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ORIGINAL PAPER

Temperature influences β-carotene production in recombinant *Saccharomyces cerevisiae* expressing carotenogenic genes from *Phaffia rhodozyma*

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Abstract Red yeast *Phaffia rhodozyma* is a prominent microorganism able to synthesize carotenoid. Here, three carotenogenic cDNAs of *P. rhodozyma* CGMCC 2.1557, *crtE*, *crtYB* and *crtI*, were cloned and introduced into *Saccharomyces cerevisiae* INVSc1. The recombinant Sc-EYBI cells could synthesize 258.8 ± 43.8 μg g^{-1} dry cell weight (DCW) of β-carotene when growing at 20 °C, about 59-fold higher than in those growing at 30 °C. Additional expression of the catalytic domain of 3-hydroxy-3-methylglutaryl-coenzyme A reductase from *S. cerevisiae* (Sc-EYBIH) increased the β-carotene level to 528.8 ± 13.3 μg g^{-1} DCW as cells growing at 20 °C, 27-fold higher than cells growing at 30 °C, although cells grew faster at 30 °C than at 20 °C. Consistent with the much higher β-carotene level in cells growing at 20 °C, transcription level of three *crt* genes and *cHMG1* gene in cells growing at 20 °C was a little higher than in those growing at 30 °C. Meanwhile, expression of three carotenogenic genes and accumulation of β-carotene promoted cell growth. These results reveal the influence of temperature on β-carotene biosynthesis and may be helpful for improving β-carotene production in recombinant *S. cerevisiae*.

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Introduction

Carotenoids are a class of pigments with important biological functions. They can be used as the coloring agents for food and feed products (Nelis and De Leenheer 1991). In addition, β-carotene is the precursor of vitamin A, thus plays an important role in the vision process. Most importantly, many carotenoids, such as β-carotene, lycopene and astaxanthin have antioxidant activity, thus can reduce cellular or tissue damage and can protect body against cancer. Although many carotenoids are currently produced mainly by chemical synthesis, a wide range of microorganisms have been found to be able to produce carotenoids naturally. For examples, *Dunaliella salina* (microalgae), *Blakeslea trispora* (filamentous fungus), *Rhodotorula glutinis* (yeast) and *Flavobacterium multivorum* (bacterium) can produce β-carotene (García-González et al. 2005; Choudhari and Singhal 2008; Wang et al. 2008; Bhosale and Bernstein 2004). *Phaffia rhodozyma* (sexual form, *Xanthophyllomyces dendrorhous*, yeast) and *Haematococcus pluvialis* (microalgae) can produce astaxanthin (Domínguez-Bocanegra et al. 2007).

Red yeast *P. rhodozyma* produces astaxanthin as a final product and also accumulates β-carotene as an intermediate. The carotenoid biosynthesis of *P. rhodozyma* is derived from the isoprenoid synthetic pathway and begins with the synthesis of dimethylallyl pyrophosphate (DMAPP) by isopentenyl pyrophosphate (IPP) isomerase encoded by *idi* (Fig. 1). Next, two molecules of IPP are sequentially added to DMAPP to give farnesyl pyrophosphate (FPP). Subsequently, another molecule of IPP is added to FPP to give

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the C20-precursor geranylgeranyl pyrophosphate (GGPP) by GGPP synthase CrtE. The first specific and crucial step of the carotenogenic pathway in *P. rhodozyma* is the synthesis of phytoene, through the condensation of two molecules of GGPP, catalyzed by the phytoene synthase activity of the bifunctional enzyme CrtYB. Phytoene is subsequently converted into lycopene by four dehydrogenation reactions catalyzed by the phytoene desaturase CrtI. Then, the lycopene is converted to γ-carotene and finally β-carotene by two cyclization reactions catalyzed by the lycopene cyclase activity of CrtYB. Finally, β-carotene is converted to astaxanthin through the addition of two hydroxyl and two keto groups, catalyzed by the astaxanthin synthase CrtS (Lodato et al. 2007; Martinez-Moya et al. 2011). In addition to CrtS, the cytochrome P450 reductase CrtR which provides CrtS with the necessary electrons for substrate oxygenation is also required for the conversion of β-carotene to astaxanthin (Alcaino et al. 2008). In *P. rhodozyma*, the prenyl pyrophosphates can also be utilized to synthesize ergosterol. In order to improve β-carotene biosynthesis, several microorganisms, such as *Escherichia coli* and *Saccharomyces cerevisiae* had been genetically modified to produce heterogeneous β-carotene by metabolic engineering strategy in recent years (Nishizaki et al. 2007; Verwaal et al. 2007).

Saccharomyces cerevisiae is a traditionally used species in brewing and baking. Due to its wide application in the food and beverage industries, it is generally recognized as safe, thus in the early 1990s it obtained a GRAS status (Ribeiro et al. 2011). Furthermore, it has the advantage of easy genetic manipulation with established host-vector systems. Most importantly, *S. cerevisiae* is able to produce GGPP, the basic building block of carotenoids (Ohto et al. 2009). Conversion of FPP into GGPP is catalyzed by GGPP synthase encoded by *BTS1* in *S. cerevisiae*. Verwaal et al. (2007) reported that through transformation with carotenogenic genes (*crtYB*, *crtI*, *crtE*) from *X. dendrorhous*, *S. cerevisiae* accumulated β-carotene successfully. Additional expression of catalytic domain of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase from *S. cerevisiae* resulted in an increase in carotenoid production level. Ukibe et al. (2009) reported that through transformation with carotenogenic genes (*crtYB*, *crtI*, *crtS*, *crtR*) from *X. dendrorhous* and overexpression of *BTS1* gene from *S. cerevisiae*, *S. cerevisiae* accumulated a small amount of astaxanthin.

