

Review

Microbial production of carotenoids – A review

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Carotenoids are natural pigments that can be synthesized by various microorganisms, including bacteria, yeasts, filamentous fungi and microalgae. These pigments comprise around 700 different structures with peculiar colors and biological properties that are beneficial to health. Advantages of biotechnological production of carotenoids include the ability of microorganisms to use low cost substrates, the optimized control of cultivation, minimized production time and the natural origin of the synthesized pigments. Techniques for separation and purification of carotenoids are well established at laboratory scale, however the development of processes that can be economically scaled-up is essential for industrial production.

Key words: Carotenoids, microorganisms, biotechnology, natural pigments.

CAROTENOIDS PRODUCED BY MICROORGANISMS

Carotenoids are natural pigments that can be synthesized by various microorganisms, including bacteria, yeasts, filamentous fungi (Berman et al., 2015) and microalgae (Henríquez et al., 2016). Carotenoids are yellow, orange or red in color and because of their proven activity as pro-vitamin A and antioxidant, they are used in food, cosmetics and feed industries (Johnson and Schroeder, 1995a). These pigments comprise around 700 different chemical structures with peculiar colors and biological properties (Stafsnes et al., 2010). The carotenoids are lipophilic isoprenoid molecules (Christaki et al., 2013) containing double bonds that form a light absorbing chromophore, which gives their staining characteristics (Figure 1). Because of these double bonds, carotenoids are sensitive to reactions such as

oxidation and isomerisation, and also to light, heat, acids and oxygen (Amorim-Carrilho et al., 2014; Mata-Gómez et al., 2014).

The production of carotenoids from microorganisms arose to compete with the production of carotenoids by chemical processes, as an alternative to synthetic additives (Bhosale, 2004). Due to the ability of various microorganisms to synthesize carotenoids, biotechnology has been considered the best alternative for the market of natural pigments, which is evident from the increase of studies about microbiological dyes (Sandmann, 2001). Advantages of biotechnological production include the ability of microorganisms to use low cost substrates, the optimized control of cultivation, minimized production time and the natural origin of the synthesized dyes (Wu

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Table 1. Productivity or yield of carotenoids depending on the choice of the substrate and microorganism.

Microorganism	Carotenoid	Substrate	Productivity	References
<i>Nannochloropsis gaditana</i>	Astaxanthin, β -carotene, canthaxanthin, neoxanthin, violaxanthin and zeaxanthin	-	393.0 - 773.7 mg.kg ⁻¹ dry biomass	Millao and Uquiche, 2016
<i>Dietzia natronolimnaea</i> HS-1	Canthaxanthin	Glucose	7.67 mg.L ⁻¹	Gharibzahedi et al., 2012
<i>Sporobolomyces ruberrimus</i> H110	Torularhodin, torulene, β -carotene and γ -carotene	Glucose and pure glycerol	0.0064 g.L ⁻¹ .h ⁻¹	Cardoso et al., 2016
<i>Paracoccus</i> bacterial strain A-581-1 (FERM BP-4671)	β -Carotene, echinenone, anthaxanthin, phoenicoxanthin, β -cryptoxanthin, Astaxanthin, asteroidenone, adonixanthin, zeaxanthin	Sources of carbon, nitrogen and inorganic substances	91.9 mg.L ⁻¹	Hirasawa and Tsubokura, 2014
<i>Chlorella zofingiensis</i>	Canthaxanthin	-	150 mg.L ⁻¹	Li et al., 2006.
<i>Gordonia amicalis</i> HS-11	1-OH-4-keto-carotene and 1-OH-carotene	n-Hexadecane	714.31/0.9 μ g.g ⁻¹ dry weight	Sowani et al., 2016
<i>Rhodotorula glutinis</i>	β -Carotene, torularhodin, torulene and γ -carotene	Glucose	206 μ g.g ⁻¹ dry weight	Davoli et al., 2004
<i>Saccharomyces cerevisiae</i> mutants	β -Carotene	Glucose	251.8 μ g.g ⁻¹ dry weight	Li et al., 2013
<i>Rhodotorula mucilaginosa</i>	β -Carotene, torularhodin torulene	Glucose, molasses, sucrose and whey lactose sugars	35.0 mg.g ⁻¹	Aksu and Eren, 2005
<i>Scenedesmus</i> sp.	β -Carotene, astaxanthin and lutein	Autotrophic	9 mg.L ⁻¹ .d ⁻¹	Pribyl et al., 2015
<i>Rhodospiridium toruloides</i> NCYC 921	β -Carotene	Glucose	0.29 g.L ⁻¹ .h ⁻¹	Dias et al., 2015

bacterioruberin is the most found carotenoid (Asker and Ohta, 1999). The *Flavobacterium* sp. is a known marine bacterium related to optimum production of zeaxanthin (Masetto et al., 2001), and *Haloferax alexandrinus* has good industrial perspective for the production of canthaxanthin (Asker and Ohta, 2002). Other bacteria such as *Agrobacterium aurantiacum* and modified *Escherichia coli* (Misawa et al., 1990),

Mycobacterium breviciae, *Mycobacterium lacticola*, *Rhodobacter sphaeroides*, *Rhodococcus maris*, *Streptomyces chrestomyceticus* and *Erwinia uredovora* also have the ability to synthesize carotenoids (Dannert, 2000).

