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

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Abstract

Background: Astaxanthin is a kind of tetraterpene with 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 microorganism is still limited by the poor efficiency of heterologous pathway.

Results: To address the bottleneck of astaxanthin yield in microbe, we developed the \textit{in vitro} and \textit{in vivo} recombination methods to optimize the combination of heterologous module of β-carotene ketolase (\textit{crtW}) and hydroxylation (\textit{crtZ}) from different species in engineered yeast strains. Finally, the astaxanthin yield of \textit{in vitro} recombination and \textit{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 \textit{in vivo} recombination with 6.05mg/g DCW of the astaxanthin yield. Moreover, it is demonstrated that the astaxanthin producing yeast of the \textit{in vivo} recombination has higher efficiency and stability than that of the \textit{in vitro} recombination.

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

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\cite{1}, has tremendous commercial value in the aquaculture, food, cosmetic and pharmaceutical industries\cite{2}. Traditional methods of synthesis of astaxanthin include chemical synthesis and extraction from natural producers, for example, the green algae or the red yeast\cite{3}. However, the biosafety concern with chemical routes and the high cost of the extraction route limit the extensive application of astaxanthin\cite{4}. Moreover, among these sources, only the algae-extracted astaxanthin is the bioactive (3S,3’S)-stereoisomer. Alternatively, microbial chassis cells have been engineered for fermentative production of astaxanthin by means of metabolic engineering techniques\cite{5}. Compared with these methods, microbial production of astaxanthin via metabolic engineering has become a promising alternative concerning the safety and economy issue. Modifying cells to improve production of a desired metabolite is a broad aim for many areas of academic and industrial biotechnology and biosciences\cite{6-9}. In recent years, heterologous productions of astaxanthin have been successfully achieved in \textit{Escherichia coli}\cite{10-15}, \textit{Saccharomyces cerevisiae}\cite{16-19}, Oleaginous Yeast \textit{Yarrowia lipolytica}\cite{20}, and \textit{Corynebacterium glutamicum}\cite{21} by introducing the biosynthesis pathway of astaxanthin. However, astaxanthin production in microorganisms still not high enough for
commercialization. The biosynthesis pathway of astaxanthin in yeast is a complex process, as shown in Fig.1. a. 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 astaxanthin biosynthesis pathway, from β-carotene to astaxanthin, is actually a metabolic web, which requires two steps of β-carotene ketolase(*crtW*) and hydroxylation(*crtZ*) [22]. It has been revealed that many bacterial *crtZ* and *crtW* could utilize β-carotene as well as its hydroxylated or ketonized products as the substrate, leading to diverse carotenoid intermediate profiles which can greatly affect astaxanthin yield and ratio[23-25]. 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 nonketolated carotenoids[26]. 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 strains, higher astaxanthin content was achieved in *S. cerevisiae* via ketolase first and hydroxylation subsequently[19]. 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 Cre/Loxp recombination system to screen the heterologous modular of *crtW* and *crtZ* in CEN/ARS plasmid[27]. The *in vivo* recombination method is using yeast homologous recombination to integrate the heterologous modular of *crtW* and *crtZ* into yeast genome. The yeast yQDD022 with highest astaxanthin yield in this study was obtained by *in vivo* recombination. Compared with the *in vitro* recombination, the more diverse yeast library was observed after yeast transformation by *in vivo* recombination method, while the concentration of *crtZ* and *crtW* fragments transformed to yQDD001 was same in these two methods. And the yQDD022 obtained by *in vivo* recombination was more stability than yQDD008 obtained by *in vitro* recombination. These results indicate that the *in vivo* combination of heterologous pathway modular of *crtW* and *crtZ* has higher efficiency and stability than the *in vitro* recombination. The combination of heterologous pathway modular 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

To construct astaxanthin biosynthesis pathway in yeast, the carotenoid biosynthesis pathway (*crtE*, *crtl*, and *crtYB* with Leu2 marker) was integrated into CAN sites of yeast BY4741, while the *crtZ* from *Agrobacterium aurantiacum* (Aa *crtZ*) and *crtW* from *Brevundimonas vesicularis DC263(B.DC 263 *crtW*) with the G418 marker were integrated into the retrotransposition of Ty1(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 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 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 (additional Tab.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 were used for PCR screening (additional Tab.2).

