In vitro and *In vivo* recombination of heterologous modules for improving biosynthesis of astaxanthin in yeast

Dan-Dan Qi
Tianjin University
Jin Jin
Tianjin University
Duo Liu
Tianjin University
Bin Jia (bin.jia@tju.edu.cn)
Tianjin University https://orcid.org/0000-0002-6245-4273
Ying-Jin Yuan
Tianjin University

Research

Keywords: In vitro recombination, In vivo recombination, Heterologous modules, Astaxanthin, Saccharomyces, Synthetic biology

Posted Date: March 26th, 2020

DOI: https://doi.org/10.21203/rs.2.21068/v2

Version of Record: A version of this preprint was published at Microbial Cell Factories on May 12th, 2020.
See the published version at https://doi.org/10.1186/s12934-020-01356-7.

Abstract

Background : Astaxanthin is a kind of tetraterpene and has strong antioxygenic property. Concerning the safety and economy issue the biosynthesis of astaxanthin has greater potential than chemical synthesis and extraction from natural producers. However, the production of astaxanthin in microorganisms is still limited by the poor efficiency of the heterologous pathway.

Results: To address the bottleneck of astaxanthin yield in microbes, we developed the in vitro and in vivo recombination methods to optimize the combination of heterologous modules of β-carotene ketolase (crtW) and hydroxylase (crtZ) from different species in engineered yeast strains. Finally, the astaxanthin yield of in vitro recombination and in vivo recombination were enhanced 2.11- to 8.51-fold and 3.05- to 9.71-fold compared to the parent strains, respectively. The highest astaxanthin producing yeast yQDD022 was obtained by the in vivo recombination with 6.05 mg/g DCW of the astaxanthin yield. Moreover, it is demonstrated that the astaxanthin producing yeast of the in vivo recombination has higher efficiency and stability than that of the in vitro recombination.

Conclusions: Recombination of heterologous modules by in vitro and in vivo provides a simple and efficient way to improve the astaxanthin yield in yeast. Both the in vitro and in vivo recombination methods enable high-throughput screening of heterologous pathways by combining crtW and crtZ from different species. And the heterologous pathway constructed by the in vivo recombination is more stable than that of the in vitro recombination. This study not only found the underlying optimal combination of crtZ and crtW , but also provided a reference to greatly enhance desired compounds accumulation by evolving heterologous pathways.

Background

Astaxanthin (3,3’-dihydroxy-β-carotene-4,4’-dione), a kind of carotenoid pigments with much higher antioxidant activity than other carotenoids and vitamin E[1] has tremendous commercial value in the aquaculture, food, cosmetic and pharmaceutical industries[2]. Traditional methods of synthesis of astaxanthin include chemical synthesis and extraction from natural producers, for example, the green algae or the red yeast[3]. However, the biosafety concern with chemical routes and the high cost of the extraction route limit the extensive application of astaxanthin[4]. Moreover, among all these sources, the algae-extracted astaxanthin and *Paracoccus carotinifaciens*-derived astaxanthin is the bioactive (3S,3’S)-stereoisomer[5, 6]. Alternatively, microbial chassis cells have been engineered for the fermentative production of astaxanthin utilizing metabolic engineering techniques[7]. Compared with these methods, microbial production of astaxanthin via metabolic engineering has become a promising alternative concerning the safety and economic issue. Modifying cells to improve the production of the desired metabolite is a broad aim for many areas of academic and industrial biotechnology and biosciences[8-11]. In recent years, the heterologous yield of astaxanthin has been successfully achieved in *Escherichia coli*[12-17], *Saccharomyces cerevisiae[5, 18-20],* Oleaginous Yeast *Yarrowia lipolytica*[21], and *Corynebacterium glutamicum*[22] by introducing the biosynthesis pathway of astaxanthin. However,

astaxanthin yield in microorganisms still not high enough for commercialization. The biosynthesis pathway of astaxanthin in yeast is a complex process, as shown in Fig. 1a. The glucose was converted into farnesyl pyrophosphate (FPP; C15) through the glycolytic pathway and mevalonate (MVA) pathway. And FPP was converted into β-carotene by the function of *crtE*, *crtYB* and *crtI*. The final reaction in the astaxanthin biosynthesis pathway, from β-carotene to astaxanthin, is a metabolic web, which requires two steps of β-carotene ketolase(*crtW*) and hydroxylation(*crtZ*) [23]. It has been revealed that many bacterial *crtZ* and *crtW* could utilize β-carotene as well as its hydroxylated or ketonic products as the substrate, leading to diverse carotenoid intermediate profiles which can greatly affect astaxanthin yield and ratio[24-26]. Choi *et al* have reported that a combination *crtW* from *Brevundimonas sp*. SD212 (*BSD212_crtW*) and *crtZ* from *Erwinia uredovora* (*Eu_crtZ*) generated more astaxanthin and fewer hydroxylated intermediates than the combination of *crtW* from *Paracoccus sp*. N81106 (PN81106_*crtW*) and Eu_*crtZ,* probably due to substrate preference for no ketonic carotenoids[27]. Meanwhile, it has also been reported that by integrating *crtW* from *Brevundimonas vesicularis.DC263* and *crtZ* from *Alcaligenes sp. strain PC-1* into a β-carotene strain, higher astaxanthin content was achieved in *S. cerevisiae* via ketolase first and hydroxylation subsequently[20]. The combination of *crtZ* and *crtW* from different species is still critical for higher astaxanthin accumulation.

In this study, we report the *in vitro* recombination and the *in vivo* recombination methods for yield improvement of astaxanthin in yeast. The *in vitro* recombination is using the *in vitro* Cre/LoxP recombination system to screen the heterologous modules of *crtW* and *crtZ* in CEN/ARS plasmids followed by yeast transformation. The *in vivo* recombination method is using yeast homologous recombination to directly integrate the heterologous modules of *crtW* and *crtZ* into the Ty1 retrotransposition sites of the yeast genome. The yeast yQDD022 with the highest astaxanthin yield in this study was obtained by *in vivo* recombination. Our study demonstrated that the transformation efficiency of *in vivo* recombination was higher than *in vitro* recombination. Moreover, *in vivo* recombination strain yQDD022 was more stable than yQDD008 obtained by *in vitro* recombination. These results indicate that the *in vivo* combination of heterologous pathway modules of *crtW* and *crtZ* has higher efficiency and stability than the *in vitro* recombination. The combination of heterologous pathway modules of *crtW* and *crtZ* is useful for fine-tuning of metabolic flux, which increased the yield of astaxanthin up to 9.71-fold compared to the ancestor strain, highlighting the use of our strategy.

Results And Discussion

Construction of astaxanthin producing strain

According to the previous studies in our lab, combinational of *crtZ* from *Agrobacterium aurantiacum* (*Aa crtZ*) and *crtW* from *Brevundimonas vesicularis DC263*(B. DC263 *crtW*) have a positive impact in astaxanthin pathway of yeast[19, 20]. Thus, *Aa crtZ* and *B. DC263 crtW* were chosen to construct the initial astaxanthin-producing strain. The high β-carotene producing strain yQDD000, with carotenoid biosynthesis pathway (*crtE*, *crtI*, and *crtYB* from the carotenoid-producing yeast *Xanthophyllomyces dendrorhous* with Leu2 marker) integrated into CAN sites of yeast BY4741, could provide enough

substrate for the synthetic of astaxanthin. The yQDD001 was obtained by the *Aa crtZ* and *B.DC 263 crtW* with the G418 marker integrated into the Ty1 retrotransposition of yQDD000 (Fig.1.b). The HPLC analysis of the β-carotene producing strain yQDD000 and engineered astaxanthin producing strain yQDD001 was shown in additional Fig.1. The β-carotene producing strain showed a onefold β-carotene peak at 20.2 min, while strain yQDD001 showed astaxanthin peak at 6.4 min along with other peaks for the identified intermediates, such as zeaxanthin (IV) at 7.5 min, canthaxanthin (III) at 10.5 min and lycopene (II) at 18.3 min. The profile of astaxanthin yield, astaxanthin titer, and cell density during fermentation in the flask with strain yQDD001 was shown in Fig. 1.c. Eventually, a yield of 0.623 mg/g DCW astaxanthin was obtained after 44 h cultivation. To further optimize the heterologous pathway, five different genes of *crtZ* and four different genes of *crtW* from diverse species were synthesized for fine-tuning of the metabolic flux of astaxanthin (Table.1). As shown in Fig.1.d, the promoter and terminator of all *crtZ* and *crtW* were FBA1p and ADH1t, TDH3p and TDH2t, respectively. And nine different PCR tags after the terminator of *crtZ* and *crtW* were used for PCR screening (additional Table.1).

