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Highly efficient biosynthesis of astaxanthin in *Saccharomyces cerevisiae* by integration and tuning of algal *crtZ* and *bkt*

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Abstract Astaxanthin is a highly valued carotenoid with strong antioxidant activity and has wide applications in aquaculture, food, cosmetic, and pharmaceutical industries. The market demand for natural astaxanthin promotes research in metabolic engineering of heterologous hosts for astaxanthin production. In this study, an astaxanthin-producing *Saccharomyces cerevisiae* strain was created by successively introducing the *Haematococcus pluvialis* β-carotenoid hydroxylase (*crtZ*) and ketolase (*bkt*) genes into a previously constructed β-carotene hyperproducer. Further integration of strategies including codon optimization, gene copy number adjustment, and iron cofactor supplementation led to significant increase in the astaxanthin production, reaching up to 4.7 mg/g DCW in the shake-flask cultures which is the highest astaxanthin content in *S. cerevisiae* reported to date. Besides, the substrate specificity of *H. pluvialis* CrtZ and BKT and the probable formation route of astaxanthin from β-carotene in *S. cerevisiae* were figured out by expressing the genes separately and in combination. The yeast strains engineered in this work provide a basis for further improving biotechnological production of astaxanthin and might offer a useful general approach to the construction of heterologous biosynthetic pathways for other natural products.

Keywords Astaxanthin · Metabolic engineering · β-Carotenoid hydroxylase · β-Carotenoid ketolase · *Saccharomyces cerevisiae* · *Haematococcus pluvialis*

Introduction

Astaxanthin (3,3′-dihydroxy-β,β′-carotene-4,4′-dione) as a natural red pigment is a member of isoprenoids found in bird, fish, and crustacean through food chain (Higuera-Ciapara et al. 2006). Due to its strong antioxidant activity and singlet oxygen quenching ability, astaxanthin can slow down aging, keep the skin healthy, boost the immune system, and prevent cardiovascular diseases (Guerin et al. 2003; Yuan et al. 2011), and thus has great potential in nutraceutical, pharmaceutical, and cosmetic industries. Currently, the commercial astaxanthin is mainly synthesized through chemical methods and only used as a colorant of aquaculture due to the concerns of its biological functions and food safety issues. Extraction from natural producers such as the green algae *Haematococcus pluvialis* or the red yeast *Xanthophyllomyces dendrorhous* has also been exploited for production of natural astaxanthin. However, cultivation of *H. pluvialis* requires strong light, while the astaxanthin content in wild-type strains of *X. dendrorhous* is generally low. Therefore, these biological approaches are not the best choice for astaxanthin production from the economic point of view.

In recent years, reports have emerged regarding the metabolic pathway engineering towards enhanced astaxanthin formation in *X. dendrorhous* (Ye et al. 2015). The highest astaxanthin productions of 9.0 mg/g DCW in shake-flask cultures (Gassel et al. 2014) and 9.7 mg/g DCW in a fermenter

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culture (Gassel et al. 2013) were achieved, respectively. However, further improvement is hindered by the lack of genetic background information and suitable tools for metabolic engineering of this microorganism. As an alternative, astaxanthin synthesis in *Escherichia coli* (Lemuth et al. 2011; Liao et al. 1999), *Saccharomyces cerevisiae* (Ukibe et al. 2009), and *Candida utilis* (Bhataya et al. 2009) has been made possible by introduction of the astaxanthin synthesis pathway into these noncarotenogenic organisms.

Metabolic engineering for heterogeneous astaxanthin production in *E. coli* has been sporadically reported in recent years. Balanced expression of carotenoid genes with a compact set of ribosome binding sites led to an astaxanthin accumulation of 5.8 mg/g DCW in *E. coli* (Zelcbuch et al. 2013). However, the inherent limitations concerning endotoxin production in bacterial systems raise biosafety issues. Therefore, *S. cerevisiae* as a generally recognized as safe (GRAS) strain is a more attractive host for the production of natural astaxanthin, especially for feed, food, and pharmaceutical applications. In our previous study, a decentralized assembly strategy was proposed for genomic integration of biosynthetic pathways, which facilitated the efficient construction of a β-carotene biosynthesis pathway in *S. cerevisiae* (Xie et al. 2014a). For this pathway, the critical steps are the sequential reactions catalyzed by phytoene synthase, phytoene desaturase, and lycopene cyclase encoded by *crtB* (*crtYB*), *crtI*, and *crtY* (*crtYB*), respectively. To be noted, *crtYB* from *X. dendrorhous* encodes a bifunctional enzyme with both phytoene synthase/lycopene cyclase activities, which converts geranylgeranyl pyrophosphate (GGPP) to phytoene or lycopene and finally to β-carotene (Fig. 1). Further conversion of β-carotene to astaxanthin requires a β-carotenoid ketolase encoded by the bacterial *crtW* (or algal *bkt*) gene for the addition of two keto groups into the 4, 4-positions and a β-carotene hydroxylase encoded by *crtZ* for the addition of two hydroxyl groups into the 3, 3-positions on the β-ionone rings. On the other hand, coexpression of *X. dendrorhous* CrtS with a cytochrome P450 reductase CrtR can also convert β-carotene to astaxanthin. However, the enzymes from different organisms appear to convert β-carotene into astaxanthin through different routes, which is accompanied with a metabolic grid of intermediates accumulation (Scaife et al. 2009). Therefore, the catalytic order as well as the substrate specificity of these enzymes still remains to be characterized in detail.

