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Advances in Astaxanthin Biosynthesis

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Abstract: *Astaxanthin is a fat-soluble and water-soluble pigment, a red carotenoid widely found in oysters, rhodococcus, and salmon. It is a powerful natural antioxidant that can effectively eliminate free radicals in the body, combat aging, clear vascular waste, reduce fatigue, regulate immunity, and exhibit a variety of biological activities. It is widely used in medicine, food, and cosmetics. Although natural astaxanthin is present in a variety of organisms, its content is very low, the extraction process is complex, the yield is small, and the price is expensive. With the identification of key genes for astaxanthin synthesis, it is possible to construct the astaxanthin biosynthesis pathway in microorganisms, synthesize the precursor substances of astaxanthin, or directly synthesize astaxanthin. Microbial synthesis of astaxanthin is environmentally friendly, cost-effective, involves small microbial size, rapid growth, strong vitality, and has garnered significant attention in the industrial production of astaxanthin. This paper reviews the recent advancements in the synthesis of astaxanthin by algae, fungi, and bacteria, as well as the efforts to increase its production by microorganisms.*

Keywords: *Astaxanthin, biosynthesis, algae, fungi, bacteria*

1. Introduction

Astaxanthin is a keto carotenoid with potent antioxidant properties. It is present in aquatic animals like shrimp and algae in various geometric and optical isomers. Astaxanthin and its isomers contribute to enhancing human health[1]. Astaxanthin has a significant impact on regulating exercise-induced inflammation and immune dysfunction, and it has positive therapeutic effects on diseases such as cancer, central nervous system diseases, diabetes, and dyslipidemia[2-7]. In addition, it also has a certain effect on counteracting mycotoxins [8].

β-carotene serves as the precursor for astaxanthin biosynthesis. In higher plants (Figure 1), the biosynthesis pathway of astaxanthin involves a three-step transformation of carotenoid by 4-deoxyxanthophyll synthase (CBFD) and 4-hydroxycyclo4-deoxyxanthophyll synthase (HBFD). These steps are catalyzed by CBFD, HBFD, and CBFD in sequence[9].

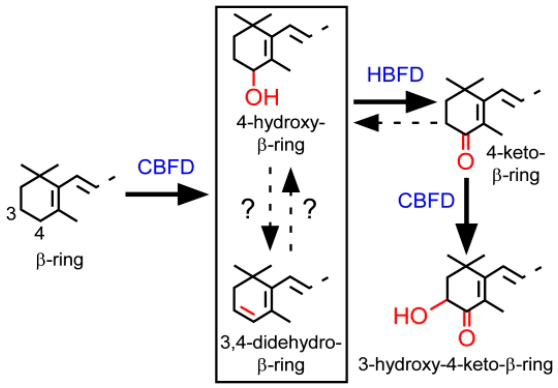

Figure 1: Astaxanthin synthesis pathway in higher plants.

In marine bacteria Paracococcus (Figure 2), the carotenoid oxygenase CrtW and the hydroxylase CrtZ catalyze the ketoylation and hydroxylation steps between β-carotene and astaxanthin, respectively[10].

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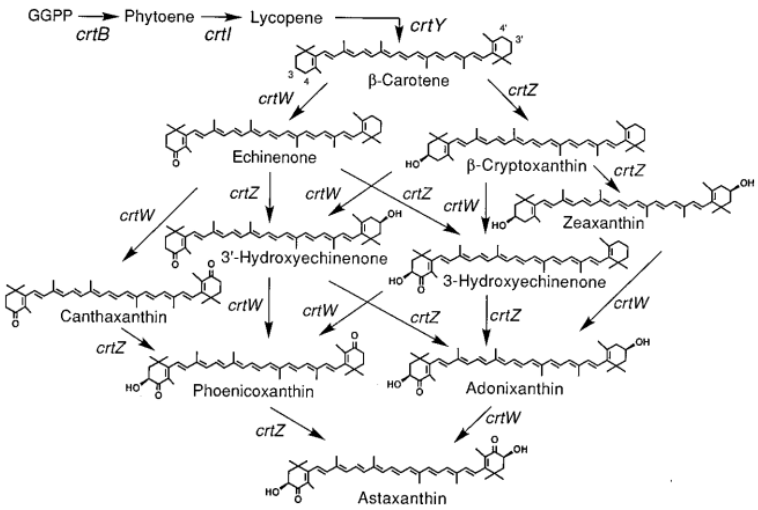

Figure 2: Astaxanthin synthesis pathway in Paracococcus.