In vitro recombination of heterologous modules *crtZ* and *crtW*

To further improve the yield of astaxanthin in yQDD001, *in vitro* recombination is used to rearrange the heterologous genes of astaxanthin in vitro[28]. Cre/LoxP is a widely used site-specific DNA recombination system derived from bacteriophage P1. The LoxP site is 34 bp in length, consisting of two 13 bp inverted repeats separated by an 8bp asymmetric spacer sequence. Cre recombinase catalyzes a site-specific recombination reaction between two LoxP sites and does not require accessory factors. Concerning the molecular mechanism of recombination, a single recombinase molecule binds to each palindromic half of LoxPsym sites, then the recombinase molecules form a tetramer, thus bringing two LoxP sites together[29]. Depending on the direction of LoxP, the Cre/LoxP system can be used to generate deletions, inversions, insertions (transpositions), or translocations. If LoxP sites encode a symmetric spacer region (loxPsym), rearrangements are orientation-independent between two loxPsym sites. The *in vitro* recombination method specifies the use of Cre recombinase for rearrangement of *crtZ* and *crtW* from different species-based optimization of constructs encoding multiple LoxPsym sites. As shown in Fig.2.a, *in vitro* recombination starts with a centromeric acceptor vector and a series of candidate genes (*crtZ* and *crtW*, represented as “donor fragments”). In addition, the acceptor vector encodes a hygromycin gene (represented as Hyg). The loxPsym sites in the acceptor vector were constructed into either side of the cassettes of Hyg and CEN/ARS. The acceptor vector was obtained by enzyme digestion of *EcoR*I and *Bam*HI before *in vitro* reaction. The cassettes of *crtZ*/ *crtW* and *Ura3*/ *His3* were located between the two LoxPsym sites in donor fragments. The donor fragments were generated by digestion of *Not*I and *Xba*I from a pUC19-based plasmid. The *crtZ*/ *crtW* donor fragments each encode a *Ura3*/ *His3* gene as a positively selectable marker. During this study, all the donor fragments of *crtZ* and *crtW* were mixed with acceptor vector as the reaction pool of *in vitro* recombination. Then the donor fragments were combined with the acceptor vector randomly under the action of Cre recombinase and to produce a pool of diverse plasmids (additional Fig.2). Then the plasmids pool was transformed into yQDD001 for generating the yeast library with different colors and sizes (Fig.2.b). The selection marker of *His*$^+$/ *Ura*$^+$ and Hyg$^+$ were

used to make sure at least one or two donor fragments during the *in vitro* recombination reaction. There were about 100 colonies on screened medium after yeast transformation. Therefore, we performed hundreds of mediums to screen for astaxanthin high producing strain to ensure the screening background was big enough. Darker red colonies were selected visually from the selected medium plates. Finally, there were nine colonies (yQDD002 - yQDD010) picking up for analysis with the darker red color. The astaxanthin yield of yQDD001-yQDD010 was assessed by HPLC. And the recombination plasmids were verified by PCRTag analysis and sequencing. The copy number of *crtZ* and *crtW* fragments in recombined plasmids was analyzed by qPCR (additional Fig.3). And the results indicated that there was only one copy number of *crtZ* or *crtW* in the yQDD002-yQDD010. As shown in Fig.2.c, astaxanthin yield of yQDD002-yQDD010 were increased to 1.24, 1.32, 2.63, 3.18, 2.20, 3.79, 5.50, 1.59 and 1.39 mg/g DCW, respectively. The *in vitro* recombination strains increased astaxanthin yield 1.98- to 8.51-fold compared with yQDD001. The results demonstrated that the additional heterologous genes of *crtZ* or *crtW* has an important effect on astaxanthin metabolic network in yeast.

In vivo recombination of heterologous modules

Theoretically, the high stability of the heterologous pathway can increase astaxanthin accumulation in yeast. The plasmids constructed by *in vitro* recombination may be lost during the fermentation process with YPD medium which may lead to the reduction of astaxanthin accumulation. There are multiple Ty1 retrotransposition sites in yeast genome[30], which can be used for the integration of multiple copies of heterologous modules by *in vivo* recombination methods. To integrate the *crtZ* and *crtW* into the genome, all the *crtZ* and *crtW* were flanked by about 500 base pair homologous sequences from the TyA (additional Fig.4). As shown in Fig.3.a, the integration cassettes of all *crtZ* and *crtW* were mixed and transferred into the yQDD001 (additional Fig.5). As shown in Fig.3.b, the *in vivo* recombination method can generate the yeast library with various color and size colonies. Finally, the darker red colonies (yQDD011-yQDD022) from *in vivo* recombination were selected for characterization. Then astaxanthin yield of selected strains was analyzed by HPLC. And the types of *crtZ* and *crtW* randomly inserted into the yeast genome were proved by PCRTag analysis. As shown in Fig.3.c, astaxanthin yield of yQDD011-yQDD022 were increased to 3.71, 3.96, 4.49, 2.81, 4.89, 5.26, 1.90, 2.00, 2.63, 3.67, 5.20, and 6.05 mg/g DCW, respectively. The *in vivo* recombination strains increased astaxanthin yield 3.05- to 9.71-fold compared with the yQDD001, respectively. And the genotypes are listed in Fig.3.c. As shown in the additional Fig.6, the copy number of *crtZ* and *crtW* of the yQDD011 to the yQDD022 were assayed by qPCR. According to the astaxanthin yield in selected strains the combination of $crtZ_1$ and $crtW_2$ have much more positive effects than other combinations on the astaxanthin pathway in the host strain. The increase of *crtZ* and *crtW* copy numbers have a positive impact on the astaxanthin pathway in the host strain. But more is not always better. There were two copies of $crtZ_4$ in yQDD017, while only one copy of *crtZ* or *crtW* in other strains. It is noted that the astaxanthin yield of yQDD017 was lower than that of yQDD020, which contained one copy of $crtZ_4$ and $crtW_4$. And the astaxanthin yield of yQDD012 was higher than yQDD017 and yQDD020, which have only one $crtZ_4$ and no *crtW* knock-in. These results indicated that the overexpression of *crtZ* or *crtW* may have a negative impact on astaxanthin yield. The

astaxanthin yield comparison between yQDD011 - yQDD015 - yQDD018 also indicated that. This may relate to the increased metabolic burden caused by the increase of *crtZ*/*crtW* copy number. Otherwise, the mutation in the genome caused by the integration of *crtZ* and *crtW* may induce astaxanthin metabolic changes. There are multiple Ty1 retrotransposition sites in the yeast genome. However, the number of strains with single copy integration were much more than the strains with multiple copies integration without any other environment pressure. To increase the integration efficiency, it is helpful to truncate the promotor of selection marker[31] or apply the Di-CRISPR[32]. Combination of those methods may further improve the efficiency of our *in vivo* recombination methods.

