Available online at www.sciencedirect.com
ScienceDirect
Chinese Journal of Natural Medicines 2020, **18**(9): 666-676
doi: 10.1016/S1875-5364(20)60005-X
Chinese Journal of Natural Medicines

•Special topic•

Engineering CrtW and CrtZ for improving biosynthesis of astaxanthin in *Escherichia coli*

LI Di[1, 2, 3, 5Δ], LI Yang[2, 3, 4Δ], XU Jiao-Yang[2, 3, 4Δ], LI Qing-Yan[2, 3, 4, 5], TANG Jin-Lei[2, 3, 4, 5], JIA Shi-Ru[1], BI Chang-Hao[2, 3, 4, 5], DAI Zhu-Bo[2, 3, 4, 5], ZHU Xin-Na[2, 3, 4, 5*], ZHANG Xue-Li[2, 3, 4, 5*]

[1] *College of Biotechnology, Tianjin University of Science and Technology, Tianjin 310018, China;*
[2] *Tianjin Institute of Industrial Biotechnology, Chinese of Academy of Sciences, Tianjin 300308, China;*
[3] *Key Laboratory of Systems Microbial Biotechnology,Chinese Academy of Sciences, Tianjin 300308, China;*
[4] *University of Chinese Academy of Sciences, Beijing 100071, China;*
[5] *National Technology Innovation Center of Synthetic Biology, Tianjin 300308, China*

Available online 20 Sep., 2020

[ABSTRACT] This study engineered *β*-carotene ketolase CrtW and *β*-carotene hydroxylase CrtZ to improve biosynthesis of astaxanthin in *Escherichia coli*. Firstly, *crtW* was randomly mutated to increase CrtW activities on conversion from *β*-carotene to astaxanthin. A *crtW** mutant with A6T, T105A and L239M mutations has improved 5.35-fold astaxanthin production compared with the wild-type control. Secondly, the expression levels of *crtW** and *crtZ* on chromosomal were balanced by simultaneous modulation RBS regions of their genes using RBS library. The strain RBS54 selected from RBS library, directed the pathway exclusively towards the desired product astaxanthin as predominant carotenoid (99%). Lastly, the number of chromosomal copies of the balanced *crtW*−crtZ* cassette from RBS54 was increased using a Cre-loxP based technique, and a strain with 30 copies of the *crtW*−crtZ* cassette was selected. This final strain DL-A008 had a 9.8-fold increase of astaxanthin production compared with the wild-type control. Fed-batch fermentation showed that DL-A008 produced astaxanthin as predominant carotenoid (99%) with a specific titer of 0.88 $g \cdot L^{-1}$ without addition of inducer. In conclusion, through constructing *crtW* mutation, balancing the expression levels between *crtW** and *crtZ*, and increasing the copy number of the balanced *crtW*−crtZ* cassette, the activities of *β*-carotene ketolase and *β*-carotene hydroxylase were improved for conversion of *β*-carotene to astaxanthin with higher efficiency. The series of conventional and novel metabolic engineering strategies were designed and applied to construct the astaxanthin hetero-producer strain of *E. coli*, possibly offering a general approach for the construction of stable hetero-producer strains for other natural products.

[KEY WORDS] Astaxanthin; RBS library; Metabolic engineering; *β*-Carotene ketolase; Cre-loxP; *Escherichia coli*
[CLC Number] Q81 **[Document code]** A **[Article ID]** 2095-6975(2020)09-0666-11

Introduction

Astaxanthin (3, 3′-dihydroxy-*β*, *β*-carotene-4, 4′-dione) is a valuable natural product that is synthesized by photosynthetic organisms and some non-photosynthetic yeasts, fungi, and bacteria *via* condensation of terpene units and subsequent oxidation reactions [1]. Preclinical studies have shown that astaxanthin has diverse pharmacological effects, such as anti-oxidative activity on low-density lipoprotein, anti-cancer activity, enhancement of the immune response, protection from ultraviolet radiation, singlet oxygen-quenching activity, and other beneficial physiological activities [2]. Consequently, it has many uses in the nutraceutical, cosmetic, food colorant, and animal feed industries, with a combined potential commercial value of US $200 million per year [1, 3-5].

Although commercial astaxanthin is mainly synthesized chemically or extracted from native producers, such as yeast *Xanthophyllomyces dendrorhous* and green algae *Haematococcus pluvialis* [6-8], heterologous production in non-carotenogenic microorganisms like *Escherichia coli* offers an alternative for the safe production of astaxanthin [9, 10]. *β*-Carotene that is the biosynthetic precursor of astaxanthin, can be synthesized by introducing four enzymes including farnesyl-diphosphate synthase (CrtE), phytoene synthase (CrtB), phytoene dehydrogenase (CrtI) and lycopene cyclase (CrtY) into *E. coli* [11], When *β*-carotene ketolase (CrtW) and *β*-

[Received on] 23-Jun.-2020
[Research funding] This work was supported by the National Key R&D Program of China (No. 2019YFA0905700) and the National Natural Science Foundation of China (No. 31870058).
[*]**Corresponding author]** Tel: 86-22-84861946, E-mails: zhu_xn@tib.cas.cn (ZHU Xin-Na); zhang_xl@tib.cas.cn (ZHANG Xue-Li)
[Δ]These authors contributed equally to this work.
These authors have no conflict of interest to declare.

carotene hydroxylase (CrtZ) are further introduced, *β*-carotene can be converted into astaxanthin by adding keto-groups and hydroxyl-groups to the *β*-ionone rings at their respective positions of *β*-carotene [3] (Fig. 1A). At present, many efforts have been done to construct an astaxanthin *E. coli* engineered strain. Lemuth *et al*. first engineered a plasmid-free *E. coli* stain, which produced astaxanthin of 1.4 $mg \cdot g^{-1}$ cell dry weight (cdw) with an up to 95% production of astaxanthin only with IPTG induction. The engineered strain that constructed by Lu *et al.* synthesized 7.4 $mg \cdot g^{-1}$ cdw astaxanthin, which is close to 97% of total carotenoids without adding inducers [10, 12-13].

Although significant efforts have been made to improve astaxanthin production, current metabolic engineering strategies for improving astaxanthin production still require improvements in two aspects. Firstly, the development of a highly active optimized CrtW with a broad substrate spectrum is necessary, since CrtW should not only efficiently introduce keto groups into *β*-carotene or ketolated carotenoid (echinenone) to synthesize canthaxanthin, but also exhibit high activity towards zeaxanthin or hydroxylated carotenoid (adonixanthin) for astaxanthin synthesis. Secondly, in the complex and interchangeable catalytic routes of astaxanthin biosynthesis, the hydroxylase CrtZ and ketolase CrtW compete for their respective substrates, which makes the balanced expression of these enzymes critical for the complete conversion of *β*-carotene to astaxanthin without the accumulation of intermediates [13].

In this study, a *crtW* mutant library was constructed by error prone PCR, and highly active CrtW mutants were selected based on both canthaxanthin and astaxanthin synthesis. The expression levels of the *crtW** (mutated *crtW*) and *crtZ* genes were balanced by screening a chromosomally integrated RBS library. After balancing the expression within the single *crtW*−crtZ* cassette, the number of its chromosomal copies was increased using a Cre-loxP based method to obtain an astaxanthin hetero-producer strain of *E. coli* without addition of inducer.

Materials and Methods

Strains, media and growth conditions

All strains used in this study are listed in Table 1. During strain construction, cultures were grown aerobically at 30, 37, or 39 °C in Luria broth (per liter: 10 g Difco tryptone, 5 g Difco yeast extract and 10 g NaCl) containing 20 $g \cdot L^{-1}$ glycerol. For analysis of astaxanthin production in shake-flask, single colonies were picked from the plate and used to inoculate 15 mm × 100 mm tubes containing 4 mL of LB with or without 34 $mg \cdot L^{-1}$ chloramphenicol, and grown at 30 ºC and 250 $r \cdot min^{-1}$ overnight. The overnight seed culture was then inoculated into 50 mL of LB (antibiotics added if need) to an initial OD_{600nm} of 0.05, and grown at 30 °C and 250 $r \cdot min^{-1}$.

