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Construction of lycopene-overproducing *Saccharomyces cerevisiae* by combining directed evolution and metabolic engineering

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ABSTRACT

Improved supply of farnesyl diphosphate (FPP) is often considered as a typical strategy for engineering *Saccharomyces cerevisiae* towards efficient terpenoid production. However, in the engineered strains with enhanced precursor supply, the production of the target metabolite is often impeded by insufficient capacity of the heterologous terpenoid pathways, which limits further conversion of FPP. Here, we tried to assemble an unimpeded biosynthesis pathway by combining directed evolution and metabolic engineering in *S. cerevisiae* for lycopene-overproduction. First, the catalytic ability of phytoene syntheses from different sources was investigated based on lycopene accumulation. Particularly, the lycopene cyclase function of the bifunctional enzyme CrtYB from *Xanthophyllomyces dendrorhous* was inactivated by deletion of functional domain and directed evolution to obtain mutants with solely phytoene synthase function. Coexpression of the resulting *CrtYB11M* mutant along with the *CrtE* and *CrtI* genes from *X. dendrorhous*, and the *tHMG1* gene from *S. cerevisiae* led to production of 4.47 mg/g DCW (Dry cell weight) of lycopene and 25.66 mg/g DCW of the by-product squalene. To further increase the FPP competitiveness of the lycopene synthesis pathway, we tried to enhance the catalytic performance of CrtE by directed evolution and created a series of pathway variants by varying the copy number of *Crt* genes. Finally, fed-batch fermentation was conducted for the diploid strain YXWPD-14 resulting in accumulation of 1.61 g/L (24.41 mg/g DCW) of lycopene, meanwhile, the by-production of squalene was reduced to below 1 mg/g DCW.

1. Introduction

Lycopene is one of the most widely used carotenoids in the market of healthcare products due to its excellent performance as an antioxidant and great potential in reduction of prostate cancer risk in humans (Agarwal and Rao, 2000; Giovannucci et al., 1995). Currently, the majority of commercial lycopene is extracted from tomato or fermented by *Blakeslea trispora*. However, producing lycopene from tomato needs a long growth cycle, while obtaining lycopene from *B. trispora* requests the addition of cyclase inhibitors for repressing the cyclase function of the bifunctional lycopene cyclase/phytoene synthase (carRA) (Rodriguez-Saiz et al., 2004), which may raise food safety issues (Mantzouridou and Tsimidou, 2008). To avoid the drawbacks mentioned above, other ways for lycopene production have to be explored.

With the development of metabolic engineering techniques, introduction of lycopene biosynthetic pathway into a heterologous host has become a promising strategy for lycopene biosynthesis. *Saccharomyces cerevisiae* is one of the platform microorganisms because of its GRAS (Generally Recognized as Safe) status and robustness in industrial fermentation. The mevalonate pathway (MVA) in *S. cerevisiae* is generally considered as an effective supplier for isoprenoid precursor, therefore, this yeast has been used as the host for production of various terpenoids (Ajikumar et al., 2008; Ignea et al., 2011; Yang et al., 2012). Generally, in order to construct a lycopene pathway in *S. cerevisiae*, at least three heterologous enzymes, geranylgeranyl diphosphate synthase (GGPPS), phytoene synthase and phytoene desaturase are required (Fig. 1).

In previous studies, carotenoid genes from carotenogenic bacterias such as *Erwinia uredovora* and *Pantoea ananatis* were used for construction of carotenoids biosynthesis pathways in heterologous hosts (Kim et al., 2011; Matthaus et al., 2014; Ye and Bhatia, 2012). However, the performance of bacterial carotenoid genes in *S. cerevisiae* is generally poor. When the *E. uredovora Crt* genes were overexpressed on 2 μ plasmids, only 113 μg/g DCW of lycopene or 103 μg/g DCW of β-carotene was obtained (Yamano et al., 1994). Even after codon usage optimization, the highest lycopene production was only 3.28 mg/g DCW in *S. cerevisiae* (Bahieldin et al., 2014). In contrast, it was reported that high-level production of lycopene could be achieved in *Escherichia coli* by expressing bacterial

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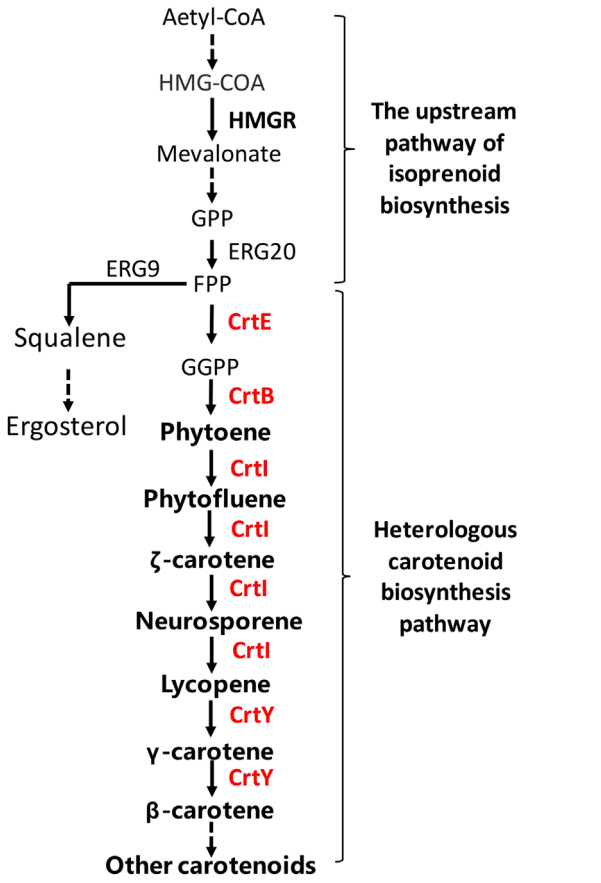

Fig. 1. Overview of carotenoid biosynthesis pathway in *S. cerevisiae*. HMGR: HMG-CoA reductase, ERG20: Farnesyl diphosphate synthase (FPPS), ERG9: Squalene synthase, CrtE: Geranylgeranyl diphosphate synthase (GGPPS), CrtB: Phytoene synthase, CrtI: Phytoene desaturase, CrtY: Lycopene cyclase. Black bold fonts represent the intermediates produced by heterologous genes. Red characters represent the heterologous enzymes required for production of lycopene. The dotted arrows represent multiple reaction steps in the pathway. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

carotenoid genes (Liao and Farmer, 2000; Vadali et al., 2005; Zhou et al., 2013), indicating that host preference might affect the optimal carotenoid biosynthesis activity of bacterial enzymes in *S. cerevisiae*. On the other hand, introduction of the fungal carotenoid genes from *Xanthophyllomyces dendrorhous* led to efficient β-carotene production in *S. cerevisiae* (Verwaal et al., 2007; Xie et al., 2014). Therefore, we speculated that carotenoid genes from eukaryotic organisms would be more suitable for carotenoid synthesis in *S. cerevisiae*.

To improve the yield of the target products, it is often necessary to enhance the availability of essential precursors. In terpenoid production, overexpression of rate-limiting enzyme tHMG1 and repression of the competing squalene pathway by weak promoter such as P_{MET3}, P_{HXT1} or P_{CTR3} are the most frequently used strategies for increasing the supply of FPP (Paddon et al., 2013; Scalcinati et al., 2012; Westfall et al., 2012). However, it should be noted that "pulling" of the metabolic flux is as important as "pushing". Insufficient capacity of the downstream pathway would also limit the final yield of the target products even under high precursor flux (Leonard et al., 2010; Lu et al., 2014). To solve this problem, strategies such as increasing the expression level of enzymes by codon optimization (Li et al., 2013), searching for genes from different sources (Kim et al., 2007) and improving enzyme properties by protein engineering (Leonard et al., 2010) have been employed.