Efficiency analysis and stability analysis of heterologous modules

For the *in vitro* recombination method, different *crtZ* and *crtW* fragments with LoxPsym sites were assembled to new plasmids in tubes by the *in vitro* Cre/LoxP system. Then the new recombined plasmids were transformed into the host strain yQDD001. For the *in vivo* recombination method, the different *crtZ*/*crtW* fragments with the homologous arm of Ty1 sites were transformed into the host strain. Then the *crtZ* and *crtW* integrated into the Ty1 retrotransposition sites of yeast genome by homologous recombination. Both the *in vitro* and *in vivo* recombination method could screen *crtZ* and *crtW* from different species randomly and could be used for the accumulate of natural products in the microorganism. In this study we worked on the efficiency analysis and stability analysis of heterologous modules in these two methods.

The colony's number of *in vitro* screening and *in vivo* screening followed yeast transformation with three biological repeats were used to assess the efficiency of *in vitro* and *in vivo* recombination. All the experimental conditions remained consistent, including the concentration of DNA fragment of *crtZ* and *crtW*, the biomass of host strain yQDD001 and other operating environments. Photograph of *in vitro* and *in vivo* screening was attached. The colony's number was listed in additional Fig. 7. There were about 100 single colonies under the *in vitro* screening after yeast transformation while there were about 550 single colonies under the *in vivo* screening. Compared with *in vitro* recombination, the more colonies of *in vivo* screening indicating the high transformation efficiency of the *in vivo* recombination.

The strains yQDD008 and yQDD022, which performed the highest astaxanthin yield from *in vitro* and *in vivo* recombination of heterologous modules respectively, were selected for characterizing the growth and stability between these two methods. Ten-fold serial dilutions of yQDD008 and yQDD022 were spotted on the SD agar plates. yQDD001 was used as control. As shown in Fig.4.a, yQDD008 and yQDD022 display darker red than the parent strain yQDD001, indicating the higher astaxanthin production. The yQDD008 and the yQDD022 were serially subcultured in YPD for 6 days and then spotted on SD agar each day (additional Fig.8). The colonies with light color were observed in the screened medium of yQDD008, while not observed in yQDD022. The ratio of colonies with light color was listed in Fig.4.b. It is demonstrated that the yQDD008 and the yQDD022 cells did not display an obvious growth defect during 6 days of subculturing. This result proved the high growth and astaxanthin producing stability of yQDD022. However, compared with yQDD001, the increase of *crtZ* and *crtW* copy number have negative effects on

yeast growth (Fig.4.c). The profile of astaxanthin titer and astaxanthin yield during fermentation in the flask with strain yQDD001, yQDD008 and yQDD022 were shown in Fig.4.d and Fig.4.e. The astaxanthin yield reached maximal value at 52 h (yQDD008 with 5.30 mg/g DCW) and 44 h (yQDD022 with 6.10 mg/g DCW). And the profile of glucose consumption in two strains was similar (additional Fig.9). The low stability may have a negative impact on the accumulation of astaxanthin in yQDD008. These results indicated the stability of strain obtained by *in vivo* recombination is higher than that by the *in vitro* recombination. And the highest astaxanthin producing yeast strain was obtained by *in vivo* recombination of $crtZ_1$ and $crtW_2$. This method can increase the astaxanthin yield of yeast significantly in a high throughput way. The *in vivo* recombination method has a great potential to increase the efficiency and copy numbers of heterologous modules in the yeast genome.

Conclusion

In this study, the exogenous genes of *crtZ* and *crtW* from different species were combined randomly in astaxanthin strain by *in vitro* and *in vivo* recombination of heterologous modules. The result indicated that the increase of *crtZ* and *crtW* copy numbers have a positive impact on astaxanthin yield increase in yeast. Compared with *in vitro* recombination, the *in vivo* recombination method improves the integration efficiency and stability of heterologous modules *crtZ* and *crtW*. Finally, the highest yield of astaxanthin yeast strain yQDD022 (6.05 mg/g DCW) in this study was obtained by *in vivo* recombination with the combination of *crtW* and *crtZ* from *Alcaligenes sp.strain* and *Agrobacterium aurantiacum*, respectively.

Traditional genetic manipulation costs lots of time to construct the heterologous pathway for screening different sources of *crtZs* and *crtW*s. However, both the *in vivo* and *in vitro* recombination method could produce a variety combination of *crtZ* and *crtW* in a short time. The advantages of the *in vitro* recombination method are fast and easy to analyze the genotype of heterologous genes which are responsible for the phenotype variation, without limitations of the host strains. The advantages of the *in vivo* recombination method are more stable for constructing and screening the heterologous pathway at the same time, which can be used for industry strains metafiction directly. We believe both the *in vitro* and *in vivo* recombination research could teach us about interaction between heterologous enzymes in building microbial cell factories. This study has the potential to improve desired compounds in yeast with efficiency and stable performance.

Methods And Materials

Strains and media

Yeast strains used in this study were described in Table.2. The astaxanthin producing strain yQDD001 (*MATa*, *His3*Δ0, *Leu2*Δ1, *met15*Δ0, Ura*3*Δ0, *HO::tR(ccu)J*, *lys::NAT*) was subjected to improve the host's compatibility with *crtZ* and *crtW* from different sources by *in vitro* and *in vivo* recombination. Selective medium for rearrangement strains were SC-Leu-Ura+G418(synthetic complete medium lacking leucine and uracil with 20 g L−1 glucose and 100ug mL^{-1} G418), SC-Leu-His+G418 (synthetic complete medium

lacking leucine and histidine with 20 g L^{-1} glucose and 100ug mL^{-1} G418) and SC-Leu-Ura-His+G418 (synthetic complete medium lacking leucine, uracil, and histidine with 20 g L^{-1} glucose). All yeast solid media were added with 20 g L^{-1} agar. *Escherichia coli* DH5α purchased from BEIJING Biomed Co., Ltd was used for plasmid transformation. *Escherichia coli* were cultivated at 37℃ in LB medium (with 10 g L^{-1} tryptone, 5 g L^{-1} yeast extract, 10 g L^{-1} NaCl and 100 ug mL^{-1} ampicillin). LB solid medium was added with 15 g L^{-1} agar.

Yeast transformation and assembly

The protocol used for yeast transformation is the LiAc/SS carrier method. Yeast colonies were inoculated into 5 ml of SC-Leu+G418 and grown overnight at 30℃. Then 200ul yeast solution was inoculated into 5 ml of new SC-Leu+G418 cultures. 5-6h after, cultures were washed out twice with ddH_2O (double-distilled water) and resuspended in 0.1 M LiAc put on ice until needed. Yeast transformation system contained 620 ul of 50% polyethylene glycol (PEG) with molecular weight 3350, 40ul salmon sperm DNA (SSDNA,100 mg ml^{-1}), 90 ul of 1M LiAc solution. Then, 50ul *in vitro* or *in vivo* recombination system was mixed with 100 ul resuspended cells. And the mixed pool was added into LiAc/SS carrier DNA/PEG mixture and stir spirally. Samples were first incubated at 30℃ for 30 min. Then heat-shocked 18min at 42℃ water-bath. 90 ul DMSO was added followed by heat-shocked. Centrifuged and resuspended cells with 400ul 5 mM $CaCl_2$, plated on selective medium after 10min. After culturing for 72 h at 30℃incubator, darker red yeast colonies were selected on synthetic medium.

