). Outside this range, partial degradation of the pigment occurs through denaturation, insolubilization, and possibly due to hydrolysis (in extreme pHs). Several solutes may aid the stabilization, as well as the use of high-temperature, short-time (HTST) processing rather than long heat-incubation times.

Table 4.7 Pigments produced by fungi in agro-industrial residues

Microorganism	Molecule	Culture medium	X_{max}	P_{max}	References
Monascus sp.	Polyketide mix	Rice, SSF	330 mg/g substrate	1.87 mg/g substrate	Carvalho et al. (2006)
Monascus sp.	Polyketide mix	Cassava bagasse, SSF		0.3 mg/g substrate	Carvalho et al. (2007)
Monascus sp.	Polyketide mix	Jackfruit seed + minerals, SSF			Babitha et al. (2006)
Monascus purpureus	Polyketide mix	Gluten and bran-free wheat flour SmF	10.34 g/L	2.46 mg/g substrate	Dominguez-Espinosa and Webb (2003)
Monascus kaoliang	Polyketide mix	Wheat meal, SSF		60.64 mg/g substrate	Lin ad Iizuka (1982)
Penicillium oxalicum	Arpink red	Molasses, yeast autolysate		1.5 g/L	Dufossé (2006)
Ashbya gossypii	Riboflavin	Corn steep liquor, peptone, soybean oil		1–5 g/L	Lim et al. (2001)

SmF submerged fermentation, *SSF* solid substrate fermentation

4.2.4 Pigments from Fungi

There are hundreds of colored pigments produced by fungi. Several of these substances are bioactive, having, e.g., antibiotic, immunomodulatory, nephrotoxic, and hepatotoxic properties. Considering that fungi are usually unable to use light, the color of the pigment could be a consequence of interaction with light of a structure with other functionalities. Several accounts on the potential for biopigments production by fungi were done by Durán et al. (2002) and others, compiling data for several classes of biopigments mainly including melanins, flavins, carotenoids, quinones, and azaphilones. If at one side this large diversity represents an untapped source of promising molecules, at the other the bioactivity is a barrier to the market. Table 4.7 presents the most important fungal pigments which are used in foods (even if *Monascus*, as already stated, is limited to oriental countries).

Riboflavin (vitamin B2) is a well-known, permitted, stable water-soluble pigment added to a multitude of products to impart yellow color and as a nutritional ingredient. Although more than 75 % of the worldwide riboflavin production is synthetic, its industrial production by fungi is well established.

An anthraquinoid pigment, Arpink red, from *Penicillium oxalicum* species has a structure similar to that of cochineal carmine and may be an important substitute to the insect-derived pigment in the future. As a stable nontoxic pigment, its use for

foods has been proposed (Dufossé 2006). A patent application claims that the compound and its derivatives have anticancer activity (Sardaryan 2006). The production by fermentation is straightforward, and its acidic functional group makes concentration very simple, via precipitation of the pigment by pH regulation.

Monascus pigments are sold either as raw fermented powders, concentrated or dried, or as fractionated extracts. Its production is done in submerged fermentation or, more usually, in solid substrate fermentation. There are dozens of substrates tested for its production, although rice and wheat meals (either integral or broken residual cereal) give the highest pigment production. After 7–10 days fermentation, the mass (*koji*, in the case of rice) may be dried or extracted with a suitable solvent. The solution may be concentrated and dried. *Monascus* pigments stability depend on pH and temperature; for pH 7–8 in aqueous media, the pigment resists processing for 30 min at 100 °C but may lose up to 20 % tinctorial strength at pH 4 (Carvalho et al. 2005). Alcoholic solutions are very stable. Monascus pigments may bind to amino groups, which may lead to increased stability in some formulations. Care must be taken for most strains produce citrinin, a yellow nephrotoxic mycotoxin; also, raw biomass may contain the anti-hypercholesteremic molecule lovastatin.