For carotenoid production in *S. cerevisiae*, our previous work demonstrated the loss of FPP through other pathways after *tHMG1* overexpression and *ERG9* repression, due to the insufficient capacity of the carotenoid pathway (Xie et al., 2015). In the present study, we tried to construct an unimpeded lycopene biosynthesis pathway in *S. cerevisiae* using the carotenoid genes from *X. dendrorhous* by protein engineering and metabolic engineering to enhance the FPP utilization efficiency. Because the *CrtYB* gene from *X. dendrorhous* encodes a bifunctional enzyme for phytoene synthesis and lycopene cyclization which results in β-carotene production, searching for a phytoene synthase with excellent specificity and activity is a key issue for successful lycopene synthesis. Therefore, as the first step we attempted to express phytoene synthase from tomato and *Pantoea agglomerans* in *S. cerevisiae*, and meanwhile tried to disrupt the lycopene cyclase functional domain of CrtYB to create a mutant enzyme with only the phytoene synthase function. In addition, directed evolution strategy was employed for enhancing the specificity and activity of the key enzymes in the lycopene biosynthesis pathway. The effects of *Crt* gene copy number and diploid strain construction on the lycopene production were also investigated. Finally, we assembled a pathway for high-level production of lycopene in *S. cerevisiae* by combining directed evolution and metabolic engineering.

2. Materials and methods

2.1. Media and culture conditions

YPD medium (1% yeast extract, 2% peptone and 2% D-glucose) was used to cultivate yeast strains for competent cell preparation or shake flask fermentation. YPD medium containing 20 μg/ml geneticin (G418) was used for selection of recombinants containing the *KanMX* marker. SD medium (Xie et al., 2014) lacking appropriate leucine, uracil, methionine or (and) histidine was used for selection of recombinants containing the corresponding auxotroph marker. SD complete medium with 100 μg/ml of 5-Fluoroorotic acid (5-FOA) was used for counter-selection of recombinants containing the *URA3* marker (Brachmann et al., 1998). For shake flask culture, single colony was picked out from agar plate and inoculated into a tube containing 5 ml of appropriate medium for overnight growth at 30 °C in a 230 rpm orbital shaker, then the precultures were inoculated into shake flask containing 50 ml of medium to an initial OD_{600} of 0.05 and grown under the same condition for 3 days.

2.2. Construction of plasmids and strains

Primers used for construction of plasmids and strains are shown in Supplementary Table S1. The *PSY1* gene (GenBank EF534739.1) and *PSY2* gene (GenBank: EU021055.1) were cloned from tomato (*Solanum lycopersicum*) cDNA using TRIzol® Reagent (invitrogen) and Reverse Transcriptase M-MLV (RNaseH-) (Takara). The *CrtB* gene (GenBank: M87280.1) of *P. agglomerans* was amplified from PAC-LYC plasmid. Two truncated *CrtYB*, *t521CrtYB* and *t422CrtYB* were created by deleting the 5′ flank sequence of wild-type *CrtYB* (Xie et al., 2014). The five genes mentioned above were used as phytoene synthase genes from different species. Detailed information on the yeast episomal and integrating plasmids construction is shown in Supplementary Tables S2 and S3. Plasmids used in this study are presented in Table 1.

To simplify the marker recycle procedure, the previously reported pMRI plasmid backbone (Xie et al., 2014) was modified by insertion of a *URA3* expression cassette between the two *loxp* sites to create pUMRI plasmids for pathway assembly. Because a low-frequency homologous recombination could occur between the two repeated *loxp* sites (*loxp-kanMX-URA3-pBR322 ori-loxp*) when yeast cells were cultured in YPD, this region could be rescued using URA3/5-FOA based counter-selection (Brachmann et al., 1998). Decentralized

Table 1
Plasmids used in this study.

Plasmid	Description	Reference
PAC-LYC	Containing *CrtE*, *CrtB*, *CrtI* gene from *P. agglomerans*	(Cunningham et al., 1994)
pMRI-31	T_{ADH1}-MCS1-P_{GAL10}-P_{GAL1}-MCS2-T_{CYC1}, HO homologous arm	(Xie et al., 2014)
PMRI-35	T_{PGK1}-MCS1-P_{HXT7}-P_{TEF1}-MCS2-T_{TPS1}, LPP1 homologous arm	(Xie et al., 2015)
pMRI-40	T_{PGK1}-MCS1-P_{GAL2}-P_{GAL7}-MCS2-T_{TPS1}, LPP1 homologous arm (GenBank: KM216416)	Lab collection
pUMRI-10	*loxp-KanMX-URA3-pbr322ori-loxp*, T_{ADH1}-MCS1-P_{GAL10}-P_{GAL1}-MCS2-T_{CYC1}, HO homologous arm (GenBank: KM216412)	Lab collection
pUMRI-11	*loxp-KanMX-URA3-pbr322ori-loxp*, T_{ADH1}-MCS1-P_{GAL10}-P_{GAL1}-MCS2-T_{CYC1}, *DPP1* homologous arm (GenBank: KM216413)	Lab collection
pUMRI-13	*loxp-KanMX-URA3-pbr322ori-loxp*, T_{ADH1}-MCS1-P_{GAL10}-P_{GAL1}-MCS2-T_{CYC1}, *GAL1-7* homologous arm (GenBank: KM216415)	Lab collection
pMRI-21-*CrtE-tHMG1*	T_{ADH1}-*CrtE*-P_{GAL10}-P_{GAL1}-*tHMG1*-T_{CYC1}	(Xie et al., 2014)
PMRI-31-*CrtYB-CrtI*	T_{ADH1}-*CrtYB*-P_{GAL10}-P_{GAL1}-*CrtI*-T_{CYC1}	(Xie et al., 2014)
P416XWP01	*CEN/ARS*, *URA3*, P_{GAL10}–P_{GAL1}, bidirectional promoter	(Xie et al., 2015)
p416XWP-P_{CYC1}-*CrtE*	*CEN/ARS*, *URA3*, P_{CYC1}-*CrtE*-T_{ADH1}	(Xie et al., 2015)
p416XWP01-*CrtYB*	*CEN/ARS*, *URA3*, P_{GAL10}-*CrtYB*-T_{ADH1}	This study
p416XWP-P_{CYC1}-*CrtI*	*CEN/ARS*, *URA3*, P_{CYC1}-*CrtI*-T_{ADH1}	This study
p416XWP01-*PSY1*	The expression of *PSY1* was under the control of P_{GAL10} on P416XWP01	This study
p416XWP01-*PSY2*	The expression of *PSY2* was under the control of P_{GAL10} on P416XWP01	This study
p416XWP01-*CrtB*	The expression of *P. agglomerans CrtB* was under the control of P_{GAL10} on P416XWP01	This study
p416XWP01-*t521CrtYB*	The expression of N-truncated *CrtYB* (521 amino acid retained) was under the control of P_{GAL10} on P416XWP01	This study
p416XWP01-*t422CrtYB*	The expression of N-truncated *CrtYB* (422 amino acid retained) was under the control of P_{GAL10} on P416XWP01	This study
pMRI-40-*CrtYB11M-tHMG1*	T_{PGK1}-*CrtYB11M*-P_{GAL2}-P_{GAL7}-*tHMG1*-T_{TPS1}	This study
pUMRI-10-*CrtE-CrtI*	T_{ADH1}-*CrtE*-P_{GAL10}-P_{GAL1}-*CrtI*-T_{CYC1}	This study
pUMRI-10-*CrtE03M-CrtI*	T_{ADH1}-*CrtE03M*-P_{GAL10}-P_{GAL1}-*CrtI*-T_{CYC1}	This study
pUMRI-10-LYC01	T_{TPS1}-*tHMG1*-P_{GAL7}-P_{GAL2}-*CrtYB11M*-T_{PGK1}-T_{CYC1}-*CrtI*-P_{GAL1}-P_{GAL10}-*CrtE03M*-T_{ADH1}	This study
pUMRI-10-LYC04	T_{TPS1}-*tHMG1*-P_{GAL7}-P_{GAL2}-*CrtYB11M*-T_{PGK1}-T_{CYC1}-*CrtI*-P_{GAL1}-P_{GAL10}-*CrtE*-T_{ADH1}	This study
pUMRI-11-*CrtE03M*	T_{ADH1}-*CrtE03M*-P_{GAL10}-P_{GAL1}-T_{CYC1}	This study
pUMRI-11-*tHMG1*	T_{ADH1}-MCS1-P_{GAL10}-P_{GAL1}-*tHMG1*-T_{CYC1}	This study
pUMRI-11-*CrtE03M-CrtI*	T_{ADH1}-*CrtE03M*-P_{GAL10}-P_{GAL1}-*CrtI*-T_{CYC1}	This study
pUMRI-11-*CrtE03M-tHMG1*	T_{ADH1}-*CrtE03M*-P_{GAL10}-P_{GAL1}-*tHMG1*-T_{CYC1}	This study
pUMRI-13-*CrtYB11M*	T_{ADH1}-*CrtYB11M*-P_{GAL10}-P_{GAL1}-T_{CYC1}	This study
pUMRI-13-*CrtI*	T_{ADH1}-P_{GAL10}-P_{GAL1}-*CrtI*-T_{CYC1}	This study
pUMRI-13-*CrtYB11M-CrtI*	T_{ADH1}-*CrtYB11M*-P_{GAL10}-P_{GAL1}-*CrtI*-T_{CYC1}	This study

