Metabolic engineering of Synechocystis sp. PCC6803 to produce astaxanthin

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ABSTRACT

Astaxanthin is a carotenoid with wide applications because of its strong antioxidant properties. It is mainly produced from the green alga Haematococcus pluvialis, but large-scale production remains a challenge. To provide another source, here, Synechocystis sp. PCC6803 was adopted as the chassis cell, and its carotenoid biosynthesis pathway was genetically extended to produce astaxanthin. The β-carotene ketolase gene (bkt) and carotenoid hydroxylase gene (crtR-B) from H. pluvialis, which wild-type Synechocystis sp. PCC6803 lacks, were optimized based on the codon preference of Synechocystis sp. PCC6803 and heterologously expressed in that bacterium to create a strain capable of producing astaxanthin. The Synechocystis sp. PCC6803 mutant synthesized 4.81 ± 0.06 mg astaxanthin per gram of cells (dry weight) after nitrogen starvation for 14 days. More than 30% of the total astaxanthin was in the free form, and 82.61% was the strong antioxidant 3S, 3’S-astaxanthin isomer. These results show the industrial application potential of metabolically-engineered Synechocystis sp. PCC6803 for astaxanthin production.

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1. Introduction

Astaxanthin (3, 3′-dihydroxy-4, 4′-diketo-β, β′-carotene) is a lipid-soluble, red-orange oxyxcarotenoid with strong antioxidant properties [1]. Commercial astaxanthin is mainly synthesized chemically or extracted from natural producers such as the green alga Haematococcus pluvialis or the red yeast Xanthophyllomyces dendrorhous [2–5]. The stereogenic carbons in the 3 and 3′ positions on the β-ionone moieties define the astaxanthin conformation as chiral [(3S, 3’S) or (3R, 3’R)] or as the meso form (3R, 3’S). H. pluvialis biosynthesizes the (3S, 3’S) isomer, whereas yeast produces the (3R, 3’R) isomer. Chemical synthesis yields the (3S, 3’S), (3R, 3’S) and (3R, 3’R) isomers (Fig. 1) [6].

H. pluvialis is the preferred source for natural astaxanthin because of its high content. In various stress conditions, H. pluvialis rapidly synthesizes and accumulates astaxanthin to 5% of the cell dry weight (DW) [7]. This process is affected by environmental factors such as light, temperature, pH, and nutrient availability [8,9]. High light, C/N ratio, and salinity all contribute to the accumulation of astaxanthin [10]. However, the production of astaxanthin by H. pluvialis is affected by the seasons and the weather; it is susceptible to bacterial and algal contamination; and it cannot take into account both cells growth and accumulation of astaxanthin [11]. These factors severely limit astaxanthin production using H. pluvialis on an industrial scale. Microbial production of astaxanthin via metabolic engineering has emerged as an attractive alternative.

Synechocystis sp. PCC6803 is a prokaryote capable of photosynthesis. It is the phototrophic organism to have its genome sequenced [12] and has the efficient and different genetic transformation systems [13,14]. Synechocystis sp. PCC6803 is a model organism for the study of metabolic engineering and also an excellent chassis for constructing cell factories by using synthetic biology [15,16]. It is both autotrophic and heterotrophic, grows rapidly, and is suitable for large-scale cultivation [17]; thus, it has various potential applications. Synechocystis sp. PCC6803 can synthesize echinenone and zeaxanthin (Fig. 2), which are precursors of astaxanthin. Based on the above characteristics, we hypothesized that Synechocystis sp. PCC6803 would be an excellent chassis for astaxanthin production. However, compared with H. pluvialis, Synechocystis sp. PCC6803 lacks the enzymes β-carotene ketolase (BKT)
and carotenoid hydroxylase (CRTR-B), encoded by the genes bkt and crtR-B respectively [19,20].

Here, we extended the carotenoid synthesis pathway in *Synechocystis* sp. PCC6803 to explore an alternative to *H. pluvialis* for astaxanthin production (Fig. 2). First, the accumulation of astaxanthin was increased by choosing efficient *Pcpc560* and *PpsbA2* promoters. *PpsbA2* and *Pcpc560* promoters were induced by high intensity of light. Through codon optimization, the bkt and crtR-B coding sequences from *H. pluvialis* were adapted for expression in *Synechocystis* sp. PCC6803. Then, culture conditions were optimized to increase astaxanthin production. Consequently, the highest amount of astaxanthin produced was $4.81 \pm 0.06 \text{ mg g}^{-1} \text{ DW}$.

