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Reduction of fatty acid flux at low temperature led to enhancement of β-carotene biosynthesis in recombinant *Saccharomyces cerevisiae*

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Abstract−Transferring the recombinant *S. cerevisiae* T73-63 from 30 °C to 4 °C resulted in 41.4% increment of β-carotene concentration (3.96 mg/g dry cell weight) relative to that of 30 °C, which was accompanied with the accumulation of fatty acid and ergosterol. The comparisons of the transcriptional levels of mevalonate pathway genes indicated that the expressions of *HMG1*, *ERG9*, *ERG19*, *ERG20* and *IDI1* at 4 °C were all higher than those of 30 °C, respectively. This suggested that increased transcriptions of mevalonate pathway genes contribute to the improvement of β-carotene production at low temperature. We also found that supplementation of 30 mg/L triclosan, an inhibitor of fatty acid synthesis, led to further 28.3% enhancement of β-carotene concentration (4.94 mg/g DCW), which was 18.8% higher than that of 30 °C with the same concentration of triclosan. The higher expressional levels of *HMG*, *ERG19* and *ERG20* and the simultaneous increment of ergosterol content (17.8%) suggested that more carbon source was transferred from fatty acid synthesis to mevalonate pathway under the circumstance of appropriately blocking fatty acid synthesis at low temperature (4 °C), which resulted in a higher increment of β-carotene production compared to that of 30 °C. The results of this study collectively suggest that the combination of reducing temperature and adding fatty acid synthesis inhibitors is a potential approach to improve the production of desirable isoprenoid compounds such as carotenoids.

Keywords: β-Carotene, Recombinant Yeast, Low Temperature, Fatty Acid, Triclosan

INTRODUCTION

Carotenoids are a class of pigments of commercial interest with important biological functions. In humans, β-carotene is the precursor for vitamin A; it may function as an anti-oxidant, has protective properties against cancer, and stimulates the immune system [1]. In recent years, genetic microorganisms producing heterogeneous β-carotene such as *E. coli*, *Saccharomyces cerevisiae* and *Candida utilis* were constructed, and their production of pigment was extensively investigated by metabolic engineering strategy [2-4]. This provides an alternative means of β-carotene production. In contrast to other hosts, *S. cerevisiae* exhibit an efficient isoprenoid metabolism and are capable of accumulating large quantities of the triterpenoid ergosterol in their membranes. Furthermore, *S. cerevisiae* strains are considered GRAS organisms, which is desirable for the production of β-carotene for pharmaceutical, nutritional and feed applications [3].

It had been proved that manipulation of mevalonate pathway for sufficient supply of precursors is crucial for enhancing the carotenoid production, such as over-expression of key enzyme 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase and down-regulating the squalene and ergosterol synthesis, which limits ergosterol accumulation and drives more intermediate FPP into the carotenoid biosynthesis [3,5]. Recently, optimizing the fermentation conditions to enhance the production of heterogeneous β-carotene has received attention, such as decreasing pH [6] and direct adding H_2O_2 [7]. Temperature is one of the most important parameters that affect the growth and survival of microorganisms. Shi et al. reported that fermentation of recombinant *S. cerevisiae* at 20 °C could obtain higher β-carotene content compared to that of 30 °C, which was ascribed to a slightly higher transcription levels of the three exogenous *crt* genes in cells growing at 20 °C [8]. During our research, the colony color of recombinant *S. cerevisiae* producing β-carotene became more orange after two days storage on the plate at 4 °C compared to those at normal temperature (30 °C). This implied that more pigment compounds accumulated in cells at low temperature. Different from 20 °C, 4 °C, the near-freezing temperature promotes the formation of a more rigid array of the lipid bilayer, which impairs the function of biological membranes. In bacteria and yeasts, cells have developed a mechanism to encounter this stress through increasing the content of ergosterol and the proportion of unsaturated fatty acids (UFAs) for maintaining the membrane integrity and fluidity [9,10]. Ergosterols and fatty acids synthesis are the branch pathways of heterogeneous β-carotene biosynthesis in yeast (Fig. 1); therefore, it is assumed that heterologous β-carotene content can be simultaneously increased when cells synthesize more ergosterols and fatty acids under cold stress. The observation that strain colony became more orange at 4 °C condition seemed to confirm this deduction. To test this hypothesis, we investigated the effects of different temperatures (30 °C and 4 °C) on β-caro-

