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Biotechnological Production of Carotenoid from Oleaginous Red yeast and Its Applications

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

The demand for carotenoids and their derivatives from natural sources is increasing rapidly due to public concern about food safety and health issues, and thus, carotenoid production from microbial fermentation is increasing significantly due to its ability to accumulate higher levels of carotene. Carotenoids, lipid-soluble pigments, are responsible for the vibrant colors in food and microorganisms. Carotenoids have the most important advantages in terms of antioxidant and anticancer activity. These possible applications are used for treating various diseases like xerophthalmia, keratomalacia, skin acne, breast cancer and tumor formation. They are widely used in the pharmaceutical, cosmetics and food industries. Due to the overall increase in the cost of carotenoids, carotenoids are produced in the pharmaceutical, food and cosmetics industries through chemical synthesis or extraction from plants. The oleaginous red yeast, *Rhodotorula minuta*, is well known for producing a high yield of carotenoids with a low production cost. Over the years, these carotenoids have been produced from oleaginous red yeast, using low-cost substrates or agricultural waste for cost-effective purposes. In this paper, we highlighted the production of carotenoids from oleaginous red yeast and its applications.

Keywords: Carotenoids; Rhodotorula; Agricultural residue; Antimicrobial activity.

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1. INTRODUCTION

Color is an important attribute that determines consumer acceptance of food and health-related drug products. Recently, the global markets for pharmaceuticals, food and cosmetics industries have been fulfilled via synthetic color pigments. Although the application of chemically synthetic color pigments is restricted because of their toxicity [1]. Carotenoids are natural pigments responsible for the colorant in fruits, flowers and roots that occur in photosynthetic systems of higher plants, algae, yeast and bacteria. These carotenoids are hydrophobic in nature and are precursors of vitamin A with beneficial effects, including boosting the immune system and reducing the risk of degenerative diseases such as cardiovascular disease, cancer, skin problems and eye deficiencies [2]. The vitamin A precursor helps in the formation of rhodopsin, which is called a photoreceptor pigment in the retina of humans, which is important in the vision process. The deficiency of vitamin A leads to xerophthalmia, keratomalacia and skin acne. Xerophthalmia is one of the major nutritional problems all over the world that leads to dryness of the conjunctiva, cornea on the surface of the eye and blindness, particularly in children [3]. Keratomalacia, in which the cornea becomes hazy and develops erosions, automatically leads to major surgery. Some skin problems, i.e., acne, have been diagnosed dermatologically due to a deficiency of vitamin A. Vitamin A is also responsible for maintaining epithelial tissues, lysosome stability and glycoprotein synthesis. Moreover, carotenoids also show anticancer activity and are important antioxidants for humans that scavenge oxygen and pyroxylin radicals. In addition, they are also useful in dietary supplements, food coloring agents, cosmetic and pharmaceutical drugs. These carotenoids are further differentiated according to their cyclic structure into four types, i.e., β-carotene, γ-carotene, torulene and torularhodin.

2. TYPES OF CAROTENOIDS

2.1 β-carotene and γ-carotene

Carotenoids' derivative form, i.e., β-carotene, has an acyclic structure with a long chain that is conjugated with double bonds. They act as an essential dietary supplement for human growth, development, immune system function, and vision. They are organic and give strong red-orange color pigments to plants, fruits, and oleaginous microorganisms [4]. Due to its high antioxidant property and bioactivity, it is also widely used in the pharmaceutical industry [3]. They also act as coloring agents for drugs and ointments. In the food industry, an orange-red pigment (β-carotene) is used in non-alcoholic beverages, edible fats, cheese, pastry and ice cream. Researchers demonstrated that β -carotene has a dual function in providing color to food while also improving its nutritional value. Cosmetics industry, it is used in sunscreen, which protects skin problems and exposure to UV radiation [1].

Commercially, these antioxidants also have the ability to reduce the risk of various skin problems and the ageing of humans. Among the numerous functions of β-carotene, the most common advantage is pro-vitamin A supply, which helps in the embryonic development, growth and vision of a new born child. It is also thought to be a cancer transporter gene inhibitor, which would result in anticancer activity [2].

β-carotene is produced naturally via oleaginous microorganisms by biotechnological processes. According to the studies, *Rhodotorula* species are natural β-carotene producers. For example, *R. glutinis* can produce β-carotene up to 25%–43% and torulene, up to 28%–30%. If grape skin were used as a carbon source, *R. glutinis* could produce 60–70% of the carotenoids. In some cheap carbon sources, such as cheese whey, sugarcane molasses, wheat and rice straw, etc. Oleaginous yeast can also accumulate a high amount of carotenoids. Another differentiated carotenoid is γ-carotene. It is also an isomer of β-carotene, which has strong antioxidant properties and is a potential precursor of vitamin A, though less than β-carotene [5]. γ- carotene concentrations are present in orange carrots with high serum concentrations. Its structure is quite equivalent to the molar amount of β-carotene due to the presence of one molecule of biologically active retinol that gets converted after central cleavage. Like other carotenoids, it has anti-carcinogenic properties. Low or high dietary intake of γ-carotene alone may enhance immune function and lower the risk of cardiovascular disease and cancer.

2.2 Torulene and Torularhodin

Torulene is derived from γ-carotene, whereas torularhodin is formed from torulene. Both exhibit the function of vitamin A and antioxidant properties [6]. They have the capacity to neutralize free radicals and also inhibit the

growth of prostate cancer and tumor formation. Torulene and torularhodin, in comparison, have higher antioxidant activity than β-carotene [7].

3. SOURCES OF CAROTENOIDS

3.1 Plants

Carotenoids are accumulated in the chloroplasts in a significant amount and are naturally synthesized by flowers, fruits and vegetables such as lily, marigold containing β-carotene, γ-carotene, antheraxanthin and lutein. In fruits, i.e., grapes, mango, melon, papaya, peach and tomatoes, lutein, β-carotene, γ-carotene, lycopene and phytopene are present. While green vegetables such as beans, broccoli, cabbage,kale, lettuce, spinach and yellow-orange vegetables contain β-carotene, lutein, etc. as shown in Table 1 [3]. In addition, xanthophylls, cryptoxanthin and zeaxanthin are also present. In purple-colored vegetables, a significant number of non-vitamin A active carotenoids are present. Saffron plants typically contain different apo-carotenoids.