In this study, the *crtE*, *crtYB* and *crtI* genes of *P. rhodozyma* CGMCC 2.1557, as well as the truncated gene encoding the catalytic domain of HMG-CoA reductase (*cHMG1*) of *S. cerevisiae* were introduced into *S. cerevisiae* INVSc1. β-carotene productions in the recombinant *S. cerevisiae* were found to be significantly different at 20 and 30 °C. In order to explain the impact of temperature on β-carotene accumulation, transcription level of these genes at different cultivation temperatures was determined.

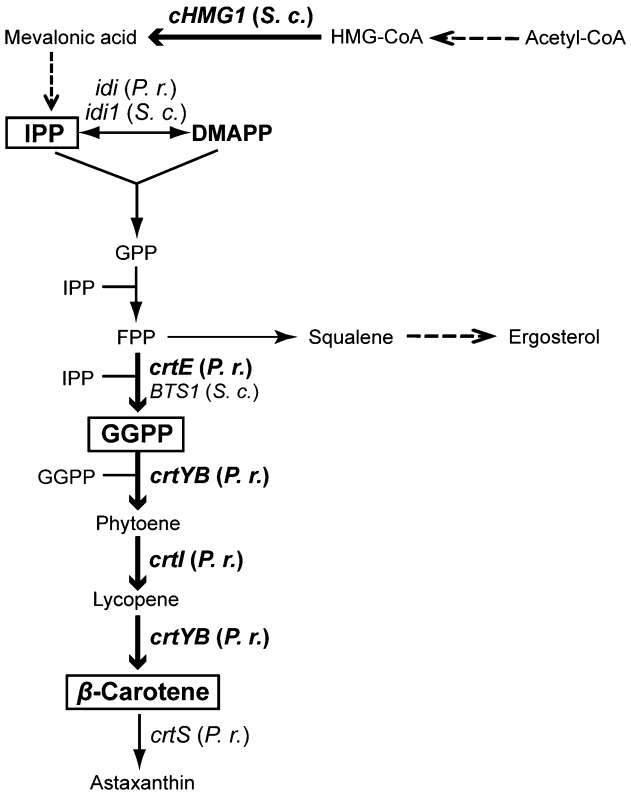

Fig. 1 Biosynthetic pathway of β-carotene in engineered *S. cerevisiae*. The *dashed lines* indicate multiple step reactions. The important genes involved in this pathway are expressed, including *crtE*, *crtYB* and *crtI* genes from *P. rhodozyma* and *cHMG1* gene from *S. cerevisiae*. *IPP* isopentenyl pyrophosphate, *DMAPP* dimethylallyl pyrophosphate, *GPP* geranyl pyrophosphate, *FPP* farnesyl pyrophosphate, *GGPP* geranylgeranyl pyrophosphate

Materials and methods

Strains and growth conditions

Red yeast *Phaffia rhodozyma* CGMCC 2.1557 was purchased from China General Microbiological Culture Collection Center (CGMCC). *P. rhodozyma* was grown at 20 °C and 200 rpm in YM medium (yeast extract 3 g L^{-1}, peptone 5 g L^{-1}, wort 3 g L^{-1} and glucose 10 g L^{-1}; pH 5.0). *Saccharomyces cerevisiae* strain INVSc1 (diploid, *MAT*a/*MAT*α *his3Δ1/his3Δ1 leu2/leu2 trp1-298/trp1-298 ura3-52/ura3-52*) was used as a host. *S. cerevisiae* strains were grown at 30 °C and 200 rpm in YPD medium or SD medium (Shi et al. 2005). YPD medium was used for the wild-type strain. SD medium was used for the screening or maintenance of transformants. *Escherichia coli* strain DH5α was used for constructing and propagating plasmids.

E. coli strains were grown in LB medium at 37 °C and 200 rpm with 100 mg L^{-1} ampicillin as required.

Extraction of total RNA from *P. rhodozyma*

RNA was extracted from *P. rhodozyma* using the method described previously (Chomczynski and Sacchi 1987) with some modifications. Briefly, the cellular material washed with diethyl pyrocarbonate (DEPC)-treated ddH_2O was suspended in same volume of breaking buffer [2 % (v/v) Triton X-100, 1 % (w/v) SDS, 100 mM NaCl, 10 mM Tris–HCl, pH 8.0, 1 mM EDTA] and phenol–chloroform (v/v, 1:1), and broken by vortexing with glass beads (diameter, 425–600 μm). The released RNA was dissolved into TE (10 mM Tris–HCl, 1 mM Na_2EDTA, pH8.0), treated subsequently with chloroform and precipitated with isopropanol. After being washed with 75 % ethanol and dried, the RNA pellet was finally dissolved in DEPC-treated water and stored at −20 °C.

cDNA synthesis

1 μg of RNA sample was treated with 1 U of RNase-free DNase I (Fermentas, Beijing, China) in 2.5 mM $MgCl_2$ for 30 min at 37 °C. The reaction was stopped by addition of EDTA at a final concentration of 5.0 mM and heating at 65 °C for 10 min. Reverse transcription was performed in a 20 μL mixture containing 1–5 μg of total RNA, 10 pmol of oligo($dT_{15–18}$), each dNTP at a concentration of 0.5 mM, 400 U of RNase inhibitor and 200 U of Moloney murine leukemia virus reverse transcriptase (RevertAid^{TM} First Strand cDNA Synthesis Kit, Tiangen Biotech, Beijing, China). The reaction mixture was pretreated at 25 °C for 5 min, incubated at 42 °C for 60 min and then heated at 70 °C for 5 min.