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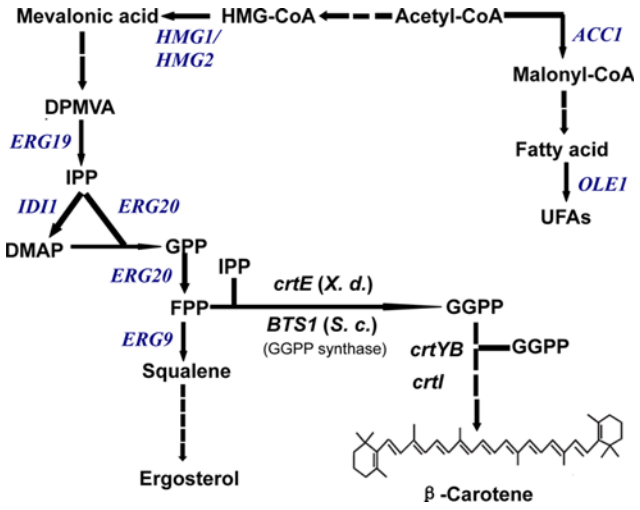

Fig. 1. Biosynthetic pathway of β-carotene in recombinant *S. cerevisiae*. The dashed lines indicate multiple step reactions. *HMG1*: HMG-CoA reductase, *ERG19*: diphosphomevalonate decarboxylase, *IDI1*: IPP:DMAPP isomerase, *ERG20*: FPP synthase. Carotenoids pathway: *CrtE*: GGPP synthase, *BTS1: S. cerevisiae* GGPP synthase, *CrtYB:* Lycopene cyclase, *CrtI:* Phytoene desaturase. DPMVA: diphosphomevalonate; IPP: isopentenyl pyrophosphate; DMAP: dimethylallyl pyrophosphate; GPP: geranyl pyrophosphate; FPP: farnesyl pyrophosphate; GGPP: geranylgeranyl pyrophosphate. UFA: unsaturated fatty acid.

tene production, ergosterol and fatty acid biosynthesis and the transcriptional profile of key mevalonate pathway genes in recombinant *S. cerevisiae* T73-63. The effects of adding triclosan, a fatty acid synthesis inhibitor, on the *β*-carotene production at both temperatures were further investigated.

MATERIALS AND METHODS

1. Yeast Strains and Medium

Recombinant *S. cerevisiae* T73-63, which was used throughout this study [11], was derived from the wine yeast strain T73-4 (*MATa*; *ura3-52*) by being transformed with the integration vectors YIplac211YB/I/E* (11,579 bp). This plasmid carries the carotenoid biosynthesis genes, including the gene *crtYB* (encodes a bifunctional phytoene synthase and lycopene cyclase), *crtI* (phytoene desaturase) and *crtE* (heterologous GGPP synthase) cloned from *Xanthophyllomyces dendrorhous*. The expressions of three genes were driven by the *S. cerevisiae* GPD strong constitutive promoter and CYC1 terminator [3]. The yeast nitrogen base (YNB) medium (0.67% amino acid-free Difco yeast nitrogen base and 2% glucose) was used in the study.