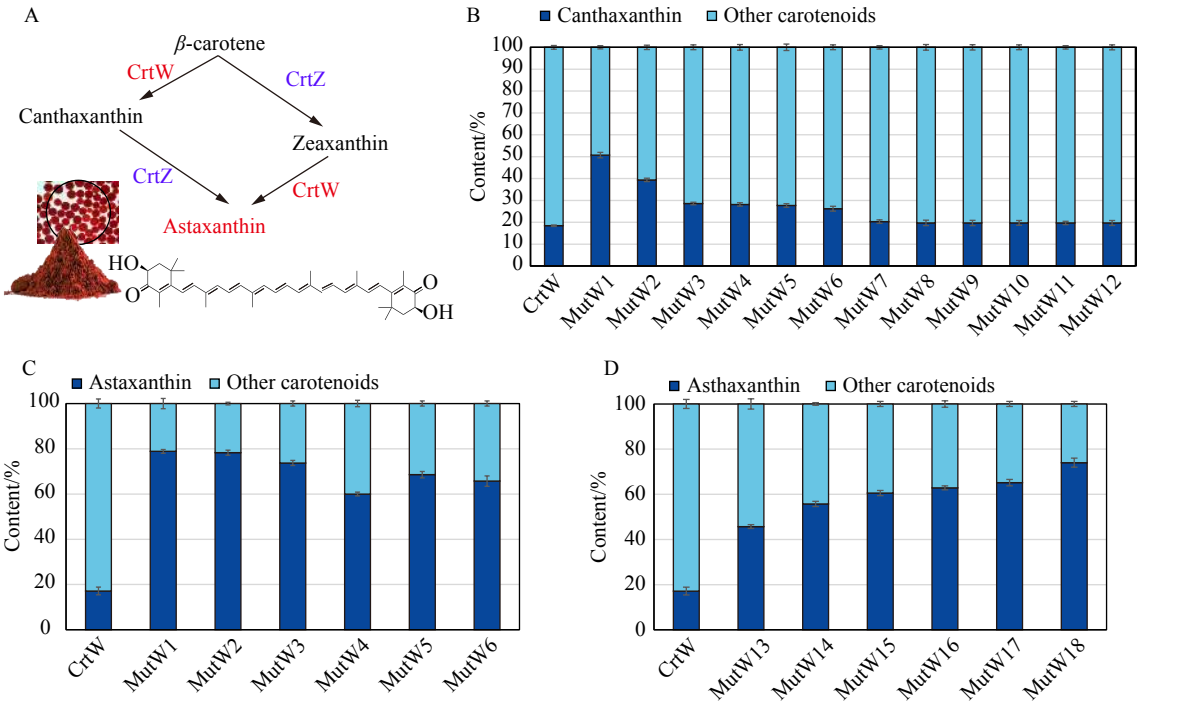

Fig. 1 Screening beneficial CrtW mutations through two platforms, one tests the synthesis of canthaxanthin, and the other one assesses the synthesis of astaxanthin. (A) The main routes of astaxanthin biosynthesizing from *β*-carotene through two key bifunctional enzymes CrtW and CrtZ. (B) Screening beneficial CrtW mutations by assaying canthaxanthin production using YL-CAR002 as platform strain. (C) CrtW mutations with higher activity of *β*-carotene converting to canthaxanthin were identified astaxanthin production using YL-Z004 as platform strain. (D) Screening beneficial CrtW mutations by assaying astaxanthin production using YL-Z004 as platform strain. CrtW, control; The means and standard deviations were calculated from triplicate data

Table 1 Strains and primers used in this work

Strains	Relative characteristics	Sources
E. coli ATCC 8739	Wild type	American Type Culture Collection
CAR005	ATCC8739, *ldhA*::M1-93-*crtEXYIB*, M1-37-*dxs*, M1-46-*idi*, M1-46-*sucAB*, M1-46-*sdhABCD*, M1-46-*talB*	[11]
YL-CAR002	CAR005, Δ*crtX*	This work
YL-Z004	YL-CAR002, *mgsA*::M1-37-*crtZ*	This work
YL-A004	YL-CAR002, *mgsA*::M1-93-*crtW**−*crtZ*	This work
RBSL54	YL-A004, promoter of *crtW** and *crtZ* from YL-A004 modulated through RBS region	This work
DL-A001	CAR005, Δ*crtX*, one copy of *crtW**−*crtZ* cassette from RBSL54 was integrated into *mgsA* site with loxP sequence	This work
DL-A008	CAR005, Δ*crtX*, multiple copies of *crtW**−*crtZ* cassette from RBSL54 through Cre-loxP system	This work
	Primers for CrtW* and CrtZ modulation Sequences (5′−3′)	
M93-cat-up	TAACAACGTTGATATAATTGAGCCCGTATTGTTAGCATGTACGTTTAAACTGTGACGGAAGATCACTTCGCA	
crtZ-sacB-down	ATTCCGATAACAGTGACCAGAACAATCAGGGCATTCCAAATCCACAACAT TTATTTGTTAACTGTTAATTGTCCT	
M93-RBSL-crtW*	TAACAACGTTGATATAATTGAGCCCGTATTGTTAGCATGTACGTTTAAACC AGGAGYNNNNNNATGACCGCCGCAGTCACAG	
crtW*-KpnI	CGCGGTACCTTACGATTCACCACGCCACAG	
KpnI-RBSL-crtZ	CGCCGGTACCCAGGAGYNNNNNNATGTTGTGGATTTGGAATG	
crtZ-down	TTACTTCCCGGGTGGCG	

After 24 h, cells were collected to measure carotenoid production.

Construction of the CrtW mutant library

The *crtW* gene from *Brevundimonas* sp. SD212 (GenBank accession number: AB181388) was artificially synthesized by Nanjing GenScript Company, Nanjing, China, with codon optimization for the *E. coli* chassis. Moreover, phenylalamine 222 was changed to leucine (F222L, Fig. S1) on the synthesized *crtW* gene, since this mutation has been reported to improve astaxanthin production [14].

The low-copy plasmid pSC102 (Table 1, Text S1) about 5 copies/cell, modified from pSC101 by adding M1-46 promoter [15] and changing to chloramphenicol resistance, was used to construct the *crtW* mutant library. The synthesized *crtW* gene was ligated into pSC102 between the *Pme*I and *Sac*I sites, resulting in the plasmid pSC102-crtW. Error-prone PCR was performed on the entire *crtW* gene with pSC102-crtW as the template and the primer pair 46-RBS-up/crtW-SacI-down. The PCR reaction was performed using Taq DNA polymerase (Transgen Biotech, China) in a reaction mixture comprising $MgCl_2$ (0.25 mmol·L^{-1}), dNTPs (0.25 mmol·L^{-1} each), $MnCl_2$ (0.5 mmol·L^{-1}), pSC102-CrtW DNA (2 ng) and the primers (0.2 μmol·L^{-1} each). The PCR program comprised 3 min at 94 °C, followed by 25 cycles comprising 94 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min each, followed by an additional 10 min at 72 °C. The purified PCR products were digested with *Pme*I, *Sac*I and *Dpn*I (NEB, USA) overnight, and ligated overnight with the pSC102 backbone digested with *Pme*I and *Sac*I. The ligated DNA was electroporated into two platform strains (see Materials and Methods: construction of two platform strains to screen beneficial CrtW mutants) to screen for *crtW* mutants. The transformed cells were spread on LB-agar plates containing 34 mg·L^{-1} chloramphenicol and incubated at 30 °C overnight. Colonies that showed intense orange or red color indicating improved ketolase activity were isolated by visual inspection. Primers used for constructing plasmids are listed in Table S2.