S. cerevisiae has been successfully engineered for astaxanthin production by introducing the heterologous biosynthetic genes (Ukibe et al. 2009). It was found that the astaxanthin level was much higher by expressing the bacterial

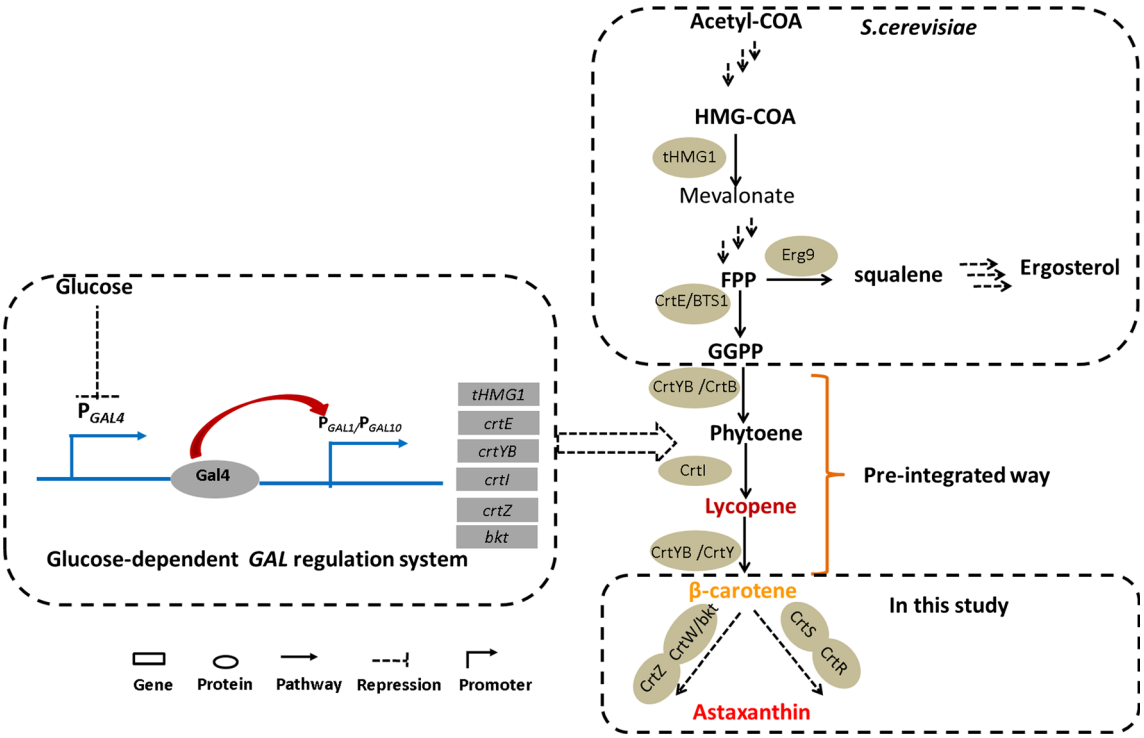

Fig. 1 The glucose-regulated biosynthetic pathway of astaxanthin in *S. cerevisiae*. The top dashed box indicates the endogenous genes of *S. cerevisiae* except for the *X. dendrorhous crtE*. The parentheses indicate the preintegrated way to produce β-carotene and the bottom box shows the pathway from β-carotene to astaxanthin catalyzed by the enzymes encoded by *bkt* and *crtZ* from bacteria or algae as well as *crtS* and *crtR* from *X. dendrorhous*. The *left dashed box* indicates the regulation system adopted. After deletion of *GAL80*, the expression of *GAL4* was controlled by adjusting the glucose supply. In the absence of glucose, Gal4p can bind to the *GAL10* and *GAL1* promoters to activate the transcription of carotenogenic genes. *HMG-COA* hydroxyl methylglutaryl coenzyme A, *FPP* farnesyl pyrophosphate, *GGPP* geranylgeranyl pyrophosphate

crtZ and *crtW* than the *X. dendrorhous crtS* and *crtR*. However, the production of astaxanthin was extremely low (29 μg/g DCW) as compared with the yield of β-carotene (390 μg/g DCW). Therefore, searching for β-carotene ketolase and hydroxylase with higher activity may be critical for the efficient conversion of β-carotene to astaxanthin. Since *H. pluvialis* produces the highest level of astaxanthin (1.5–3.0 % dry weight) among all organisms (Lorenz and Cysewski 2000), we hypothesized that the catalytic activities of the *H. pluvialis* β-carotene hydroxylase and ketolase might be higher than those from other sources. A recent study found that integrating a *H. pluvialis* β-carotene hydroxylase gene into the predesigned carotenoid-biosynthesis pathway in *Kluyveromyces marxianus* indeed gave higher carotenoid production than using *crtS* and *crtR* from *X. dendrorhous*. However, due to the low copy number of gene cassettes integrated into the genome, its total carotenoid productivity was still quite low (about 162 μg/g DCW) as compared with previous studies (Chang et al. 2014).

In the present study, we cloned the β-carotene hydroxylase and ketolase genes (*crtZ* and *bkt*) from *H. pluvialis* and introduced them into *S. cerevisiae* by the decentralized assembly strategy to construct an astaxanthin producer based on a β-carotene-hyperproducing parent strain (YXWP-53) recently developed in our group (Xie et al. 2014a). The expression of carotenoid biosynthetic genes was under the control of *GAL* promoters in *S. cerevisiae* which were regulated by glucose using a modified *GAL* regulation system. Furthermore, the efficiency of *H. pluvialis* β-carotene hydroxylase and ketolase in astaxanthin biosynthesis was analyzed and the substrate selectivity was characterized. Subsequently, the strategies of codon optimization, gene copy number adjustment and cofactor supply were employed to overcome the rate-limiting steps from β-carotene to astaxanthin and meanwhile balance the expression of *crtZ* and *bkt*. Finally, the astaxanthin production was quantified in shake-flask cultures.

Materials and methods

Strains, culture media, reagents

H. pluvialis Flotow N-212 (Gu et al. 2014) was kindly provided by Professor Guangce Wang from Institute of Oceanology, Chinese Academy of Sciences, and used as the source of template for the complementary DNA (cDNA) amplification of *crtZ* and *bkt*. *H. pluvialis* Flotow N-212 was grown at 22 °C with a 12-h:12-h light–dark cycle in the MCM medium (Borowitzka et al. 1991). All the *S. cerevisiae* strains used in this study are summarized in Table S1, among which, *S. cerevisiae* YXWP-53 was used as the host for constructing the astaxanthin biosynthetic pathway. *S. cerevisiae* strains were cultivated at 30 °C in YPD medium (1 % yeast extract, 2 % peptone, and 2 % D-glucose) and 200 μg/mL geneticin (G418) was supplemented for selection of the KanMX marker. Plasmids or gel-purified DNA fragments were transformed into *S. cerevisiae* by electroporation at 1.5 kV in an electroporation cuvette with 0.2 cm gap using an Eppendorf Electroporator 2510. *E. coil* DH5α (Invitrogen) was used as the host for propagation of recombinant plasmids, and cultivated in Luria-Bertani (LB) complete medium (0.5 % Bacto yeast extract, 1 % Bacto-tryptone [Difco Laboratories], 1 % NaCl, pH 7.0) at 37 °C, with supplementation of 50 μg/ml kanamycin for selection.