Currently, the sources of astaxanthin include extraction, chemical synthesis, and the biosynthesis of waste from aquatic processing. The utilization of aquatic processing waste extraction can maximize waste utilization, recover essential nutrients for algae from wastewater, and generate astaxanthin, which can address environmental pollution issues [11]. This method is cost-effective, but the raw materials are susceptible to degradation. Chemical synthesis of astaxanthin involves numerous steps, complex processes, high equipment requirements, and increased synthesis costs. Constructing the astaxanthin biosynthesis pathway in microorganisms can not only streamline complex synthesis steps but also provide an environmentally friendly production method. The analysis of the astaxanthin biosynthetic metabolic pathway can improve the understanding of the heterologous synthesis of astaxanthin by microorganisms, reduce the production of impurities, and fundamentally lower the production cost.

2. Algae

In recent years, studies have shown that the cost of producing astaxanthin through algal synthesis has decreased, with the production cost reaching as low as $718 / kg, which is lower than the cost of chemically synthesized astaxanthin[12]. The methods for separating astaxanthin mainly include solvent extraction, ultrasonic-assisted extraction (UAE), microwave-assisted extraction (MAE), and supercritical fluid extraction (SFE)[13]. Aye Aye Myint et al. developed a technology for extracting astaxanthin by breaking the cell wall and directly recovering it using ball milling and ethyl acetate. This method eliminates the need for high-energy and high-cost drying steps, and enhances the extraction yield of astaxanthin[14].

Under the synergistic effect of nitrogen starvation and high light intensity, the biosynthesis of astaxanthin can be enhanced[15]. Lee KH et al. utilized the phototaxis of Rhodophylla pluvialis to develop a screening method that demonstrated a positive relationship between photosynthetic efficiency and phototaxis. This approach led to a 1.26-fold increase in astaxanthin production (55.12±4.12 mg g-1), laying the groundwork for the industrial utilization of astaxanthin production by Rhodophylla pluvialis[16]. The production of astaxanthin by Haematococcus pluvialis algae involves two stages: microalgae growth and astaxanthin production. Under the condition of maintaining the same medium, the first stage grows under low light to achieve optimal biomass, while the second stage utilizes high light and increases carbon dioxide content to alter the C/N balance and boost the production of astaxanthin[17]. In addition, specific wavelengths of light-emitting diodes are utilized for different purposes: red LEDss are used for microalgal cell growth, while blue LEDs induce astaxanthin biosynthesis. The alteration of the wavelength can significantly enhance astaxanthin production compared to continuous illumination using red LEDs[18]. Astaxanthin accumulation can also be enhanced by adjusting the pH value and increasing light intensity. Han SI et al. adjusted the pH value using H_2SO_4 and KOH, resulting in an increase in astaxanthin production to 39±6.92%. When the light intensity was increased while changing the pH value, the production of astaxanthin increased by 65±0.541%. Total production increased by 105±6.66% compared to the initial production[19]. Wang et al. increased the astaxanthin yield of Ravivium rhododendri through a three-stage mutagenesis breeding. First, ultraviolet light was used to irradiate the mutant UV11-4 in order to increase the dry weight of cells. Then, based on the ultraviolet mutant, ethyl

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methanesulfonic acid (EMS) was used to shorten the incubation period and prolong the logarithmic growth phase. Finally, diphenylamine (DPA) was used to screen high-yielding astaxanthin strains on solid medium by observing the change in colony color. The astaxanthin yield of the mutant was 1.7 times that of the wild type[20].

The production of astaxanthin was induced by the combination of melatonin and butylenediamine, and the production of carotene, gamma-aminobutyric acid and reactive oxygen species was regulated, and the yield was increased by 1.71[21]. The connection of polyvinylimine with chitosan can increase the content of reactive oxygen species in the cells of rainious red algae, thereby up-regulating the transcription of enzymes involved in astaxanthin biosynthesis and increasing astaxanthin production. The production efficiency of astaxanthin can reach more than 90%. In addition, polyvinylimine and chitosan can also be reused[22]. It was found that under the induction of succinic acid, endogenous hydrogen sulfide improved astaxanthin production by mediating related gene transcription levels and reactive oxygen signaling. Under the treatment of 1.0 mM succinic acid and 100 μM sodium hydrosulfide, astaxanthin concentration reached 44.96 mg L-1 with 163.55 pg cell-1, and the production was amplified. Biomass was increased to 2.14 g L-1 and astaxanthin was increased to 66.25 mg L^{-1}[23]. Yu C et al. increased astaxanthin production by batch feeding succinic acid (SA). On day 0, the first addition of SA increased astaxanthin production by 71.61%. The contents of astaxanthin and lipid reached the maximum on the 7th day, in which the output of astaxanthin reached 35.88 mg g-1 and the output of lipid reached 54.79%. Chlorophyll, carbohydrate, and protein levels were reduced, but intracellular levels of SA and reactive oxygen species (ROS), transcription levels of astaxanthin and fatty acid biosynthesis, and genes associated with antioxidant systems were increased. In addition, large-scale culture in the bioreactor further increased astaxanthin production of pluvium[24]. Supplementation of light, inorganic carbon and nitrate can effectively enhance Marine microalgae Tetraselmis sp. Astaxanthin production of Chlorella sorokiana and freshwater microalgae[25]. Zhao Y et al., treated with 2mg L^{-1} butylhydroxytoluene (BHT), increased astaxanthin by 71.13% in algae cells. This method is conducive to the production of astaxanthin in rainflood algae, which is a signaling molecular response of rainflood algae against abiotic stress conditions[26].