In vitro recombination

As shown in the additional Fig.2, the donor fragments and acceptors were cut from the plasmids by the enzyme. The 50 ul reaction system of *in vitro* recombination contained 1000 ng acceptor vector, the donor fragments pool of *crtZ* and *crtW* from different sources (1000 ng, respectively) and 2 ul of high concentration Cre recombinase (NEB, M0298M). Refer to previous studies of Zhu et al[28]the Cre recombinase reaction was set up as incubated at 37℃ for 4 h. The Cre enzyme was heat-inactivated for 10 min at 70 ℃. Then the reaction pools were transformed to hosts strains yQDD001 for genotype and phenotype testing. SC−Leu-Ura+G418, SC-Leu-His+G418 and SC-Leu-Ura-His+G418 medium were used to select for recombined constructs. To ensure the quality of color screening, all the yeast transformation were diluted 5-fold and repeated three times in this study. There are thousands of colonies generated for screening high producing strains.

In vivo recombination

As shown in additional Fig.5, The fragments of *crtZ* and *crtW* were cut from the plasmids by the *Not*I enzyme. Refer to the system of *in vitro* recombination, the 50 ul system of *crtZ* and *crtW* inserted randomly in genome contained fragments of *crtZ* and *crtW* from different sources (1000 ng, respectively). Then the fragments pool was transformed into the hosts yQDD001. SC−Leu-Ura+G418 or SC-Leu-His+G418 and SC-Leu-Ura-His+G418 medium are used to select for recombined constructs. To ensure the

quality of color screening, all the yeast transformation were diluted 5-fold and repeated three times in this study. There are thousands of colonies generated for screening high producing strains.

Shake flask cultivation for astaxanthin production

For shake flask culture, recombinant yeast colonies were inoculated into 5 mL SC-Leu+G418, SC-Leu-Ura+G418, SC-Leu-His+G418, or SC-Leu-Ura-His+G418 liquid medium respectively at 250 r.p.m., 30℃ for 24 h. Then the preculture was inoculated into the corresponding fresh SC defective medium (50 mL) with an initial OD600 of 0.2 for further 14 h cultivation (OD600 ≈ 5.0). Then seed culture was transferred into 50 mL fresh YPD-40 medium (40 g L^{-1} glucose, 20 g L^{-1} tryptone and 10 g L^{-1} yeast extract) at an initial OD600 of 0.1 grown for 84 h with the condition of 250 r.p.m.,30℃. Each sample was performed on technical triplicates.

Growth curve assay

The single colony was cultured to saturation in 5 mL YPD medium at 30 °C. The cultures were inoculated into a 250 mL shake flask containing 50 mL of YPD medium with initial OD_{600} at 0.1, and cultured at 30 °C, 220 rpm. The OD value was measured at appropriate intervals. Growth curves were plotted using Origin software.

Analysis of astaxanthin production by HPLC

1ml of the saturated culture was centrifuged for 2 min at 12000 g. Cells were washed with 1ml ddwater twice and resuspended in 1 ml of 3 M HCl. The resuspended cells were heated in the boiling water bath for 2 min and then cooled in ice-bath for 3 min, repeating three times. Then the samples were washed twice with ddwater to wash out HCl and harvested by centrifugation at 12000g for 2min. After removal of the supernatant, the cells were resuspended in 500ul acetone and vortexed for 20 min. Acetone extracts were centrifuged (13000g, 15min) and filtered with a 0.22 um filter for subsequent. Astaxanthin yield of samples was determined by HPLC (Waters 2695) equipped with HyPURTY C18 column (150mm ×4.6 mm, Thermo Scientific) and UV detection at 450 nm and 470 nm at 25 ℃. The following two buffers were used：A buffer, acetonitrile/Water (9:1 vol/vol) and B buffer, methanol/2-propanol (3:2 vol/vol). The flow rate of the mobile phase was 1 mL/min., and the solvent gradient was as follows: from 0 to 15 min for 100% to 10% of A buffer and 0% to 90% of B buffer, and then from 16 to 30 min for 10% of A buffer and 90% of B buffer；then from 31-35 min for 10% to 100% of A buffer and 90% to 0% of B buffer, at last from 35 to 55 min for 100% of A and 0% of B buffer. Each sample was performed on technical triplicates.

PCRtag analysis

15 ul PCR reaction system contained 7.5 ul 2×rapid Taq master mix (Vazyme), 0.3 uL forward primer (10 uM), 0.3 ul reverse primer (10 uM), 1 uL genome DNA, and 4.9 uL ddH2O. The procedure: 95 ℃/3 min, 30 cycles of (95℃/15s, 53℃/30 s,72 ℃/15 s), and 72 ℃/5 min. Agarose gel electrophoresis was used for PCR analysis. All primers used in this study were listed in additional Table.2.

Screening and verification of selected strains

For preliminary screening, darker red and big colonies were selected on selective media. Candidate strains were verified on SD media (synthetic complete medium with 20 g L−1 glucose) using a 10-fold serial dilution assay.

Extraction of the yeast genomic DNA

Strains were cultured overnight to saturation. Centrifuged at 12, 000 rpm to harvest cells. 200 µl breaking buffer (500 $mM.L^{-1}$ NaCl, 200 $mM.L^{-1}$ Tris-HCl, 100 $mM.L^{-1}$ EDTA, 1% SDS), 200 µl silica sand and 200 µl phenol/chloroform/isoamyl alcohol (25:24:1) were added to cells tube. Disrupted cells by the vortex mixer for 20 minutes. Then added 1 mL cold ethanol to the supernatant, mixed and centrifuged at 4 ℃ for 10 minutes. The precipitate was washed with 75% cold ethanol and dried at 37 ℃. 200 µl ddH_2O was added to dissolve the yeast genome DNA. Stored the genome DNA at -20 ℃.

Quantitative real-time PCR (qPCR) analysis

qPCR was applied to quantify copy numbers of the gene in engineered strains. The template in the qPCR analysis was yeast genomic DNA. Reference primers were selected from gene ALG9. and the target primers were selected from the cassettes of *crtZ* and *crtW* respectively. Strain with a single copy of *crtZ* and *crtW* was used as the reference strain. The copy numbers were determined by comparing the Ct values of *crtZ* or *crtW* and the reference gene ALG9 using the $2^{-\Delta\Delta C\ t}$ method. The relative ratio of *crtZ* or *crtW* was calculated as $2^{-\Delta t(crtZ)}$ or $2^{-\Delta Ct(crtW)}$. Unique Aptamer TM qPCR SYBR Green Master Mix (Beijing Novogene Bioinformatics Technology Co., Ltd) was used for the qPCR reaction, and the equipment was Quantagene q225 (Novogene). The reaction procedure was performed as follows: precycling,95℃/300 s, 40 cycles of (95℃/10 s, 57℃/20 s, 72℃/20 s), melt curve, which started from 60℃to 95℃.

Abbreviations

crtW: β-carotene ketolase; *crtZ*: β-carotene hydroxylation; HPLC: High-pressure liquid chromatography.

Declarations

Author contributions

DDQ, JJ, BJ and YJY designed the experiments. DDQ and JJ performed the experiments. DDQ and BJ wrote the manuscript. BJ, DL and YJY edited the manuscript. All authors read and approved the final manuscript.

Acknowledgments

The authors are grateful for the financial support from the National Natural Science Foundation of China 31800719, 21750001, 21621004, 21706186.

Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

All data generated or analyzed during this study are included in this article.

Consent for publication

Not applicable

Ethics approval and consent to participate

Not applicable.