Construction of two platform strains to screen beneficial CrtW mutants

The *crtX* gene (GenBank accession number: JX876608), encoding zeaxanthin glucosyltransferase that converts zeaxanthin to zeaxanthin *β*-di-glucoside, was deleted in *β*-carotene producing strain CAR005 [11] by two-step recombination method [16-18] (Text S1), resulting in the strain YL-CAR002. For screening *crtW* mutagenesis, the YL-CAR002 was one platform strain since it produced *β*-carotene that could be converted to canthaxanthin through CrtW. The other platform strain YL-Z004 was constructed by introducing the *crtZ* gene from *Pantoea agglomerans* CGMCC No. 1.2244 (GenBank accession number HQ003247) into the *mgsA* site of YL-CAR002 through the two-step recombination method, further modulating expression of *crtZ* gene using promoter M1-37 [15] using the same two-step recombination [16-18]. Zeaxanthin produced by strain YL-Z004 could be converted to astaxanthin through CrtW.

Site-directed mutagenesis

The point mutant A6T was introduced into *crtW* on pSC102-CrtW *via* the Fast Mutagenesis System (Transgen Biotech, China) using the primer pair A6T-F/A6T-R. The res-

ulting plasmid pA6T was confirmed by DNA sequencing. The plasmid containing point mutant such as P12Q, T105A, M147T, A152T, and L239M was individually constructed in the same manner. A combinatorial mutant was constructed by introducing the A6T mutation into pMutW1 containing the T105A and L239M double mutations using the primer pair A6T-F/A6T-R. The plasmid pMutW* with three amino-acid substitutions was confirmed by sequencing.

Simultaneous modulation of crtW and crtZ using an RBS library*

The *crtW** gene from pMutW* was integrated into YL-Z004 before *crtZ* gene through the two-step recombination method, and the expression of *crtW** gene was modulated by promoter M1-93 [15] using the same two-step recombination method. The resulting strain YL-A004 was served as the starting strain for simultaneous modulating *crtW** and *crtZ* genes through an RBS library, which was a novel method based on the two-step recombination. Cassettes containing *crtW** and *crtZ* genes with an RBS library were PCR amplified, and 6 random nucleotides in this gene's RBS region was embedded on forward primers (Table 1). Forward primer M93-RBSL-crtW* and reverse primer crtW*-KpnI were used to amplify *crtW** cassette with RBS library using plasmid pMutW* as template. Through the same manner, cassette containing *crtZ* gene with RBS library were obtained using forward primer KpnI-RBSL-crtZ and reverse primer crtZ-down to amplify using pACYC184-M-crtZ as template. These two cassettes were digested with *Kpn*I and *Dpn*I (NEB, USA) and ligated using the Quick ligation kit (NEB, USA). After cleaning up using the MinElute PCR Purification Kit (Qiagen, Germany), the ligation cassette *crtW**−*crtZ* with RBS library were used to transform JL-A004 competent cells with *Cat-SacB* cassette, which has been inserted into chromosome at *mgsA* site by the two-step recombination method. The *Cat-SacB* was replaced by the cassette *crtW**−*crtZ* library, and colonies with no chloramphenicol resistance that grew on sucrose plates were picked for PCR verification. The verified colonies were then subjected to measurements of astaxanthin production. Primers used for modulation of *crtW**−*crtZ* through the RBS library were listed in Table 1.

Multiplication of the chromosomal gene copy number of the crtW-crtZ cassette using the Cre-loxP technique*

The *crtW**−*crtZ* cassette from the astaxanthin producing strain RBS54 was integrated into the chromosome, and its copy number was multiplied using the Cre-loxP technique [19]. First, the loxP sequence was integrated into the *mgsA* site of YL-CAR002 *via* two-step recombination to obtain the starting strain. Second, a suicide plasmid containing the R6K replicon, kanamycin and chloramphenicol resistance genes, a loxP sequence and the *crtW**−*crtZ* cassette was assembled *via* Golden Gate Assembly [20], which was termed pR6K-KC-loxP-crtW*Z (Text S1). Third, the *cre* gene was synthesized and inserted into the pKD46 backbone to replace the Red recombinase gene, yielding the helper plasmid pKDCre. The *cre* gene was arabinose-inducible, and the helper plasmid was temperature-sensitive. Finally, the suicide plasmid pR6K-KC-loxP-crtW*Z and pKDCre were used to co-transform the starting strain to induce the integration of the *crtW**−*crtZ* cassette *via* loxP recombination. The helper plasmid was kept in the integrated strain while arabinose and high concentrations of antibiotics were added to the culture to induce more rounds of loxP-based recombination for the multiplication of the chromosomal copy number of the *crtW**−*crtZ* cassette. Colonies with intense color and high resistance were selected for screening of strains with higher astaxanthin accumulation.

Estimation of chromosomal DNA cassette copy numbers

The Wizard genomic DNA kit (Promega, Madison, WI, USA) was used to extract chromosomal DNA from overnight cultures according to the manufacture's protocol. Chromosomal DNA cassette copy numbers were determined *via* quantitative RT-PCR (qRT-PCR) with the DNA extracts as templates. The *crtW** gene on the integration cassette and the 16sRNA gene on the chromosome were selected as the target and reference gene, respectively. The strain containing one copy of *crtW** was used as the control.

The copy numbers were quantified by comparing the C_t values of the target and reference genes using a previously described method [18]. qPCR was performed by BGI, China, using the FastStart Essential DNA Green Master kit (Roche, Basel, Switzerland) on a Roche Light Cycler 96 System. The oligonucleotide primers used for qPCR (Table S2) were designed using Beacon Designer 7.0 software (PREMIER Biosoft International, USA).

Carotenoids analysis

Carotenoids was extracted using acetone as described in our previous work [11]. Samples (2 mL) were withdrawn from culture after 24 h incubation. The cell pellets were washed with cold water and extracted with the same volume of acetone. HPLC analysis was performed on a Series 1200 system (Agilent, USA) equipped with a C_{18}-reverse-phase column (250 mm × 4.6 mm, 5 μm particles, Waters, Ireland), with a diode array detector. Products were analyzed by loading 20 μL of supernatant and the spectra of the eluted carotenoids were detected at 476 nm. The mobile phase containing A (methanol/acetonitrile/dichloromethane = 21 : 21 : 8) and B (methanol/water = 1 : 9) was used at a flow rate of 0.8 $mL \cdot min^{-1}$ and the column was kept at 30 °C. The solvent gradient encompassed 80%−100% A, 20%−0% B from 0 to 18 min, 100% A−0% B from 18−38 min, and 100%−80% A, 0%−20% B from 38−50 min. Standard curves were generated using commercial standards: astaxanthin, canthaxanthin, and zeaxanthin, echinenone, adonirubin, adonixanthin were purchased from CaroteNature (Lupsingen, Switzerland) and *β*-carotene was purchased from Sigma (Sigma-Aldrich, USA). Cell dry weight (cdw) was calculated from the optical density at 600 nm according to the equation 1 OD_{600} = 0.323 g $cdw \cdot L^{-1}$. The results represent the means ± SD of three independent experiments.

Fed-batch fermentation

Strains DL-A008 were used to produce astaxanthin

through fed-batch fermentation. The medium used for seed preparation and fermentation was described previously [11], and the glycerol was provided about 32 $g \cdot L^{-1}$ for fermentation medium. The seed culture was prepared by inoculating several colonies into a 250 mL flask containing 50 mL culture medium and incubating at 30 ºC with shaking at 250 $r \cdot min^{-1}$ for 14 h. The seed was then transferred to a 7 L-fermentor (Infors Biotechnology Co., Ltd.) containing 3 L-medium with an initial OD_{600} = 0.2. Fermentation was carried out at 30 ºC with an air flow of 3 $L \cdot min^{-1}$. Dissolved O_2 was maintained at 30% by adjusting the agitation speed from 300 to 1200 $r \cdot min^{-1}$. The pH was maintained at 7.0 by automatic addition of 5 $mol \cdot L^{-1}$ NH_4OH. A fed solution containing (per liter) 500 g glycerol, 15 g peptone, 30 g yeast extract, and 30 g $MgSO_4 \cdot 7H_2O$, was added to the fermentator at average rate of 20 $mL \cdot h^{-1}$.