Different types of carotenoids are produced from different sources depending on their genotype, maturation phase, cultivation, climatic conditions, post-harvest and processing practices. Carotenoid-rich sources, mainly fruits and vegetables, have received a lot of attention recently due to the health-related benefits expressed after their regular consumption. In addition to their key roles in photosynthesis, photo-protection, and response to environmental stress, carotenoids are involved in interactions with pathogens and symbiotic organisms. Moreover, these pigments play important roles in germination, photomorphogenesis, fruit development and different signaling and regulatory processes. Carotenoids are very abundant in flowers and fruits, conferring yellow, orange and red colors to attract insects and animals for pollination or seed dispersal [6].

Table 1. Natural Sources of carotenoids [3]

Sources of Carotenoids	Carotenoids
Plants	
Flowers	
Adonis, Chrysanthemum, lily, marigold, *Oncidium, Osmanthus*	β-carotene, γ-carotene, Antheraxanthin lutein
Fruits	
Capsicum, cashew apple, citrus, grape,Mango, melon, papaya, peach, tomato	β-carotene, lutein, γ-carotene,Phytopene, lycopene
Seeds	
Arabidopsis, canola, maize, pumpkin, red millets, sunflowers, wheat	β-carotene, xanthophyll, lutein
Vegetables	
Green vegetables	
Beans, broccoli, cabbage, kale, lettuce, spinach etc.	β-carotene, lutein
Yellow-orange vegetables	
Carrot, corn	lutein, β-carotene,cryptoxanthin, zeinoxanthin
Red-purple vegetables	Non-vitamin A active,
Tomato, watermelon, red paprika	capsanthin, carotenoids, lycopene
Oleaginous microorganism	
Oleaginous red yeast	Torulene, Torularhodin and β-Carotene
Fungi	β-carotene, Lycopene, Astaxanthin
Micro-algae	Xanthophyll, Zeaxanthin,lutein, Antheraxanthin
Bacteria	β-carotene, Salinixanthin,Astaxanthin, Zeaxanthin
Animals	.
Marine animals	β-carotene, fucoxanthin, Peridinin, Diatoxanthin,Alloxanthin, Astaxanthin
Reptiles	Lutein, β-carotene

3.2 Oleaginous Microorganisms

Natural carotenoids are found in plants and vegetables in the form of carotene and xanthophyll. According to the research, carotenoids are now the most important feedstocks for pharmaceutical, food and cosmetics industries, but due to limited resources and high costs, they cannot meet the demands economically [8]. Therefore, enormous efforts have been initiated to develop advanced strains for higher carotenoid production for pharmaceutical-based chemicals and food additives [9]. Oleaginous microorganisms such as fungi, bacteria, micro-algae, and yeast have the capability of accumulating higher carotenoids [8].

3.2.1 Filamentous fungi

The increasing demands for pigments of natural origin particularly in the food sector, further increases the interest to investigate filamentous fungi as a potential pigment producer. Several carotenoids such as β-Carotene, lycopene, canthaxanthin, cryptoxanthin and astaxanthin are important industrially as components of animal feeds for coloration and as precursors of vitamin A and other drugs related products for human. A few strains of ascomycetes filamentous fungi are being considered as potential pigment producers include, some strains of *Talaromyces (e.g. T. purpurogenus*and *T. atroroseus* producing red pigments, *Herpotrichia rhodosticta* produce orange pigment. These strains are non mycotoxigenic and non- pathogenic to humans. After two centuries of research, *Neurospora sp*. are able to accumulate orange pigments such as *N. crassa*, that have been investigated for biosynthesis of carotenoid and its regulation specially mixture of carotene pigments such as γ-carotene and neurosporaxanthin. However the higher production of mycotoxins is a major problem which limits the industrial application of these strains of fungi [10].

3.2.2 Microalgae

Microalgae are small groups of algae that exist in nature as a single cell and are rich in carotenoids because of their rapid growth and strong adaptivity in different cultural mediums and leads as a main raw material for carotenoids. There are many microalgae such as *Haematococcus lacustris, H. rubicundus, Bracteacoccus aggregatus.* The main carotenoids are astaxanthin and β-Carotene. The highest fucoxanthin production of 26.6 mg/g DW from *Mallomona ssp* is being investigated. freshwater microalgae *Chlorella zofingiensis* yield, astaxanthin content of 5.32-6.02 mg/g DW. However, the disadvantages of microalgae is that they require continuous sunlight for growth and high levels of carbon substrate. [11]

3.2.3 Bacteria

Many promising carotenoid producing bacterial strains have been isolated throughout freshwater and marine waters that possess diverse carotenoid such as β-cryptoxanthin, canthaxanthin, zeaxanthin, astaxanthin and salinixanthin, that contribute to the red or orange pigments that help protect bacterial cells from the extremely humid desert sunlight. Carotenoids production from bacterial sources is an alternative for plant based carotenoid due to their short life span, easily maintaining cultural conditions. Moreover, bacterial strains can produce high- level of carotene using low cost substrate. Popular bacterial strains are *Flavobacterium spp., Agrobacterium app., Pseudomonas aeruginosa* and *Chromobacterium spp.* They are used in various applications as carotenoid producers in the field of food , feed and as a prophylactic agent for multiple diseases. According to the report, recombinant strains of *E.coli*, accumulate lycopene of 220 mg L-1 from 27 g dry cell weight L-1 during fed-batch fermentation. The disadvantages of bacteria is, it cannot fulfill the higher demand of carotenoid in industrial sectors [12]

3.2.4 Oleaginous yeast

However, fungi, bacteria and micro-algae have limited efficiencies in producing carotenoid due to higher costs and time-consuming problems. Oleaginous yeast have a capability of producing carotenoids up to 40% of the total biomass weight, including *Rhodosporidium/Rhodotorula* and *Sporidiobolus/Sporobolomyce.* Interestingly, the advantage of using oleaginous yeast is that it is easily scaled-up, has a higher growth rate and can be grown on residual substrate from an agricultural source while producing lipids and carotenoids simultaneously [13]. *R. minuta* and other red yeast mainly produce β-carotene, torulene, and torularhodin. *Rhodotorula* yeast strain requires growth parameters such as carbon, nitrogen sources, low-cost substrate and culture conditions to produce more carotenoid. In the production medium, for example, *Rhodotorula glutinis* and *Rhodotorula graminis* produce 11–15% of the total carotenoid. On the other hand, *Rhodosporidium* species, due to their

flexible substrate adaptability, are considered a promising candidate for carotene production. *R. toruloides* yields γ-carotene with of 8.6 ± 0.2 mg/g DW [14]. *R. toruloides* NRRL Y-1091 also produces a high yield of carotene, with the highest carotenoid production of 24.58 ± 1.88 mg/L [15]. *R. toruloides* and *Rhodosporidium diobovatum* red yeast strains also have the capability of producing a high yield of carotenoid and its derivatives. The composition and yield of different strains are affected by medium composition, environmental conditions, and carbon nitrogen ratio [15].