assembly strategy as described previously was employed for construction of yeast recombinant strains (Xie et al., 2014). The *GAL80* gene in the *GAL* regulation system was disrupted in all the strains constructed in this study. Therefore, the expression of genes under the control of *GAL* promoters could be repressed by high glucose condition and induced by low glucose or glucose-free condition (Xie et al., 2015). The flowchart of strain construction is shown in Fig. 2. The *Marker-HIS3* and *Marker-MET15* auxotroph markers were co-transformed into the host strain followed by screening on SD-HIS-LUE-MET-URA agar plate for prototrophic strain construction. The diploid yeast strains were generated by mating two haploid strains according to the procedure introduced in Yeast Protocols Handbook (Clontech). The detailed genotype of yeast strains used and constructed in this study is given in Table S4.

2.3. Construction of yeast mutant library

Plasmids or linearized DNA fragments were introduced into *S. cerevisiae* by electrotransformation (Eppendorf Eporator 2510, Germany). The plasmids p416XWP01-*CrtYB*, p416XWP-P_{CYC1}-*CrtE* and p416XWP-P_{CYC1}-*CrtI* were used for directed evolution of CrtYB, CrtE and CrtI, respectively. DNA fragments containing about 40–100 bp of homologous sequence with the linearized plasmid were generated by error-prone PCR (Cirino et al., 2003). For directed evolution of CrtYB, we chose the first 750 bp of *CrtYB* where the phytoene cyclase is located to carry out error-prone PCR, meanwhile, the phytoene synthase region was amplified by high-fidelity PCR to avoid mutagenesis (Fig. S1). The error-prone PCR products of *CrtE* and *CrtI* were amplified using P_{CYC1}F2/ADH1tR2 from p416XWP-P_{CYC1}-*CrtE* and p416XWP-P_{CYC1}-*CrtI*, respectively. p416XWP01-*CrtYB*, p416XWP-P_{CYC1}-*CrtE* and p416XWP-P_{CYC1}-*CrtI* were digested by *Not*I/*Sac*I for linearization. PCR products were mixed with the corresponding linearized plasmid and co-transformed into the host strain for library construction (Raymond et al., 1999). Positive colonies were selected out according to color change. The detailed procedure is described in Fig. S2.

2.4. Fed-batch fermentation

For larger-scale cultures, a 5 L fermentor (Shanghai Huihetang Bioengineering Equipment Co. Ltd, China) containing 2.5 L of fermentation medium was adopted. The recombinant yeast strain precultured in YPD shake flask was used as the inoculum and the inoculation volume was 10%. The composition of the fermentation medium used in this work was the same as described previously (van Hoek et al., 2000). A two-stage feeding process was adopted for achieving the goal of high-cell density fermentation. First, a 1 L of feeding solution I consisting of 500 g/L glucose, 9 g/L KH_2PO_4, 2.5 g/L $MgSO_4$, 3.5 g/L K_2SO_4, 0.28 g/L Na_2SO_4, 10 ml/L trace metal solution and 12 ml/L vitamin solution was used to sustain fast cell growth. After that, another 500 mL of feeding solution II containing 250 g/L glucose and 250 g/L glycerol as carbon sources was used to induce the lycopene accumulation.

2.5. Analysis of carotenoids and squalene

The carotenoids and squalene were co-extracted using hot HCl-acetone as described previously (Xie et al., 2014). Total carotenoids were measured by a spectrophotometer at a wavelength of 472 nm and calculated using an extinction coefficient of 3450 (A1%=3450)