2. Fermentation Conditions

Yeast strains were pre-cultured aerobically in YPD medium at 200 rpm for about 15 h to late-exponential-phase. The initial yeast inoculum of 5×10^5 CFU/mL was inoculated in 500-ml flasks containing 200-ml YNB medium and continuously agitated (180 rpm) at 30 °C. Since the optimal fermentation temperature of the parent wine T73-4 is 28-30 °C, we chose 30 °C as fermentation temperature of the recombinant *S. cerevisiae* T73-63. In cold stress experiment, the yeast cells were grown at 30 °C until 12 h when cells reached mid stage of logarithmic growth. Subsequently, 200 mL YNB broth was divided into two flasks, each of which contained 100 mL fermentation medium and continued to culture at 30 °C and 4 °C for 40 or 50 h, respectively. Considering that temperature can influence dissolved oxygen level in the medium, the cultivation is static. To inhibit the fatty acid synthesis, fatty acid pathway inhibitor triclosan was dissolved in water to make a stock solution (concentration 120 mg/mL, pH adjusted to 9.0 by NaOH; sterilized by filtering) and added into the fermentation medium when the temperature was shifted to 4 °C. Samples were taken at the indicated time points and analyzed for cell growth, ergosterol, fatty acids and *β*-carotene content. All experiments were independently repeated three times, and data in the figures and table are expressed as the averages± standard deviations.

3. Analytical Methods

To determine dry cell weight (DCW), the turbidity in the fermentation broth was measured with a spectrophotometer (DU-70 Beckman Inc., Fullerton, CA) at 660 nm. Dry weight cell concentration was estimated from $OD_{660\,nm}$ by using the following equation: Dry cell weight (g/L)=0.294*$OD_{660\,nm}$+0.602. Content of *β*-carotene was determined according to our previous study [5,6]. Ergosterol content was measured by the method described by Shang et al. [12]. Fatty acids of whole yeast cells were extracted and methyl-esterified as described by Zhang et al. [13]. Fatty acid methyl esters were analyzed by GC-MS (ISO, Thermo, USA) on Thermo Scientific TRACE TR-WaxMS capillary GC column (30 m×0.32 mm×0.25 μm) under a helium carrier gas (1 ml/min). The lipid standards (fatty acid methyl ester mixtures) were purchased from Wako Chemicals or Sigma. Total fatty acid concentrations were calculated as the sum of C8 to C22 (saturated and unsaturated). The content of ergosterol and fatty acid was expressed as mg/g dry cell weight.

4. Quantification of mRNA

For the confirmation of specific gene expression, real-time RT-

Table 1. Primers pairs used for quantitative real-time PCR

Gene	Primer sequence (5′-3′)	Amplicon size
HMG1	FW : CTCCAACACTCTATTTCAAG	205
	RV : CCTCCATTTCGTTCCATCAG	
ERG19	FW : TGAAGATGGTCATGATTCCAT	205
	RV : GGCTTTACGCATGACTTCAA	
IDI1	FW : GGACTAACACATGCTGCTCT	207
	RV : ATGGTTCATTGCTTGGTGCCA	
ERG20	FW : ACTGGTATGCCCACTCATTG	188
	RV : ATCGGCGACCAAGAAGTAAG	
ERG9	FW : CCACGTCTCCATATCTCTTG	201
	RV : CGTGGAAGTGACGCAACAAG	
ACC1	FW : GAGGCACGTCGTTTCTTCTT	170
	RW : GTTGCGACTTGCCTATCATC	
OLE1	FW : GACGCTCGTAGAGGTCTATG	299
	RV : GGTTGGGTACCGATGTAATG	
ACT1	FW : TCCCAGGTATTGCCGAAAGAATG	145
	RV : TGTTGGAAGGTAGTCAAAGAAGCC	