Results

Construction a platform strain to screen beneficial CrtW mutations by assaying canthaxanthin production

Strain CAR005, carrying the *β*-carotene synthetic gene operon (*crtEXYIB* from *P. agglomerans* CGMCC No. 1.2244) on chromosome, constructed by our group and produced 30.2 $mg \cdot g^{-1}$ cdw *β*-carotene [11]. To inactivate competitive pathway of astaxanthin synthesis, the *crtX* gene (encoding zeaxanthin glucosyltransferase that converts zeaxanthin into zeaxanthin *β*-di-glucoside) [21] was inactivated by deletion. The resulting strain YL-CAR002 producing *β*-carotene of 30 $mg \cdot g^{-1}$ cdw, was used as the platform strain to screen CrtW mutations.

In order to screening higher activity of CrtW by visual color, the *crtW* gene from *Brevundimonas* sp. SD212 was synthesized with phenylalamine 222 changing to leucine (Genscript, China) and cloned into a low-copy plasmid pSC102 (Table S1), and its expression was controlled by low-strength promoter M1-46 (expression strength is 1.7-fold of induced *lacZ* promoter) that generated by our previous work [15, 18], resulting in plasmid pSC102-crtW. When pSC102-crtW was transformed into YL-CAR002, cells were light-orange and produced a mixture of carotenoids with 18% canthaxanthin. The color background and the low canthaxanthin content would allow screening out higher CrtW activity with converting more *β*-carotene into canthaxanthin.

Error-prone PCR was performed to the whole *crtW* gene from ATG to TAA on pSC102-crtW. The digested error prone PCR product was purified and ligate to *Pme*I/*Sac*I sites of pSC102-crtW to replace the wild-type *crtW* gene. The ligated DNA was electroporated into *β*-carotene producing strain YL-CAR002. Approximately 7000 colonies were obtained from this *crtW* mutagenesis library. One hundred and five orange or orange-red colonies were isolated. Further, canthaxanthin production were determined with shake-flask fermentation and identified by HPLC. As a result, 12 colonies showed higher canthaxanthin production than wild-type control. There were six mutants (Mut1 to Mut6) produced more than 26% canthaxanthin comparing to 18% canthaxanthin produced by the wild-type control. The highest was Mut1 mutant that produced 51% canthaxanthin (Fig. 1B).

Construction another platform strain to screen beneficial CrtW mutations by assaying astaxanthin production

A *crtZ* gene from *P. agglomerans*, encoding *β*-carotene hydroxylase, was integrated into *mgsA* site of *β*-carotene producing strain YL-CAR002 by two-step recombination, and its expression was controlled under the middle-strength promoter M1-37 (expression strength is 2.5-fold of induced *lacZ* promoter) that constructed by our previous work [15, 18]. The resulting strain YL-Z004 produced zeaxanthin, and was used as another platform strain to screen beneficial CrtW mutations. When pSC102-crtW with low expression *crtW* gene was transformed into YL-Z004, cells were yellow and produced a mixture of carotenoids with 17% astaxanthin, which could allow to screen out higher CrtW activity with converting more zeaxanthin into astaxanthin.

First, six CrtW mutations (MutW1−6) from canthaxanthin screening library, which have higher CrtW activity with converting *β*-carotene to canthaxanthin, were further investigated if these mutations can also improve astaxanthin production using zeaxanthin as substrate. Plasmids with *crtW* mutations were introduced into zeaxanthin producing strain YL-Z004 and analyzed astaxanthin production by shake-flask fermentation. HPLC analysis indicated that more than 60% astaxanthin produced by the six mutants (Fig. 1C), suggesting that CrtW mutants with improved canthaxanthin production also had better astaxanthin production. The best mutant MutW1 produced 2.76 $mg \cdot g^{-1}$ cdw astaxanthin with 79% astaxanthin comparing to 17% astaxanthin produced by the wild-type control. The second best mutant MutW2 produced 78% astaxanthin (Table 2 and Fig. 1C).

Second, the error prone PCR product of *crtW* mutagenesis library was introduced into zeaxanthin producing strain YL-Z004 to screen CrtW mutations with higher activity on converting zeaxanthin to astaxanthin. Out of approximately 100 000 colonies obtained, 59 orange or orange/red colonies were selected. Five colonies were confirmed to improve astaxanthin production by HPLC analysis (Fig. 1D). The highest specific production value was achieved by the MutW18 mutant, which produced 2.59 $mg \cdot g^{-1}$ cdw astaxanthin with 74% comparing to 17% astaxanthin produced by the wild-type control (Table 2 and Fig. 1D).

Combinatorial analysis of beneficial crtW mutations for increased astaxanthin production

The three mutants MutW1, MutW2 and MutW18, having higher CrtW activity to produce astaxanthin, were identified by DNA sequencing. Each mutant was contained at least four mutations. MutW1 had two non-silent (T105A and L239M) and three silent mutations, and MutW2 had one non-silent (M147T) and five silent mutations, and MutW18 had three non-silent (A6T, P12Q and A152T) and one silent mutation (Table 3). All the silent mutations did not appear to significantly change the codon usages, and *crtW* mutation gene expression levels in the three mutants were similar with the wild-type gene by qRT-PCR analysis (data not shown).

These results suggested that the improved astaxanthin production of these mutants was mostly due to increased CrtW catalytic, rather than increased transcription levels of mutated *crtW* genes.

Next, the six single mutation including A6T, P12Q, A152T, T105A, M147T and L239M of CrtW were selected to analyze their contribution to the phenotype of enhanced astaxanthin production. The single mutated *crtW* genes were individually introduced into plasmid pSC102-crtW (Table S1) by site-directed mutagenesis, generating six plasmids containing single mutated *crtW* gene. These plasmids were transformed into zeaxanthin producing strain YL-Z004 and analyzed astaxanthin producing by shake-flask fermentation. Compared with wild-type CrtW, each CrtW mutant had higher astaxanthin production, and the best mutant A6T produced 60% astaxanthin comparing to 17% astaxanthin produced by the wild-type control (Fig. 2). The A6T mutation, conferring a higher efficient conversion from zeaxanthin to astaxanthin, was further integrated into the MutW1, which was contained the two non-silent mutations T105A and L239M, generating the combinatorial mutant MutW*. This mutant produced 92% astaxanthin with 5.35-fold higher than the wild-type control (Fig. 2 and Table 2). This further demonstrated that beneficial CrtW mutations could be combinatory and lead a significant effect on astaxanthin production. The mutated *crtW* gene from the strain MutW* was designated as *crtW**.

A novel method for the simultaneous modulation of chromosomal crtW and crtZ genes for improving astaxanthin production*

To enhance strain stability and further increase the astaxanthin production, the mutated *β*-carotene ketolase CrtW and *β*-carotene hydroxylase CrtZ were integrated into chromosome of *β*-carotene engineered *E. coli*. The *crtW*−crtZ* cassette including the RBS sequence, was integrated into YL-CAR002 and modulated with strong promoter M1-93 (expression strength is 5-fold of induced *lacZ* promoter) [15], resulting strain YL-A004. This strain produced astaxanthin of 1.83 mg·g^{-1} cdw, which represented 52% of the total carotenoids produced. This strain seemed not to be an ideal producer since it also produced 22% canthaxanthin. The main reason is probably that the expressions of *crtW** and *crtZ* genes were unbalance in the synthetic pathway.