Another microbial pigment producer whose metabolites are bioactive is *Pycnoporus sanguineus*, a ubiquitous wood-growing fungus which produces phenoxazine analogs with antimicrobial activity.

4.2.5 *Bacterial Pigments: Prodigiosin, Violacein, Pyocyanin, etc.*

There are several well-studied bacterial pigments but these are not introduced in market because of its antimicrobial and eventual toxic activity, as is the case of prodigiosin and violacein. However, these pigments may have niche food uses (e.g., avoid fungal proliferation on the surface of meat products) or nonfood uses, as in textiles. At the other side, some bacteria produce harmless carotenoids. Examples of bacterial pigments produced using fermentation are presented in Table 4.8.

Prodigiosins are a class of tripyrrole antibiotic pigments produced by several microorganisms such as *Serratia marcescens* and *Hahella chejuensis*. These substances received recent renewed attention because of its reported immunosuppressant and anticancer properties (Williamson et al. 2006; Gulani et al. 2012) and potential involvement in the reduction of algal proliferation in algal blooms (Kwon et al. 2010). *Serratia* cultures produce almost 500 mg/L of prodigiosin in 2 days, at 30 °C. Giri et al. (2004) obtained excellent production in peanut seed broth (38.75 g/L), which indicated that peanut (and perhaps soy) processing residues could be an adequate substrate for the pigment.

Violacein is a purple diindole-pyrrole pigment derived from tryptophan. It is soluble in ethanol, and its biosynthesis and potential uses are still being studied. The same applies to the blue phenazine pigment pyocyanin, produced by *Pseudomonas aeruginosa*. Pyocyanin is a highly reactive metabolite which, being toxic to mammal

Table 4.8 Selected pigments produced by bacteria

Microorganism	Molecule	Culture medium	X_{max} (g/L)	P_{max} (mg/L)	References
Serratia marcescens	Prodigiosin	Maltose and peanut oil based	–	535	Gulani et al. (2012)
Serratia marcescens	Prodigiosin	Powdered peanut medium	–	39,000	Giri et al. (2004)
Hahella chejuensis, mutant	Prodigiosin	Sucrose and peptone based	–	2,600	Kim et al. (2008)
Chromobacterium violaceum	Violacein	Glucose and peptone based	21	430	Mendes et al. (2001)
Paracoccus carotinifaciens	Astaxanthin, canthaxanthin	Glucose and peptone based	–	25–40 (mg/g)	Hirschberg et al. (1999); Tanaka et al. (2011)

cells, cannot be used in foods; however, it is possible that its conjugation to proteins in leather and other materials may stabilize it, permitting its use as a textile pigment.

Paracoccus carotinifaciens is a bacterium which accumulates a mix of carotenoids. Recent patents cover proprietary isolates and mutant strains, and although the carotenoid content is not superior to that of selected microalgae, the specific growth rate is probably high, as common in bacteria. This biomass hit market relatively quickly and is already permitted as a fish feed supplement in the USA.

4.3 Biopigment Production and Formulation

Among the biopigments discussed in this chapter, few are produced in industrial scale; in some cases, the technology is relatively new and scaling up is being developed (as is the case for phycobilins), or there are regulatory issues (as with *Monascus* pigments in Europe and in the USA). The following section presents suitable, laboratory-tested conditions for producing and concentrating selected pigments.

Although the pigments discussed here vary widely, there are some general considerations which apply: we may be interested in the integral biomass or in a concentrated pigment. The substances of interest may be intra- or extracellular (or both), predominantly lipo- or hydrosoluble, and probably thermolabile and prone to oxidation. Finally, biomasses destined for feeds and supplements may have poor digestibility, requiring a cell disruption step. Four selected cases of biopigment production systems follow:

4.3.1 Production

β-carotene from *Blakeslea trispora*: inocula of spores of mating types + and − are used in the ratio 1:10 (e.g., 10^4 spores of type + and 10^5 spores of type −) (Varzakakou et al. 2010) in a suitable culture medium, such as (in g/L) D-glucose 50, olive oil 5.4, soybean oil 5.4, sunflower oil 5.4, corn steep liquor 80.0, span 20 10.0, Tween 80 0.1, casein hydrolysate 2.0, yeast extract 1.0, L-asparagine 2.0, KH_2PO_4 1.5, $MgSO_4 \cdot 7H_2O$ 0.5, BHT 0.02, and thiamine-HCl 0.005, as proposed by Papaioannou and Liakopoulou-Kyriakides (2010). After 8 days in aerated culture with moderate agitation at 26 °C, the cells are separated from the broth by filtration, partially dehydrated and slowly extracted with a suitable solvent such as chloroform. The solvent is separated from the biomass and evaporated; the dry raw extract is further processed. From 62 kg of corn steep liquor and 38 kg of glucose, there should be produced 20 kg of biomass which, extracted with 50 L chloroform, would give approximately and 8 kg extract containing 90 g of β-carotene.

Carotenoids from *Haematococcus*: This microalga may be cultivated in synthetic mineral media or in residues if sufficient nitrogen and phosphorus is present. An initial cell count of 10^3 cells/mL is a suitable inoculum to a basal medium containing (in g/L): KNO_3 2, K_2HPO_4 0.2, $MgSO_4 \cdot 7H_2O$ 0.2, soil extract 30 mL (according to SAG 2012), and micronutrients. Cultivation is done at 25 °C for 10–15 days, at moderate irradiation. Biomass should reach 2–3 g/L, and at this point the irradiation should be increased to 30–40 kLux with the addition of NaCl reaching a concentration of 0.4–0.6 %, conditions which induce carotenogenesis. After 5 days, carotenoid-rich biomass is separated by centrifugation. The biomass is thermally processed in order to enhance the digestibility of the algae meal (for instance, right before spray drying). From 100 kg of salts, there may be produced 125 kg of biomass with 1.65 kg of astaxanthin, requiring a production area (for a pond) of 200 m^2.

Phycocyanin from *Arthrospira* sp.: this cyanobacterium may be suitably cultivated in agro-industrial wastes, provided that there is enough nitrogen and phosphorus and that the pH is increased (if necessary) to around 9, which usually requires addition of bases. Growth in *manipueira*, for example, produces at least 2–3 g/L of biomass (or 10–15 g/L of residue, since it is diluted to 20 %). From 1,000 L of residue, diluted to 20 % and inoculated with a previous culture to an initial concentration of 0.1 g/L, approximately 12.5 kg of biomass may be obtained after 10–15 days, depending on irradiation and eventual contamination. This biomass is harvested by flocculation with a cationic copolymer such as a polyacrylamide-amine, followed by filtration and drying of the paste, in the case of algae meal for broilers. For phycocyanin production, the wet biomass is resuspended in 100 L of 0.1 M phosphate buffer and successively frozen and thawed (4 cycles). The phycobilins are liberated to the buffer, which is then centrifuged and desalinized by ultrafiltration and dialysis, giving a raw extract with 1.25–1.5 g of phycocyanin. Further purification is possible using fractional precipitation with ammonium sulfate. Such a process requires a production area of 25 m^2.

Pigments from *Monascus*: this fungus may be cultivated over residual cereals from mill processing, e.g., broken rice. The cereal is mixed with one part water and autoclaved, giving a product with approximately 56 % water. This cooked rice is inoculated with a spore suspension or with a fermented powder previously obtained, and is then cultivated for 7–10 days at 30 °C. In this solid substrate fermentation, it is important to maintain aeration through the medium. After cultivation, the material may be dried for production of a meal or extracted (without drying) with 95 % ethanol (2–3 L:kg of rice initially used). The extract is then filtered, concentrated by evaporation, and the viscous precipitate that forms may be processed into a powder or a liquid formulation. From 100 kg of broken rice, an extract or powder with approximately 200 g of mixed pigments may be produced. Further fractionation is uncommon but may be done by chromatography.