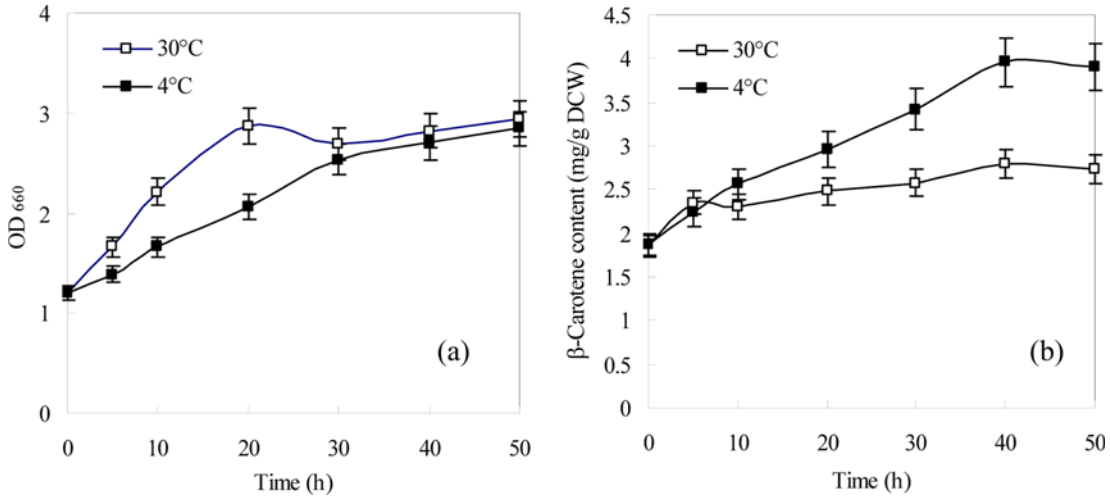

Fig. 2. Effect of temperature downshift on cell growth (a) and β-carotene production (b) by recombinant *S. cerevisiae* T73-63.

PCR analysis was performed. Real-time PCR was carried out in an Applied Biosystem 7300 Real Time PCR system (Applied Biosystems, USA). Amplification assay was performed using Real-MasterMix (SYBR Green) kit (Tiangen Biotech) with the quantitative PCR (qPCR) primers (Table 1). All target genes were obtained from SGD (http://www.yeastgenome.org/). The actin gene, *ACT1*, was used as the control gene due to its stable expression at low temperature [14,15]. 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 [16]. Each RT-PCR analysis was run in triplicate to test consistency.

RESULTS

1. Effects of Temperatures Downshift on Cell Growth and *β*-Carotene Production

To evaluate the effect of low temperature (4 °C) on *β*-carotene production, the mid-logarithmic growth of cells cultivated for 12 h at 30 °C was transferred to 4 °C condition. As shown in Fig. 2, cell growths were inhibited by reducing temperature indicated by the decreased biomass (OD_{660}) relative to those of 30 °C. No significant differences of biomass were observed after 30 h. However, lowering the temperature resulted in a significant stimulation of *β*-carotene production. Their content was gradually increased with fermentation time and the highest content (3.96 mg/g DCW) was obtained at 40 h, which was 41.4% higher than that of 30 °C. These results suggested that low temperature as one stress condition (inhibition of cell growth) is favorable for *β*-carotene biosynthesis.

2. Effects of Temperatures Downshift on Ergosterol and Fatty acids Synthesis

It is known that yeast cells can increase the content of ergosterol and the proportion of unsaturated fatty acids to protect the cells from cold stress damage [9,10]. The intracellular contents of ergosterol and fatty acid at both temperatures were therefore determined and the results shown in Fig. 3 and Table 2, respectively. Similar to *β*-carotene content, the intracellular content of ergosterol at 4 °C also increased compared to those of 30 °C. Especially at 5 h and

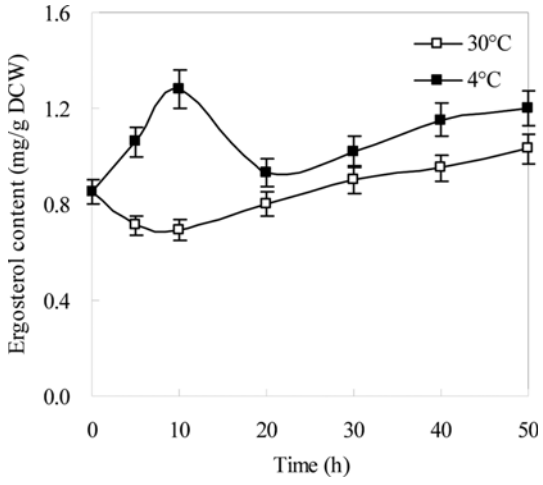

Fig. 3. Effect of temperature downshift on ergosterols biosynthesis by recombinant *S. cerevisiae* T73-63.