The production of carotenoids by non-photosynthetic bacteria is influenced by the composition of the culture medium and also by temperature, agitation speed and aeration

(Roukas et al., 2002). Studies have shown that carbon and nitrogen sources (Naveena et al., 2006), inorganic salts (Fang et al., 2010), chemical agents (Bhosale et al., 2004) and metal ions (Giotta et al., 2006) result in higher or lower synthesis of pigments.

Production of carotenoids is directly associated with light, which sometimes favors or inhibits the production of some types of carotenoids in

different microorganisms. For example, under intense light, the synthesis of carotenoids by *Spirulina platensis* was enhanced (Liu, 1984) and the *Flavobacterium* sp. was positive for the production of zeaxanthin (Arakawa et al., 1977). Studies suggest that the production of pigments by chemotrophic bacteria such as *Rhodospseudomonas spheroides* and *H. salinarum* is a way of protection of the cell against the harmful effects of light (Dundas and Larsen, 1962).

Carotenoids produced by yeasts and filamentous fungi

Among the microorganisms capable of synthesizing carotenoids are yeasts and filamentous fungi. The best known genera of carotenoid producing yeasts are *Rhodotorula*, *Rhodospiridium*, *Sporobolomyces* (Cardoso et al., 2016), *Phaffia* (Johnson and Lewis, 1979) and *Sporidiobolus* (Buzzini et al., 2007). The compositions of carotenoids are similar, consisting of β -carotene, γ -carotene, torulene and torularhodin. The torulene is the carotenoid of higher occurrence in yeasts (Zoz et al., 2015).

Results of many studies indicate that carotenoids production by yeasts can become industrially viable by using by-products as carbon sources (Buzzini et al., 2007); this also reduces the environmental problems linked to waste and effluent emissions (Buzzini, 2001). According to the literature the yeast *Rhodotorula glutinis* 22P together with *Lactobacillus helveticus* 12A presented yields of around 8.4 mg/L of carotenoids (Frengova et al., 1995), besides, *Phaffia rhodozyma* presented optimum yield of astaxanthin and β -carotene (Johnson and Schroeder, 1995b).

In addition to the light that is related to carotenoids production, the pH is another factor that affects the production yield (Frengova et al., 1994). According to studies with *P. rhodozyma*, the ideal pH for growth was 5.8, while the highest astaxanthin production was in pH 5.0 (Johnson and Gil-Hwan, 1990). Other studies with the yeast *Xanthophyllomyces dendrorhous* achieved a maximum concentration of 27 mg/L of astaxanthin under controlled conditions, pH 6.0 in the first 80 hours, followed by pH 4.0 in 144 hours of growth culture (Hu et al., 2006).

The production of pigments by fungi dates to hundreds of years, in the Asian continent (Mapari et al., 2005). The ascomycete *Monascus purpureus* was so named because of its reddish color in rice contaminated with this fungus (Dufossé, 2006). The pigments produced by *Monascus* can be yellow, orange and red; the red pigments being more interesting for industrial applications (Mukherjee and Singh, 2011). A company in the Czech Republic isolated a red coloring *Penicillium oxalicum* in submerged culture with sucrose and molasses (Dufossé et al., 2014). The European countries have used the fungus *Blakeslea trispora* for the industrial production of

pigments such as β -carotene and lycopene (Joshi et al., 2003).

Usually the fungi grow at temperatures between 25 and 30°C (Garbayo et al., 2003; Estrada et al., 2009; Csernetics et al., 2011). Studies have demonstrated that the fungus *Gibberella fujikuroi* is influenced by light in its mycelial growth, in the presence of light there is production of orange carotenoids and in the dark there is no carotenoids production (Garbayo et al., 2003). As previously mentioned, the production of carotenoids is influenced by factors such as light, pH, temperature and culture medium (Burja et al., 2006; Ramirez et al., 2001; Santos et al., 2016).

Carotenoids produced by microalgae

The growing demand for natural alternatives for the industry and the extensive research on strains of microalgae makes them potentially attractive. Especially, because they produce special carotenoids in specific stress conditions (Gateau et al., 2016).