All restriction enzymes, T4 DNA ligase, and primers were purchased from Sangon Biotech (Shanghai, China). PrimeSTAR HS DNA polymerase was purchased from Takara (Dalian, China).

Gene amplification and plasmid construction

The total RNA of *H. pluvialis* Flotow N-212 was isolated using the RNAiso Plus Kit (Takara, Dalian, China) and genomic DNA contamination in the RNA samples was eliminated by DNase I (Fermentas). Then, cDNA was synthesized from messenger RNA (mRNA) using a PrimeScript™ 1st Strand cDNA Synthesis Kit (Takara Bio, Dalian, China) with oligo (dT18). The *crtZ* (KP866868) and *bkt* (D45881.1) genes were amplified from the cDNA of *H. pluvialis* Flotow N-212 using primers designed based on the conserved regions of these genes among different algal species, and analyzed by the Codon Adaptation Tool (JCAT) (http://www.jcat.de/) according to the codon preference of *S. cerevisiae*. The *OcrtZ* (KP866869) and *Obkt* (KP866870) sequences with optimized codons were then synthesized by Sangon Biotech (Shanghai, China) and cloned into pUMRIs, which were modified from the previously reported pMRIs (Xie et al. 2014a) by insertion of a *URA3* cassette to facilitate 5-fluoroorotic acid counterselection and thus simplify the marker recycling process. The constructed plasmids were subsequently inserted into the yeast chromosome to build an astaxanthin synthesis pathway. The yeast cells carrying plasmids with the *URA3* selectable marker were selected and cultivated in synthetic complete drop-out medium with 2 % D-glucose and without uracil (SD-URA$^-$). Details of primers and recombinant plasmids are shown in Table S2 and Table 1, respectively.

Fluorescence microscopy and flow cytometric analysis

The fluorescent protein accumulation rate was used as a reporter for the expression level of the genes. Fluorescence images of colonies expressing the fusion protein of EGFP (amplified from PUG23) and CrtZ, OCrtZ, BKT, or OBKT were taken using a Nikon ECLIPSE Ti microscope. For further quantitative analysis, *S. cerevisiae* transformants were inoculated to an initial OD_{600} of 0.05 and cultured overnight at

Table 1 Plasmids constructed in this study

Plasmids[a]	Genotype/description	Reference
PUG23	GFP fusion vector	AF298783.1
pUMRI-11	T_{ADH1}-MCS1-P_{GAL10}-P_{GAL1}-MCS2-T_{CYC1}, *DPP1* homologous arm	KM216413
pUMRI-13	T_{ADH1}-MCS1-P_{GAL10}-P_{GAL1}-MCS2-T_{CYC1}, *GAL1-7* homologous arm	KM216415
pUMRI-15	T_{ADH1}-MCS1-P_{GAL10}-P_{GAL1}-MCS2-T_{CYC1}, LPP1 homologous arm	This study
pUMRI-11-*OcrtZ*	T_{ADH1}-*OcrtZ*-P_{GAL10}-P_{GAL1}-MCS2-T_{CYC1}	This study
pUMRI-11-*Obkt*	T_{ADH1}-MCS1-P_{GAL10}-P_{GAL1}-*Obkt*T_{CYC1}	This study
pUMRI-11-*crtZ~EGFP*	T_{ADH1}-*EGFP~crtZ*-P_{GAL10}-P_{GAL1}-MCS2-T_{CYC1}	This study
pUMRI-11-*bkt~EGFP*	T_{ADH1}-MCS1-P_{GAL10}-P_{GAL1}-*bkt~EGFP*-T_{CYC1}	This study
pUMRI-11-*OcrtZ~EGFP*	T_{ADH1}-*EGFP~OcrtZ*-P_{GAL10}-P_{GAL1}-MCS2-T_{CYC1}	This study
pUMRI-11-*Obkt~EGFP*	T_{ADH1}-MCS1-P_{GAL10}-P_{GAL1}-*Obkt E~GFP*-T_{CYC1}	This study
pUMRI-11-*EGFP*	T_{ADH1}-*EGFP*-P_{GAL10}-P_{GAL1}-MCS2-T_{CYC1}	This study
pUMRI-11-*crtZ-bkt*	T_{ADH1}-*crtZ*-P_{GAL10}-P_{GAL1}-*bkt*-T_{CYC1}	This study
pUMRI-11-*OcrtZ-Obkt*	T_{ADH1}-*OcrtZ*-P_{GAL10}-P_{GAL1}-*Obkt*-T_{CYC1}	This study
pUMRI-13-*OcrtZ-Obkt*	T_{ADH1}-*OcrtZ*-P_{GAL10}-P_{GAL1}-*Obkt*-T_{CYC1}	This study
pUMRI-15-*OcrtZ-Obkt*	T_{ADH1}-*OcrtZ*-P_{GAL10}-P_{GAL1}-*Obkt*-T_{CYC1}	This study

[a] The nomenclature of plasmids: for example, pUMRI-11-*crtZ-bkt* represents insertion of *crtZ* and *bkt* into the pUMRI-11 backbone. *crtZ* and *bkt* represent wild-type genes from *H. pluvialis*. *OcrtZ* and *Obkt* represent codon-optimized *crtZ* and *bkt* genes

30 °C, collected by centrifugation in a 2-ml round-bottomed plastic tube and washed twice with phosphate buffer (100 mM, pH=7.0). Then, cells were diluted in the phosphate buffer to about 1×10^6 cells/ml for fluorescence measurements using a BD-FACS Calibur Flow Cytometer. In all, about 50,000 cells were counted in each flow cytometry experiment.