Funding

This work was funded by the National Natural Science Foundation of China□21750001, 21621004, 31800719, 21706186□

References

1. Igielska-Kalwat J, Gościańska J, Nowak I: **Carotenoids as natural antioxidants.** *Postępy Higieny i Medycyny Doświadczalnej* 2015, **69:**418-428.
2. Ambati RR, Phang SM, Ravi S, Aswathanarayana RG: **Astaxanthin: sources, extraction, stability, biological activities and its commercial applications–a review.** *Mar Drugs* 2014, **12:**128-152.
3. Kang CD, Lee JS, Park TH, Sim SJ: **Comparison of heterotrophic and photoautotrophic induction on astaxanthin production by Haematococcus pluvialis.** *Appl Microbiol Biotechnol* 2005, **68:**237-241.
4. Higuera-Ciapara I, Felix-Valenzuela L, Goycoolea FM: **Astaxanthin: a review of its chemistry and applications.** *Crit Rev Food Sci Nutr* 2006, **46:**185-196.
5. Zhou P, Ye L, Xie W, Lv X, Yu H: **Highly efficient biosynthesis of astaxanthin in Saccharomyces cerevisiae by integration and tuning of algal crtZ and bkt.** *Appl Microbiol Biotechnol* 2015, **99:**8419-8428.
6. Hayashi M, Ishibashi T, Maoka T: **Effect of astaxanthin-rich extract derived from Paracoccus carotinifaciens on cognitive function in middle-aged and older individuals.** *J Clin Biochem Nutr* 2018, **62:**195-205.
7. Ye VM, Bhatia SK: **Pathway engineering strategies for production of beneficial carotenoids in microbial hosts.** *Biotechnol Lett* 2012, **34:**1405-1414.
8. Li Z, Guo X, Feng X, Li C: **An environment friendly and efficient process for xylitol bioconversion from enzymatic corncob hydrolysate by adapted Candida tropicalis.** *Chemical Engineering Journal* 2015, **263:**249-256.

9. Moon TS, Dueber JE, Shiue E, Prather KLJ: **Use of modular, synthetic scaffolds for improved production of glucaric acid in engineered E. coli.** *Metabolic Engineering* 2010, **12:**298-305.
10. Yazdani SS, Gonzalez R: **Anaerobic fermentation of glycerol: a path to economic viability for the biofuels industry.** *Curr Opin Biotechnol* 2007, **18:**213-219.
11. Zhang W, Li Y, Tang Y: **Engineered biosynthesis of bacterial aromatic polyketides in Escherichia coli.** *Proc Natl Acad Sci U S A* 2008, **105:**20683-20688.
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.** *Biotechnol Bioeng* 2009, **103:**944-955.
13. Lemuth K, Steuer K, Albermann C: **Engineering of a plasmid-free Escherichia coli strain for improved in vivo biosynthesis of astaxanthin.** *Microb Cell Fact* 2011, **10:**29.
14. Zelcbuch L, Antonovsky N, Bar-Even A, Levin-Karp A, Barenholz U, Dayagi M, Liebermeister W, Flamholz A, Noor E, Amram S, et al: **Spanning high-dimensional expression space using ribosome-binding site combinatorics.** *Nucleic Acids Research* 2013, **41:**e98-e98.
15. Lu Q, Bu YF, Liu JZ: **Metabolic Engineering of Escherichia coli for Producing Astaxanthin as the Predominant Carotenoid.** *Mar Drugs* 2017, **15**.
16. Ma T, Zhou Y, Li X, Zhu F, Cheng Y, Liu Y, Deng Z, Liu T: **Genome mining of astaxanthin biosynthetic genes from Sphingomonas sp. ATCC 55669 for heterologous overproduction in Escherichia coli.** *Biotechnol J* 2016, **11:**228-237.
17. Zhang C, Seow VY, Chen X, Too HP: **Multidimensional heuristic process for high-yield production of astaxanthin and fragrance molecules in Escherichia coli.** *Nat Commun* 2018, **9:**1858.
18. Zhou P, Xie W, Li A, Wang F, Yao Z, Bian Q, Zhu Y, Yu H, Ye L: **Alleviation of metabolic bottleneck by combinatorial engineering enhanced astaxanthin synthesis in Saccharomyces cerevisiae.** *Enzyme Microb Technol* 2017, **100:**28-36.
19. Jin J, Wang Y, Yao M, Gu X, Li B, Liu H, Ding M, Xiao W, Yuan Y: **Astaxanthin overproduction in yeast by strain engineering and new gene target uncovering.** *Biotechnology for biofuels* 2018, **11:**230.
20. Wang R, Gu X, Yao M, Pan C, Liu H, Xiao W, Wang Y, Yuan Y: **Engineering of β-carotene hydroxylase and ketolase for astaxanthin overproduction in Saccharomyces cerevisiae.** *Frontiers of Chemical Science and Engineering* 2017, **11:**89-99.
21. Tramontin LRR, Kildegaard KR, Sudarsan S, Borodina I: **Enhancement of Astaxanthin Biosynthesis in Oleaginous Yeast Yarrowia lipolytica via Microalgal Pathway.** *Microorganisms* 2019, **7**.
22. Henke NA, Heider SA, Peters-Wendisch P, Wendisch VF: **Production of the Marine Carotenoid Astaxanthin by Metabolically Engineered Corynebacterium glutamicum.** *Mar Drugs* 2016, **14**.
23. Martin JF, Gudina E, Barredo JL: **Conversion of beta-carotene into astaxanthin: Two separate enzymes or a bifunctional hydroxylase-ketolase protein?** *Microb Cell Fact* 2008, **7:**3.
24. Chang J-J, Thia C, Lin H-Y, Liu H-L, Ho F-J, Wu J-T, Shih M-C, Li W-H, Huang C-C: **Integrating an algal β-carotene hydroxylase gene into a designed carotenoid-biosynthesis pathway increases carotenoid**

production in yeast. *Bioresource technology* 2015, **184:**2-8.

25. Sarria S, Wong B, Garcia Martin H, Keasling JD, Peralta-Yahya P: **Microbial synthesis of pinene.** *ACS Synth Biol* 2014, **3:**466-475.
26. Chen Y, Xiao W, Wang Y, Liu H, Li X, Yuan Y: **Lycopene overproduction in Saccharomyces cerevisiae through combining pathway engineering with host engineering.** *Microb Cell Fact* 2016, **15:**113.
27. Choi SK, Nishida Y, Matsuda S, Adachi K, Kasai H, Peng X, Komemushi S, Miki W, Misawa N: **Characterization of beta-carotene ketolases, CrtW, from marine bacteria by complementation analysis in Escherichia coli.** *Mar Biotechnol (NY)* 2005, **7:**515-522.
28. Wu Y, Zhu R-Y, Mitchell LA, Ma L, Liu R, Zhao M, Jia B, Xu H, Li Y-X, Yang Z-M: **In vitro DNA SCRaMbLE.** *Nature communications* 2018, **9:**1935.
29. Nagy A: **Cre recombinase: the universal reagent for genome tailoring.** *Genesis* 2000, **26:**99-109.
30. Han JY, Song JM, Seo SH, Wang C, Lee SG, Lee H, Kim SW, Choi ES: **Ty1-fused protein-body formation for spatial organization of metabolic pathways in Saccharomyces cerevisiae.** *Biotechnol Bioeng* 2018, **115:**694-704.
31. Lian J, Jin R, Zhao H: **Construction of plasmids with tunable copy numbers in Saccharomyces cerevisiae and their applications in pathway optimization and multiplex genome integration.** *Biotechnol Bioeng* 2016, **113:**2462-2473.
32. Shi S, Liang Y, Zhang MM, Ang EL, Zhao H: **A highly efficient single-step, markerless strategy for multi-copy chromosomal integration of large biochemical pathways in Saccharomyces cerevisiae.** *Metab Eng* 2016, **33:**19-27.