4.3.2 Formulation

The distribution (in the mass transfer sense) of a pigment in a product matrix is determined by its structure and formulation. Therefore, besides producing the molecule, it is usually desirable to have several physicochemically distinct presentable forms of the pigment, e.g., hydrosoluble and liposoluble concentrates, solid dispersible powders, and solid additives for feed. Microencapsulation and/or additives may be used to protect the pigment. Of the products to which biopigments may be applied, perhaps foods represent the most complex class, because of its naturally complex composition; however, the considerations below may be also applied to drugs and cosmetics.

Hydrosoluble pigments: Riboflavin, phycocyanin, cupric chlorophyllin, and other hydrosoluble pigments may be directly added to foods, in accordance with the *Codex Alimentarius* regulations. These pigments may be prepared and stored as dry powders or liquid concentrates, eventually with dispersing agents. Dry powders are preferred because of its low water activity and high stability.

Liposoluble pigments: Carotenoids, several xanthophylls, and chlorophyll dissolve poorly in water but may readily be dissolved in hot oils, fats, or highly concentrated alcoholic solutions (e.g., spirits). For lipid-rich foods, these pigments may be applied directly, but for foods with high water contents, the application must be done carefully to avoid phase separation. For example, application of β-carotene in emulsions such as sausages may lead to segregation of the pigment to lipid droplets and lead to a heterogeneous aspect. Prior assessment of color stability is necessary. Formulations for these pigments are usually solutions in oil (e.g., lycopene in soy oil) or dry powders.

There are commercial hydrosoluble preparations of these pigments: these are stable emulsions with edible oil micelles stabilized by USP/FDA/EC-approved emulsifiers. These are lipo- and hydrosoluble, mostly intended for use in beverages.

Microencapsulation consists of preparing a mixture of the pigment with a suitable support which surrounds or dissolves it: for example, hydrophobic carotenoids may be mixed with cyclodextrins forming water-soluble complexes; hydrophilic pigments

such as phycocyanin may be emulsified in light oil with the aid of a lecithin, followed by spray drying. For example, one commercial astaxanthin formulation contains the extract dispersed into soy protein and contains alginate and hydroxypropyl cellulose, besides antioxidants to protect the material during processing and storage.

4.4 Future Developments

There are several ways in which biopigment research should focus: vegetable and animal cell cultures may lead to the production of already permitted pigments (for example, anthocyanins from grapes) using "cell reactors." Genetic tools are much more likely to be used in order to develop genetically modified hosts which will produce the desired pigment with very high productivities. For example, Das et al. (2007) report that yields of up to 18, 49, and 11.4 mg/g DCW of lycopene, β-carotene and astaxanthin were obtained in specific studies where *E. coli* was modified for production of carotenoids. The elucidation of the composition of structural pigments such as helically coiled cellulose in *Pollia* fruits (Vignolini et al. 2012) may lead to exciting new GMO structural pigment producers—today, pearlescent effects are obtained using mica, a mineral.

Molecular tools may be used for knockout of genes for toxin production in fungi, to enhance concentration through multiple gene copies, and regulating of pathways: mutagenesis as in *Ashbya gossypii* and further understanding of pheromone and carotene biosynthesis regulation in *Phycomyces blakesleeanus* (Tagua et al. 2012) is leading to enhanced production of carotenoids.

On the more traditional side, the fungal, bacterial, and microalgal diversity is yet to be explored, and new tools for molecule identification may be used for high throughput screening of ambiental samples. The fact that one of the newest biomasses permitted for feed use is a newly bacterial isolate (*Paracoccus* sp.) shows the potential of bioprospection. At the other side, species with known behavior may be mutated, with selective pressure, in order to develop better pigment producers—as it has been done successfully with *Haematococcus* algae.

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