Construction of astaxanthin-producing yeast strains

All strains in this work were constructed based on the YXWP-53 strain developed previously (Xie et al. 2014a). Firstly, the *URA3* marker in the *GAL80* locus was replaced by the disruption cassette consisting of a *LEU2* selection marker and the upstream and downstream homologous regions of the *GAL80* ORF, and then, the resulting strain was selected on a SD (LEU^-) plate. The disruption cassette was constructed by overlap extension PCR using the primers listed in Table S2. The genes were integrated into different genomic loci (*DPP1*, *LPP1*, *GAL1-7*) (Xie et al. 2014b) by transforming the SfiI-linearized pUMRI vectors to generate new strains containing different copies of *OcrtZ* and *Obkt* for astaxanthin production. The detailed construction process of all the yeast strains is shown in Fig. 2.

Cultivation in shaking flasks

The engineered yeast strains were firstly grown overnight in tubes containing 5-ml medium at 30 °C in a rotary shaker (250 rpm), then inoculated into 250-ml Erlenmeyer flasks containing 50-ml YPD medium to an initial OD_{600} of 0.05 and cultured under the same conditions for 84 h. The cell density was measured at 600 nm on a spectrophotometer (UNIC, Shanghai, China) .

To study the effect of the Fe^{2+} cofactor on astaxanthin production, the YPD growth medium was supplemented with varying Fe^{2+} concentrations in the range of 0–0.9 mM.

Analysis of carotenoids

The extraction and assay of carotenoids were performed as previously described (Xie et al. 2014a). Carotenoids were extracted from the HCl-heat-treated cells with 4 ml acetone and used for analysis by reverse-phase high-performance liquid chromatography (SHIMADZU LC-20 AT) equipped with an Amethyst C18-H column (4.6×150 mm, 5 μm, Sepax Technologies, Inc.), and signals were detected by a UV/VIS detector at 470 nm. Samples were eluted with the following gradient program with solvent A: 90 % aqueous acetonitrile (HPLC grade), and solvent B: methyl alcohol-isopropyl alcohol (3:2, *v*/*v*, HPLC grade) as the mobile phase at a flow rate of 1.0 ml/min: 0–90 % solvent B (0–15 min), 90 % solvent B (15–30 min), 90–0 % solvent B (30–35 min). The authentic carotenoid standards and their retention times were as follows: astaxanthin, 7.6 min; canthaxanthin, 11.5 min; echinenone, 17.6 min; and β-carotene, 24.5 min.

The contents of total carotenoids were measured by a spectrophotometer at 450 nm and calculated using an extinction coefficient of 2500 ($A^{1\%}$=2500) (Scott 2001).

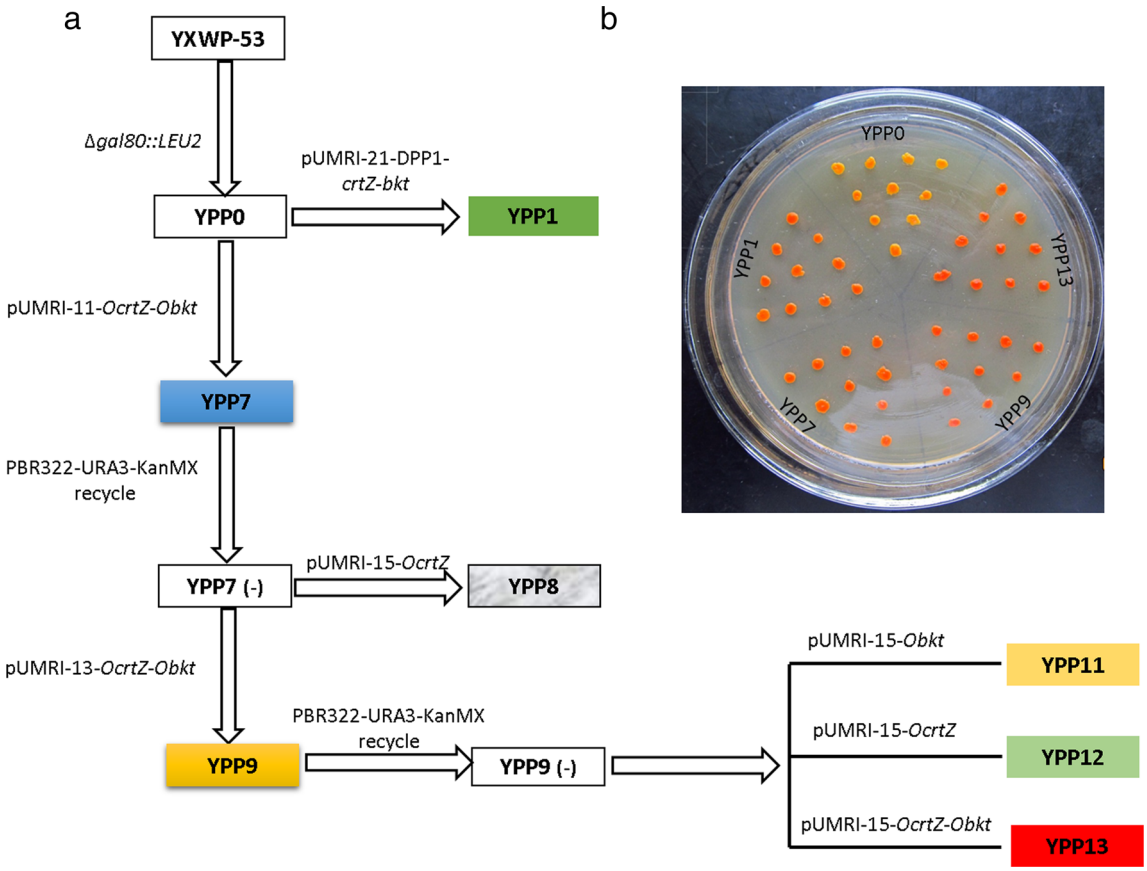

Fig. 2 Construction and phenotype of strains. **a** The construction process of all the strains. **b** The colony color of typical strains on YPD plate