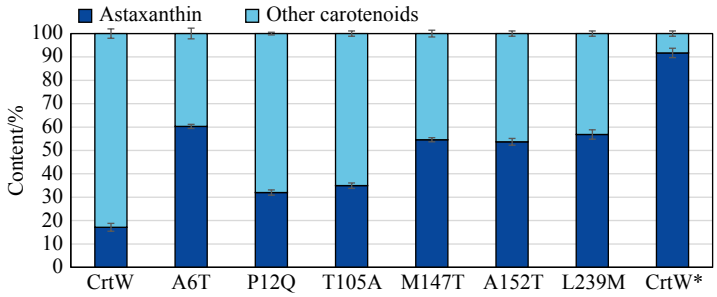

Fig. 2 Combinatory analysis of beneficial *crtW* mutations for increased astaxanthin production. Effects of the point mutation (A6T, P12Q, A152T, T105A, M147T, A152, L239) and combinatorial mutation (A6T, T105A and L239M, CrtW*) of CrtW on astaxanthin production. CrtW, control; The means and standard deviations were calculated from triplicate data

Table 2 Astaxanthin production of the engineered *E. coli* strains

Strain	Genetic characteristics	Astaxanthin production		Relative increase of astaxanthin production
		Astaxanthin (mg·g^{-1} cdw)	Astaxanthinratio (%)	
Plasmid-based astaxanthin strains				
Wild-type	YL-Z004, pSC102-crtW	0.6 ± 0.10	17%	1
MutW1	YL-Z004, pSC102-MutW1	2.76 ± 0.11	79%	4.60
MutW2	YL-Z004, pSC102-MutW2	2.74 ± 0.36	78%	4.57
MutW18	YL-Z004, pSC102-MutW18	2.59 ± 0.11	74%	4.32
MutW*	YL-Z004, pSC102-crtW*	3.21 ± 0.18	92%	5.35
Chromosome-based astaxanthin strains				
YL-A004	YL-CAR002, *mgsA*::M1-93::*crtW*−crtZ*	1.83 ± 0.07	52%	3.05
RBS54	*ctrW** and *crtZ* from YL-A004 were modulated through RBS region	3.46 ± 0.04	99%	5.77
DL-A001	One copy of *crtW*−crtZ* cassette, as the starting strain to multiple copies	3.5 ± 0.06	99%	5.83
DL-A008	Multiple copies of *crtW*−crtZ* cassette	5.88 ± 0.36	99%	9.8

Table 3 CrtW mutations isolated from the mutagenesis library

Mutants	Non-silent mutation		Silent mutation	
	Amino acid change	Nucleotide change	Amino acid unchanged	Nucleotide change
MutW1	T105A, L239M	A313G, C715A	A55A, L169L, A174A	T165C, G507A, A522G
MutW2	M147T	T440C	P8P, L21L, I25I, Y95Y, L168L	G24C,C61T,T75A,T285C,C502T
MutW18	A6T, P12Q, A152T	G16A, C35A, G454A	P78P	G234A

To balance the expression between *crtW** and *crtZ* genes, we decided to design a simple method for the simultaneous modulation of multiple genes on the chromosome (Fig. 3A). One cassette containing the *crtW** gene and an RBS library was amplified using the primer pair M93-RBSL-crtW*/crtW-KpnI, and 6 random nucleotides in this gene's RBS region were embedded in the forward primer M93-RBSL-crtW*(Table 1). The other cassette containing an RBS library controlling the *crtZ* gene was prepared in the same manner. These two cassettes were digested and ligated together to generate a combinatorial RBS library to modulate *crtW** and *crtZ* genes simultaneously. After a two-step recombination procedure, this library was integrated and replaced the *Cat-SacB* cassettes that has been inserted between the promoter M1-93 and *crtZ* gene on chromosome (Fig. 3A), resulting in a library of astaxanthin-producing strains with simultaneously modulated *crtW** and *crtZ* genes (Fig. 3A). Nine colonies with red-orange coloration were selected from the library for carotenoid production measurement, and most of them produced more astaxanthin than the strain YL-A004 with one copy of *crtW**−*crtZ* cassette (Fig. 3B). The best strain RBS54 produced astaxanthin as predominant carotenoid (99%) with a specific titer of 3.46 mg·g^{-1} cdw (Table 2). This result suggested a *crtW**−*crtZ* cassette with better balanced expression was screened out through the method of multiple genes simultaneously modulated on chromosomal. Comparing to the control strain YL-A004, the transcription levels of the *crtW** and *crtZ* in RBS54 were 3.2-fold and 1.6-fold higher, respectively. These results indicated that high expression levels of *crtW** gene and middle expression level of *crtZ* gene would be balanced and able to contribute more astaxanthin production from *β*-carotene.

Genomic integration of multiple cassette copies for improving astaxanthin production

For further improvement of astaxanthin production, the Cre-loxP integration system [19, 22] was employed to multiply the chromosomal copy number of *crtW**−*crtZ* cassette. The loxP sequence was embedded at the *mgsA* site on *β*-carotene strain YL-CAR002. Subsequently, a single copy of the *crtW**−*crtZ* cassette from the astaxanthin high-producer RBS54 was integrated alongside the kanamycin and chloramphenicol resistance genes into the chromosome at the *mgsA* site *via* Cre-loxP recombination, generating the starting strain DL-A001 with two loxP sequences (Fig. 4A). In the presence of the Cre recombinase helper plasmid, the recombination between loxP sequences occurred repeatedly and generated a library of strains with different copy numbers of the *crtW**−*crtZ* cassette on the chromosome. Under high antibiotic selection pressure, only the recombinants containing multiple copies of the kanamycin and chloramphenicol resistance genes survived, which simultaneously selected for strains with high copy numbers of the *crtW**−*crtZ* cassette (Fig. 4A).

With the concentration of antibiotics increasing, the astaxanthin of recombinants from this process were improved gradually (Fig. 4B). When 600 μg·mL^{-1} kanamycin and 408 μg·mL^{-1} chloramphenicol as selection pressure were added, strain DL-A008 was obtained. This recombinant strain produced 5.88 mg·g^{-1} cdw astaxanthin, while the starting strain DL-A001 with single copy of *crtW**−*crtZ* cassette produced 3.5 mg·g^{-1} cdw. A 70% increase of astaxanthin titer was over the starting strain DL-A001. Moreover, there was no detectable canthaxanthin, zeaxanthin or other carotenoids in the engineered strain DL-A008. The copy number of the *crtW**−*crtZ* cassette was found to be approximately 30 copies in this strain by qPCR detecting (data not shown).

Further, the stability of strain DL-A008 was estimated through continuously culturing in the absence of antibiotics for ten passages. The astaxanthin production was found to be stably maintained at around 5.8 mg·g^{-1} cdw, which indicated that the multi-copy strain was stable enough for large-scale industrial applications.

Fed-batch fermentation of strain DL-A008 for astaxanthin production

Fed-batch fermentation of strain DL-A008 was performed in a 7 L-fermentor with pH controlled at 7.0 (Fig. 5). Fed solution was provided at 15 h after inoculation, and the concentration of glycerol in the fermentor was controlled under 0.5 g·L^{-1} through adjusting the speed of fed solution. Cell growth reached stationary stage after 56 h, and the final OD_{600} was 455 (equaled to 147 g·L^{-1} cdw biomass). Production of astaxanthin continued to increase until 66 h, and the maximum titer was 0.88 g·L^{-1} (Fig. 5). Without adding inducer, the astaxanthin was produced as the predominant carotenoid (99%) with a specific content of 6 mg·g^{-1} cdw.

Discussion

Finding highly active CrtW enzymes with a broad substrate spectrum is necessary for efficient production of astaxanthin, since CrtW should not only efficiently introduce keto groups into *β*-carotene or ketolated *β*-carotene for canthaxanthin production, but also exhibit high activity towards hydroxylated carotenoids including zeaxanthin and adonixanthin. For this purpose, we designed two platforms for