Tables

Table 1 heterologous modules *crtZ* and *crtW* used in this study

Name of *crtZ/crtW*	Microbial source	Called in this study
Aa crtZ	*Agrobacterium aurantiacum*	$crtZ_1$
B. DC263 crtZ	*Brevundimonas vesicularis DC263*	$crtZ_2$
B.SD212 crtZ	*Brevundimonas sp. SD212*	$crtZ_3$
HpChyb crtZ	*Haematococcus pluvialis*	$crtZ_4$
SsP2 crtZ	*Sulfolobus solfataricus P2*	$crtZ_5$
Aa crtW	*Agrobacterium aurantiacum*	$crtW_1$
Asp crtW	*Alcaligenes sp.strain*	$crtW_2$
B. DC263 crtW	*Brevundimonas vesicularis DC263*	$crtW_3$
GvccrtW	*Gloeobacter violaceus PCC 7421*	$crtW_4$

Table 2 Strains used in this study

Strain and plasmid	Description	Sources
yQDD000	By4741 with a carotenoid pathway (Leu2 Marker) into the YEL063C/CAN1 locus in chromosome V	This lab
yQDD001	yQDD000 with *Aa crtZ* and *B.DC 263 crtW* (with the G418 marker) were integrated into the retrotransposition of Ty1	This study
yQDD002	*In vitro* recombined strain from yQDD001 with $crtZ_2$	This study
yQDD003	*In vitro* recombined strain from yQDD001 with $crtZ_5$	This study
yQDD004	*In vitro* recombined strain from yQDD001 with $crtZ_3$	This study
yQDD005	*In vitro* recombined strain from yQDD001 with $crtZ_1$	This study
yQDD006	*In vitro* recombined strain from yQDD001 with $crtW_2$	This study
yQDD007	*In vitro* recombined strain from yQDD001 with $crtW_4$	This study
yQDD008	*In vitro* recombined strain from yQDD001 with $crtW_3$	This study
yQDD009	*In vitro* recombined strain from yQDD001 with $crtZ_5$ and $crtW_1$	This study
yQDD010	*In vitro* recombined strain from yQDD001 with $crtZ_3$ and crtW_4	This study
yQDD011	*In vivo* recombined strain from yQDD001 with $crtZ_2$	This study
yQDD012	*In vivo* recombined strain from yQDD001 with $crtZ_4$	This study
yQDD013	*In vivo* recombined strain from yQDD001 with $crtZ_5$	This study
yQDD014	*In vivo* recombined strain from yQDD001 with $crtW_4$	This study
yQDD015	*In vivo* recombined strain from yQDD001 with $crtW_1$	This study
yQDD016	*In vivo* recombined strain from yQDD001 with $crtW_3$	This study
yQDD017	*In vivo* recombined strain from yQDD001 with $crtZ_4$ and $crtW_4$	This study
yQDD018	*In vivo* recombined strain from yQDD001 with $crtZ_2$ and $crtW_1$	This study
yQDD019	*In vivo* recombined strain from yQDD001 with $crtZ_1$ and $crtW_3$	This study
yQDD020	*In vivo* recombined strain from yQDD001 with $crtZ_4$ and $crtW_4$	This study
yQDD021	*In vivo* recombined strain from yQDD001 with $crtZ_5$ and $crtW_1$	This study
yQDD022	*In vivo* recombined strain from yQDD001 with $crtZ_1$ and crtW_2	This study

Figures

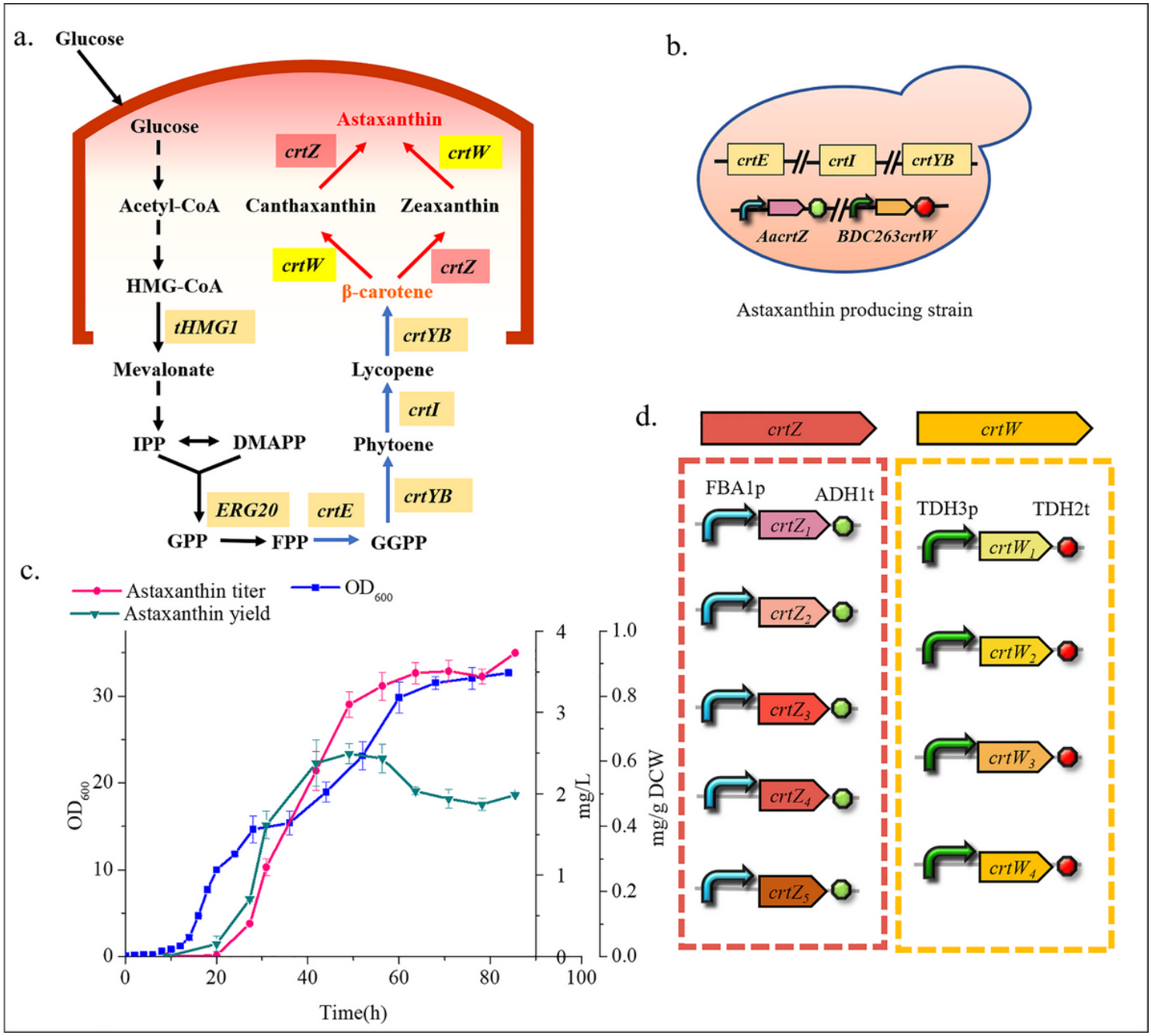
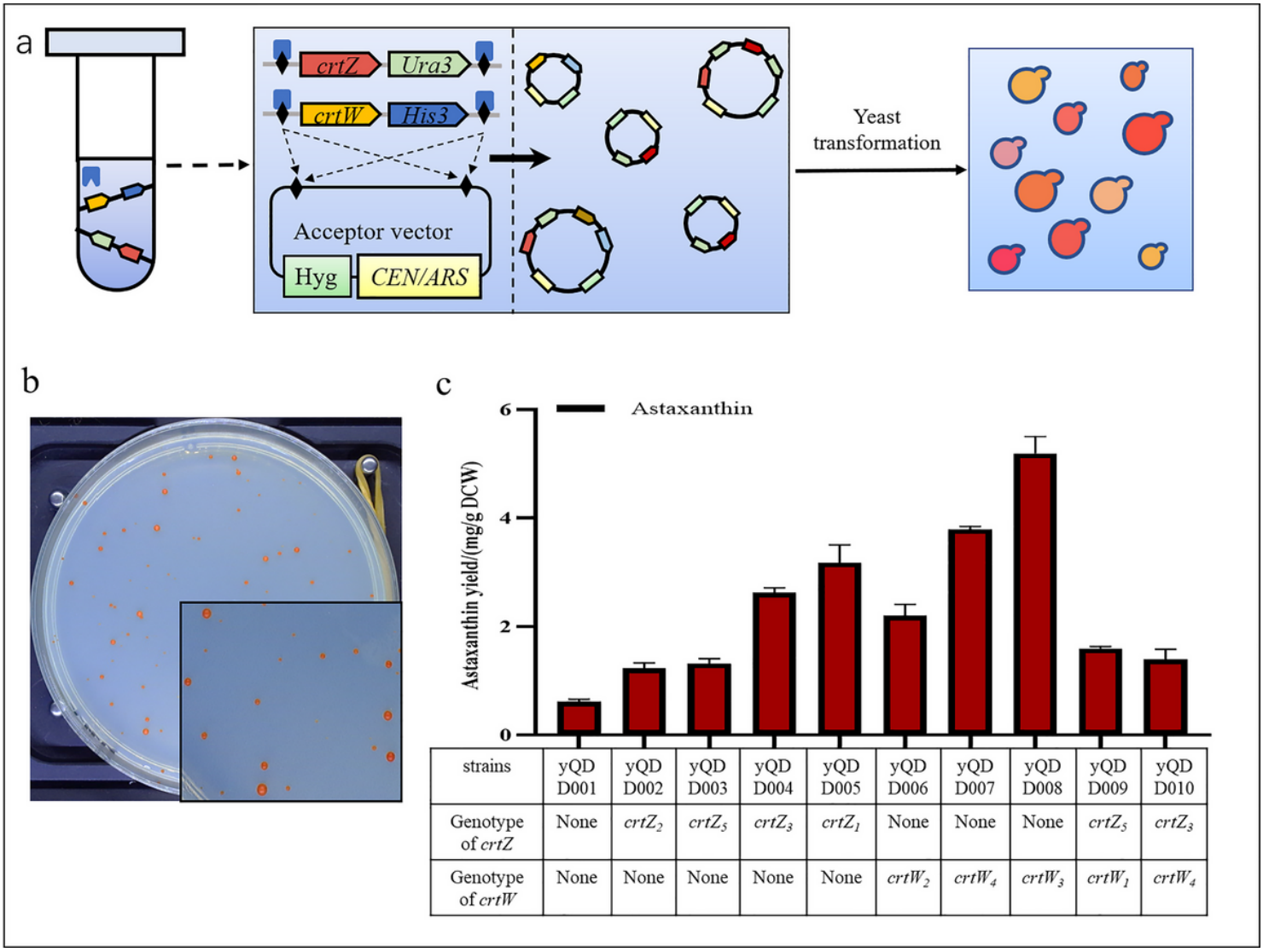


