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

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Abstract

Background: Astaxanthin is a kind of tetraterpene and has strong antioxygenic property. The biosynthesis of astaxanthin in engineered microbial chassis has greater potential than its chemical synthesis and extraction from natural producers in an environmental-friendly way. However, the cost-offsetting production of astaxanthin in engineered microbes is still constrained by the poor efficiency of astaxanthin synthesis pathway as a heterologous pathway.

Results: To address the bottleneck of limited production of astaxanthin in microbes, we developed *in vitro* and *in vivo* recombination methods respectively in engineered yeast chassis to optimize the combination of heterologousβ-carotene ketolase (*crtW*) and hydroxylase (*crtZ*) modules that were selected from different species. As a result, the *in vitro* and *in vivo* recombination methods enhanced the astaxanthin yield respectively to 2.11~8.51 folds and 3.0~9.71 folds compared to the initial astaxanthin pathway, according to the different combination of particular genes. The highest astaxanthin producing strain yQDD022 was constructed by *in vivo* method and produced 6.05 mg/g DCW of astaxanthin. Moreover, it was proved that the *in vivo* recombination method showed higher DNA-assembling efficiency than the *in vitro* method and contributed to higher stability to the engineered yeast strains.

Conclusions: The *in vitro* and *in vivo* recombination methods of heterologous modules provide simple and efficient ways to improve the astaxanthin yield in yeast. Both the two methods enable high-throughput screening of heterologous pathways through recombination of certain *crtW* and *crtZ* derived from different species. This study not only exploited the underlying optimal combination of *crtZ* and *crtW* for astaxanthin synthesis, but also provided a general approach to evolve a heterologous pathway for the enhanced accumulation of desired biochemical products.

Background

Astaxanthin (3,3'-dihydroxy-β-carotene-4,4'-dione), a kind of carotenoid-derivative pigment with much higher antioxidant activity than other carotenoids and vitamin E [1], is commercially valuable in the aquaculture, food, cosmetic and pharmaceutical industries [2]. Traditional methods of astaxanthin production include chemical synthesis and extraction from natural producers, for example, the green algae or the red yeast [3]. However, the biosafety concerns with chemical routes and the high cost of the extraction route limits the extensive application of astaxanthin [4].Thus far, it has been found that the astaxanthin extracted from algaes and *Paracoccus carotinifaciens* are the bioactive (3S,3'S)-stereoisomer [5, 6]. Alternatively, microbial chassis cells have been engineered for the fermentative production of astaxanthin by utilizing metabolic engineering techniques [7]. This way has become a promising alternative to produce terpene-derivatives that meet the safety and economic concerns. Modifying microbial cells to improve the production of the desired endogenous or exogenous metabolites is a broad aim at many areas of academic, industrial biotechnology and biosciences [8-11]. In recent years, the heterologous biosynthesis of astaxanthin has been successfully achieved in *Escherichia coli* [12-17], *Saccharomyces cerevisiae[5, 18-20],Yarrowia lipolytic* [21], and *Corynebacterium glutamicum* [22]

by introducing particular biosynthesis pathways.. However, the astaxanthin yields in these engineered microbes were still not high enough for cost-worthy commercialization. The total biosynthesis pathway of astaxanthin in yeast is complex and full of branches, as shown in Fig. 1a. Glucose is converted into farnesyl pyrophosphate (FPP; C15) through the glycolytic pathway and mevalonate (MVA) pathway, and FPP is converted into β-carotene by the reaction of *crtE*, *crtYB* and *crtI*. The final synthesis of astaxanthin from β-carotene is a metabolic web containing several branches, according to the different participation steps and orders of the β-carotene ketolase(*crtW*) and hydroxylation(*crtZ*) [23]. It has been revealed that many bacterial *crtZ*s and *crtW*s 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* has 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 none-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 producing strain, higher astaxanthin yield was achieved in *S. cerevisiae* via ketylation first and hydroxylation subsequently [20]. The combination of *crtZ* and *crtW* from different species is still critical for the enhanced astaxanthin accumulation.