PCR amplification and plasmids construction

The three carotenoid genes, *crtE*, *crtYB* and *crtI* were amplified from cDNA of *P. rhodozyma* by using Ex Taq polymerase (Takara Bio, Dalian, China). Primers were designed using the *X. dendrorhous* ATCC 24230 mRNA sequence reported by NCBI and are listed in Table 1. All primers were synthesized by Generay Biotech (Shanghai, China). The DNA sequence encoding the catalytic domain of HMG-CoA reductase (*cHMG1*) was amplified from genomic DNA of *S. cerevisiae* INVSc1 by using Ex Taq polymerase. The *cHMG1* has an engineered Met start codon and continues with the natural coding region of *HMG1* from codon 531 (Asp) to the natural stop codon (Donald et al. 1997). The amplified PCR product *Xho*I-*Nco*I-*crtI*-*Kpn*I-*Nhe*I was ligated into *Xho*I/*Nhe*I restriction sites of the vector pFA6a-XN-HIS3-KN and transformed into *E. coli* DH5α, yielding *E. coli* DH5α/pFA6a-*crtI*, thus replaced *HIS3* gene with *crtI* gene. The vector pFA6a-XN-HIS3-KN was derived from a gene-deletion vector pFA6a-HIS3MX6 (Wach et al. 1997) by inserting the *Xho*I-*Nco*I and *Kpn*I-*Nhe*I restriction sites into the 5′- and 3′-termini of *HIS3* gene, respectively. The obtained plasmid pFA6a-*crtI* contained the *crtI* gene expression cassette *TEF* promoter (P_{TEF})-*crtI*-*TEF* terminator (T_{TEF}). The P_{TEF} and T_{TEF} of *Eremothecium gossypii* were obtained from the initial vector pFA6a-HIS3MX6. Similarly, the amplified PCR products *Nco*I-*crtE*-*Kpn*I, *Xho*I-*crtYB*-*Kpn*I and *Nco*I-*cHMG1*-*Nhe*I were ligated into *Nco*I/*Kpn*I, *Xho*I/*Kpn*I and *Nco*I/*Nhe*I restriction sites of the plasmid pFA6a-*crtI*, respectively and transformed into *E. coli* DH5α, thereby resulting the plasmids pFA6a-*crtE*, pFA6a-*crtYB* and pFA6a-*cHMG1*. The plasmids used in this study are listed in Table 2.

Subsequently, the four gene expression cassettes P_{TEF}-gene-T_{TEF} were ligated into *E. coli*/*S. cerevisiae* shuttle vectors (Gietz and Sugino 1988) to construct gene expression plasmids. Firstly, the pFA6a-*crtE* was digested with *Sal*I and *Eco*RI, and ligated into YEplac195 which was similarly digested, resulting in the plasmid YEplac195-*crtE*. Secondly, the pFA6a-*crtYB* was digested with *Bam*HI and *Eco*RI, and ligated into pRS426 which was similarly digested, resulting in the plasmid pRS426-c*rtYB*. Thirdly, the *Bam*HI-P_{TEF}-*crtE*-T_{TEF}-*Spe*I fragment digested from pFA6a-*crtE* and *Spe*I-P_{TEF}-*crtYB*-T_{TEF}-*Eco*RI fragment digested from pRS426-c*rtYB* were ligated into the *Bam*HI/*Eco*RI restriction sites of the vector YEplac195, resulting in the plasmid YEplac195-*crtE*-c*rtYB*. Next, the PCR product *Sal*I-P_{TEF}-*crtI*-T_{TEF}-*Sal*I was digested with *Sal*I and ligated into *Sal*I restriction site of vector YEp351, resulting in the plasmid YEp351-*crtI*. Finally, the pFA6a-*cHMG1* was digested with *Bam*HI and *Eco*RI, and ligated into pRS200 which was similarly digested, resulting in the plasmid pRS200-*cHMG1*. All plasmids were verified by restriction enzyme digestion and target gene amplification. All the restriction enzymes of *Xho*I, *Nco*I, *Kpn*I, *Nhe*I, *Sal*I, *Eco*RI, *Bam*HI, *Spe*I and T4 DNA ligase were purchased from Fermentas. The *crtE* and *crtYB* genes cloned into vector YEplac195, as well as *crtI* gene cloned into vector YEp351 were then sequenced by the Sangon Company (Shanghai, China) using universal pUC primers. The constructs and empty vectors were transformed into *S. cerevisiae* INVSc1 by lithium acetate method (Gietz and Woods 2002) and selected on SD plates with appropriate amino acids, yielding the recombinant strains INVSc1/YEplac195-*crtE* (Sc-E), INVSc1/pRS426-c*rtYB* (Sc-YB), INVSc1/YEp351-*crtI* (Sc-I), INVSc1/pRS200-*cHMG1* (Sc-H), INVSc1/YEplac195-*crtE*-c*rtYB*/YEp351-*crtI* (Sc-EYBI) and INVSc1/YEplac195-*crtE*-c*rtYB*/YEp351-*crtI*/pRS200-*cHMG1* (Sc-EYBIH), as well as the control strains INVSc1/YEplac195