The composition and productivity of carotenoids in algae is greatly influenced by environmental conditions (D'Alessandro and Filho, 2016), such as salinity and nutrients available in the culture medium (Beihui and Kun, 2001; Bocanera et al., 2004; Fazeli et al., 2006; Abe et al., 2007; Raja et al., 2007; Rao et al., 2007). The green microalgae can produce the following carotenoids: Xanthin, violaxanthin, neoxanthin, α -carotene, β -carotene, lutein and others. For example, *Chlorella* contains 93% of lutein, 2.6% of α -carotene and β -carotene, 1.3% of zeaxanthin, 0.2% of xanthophylls and 0.2% of β -cryptoxanthin (Inbaraj et al., 2006). The main traded microalgae are *Arthrospira (Spirulina)*, *Chlorella*, *Dunaliella salina* and *Aphanizomenon flos-aquae* (Spolaore et al., 2006).

Spirulina is a prokaryotic microalga, also classified as cyanobacteria, produced in several countries, the largest producer being China. It is used commercially due to its metabolic products such as phycocyanin, used as food additive. One of the possible process configurations for *Spirulina* production utilizes heterotrophic fermentation reactors containing sugars in the absence of light (Lu et al., 2011). The Chinese production uses the combination of bicarbonate and air to provide CO₂ to produce *Chlorella vulgaris* and *Spurulina* in an autotrophic process (Chen et al., 2016).

Traditionally grown in Japan, *Chlorella* recently gained prominence in China. Industrially, it presents higher yield than *Spirulina*, however, the production process has to be carefully controlled in order to avoid contamination. Centrifugation methods are used to harvest the algal biomass, which is after spray-dried and can be commercialized in the form of powder, tablets or capsules (Chen et al., 2016).

The salt-tolerant microalga *Dunaliella salina* is famous for commercially producing β -carotene (Raja et al., 2007).

India presents the largest production of carotenoids derived from microalgae, followed by Australia, the United States and China (Dufossé et al., 2005). Researches indicated the production of carotenoids by *Botryococcus braunii*, and confirmed the presence of canthaxanthin, astaxanthin and β -carotene (Abe et al., 2007). The microalga *Haematococcus* is of high commercial interest due to the production of astaxanthin, the main producers being the United States, Japan and India (Dufossé et al., 2005). The microalga *Botryococcus* sp., found in sweet pond water in Mahabalipuram, Tamil Nadu, India, showed high lutein and β -carotene contents. The authors suggest further studies to optimize the growing process of *Botryococcus* due to its high industrial potential (Rao et al., 2007).

EXTRACTION, PURIFICATION AND IDENTIFICATION OF CAROTENOIDS

Although the evolution of biotechnology contributed to the optimization of the synthesis of carotenoids, there is still need for research to improve the process efficiency and commercial gain. The fermentation process is followed by separation and purification methods to recover the pigments, and these usually represent the major production costs. After the extraction methods are set according to the characteristic of the sample, procedures to obtain the pure carotenoid are followed (Feltl et al., 2005). Most of the studies involving carotenoid extraction and purification were performed at laboratory scale.

In order to release the intracellular carotenoids, it is necessary break the cell (Valduga et al., 2009) to extract its components. It is at this stage that comes the challenge of recovering the compounds extracted with minimum possible damage due to the high sensitivity of the molecule out of its environment (Pennacchi et al., 2014).

Carotenoid extraction techniques use organic solvents to disperse the substances, the most commonly used solvents are acetone, chloroform, dichloromethane, hexane, cyclohexane, methanol, ethanol, isopropanol, benzene, carbon disulfide, diethyl ether and the technology of Supercritical Fluid Extraction (SFE) with carbon dioxide, which has been diffused in recent works. Purification can be carried out by conventional procedures such as adsorption column chromatography, differential extraction, countercurrent extraction and differential crystallization (Mezzomo and Ferreira, 2016). The development of methods for the separation of carotenoids from the *Paracoccus* bacterium was performed by precipitation from the culture of the producer bacterium, centrifugation, filtration or decantation at acidic pH. Carotenoids were then quantified by high performance liquid chromatography (HPLC) (Hirasawa and Tsubokura, 2014).

Most studies involving carotenoids used chromatography

techniques coupled with other techniques that confer selectivity and separation efficiency. The purification and identification of carotenoids can be performed by liquid chromatography coupled to mass spectrometry (LC-MS) (Oliver and Palou, 2001; Ravanello et al., 2003; Stafsnes et al., 2010; Davoli et al., 2007), comparing the mass spectra with standards or databases (Martínez-Laborda et al., 1990). If they are not available, coupling the methods of Diode Array Detectors (DAD), Photodiode Array (PDA) or UV-VIS (Fong et al., 2001) with chromatography LC-MS/MS contributes with the results.