Results

Construction of astaxanthin synthetic pathway in *S. cerevisiae*

Based on an initial effort to produce β-carotene in *S. cerevisiae* (Xie et al. 2014a), the yeast strain YXWP-53 was further engineered to confer a novel biosynthetic pathway for the production of astaxanthin. Firstly, the *URA3* marker in the *GAL80* locus of YXWP-53 was replaced by an *LEU2* marker to facilitate the next round of assembly. The resulting strain YPP0 produced about 10.5 mg/g DCW of β-carotene after 84 h cultivation in YPD shake flask. To further convert β-carotene to astaxanthin, we adopted the decentralized assembly strategy proposed in the previous work (Xie et al. 2014a) and extended the glucose-responding β-carotene synthesis pathway to an astaxanthin pathway by integration of *H. pluvialis crtZ* and *bkt* genes encoding β-carotene ketolase and hydroxylase, respectively. The *crtZ* and *bkt* genes were transformed into the *S. cerevisiae* chromosome using the linearized PUMRI-11-*crtZ-bkt* vector. The expression of all carotenoid genes (eight open reading frames) was controlled by *GAL1*/*GAL10* promoters which were regulated under the modified *GAL* regulation system in response to the variation of glucose concentration in the medium (Xie et al. 2014a). The result of HPLC analysis showed that 0.385 mg/g DCW of astaxanthin was accumulated when the wild-type CrtZ and BKT were expressed in YPP1. Meanwhile, it produced 9.480 mg/g DCW of β-carotene.

Utilization of codon-optimized *crtZ* and *bkt* genes for astaxanthin production

To determine the expression levels of *H. pluvialis crtZ* and *bkt* in *S. cerevisiae*, the genes were fused with the *EGFP* gene and integrated into the *DPP1* site in the chromosome of YPP0 by linearized pUMRI-11-*crtZ~EGFP* and pUMRI-11-*bkt~EGFP*, respectively. Fluorescence microscopy and flow cytometry analyses revealed relatively low fluorescence intensity of these strains as compared with the strain expressing solely the *EGFP* gene at the same site, indicating the low expression level of the fused proteins. As a negative control, the fluorescence was hardly visible in the control yeast cells transformed with the empty vector PUMRI-11 (Fig. S1). Further analysis of the codon adaptation index (CAI value) showed that the genes from *H. pluvialis* were not perfectly matched with the host *S. cerevisiae* because of codon usage bias. Moreover, the downstream sequence of *bkt* gene from *H. pluvialis* is GC-rich (70~80 %), which might terminate transcription, while the average GC content of *crtZ* gene is

as high as 62.5 %, which may also lead to low-efficiency translation. Therefore, the β-carotene ketolase and hydroxylase of *H. pluvialis* were not well expressed in *S. cerevisiae* in their original form.

After adapting the codon usage of algal genes to the *S. cerevisiae* expression host using JCat (Java Codon Adaptation Tool) (Grote et al. 2005), the average GC contents of the codon-optimized *crtZ* and *bkt* genes (named *OcrtZ* and *Obkt*, respectively) were brought down to 46 and 47 %, respectively. And, the CAI values of *OcrtZ* and *Obkt* were increased to rather high levels of about 0.90 and 0.85. Both of them were integrated into the chromosome of YPP0 by linearized pUMRI-11-*OcrtZ-Obkt* and the new strain was designated YPP7. As a result, the astaxanthin yield was increased by about 4-fold (from 0.385 to 1.587 mg/g DCW), while the β-carotene yield was decreased by about 50 % (from 9.480 to 4.807 mg/g DCW) in comparison with the parent strain YPP1 (Fig. 3a).

To check the variation in expression level, codon-optimized *OcrtZ* and *Obkt* genes were also fused with the *EGFP* gene, and subjected to flow cytometric analysis. The result suggested a 9.4-fold higher expression level of *OcrtZ* than the wild-type *crtZ* and a 26.7-fold higher expression level of *Obkt* than the wild-type *bkt* (Fig. 4).

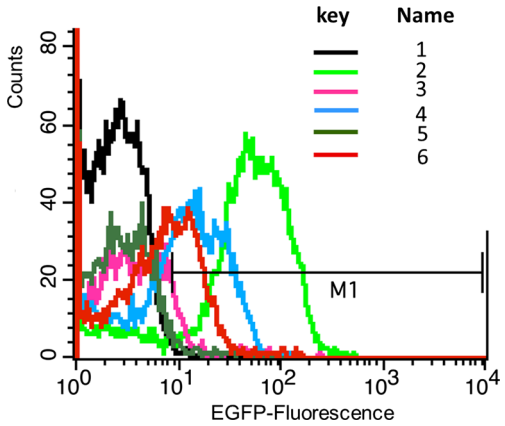

Fig. 4 Fluorescence measurements of cells expressing *EGFP*, *crtZ~EGFP*, *OcrtZ~EGFP*, *bkt~EGFP*, and *Obkt~EGFP* by flow cytometry. The following expression vectors 1. pUMRI-11, 2. pUMRI-*EGFP*, 3. pUMRI-11-*crtZ~EGFP*, 4. pUMRI-11-*OcrtZ~EGFP*, 5. pUMRI-11-*bkt~EGFP*, 6. pUMRI-11-*Obkt~EGFP* were integrated in YPP0

Substrate selectivity of CrtZ and BKT from *H. pluvialis*

To clarify the products and therefore figure out the substrate specificity of *H. pluvialis* β-carotene ketolase and hydroxylase when expressed in *S. cerevisiae*, the plasmids pUMRI-11-*Obkt*, pUMRI-11-*OcrtZ*, and pUMRI-11-*OcrtZ-Obkt* carrying the codon-optimized *crtZ* and *bkt* genes were introduced separately and in combination into the β-carotene producing strain YPP0, respectively. The transformed cells harboring pUMRI-11-*Obkt* accumulated echinenone and canthaxanthin, while those possessing pUMRI-11-*OcrtZ* produced a trace amount of hydroxyl-β-carotenoid. When the pUMRI-11-*Obkt-O*crtZ plasmid carrying both *Obkt* and *OcrtZ* was introduced, astaxanthin was detected in the cell extracts in addition to the intermediates between β-carotene and astaxanthin (Fig. 5a). The results indicated the *H. pluvialis* β-carotene ketolase preferentially convert β-carotene to echinenone and canthaxanthin, whereas the *H. pluvialis* β-carotene hydroxylase showed a priority in converting canthaxanthin to astaxanthin.