Table 1 Primers for amplifying target genes from yeast and for quantitative PCR

Primer	Oligonucleotide sequence	Restriction site
For gene cloning		
crtI-F	GATA**CTCGAG**CA**CC**<u>**ATG**</u>**G**<u>GAAAAGAACAAGATCAGG</u>	*Xho*I- *Nco*I
crtI-R	GATC**GCTAGCGGTACC**<u>TCA</u><u>GAAAGCAAGAACACCAACG</u>	*Nhe*I-*Kpn*I
crtE-F	GACTCA**CC**<u>**ATG**</u>**G**<u>AAGATTACGCGAACATCCTC</u>	*Nco*I
crtE-R	GATA**GGTACC**<u>TCA</u><u>CAGAGGGATATCGGC</u>	*Kpn*I
crtYB-F	GATC**CTCGAG**<u>CCCAACAGATAGAGTTTTTGTC</u>	*Xho*I
crtYB-R	GATA**GGTACC**<u>TTA</u><u>CTGCCCTTCCCATCC</u>	*Kpn*I
hmg1-F	GCTG**CC**<u>**ATG**</u>**G**<u>ACCAATTGGTGAAAACTGAAG</u>	*Nco*I
hmg1-R	GACC**GCTAGC**<u>TTA</u><u>GGATTTAATGCAGGTGACGG</u>	*Nhe*I
SalI-P_{TEF}-F	CAAA**GTCGAC**CGCCAGATCTGTTTAGCTTG	*Sal*I
SalI-T_{TEF}-R	GCCA**GTCGAC**CTGGATGGCGGCGTTAGTATC	*Sal*I
For quantitative PCR		
18S-qF	GTCGGGGGCATCAGTATTC	
18S-qR	GACGGTATCTGATCATCTTCG	
kgd1-qF	GACCCATCTTCAGTCCATG	
kgd1-qR	CTAAGGGAGCTGCTTCGGTAC	
crtI-qF	ATGGGAAAAGAACAAGATCAGG	
crtI-qR	CCATCTCGCTCGATTAAAGAG	
crtE-qF	GATTACGCGAACATCCTCAC	
crtE-qR	GATCCTCCTTCTTGACATCC	
crtYB-qF	CTCTCGCATATTACCAGATCC	
crtYB-qR	CTGATGATCCATGAGTCCCAT	
hmg1-qF	CGAGCTCATCAGGACCTTC	
hmg1-qR	GAATAACCAAGGCAGCGAC	

The restriction sites are in boldface. The sequences corresponding to the genomic DNA of *P. rhodozyma* are underlined. The start and stop codons are boxed

Table 2 Plasmids used in this study

Plasmid	Description	Source
pFA6a-HIS3MX6	*S. cerevisiae* gene deletion vector, *HIS3*, Ap^r	Wach et al. (1997)
pFA6a-XN-HIS3-KN	*Xho*I-*Nco*I and *Kpn*I-*Nhe*I sites were inserted into 5′- and 3′-termini of *HIS3* gene in pFA6a-HIS3MX6	This study
pRS426	*E. coli/S. cerevisiae* shuttle vector, *URA3*, 2 μm, Ap^r	ATCC
YEplac195	*E. coli/S. cerevisiae* shuttle vector, *URA3*, 2 μm, Ap^r	ATCC
YEp351	*E. coli/S. cerevisiae* shuttle vector, *LEU2*, 2 μm, Ap^r	ATCC
pRS200	*E. coli/S. cerevisiae* shuttle vector, *TRP1*, *CEN*, Ap^r	ATCC
pFA6a-*cHMG1*	DNA fragment for catalytic domain of Hmg1 from *S. cerevisiae* in pFA6a-XN-HIS3-KN	This study
pFA6a-*crtE*	cDNA of *crtE* from *P. rhodozyma* in pFA6a-XN-HIS3-KN	This study
pFA6a-*crtI*	cDNA of *crtI* from *P. rhodozyma* in pFA6a-XN-HIS3-KN	This study
pFA6a-*crtYB*	cDNA of *crtBY* from *P. rhodozyma* in pFA6a-XN-HIS3-KN	This study
pRS426-*crtYB*	P_{TEF}-*crtYB*-T_{TEF} in pRS426	This study
YEplac195-*crtE*	P_{TEF}-*crtE*-T_{TEF} in YEplac195	This study
YEplac195-*crtE-crtYB*	P_{TEF}-*crtE*-T_{TEF} and P_{TEF}-*crtYB*-T_{TEF} in YEplac195	This study
YEp351-*crtI*	P_{TEF}-*crtI*-T_{TEF} in YEp351	This study
pRS200-*cHMG1*	P_{TEF}-*cHMG1*-T_{TEF} in pRS200	This study

(Sc-195), INVSc1/pRS426 (Sc-426), INVSc1/YEp351 (Sc-351), INVSc1/pRS200 (Sc-200) and INVSc1/YEplac195/YEp351/pRS200 (Sc-vector).