10 h, the values were 75.0% and 82.8% higher than those of 30 °C, respectively. In *Saccharomyces cerevisiae*, the major fatty acids in yeast are palmitic acid (C16:0), palmitoleic acid (C16:1), stearic acid (C18:0) and oleic acid (C18:1). We found that fatty acid syntheses were improved after lowering the temperature. The total fatty acids content (C8-C22) of 4 °C *vs* 30 °C was 52.05 *vs* 42.63 mg/g, 65.02 *vs* 41.49 mg/g and 67.69 *vs* 40.48 mg/g at 20 h, 30 h and 40 h, respectively. Note that the increment of fatty acid content was mainly caused by the enhancement of UFAs content (C16:1 and C18:1) not by fatty acids (FAs) because the total UFAs content of 20 h, 30 h and 40 h at 4 °C was 60.94%, 109.44% and 135.75% higher than those of 30 °C, respectively. While, no considerable differences were found in total FAs content. As a result, the UFA/SFA ratios were accordingly increased. These results well confirmed the important roles of ergosterol and UFAs for yeast cells to encounter cold stress.

3. Effects of Temperatures Downshift on Transcriptions of Mevalonate Pathway and Fatty Acid Synthesis Genes

To gain some insight into the mechanism of cold stress improv-

Table 2. Fatty acid composition of recombinant *S. cerevisiae* T73-63 cultured at 30 °C and 4 °C

Fatty acid (mg/g)	10 h	20 h	30 h	40 h
30 °C				
MCFA	4.16±0.26	7.28±0.35	7.07±0.30	9.81±0.46
SFA	12.92±0.66	17.48±1.05	16.45±0.96	14.33±0.82
UFA	5.15±0.35	16.23±1.12	15.89±1.36	14.77±0.94
UFA/SFA	0.40	0.93	0.97	1.03
Total FA	24.28±1.27	42.63±2.52	41.49±2.62	40.48±2.22
4 °C				
MCFA	5.12±0.37	9.50±0.56	12.20±0.71	12.35±0.86
SFA	12.33±0.62	15.10±1.10	18.02±1.35	18.70±1.41
UFA	7.32±0.39	26.12±1.86	33.28±2.06	34.82±2.12
UFA/SFA	0.59	1.73	1.85	1.86
Total FA	26.94±1.38	52.05±3.51	65.02±4.12	67.69±4.39

Total fatty acid concentrations were calculated as the sum of C8 to C22 (saturated and unsaturated). MCFA is the sum of C8 to C14; SFA is the sum of C16 and C18; UFA is the sum of C16:1 and C18:1

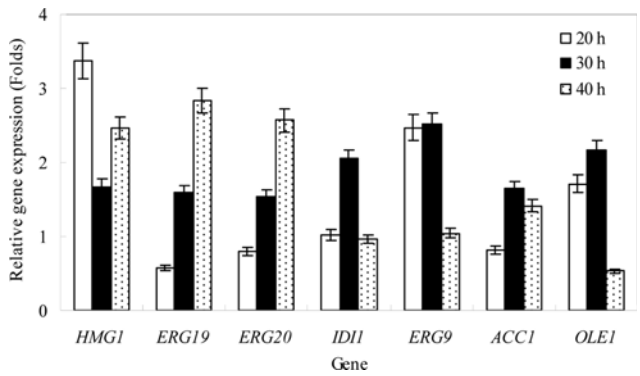

Fig. 4. Effect of temperature downshift on relative genes expressions (4 °C *vs* 30 °C) in mevalonate pathway and fatty acid biosynthesis pathway at different cultivation time.