In addition to modifying the production conditions of strains to enhance astaxanthin yield, screening strains with high astaxanthin production is also an efficient method. Yang HE et al. used radiation to screen mutant strains, and the screened mutants exhibited higher astaxanthin yield under high salt and high light intensity. By increasing reactive oxygen species and overexpressing astaxanthin-synthesizing genes (lyc, crtR-b, bkt2), the production of astaxanthin increased by 241%[27].

Plant hormones can stimulate cell growth and enhance metabolism. In the synthesis of astaxanthin in green algae culture, auxin is the most effective stimulant for astaxanthin accumulation. Indole-propionic acid (10 mg/L) and indole-acetic acid (7.8 mg/L) were utilized to achieve a maximum content of 13.1 mg/g and a yield of 89.9 mg/L[28].

3. Fungus

Yeast genetic engineering is straightforward and was initially developed as a cell factory in eukaryotes. The construction of intricate biosynthetic pathways and synthetic compounds in yeast has been a popular topic in recent years. The production of astaxanthin by Saccharomyces cerevisiae promoted astaxanthin biosynthesis by regulating lipid synthesis. Li M et al. used the three-function CRISPR system to screen the gene pool related to lipid metabolism. After moderately regulating lipid synthesis, the production of astaxanthin reached 9.79 mg/g DCW. The production of astaxanthin was further increased to 10.21 mg/g DCW by optimizing the hydroxylase, and other factors. Finally, 446.4 mg/L of astaxanthin was produced by optimizing the fermentation conditions in the fed-batch fermentation[29]. Ma Y et al. successfully engineered the astaxanthin biosynthesis pathway within yeast suborganelles, addressing challenges such as enzyme and substrate isolation, metabolic interference, and others. Their study revealed that the co-expression of β-carotene ketolase and hydroxylase outperformed the individual enzyme expression. Furthermore, they demonstrated that the synthetic pathway could be expressed independently or in combination within liposomes, endoplasmic reticulum, or peroxisomes. The fusion of CrtZ from H. pluvialis and CrtW from Paracococcus sp into the endoplasmic reticulum targeted the suborganelles' astaxanthin synthesis pathway to accelerate the conversion of β-carotene to astaxanthin and reduce the accumulation of keto-carotenoid intermediates. The production of astaxanthin reached 858 mg/L in batch feeding fermentation. 141 times greater than the initial strain[30]. Lin YJ et al. developed strain Sm23 of Kluyveria marsii for astaxanthin biosynthesis, which achieved a production of 31 μg/g DCW. They increased the copy number of Hpchyb and bkt, the key genes involved in astaxanthin biosynthesis, and