Carotenoid production by *S. cerevisiae*

For the carotenoid production experiments, the recombinant *S. cerevisiae* cells were precultured in synthetic complete (SC) medium lacking appropriate amino acids at 30 °C and 200 rpm for 16 h. The preculture was inoculated into 25 mL of SC medium to a final absorbance (A_{600}) of 0.1 and shaken at 30 °C for 120 h, or at 20 °C for 120 h, or firstly at 30 °C for 48 h and subsequently at 20 °C for 72 h. For blocking the ergosterol biosynthetic pathway, fluconazole was added to a final concentration of 60 mg L^{-1} after cells were cultivated at 30 °C for 12 h, and then cells were cultivated continuously at 30 °C for 36 h and finally at 20 °C for 72 h.

Carotenoids were extracted from the cultured cells with hexane as described previously (Verwaal et al. 2007) and used for analysis by high-performance liquid chromatography (HPLC). HPLC separation and quantization were performed on a symmetry C_{18} column (3.9 × 150 mm) eluted isocratically with acetonitrile–dichloromethane (v/v, 75:25) at a flow rate of 1 mL min^{-1}. The separated carotenoids were detected at 450 nm and spectra were recorded online. The dry cell weight (DCW) of a sample was measured by taking the same culture volume as used for the carotenoid extraction, drying it by vacuum oven at 37 °C, and measuring the weight of the dried yeast cells on an analytical balance.

Quantification of mRNA in recombinant *S. cerevisiae*

The mRNA transcription levels of *cHMG1* and *crt* genes in recombinant *S. cerevisiae* during fermentation were determined by real-time PCR as described previously (Shi and Li 2011). *S. cerevisiae* was precultured in SC medium at 30 °C for 16 h and then cultured in SC medium at 30 or 20 °C for 16 or 24 h. Total RNA was extracted from the harvested cells. DNA was digested with DNase I and mRNA was reverse transcribed using a RevertAid^{TM} First Strand cDNA Synthesis Kit (Tiangen Biotech) with oligo($dT_{15–18}$) primers. Real-time PCR was carried out in an ABI StepOne real-time PCR system (Applied Biosystems, USA). Amplification assay was performed using RealMasterMix (SYBR Green) kit (Tiangen Biotech) with the quantitative PCR (qPCR) primers (Table 1). The specificity of the PCR products was confirmed by melting curve analysis and relative gene expression analysis was performed by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001).

Results

In order to express the *P. rhodozyma* enzymes involved in β-carotene biosynthesis in *S. cerevisiae*, the expression

plasmids YEplac195-*crtE*-c*rtYB* and YEp351-*crtI* were introduced into *S. cerevisiae* INVSc1 cells, yielding the recombinant strain Sc-EYBI. Sc-EYBI was further transformed with the expression plasmid pRS200-*cHMG1*, yielding the strain Sc-EYBIH. The *crtE*, *crtYB* and *crtI* cDNAs of *P. rhodozyma* CGMCC 2.1557 cloned into YEplac195 and YEp351 were sequenced before these plasmids were transformed into INVSc1. The sequence identities of *crtE*, *crtYB* and *crtI* cDNA corresponded to the *X. dendrorhous* ATCC 24230 cDNAs 1129/1131, 2018/2022 and 1736/1749, respectively, with two differences (G988A and C1116T) existing in *crtE* cDNA, four differences (C66T, T187C, C425T and C1359T) existing in *crtYB* cDNA and thirteen differences (A493G, G668A, C1281A, C1308T, C1407T, T1413C, G1503A, G1561A, A1578G, A1623G, T1644C, G1679A and G1695A) existing in *crtI* cDNA. The sequence identities of CrtE, CrtYB and CrtI proteins between *X. dendrorhous* ATCC 24230 and *P. rhodozyma* CGMCC 2.1557 strains were 375/376, 671/673 and 577/582, respectively, with one variation (E330 K) existing in CrtE protein, two variations (Y63H and S142L) existing in CrtYB protein and five variations (I165V, G223D, H427Q, D521N and R560K) existing in CrtI protein. The β-carotene production in expression strains Sc-EYBI and Sc-EYBIH were compared with that in the control strain Sc-vector.

Temperature influences the cell growth in recombinant *S. cerevisiae*

Cell growth of the recombinant *S. cerevisiae* strains was investigated first. Compared to the control strain Sc-vector and wild-type strain INVSc1, the *crt* genes expressing strain Sc-EYBIH exhibited different growth pattern (Fig. 2a, b), unlike previous reports that over-expression of carotenogenic genes derived from *X. dendrorhous* in *S. cerevisiae* did not influence cell growth (Verwaal et al. 2007; Yan et al. 2012). All the INVSc1, Sc-vector and Sc-EYBIH strains grew faster at 30 °C and slower at 20 °C. Compared to the wild-type strain INVSc1, the empty-vector control Sc-vector grew slowly and the final DCW of Sc-vector culture was about 60 % of that of INVSc1 culture. At the early period of cultivation, the growth rate of Sc-EYBIH was slower than that of Sc-vector. It took Sc-EYBIH much more time to grow into log phase (10 h) and stationary phase (48 h), i.e. 2–8 and 23–33 h more than that control strain. While after mid-log phase of cultivation, Sc-EYBIH grew faster, regardless of the temperature. After 81 h, the DCW of Sc-EYBIH culture (2.9 ± 0.2 mg mL^{-1} at 30 °C and 1.8 ± 0.3 mg mL^{-1} at 20 °C) was nearly equal to that of INVSc1 culture (2.8 ± 0.2 mg mL^{-1} at 30 °C and 1.9 ± 0.2 mg mL^{-1} at 20 °C) and was much higher than that of Sc-vector culture (1.8 ± 0.2 mg mL^{-1} at 30 °C and 1.2 ± 0.1 mg mL^{-1} at 20 °C), showing the promoting effect of the expressed *crtE*, *crtYB*, *crtI* and *cHMG1* genes on the final cell growth of recombinant *S. cerevisiae*. However, the single gene expression strains Sc-E, Sc-YB, Sc-I and Sc-H showed similar growth pattern and cell density with that of their control strains Sc-195, Sc-426, Sc-351 and Sc-200, respectively (data not shown).