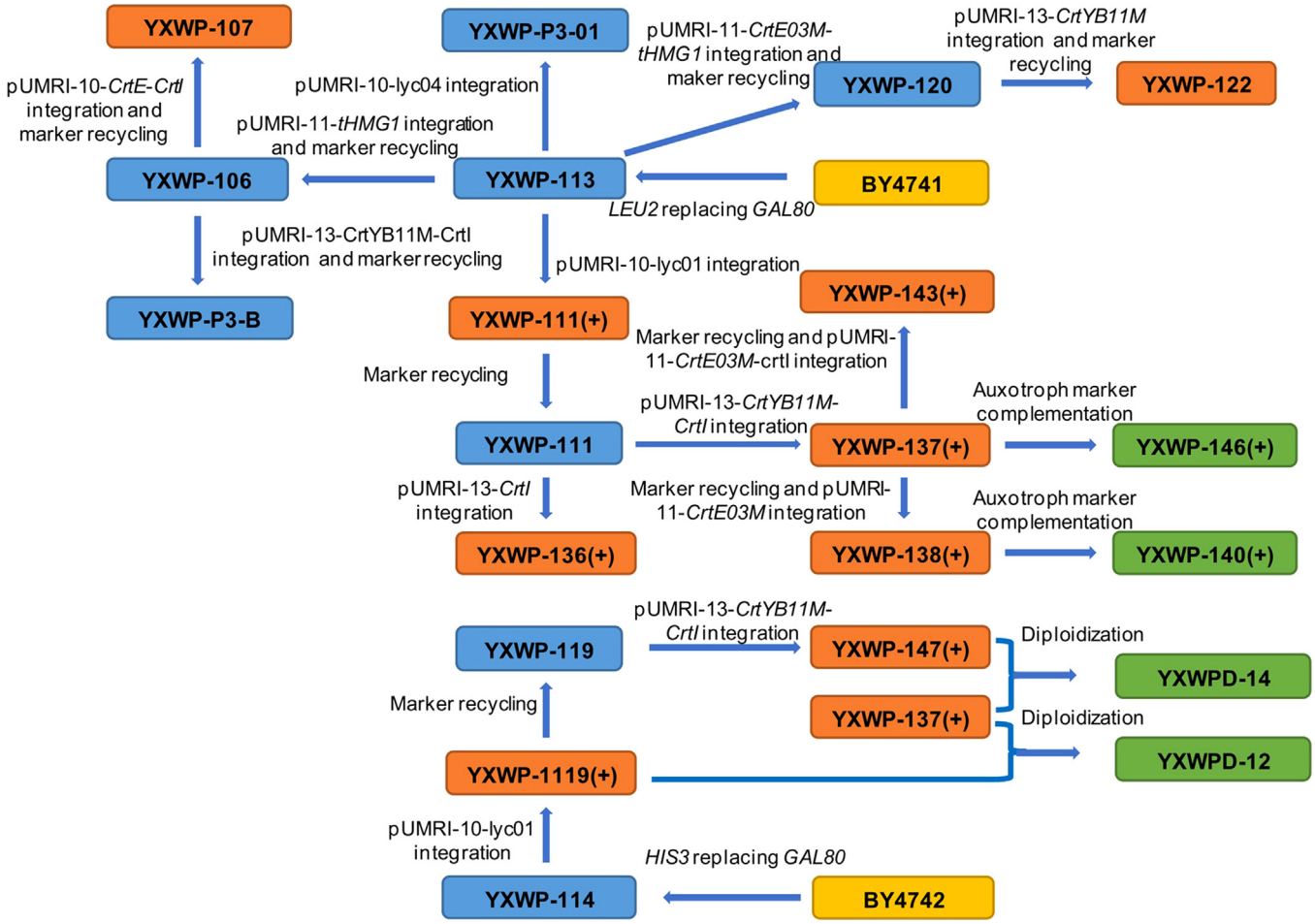

Fig. 2. Flowchart of yeast strain construction in this study. The *loxp-kanMX-URA3-pBR322 ori-loxp* region in pUMRI series plasmids was rescued using URA3/5-FOA-based counter-selection.

(Scott, 2001). The lycopene and β-carotene contents of the extracts were determined using an HPLC system (SHIMADZU LC-20 AT) equipped with a Wondasil C18 column (4.6 mm × 150 mm, 5 μm particle size, GL Sciences, Tokyo, Japan) and a UV/VIS detector. The signals were detected at 450 nm. The mobile phase consisted of acetonitrile-methanol-isopropanol (50:30:20 v/v) with a flow rate of 1 mL/min at 40 °C. Squalene was analyzed by the same HPLC equipment with 100% acetonitrile as the mobile phase and the detection wavelength was 195 nm. Standards of lycopene, β-carotene and squalene were purchased from Sigma (Sigma Aldrich, St. Louis, MO).

3. Results and discussion

3.1. Lycopene production using phytoene synthases from different species

For efficient synthesis of target products, biosynthetic pathway (s) with high capability of precursor utilization are always required. In previous study, combined expression of the β-carotene biosynthesis pathway genes *CrtE*, *CrtYB* and *CrtI* from *X. dendrorhous* and the *tHMG1* gene from *S. cerevisiae* resulted in high production of carotenoids consisting of 32.56% of β-carotene, 28.32% of lycopene and 39.06% of other carotenes (Xie et al., 2014). Here, we intended to assemble a lycopene biosynthetic pathway in *S. cerevisiae* using CrtE and CrtI from *X. dendrorhous* together with a proper phytoene synthase.

The *PSY1* and *PSY2* in the lycopene biosynthesis pathway could confer tomato with deep red color (Giorio et al., 2008), the *CrtB* gene from *P. agglomerans* also showed an excellent performance in lycopene synthesis of *E. coli* (Yoon et al., 2007). These three genes were therefore chosen as candidate phytoene synthase genes of plant and bacterial sources, respectively. In addition, the *CrtYB* gene from *X. dendrorhous* encodes a bifuncational enzyme containing two lycopene cyclase functional domains in the N-terminus and a phytoene synthase functional domain in the C-terminus (Verdoes et al., 1999). In order to create a CrtYB mutant with solely phytoene synthase function, two truncated CrtYB, t521CrtYB and t422CrtYB, were obtained by deleting one or both lycopene cyclase domain(s) of CrtYB, respectively, which were then used as candidate phytoene synthases of fungal source.

The expression of the five candidate phytoene synthase genes in the *S. cerevisiae* strain YXWP-107 harboring previously integrated *tHMG1*, *CrtE* and *CrtI* genes resulted in pink-red colonies on SD-URA-LEU agar plate (Fig. 3A), and the synthesis of lycopene was further confirmed by HPLC (Fig. S3). Among the five phytoene synthases, CrtB had the best performance in lycopene production, while the carotenoid accumulation in the strains expressing PSY1, PSY2, t521CrtYB or t422CrtYB was rather low (Fig. 3). The poor performance of PSY1 and PSY2 might be ascribed to the incorrect subcellular location of these enzymes since they are originally localized in the chloroplast of tomato (Giorio et al., 2008), while there is no such organelle in yeast. Furthermore, the codon bias between plant and yeast might affect the expression of *PSY1* and *PSY2*, resulting in low lycopene production. Although the lycopene

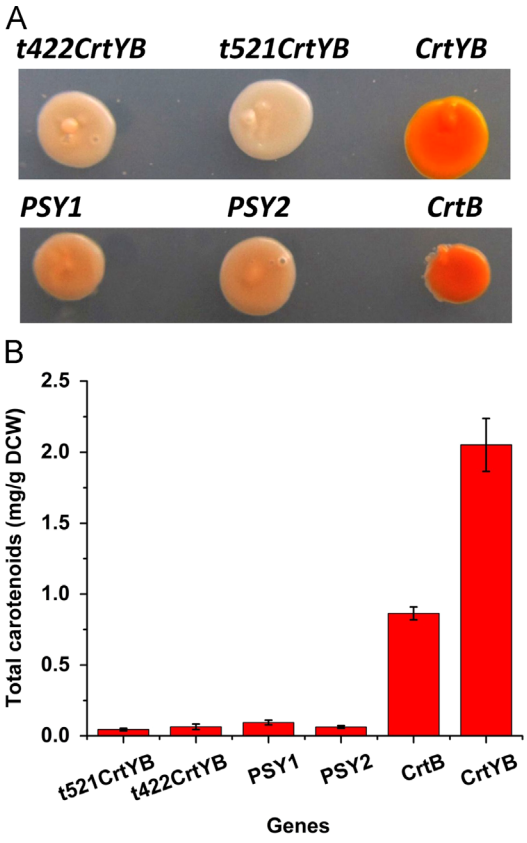

Fig. 3. Effects of expression of different phytoene synthase genes on lycopene production in *S. cerevisiae*. (A) *PSY1*, *PSY2*, *CrtB*, *t521-CrtYB*, *t422-CrtYB* and *CrtYB* were expressed in YXWP-107. The colonies were grown on SD-URA-LEU agar plate for three days. (B) Analysis of carotenoid production after expression of *t521CrtYB*, *t422CrtYB*, *PSY1*, *PSY2*, *CrtB* and *CrtYB* in the host strain YXWP-107. Strains were cultured in shake flasks with SD-URA-LEU medium for 3 days. The error bars represent SD value of triplicate determinations.