2. Materials and methods

2.1. Culture of *Synechocystis* sp. PCC 6803

*Synechocystis* sp. PCC6803 was obtained from the Biotechnology Research Center of the Shandong Academy of Agricultural Sciences, China. *Synechocystis* sp. PCC6803 was cultured in BG11 medium [21] at
30 ± 2 °C [22]. The normal light intensity was 50 μmol photon m−2 s−1 with a 12-h/12-h light/dark cycle. Absorbance was measured at 730 nm every 24 h to draw growth curves.

**Escherichia coli** strain DH-5α (Invitrogen, China) was used for DNA cloning and plasmid construction, and cultured in LB culture at 37°C in a shaker (160 rpm).

### 2.2. Construction of homologous recombination vectors

Genomic DNA of *Synechocystis* sp. PCC6803 was extracted using a Plant DNA Isolation Reagent (Takara, China). Genomic DNA of *E. coli* was extracted using a Rapid Bacterial Genomic DNA Isolation Kit (Sangon, China).

The open reading frames (ORFs) of the bkt gene (GenBank: AY603347.1) and the crtR-B gene (GenBank: AF162276.1) from *H. pluvialis* were optimized according to the codon preference of *Synechocystis* sp. PCC6803, and synthesized by GenScript Company (China). Promoters P_psc560 and P_pshA2 were amplified from *Synechocystis* sp. PCC6803 genomic DNA using primer pairs P_psc560-Sall-F/P_psc560-Sall-R and P_pshA2-Promoter-Sall-F/P_pshA2-Promoter-R, respectively.

To construct a vector containing the bkt gene, the bkt ORF was amplified using primers bkt-F and bkt-R and fused with the P_psc560 promoter using fusion PCR, and then inserted into pSS1285UD [23] between the EcoRI and Sall sites, forming plasmid pSS1285UD-bkt. The crtR-B ORF amplified with primers crtR-B-F/crtR-B-R was fused with the P_pshA2 promoter using fusion PCR and inserted into pSKT1T2 [24] between the EcoRI and Sall sites, forming pSKT1T2-crtR-B. Descriptions of control elements utilized in this study are listed in the Table 3.

### 2.3. Transformation of *Synechocystis* sp. PCC6803 and selection

*Synechocystis* sp. PCC6803 in late logarithmic growth phase (OD_730 = 0.6–0.8) was harvested by centrifugation and transformed with the plasmids described above using the natural transformant procedure described by Chen et al. [24]. Transformed *Synechocystis* sp. PCC6803 cultures were selected on BG11 solid medium containing 5 μg ml−1 gentamycin and 25 μg ml−1 kanamycin. Positive colonies were tested further. Cultures transformed with pSS1285UD and pSKT1T2 were used as the negative control. Ampicillin, gentamicin and kanamycin were purchased from Sigma-Aldrich (China).

### 2.4. Characterization of bkt/crtR-B expressing strain

For the DNA level verification of mutant *Synechocystis* sp. PCC6803,
Using a Plant RNA Kit (Omega, China), and reverse transcription to Sac I, at 37°C for 4–5h, and about 1μg genomic DNA was digested with Bam HI. Southern blotting was carried out according to the instructions of the Table 1 were used to confirm the presence of the transgenes, and PCR and Southern blotting were conducted. Specific primers listed in Table 1 were used to confirm the presence of the transgenes, and PCR with the total RNA as the template was used as the negative control.

2.5. Stress treatment

Mutant Synechocystis sp. PCC6803 in the logarithmic growth phase (OD\textsubscript{730} = 0.6–0.8) were harvested, and adjusted to OD\textsubscript{730} = 0.5. The mutant cultures were treated in the following conditions for 14 days: 1%, 10%, 20%, 50%, and 100% of the normal NaNO\textsubscript{3} concentration (1285D) for homologous recombination, the gentamicin resistance gene upstream fragment (1285U) and a 1000-bp downstream fragment were constructed (Fig. 3a, b). p5S1285UD-\textit{bkt} contains a 1000-bp promoter and the \textit{psbA2} ORF were used as homologous fragments. In p5S1285UD-\textit{bkt} and \textit{cpc560} promoter and the TI1T2 terminator. In pSKT1T2-\textit{crtR-B}, the \textit{psbA2} promoter and the \textit{psbA2} ORF were used as homologous fragments, and the \textit{psbA2} promoter controlled expression of the \textit{cpc560} resistance cassette.