Temperature influences the β-carotene accumulation in recombinant *S. cerevisiae*

The β-carotene accumulation in recombinant *S. cerevisiae* was investigated. As expected, the wild-type *S. cerevisiae* strain INVSc1 and the control strain Sc-vector did not accumulate β-carotene, because they do not possess the

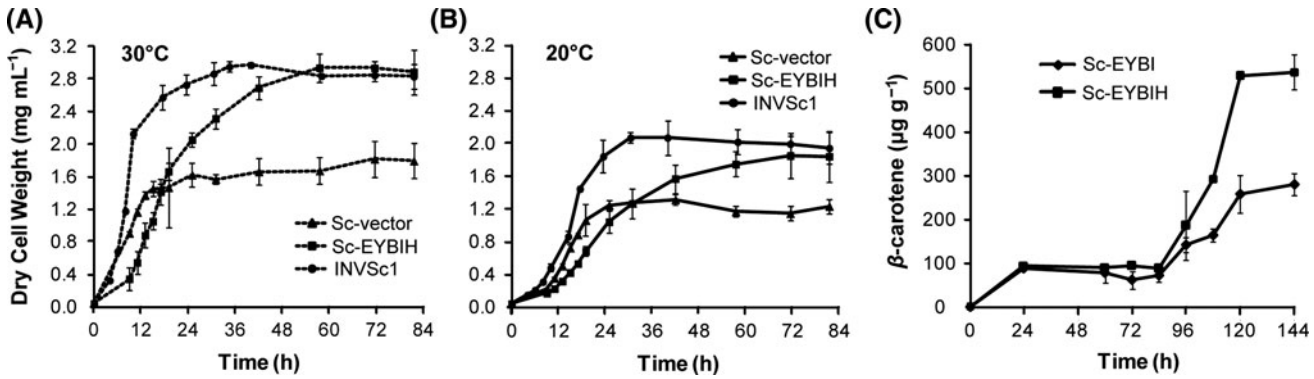

Fig. 2 Cell growth and β-carotene accumulation of *S. cerevisiae* strains. **a** Cell growth of *S. cerevisiae* at 30 °C (*dotted lines*). **b** Cell growth of *S. cerevisiae* at 20 °C (*solid lines*). *Circles* wild-type strain INVSc1; *triangles* control strain Sc-vector; *squares crt* genes expressing strain Sc-EYBIH. **c** Time course of β-carotene accumulation in *crt* genes expressing strains Sc-EYBI (*diamonds*) and Sc-EYBIH (*squares*) at 20 °C. *S. cerevisiae* cells that were precultured in SC medium at 30 °C for 16 h were inoculated into SC medium to give an A_{600} of 0.1 and cultivated aerobically at 30 or 20 °C. Cell growth was monitored by measuring the dry cell weight of cultured cells. β-carotene was extracted from the cultured cells and its concentration was measured by HPLC. Averages in three independent experiments are provided

β-carotene biosynthetic pathway, whereas the *crt* genes expressing strains Sc-EYBI and Sc-EYBIH could accumulate β-carotene. Overexpression of *crtE*, *crtYB* and *crtI* resulted in yellow colonies on SC-Ura-Leu plate at 20 °C. Additional overexpression of *cHMG1* from *S. cerevisiae* resulted in orange colonies (picture not shown). HPLC analysis confirmed that when cultured at 30 °C for 5 days, small amount of β-carotene was produced in Sc-EYBI cells (4.3 ± 2.5 μg g^{-1} DCW) and a little higher amount of β-carotene was produced in Sc-EYBIH cells (18.6 ± 7.6 μg g^{-1} DCW). Interestingly, when initially cultivated at 30 °C for 2 days and subsequently cultivated at 20 °C for 3 days, the recombinant Sc-EYBI and Sc-EYBIH accumulated 40.3 ± 1.2 and 150.9 ± 46.0 μg g^{-1} DCW of β-carotene, respectively, 8.4- and 7.1-fold higher than that cultivated at 30 °C all the time. Furthermore, when 60 mg L^{-1} of fluconazole was utilized, β-carotene concentration in Sc-EYBIH increased to 236.9 ± 17.8 μg g^{-1} DCW. Fluconazole is an inhibitor of ergosterol biosynthetic pathway and biosynthesis of ergosterol also requires prenyl pyrophosphates as a precursor (Fig. 1). Most interestingly, when cultured at 20 °C for 5 days, the recombinant *S. cerevisiae* cells accumulated much higher levels of β-carotene, with Sc-EYBI 258.8 ± 43.8 μg g^{-1} DCW and Sc-EYBIH 528.8 ± 13.3 μg g^{-1} DCW.