In this study, we report the *in vitro* recombination and the *in vivo* recombination methods for improvement of astaxanthin yield in yeast. The *in vitro* method 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* method is using yeast homologous recombination system (HR) to directly integrate the heterologous modules of *crtW* and *crtZ* into the Ty1 retrotransposon sites in the yeast genome. The yeast 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 was more stable than the *in vitro* recombination strain. These results indicate that the *in vivo* combination of heterologous pathway modules 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 significantly 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, combination of *crtZ* from *Agrobacterium aurantiacum* (*Aa crtZ*) and *crtW* from *Brevundimonas vesicularis DC263*(B. DC263 *crtW*) has a positive impact on the astaxanthin pathway in yeast [19, 20]. Thus, *AacrtZ* and *B. DC263crtW* 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 the CAN sites of yeast strain BY4741, could provide amounts of substrate for the synthesis of astaxanthin. The yQDD001 was constructed through the co-integration of the *Aa crtZ* and *B.DC 263crtW* accompanied with the G418 marker integrated into the Ty1 retrotransposon of yQDD000 (Fig.1.b). The HPLC detection of the carotenoid products extracted from the strain yQDD000 and yQDD001 was shown in additional Fig.1. The β-carotene producing strain showed an onefold β-carotene peak at 20.2 min, while strain yQDD001 showed astaxanthin peak at 6.4 min along with other peaks of 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 the fermentation of the strain yQDD001 in the 50mL flask 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, we selected five alternative *crtZ* from five other species and four *crtW* from four species to test whether their participation into the pathway would improve astaxanthin production (Table.1). We located 1 pair of the promoter FBA1p and terminator ADH1t for the expression of *crtZ* and another pair of TDH3p and TDH2t for the expression of *crtW* (Fig.1.d). We designed nine PCR tags for screening each gene of crtZ or crtW by inserting the tags downstream of each gene's terminator (additional Table.1).

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

To further improve the yield of astaxanthin in yQDD001, *in vitro* recombination was used to rearrange the heterologous genes of astaxanthin in vitro [28]. Cre/LoxP was a widely used site-specific DNA recombination system derived from bacteriophage P1. The LoxP site was 34 bp in length, consisting of two 13 bp inverted repeats separated by an 8bp asymmetric spacer sequence. Cre recombinase catalyzed a site-specific recombination reaction to two LoxP sites and did not require accessory factors. Concerning the molecular mechanism of recombination, a single recombinase molecule binded to each palindromic half of LoxPSym sites, then the recombinase molecules formed a tetramer, thus bringing two LoxP sites together [29]. Depending on the direction of LoxP, the Cre/LoxP system could be used to generate deletions, inversions, insertions (transpositions), or translocations. If LoxP sites encoded a symmetric spacer region (LoxPSym), rearrangements were orientation-independent between two LoxPSym sites. The *in vitro* recombination method specified the use of Cre recombinase for rearrangement of *crtZ* and *crtW* expression constructs each containing a pair of LoxPSym sites besides the gene's transcriptional unit along with an accompanied Ura3/His3 marker (Fig.2.a). The *in vitro* recombination started with a centromeric acceptor vector and a series of candidate genes (*crtZ* and *crtW*, represented as "donor fragments"). In addition, the acceptor vector encoded a hygromycin resistance gene (represented as Hyg^R). Tow LoxPSym sites were located in the outsides of the cassettes of Hyg^R and CEN/ARS in the acceptor vector. The acceptor vector was digested by *EcoR*I and *Bam*HI and the linearized fragment was purified for the preparation of *in vitro* recombination reaction. The donor fragments were generated by digestion of *Not*I and *Xba*I from their pUC19-based plasmids. During this study, all the donor fragments of *crtZ* and *crtW* were mixed with acceptor vector as the reaction pool of *in vitro* recombination. The donor fragments were recombined with the acceptor vector randomly under the action of Cre recombinase and produced a pool with diverse plasmids (additional Fig.2). Then the plasmids pool was transformed into