3.2.4.1 Rhodotorula minuta

Rhodotorula minuta is a unicellular oleaginous microorganism of the basidiomycetes phylum that has distinctive yellow, orange, and red colonies. These *R. minuta* species are known for synthesizing natural carotenoids under certain cultural conditions, and the main carotenoids produced are torulene, torularhodin, and a minute quantity of β-carotene. They are important in food, cosmetic, and pharmaceutical industries [16]. The oleaginous red yeast has a faster growth rate as compared to similar oleaginous yeasts like *Lipomyces starkeyii and R. glutinis.* According to the studies for the production of pigments from red yeast, various dairy waste substrates, agricultural residues and molasses were used [17]. Researchers favored *R. minuta* oleaginous red yeast, one of the most efficient carotenoid producers due to its ability to grow in harsh environmental conditions where other red yeasts cannot. That's why it is a more suitable oleaginous microorganism for a biotechnological application, both in pharmaceutical and food industries on a commercial basis.

3.2.4.2 Rhodosporidium toruloides

R.toruloides is an aerobic, dimorphic, and non-pathogenic, oleaginous pink yeast that is naturally present in different environmental sources, e.g., soil, wood pulp, conifers, seawater plants and dry leaves. When excess carbon sources are used during cell culture growth, this oleaginous yeast can accumulate carotenoids and their derivatives to more than 30–40% of the total biomass dry weight [13, 18]. There are various carbon sources such as lignocellulosic biomass, crude glycerol, vegetable and animal waste, sugarcane molasses and cheese whey, etc. *R. toruloides* is able to utilize different types of carbon sources for carotenoid production and shows robustness in terms of resistance against inhibitors produced after pretreatment of lignocellulosic biomass [19]. According to several studies, growing red yeast using cheap carbon and nitrogen sources is the most efficient nutrient for the growth of the cell [20]. They produce carotenoids at a higher level, which makes them promising feedstocks for pharmaceuticals, food colorants and cosmetics. Therefore, many researchers are working on increasing carotenoid production using engineered strains of oleaginous yeasts [17]. For example, carob pulp syrup and sugarcane molasses were used for carotenoid and lipid production using the *R. toruloides* NCYC 921 strain, which contains a total sugar concentration of 75 g/L and produces lipid productivity (1.90 g/L) and carotene productivity (9.79 g/L h^{-1}) [21]. In another report, at high salt concentration, *R. toruloides* increases lipid and carotenoid production processes, and it was observed that 27.2% (w/v) increased in carotenoid and 36.2% (w/v) in lipid production under osmotic stress [22]. *R. toruloides*was grown in a variety of food waste extracts to increase lipid and β-carotene production, yielding good results of 1.60 mg/L carotene and 9.6 g/L lipid production [23].

3.2.4.3 ***Rhodotorula glutinis***

R. glutinis is an important oleaginous red yeast that can accumulate a variety of valuable compounds including microbial lipid, pigmented carotenoid, and enzymes. They are non-pathogenic and mostly found in soil, fruits, dry leaves and plants, animal waste and milk products. *R. glutinis* is aerobic and mesophillic in nature [24]. Certain carbon sources (glucose, molasses, fructose, glycerol) and nitrogen sources (ammonium sulphate, chloride, yeast extract, or monosodium glutamate wastewater) are used for lipid production and carotene. Additionally, researchers are working on *R. glutinis* to increase microbial lipid and carotene production either by improving the culture medium or by genetically modifying the *R. glutinis* strain. It was demonstrated that *R. glutinis* has the potential capability of producing lipid and carotenoid production on a large scale.

4. CHEMICAL SYNTHESIS OF CAROTENOID

Carotenoids are synthesized on an industrial scale. Among them are β-carotene (-apo-8′-carotenal -apo-8′-carotene), lycopene, xanthophyll (canthaxanthin, astaxanthin and cytranaxanthin). These chemicals are

synthesized from two reactions, i.e., Wittig reactions and Grignard reaction. Grignard compounds, for producing carotenoids and their derivatives. The Wittig reaction produces alkenes and triphenylphosphine oxides with the chemical reaction of aldehydes or ketones with triphenylphosphine salt. In addition, this salt is called a "wittig reagent" in the reaction. The Wittig reaction is most often used to combine di-aldehyde or ketone molecules containing 10 carbon atoms and two phosphonium salt molecules, each containing 15 carbon atoms, as shown in Fig. 1. Then the reaction is subjected to an isomerisation reaction, producing compounds of 40 carbon atoms including β -carotene, astaxanthin or lycopene.

The other chemical synthesis of carotenoids is the grignard reaction, where the addition of organomagnesium halide, also known as the grignard reagent, is shown in Fig. 2. In this chemical reaction, one di-ketone molecule and two methanol molecules combine together and, thereafter, carotenoid is produced with 40 carbon [6]. Among other methods of producing β-carotene, the selective condensation reaction of carbonyl compounds and the homo-dimerisation reaction with dehydration and elimination can also produce the carotenoid. However, the chemical synthesis of carotenoids is harmful for humans as they are toxic and unable to be consumed in food and feed. As a result, researchers are looking for another method of producing natural carotenoids from oleaginous microorganisms capable of accumulating carotenoid and its derivatives, such as β-carotene, α-carotene, lutein and so on.