Figure 1

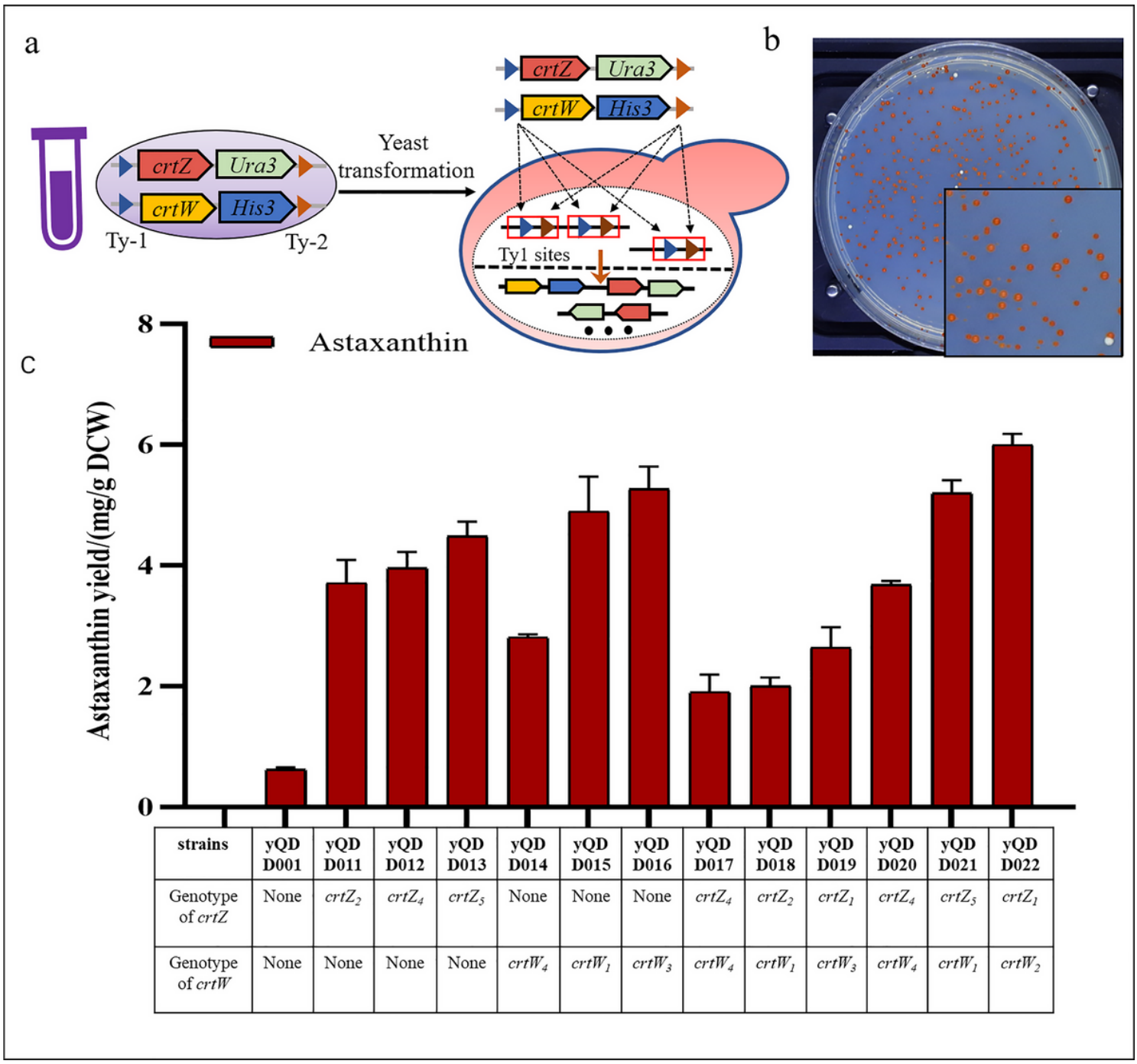
a Biosynthesis pathway of astaxanthin in yeast. The pathway from β-carotene to astaxanthin was boxed through the red line and engineered in this study. b Astaxanthin producing strain. The modular grene cassettes of crtE, crtI, crtYB were inserted into the CAN sites of BY4741, while the Aa crtZ and B. DC263 crtW were integrated into the retrotransposition of Ty1. c Profile of astaxanthin yield (green), astaxanthin titer (pink), and cell density (blue) during fermentation of astaxanthin producing strain yQDD001. d Sketch map of crtZ and crtW expression cassettes. Expression modules of all crtZ were assembled with FBA1 promoter and ADH1 terminator. And the expression modules of all crtW were assembled with TDH3 promoter and TDH2 terminator.