**In vitro recombination of heterologous modular 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. 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”). The acceptor vector has two *loxP*sym sites and encode a hygromycin gene (represented as Hyg) and was digested by restriction enzyme of EcoRI and BamHI before *in vitro* reaction. The donor fragments are generated by digestion of NotI and XbaI from an 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 color and size (Fig.2.b). The selection marker of His+/Ura+ and Hyg+ are used to make sure at least one or two donor fragments during the *in vitro* recombination reaction. Darker red colonies were selected visually from the selected medium plates. Finally, there were ten colonies (yQDD002 - yQDD010) picking up for analysis with darker red color. The astaxanthin yield of yQDD001-yQDD010 were assessed by HPLC. And the recombination plasmids were verified by PCRTag analysis and sequencing. 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. It is indicated that the increase of *crtZ* or *crtW* copy number have positive impact on astaxanthin biosynthesis in yeast. The genotype of plasmids in yQDD007 and yQDD008, in which higher astaxanthin yield were measurement, was *crtW*$_4$ and *crtW*$_3$ respectively. These results indicated that the β-carotene ketolase is the key step of the metabolic network, overexpression of the β-carotene ketolase has significant effect on increasing astaxanthin yield.

**In vivo recombination of heterologous modular**

Theoretically, high stability of heterologous pathway can increase astaxanthin accumulation in yeast. It is possible that the plasmids constructed by *in vitro* recombination may be lost during the fermentation process with YPD medium which decreases the astaxanthin accumulation. There are multiple Ty1
retotransposition sites in yeast genome[28], which can be used for integration of multiple copy of heterologous modular by in vivo recombination methods. To integrate the crtZ and crtW into genome, all the crtZ and crtW are flanked by 500 base pair homologous sequences from the TyA (additional Fig.3). As shown in Fig.3a, the integration cassettes of all crtZ and crtW were mixed together and transferred into the yQDD001 (additional Fig.4). As shown in Fig.3.b, the in vivo recombination method can generate the yeast library with various color and size colonies. The number of yeast colonies of the in vivo recombination is above 6.7-fold compared with that of the in vitro recombination, while the each DNA fragment of crtZ and crtW was 1 microgramme for both the in vivo and in vitro recombination. It is indicated the high transformation efficiency of the in vivo recombination. Finally, the darker red colonies (yQDD011-yQDD022) were selected for characterization. Then astaxanthin yield of selected strains were analyzed by HPLC. And the types of crtZ and crtW randomly inserted into the yeast genome were proved by PCRTag analysis. As shown in Fig3.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 6.05mg/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.3c. As shown in additional Fig.5, the copy number of crtZ and crtW of the yQDD011 to the yQDD022 were assayed by qPCR. There have two copies of crtZ4 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 crtZ4 and crtW4. These results indicated that the overexpression of crtZ4 have negative impact on astaxanthin yield.

Stability analysis of heterologous modular

The strains yQDD008 and yQDD022, which performed highest astaxanthin yield from in vitro and in vivo recombination of heterologous modular respectively, were selected for characterized 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 obviously darker red than the parent strain yQDD001, indicating the high 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.6). The colonies with light color were observed in screened medium of yQDD008, while not observed in yQDD022. The ratio of colonies with light color were 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 the yeast growth (Fig.4.c). The profile of astaxanthin titer and astaxanthin yield during fermentation in flask with strain yQDD008 and yQDD022 was 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 44h (yQDD022 with 6.10 mg/g DCW). And the profile of glucose consumption in two strains were similar (additional Fig.7). It is probably the low stability has impact on the yield of astaxanthin in yQDD008 compared to yQDD022. 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

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**vivo recombination of *crtZ*<sub>1</sub> and *crtW*<sub>2</sub>. This method can increase the astaxanthin yield of yeast significantly in a high throughput way. The *in vivo* recombination method has great potential in increase the efficiency and copy numbers of heterologous modules in yeast genome.