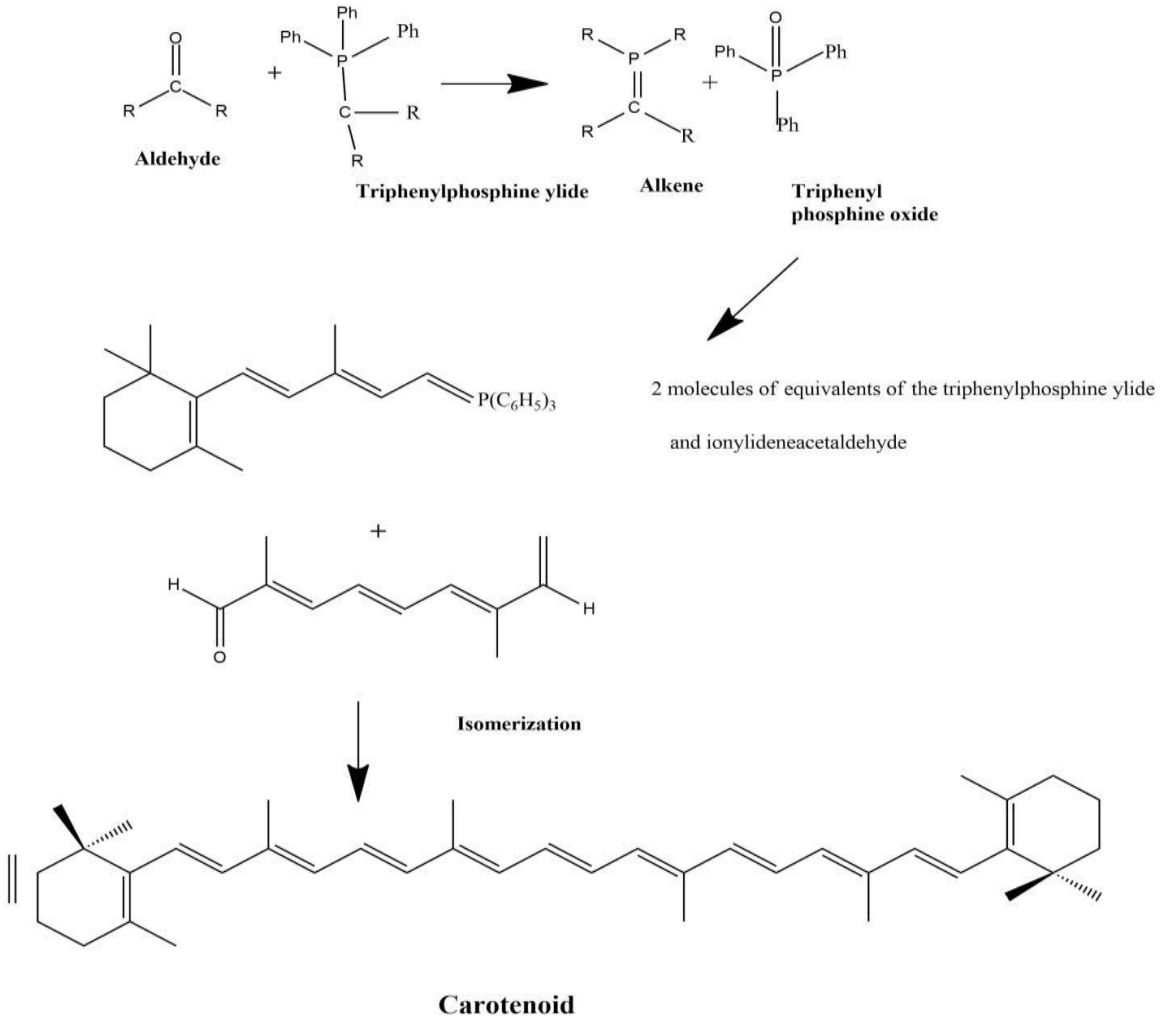

Fig. 1. Wittig condensation reaction of Carotenoid

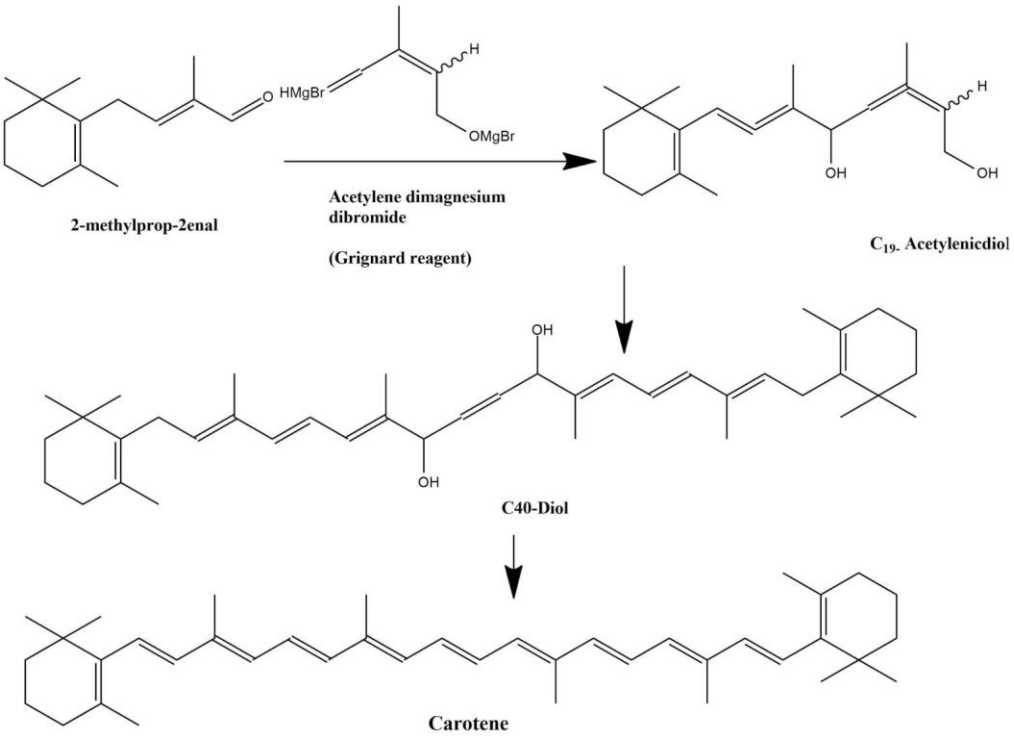

Fig. 2. Grignard reaction of Carotenoids

5. BIOSYNTHETIC PATHWAYS OF CAROTENOIDS

Carotenoids are terpenoid compounds with 40 carbon atoms based on their structure .β-carotene (γ-carotene, torulene, toruhorludin) and xanthophyll (salinixanthin, Astaxanthin, Zeaxanthin, Canthaxanthin) are the most common and natural carotenoids [1]. Carotenoids are synthesized by prokaryotes and eukaryotic microorganisms, and biosynthesis occurs in two different pathways: via the mevalonate pathway (MVA) and the methylerythritol 4-phosphate pathway (MEP) [6]. Commonly, eukaryotes such as yeast, fungi, and micro-algae synthesize carotenoids by MVA pathways with the condensation of 3 molecules of acetyl-CoA to synthesize mevalonic acid via acetoacetyl-CoA and get converted into 3-hydroxy-3-methyl-glutaryl (HMG-CoA) with the help of the enzyme hydroxymethylglutaryl-CoA synthase. This HMG-CoA with hydroxymethylglutaryl-CoA reductase enzyme gets converted into Mevalonic acid and biocatalytic reactions mediated by mevalonate kinase form mevalonate 5 phosphate (MVP), which in turn originates into the mevalonate pyrophosphate (MVPP) mediated by phosphomevalonate kinase, and then the formation of IPP occurs as shown in Fig. 3. Isopentenyl pyrophosphate (IPP), isomerized to dimethylallyldiphosphate (DMAPP). After DMAPP, the phenyl transferase enzyme converts it into geranyl pyrophosphate to geranylgeranyl pyrophosphate, which further gives phytoene, which is mediated by phytoenedesaturase enzyme to form neurosporene, further classified