Varying gene copy numbers of *OcrtZ* and *Obkt* for enhanced astaxanthin production

In order to further increase astaxanthin production, optimization of the ratios between β-carotene ketolase and

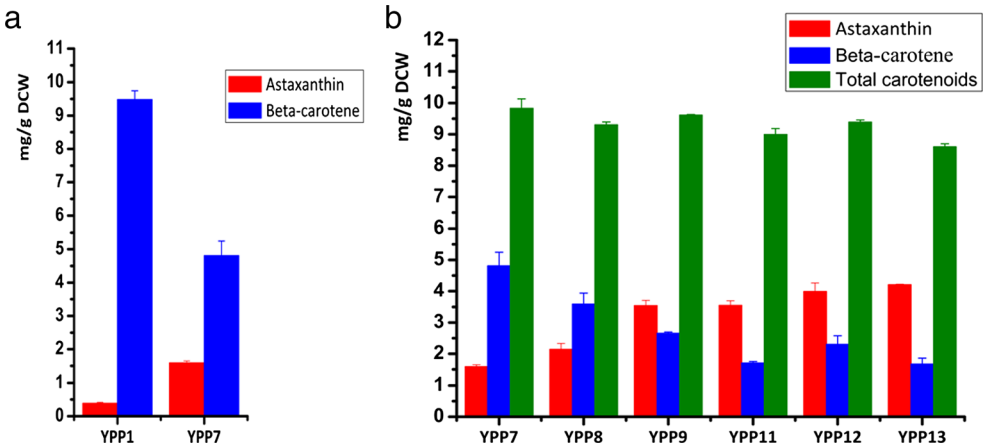

Fig. 3 Effect of codon optimization (**a**) and gene copy number adjustment (**b**) on the production of carotenoids. All strains were inoculated into 250-ml Erlenmeyer flasks with 50-ml YPD medium to an initial OD_{600} of 0.05 and cultivated for 84 h. Total carotenoids in cell extract were quantified by absorbance at 450 nm using a spectrophotometer, while astaxanthin and β-carotene were measured by HPLC. The *error bars* represent standard deviations calculated from triplicate experiments

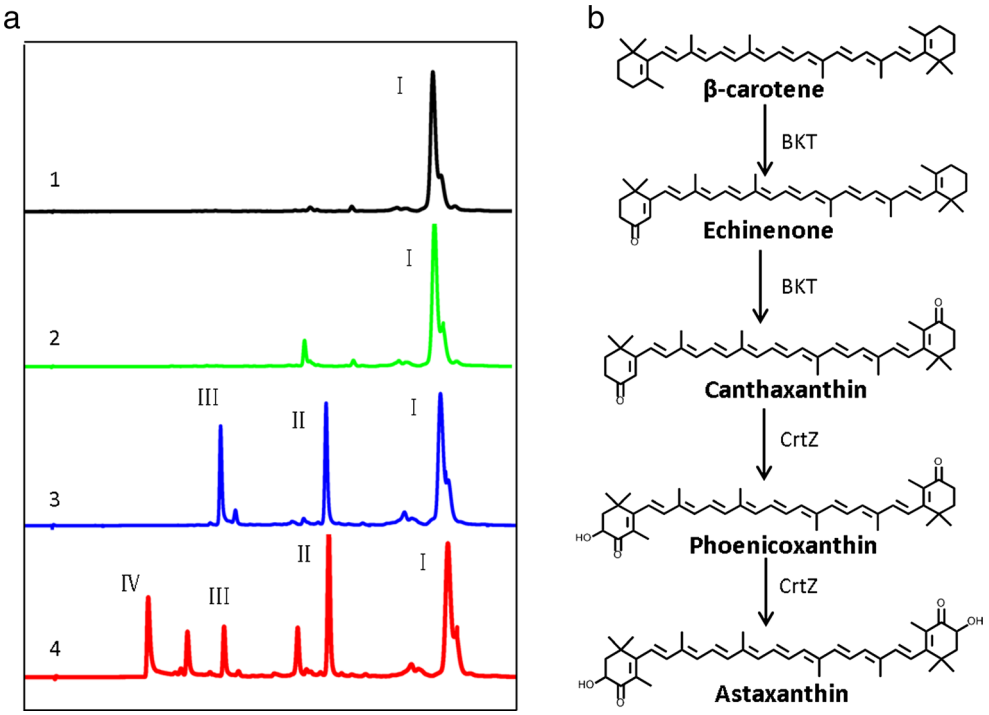

Fig. 5 Substrate selectivity of CrtZ and BKT from *H. pluvialis*. **a** HPLC analysis of carotenoids in the transformed cells which were constructed by introducing the following integrative plasmids into YPP0. *1*, Empty vector pUMRI-11; *2*, pUMRI-11-*OcrtZ*; *3*, pUMRI-11-*Obkt*; *4*, pUMRI-11-*OcrtZ*-*Obkt*. *I*, β-Carotene; *II*, echinenone; *III*, canthaxanthin; *IV*, astaxanthin. **b** The proposed pathway for astaxanthin biosynthesis catalyzed by *H. pluvialis* β-carotene ketolase and hydroxylase in *S. cerevisiae*

hydroxylase might be helpful for elimination of the bottleneck. For this purpose, a series of vectors carrying varied gene copy numbers of *OcrtZ* and *Obkt* were constructed. The strain YPP7 containing only a single copy each of *Obkt* and *OcrtZ* genes produced about 1.587 mg/g DCW of astaxanthin, while in the strain YPP8 containing one copy of *Obkt* and two copies of *OcrtZ*, the production of astaxanthin was improved by 34.8 %. This result indicated that the hydroxylation step was rate-limiting. Subsequently, a 2.2-fold higher production of astaxanthin (3.541 mg/g DCW) was achieved by integrating two copies each of *Obkt* and *OcrtZ* in YPP9. With the increasing gene copy numbers of *OcrtZ* and *Obkt*, the oxygenation reactions from β-carotene to astaxanthin were accelerated. These results indicated that overexpression of *OcrtZ* and *Obkt* was essential for enhancing astaxanthin production in carotenogenic *S. cerevisiae* (Fig. 3b).