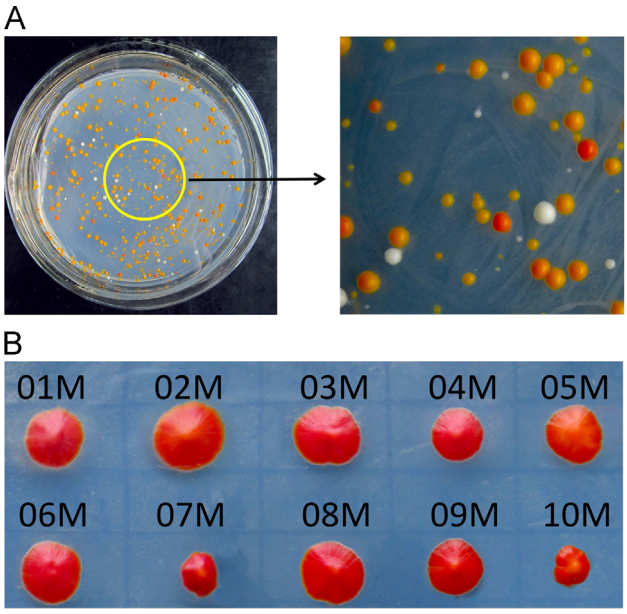

Fig. 4. The results of CrtYB directed evolution. (A) The expression of wild-type CrtYB resulted in colonies with yellow coloration, the null mutation of CrtYB resulted in white colonies, while only the colonies expressing CrtYB mutants where the lycopene cyclase functional domain was impaired or disrupted would show the red color change, which could then be easily distinguished from the library. (B) Ten red mutants isolated from the library. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

cyclase and phytoene synthase function domains in CrtYB seemed to be structurally independent from each other, the simple deletion of lycopene cyclase domain severely impaired the phytoene synthase function, which is consistent with the phenomenon of cyclase domain deletion of CrtYB in *E. coli* (Niklitschek et al., 2008; Verdoes et al., 1999). Because the crystal structure of CrtYB is not available, the underlying mechanism for the impaired phytoene synthase activity after deleting the lycopene cyclase functional domain remains unrevealed. However, it should be noted that although the carotenoid production in *t521CrtYB* and *t422CrtYB*-expressing strains was extremely low, the total carotenoid production in *S. cerevisiae* strain expressing the wild-type *CrtYB* was about 2.4-times higher than that in *CrtB*-expressing strain (Fig. 3B), indicating CrtYB might have a higher phytoene synthase activity than CrtB when expressed in *S. cerevisiae*.

3.2. Directed evolution of CrtYB to inactivate the cyclase functional domain

Directed evolution is a widely used strategy for altering the catalytic characteristics of enzymes. Generally, with a proper screening method, the goal of "you get what you screen for" could be achieved using this strategy (Schmidt-Dannert and Arnold, 1999). Thus, as an alternative strategy for obtaining a phytoene synthase enzyme with high activity and specificity, directed evolution was employed to inactive the lycopene cyclase function but retain the phytoene synthase function in CrtYB.

Ten CrtYB variants exhibiting red coloration were screened out from a library of about 1000 transformants based on the color change (Fig. 4). To check whether the lycopene cyclase function was completely inactivated in these ten mutants, their carotenoids production was analyzed by HPLC. Meanwhile, the strains expressing the wild-type *CrtYB*, *CrtB* and null *CrtYB* were used as controls. Among the ten isolated mutants, carotenoids produced by the four strains expressing *CrtYB01M*, *CrtYB03M*, *CrtYB04M* or *CrtYB06M* gave products with the same peaks and retention time with the *CrtB*-expressing strain, indicating that the lycopene cyclase function of these *CrtYB* variants was totally disabled (Fig. 5), whereas β-carotene production was still detected in strains expressing the other six CrtYB variants. Moreover, an unknown carotenoid produced by neither the wild-type CrtYB nor CrtB was also detected. According to the λ_{max}, value of %III/II (Rodriguez-Amaya, 1999) and the carotenoid biosynthesis pathway, we inferred that this novel carotenoid might be γ-carotene (Fig. S4), the intermediate between lycopene and β-carotene (Fig. 1). The impairment but not inactivation of the cyclization function of CrtYB by mutation might affect the cyclization process from lycopene to β-carotene, leading to the accumulation of γ-carotene.

Sequencing results of the ten mutants revealed that the mutagenesis mainly occurred within the region of first 90 amino acids (Table 2 and Fig. S5). Especially, three of them occurred in the first "hPhEEhhhhhh" motif (h represents any hydrophobic residue), which is a characteristic motif in lycopene cyclases (Moise et al., 2014), suggesting that the first lycopene cyclase domain might be more important for lycopene cyclization although there are two such functional domains in CrtYB (Fig. S5). More importantly, we noticed that single residue mutation (D52G, W61R or E83K) could completely destroy the cyclization ability of the bifunctional CrtYB. Because those three residues are highly conserved in most fungal lycopene cyclase/phytoene synthase (Fig. S6), mutation of these sites in the bifunctional carRA of the currently commercialized lycopene producer *B. trispora* might also result in mutants with solely phytoene

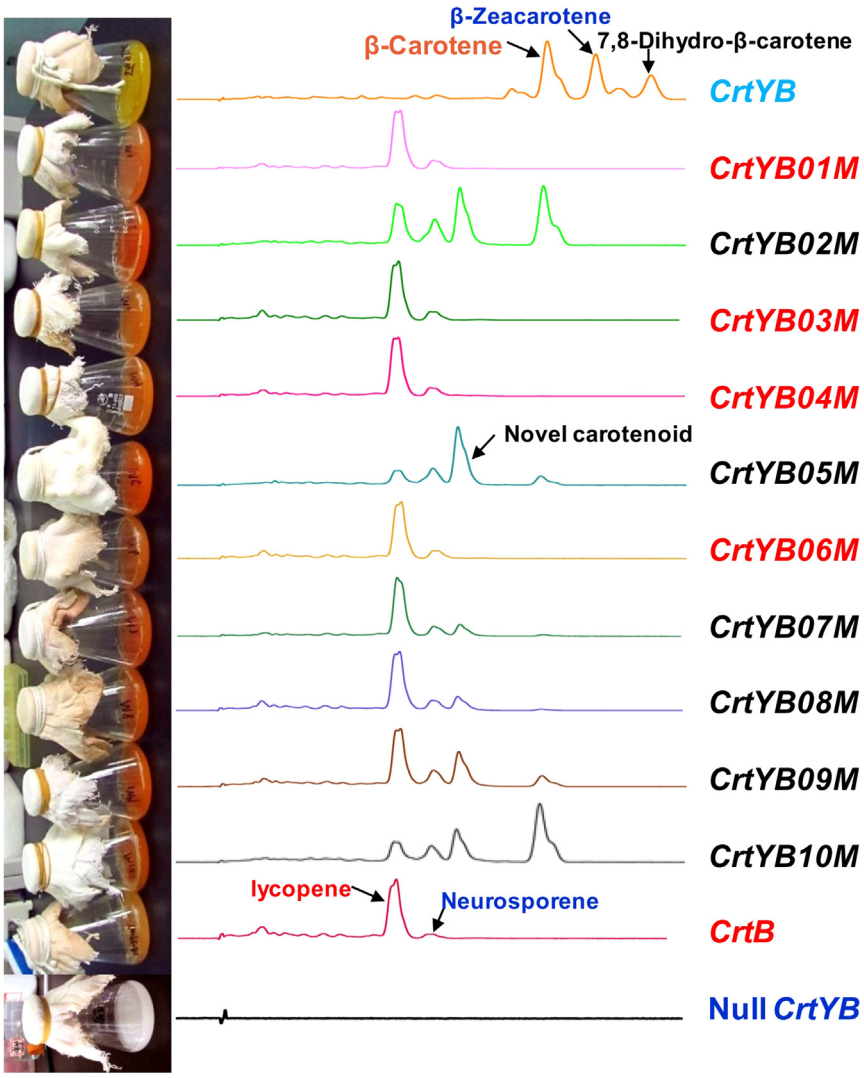

Fig. 5. HPLC analysis of the carotenoid components in *S. cerevisiae* strains expressing the *CrtYB* mutants, wild-type *CrtYB*, *CrtB* and null *CrtYB*. YXWP-107 was used as the host, and the cultures in the conical flasks were corresponding recombinants.