into lycopene and β-zeacarotene which gets converted into γ-carotene to β-carotene, torulene and torularhodin, a carotenoid compound [6]. While in prokaryotes, the MEP pathways create IPP and DMAPP through a primary condensation reaction between pyruvate and glyceraldehyde-3-phosphate. It begins with the glycolysis cycle, where a 6 carbon source (glucose) is converted into 2 pyruvate, which enters the mitochondrial matrix from the cytoplasm of the cell. This acetyl-CoA is further oxidized and moves towards the cytoplasm of the cell for catalyzing the first reaction with glyceraldehyde-3 phosphate in MEP pathways, where 1-deoxy-D-xylulose-5-Phosphate convert to form 4 diphosphocytidyl-2C-methyl-D-erythritol -2-phosphate and from 4-diphosphocytidyl-2C-methyl-D-erythritol-5-phosphate gets converted into isopentenyl pyrophosphate which give reversible reaction to Dimethylallyldiphosphate and the chain elongation occurs through the condensation

mechanism of IPP to DMAPP. The short-chain-length of DMAPP is mediated by phenyl transferase synthesis, which forms geranyl pyrophosphate and geranylgeranyl pyrophosphate, which are the precursors of carotenoids. Colorless C40 phytoene is formed by the condensation of two molecules of geranylgeranyl pyrophosphate, which is catalyzed by phytoene synthase. Subsequent desaturation of phytoene by phytoenedesaturase further produces neurosporene, red-coloured lycopene and β-Zeacarotene. The synthesis of cyclic carotenoids of lycopene and the dehydration of β-Zeacarotene further produces β-carotene, toluene, and torularhodin production, which in turn can be converted into other types of carotenoids derivatives.