yQDD001 for generating the yeast colony library which presented different colors and sizes (Fig.2.b). The selection marker for *His*$^{+}$/ *Ura*$^{+}$ and Hyg^{R+} were used to make sure at least one or two donor fragments were recombined into the acceptor vector during the *in vitro* recombination reaction. There were about 100 colonies appeared on the plates after yeast transformation. We picked almost every colony to screen for the potential highest astaxanthin producing strain. According to the visual judgement, darker red colonies were selected from the whole colonies. Finally, there were nine darker-red colonies (yQDD002 - yQDD010) were optimally selected and the astaxanthin tier in these strains were detected by HPLC. The recombination plasmids were verified by PCR-Tag analysis and sequencing. The copy number of *crtZ* and *crtW* fragments in recombined plasmids was analyzed by qPCR (additional Fig.3). 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 yields 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 the astaxanthin yield by 1.98- to 8.51-fold compared with yQDD001. The results demonstrated that the additional heterologous genes of *crtZ* or *crtW* had positive effect on the astaxanthin synthesis in yeast.

In vivo recombination of heterologous modules

Guided by the industrial experience of microbial fermentation, it was supposed that the high stability of the recombined heterologous pathway could increase astaxanthin accumulation in yeast. The plasmids constructed by *in vitro* recombination might be lost during the fermentation process with YPD medium which might lead to the reduction of astaxanthin accumulation. There were multiple Ty1 retrotransposon sites in yeast genome [30], which could 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 selected 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 generated the yeast library with various color and size colonies. Finally, the darker red colonies (yQDD011-yQDD022) from *in vivo* recombination were selected for characterization and their production of astaxanthin was detected by HPLC. The particular specie-derived genes of *crtZ* or *crtW* randomly inserted into the yeast genome were identified by the designed PCRTag analysis (additional Table.1). As shown in Fig.3.c, the astaxanthin yields in strain 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 of *crtZ*/*crtW* were 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 $crtW_2$ had much more positive effects than other combinations on the astaxanthin pathway in the host strain. The increase in *crtZ* and *crtW* copy numbers had a positive impact on the astaxanthin pathway in the host strain. There were two copies of $crtZ_4$ in yQDD017, while only one copy of *crtZ* or *crtW* in other strains. It was noted that the astaxanthin yield of yQDD017 was lower than that of yQDD020, which contained one copy of $crtZ_4$ and $crtW_4$. The astaxanthin yield of

yQDD012 was higher than yQDD017 and yQDD020, which had only one $crtZ_4$ but no *crtW*. These results indicated that the overexpression of *crtZ* or *crtW* might have a negative impact on astaxanthin yield. The astaxanthin yield comparison between yQDD011 - yQDD015 - yQDD018 also indicated that. This might relate to the increased metabolic burden caused in the increase of *crtZ*/ *crtW* copy number. Otherwise, the mutation in the genome caused by the integration of *crtZ* and *crtW* might induce astaxanthin metabolic changes. There were multiple Ty1 retrotransposon sites in the yeast genome. However, the number of strains with single copy integration was much more than the strains with multiple copies integration without any other environment pressure. It was helpful to truncate the promotor of selection marker [31] or apply the Di-CRISPR[32] to increase the integration efficiency. Combination of those methods may further to 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, and 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, and the *crtZ* and *crtW* integrated into the Ty1 retrotransposon sites by homologous recombination. Both the *in vitro* and *in vivo* recombination method could screen *crtZ* and *crtW* derived from different species randomly and could be used for the accumulation 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 the *in vitro* screening and the *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, the biomass of host strain yQDD001 and other operating environments. Photograph of the *in vitro* and the *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, more colonies appeared on the plates after *in vivo* recombination method, indicating the higher transformation efficiency of the *in vivo* recombination.

The strains yQDD008 and yQDD022, which performed the highest astaxanthin yield from the *in vitro* and the *in vivo* recombination 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 subculture in YPD for 6 days and then spotted on SD solid medium each day (additional Fig.8). The colonies of 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. This result proved

the high stability of heterogeneous pathway recombined by *in vivo* method. However, compared with yQDD001, the increase of *crtZ* and *crtW* copy number had negative effects on yeast growth (Fig.4.c). The profile of astaxanthin titer and astaxanthin yield during fermentation in the 50 mL 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 glucose consumption profile of two strains was similar (additional Fig.9). The low stability of recombined plasmids by *in vitro* method may have a negative impact on the accumulation of astaxanthin in yQDD008. These results indicated that the stability of strains 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* derived from different species were combined randomly in astaxanthin producing strain by *in vitro* and *in vivo* method. The results indicated that the increase of *crtZ* and *crtW* copy numbers have a positive impact on the improvement of astaxanthin yield in yeast. Compared with *in vitro* recombination, the *in vivo* recombination method showed higher integration efficiency and higher stability of the heterologous modules. Finally, the strain yQDD022 with the highest yield of astaxanthin (6.05 mg/g DCW) in this study was obtained by *in vivo* recombination, which with the integration of *crtW* and *crtZ* from *Alcaligenes sp.strain* and *Agrobacterium aurantiacum*, respectively.