**Conclusion**

In this study, the *crtZ* and *crtW* from different species were combined randomly in astaxanthin strain by *in vitro* and *in vivo* recombination of heterologous modular. The result indicated that the increase of *crtZ* and *crtW* copy number have 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 modular *crtZ* and *crtW*. Finally, the highest yield of astaxanthin yeast strain yQDD022 (6.05mg/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. This study has the potential to improve desired compounds in yeast with stable performance.

**Methods And Materials**

**Strains and media**

Yeast strains used in this study were described in Tab.1. The astaxanthin producing strain yQDD001 (*MATa, His3Δ0, Leu2Δ1, met15Δ0, Ura3Δ0, HO::tR(ccu)J, lys::NAT*) was subjected to improve the hosts 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<sup>-1</sup> glucose and 100ug mL<sup>-1</sup> G418), SC-Leu-His+G418 (synthetic complete medium lacking leucine and histidine with 20 g L<sup>-1</sup> glucose and 100ug mL<sup>-1</sup> G418) and SC-Leu-Ura-His+G418 (synthetic complete medium lacking leucine, uracil and histidine with 20 g L<sup>-1</sup> glucose ). The All yeast solid media were added with 20 g L<sup>-1</sup> agar. *Escherichia coli* DH5α purchased from BEIJING Biomed Co., Ltd were used for plasmids transformation. *Escherichia coli* were cultivated at 37°C in LB medium (with 10 g L<sup>-1</sup> tryptone, 5 g L<sup>-1</sup> yeast extract, 10 g L<sup>-1</sup> NaCl and 100 ug mL<sup>-1</sup> ampicillin). LB solid medium were added with 15 g L<sup>-1</sup> agar.

**Yeast transformation and assembly**

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°C. 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<sub>2</sub>O (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<sup>-1</sup>), 90 ul of 1M LiAc solution. Then, 50ul *in vitro* or *in vivo* recombination system were 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°C for 30 min. Then heat-shocked 18min at
42°C water-bath. 90 ul DMSO was added followed by heat-shocked. Centrifuged and resuspended cells with 400ul 5 mM CaCl₂, plated on selective medium after 10min. After culturing for 72 h at 30°C incubator, darker red yeast colonies were selected on synthetic medium.

**In vitro recombination**

As shown in additional Fig.2, the donor fragments and acceptor were cut from the plasmids by 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). Reference to previous studies of Zhu et al[27] the Cre recombinase reaction was set up as incubated at 37°C for 4 h. The Cre enzyme was heat inactivated for 10 min at 70°C. 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.

**In vivo recombination**

As shown in additional Fig.4, The fragments of *crtZ* and *crtW* were cut from the plasmids by NotI 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.

**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°C 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 trptone 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°C. Each sample were performed on technical triplicates.

**Growth curve assay**

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 OD₆₀₀ 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**
1 ml of the saturated culture was centrifuged for 2 min at 12000 g. Cells were washed with 1 ml ddwater twice and resuspended in 1 ml of 3 M HCl. The resuspended cells were heated in boiled 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 12000 g for 2 min. After removal of the supernatant, the cells were resuspended in 500 ul acetone and vortexed for 20 min. Acetone extracts were centrifuged (13000 g, 15 min) 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 (150 mm × 4.6 mm, Thermo Scientific) and UV detection at 450 nm and 470 nm at 25 °C. The following two buffers was 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 were 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 °C/3 min, 30 cycles of (95°C/15s, 53°C/30 s, 72 °C/15 s), and 72 °C/5 min. Agarose gel electrophoresis was used for PCR ganalysis. All primers used in this study were listed in additional Tab.3.