β-carotene accumulation in recombinant Sc-EYBI and Sc-EYBIH strains at 20 °C was then monitored over time. β-carotene concentration increased to about 90 μg g^{-1} DCW after 24 h of cultivation, maintained at such level thereafter until 84 h, then increased rapidly during 84–120 h, and kept at the highest level until 144 h (Fig. 2c). The accumulation rate of β-carotene in Sc-EYBIH was quite higher than that in Sc-EYBI. Finally, the β-carotene concentration reached to 280.2 ± 25.0 μg g^{-1} DCW in Sc-EYBI and 537.6 ± 40.7 μg g^{-1} DCW in Sc-EYBIH.

Transcription level of genes in recombinant *S. cerevisiae* growing at different temperatures

In order to explain why temperature influences β-carotene biosynthesis in recombinant *S. cerevisiae*, transcription level of *crt* genes and *cHMG1* gene in the cells growing at 20 and 30 °C was measured. Meanwhile, in order to understand the reason for the different accumulation level of β-carotene in two recombinant strains, transcription level of *crt* genes between the strains Sc-EYBI and Sc-EYBIH, as well as transcription level of *cHMG1* gene among the strains Sc-vector, Sc-EYBI and Sc-EYBIH were also analysed. mRNA levels of *crt* and *cHMG1* genes were quantified using the $2^{10-\Delta Ct}$ value from real-time PCR, because the $2^{-\Delta Ct}$ value was too low when comparing the target genes with the housekeeping 18S rRNA gene. Relative mRNA levels of these genes between cells growing at 20 and 30 °C were quantified using the $2^{-\Delta\Delta Ct}$ value.

When the strain Sc-EYBI was cultivated at 30 and 20 °C, mRNA level of *crt* and *cHMG1* genes at 24 h was 1.5–2.8 and 0.9–2.2 times of that at 16 h, respectively (Table 3). When the strain Sc-EYBIH was cultivated at 30 and 20 °C, transcription level of these four genes at 24 h was 0.5–1.0 and 10–35 times of that at 16 h, respectively. Compared to each gene in P_{TEF}-gene-T_{TEF} cassette, the housekeeping gene *KGD1* encoding α-ketoglutarate dehydrogenase of tricarboxylic acid (TCA) cycle was transcribed weakly at both 30 and 20 °C, indicating that genes were transcribed well under the *TEF* promoter and *TEF* terminator. Transcription level of *KGD1* at 20 °C was weaker than that at 30 °C, in accord with the weaker cell growth at 20 °C. As Sc-EYBIH did not grow well at 16 h, 20 °C (Fig. 2b) and β-carotene accumulation was very low before 24 h (Fig. 2c), mRNA level of these genes at 24 h was then compared. Between the two expression strains Sc-EYBI and Sc-EYBIH, the three *crt* genes were transcribed at nearly same level, especially at 30 °C, with *crtYB* transcribed a little weakly in both strains, and *crtE* and *crtI* transcribed a little strongly in both strains (Table 3). However, the mRNA level of *cHMG1* was quite different among the three strains, with the strain Sc-EYBIH much higher than the other two strains Sc-vector and Sc-EYBI (Table 3), indicating that the *cHMG1* was transcribed well in Sc-EYBIH by the centromere vector pRS200, while the original chromosomal *HMG1* was transcribed weakly in Sc-EYBI and Sc-vector. The much higher transcription level of *cHMG1* gene in Sc-EYBIH than in Sc-EYBI might result in the relatively higher β-carotene accumulation in Sc-EYBIH, since the transcription levels of three *crt* genes are similar between them. Interestingly, mRNA levels of *cHMG1*, *crtE*, *crtYB* and *crtI* in Sc-EYBI and Sc-EYBIH cultured at lower temperature 20 °C were a little higher than that cultured at higher temperature 30 °C (Table 3), which might be the partial reason for the higher β-carotene production at 20 °C.

Discussion

In this study, *S. cerevisiae* INVSc1 was engineered to synthesize β-carotene by introducing carotenogenic genes from *P. rhodozyma* and expressing catalytic domain of HMG-CoA reductase gene *cHMG1*. Unlike the slower growth of empty vector control strain Sc-vector, cell growth of engineered strain Sc-EYBIH recovered to nearly the same level of wild-type strain INVSc1, especially after mid-log phase (Fig. 2a, b), likely due to the expressed four carotenogenic genes and final product β-carotene, which could protect cells against oxidative damage because it has antioxidant activity. Sc-EYBIH exhibited higher β-carotene production than the strain Sc-EYBI (Fig. 2c), demonstrating the importance of

Table 3 The expression level of *crt* and *cHMG1* genes in recombinant *S. cerevisiae*