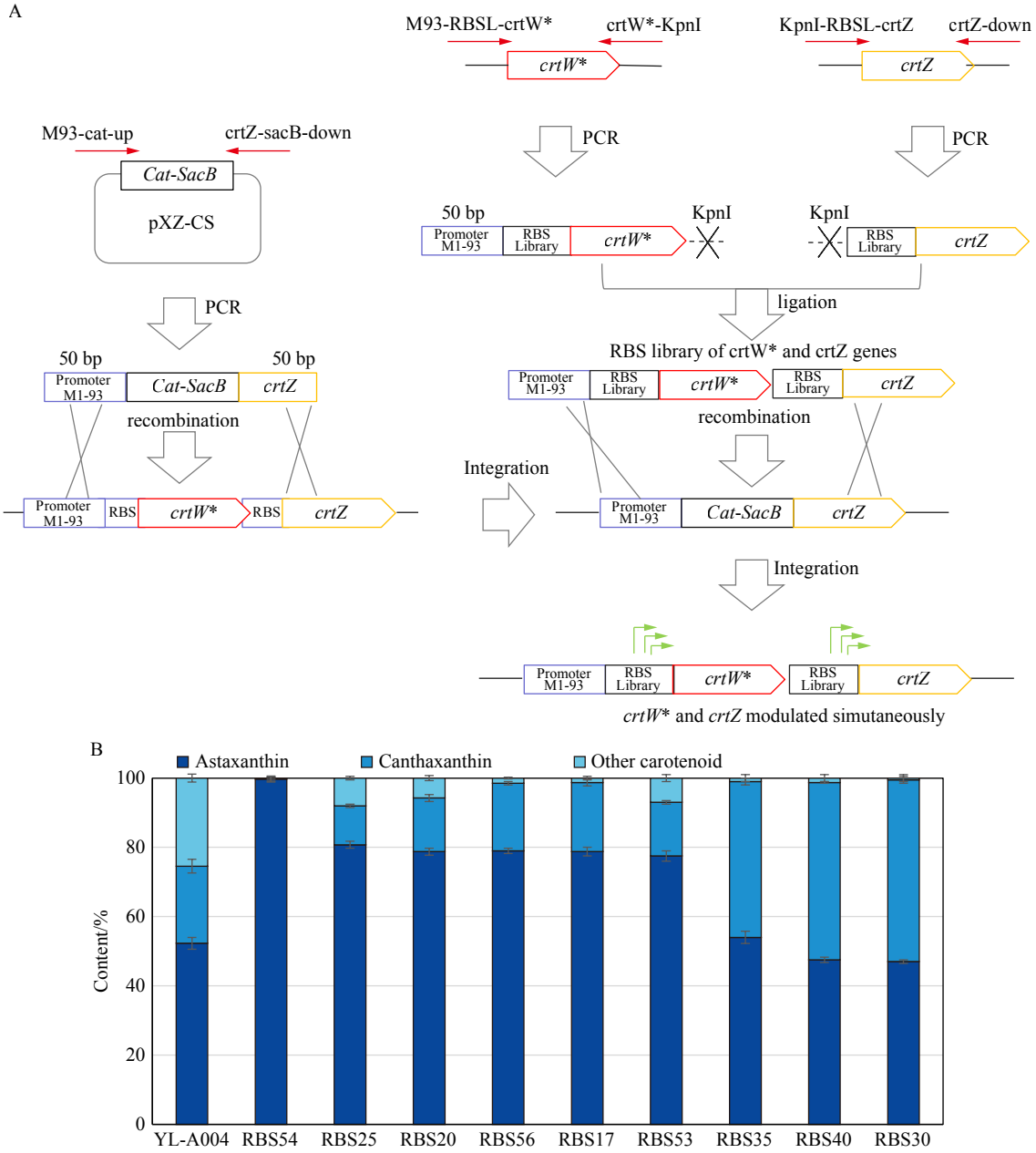

Fig. 3 Simultaneous modulation of *crtW (A6T, T105A and L239M) and *crtZ* genes on chromosomal. (A) Schematic illustration of a novel method for simultaneous modulation of *crtW** and *crtZ* genes on chromosomal. Cassettes containing *crtW** and *crtZ* genes with an RBS library were PCR amplified, and 6 random nucleotides in this gene's RBS region was imbedded on forward primers M93-RBSL-crtW* and KpnI-RBSL-crtZ (Table 1). These two cassettes were digested and ligated together to generate a combinatory RBS library of *crtW** and *crtZ* gene. After a two-step recombination, this library was integrated upstream of ATG locus of *crtZ* gene, resulting an astaxanthin producing strain library with simultaneously modulated *crtW** and *crtZ*. Promoter M1-93: constructed by our previous work and 5-fold higher expression than that of induced *lacZ*. (B) Relative astaxanthin production of *E. coli* strains with simultaneously modulated *crtW** and *crtZ* genes. The means and standard deviations were calculated from triplicate data**

screening of beneficial CrtW mutations. One platform analyzed the canthaxanthin synthesis based on a *β*-carotene producing strain, and the other one analyzed the astaxanthin synthesis based on a zeaxanthin producing strain.

A low-copy plasmid pSC102 (about 5 copies per cell) was selected to construct CrtW mutation library. When pSC102 containing the wild-type *crtW*, which was controlled under the relative low strength promoter M1-46 (expression strength is 1.7-fold of *lacZ* induced) was transformed into the zeaxanthin producing strain, the colony's color was orange, which was too high as background. For better visual screening, a lower-strength promoter was used by promoter library selection. The -35 region of this promoter was changed to TGTTGA comparison to TTGACA of M1-46. When this pro-

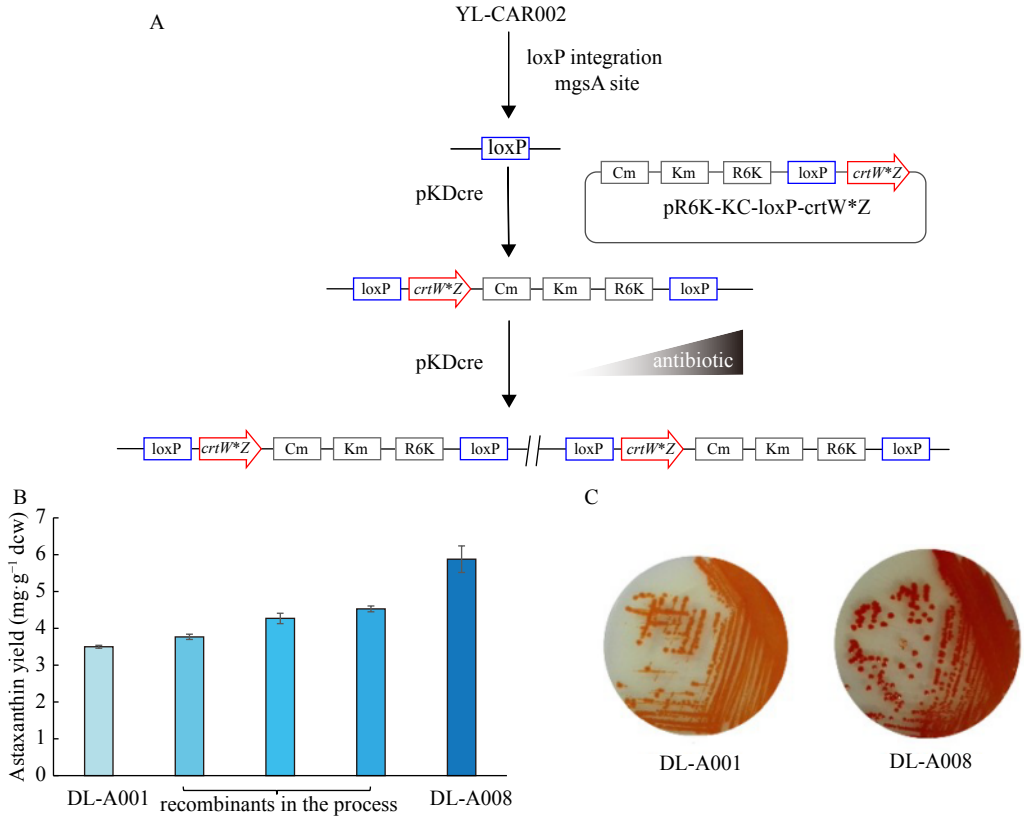

Fig. 4 (A) Schematic illustration of chromosomal copies multiplication of *crtW*-crtZ* cassette with the Cre-loxP technique. The loxP sequence was embedded at *mgsA* site to generate the starting strain. Subsequently, a single copy of *crtW*-crtZ* cassette with kanamycin and chloramphenicol resistance genes was integrated into *mgsA* site *via* Cre-loxP recombination. In the presence of Cre recombinase helper plasmid and under a high antibiotic pressure, only the recombinants containing multiple copies of kanamycin and chloramphenicol resistance genes from loxP recombination would survive. (B) Astaxanthin production of *E. coli* strains containing various copy numbers of *crtW*-crtZ* cassettes during the process of Cre-loxP. (C) The color intensity of DL-A001 and DL-A008 on culture plates