**Screening and verification of selected strains**

For preliminary screening, darker red and big colonies were selected on the selective media. Then candidate strains were verified on SD media (synthetic complete medium with 20 g L−1 glucose) using 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 vortex mixer for 20 minutes. Then added 1 mL cold ethanol to the supernatant, mixed and centrifuged at 4 °C for 10 minutes. Precipitate was washed with 75% cold ethanol and dried at 37 °C. 200 μl ddH2O was added to dissolve the yeast genome DNA. Stored the genome DNA at -20 °C.

**Quantitative real-time PCR (qPCR) analysis**

qPCR was applied to quantify copy numbers of gene in engineered strains. The template in 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 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 $2^{-\Delta C_{t}(crtZ)}$ or $2^{-\Delta C_{t}(crtW)}$. 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: pre-cycling, 95°C/300 s, 40 cycles of (95°C/10 s, 57°C/20 s, 72°C/20 s), melt curve, which started from 60°C to 95°C.

**Abbreviations**

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

**Declarations**

**Authors’ 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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| Strain and plasmid | Description                          | Sources     |
|--------------------|--------------------------------------|-------------|
| yQDD000            | β-carotene producing strain          | This lab    |
| yQDD001            | Engineered astaxanthin producing strain | This study |
| yQDD002            | *In vitro* recombined strain         | This study  |
| yQDD003            | *In vitro* recombined strain         | This study  |
| yQDD004            | *In vitro* recombined strain         | This study  |
| yQDD005            | *In vitro* recombined strain         | This study  |
| yQDD006            | *In vitro* recombined strain         | This study  |
| yQDD007            | *In vitro* recombined strain         | This study  |
| yQDD008            | *In vitro* recombined strain         | This study  |
| yQDD009            | *In vitro* recombined strain         | This study  |
| yQDD010            | *In vitro* recombined strain         | This study  |
| yQDD011            | *In vivo* recombined strain          | This study  |
| yQDD012            | *In vivo* recombined strain          | This study  |
| yQDD013            | *In vivo* recombined strain          | This study  |
| yQDD014            | *In vivo* recombined strain          | This study  |
| yQDD015            | *In vivo* recombined strain          | This study  |
| yQDD016            | *In vivo* recombined strain          | This study  |
| yQDD017            | *In vivo* recombined strain          | This study  |
| yQDD018            | *In vivo* recombined strain          | This study  |
| yQDD019            | *In vivo* recombined strain          | This study  |
| yQDD020            | *In vivo* recombined strain          | This study  |
| yQDD021            | *In vivo* recombined strain          | This study  |
| yQDD022            | *In vivo* recombined strain          | This study  |

Table 1 Strains used in this study

**Figures**
Figure 1

a. Biosynthesis pathway of astaxanthin in yeast. The pathway from β-carotene to astaxanthin was boxed through red line and engineered in this study. b. Astaxanthin producing strain. The modular gene 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.
In vitro recombined of heterologous modular crtZ and crtW a Workflow of the in vitro recombination to evolve the heterologous pathway of astaxanthin in yeast. Each fragment of crtZ and crtW carry a Ura3 and His3 marker and two loxPsym sites, respectively. The acceptor vector encodes two loxPsym sites. the acceptor vector and the pool of donor constructs 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. The reaction products were transformed into yQDD001 produced the yeast library with different color and size. b Astaxanthin yield measurement in shake flask of in vitro SCRaNbLEd strains by HPLC. And the gene type of yQDD002 - yQDD011 were 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 unit as mg/g DCW.
In vivo recombination of heterologous modular. 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. The fragments inserted into the Ty1 sites of yeast genome randomly to produce the yeast library with various combination of crtZ and crtW. b Yeast colonies of in vivo recombination method screened in selected medium. The yeast library contained various yeast with different color and size. c Astaxanthin yield measurement in shake flask of in vivo recombination evolved strains by HPLC. The genotype of yQDD011 - yQDD022 were proved by PCRTag analysis.
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 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 were counted. The ratio of unstable strains in every 12 generation were record and listed. 

c. Growth curve of yQDD008 and yQDD022. d Profile of astaxanthin titer during fermentation with strain yQDD008 and yQDD022. e Profile of astaxanthin yield during fermentation with strain yQDD008 and yQDD022.

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