2.6. Pigment analysis

Wild-type \textit{Synechocystis} sp. PCC6803, the negative controls, and \textit{bkt}/\textit{crtR-B} cultures grown in BG11 medium and under different stress treatments were harvested, and the pigments were extracted with acetone as described by Baroli et al. [25]. Pigments were analyzed by high-performance liquid chromatography (HPLC) using carotenoid standards obtained from Sigma (China). HPLC was conducted on a Thermo Fisher UliMate-3000 liquid chromatograph equipped with a UV–vis detector. The pigments were analyzed (20μl aliquots) using a reverse-phase C18 column, Acclaim 120 A (5 μm × 4.6 mm × 250 mm), at 25°C. The solvent system and gradient procedure were as described by Baroli et al. [25]. For total astaxanthin contains free style astaxanthin and astaxanthin esters, the extracted pigments were catalyzed using cholesterol esterase as described by Su et al. [26] and analyzed by HPLC as above to profile the astaxanthin composition. Further separation of optical isomers of astaxanthin was according to the method of Wang et al. [27]. Baseline separation of stereomers of astaxanthin used a Chiralpack IC column (25 × 4.6 cm; Daicel Chiral Technologies, China). Peaks were identified by comparing the retention times for samples with 9-cis-astaxanthin and racemic astaxanthin [1:2:1 mixture of (3S, 3′S), (3R, 3′S), and (3R, 3′R)] isomer standards, purchased from Carotenature (Lupsingen, Switzerland). The solvent system and gradient procedure were as described by Su et al. [26].

2.7. Statistical analysis

All experiments were performed in triplicate, and the data are presented as the means of three experiments with standard deviations. Post-hoc Tukey tests were carried out using the SPSS (version 24) package. Differences between means were considered statistically significant when p < 0.05.

3. Results

3.1. The \textit{Synechocystis} sp. PCC6803 \textit{mutant bkt}/\textit{crtR-B} produces astaxanthin

The integrative expression vectors p5S1285UD-\textit{bkt} and pSKT1T2-\textit{crtR-B} were constructed (Fig. 3a, b). p5S1285UD-\textit{bkt} contains a 1000-bp upstream fragment (1285U) and a 1000-bp downstream fragment (1285D) for homologous recombination, the gentamicin resistance gene cassette from pFastBac1 (Invitrogen), and the \textit{bkt} gene regulated by the \textit{P}_{gpc560} promoter and the TI1T2 terminator. In pSKT1T2-\textit{crtR-B}, the \textit{P}_{psbA2} promoter and the \textit{psbA2} ORF were used as homologous fragments, and the \textit{psbA2} promoter controlled expression of the \textit{crtR-B} gene. A kanamycin resistance cassette carrying npt neomycin phosphotransferase was adopted in pSKT1T2-\textit{crtR-B} as the selective marker.

After transformation and five rounds of selection, 20 putative transformed clones were further examined. First, PCR analysis was conducted with specific primers, as shown in Fig. 4a and c. One of the positive \textit{Synechocystis} sp. PCC6803 mutants was named \textit{bkt}/\textit{crtR-B} and...
An expected 4.9-kb band was amplified with primers 1285U-F/1285D-R from \textit{bkt}/\textit{crtR-B} expressing strain, compared with a 2.0-kb band observed in the negative control (Fig. 4b). A 4.1-kb fragment was amplified with primers \textit{P}psbA2\textit{Promoter-SalI-F}/\textit{P}psbA2\textit{R}, compared with a 1.5-kb fragment from the negative control (Fig. 4d). This demonstrated that the fragment in the homology arm has entered the genome of the \textit{Synechocystis} sp. PCC6803 by double exchange. This indicated successful introduction of \textit{bkt} and \textit{crtR-B} into \textit{Synechocystis} sp. PCC6803. The integration of \textit{bkt} and \textit{crtR-B} was confirmed by Southern blotting of genomic DNA digested with \textit{BamHI}/\textit{EcoRI} and \textit{XhoI}/\textit{SacI}. As shown in Fig. 5a, bands representing \textit{bkt} and \textit{crtR-B} were visible in the DNA of the \textit{bkt}/\textit{crtR-B} expressing strain, which suggested that \textit{bkt} and \textit{crtR-B} were integrated into the specific directed sites of the mutant \textit{bkt}/\textit{crtR-B}.