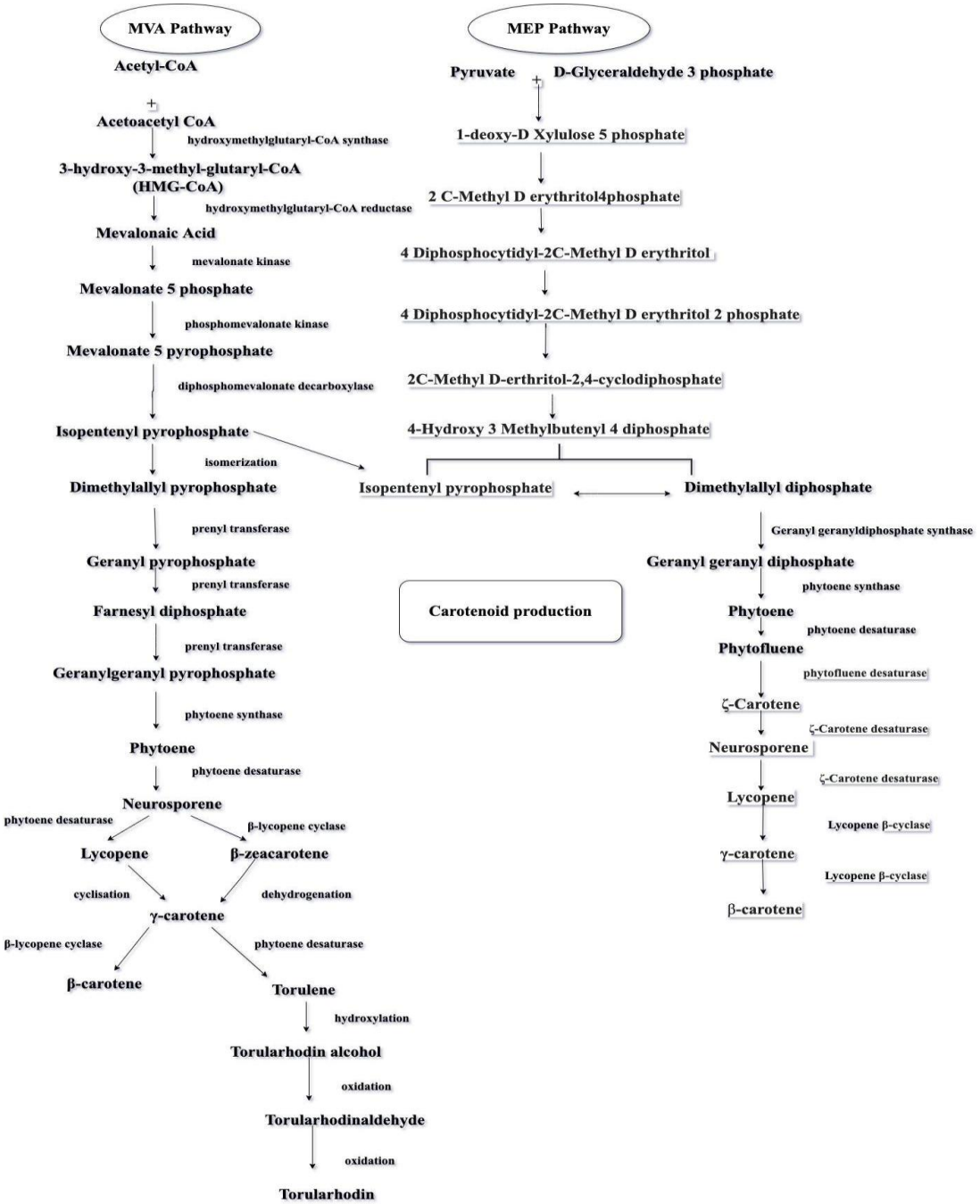

Fig. 3. Biosynthetic pathways of carotenoid production by MVA and MEP pathway

6. METHODS OF EXTRACTION OF CAROTENOIDS

6.1 Organic-solvent Extraction

Carotenoids are hydrophobic in nature and are usually extracted from organic solvents. Generally, organic solvents are of two types: polar solvents and non-polar solvents. Polar solvents, i.e., acetone, ethanol and ethyl acetate, for extracting derivatives of carotenoids, are easy and simple methods for laboratory scale extraction applications. While non-polar solvents like petroleum, hexane and tetra-hydrofuran are excellent choices for extracting precursors of carotene. However, excess use of toxic solvents should usually be avoided because they become toxic for the environment and can cause harm to human health [4].

6.2 Saponification

Mainly higher production of carotene is found in oleaginous microorganisms after plants and this carotene exists in free form and has triacylglycerol biosynthesis ability. In order to release carotenoids in ester form, saponification reactions containing alcohol and KOH or NaOH are used. This alcohol saponified the compound and then removed the ester bond to create functional groups. The saponification reaction requires specific conditions, i.e., temperature, time, and alkali concentration, which are the main factors that affect the yield of carotenoids [1]. In Liu et al (2021) report, it was found that the production of carotenoids in *R. toruloides* by using a saponification method in which alcohol and KOH had a higher recovery rate (78.7 ±2.4%) [4].

6.3 Microwave and Ultrasound Assisted Extraction

A variety of combined extraction technologies have been developed in recent years. Microwave and ultrasonic-assisted extraction technologies were used for direct and rapid extraction of carotenoids and their precursors in order to overcome the problems of low yield, toxicity and high extraction cost of carotenoid production. However, single microwave or ultrasonic extraction can no longer meet the needs of production, so a variety of combined extraction technologies have been developed. For example, it was reported that in *Phaffia rhodozyma*, lactic acid was used to destroy the cell clusters and then carotene derivatives were extracted via ultrasonic extraction, which was then used further in the food and feed industries [25].

6.4 Green Solvents as Ionic Liquids

The organic solvents, for example, methanol, ethanol, acetone and hydrochloric acid, etc., are extremely flammable, volatile, and toxic, and can also cause environmental pollution. Thus, the use of these organic reagents is avoided in the extraction of carotenoids. Ionic liquids (ILs) are a new type of green solvent with many characteristics, including environmentally friendly, non-volatility, non-flammable, thermal stability, control of lead miscibility and the ability to maintain the composition of anionic and cationic salts. Lewis acidic ionic liquid and bronsted acidic ionic liquid are two types of liquids that are commonly used [4].

6.5 Enzyme-assisted Extraction

Conventionally, solvent extraction methods were used for extracting the carotenoids, but due to heat production during this extraction process, it affected the stability of carotenoids. In order to obtain a high yield of carotene precursor and improve its stability, enzyme pretreatment was used. They are non-toxic when used in food and feed industries as compared to organic solvents. Since enzymes are environmentally friendly, renewable, and increase production rate and reduce the use of solvents, their disadvantages are that they are expensive and require optimal operating conditions, which makes researchers find an alternative solution [1].

6.6 Supercritical Fluid Extraction

The new technology, named "supercritical fluid extraction technology," has been developed due to the high demand for carotenoids and their precursors in the last few years. Supercritical fluid is the most preferred extraction method in the pharmaceutical and food industries as it uses non-toxic solvents, namely water and carbon dioxide. This carbon dioxide has an ambient critical temperature range of 31 °C and carotenoids can be extracted from microorganisms under 35°C. The density of supercritical CO_2 is around 200 bar, which is close to hexane and the properties are similar for extraction. It is also a green alternative to conventional carotenoids extraction methods. More importantly, supercritical fluid extraction technology has higher permeability and diffusibility, so it can obtain carotenoids faster and higher.

7. FACTOR AFFECTING THE PRODUCTION OF CAROTENOIDS

7.1 Low Cost Feedstock

Earlier, β-carotene was extracted from carrots and fruits of palm trees, but for commercial purposes, it could not meet the higher demand in humans [1, 26]. However, the alternative i.e., oleaginous microorganisms, is used for carotenoid production, but due to its expensive carbon sources, large area for cultivation and limited growth, it cannot fulfill the demands. Recently, oleaginous red yeasts have been shown to be potentially more beneficial and effective for carotenoid production as compared to other microorganisms. *Rhodosporidium toruloides*, in particular, have the ability to accumulate natural β-carotene, torulene, and torularhodin [5,27]. *R. toruloides* can assimilate various lignocellulosic substrates for carotenoids production, including corn stover, sugarcane bagasse, rice, wheat straw, banana peels and food wastes [28]. In another study, camelina meal hydrolysates were used as a lignocellulosic biomass for the cultivation of carotenoids by *Rhodosporidium toruloides* in culture medium, and it was found to produce 13 ± 2.6 mg/L of carotenoids. The wheat straw hydrolysate was also used as a carbon source for cultivating *R. toruloides* in culture medium and, after pretreatment, it produced 24.58 ± 1.88 mg/L of carotenoid [29].