With further increasing copy number of *Obkt* and *OcrtZ*, however, no linear correlation between astaxanthin productivity and gene copy number was found. To our surprise, when three copies each of *Obkt* and *OcrtZ* were introduced, the astaxanthin level in the transformed cells YPP13 was not significantly increased (4.203 mg/g DCW) as expected, while the production of β-carotene was obviously decreased, leading to a much higher astaxanthin proportion (50 %) in the final product of the resultant YPP13.

Effect of Fe^{2+} cofactor on the production of astaxanthin

The effects of Fe^{2+} addition on the astaxanthin production by strains carrying different copies of *OcrtZ* and *Obkt* were investigated. The result showed that all the astaxanthin-producing strains had a significant response to the changes of Fe^{2+} concentration in the culture. When cultivated in Fe^{2+}-rich mediums, all transformant strains gave higher astaxanthin production than in the control medium without Fe^{2+}. In YPP7, the response was found in the Fe^{2+} concentration range of 0–0.65 mM. Further increase of the Fe^{2+} amount showed a saturation effect. Finally, an astaxanthin yield 1.9-fold higher than the control value was achieved. In recombinant strains with increasing copies of *Obkt* and *OcrtZ*, the enhancing effect of Fe^{2+} on astaxanthin production became continuously less significant (Fig. 6).

Discussion

In this study, an astaxanthin biosynthetic pathway was assembled by integrating the *crtZ* and *bkt* genes from *H. pluvialis* into the β-carotene-producing *S. cerevisiae*, resulting in an astaxanthin yield of 0.385 mg/g DCW, which was about 13-fold higher than the first astaxanthin-producing *S. cerevisiae* strain constructed by Ukibe and coworkers (Ukibe et al. 2009). This could probably be ascribed to the sufficient supply of β-carotene precursors in our work and the higher catalytic activities of the *H. pluvialis* β-carotene ketolase and hydroxylase as compared with their counterparts from bacteria. However, the low quantity of astaxanthin and high amount of β-carotene accumulated in this strain (YPP1) suggested that the subsequent oxygenation reactions converting β-carotene to astaxanthin appeared to be rate-limiting.

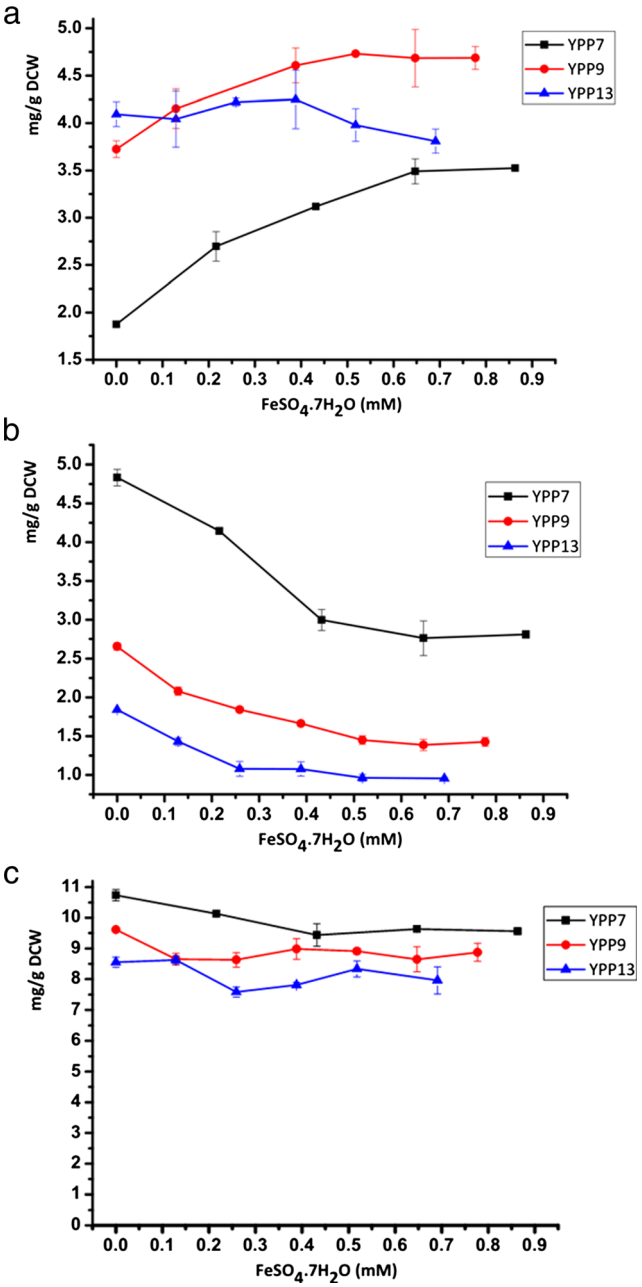

Fig. 6 Effect of Fe^{2+} addition on the carotenoid production by YPP7, YPP9, and YPP13 strains. Transformants were cultivated in YPD medium with different Fe^{2+} concentrations for 84 h. **a** astaxanthin; **b** β-carotene; **c** total carotenoids. The *error bars* represent standard deviations calculated from triplicate experiments

Fluorescence microscopy of EGFP-fused proteins revealed that the *crtZ* and *bkt* genes were expressed at low levels in *S. cerevisiae*. As reported in many studies, the expression of heterologous genes in the desired host is often limited, which is not only because of the infrequently used codons but also due to the possible formation of a secondary structure of the transcripts which is unfavorable for translation (Gustafsson et al. 2004; Kim et al. 1997). Thus, aside from adoption of multicopy expression plasmids and strong transcriptional promoters (Alper et al. 2005) Chen et al. 2012), a common strategy to improve heterologous gene expression is to optimize the sequence of mRNA by decreasing the GC content and choosing high-frequency codons without modifying the amino acid sequence of the encoded protein. As shown in Fig. 3a, when the codon-optimized *crtZ* and *bkt* genes were introduced into the *S. cerevisiae* genome, the astaxanthin yield was increased by 4-fold. This result suggested that increasing the expression levels of the heterologous β-carotene ketolase and hydroxylase by codon optimization could be an effective strategy for enhancing the productivity of astaxanthin in *S. cerevisiae*.