To analyze the transcription of the \textit{bkt} and \textit{crtR-B} genes, total RNA was extracted from \textit{bkt}/\textit{crtR-B} cells. As shown in Fig. 5b, bands of approximately 410 and 258 bp, representing \textit{bkt} and \textit{crtR-B} respectively, were amplified from cDNA from \textit{bkt}/\textit{crtR-B}, while no band was observed in the negative control. These results showed that the \textit{bkt} and \textit{crtR-B} genes integrated into the genome were being transcribed.

According to carotenoid profiling results (Fig. 6), \textit{bkt}/\textit{crtR-B} cells produced adonixanthin, canthaxanthin, and astaxanthin, while the negative control did not. The content of free astaxanthin was 0.51 ± 0.01 mg g⁻¹ (DW) and the total astaxanthin content was 1.12 ± 0.01 mg g⁻¹ (DW), thus, 45.54% of the total astaxanthin was in the free form (Table 2).
Based on retention times of standards, the astaxanthin produced by bkt/crtR-B contained 3S, 3′S-astaxanthin and 9-cis-astaxanthin (Fig. 7); 82.61% of the astaxanthin was 3S, 3′S-astaxanthin.

3.2. Stress increased astaxanthin production

Nitrogen deficiency treatment increased the production of astaxanthin, and the effects of different N concentrations were different. With 10% N after 14 days, bkt/crtR-B culture turned orange (Fig. 8c), and the content of free astaxanthin reached 1.65 ± 0.03 mg g⁻¹ (DW) (Table 2), and the total astaxanthin content reached 4.81 ± 0.06 μg g⁻¹ (DW) (i.e., 34.33% of the astaxanthin was in the free form). This proved to be the most effective nitrogen concentration for astaxanthin production. With 1% N, the growth of bkt/crtR-B was greatly affected, and the growth rate of cells under other nitrogen starvation conditions was not much different (Fig. 9a).

In high light conditions, free astaxanthin formed more rapidly, reaching 1.57 ± 0.01 mg g⁻¹ (DW) after 14 days (Fig. 8b) (37.35% free astaxanthin). A significant increase in astaxanthin content was found between the 4th and 6th days of high light exposure (Fig. 8b).

4. Discussion

*H. pluvialis* has two ways to synthesize astaxanthin from β-carotene (Fig. 2). In one pathway, BKT catalyzes conversion of echinenone to canthaxanthin, and then canthaxanthin is hydroxylated to astaxanthin by CRTR-B. In the second pathway, BKT catalyzes transformation of zeaxanthin to adonixanthin, and then BKT converts adonixanthin to astaxanthin by ketolation. In *H. pluvialis*, most astaxanthin (98%) is esterified to monester (> 90%) and diester [28], which creates many problems in astaxanthin extraction and application. Free astaxanthin is a better antioxidant than the esterified form, and free astaxanthin is preferentially absorbed or selectively transported through the body [29]. Industrially, esterified astaxanthin is saponified to free astaxanthin after extraction from *H. pluvialis*. Here, the bkt and crtR-B genes from *H. pluvialis* were expressed in *Synechocystis* sp. PCC6803, which resulted in astaxanthin production in the mutant strain bkt/crtR-B. It is interesting and attractive that > 34.34% of the astaxanthin in bkt/crtR-B was in the free form.

There are a variety of astaxanthin isomers, such as all *trans* ([3S, 3′S], [3R, 3′S], and [3R, 3′R]), 9-cis, and others. 3S, 3′S-astaxanthin and 9-cis-astaxanthin exhibits especially strong antioxidant activity both *in vitro* and *in vivo*, followed by [3R, 3′R]-astaxanthin and [3R, 3′S]-astaxanthin [6]. bkt/crtR-B produced 3S, 3′S-astaxanthin and 9-cis-astaxanthin, and 82.61% of the astaxanthin was 3S, 3′S-astaxanthin, higher than the proportion produced by *H. pluvialis* (72.8%) [30]. Therefore, the astaxanthin-producing *Synechocystis* sp. PCC6803 mutant developed in this work has wide application potential.