S. cerevisiae strains	Culturing time	Gene analysed	30 °C		20 °C		$2^{-\Delta\Delta Ct}$
			$\overline{Ct}$	$2^{10-\Delta Ct}$ (A)	$\overline{Ct}$	$2^{10-\Delta Ct}$ (B)	B/A
Sc-vector	24 h	*cHMG1*	20.91 ± 0.00	6.3	20.95 ± 0.01	0.49	0.08
		18S rRNA	13.57 ± 0.57	1,024	9.92 ± 0.03	1,024	
Sc-EYBI	16 h	*crtE*	13.89 ± 0.04	13.0	13.36 ± 0.46	24.5	
		crtYB	15.80 ± 0.13	3.5	13.73 ± 0.30	19.0	
		crtI	13.72 ± 0.43	14.7	12.76 ± 0.27	37.1	
		cHMG1	20.89 ± 0.11	0.10	20.02 ± 0.53	0.24	
		KGD1	17.71 ± 0.20	0.92	18.94 ± 0.00	0.51	
		18S rRNA	7.60 ± 0.32	1,024	7.98 ± 0.03	1,024	
Sc-EYBI	24 h	*crtE*	14.07 ± 0.72	32.1	14.89 ± 0.07	32.6	1.02
		crtYB	15.91 ± 0.05	9.0	15.87 ± 0.06	16.6	1.85
		crtI	14.60 ± 0.50	22.2	14.69 ± 0.24	37.4	1.69
		cHMG1	20.90 ± 0.02	0.28	20.81 ± 0.15	0.54	1.91
		18S rRNA	9.08 ± 0.29	1,024	9.92 ± 0.04	1,024	
Sc-EYBIH	16 h	*crtE*	12.77 ± 0.15	34.7	15.93 ± 0.02	1.6	
		crtYB	14.75 ± 0.19	8.8	15.99 ± 0.35	1.5	
		crtI	12.92 ± 0.04	31.3	13.83 ± 0.19	6.7	
		cHMG1	11.87 ± 0.04	64.6	13.77 ± 0.14	7.0	
		KGD1	18.77 ± 0.15	0.54	19.07 ± 0.32	0.18	
		18S rRNA	7.89 ± 0.06	1,024	6.57 ± 0.41	1,024	
Sc-EYBIH	24 h	*crtE*	13.83 ± 0.14	32.8	14.18 ± 0.23	55.0	1.67
		crtYB	15.76 ± 0.20	8.6	15.88 ± 0.09	16.9	1.96
		crtI	14.54 ± 0.32	20.0	13.85 ± 0.12	69.2	3.46
		cHMG1	13.74 ± 0.19	34.9	12.91 ± 0.02	132.8	3.80
		18S rRNA	8.86 ± 0.09	1,024	9.96 ± 0.01	1,024	

Cells were cultured at 20 or 30 °C in SC medium for 16 or 24 h. Total RNA was extracted from the harvested cells, cDNA was synthesized with oligo(dT_{15-18}) primers, and real-time PCR was performed. The specificity of the PCR products was confirmed by melting curve analysis. mRNA level of target gene was determined by the $2^{10-\Delta Ct}$ value. Relative gene expression level between cells growing at 20 and 30 °C was quantified using the $2^{-\Delta\Delta Ct}$ value. Ct is the minimum number of cycles achieved when the accumulated PCR products generate a detectable fluorescence signal. Values are the mean ± standard error of three experiments

cHmg1 for β-carotene biosynthesis, as reported previously by Verwaal et al. (2007) and Yan et al. (2012). HMG-CoA reductase catalyzes a rate-limiting step in mevalonate pathway (Ohto et al. 2009). It is regulated at all the levels of transcription, translation, post-translational modification and degradation. *S. cerevisiae* HMG-CoA reductase Hmg1 contains the regulatory domain at the amino-terminal region and the catalytic domain at the carboxyl-terminal region. Here, expression of the catalytic domain of Hmg1 resulted in 3.3- and 1.0-fold increment of β-carotene concentration at 30 and 20 °C, respectively. Sc-EYBIH strain can also be improved to synthesis astaxanthin. Furthermore, blockage of ergosterol biosynthetic pathway by fluconazole increased the β-carotene concentration by 57 %, suggesting that redirection of precursor FPP to carotenoid biosynthetic pathway was a successful strategy to increase the β-carotene production.

Interestingly, Sc-EYBIH grew faster at 30 °C and slower at 20 °C (Fig. 2a, b), but accumulated much more β-carotene at 20 °C than at 30 °C, which might be partially due to a little higher transcription level of *crt* genes and *cHMG1* gene at 20 °C (Table 3). However, other reason might also exist, because the β-carotene accumulation at 20 °C was much higher than that at 30 °C. The most possible reason might be the higher enzyme activities of CrtE, CrtYB and CrtI proteins at 20 °C, since these proteins are derived from *P. rhodozyma* which can only survive and accumulate carotenoid at lower temperature 20 °C, but can not survive and accumulate carotenoid at higher temperature 30 °C. However, the enzyme activities of these proteins as well as the thermal control mechanism of β-carotene biosynthesis in *P. rhodozyma* CGMCC 2.1557 and recombinant Sc-EYBIH need to be researched further, since the thermal control mechanism of several

microorganisms had been revealed (Shapiro and Cowen 2012). β-carotene production in Sc-EYBIH is not as high as that reported previously in other recombinant *S. cerevisiae* (Verwaal et al. 2007; Yan et al. 2012) or *E. coli* strain (Yoon et al. 2007). Other metabolic engineering and processing strategies need to be researched on, such as integrative expressing of carotenogenic genes and increasing the activity of Crt proteins in *S. cerevisiae*.

In conclusion, β-carotene production in recombinant Sc-EYBIH was quite higher than that in Sc-EYBI. Most importantly, temperature influenced β-carotene production greatly. Although the recombinant strains grew faster at 30 °C and slower at 20 °C, they accumulated much more β-carotene at 20 °C than at 30 °C, in accordant with a little higher transcription level of *crt* genes and *cHMG1* gene at 20 °C, illustrating the impact of temperature on β-carotene biosynthesis in recombinant *S. cerevisiae*. Such results would be helpful for regulating carotenoid biosynthesis in recombinant *S. cerevisiae*.

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