Traditional genetic manipulation costs lots of time to construct the heterologous pathway to 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 obtain 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. Both the *in vitro* and *in vivo* recombination research could provide reference to the recombination of heterologous pathway in building microbial cell factories. This study has great reference values to improve the accumulation of 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 mins. 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 vectors, 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 into 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 was 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 was 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 cultivations (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 colonies were 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 into 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 were 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 systems 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 for 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℃.

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.

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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.

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Abbreviations

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

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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

Strains and plasmids	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

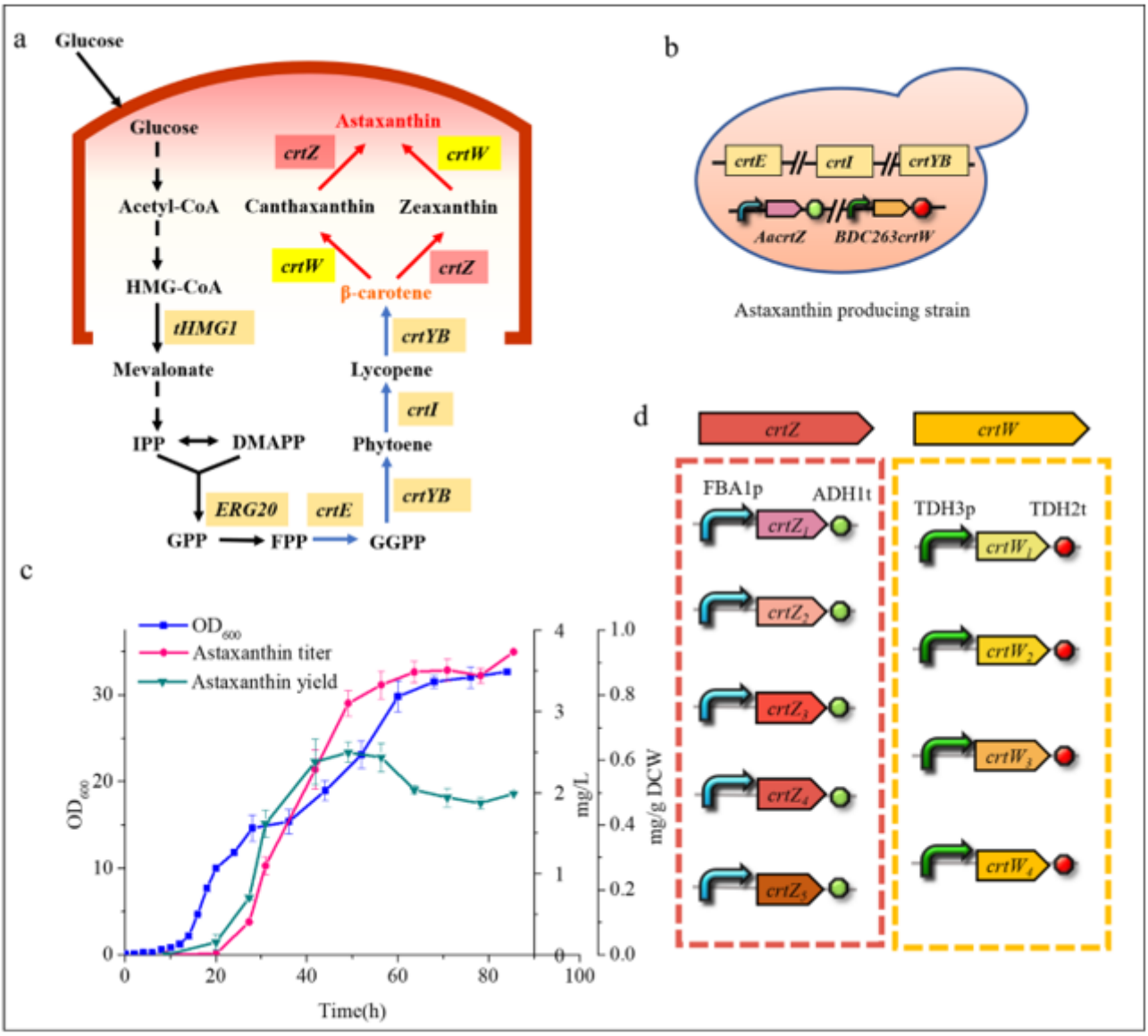
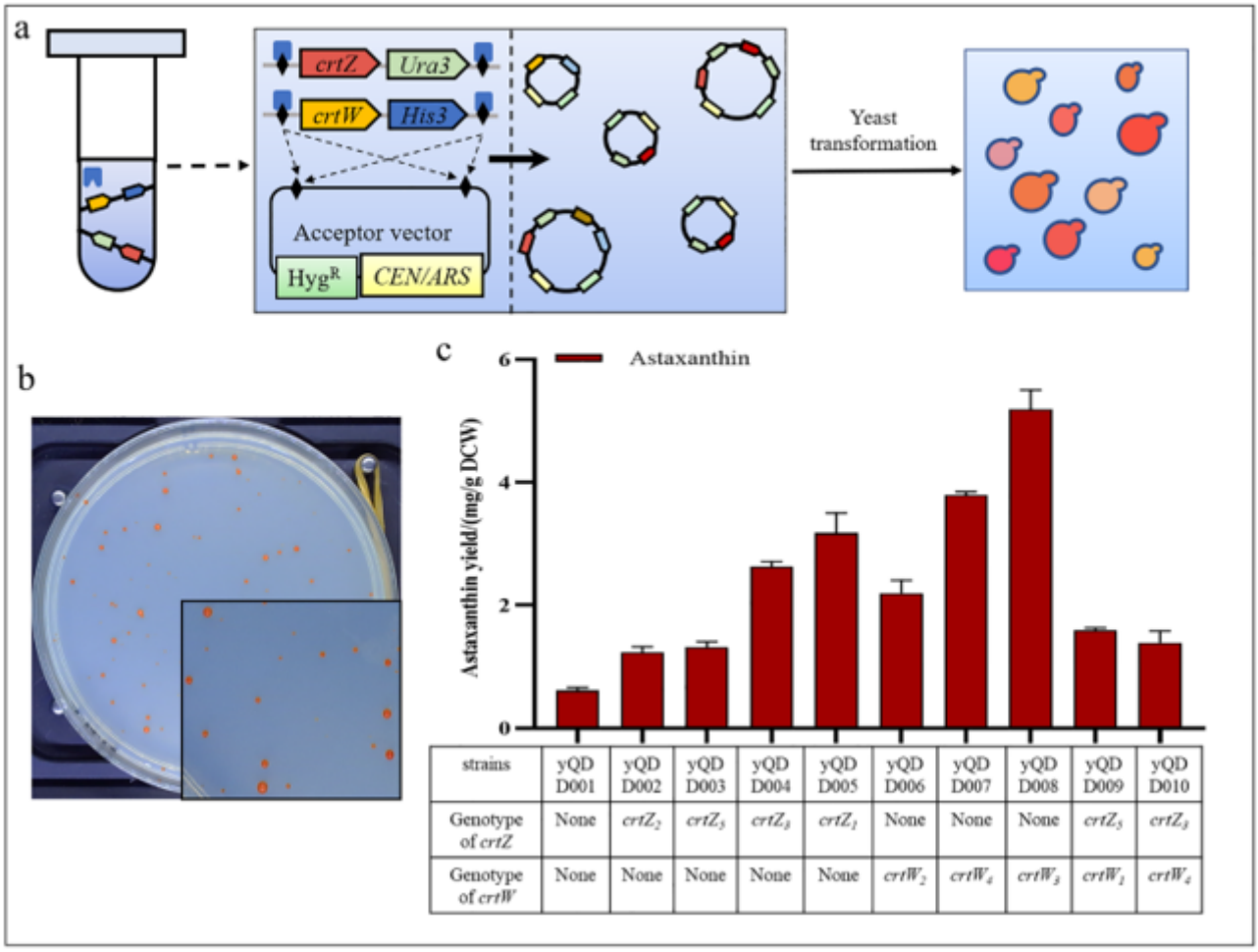