Many researches were conducted with metabolic engineering methods for astaxanthin biosynthesis in different species such as *E. coli* [31], the yeast *S. cerevisiae* [32], *Synechocystis* sp. PCC6803 and so on, and the astaxanthin product in engineered *E. coli* reached 15.1 mg/g DCW which was the highest production in metabolic engineered species. But this production is still lower than that of *H. pluvialis*, and cannot meet the industry need now. Many engineering and production works are needed for higher astaxanthin yields and better astaxanthin

### Table 2

<table>
<thead>
<tr>
<th>Growth conditions</th>
<th>Free astaxanthin (mg g⁻¹ DW)</th>
<th>Total astaxanthin (mg g⁻¹ DW)</th>
<th>The ratio of free astaxanthin (%)</th>
<th>Titer of astaxanthin (mg L⁻¹ day⁻¹)</th>
<th>Cell density (g L⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>bkt/crtR-B</td>
<td>0.51 ± 0.01</td>
<td>1.12 ± 0.01</td>
<td>45.54%</td>
<td>0.30 ± 0.01</td>
<td>7.70 ± 0.03</td>
</tr>
<tr>
<td>bkt/crtR-B (10%N)</td>
<td>1.65 ± 0.03</td>
<td>4.81 ± 0.06</td>
<td>34.34%</td>
<td>0.71 ± 0.01</td>
<td>5.92 ± 0.02</td>
</tr>
<tr>
<td>bkt/crtR-B (HL)</td>
<td>1.57 ± 0.01</td>
<td>4.20 ± 0.01</td>
<td>37.35%</td>
<td>0.94 ± 0.01</td>
<td>7.48 ± 0.05</td>
</tr>
</tbody>
</table>

Fig. 6. High-performance liquid chromatography (HPLC) analysis of pigments. 1: Astaxanthin; 2: myxoxathophyll; 3: adonixanthin; 4: zeaxanthin; 5: canthaxanthin; 6: chlorophyll a; 7: echinone; 8: β-carotene.
product.

That the carotenoid biosynthetic pathway of Synechocystis sp. PCC 6803 has been extended by introducing the 4,4’ β-carotene oxygenase (CrtW) and 3,3’ β-carotene hydroxylase (CrtZ) genes from Brevundimonas sp. SD-212 [33]. Astaxanthin accumulation was 50% of total carotenoids when CrtZ was cloned under upstream of CrtW which indicated the importance of the regulator regions for the foreign genes’ expression. A Synechocystis mutant was engineered with an insertion of a β-carotene di-ketolase gene crtW148 from Nostoc punctiforme, insertion of an additional copy of the endogenous β-carotene hydroxylase gene crtR from Synechocystis sp. PCC6803, and an open reading frame disruption of the endogenous β-carotene mono-ketolase gene crtO [34]. These manipulations generated a mutant capable of astaxanthin titers that reached production rates of 1.11 ± 0.07 mg L⁻¹ day⁻¹. We introduced the bkt and crtR-B genes from H. pluvialis in Synechocystis sp. PCC6803. These manipulations generated a mutant with astaxanthin titers that reached production rates of 0.94 ± 0.01 mg L⁻¹ day⁻¹. Albers’ experiment has provided us with a new idea, and regulation at the genetic level can further increase the production of astaxanthin.

Lemuth et al. [35] found that increasing the activity ratio of BKT to CRTR-B favored the conversion of β-carotene to astaxanthin. Thus, to generate more astaxanthin, we could integrate many copies of the bkt gene into the chromosome of Synechocystis sp. PCC6803, better optimize the culture conditions, and select a stronger promoter.

Final cell concentration of H. pluvialis for the 1000 l bioreactor is 5–7×10⁵ cells ml⁻¹ and pond reddening days of H. pluvialis is 8–9 days [36]. Final cell concentration of Synechocystis sp. PCC6803 is 15–32×10⁷ cells ml⁻¹, reddening days of Synechocystis sp. PCC6803 is 14 days.

5. Conclusions

Here, we extended the carotenoid synthesis pathway in Synechocystis sp. PCC6803 to explore an alternative to H. pluvialis for astaxanthin production. Natural transformation was used to introduce the genes bkt and crtR-B into Synechocystis sp. PCC6803 for synthesis of astaxanthin. PCR analysis, Southern blotting and RNA analysis indicated that bkt and crtR-B were integrated into Synechocystis sp. PCC6803 by homologous recombination and expressed. We were able to obtain 4.81 ± 0.06 mg g⁻¹ (DW) by manipulating the culture
conditions, of which 34.34% was free astaxanthin.

Author contributions

Yaming Liu, Yulin Cui, Jun Chen, Song Qin and Gao Chen designed and supervised the research. Yaming Liu conducted experiments. Yaming Liu and Yulin Cui analyzed and interpreted the data and drafted the manuscript. Yulin Cui, Song Qin and Gao Chen critically reviewed the manuscript. All authors read and approved the final version of the manuscript.

Declaration of Competing Interest

The authors declare that there is no conflict of interest. No conflicts, informed consent, human or animal rights are applicable to this work.

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