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obtained four enhanced strains. To further enhance production, the Hpchyb gene was modified through site-specific mutation. Finally, the production of astaxanthin reached 9972 μg/g DCW in a 5L fermenter[31]. Tramontin LRR et al. designed an oil-producing yeast, optimized the β-carotene synthesis pathway, introduced the β-ketolase and β-hydroxylase coding genes from bacteria and algae, and enabled it to synthesize astaxanthin. Ultimately, the yield of astaxanthin reached 285±19 mg/L[32]. The enhancement of carbon utilization in astaxanthin production is crucial for the rhodogenic industry. A potential carbon regulator that can increase astaxanthin content is disodium 2-oxoglutarate (2-OG-2Na). After treatment with 2-OG-2Na, the ipi, bkt, and crtR-b genes are up-regulated. Meanwhile, the levels of carotenoids and astaxanthin increased by 15.4% and 14.0%, respectively, after 120 hours[33]. Studies have shown that under titanium dioxide (TiO2) stress, it can promote cell division and change cell morphology, accelerate the absorption of glucose by yeast, promote the reuse of ethanol, and increase the formation of acetyl-CoA and ATP. Transferring more carbon flux to the astaxanthin synthesis pathway reduces the accumulation of carotenoids in the astaxanthin branching pathway, leading to increased astaxanthin production[34]. Jin Jin et al. utilized microbial metabolic engineering technology to enhance the synthesis of astaxanthin in Saccharomyces cerevisiae. They replaced CrtZ from Agrobacterium aurantiacum with allogenic module engineering, resulting in a 1.78-fold increase in astaxanthin production. Subsequently, they further improved astaxanthin production by 0.83 times through room temperature plasma mutagenesis technology. At a fermentation volume of 5 L, the astaxanthin yield reached 217.9 mg/L[35]. Zhou P et al. achieved significant progress in the enhancement of β-carotene ketenase and β-carotene hydroxylase mutants through protein engineering and directed evolution. They developed a high-efficiency astaxanthin synthesis strain of Saccharomyces cerevisiae and improved the conversion of β-carotene to astaxanthin. In addition, a temperature-responsive dynamic control system, Gal4m9, was developed. The cell growth was separated from the astaxanthin synthesis, resulting in a yield of 235 mg/L of astaxanthin through two-stage fermentation[36]. Jiang et al. modified the genome of Saccharomyces cerevisiae using H_2O_2 adaptive evolution and room temperature plasma mutagenesis technology . After multiple rounds of treatment, the production of astaxanthin increased fourfold compared to that of the initial strain. This increase promoted product accumulation, reduced intracellular reactive oxygen species, and boosted production. Under a 5-liter fermentation system, the yield of astaxanthin reached 404.78 mg/L[37].

The production of astaxanthin can also be increased by adjusting the nutrient composition in the medium. When 10mL/ L soybean oil is added to the medium, the fatty acids in red yeast can be significantly increased due to the stimulation of soybean oil. The production of palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1n9), linoleic acid (C18:2n6), α-linolenic acid (C18:3n3), and γ-linolenic acid, which are closely related to the formation of astaxanthin esters, increased, thereby raising the content of astaxanthin esters. As a result, the production of astaxanthin reached 7.35mg L-1[38]. Miao L et al. obtained a strain MK19 with high astaxanthin production through mutagenesis breeding and studied the cause of the increase in astaxanthin at the gene expression level. The results revealed an increase in the expression of crtE, crtI, pbs, and ast, as well as a decrease in the biosynthesis of fatty acids and ergosterol. This led to the accumulation of precursors and their transfer to the astaxanthin synthesis pathway. Enhance astaxanthin production[39].

Limulus limulus is a heterotrophic eukaryotic microorganism with a high growth rate, making it an ideal candidate for astaxanthin cell production. However, the biosynthesis process of astaxanthin in horseshoe crabs is not fully understood. Overexpression of the crtZ gene by Yoshimi T and colleagues increases the production of astaxanthin, thereby enhancing our understanding of the process of converting β-carotene into astaxanthin. Compared to wild-type strains, there is a significant increase in the production of astaxanthin[40]. Limulus horseshoe crabs can simultaneously produce docosahexaenoic acid (DHA) and astaxanthin. Kubo Y and others overexpressed CarS to enhance the synthesis of β-carotene, resulting in a large amount of DHA and carotenoid production, thereby increasing astaxanthin production. The increase in astaxanthin production does not affect DHA production[41].

4. Bacteria

All The cyanobacteria, capable of utilizing carbon dioxide and serving as ideal microbial cell factories, have developed efficient astaxanthin biosynthesis pathways in the cyanobacteria Synechocystis sp. PCC 6803. These pathways have been integrated into the endogenous metabolism, resulting in an increase in astaxanthin production by more than 500 times[42]. Hanyu Liang et al. built the astaxanthin biosynthesis pathway in cyanobacteria by silencing endogenous CrtO genes and introducing HpBKT, CrtZ, and CrtR to synthesize astaxanthin. Compared to green algae and plants, cyanobacteria have a simpler synthesis pathway, producing only a small amount of carotenoids without interference from endogenous genes. No

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organic carbon source is required [43].