A recent study analyzed the pigment canthaxanthin using an UV-HPLC method, with separation in a Lichrospher 100 RP-18 silica column, the isocratic mobile phase used was acetonitrile and methanol (80:20, v/v) at a flow rate of 2 mL/min (Gharibzahedi et al., 2012). Also, the carotenoids produced by *Haloflex alexandrinus* TMT strain were analyzed by HPLC, and the carotenoids β -carotene, 3-hydroxyechinenone, γ -carotene, cis-astaxanthin, lycopene, anhydrobacterioruberin, bacterioruberin isomer, bacterioruberin and canthaxanthin were identified (Asker et al., 2002). The separation and purification of canthaxanthin from the microalga *Chlorella zofingiensis* was performed using a high speed countercurrent chromatography technique (HSCCC), which successfully yielded 98.7% of purity from 150 mg of crude extract (Li et al., 2006).

The bacteria of the genus *Micrococcus* produce different colored pigments, yellow, green and red. It is known that the main pigments of *Micrococcus roseus* have been purified by the HPLC system and the molecular weight of the samples has been determined by mass spectra. Samples were analyzed on a C-18 column, eluting with 80 to 100% ethanol at 470 nm with photodiode detector, under controlled conditions. The main carotenoid detected was β -carotene (Shivaji et al., 1991).

Pigments of the bacteria *Micrococcus luteus* and of the yeast *R. glutinis* were purified with HPLC, using binary solvents such as ethyl-methyl ether and tert-butyl ether, previously filtered on a cellulose 0.2 μ m filter and with the reverse polymer phase C-30 at 10°C. The substances of interest were detected and identified applying a PDA and using apo-CAR as internal standard. It was possible to identify key carotenoids, including cis and trans isomers (Kaiser et al., 2007).

The purification of pigments from the yeast *Phaffia rhodozyma* was performed by chromatography using acetone and identification was performed by electronic absorption mass spectroscopy, confirming the synthesis of astaxanthin by the yeast (Johnson and Lewis, 1979). The production of lycopene by *Yarrowia lipolytica* was confirmed using HPLC with various compositions of the mobile phase, water, methanol, acetonitrile and ethyl acetate (Matthäus et al., 2014).

Studies with *Saccharomyces cerevisiae* ULI3 succeeded in converting β -carotene to β -apo-100-

carotenal, by the action of β -carotene-9,100-oxygenase enzyme (ScBCO₂), altering two different biochemical pathways, making it apt for enzymatic biotransformation. Analyses of ultra-HPLC-ion trap MS equipped with an atmospheric pressure chemical ionization ion source, LC-MS analysis and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) showed that the enzyme has the potential to produce apocarotenoid precursors of biotechnological interest (Wei et al., 2015).

The red yeast *R. glutinis* produces pigments such as β -carotene, torulene and torularhodin. The percentage of the carotenoids was determined with UV-VIS diode array spectrophotometer, separated by reversed phase HPLC beyond thin layer chromatography (TLC) (Kim et al., 2007). A study with the new carotenogenic bacteria *Jejuia pallidilutea* strain 11shimoA1 was performed, with Fast Atom Bombardment Mass Spectroscopy (FAB-MS), nuclear magnetic resonance (NMR), circular dichroism (CD), DNA molecular analysis and the quantitative analysis of carotenoids was subjected to HPLC (Takatani et al., 2014).

For a mutant *Paracoccus* sp. strain TSAO538 that selectively synthesizes canthaxanthin, the composition of the carotenoids was analyzed by reverse phase HPLC with Sherisorb ODS2 column and diode detectors and a solvent containing proportions of ethyl acetate, acetonitrile and water. TLC was performed on Kieselgel 60 F254 silica plates with diethyl ether and hexane or ethyl acetate. The absorption spectra were recorded using UV/VIS spectrophotometer with redistilled acetone and diethyl ether, and subsequently a mass spectrometry analysis was performed (Tanaka and Kawasaki, 2013).

Researchers have used recombinant enzymes to release carotenoid precursors, identified by Gas Chromatography-Flame Ionization Detector (GC-FID) and Gas Chromatography-Mass Spectrometry (GC-MS), analyzed by means of HPLC-DAD and HPLC-MS (Zorn et al., 2009).

Pigments from the *Monascus* fungus were purified by thin layer chromatography (Feng et al., 2012), or in silica gel column with CH₂Cl₂ / acetone 99:1 or chloroform-ethanol 9: 1 v/v (Vidyalakshmi et al., 2009).

As can be seen, separation and purification methods for analytical purposes are well established for carotenoids. However, the development of purification processes that can be economically scaled-up is essential for industrial production, and should be the focus of future researches.

Conflicts of Interests

The authors have not declared any conflict of interests.

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