Table 2
DNA and amino acid changes in the CrtYB mutants.

Mutants	Base change[a]	Corresponding residual change[b]
CrtYBM1	T181C T630C	W61R S210S
CrtYBM2	T638C T726C	I213T I309I
CrtYBM3	G247A	E83K
CrtYBM4	A155G	D52G
CrtYBM5	T119C	L40P
CrtYBM6	A88G T181C	T30A W61R
CrtYBM7	T628C	S210P
CrtYBM8	C239A	P80Q
CrtYBM9	A245G	E82G
CrtYBM10	T44C G121A	L15P V41I

[a] The number represents the position of the nucleotide in the ORF of *CrtYB*.
[b] The number represents the position of residual change in CrtYB.

synthase activity, so as to avoid the addition of unsafe chemical inhibitors during lycopene production.

To investigate the phytoene synthase activity of the four CrtYB mutants with completely disrupted cyclase function, we further compared the effects of expressing *CrtYB01M*, *CrtYB03M*, *CrtYB04M*, *CrtYB06M* and the *CrtB* gene on lycopene production in YXWP-107. Shake flask experiments demonstrated *CrtYB01M* overexpression resulted in 2.53 mg/g DCW of lycopene production, which was about 3-fold higher than that of the *CrtB*-overexpressing strain (Fig. 6). To facilitate the subsequent cloning operation, a silent mutation G1221A was created by site-directed mutagenesis in *CrtYB01M* to remove the *Sac*I restriction site, generating the mutant *CrtYB11M*.

3.3. Genomic integration of the lycopene biosynthesis pathway

pUMRI-10-LYC04 harboring the *CrtYB11M*, *CrtE*, *CrtI* genes and the MVA rate-limiting step gene *tHMG1* was integrated into the yeast genome, constructing a lycopene-producing strain YXWP-P3-01. The overexpression of *tHMG1* could increase the supply of the FPP precursor for terpenoid biosynthesis (Misawa, 2011; Paradise et al., 2008), but the accumulated FPP might also be converted to squalene, ergosterol, farnesol and other by-products if it could not be effectively metabolized by the target pathways (Asadollahi et al., 2010; Xie et al., 2015). We therefore measured the contents of lycopene and the major by-product squalene in YXWP-P3-01 after 72 h of shake-flask culture in YPD medium. It turned out that squalene was accumulated to as high as 25.66 mg/g DCW while only 4.47 mg/g DCW of lycopene was produced (Fig. 8), suggesting the competitiveness of ERG9 responsible for squalene biosynthesis

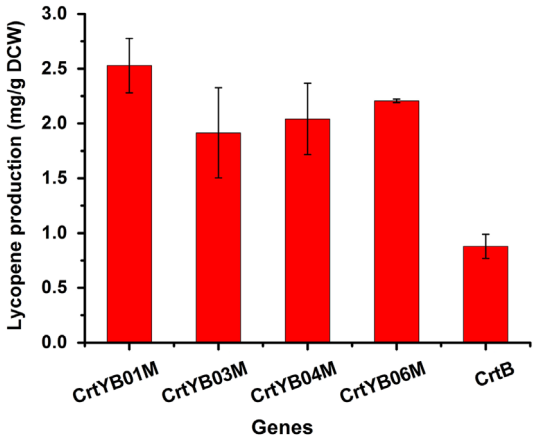

Fig. 6. Comparison of lycopene production among positive *CrtYB* mutants and *CrtB*. The expression of all the genes was under the control of P_{GAL10} promoter on p416XWP01 plasmid. YXWP-107 harboring corresponding plasmids were cultured in shake flasks with SD-URA-LEU medium for three days, respectively. All data presented are the average values of three biological replicates.

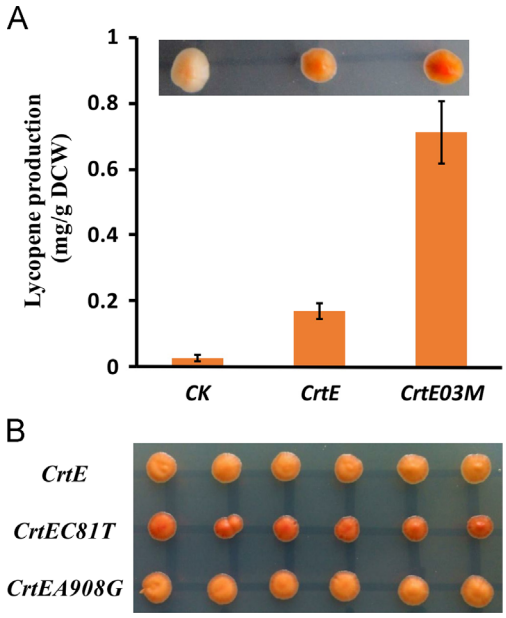

Fig. 7. The results of CrtE directed evolution. (A) The SD-URA agar plate showing the colonies of YXWP-P3-B transformed with p416XWP-P_{CYC1} (CK), p416XWP-P_{CYC1}-*CrtE* (*CrtE*) and p416XWP-P_{CYC1}-*CrtE03M* (*CrtE03M*). The bar chart was lycopene production of the corresponding strains cultured in shake flask containing SD-URA-LEU medium for 3 days. (B) The comparison of lycopene accumulation in YXWP-P3-B expressing *CrtE*, *CrtEC81T* and *CrtEA908G*. (For interpretation of the references to color in this figure, the reader is referred to the web version of this article.)

was higher than that of CrtE; consequently, most of the enhanced metabolic flux from MVA pathway was actually lost to the sterol biosynthesis pathway instead of the desired carotenogenic pathway.

3.4. Directed evolution of CrtE for improvement of lycopene production

As the direct precursor for diterpene and tetraterpene biosynthesis, the GGPP supply determines the flux from FPP to target products. To redirect more FPP for synthesis of the target terpenoid in *S. cerevisiae*, fusion of ERG20 and heterologous terpenoid synthase and down-regulation of the squalene pathway were often employed in previous studies (Albertsen et al., 2011; Scalcinati et al., 2012; Westfall et al., 2012). Here, we tried to improve the catalytic ability of CrtE by directed evolution for enhancing the synthesis of GGPP. The YXWP-P3-B strain overexpressing *tHMG1*, *CrtYB11M* and *CrtI* was used as the host, and the *CrtE* mutation library was generated by random PCR mutagenesis followed by in vivo gap repair with linearized p416XWP-P_{CYC1}. The weak promoter P_{CYC1} was used to limit the CrtE expression at a relatively low level. This strategy could facilitate easy distinguishing of positive mutants with enhanced color intensity by visual inspection, meanwhile avoid the color saturation problem of color-based screening systems (Wang et al., 2000). Four colonies with enhanced red phenotype were isolated from 3000 colonies, but only the mutant *CrtE03M* was confirmed to be real positive after repeated verification by re-transforming the extracted plasmids into YXWP-P3-B. The result of shake flask fermentation indicated that the lycopene accumulation in *CrtE03M*-expressing strain was four times higher than that expressing the wild-type *CrtE* (Fig. 7A).