strains	yQD D001	yQD D002	yQD D003	yQD D004	yQD D005	yQD D006	yQD D007	yQD D008	yQD D009	yQD D010
Genotype of *crtZ*	None	$crtZ_2$	$crtZ_5$	$crtZ_3$	$crtZ_1$	None	None	None	$crtZ_5$	$crtZ_3$
Genotype of *crtW*	None	None	None	None	None	$crtW_2$	$crtW_4$	$crtW_3$	$crtW_1$	$crtW_4$

Figure 2

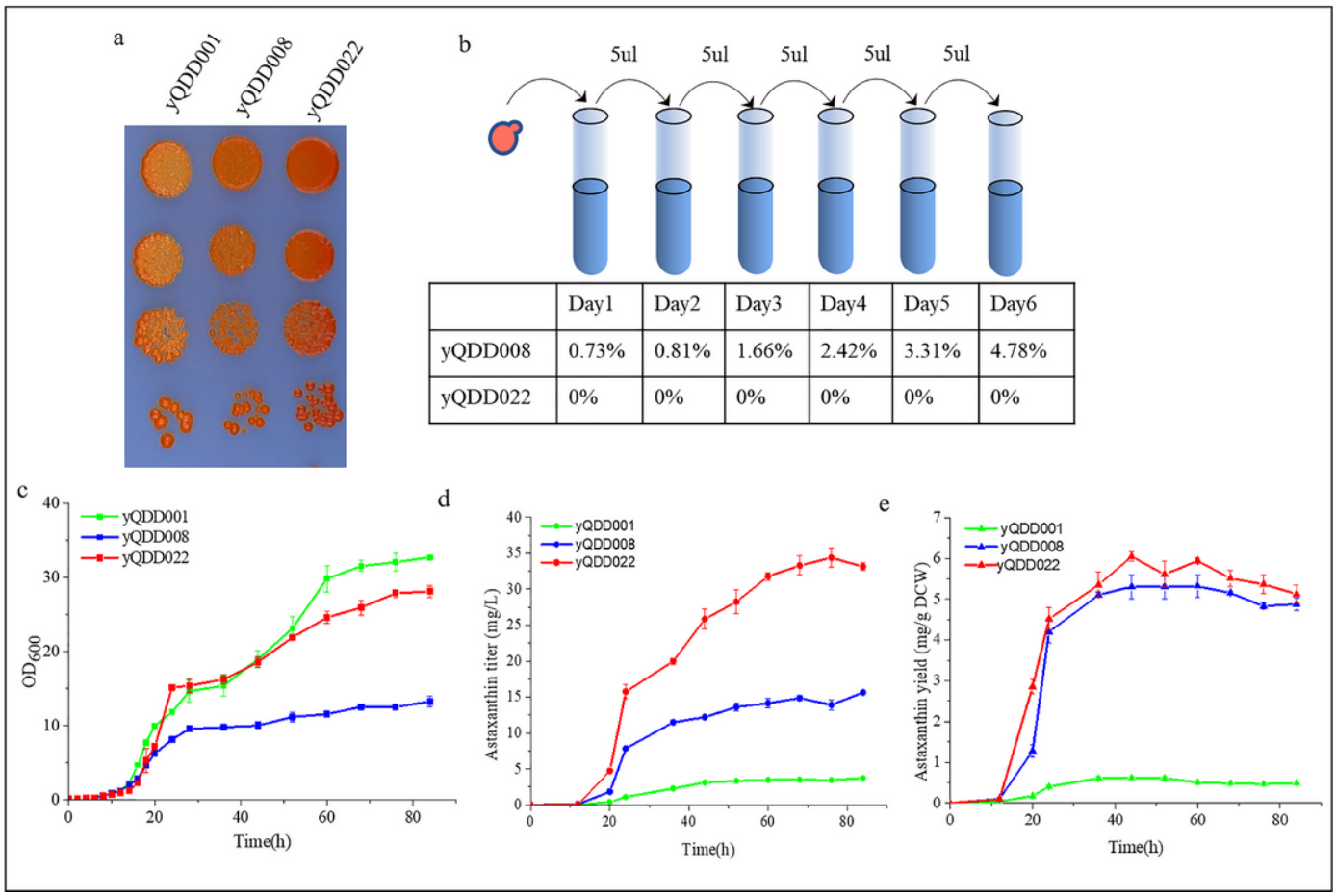
In vitro recombined of heterologous modules crtZ and crtW a Workflow of the in vitro recombination to evolve the heterologous pathway of astaxanthin in yeast. The acceptor vector and the pool of donor constructs (crtZ and crtW) are mixed with Cre recombinase in vitro. The donor fragments will be randomly inserted into loxPsym sites of the acceptor vector assemble into various new plasmids. b The reaction products were transformed into yQDD001 produced the yeast library with different colors and sizes. c Astaxanthin yield measurement in shake flask of in vitro recombined strains by HPLC. And the gene type of yQDD002 - yQDD010 was proved by PCRTag analysis. The error bars represent standard deviations calculated from duplicate experiments. “Astaxanthin yield” was determined as “the astaxanthin content in single-cell” with the unit as mg/g DCW.



strains	yQD D001	yQD D011	yQD D012	yQD D013	yQD D014	yQD D015	yQD D016	yQD D017	yQD D018	yQD D019	yQD D020	yQD D021	yQD D022
Genotype of *crtZ*	None	$crtZ_2$	$crtZ_4$	$crtZ_5$	None	None	None	$crtZ_4$	$crtZ_2$	$crtZ_1$	$crtZ_4$	$crtZ_5$	$crtZ_1$
Genotype of *crtW*	None	None	None	None	$crtW_4$	$crtW_1$	$crtW_3$	$crtW_4$	$crtW_1$	$crtW_3$	$crtW_4$	$crtW_1$	$crtW_2$

Figure 3

In vivo recombination of heterologous modules. a Each fragments crtZ/crtW carry a Ura3/His3 marker and two homologous arms with Ty1. All crtZ and crtW fragments were mixed up to transform into yQDD001. b The fragments inserted into the Ty1 sites of yeast genome randomly to produce the yeast library with various combinations of crtZ and crtW. c Yeast colonies of in vivo recombination method screened in the selected medium. The yeast library contained various yeast with different colors and sizes. c Astaxanthin yield measurement in shake flask of in vivo recombination evolved strains by HPLC. The genotype of yQDD011 - yQDD022were proved by PCRTag analysis.



	Day1	Day2	Day3	Day4	Day5	Day6
yQDD008	0.73%	0.81%	1.66%	2.42%	3.31%	4.78%
yQDD022	0%	0%	0%	0%	0%	0%

Figure 4

The comparison between in vitro and in vivo evolution of heterologous modular pathway of astaxanthin. a. Phenotype verification of yQDD008 and yQDD022. The parent strain yQDD001 was used as control strains. The photograph was attached to illustrate the visual color of the related strains. b Stability assay of yQDD008 and yQDD022. Yeast Cultures in YPD after 6 days were plated on SD agar and the number of unstable strains was counted. The ratio of unstable strains in every 12 generations was recorded and listed. c Growth curve of yQDD001, yQDD008 and yQDD022. d Profile of astaxanthin titer during fermentation with strain yQDD001, yQDD008 and yQDD022. e Profile of astaxanthin yield during fermentation with strain yQDD001, yQDD008 and yQDD022.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- supplementaryMaterial.docx