7.2 Light

Light intensity is the most important parameter in the synthesis of carotenoids in oleaginous microorganisms (fungi, microalgae, yeast and bacteria). The biosynthetic pathway and production of carotenoids are affected by light and, more importantly photo-induction, which enhances the carotenoids yields by promoting cell growth density and the activity of enzymes involved in carotenoid biosynthesis. According to the report, in *R. glutinis*, when light is facilitated the yield of torularhodin increases by 18% and the production of β-carotene increases by up to 14%. Carotenoid production is affected by white light, mostly UV-light or LED light. In addition, carotenoid production in the *R*. *mucilaginosa* strain showed a high yield of carotenoid in the presence of white light [30]. They also stated that the higher production of torularhodin in yeast improves with UV light exposure. Another report showed that β-carotene production results in a concentration of 24.6 μg^{-1} under two LED lights, whereas without light intensity, the concentration decreases to 14.69 μg^{-1}. The synthesis of torularhodin increases when yeast cells are exposed to white light. When *R. glutinis* is exposed to white light irradiation, it increases the carotenoid production, mostly toluene and torularhodin [3]. However, increases in microbial growth and the activities of enzymes are directly related to the increase in carotenoid biosynthesis [30].

7.3 Temperature and pH

Temperature is the most important factor for the biosynthesis of carotenoid pigments. It influences cell growth and enzyme concentrations in cell metabolism [31]. At low temperatures, *R. glutinis* and *R. vulgaris* increase β-carotene content [29]. In another report, the synthesis of torularhodin depends on the corresponding enzyme when it is less active than β-carotene synthesis at low temperatures [29]. The increase or decrease in temperature mostly depends on the growth of oleaginous microorganisms and their synthesis of carotenoid. Temperature increases from 29°C to 30°C increase the overall production of carotenoid and biomass yield in *R. glutinis* [4]. Temperature ranges of 20°C to 30°C are best suited for *Rhodotorula* species growth, according to the report, while higher temperatures, i.e., 32 °C or above, reduce the amount of carotenoids synthesis [5]. The carotenoid pigments i.e., β-carotene and torulene production, decrease at 31°C temperature. Whereas, torularhodin production increases at 31°C.

The pH range is also an important parameter for optimizing the growth of oleaginous yeast for carotenoid production. According to the report, at an initial pH of 5, under various temperature ranges, i.e., (22°C to 28°C), it exhibits the maximum amount of carotenoid pigments in *Rhodotorula* yeast. However, at higher temperatures, i.e., above 30°C, it lowers the yield of carotenoid. It was stated that the biomass growth of oleaginous yeast was influenced when the pH range was between 5 and 7. The highest carotenoid production was at pH 5. At pH 4, however, carotenoid production decreases. The effect of pH on the yield of carotenoids derived from *R. glutinis* was greatest when the pH of the culture medium was 3.0. The carotenoid yield was 115.8 μg^{-1} DW, while at pH 4.0–7.0, the yield of carotenoid was very high (191.7–202.9 μg^{-1} DW) [5].

7.4 Carbon and Nitrogen Sources (C/N ratio)

Biosynthetic metabolism of *Rhodotorula* species depends on the carbon source and nitrogen sources in the culture medium. The carbon to nitrogen ratio is one of the major factors that assimilate the production of carotenoid pigments. According to the investigation, it was stated that high C/N ratios are advantageous for both biomass and carotenoid yield. Thus, different experiments are performed to observe the impact of different C/N ratios i.e., (C/N 70 and 120), glucose concentration, initial pH value, and nitrogen content on the microbial cell growth for a higher yield of carotenoid pigments by *R. glutinis* [31]. Furthermore, the results showed that carotenoid production increases at high C/N ratios when the pH value is greater than (> 2), whereas it decreases at low pH 2. Since the optimum pH levels for carotenoids are around pH 6 [32]. Continuous optimization of the cultural medium in research found that the continuous supplement of organic and inorganic nitrogen sources promoted the production of carotenoids in oleaginous yeast *R. glutinis* (β-carotene, torularhodin, and torulene) by about 25.83%, 11.88%, and 24.50% respectively.

7.5 Metal Ions

Many metal ions such as Ba^{2+}, Fe^{2+}, Mg^{2+}, Ca^{2+}, Zn^{+2} are capable of accumulating carotenoid pigment in *Rhodotorula* species. According to the report, the effect of metal ions in *R. graminis* has been investigated and shows that Al^{3+} and Zn^{2+} metal ions increase the production of both γ-carotene and β-carotene. Metal ions Mn^{2+} and Zn^{2+}, on the other hand, decrease the production of bio-pigments such as torulene and torularhodin. In another study, after adding 0.1 g/L of calcium chloride, magnesium sulphate or ferric sulphate to the production medium of *Rhodotorula* species, it had a slight influence on cell growth when compared to the control. The carotenoid production with added metal ions showed excellent growth compared to those without metal ions. As per the report, the most suitable metal ion is Fe^{2+}, whose carotenoid concentration was up to 37.6%. Magnesium and calcium ions increase carotenoid concentration in *R. glutinis* by 21.4% and 9.2%, respectively. Whereas, ferric and magnesium ions increase the total carotenoid concentration by 23.6% and 9.1%, respectively. β-carotene increases after adding metal ions to cell concentration, while torularhodin decreases after involvement of metal ions. The torulene pigment also increases when Fe^{2+} and Mg^{2+} ions are added to the cell, but calcium ions decrease the growth of torulene [4]. The use of metal ions in all microbial growth has a relatively larger impact on increasing carotenoid production. For example, sodium, calcium and zinc. While potassium and magnesium are the active groups of certain enzymes in carotenoid biosynthetic pathways, they have the capability of regulating cell metabolism and cell membrane permeability in microorganisms [3, 33].

8. APPLICATION OF CAROTENOIDS

8.1 Antimicrobial Activity

The secondary metabolites of a few pigmented yeast strains have been observed for their antibacterial and antifungal activities. In general, the antibacterial activity was assessed in a qualitative or quantitative method. The carotenoids were extracted from *R. mucilaginosa, R. rubra* and *R. glutinis*, sp, where the major concentrations of carotenoids were toluene and torularhodin. *Bacillus*, *Salmonella*, *Pseudomonas* and *Escherichia* are of clinical interest for antimicrobial tests.