In previous studies, directed evolution was successfully used to generate *Taxus canadensis* GGPPS mutants for enhancing the production of levopimaradiene (Leonard et al., 2010) and *Archaeoglobus fulgidus* GGPPS mutants for increasing the lycopene production (Liao et al., 1999) in *E. coli*. In these two cases, the improvements of activity or expression level of GGPPS were recognized as the reasons for final production increments. In our work, we found that two bases were mutated in the coding region of *CrtE03M*, among which, C81T was a silent mutation (CAC→CAU), while A908G led to H303R substitution. To investigate which mutation was responsible for the improvement of lycopene production; two single mutants CrtEC81T and CrtEA908G were generated. Interestingly, no difference in lycopene production was observed between the wild-type CrtE and CrtEA908G, while the CrtEC81T with a silent mutation gave an enhanced lycopene accumulation (Fig. 7B). According to the codon bias of *S. cerevisiae* (http://www.kazusa.or.jp/codon/cgi-bin/showcodon.cgi?species=4932), the usage frequency of CAU is higher than that of CAC (13.6% vs. 7.8%). Therefore, the silent mutation in *CrtE* might have improved its expression at protein level and consequently enhanced the flux from FPP to GGPP and lycopene.

We then used *CrtE03M* to replace the wild-type *CrtE* in pUMRI-10-LYC04 and constructed YXWP-111(+). After the new strain was cultured in shake flasks containing YPD medium for 3 days, a lycopene production of 9.67 mg/g DCW was achieved, which was about 2.2 folds that of YXWP-P3-01. Meanwhile, the squalene accumulation was decreased to 10.23 mg/g DCW (Fig. 8), suggesting that the positive mutant *CrtE03M* indeed rerouted the flux of FPP and promoted the lycopene production.

3.5. Adjusting copy number of Crt genes for further improvement of lycopene production

In the directed evolution of CrtE, we noticed that the genomic integration of *CrtE03M* and expression under control of the strong promoter P_{GAL10} led to lower increment folds of lycopene production (Fig.8) as compared to that expressed on plasmid under the control of the weak promoter P_{CYC1} (Fig. 7A). In addition, when compared with YXWP-P3-01, the accumulation of the target product lycopene was only increased by 5.2 mg/g DCW while the production of squalene was reduced by about 15 mg/g DCW in YXWP-111(+). Those results suggested that the improvement of lycopene production might have been limited by the steps of phytoene synthesis or desaturation. In a previous study, the inadequate catalysis ability of CrtI was recognized as a rate-limiting step in β-carotene production by *S. cerevisiae* (Verwaal et al., 2007).

Here, the directed evolution strategy was also employed to improve the activity of CrtI in the host strain YXWP-122, but

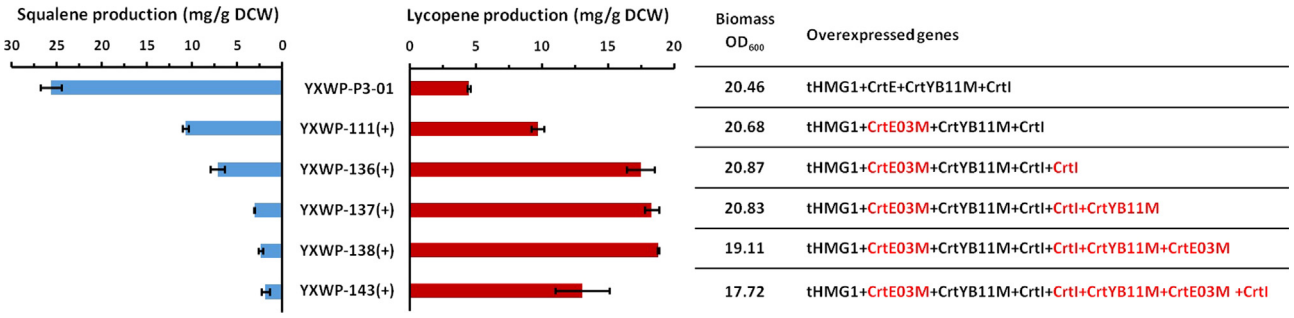

Fig. 8. Lycopene and squalene production by recombinant yeast strains harboring different copies of *Crt* genes. The strains were cultivated for 3 days in YPD media. The data were obtained from at least three independent cultures. (For interpretation of the references to color in this figure, the reader is referred to the web version of this article.)

unfortunately no positive mutant was obtained. The CrtI adopted in our work is in charge of four successive desaturation cycles for converting phytoene to lycopene (Niklitschek et al., 2008), while the same process is catalyzed by four different enzymes in plants (Moise et al., 2014). A previous work also demonstrated directed evolution of the *Pantoa ananatis* CrtI did not enhance the catalytic ability for lycopene production but rather led to formation of other carotenoids (Schmidt-Dannert et al., 2000). The multiple catalysis steps by CrtI make the catalytic mechanism of this enzyme extremely complicated, which might be one of the reasons for our failure in obtaining positive mutants.

In order to further enhance the flux of the downstream pathway for lycopene biosynthesis, alternatively, we tried to create more pathway variants by adjusting the copy number of *Crt* genes. First, we added one copy of *CrtI* into YXWP-111(+), the lycopene production in the resulting strain YXWP-136(+) was improved by 80.6%, to 17.46 mg/g DCW, and the by-product squalene was decreased by 30%, to 7.17 mg/g DCW. Further integration of another copy of *CrtYB11M* or another copy of *CrtYB11M* together with *CrtE03M* only led to a marginal increase in lycopene production of the corresponding strains YXWP-137(+) and YXWP-138(+) when compared with YXWP-136(+), although a further decrement of squalene production was observed in those strains. To investigate whether *CrtI* was still the rate-limiting step in YXWP-138(+), another copy of *CrtI* was introduced into YXWP-138(+), constructing the strain YXWP-143(+). However, both the lycopene production and the biomass were decreased in this strain (Fig. 8). Comparing the color change among the series of strains from YXWP-111(+) to YXWP-143(+) in shake flasks, we observed that the cells turned red earlier with the increment of *Crt* gene copy number (data not shown). However, too early accumulation of lycopene in the cell membrane system might be toxic to cells (Sung et al., 2007; Verwaal et al., 2010; Yoon et al., 2008). Furthermore, overexpression of too many genes could also impair cell growth due to metabolic burden (Karim et al., 2013). These two reasons were considered to be responsible for the decrement in the final biomass and lycopene production of the YXWP-143(+) strain.

3.6. Construction of prototrophic strains for lycopene production

Lycopene can only be stored in the membrane system due to its high hydrophobicity. Thus, to achieve a high titer of lycopene, high biomass is necessary. However, the strains constructed above were auxotrophic thus not suitable for high-cell density fermentation. We therefore complemented the markers in YXWP-137(+) and YXWP-138(+) to generate two prototrophic haploid strains YXWP-146(+) and YXWP-140(+), respectively. Because diploid strains can generally tolerate higher stresses as compared to haploid strains (Li et al., 2010), two prototrophic diploid strains YXWPD-12 and YXWPD-14 were constructed by mating YXWP-137(+) with YXWP-119(+) and YXWP-147(+), which were constructed from the YXWP-114 strain by overexpressing the same genes with YXWP-111(+) and YXWP-137(+), respectively. The final biomass (OD_{600}) and lycopene production (mg/g DCW and mg/L) of these four prototrophic strains were compared in shake flask culture conditions. The diploid strain YXWPD-14 gave a superior cell growth ability (23.408 OD_{600}) and the best lycopene production (23.23 mg/g DCW, 159.56 mg/L) (Table 3), and was consequently chosen for subsequent fed-batch fermentation to obtain even higher lycopene production.

Table 3
Comparison of biomass and lycopene production by haploid and diploid prototrophic strains. All strains were cultured in shake flasks with YPD medium for 3 days. Data represent the average and SD of three independent experiments.