Ma et al. utilized spinosinomonas ATCC 55669 to produce astaxanthin. They employed the PacBio-Illumina combination method to identify the astaxanthin biosynthesis pathway and constructed a highly efficient targeted carotenoid synthesis pathway in Escherichia coli. Ultimately, they obtained a highly efficient synthetic astaxanthin production strain[44]. Asker D et al. discovered a new strain of astaxanthin-producing bacteria through high-throughput screening of such bacteria. Through phylogenetic analysis of the 16S rRNA gene, it was determined that the organism belonged to the genus Brevimonomonas and was named Brevundimonas sp. The carotenoid content of strain N-5 was determined using HPLC-DAD and HPLC-MS methods. The results showed that the total carotenoid content was 601.2 μg g-1 in stem cells, with a significant amount of optically pure astaxanthin (3S, 3'S) isomer at 364.6 μg g-1 in stem cells. Increasing the aeration of the culture can boost astaxanthin production to up to 85% of the total carotenoids[45]. Gong Z et al. utilized the CAR026 strain of Escherichia coli, which has metabolic flow balance, as the initial strain for synthesizing astaxanthin. They employed β-carotene ketoenzyme (CrtW) and β-carotene hydroxylase (CrtZ) to convert β-carotene into astaxanthin, and enhanced astaxanthin production by amplifying the copy number of crtY. After regulating the molecular chaperone groES-groEL, the final astaxanthin production can reach 1.18 g/L[46]. Lemuth K et al. utilized λ-Red recombination technology to integrate the astaxanthin biosynthesis pathway gene into the genome of Escherichia coli, and developed an astaxanthin production strain without the use of a plasmid. By regulating the expression level of the astaxanthin synthesis gene, various promoters were utilized to enhance the production of astaxanthin. Firstly, the biosynthesis of isopentenyl diphosphate (IPP) was enhanced to increase the yield of β-carotene. Subsequently, astaxanthin was synthesized under the catalysis of β-carotene ketolase crtW148 and β-carotene hydroxylase (crtZ). Finally, the yield of astaxanthin reached 1.4 mg/g CDW[47]. Lu Q et al. also constructed an astaxanthin-producing strain, Escherichia coli ASTA-1, which did not contain a plasmid or antibiotic labeling. By comparing the conversion efficiency of β-carotene ketolase from different sources with that of recombinant Escherichia coli astaxanthin, it was found that the combination of B. sp. SD212 crtW and P. ananatis crtZ was the most effective for the synthesis and production of astaxanthin. With astaxanthin as the primary carotenoid, the specific content reached 7.4±0.3 mg/g DCW. Subsequently, the production of astaxanthin increased with higher levels of reactive oxygen species. The coordinated expression of membrane-related genes and oxidative stress-related genes led to a further increase in astaxanthin production to 11.92 mg/g of stem cell weight[48,55]. Zhang C et al. utilized a multi-variable modular approach to optimize the astaxanthin biosynthesis pathway and enhance astaxanthin production[49]. Tao L et al. developed a color screening method and utilized directed evolution to identify superior mutants capable of enhancing astaxanthin production. They also created a random mutant library of the β-carotene ketonase gene (crtW) to support their research. Six mutants (H96L, R203W, A205V, A208V, F213L, and A215T) were identified as increasing astaxanthin production[50]. Li D et al. obtained A6T, T105A, and L239M mutants by constructing a random mutant library of the β-carotene ketoenzyme CrtW, resulting in a 5.35-fold increase in astaxanthin production. Subsequently, the researchers utilized the RBS library to simultaneously regulate the expression levels of crtW and crtZ in order to increase the copy number. The yield of astaxanthin was 9.8 times higher than that of the wild type[51]. Nogueira M et al. expressed crtZ and CrtW as a fusion protein and observed that only when CRTZ was linked as an N-terminal module, the two enzymes expressed as a fusion exhibited their respective catalytic activities. The expression of astaxanthin in this fusion mode was 1.4 times higher than when expressed alone[52]. Ye L et al. utilized a flexible connector to link CrtW and CrtZ, resulting in a 215.4% increase in the production of astaxanthin[53]. Park SY et al. enhanced astaxanthin production through the use of various fusion tags, overexpression of relevant genes, and optimization of fermentation conditions. As a result, they achieved a production level of 385.04 mg/L[54].

5. Summary and prospect

Astaxanthin is increasingly being used in medicine, food, and cosmetics, leading to a growing market demand. The biosynthesis of astaxanthin can fulfill the goal of rapid and efficient production. The construction of the astaxanthin synthesis pathway in microorganisms can reduce the generation of by-products and minimize environmental pollution. Currently, the biosynthetic pathway of astaxanthin has been analyzed and utilized for microbial synthesis, but the yield of astaxanthin remains low. Currently, with the rapid advancement of synthetic biology and metabolic engineering, techniques such as synthetic biology, metabolic engineering, and directed evolution are employed to develop chassis cells, optimize metabolic pathways, and modify key enzymes in metabolic pathways using both rational and irrational methods. These methods aim to enhance enzyme expression and activity, lower production costs, and

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boost the yield of astaxanthin in microbial synthesis.

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