The MIC minimum inhibitory concentration was tested against *Salmonella choleraesius ATCC 6539, Staphyloccus aureus ATCC 13,565, Pseudomonas aeruginosa ATCC 25,442, Listeria monocytogenes ATCC 19,117 and Escherichia coli ATCC 11,229* for carotenoids from *R.mucilaginosa CCMA 0156* [12]. One of the most relevant applications of purified carotenoids as antimicrobials is their use as antimicrobial coatings in some titanium-medical products, thus improving the effectiveness of torularhodin in implants and protecting humans against bacteria. Soliman et al. (2018) showed a MIC of 1 g/mL of silver nanoparticles added to *Rhodotorula sp* [12]. ATL72 culture it shows antimicrobial activity against *E. coli* and *Bacillus*sp Also, Cunha et al. (2018) showed the lowest MIC reported for the same type of silver nanoparticles added to yeast carotenoids cultures and showed antimicrobial activity against *Candida parapsilosis*[10].

8.2 Anticancer Activity

Very promising findings were obtained for inhibiting different types of cancerous cells in humans using carotenoids and their derivatives. α-carotene, β-carotene, lycopene, torulene, torularhodin and some other derivatives exhibit

anticancer activity for prostate, breast, colon, lung, oral, gastric and skin cancers, in addition to hepatoma, leukemia, etc. Certain antioxidant response elements are activated within cells to inhibit the androgen receptor activity of cancer cells. These carotenoids are lycopene, phytoene, and phytofluene and they are further used as therapies for prostate and breast cancer cells. The experiments were performed where ER-negative Hs578T, MDA-MB-231 human breast cancer cells, and estrogen receptor (ER) positive MCF-7 were treated with carotenoids. Among them, beta-carotene significantly reduced the growth of Hs578T cells and MCF-7, and this lycopene inhibited the growth of MCF-7, MDA-MB-231 cells, and MCF-7 [6]. According to the report, Astaxanthin, a derivative of carotenoids, also inhibits the growth of breast cancer cell lines, and it is found that estrogen receptors are the major key factor. The carotene-enriched food was found to be effective against lung and stomach cancer. More recently, tumor metastasis mechanisms in humans are inhibited by lycopene by proliferating diverse cancer cell lines and slowing cell-cycle progression. Some recent reviews discuss the mechanisms of carotenoid cell-cycle arrest, anticancer activity, apoptosis-inducing effect and anti-metastasis effect. They also act as chemo-protective agents against malignant transformation and cellular mutagenesis. β-carotene, α-carotene and canthaxanthin can inhibit 3-methylcholanthrene against malignant transformation or X-ray treatment in the fibroblast cell line [6].

8.3 Antioxidant and Anti-inflammatory Activity

The world is facing severe problems in chronic health complications like cancer, cardiovascular diseases, diabetes, hypertension and other malfunctions. The primary nutrients provided by fruits and vegetables can be the solution to health-related problems as they contain carotenoid pigments. These carotenoids are rich in antioxidants, which is the main factor responsible for their protective effects against chronic diseases. Antioxidants provide additional protection against increased oxidative stress and help in scavenging two of the reactive oxygen species, singlet molecular oxygen ($^1O2$) and peroxyl radical. β -carotene plays a major role in radical transfer chains, as it has a triplet energy level close to that of singlet oxygen molecules and can transfer energy to the cells. In addition, zeaxanthin, cryptoxanthin and γ-carotene also have an ability to inhibit reactive oxygen species (ROS) in human retinal cells or cancerous cells [6]. These antioxidants have the advantages of lowering the amount of free radicals in our bodies preventing direct cell damage caused by the chain reaction and reducing the signs of ageing by preventing oxidation in our skin cells. Carotenoid activity is important for wound healing procedures in the human body and has the capability of inhibiting various carcinogenic diseases and may act as a drug or substance that may activate the immune response in an organism. The immune responsive compounds in the human body i.e., cytokinins and interleukins are produced by B lymphocytes, keratinocytes, T lymphocytes, macrophages and the endocrine system, which release enzymes and antibodies that perform inflammatory action. Interleukin-1 and interleukin-2 are cytokine groups that aid in the regulation of immune and anti-inflammatory responses in human organs in response to infections, as well as acting as mediators to induce healing. They also control the biochemical pathway and prevent the diseases from further spreading [6]. The study revealed that lycopene, a carotenoid precursor acts as a mediator that limits the release of excess glucose metabolism in human cells by effectively reducing the oxidation and degradation of free radicals and non-free radicals i.e., ROS, H_2O_2, O_2, R., etc., that results in negative effects in the mitochondria of human retinal cells. However, these carotenoids block the effects of ROS with the help of antioxidant proteins (superoxide dismutase (SOD), catalase, peroxiredoxin (Prx), glutathione reductase (GR) and thioredoxin reductase (TR)), which then activates the proliferator-activated receptor in bone health, which directly influences anti-inflammatory transcription factors and decreases the risk of osteoporosis. According to the report, the osteoporosis risk in postmenopausal women can be decreased by lycopene. The same report identifies the effects as both lycopene and β-carotene, and β-cryptoxanthin are involved in bone health [34].

9. CONCLUSIONS

Carotenoids are beneficial color pigments that protect against chronic diseases and enhance the immune system of humans. There is a keen interest in the methods of obtaining carotene and its derivatives through natural sources for the food, cosmetic, and pharmaceutical industries [35,36]. For many years, they have been widely used as food additives, anti-inflammatory drugs, cosmetics and natural food colorants. As a

matter of concern due to global warming, environmental pollution and health issues, demands for natural carotenoids in the global markets are also increasing. Due to high demand, the main quantity of these compounds are naturally synthesized from fruits and vegetables through physicochemical methods. Currently, the production of carotenoids and their precursors is more expensive than synthetic forms. Therefore, lots of new approaches have recently been developed. Oleaginous microorganisms, especially *Rhodotorula* red yeast (*Rhodotorula minuta, Rhodotorula glutinis, Rhodosporidium*sp, etc.), are capable of accumulating natural carotenoids. These oleaginous red yeasts produce a high yield of β-carotene, lutein and compete with the composition of carotenoids produced from fruits and vegetables. However, the production of carotenoids from oleaginous yeast requires carbon sources and because of that, an alternative lignocellulosic biomass has been used. Therefore, researchers are heading towards genetic modification or gene modeling of yeast to gain a high yield of carotenoids.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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