Figure 1

a Biosynthesis pathway of astaxanthin in yeast. The pathway from β-carotene to astaxanthin was boxed in the red line and engineered in this study. b Astaxanthin producing strain. The modular gene cassettes of crtE, crtI, crtYB were inserted into the CAN site of BY4741, while the Aa. crtZ and B. DC263 crtW were integrated into the retrotransposon 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	$crtZ_2$	$crtZ_3$	$crtZ_3$	$crtZ_1$	None	None	None	$crtZ_3$	$crtZ_3$
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, assembled into various new plasmids. b The reaction products were transformed into yQDD001, and produced the yeast library with different colors and sizes. c Astaxanthin yield measurement of in vitro recombined strains. And the genotype 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.

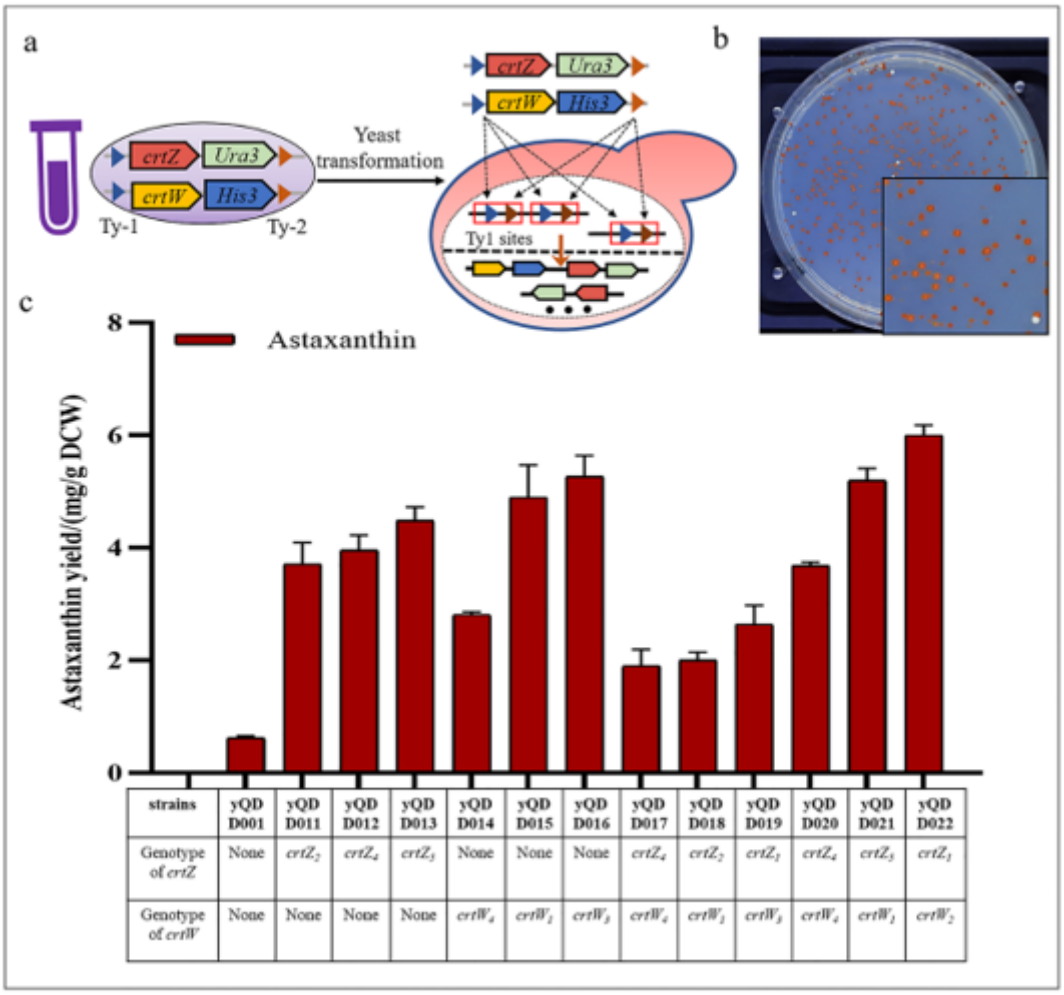
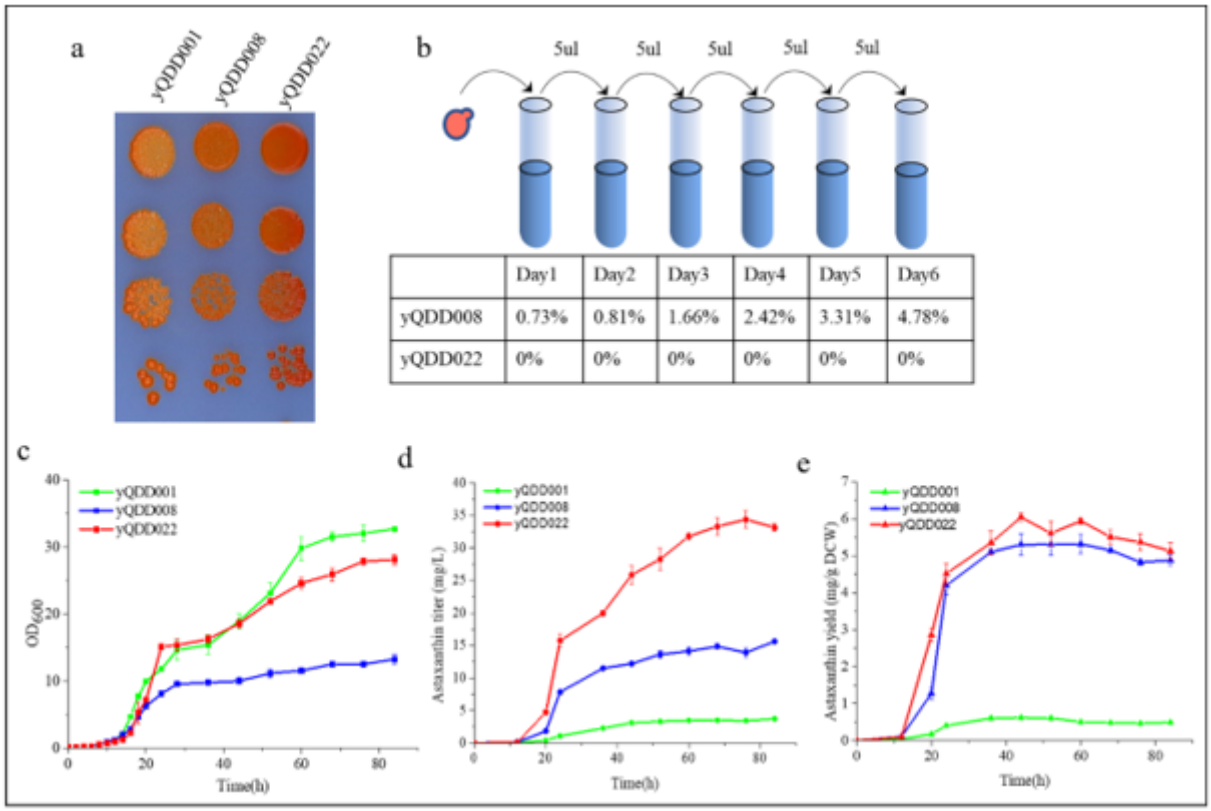


Figure 3

In vivo recombination of heterologous modules. a Each fragments crtZ/crtW carry an Ura3/His3 marker and two homologous arms with Ty1 (Ty-1Ty-2). All crtZ and crtW fragments were mixed up to transform into yQDD001. The fragments inserted into the Ty1 sites of yeast genome randomly to produce the yeast library with various combinations of crtZ and crtW. b 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 of in vivo recombination evolved strains. 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 the in vitro and the 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 assays of yQDD008 and yQDD022. Yeast cultures in YPD after 6 days were plated on SD agar and the number of light-color strains was counted. The ratio of unstable strains in every 12 generations was recorded and listed. c Growth curves 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

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