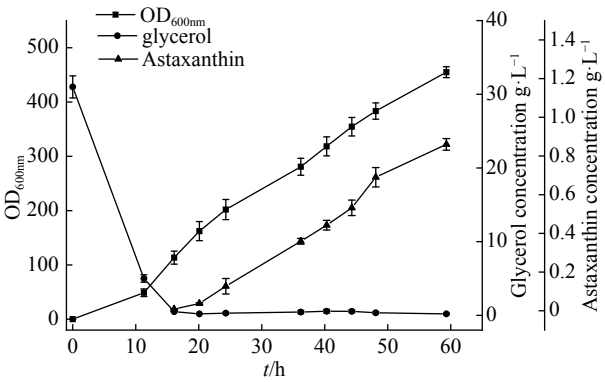

Fig. 5 Fed-batch fermentation profile of strain DL-A008. The initial fermentation medium contained 32 g·L^{-1} glycerol. The pH was controlled at 7.0. The data points represent averages and standard deviations of three replicates

moter was controlled *crtW* gene expression in zeaxanthin producing strain, cells were yellow which allowed screening of improved *crtW* mutants according to color change such as orange or red. Thus, twenty CrtW mutants with possible higher activity were easily screened from a *crtW* mutant library (about 10 000 colonies). However, these mutated *crtW* did not really improve CrtW activity, since astaxanthin titers (data not shown) were still lower than that of wild-type strain when the lower-strength promoters were changed back to M1-46 to express the mutated *crtW* gene. The results of sequencing also showed that the most mutated loci were located at *N*-terminal of CrtW protein and were silent mutations. Therefore, we proposed that these mutated *crtW* in 5′-terminal nucleotides changing would be increased the mRNA stability, leading more CrtW to convert zeaxanthin into astaxanthin and to contribute color change to orange. Moreover, it was hard to screen high CrtW activity using the lower-strength promoter. The probable reason was that using the lower-strength promoter generated less mRNA, which was more easily degraded due to mRNA itself instability even if CrtW mutants with high activity. Thus, we sought to the low-strength promoter M1-46 to express and screen *crtW* mutants, although background color was relatively high.

Constructing *crtW* mutagenesis library on low-copy plasmid through error PCR and using two platform strains to screen and assay mutated CrtW activity, three mutants MutW1, MutW2 and MutW18 were isolated, which produced 79%, 78% and 74% astaxanthin comparing to 17% astaxanthin produced by wild-type control (Table 2). All the

mutants contained at least one mutation, and some contained multiple mutations in the gene, which did not appear to change codon usage preference significantly. That suggested that the improved astaxanthin production in the mutants was mostly due to change of the CrtW properties rather than the increase of *crtW* gene expression. Total six non-silent mutations were analyzed their contribution to the phenotype of enhanced astaxanthin production through site-directed mutagenesis technology. The best point mutant A6T, produced 60% astaxanthin, was combinated with both T105A and L239M mutations that from MutW1. The resulting mutant, which was designed as MutW*, produced 92% astaxanthin with 5.35-fold higher than the wild-type control. This result demonstrated that beneficial CrtW mutations could be combinatory and lead a significant effect on astaxanthin production. The mutated CrtW from the strain MutW* was designated as CrtW*.

CrtW was reported to belong to a superfamily of integral membrane proteins that catalyze oxidative reactions [23, 24]. However, there was no three-dimensional structure information on these membrane proteins, and the only noticeable features included their four transmembrane domains and three conserved histidine-rich motifs involved in Fe^{2+} binding [1]. By comparing the amino acid sequence of CrtW* to that of the parent CrtW (Fig. S1), it was found that the T105A mutation was close to the second conserved His motif in the middle of the CrtW* protein, which might help improve Fe^{2+} binding since the alanine residue represents a smaller steric hindrance than a threonine. The mutations A6T and L239M were located at the *N*- and *C*-termini of CrtW*, respectively. Thus, they were far away from transmembrane domains I or III, and were located inside the cell membrane facing the cytoplasmic compartment. This indicates that these mutations might contribute to substrate binding. The high astaxanthin production of CrtW* appears to be the result of the synergetic effect of all three mutations.

Although the engineered strain MutW* could synthesize astaxanthin 3.21 mg·g^{-1} with 92% of carotenoids (Table 2), this astaxanthin produced system was based on plasmid since the *crtW** gene from MutW was expressed on pSC102. However, the plasmid expression systems were found to be segregationally instable and cause growth burden on the host cells, leading to decreased productivity of cell factories [25] Therefore, the *crtW** and *crtZ* were integrated into *β*-carotene producing strain YL-CAR002 to obtain a stable genetic strain. The result strain YL-A004 produced 52% astaxanthin, especially containing 22% canthaxanthin. It was assumed that the accumulation of intermediates could be caused by unbalance expression of *crtW** and *crtZ* gene [13]. MAGE [26, 27] is one of the major strategies that have been employed to balance multiple genes expression on chromosome. It enabled modifying several loci simultaneously and greatly enhanced the ability to engineer complex pathways. However, ssDNA donor in MAGE was necessary to target the lagging strand of the replication fork, needed to be phosphorylated and introduced into host cells repetitively, which increased experiment cost and complexity. In addition, MAGE was unable to introduce sequences longer than 20 nt, and the integration efficiency of multiple loci was low. Thus, it is necessary to develop a simple technique to modulate multiple genes on chromosome simultaneously with high efficiency. Here, we designed a novel strategy to simultaneously modulate *crtW** and *crtZ* gene on chromosome by RBS library. Six random nucleotides were designed on the forward primers on RBS regions of *crtW** and *crtZ* genes, and two cassettes of these two genes containing an RBS library was amplified, ligated and integrated into *β*-carotene producing strain. This method based on two-step recombination was time-saving and low cost to modulate simultaneously multiple genes compared to MAGE. The resulting strain RBS54, which balanced expression levels of *crtW** and *crtZ*, yielded astaxanthin as the predominant carotenoid (99%) with a specific content of 3.46 mg·g^{-1} cdw on chromosome without an addition of inducer. This result also supported that the balanced expression of CrtW and CrtZ become an extremely critical for completely conversion of *β*-carotene to astaxanthin without accumulation of the intermediates [13].

The balanced *crtW**−*crtZ* cassette from RBS54 was integrated and increased copy-number on chromosome through a Cre-loxP based technique, and the resulting strain DL-A008 produced 6 mg·g^{-1} cdw astaxanthin as predominant carotenoid (99%). More efficient strategies including multidimensional heuristic process (MHP) [28], membrane and oxidative stress engineering [29, 30], would be employed for further improving cell growth and astaxanthin productivity in our further work.

Conclusions

In this study a series of metabolic engineering strategies was designed and applied to construct an astaxanthin heteroproducer strain of *E. coli* as summarized in Table 2. Beneficial mutations that increase the catalytic efficiency of CrtW were screened from mutant library, and their contribution to the improvement was analyzed. The mutations A6T, T105A and L239M (*crtW**) were combined, and their combination improved the production of astaxanthin 5.35-fold than the parent strain carrying wild-type *crtW*. Furthermore, a novel method was developed for the simultaneous modulation of multiple genes on the chromosome, which helped to obtain strain RBS54 with a balanced expression of *crtW** and *crtZ*, yielding astaxanthin 3.46 mg·g^{-1} cdw, a 5.77-fold than the parent strain. For further improvement, the number of chromosomal copies of the *crtW**−*crtZ* cassette was multiplied using a Cre-loxP based technique, yielding a strain with 30 copies and a 9.8-fold increase of production. This final strain DL-A008 had a specific astaxanthin production value of 5.88 mg·g^{-1} cdw. Fed-batch fermentation show that DL-A008 produced astaxanthin as predominant carotenoid (99%) with a specific content of 0.88 g·L^{-1} without addition of inducer. The series of metabolic engineering strategies that were designed and applied in this study can be considered a general approach for various metabolic engineering projects, and we

hope that it will yield many more improved strains in the future.