For further improvement of astaxanthin production in *S. cerevisiae*, the exploration of substrate selectivity for *H. pluvialis* enzymes expressed in this host would provide valuable guidance. Previous studies indicated that the β-carotene ketolase and hydroxylase from different organisms show differences in substrate specificity. In general, β-carotene hydroxylase could form zeaxanthin from β-carotene via β-cryptoxanthin, as well as astaxanthin from canthaxanthin via phoenicoxanthin (adonirubin), while β-carotene ketolase synthesizes canthaxanthin via echinenone from β-carotene and 4-ketozeaxanthin (adonixanthin) together with part of astaxanthin from zeaxanthin. Therefore, a number of intermediates might be produced when β-carotene is further converted by β-carotene hydroxylase and ketolase, such as adonixanthin, echinenone, canthaxanthin, and zeaxanthin (Martín et al. 2008). Expression of the *H. pluvialis* β-carotene hydroxylase in *E. coli* indicated that it may use either β-carotene or ketocarotenoids such as canthaxanthin as substrates, to form zeaxanthin or astaxanthin, respectively (Linden 1999). However, in our experimental system, the comparison of carotenoid biosynthesis productivities for the β-carotene hydroxylase revealed that the utilization capacity of β-carotene was not able to compete with that of the ketocarotenoids (Fig. 5a). Additionally, it is in accordance with the results from a previous study on *H. pluvialis* β-carotene ketolase, which showed that the ketolase had a higher catalytic specificity towards nonhydroxylated substrates such as β-carotene and echinenone (Lotan and Hirschberg 1995). These findings led to the deduction of a putative astaxanthin biosynthetic pathway in *S. cerevisiae* with the most favorable route from β-carotene to astaxanthin under the catalysis of *H. pluvialis* β-carotene hydroxylase and ketolase, where β-carotene undergoes ketolation first, followed by hydroxylation, and finally forms astaxanthin (Fig. 5b). This is for the first time that the order of astaxanthin biosynthesis steps catalyzed by *H. pluvialis* β-carotene hydroxylase and ketolase is clarified by evident experimental data, although similar speculation was suggested by a more indirect approach using diphenylamine as an enzyme inhibitor (Fan et al. 1995).

For efficient biosynthesis of astaxanthin based on a β-carotene hyperproducer, the complete conversion of β-

carotene to astaxanthin is obviously critical, in which the ratio between the β-carotene hydroxylase and ketolase might play a key role. Therefore, the influence of varied gene copy numbers of *OcrtZ* and *Obkt* on astaxanthin production was studied. As shown in Fig. 3b, by increasing the gene copy numbers of *crtZ* and *bkt* to two each, the oxygenation reactions from β-carotene to astaxanthin were accelerated and the astaxanthin yield was improved, while further increased gene copy number led to insignificant enhancement in astaxanthin production and repressed β-carotene accumulation. These results suggested that proper overexpression of gateway enzymes indeed alleviated the metabolic bottleneck of the astaxanthin production pathway, while excessive expression of these enzymes might rather cause cellular burden, weaken the performance of the engineered strain and impair cell growth.

Instead of exclusively regulating the amounts of the key enzymes, improving the activities of the β-carotene ketolase and hydroxylase might be an efficient alternative for further enhancement of astaxanthin biosynthesis. As revealed by molecular data analysis, both the β-carotene ketolase and hydroxylase contain a number of histidine residues which are conserved in the formation of the reported Fe^{2+} binding motifs (HX3H, HX2HH, or HX2HH) (Linden 1999; Ye et al. 2006), suggesting Fe^{2+} as a cofactor involved in the catalysis performed by these enzymes. Therefore, the influence of iron cofactor addition on the activities of these enzymes was explored. The decreased increment in astaxanthin accumulation with the increased copy number of *crtZ* and *bkt* suggested that the astaxanthin formation was drastically stimulated in a Fe^{2+}-concentration-dependent manner when sufficient β-carotene supply was available, while this response became insignificant when the β-carotene supply ran short due to the enhanced downstream metabolic flux. To date, two possible explanations have been proposed for the role of Fe^{2+} in enhancing astaxanthin formation. The addition of Fe^{2+} was presumed to result in the generation of active oxygen species which played an important role in the activation of carotenogenesis via an iron-involved Fenton reaction as indicated from the regulation kinetics of astaxanthin biosynthesis (Kobayashi et al. 1993). In addition, based on the amino acid sequences, both β-carotene ketolase and hydroxylase have high similarities with oxygen-dependent and iron-containing integral membrane enzymes, which supports the hypothesis that these enzymes belong to the family of iron-dependent integral membrane proteins. Thus, Fe^{2+} might be beneficial to the activity of astaxanthin biosynthetic enzymes as previously suggested by in vitro assay systems (Fraser et al. 1997).

In this study, an ultimate astaxanthin yield of 4.7 mg/g DCW was achieved in the shake-flask cultures of YPP9 with addition of 0.52 mM Fe^{2+}, which is the highest astaxanthin content in *S. cerevisiae* reported to date. This work demonstrates the potential of *S. cerevisiae* in astaxanthin synthesis, and the recombinant strains constructed can serve as the starting strains for further improvement in biotechnological production of astaxanthin. Future efforts toward enhanced performance may include directed evolution of the rate-limiting enzymes, increasing the precursor supply and repressing the competing pathways.

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Conflict of interest The authors declare that they have no conflict of interest.

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