Strains	Biomass (OD_{600})	Lycopene (mg/g DCW)	Lycopene (mg/L)
YXWP-146(+)	20.935 ± 0.078	17.685 ± 0.225	96.661 ± 1.481
YXWP-140(+)	20.232 ± 0.247	16.305 ± 1.382	78.887 ± 3.347
YXWPD-12	22.712 ± 1.430	16.001 ± 2.324	95.653 ± 15.780
YXWPD-14	23.408 ± 0.189	23.225 ± 0.595	159.559 ± 10.470

3.7. Lycopene overproduction by High-cell-density fermentation

Our previous work demonstrated the stages of cell growth and product accumulation during high-density cell fermentation could be well separated from each other by employing a modified *GAL* regulation system and controlling the supply of glucose (Xie et al., 2014; Xie et al., 2015). Here, a similar feeding process was adopted (Fig. 9A). After 1 L of feeding solution I containing 500 g glucose was fed into the bioreactor in the first stage, the biomass reached OD_{600}=230, while the lycopene in cells was only about 4.3 mg/g DCW. During the second feeding stage with 0.5 L feeding solution II containing 125 g glucose and 125 g glycerol, although the biomass only showed a slight increment, the lycopene accumulation was dramatically increased. After the exhaustion of the feeding solution II at 96 h, a lycopene production of 1.43 g/L and 22.03 mg/g DCW was achieved. Although the feeding process was stopped after 96 h, lycopene was accumulated continuously to 1.61 g/L and 24.41 mg/g DCW until the completion of fermentation (Fig. 9B–D). To be noted, the by-product squalene in cells was kept below 1 mg/g DCW during the whole process of fermentation (Fig. 9B), suggesting that the FPP flux was efficiently pulled to GGPP and lycopene biosynthesis after we strengthened the downstream lycopene pathway.

Although the lycopene content in this work (24.41 mg/g DCW) was still lower than the *E. coli* production system (32 mg/g DCW) (Kim et al., 2011), it was much higher than previous reports on lycopene production from engineered *S. cerevisiae* strains (Bahieldin et al., 2014; Yamano et al., 1994), or the natural producers such as

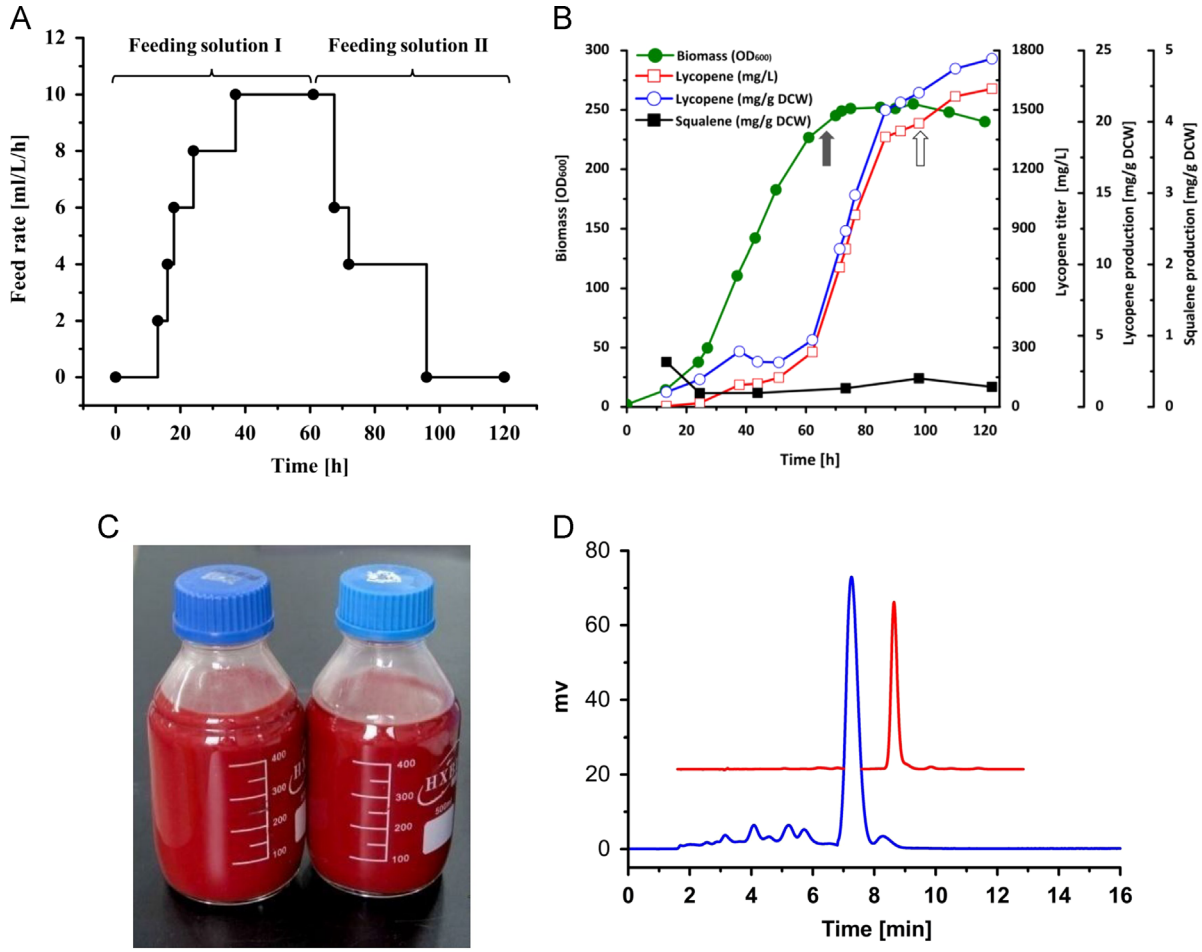

Fig. 9. High-density fermentation for lycopene production. (A) Feeding profile in the fed-batch fermentation. The feed rate ml/L/h represents the volume of feeding solution was added into per liter of fermentation broth in per h at a constant flow-rate. (B) The lycopene, squalene and biomass accumulation of strain YXWPD-14 during the fed-batch fermentation. The solid arrow represents the time point for completion of the first feeding stage, and the hollow arrow represents the time point for completion of the second feeding stage. (C) The red liquid in the bottles was the lycopene fermentation broth of YXWPD-14. (D) HPLC analysis of the carotenoid extracts from YXWPD-14. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

tomato (Agarwal et al., 2001) and *B. trispora* (Mantzouridou and Tsimidou, 2008). In previous studies, media optimization was shown to successfully enhance lycopene production (Kim et al., 2011; Yoon et al., 2008). We believe that the lycopene production of the strain constructed in this work would also benefit from a further media optimization.

4. Conclusion

An ideal microbial factory should have excellent capability of accumulating target products as well as achieving high-cell density. Repressed expression of squalene synthase could result in accumulation of FPP for target metabolite biosynthesis but meanwhile possibly lead to impaired cell growth. In addition, too early excessive accumulation of lycopene was demonstrated to damage the cytoplasmic membrane and cause cell death due to the antifungal effects of lycopene (Sung et al., 2007). Therefore, different from the strategy of rerouting the FPP flux by repressing the competing pathway, in this work, the strategies of directed evolution and copy number variation of the *Crt* genes were employed to enhance the competitiveness of the target lycopene synthesis pathway. Additionally, the modified *GAL* regulation system was used for controlling the lycopene accumulation in the high-cell fermentation process. The final lycopene production of 24.41 mg/g DCW (1.61 g/L) achieved in this study was the highest ever reported from an engineered yeast strain, demonstrating the efficiency of this combinational strategy of directed evolution and metabolic engineering for construction of unimpeded biosynthetic pathways, especially for the production of molecules with cell toxicity.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ymben.2015.04.009.

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