Supplemental file

All the supporting information of this paper, including Text S, Table S, Figure S, can be requested by sending E-mails to the corresponding authors.

Competing interests

This work has been included in patent applications by Tianjin Institute of Industrial Biotechnology, Chinese Academy of Sciences.

References

[1] Ye RW, Stead KJ, Yao H, *et al*. Mutational and functional analysis of the beta-carotene ketolase involved in the production of canthaxanthin and astaxanthin [J]. *Appl Environ Microbiol*, 2006, **72**(9): 5829-5837.

[2] Lauver DA, Driscoll EM, Lucchesi BR. Disodium disuccinate astaxanthin prevents carotid artery rethrombosis and *ex vivo* platelet activation [J]. *Pharmacology*, 2008, **82**(1): 67-73.

[3] Choi SK, Matsuda S, Hoshino T, *et al*. Characterization of bacterial beta-carotene 3, 3'-hydroxylases, CrtZ, and P450 in astaxanthin biosynthetic pathway and adonirubin production by gene combination in *Escherichia coli* [J]. *Appl Microbiol Biotechnol*, 2006, **72**(6): 1238-1246.

[4] Choi SK, Nishida Y, Matsuda S, *et al*. Characterization of beta-carotene ketolases, CrtW, from marine bacteria by complementation analysis in *Escherichia coli* [J]. *Mar Biotechnol (NY)*, 2005, **7**(5): 515-522.

[5] Cision Prweb. *Carotenoids: a global strategic business report, in Global Industry Analysts I* [EB/OL]. (2007-07-25) [2020-06-16].https://www.prweb.com/releases/carotenoids/beta_carotene/prweb541757.htm

[6] Guerin M, Huntley ME, Olaizola M. Haematococcus astaxanthin: applications for human health and nutrition [J]. *Trends Biotechnol*, 2003, **21**(5): 210-216.

[7] Rodriguez-Saiz M, De la Fuente JL, Barredo JL. *Xanthophyllomyces dendrorhous* for the industrial production of astaxanthin [J]. *Appl Biochem Biotechnol*, 2010, **88**(3): 645-658.

[8] Gassel S, Breitenbach J, Sandmann G. Genetic engineering of the complete carotenoid pathway towards enhanced astaxanthin formation in *Xanthophyllomyces dendrorhous* starting from a high-yield mutant [J]. *Appl Microbiol Biotechnol*, 2014, **98**(1): 345-350.

[9] Ma T, Zhou Y, Li X, *et al*. Genome mining of astaxanthin biosynthetic genes from *Sphingomonas* sp. ATCC 55669 for heterologous overproduction in *Escherichia coli* [J]. *Biotechnol J*, 2016, **11**(2): 228-237.

[10] Lu Q, Bu YF, Liu JZ. Metabolic engineering of *Escherichia coli* for producing astaxanthin as the predominant carotenoid [J]. *Mar Drugs*, 2017, **15**(10): 296.

[11] Zhao J, Li Q, Sun T, *et al*. Engineering central metabolic modules of *Escherichia coli* for improving beta-carotene production [J]. *Metab Eng*, 2013, **17**: 42-50.

[12] Scaife MA, Burja AM, Wright PC. Characterization of cyanobacterial beta-carotene ketolase and hydroxylase genes in *Escherichia coli*, and their application for astaxanthin biosynthesis [J]. *Biotechnol Bioeng*, 2009, **103**(5): 944-955.

[13] Lemuth K, Steuer K, Albermann C. Engineering of a plasmid-free *Escherichia coli* strain for improved *in vivo* biosynthesis of astaxanthin [J]. *Microb Cell Fact*, 2011, **10**: 29.

[14] Tao LA, Wilczek J, Odom JM, *et al*. Engineering a beta-carotene ketolase for astaxanthin production [J]. *Metab Eng*, 2006, **8**(6): 523-531.

[15] Lu J, Tang J, Liu Y, *et al*. Combinatorial modulation of *galP* and *glk* gene expression for improved alternative glucose utilization [J]. *Appl Microbiol Biotechnol*, 2012, **93**(6): 2455-2462.

[16] Jantama K, Zhang X, Moore JC, *et al*. Eliminating side products and increasing succinate yields in engineered strains of *Escherichia coli* [J]. *Biotechnol Bioeng*, 2008, **101**(5): 881-893.

[17] Zhang X, Jantama K, Moore JC, *et al*. Production of L-alanine by metabolically engineered *Escherichia coli* [J]. *Appl Microbiol Biotechnol*, 2007, **77**(2): 355-366.

[18] Shi A, Zhu X, Lu J, *et al*. Activating transhydrogenase and NAD kinase in combination for improving isobutanol production [J]. *Metab Eng*, 2013, **16**: 1-10.

[19] Santos CNS, Regitsky DD, Yoshikuni Y. Implementation of stable and complex biological systems through recombinase-assisted genome engineering [J]. *Nat Commun*, 2013, **4**: 2503.

[20] Engler C, Gruetzner R, Kandzia R, *et al*. Golden gate shuffling: a one-pot DNA shuffling method based on type IIs restriction enzymes [J]. *PLoS One*, 2009, **4**(5): e5553.

[21] Yokoyama A, Shizuri Y, Misawa N. Production of new carotenoids, astaxanthin glucosides, by *Escherichia coli* transfomants carring carotenoid biosynthetic gene [J]. *Tetrahedron Lett*, 1998, **39**: 3709-3712.

[22] Yin J, Wang H, Fu XZ, *et al*. Effects of chromosomal gene copy number and locations on polyhydroxyalkanoate synthesis by *Escherichia coli* and *Halomonas* sp. [J]. *Appl Microbiol Biotechnol*, 2015, **99**(13): 5523-5534.

[23] Bouvier F, Keller Y, d'Harlingue A, *et al*. Xanthophyll biosynthesis: molecular and functional characterization of carotenoid hydroxylases from pepper fruits (*Capsicum annuum* L.) [J]. *Biochim Biophys Acta*, 1998, **1391**(3): 320-328.

[24] Shanklin J, Whittle E, Fox BG. Eight histidine residues are catalytically essential in a membrane-associated iron enzyme, stearoyl-CoA desaturase, and are conserved in alkane hydroxylase and xylene monooxygenase [J]. *Biochemistry*, 1994, **33**(43): 12787-12794.

[25] Keasling JD. Synthetic biology for synthetic chemistry [J]. *ACS Chem biol*, 2008, **3**(1): 13.

[26] Wang HH, Isaacs FJ, Carr PA, *et al*. Programming cells by multiplex genome engineering and accelerated evolution [J]. *Nature*, 2009, **460**(7257): 894-898.

[27] Ng CY, Farasat I, Maranas CD, *et al*. Rational design of a synthetic Entner-Doudoroff pathway for improved and controllable NADPH regeneration [J]. *Metab Eng*, 2015, **29**: 86-96.

[28] Zhang C, Seow VY, Chen X, *et al*. Multidimensional heuristic process for high-yield production of astaxanthin and fragrance molecules in *Escherichia coli* [J]. *Nat Commun*, 2018, **9**(1): 1858.

[29] Park SY, Binkley RM, Kim WJ, *et al*. Metabolic engineering of *Escherichia coli* for high-level astaxanthin production with high productivity [J]. *Metab Eng*, 2018, **49**: 105-115.

[30] Lu Q, Liu JZ. Enhanced astaxanthin production in *Escherichia coli via* morphology and oxidative stress engineering [J]. *J Agric Food Chem*, 2019, **67**(42): 11703-11709.

Cite this article as: LI Di, LI Yang, XU Jiao-Yang, LI Qing-Yan, TANG Jin-Lei, JIA Shi-Ru, BI Chang-Hao, DAI Zhu-Bo, ZHU Xin-Na, ZHANG Xue-Li. Engineering CrtW and CrtZ for improving biosynthesis of astaxanthin in *Escherichia coli* [J]. *Chin J Nat Med*, 2020, **18**(9): 666-676.