ing β-carotene production, the transcriptional profiles of several key mevalonate pathway genes at both temperatures were determined, including *HMG1*, *ERG19*, *ERG20*, *IDI1* and *ERG9*. In addition, *ACC1* and *OLE1* involved in fatty acid biosynthesis were also investigated. *ACC1* encoding acetyl CoA carboxylase is the first key regulated gene involved in fatty acid biosynthesis, while *OLE1* encoding the stearoyl-CoA desaturase enzymes catalyze the desaturation of palmitic acids (C16:0) and stearic acids (C18:0) to produce palmitoleic acids (C16:1) and oleic acids (C18:1), respectively. The genes' relative expression ratios (4 °C *vs* 30 °C) of 20 h, 30 h and 40 h are presented in Fig. 4. The expressions of *HMG1*, *ERG20*, *IDI1*, *ERG9* and *OLE1* were all increased in varying levels after reducing temperature compared to those of 30 °C. Especially for *HMG1*, 3.37, 1.67 and 2.46-fold were observed at 20 h, 30 h and 40 h, respectively. The highest ratios of *ERG19* (2.83-fold at 40 h), *ERG20* (2.57-fold at 40 h) and *IDI1* (2.05-fold at 30 h) were also found. In addition, the expressions of *ERG9* involved in ergosterol synthesis (2.47 and 2.51 at 20 and 30 h, respectively) and *OLE1* responsible for unsaturated fatty acid synthesis (1.71 and 2.16 folds at 20 h and 30 h, respectively) were simultaneously induced, which was consistent with the increment of ergosterol and UFAs content at low temperature. There was a remarkable decrease in *OLE1* ratio at 40 h. This might be because excessive accumulation in UFAs content before 40 h generates a signal to repress *OLE1* transcription, because it had been proved that *OLE1* is subject to tight feedback control by intracellular UFAs content at transcription level [9,10]. *HMG1*, *ERG19*, *ERG20* are the key mevalonate genes that control the flux of pathway [17]. Over-expression of *HMG1* and *ERG20* can significantly enhance isoprenois production (including β-carotene) of recombinant *S. cerevisiae* [3,18]. Therefore, it can be concluded that the higher expression levels of mevalonate pathway genes contribute to the increment of β-carotene production at low temperature. We also compared the transcription levels of the three exogenous *crt* genes *crtYB*, *crtE* and *crtI* at both temperatures. The results showed that expressions of three genes were also increased at low temperature (2.71, 2.54 and 1.56 folds at 30 h, respectively, and 2.53, 1.76 and 2.36-fold at 40 h, respectively), which was consistent with the results of Shi et al. [8]. This can be ascribed to the fact that the expressions of carotenoid biosynthesis genes were under the control by the *S. cerevisiae* GPD strong constitutive promoter. Lowering the temperature to 4 °C proved to significantly induce *GPD1* gene and activate the glycerol pathway [19]. These observations suggested that *crt* genes cooperate with increment of mevalonate pathway flux to convert more carbon source to β-carotene.

4. Effect of Adding Triclosan on β-Carotene Production

The obtained results indicated that the content of β-carotene, ergosterol and fatty acid synthesis (mainly UFAs) was simultaneously enhanced by lowing the temperature. Fatty acids and ergosterol are two branch pathways of β-carotene biosynthesis sharing the precursors acetyl-CoA and FPP, respectively. Inhibition of the fatty acid improving astaxanthin production in *Phaffia rhodozyma* had been reported previously [20]. Our previous work confirmed that over-expressing *HMG1* coupled with addition of ergosterol synthesis inhibitor can achieve a higher increment of β-carotene content [5]. Hence, it is assumed that like *P. rhodozyma*, inhibiting fatty acid synthesis could push more precursors acetyl-CoA to carotenoid pathway and improve β-carotene contents in recombinant yeast, while at low temperature, this inhibition could result in a higher

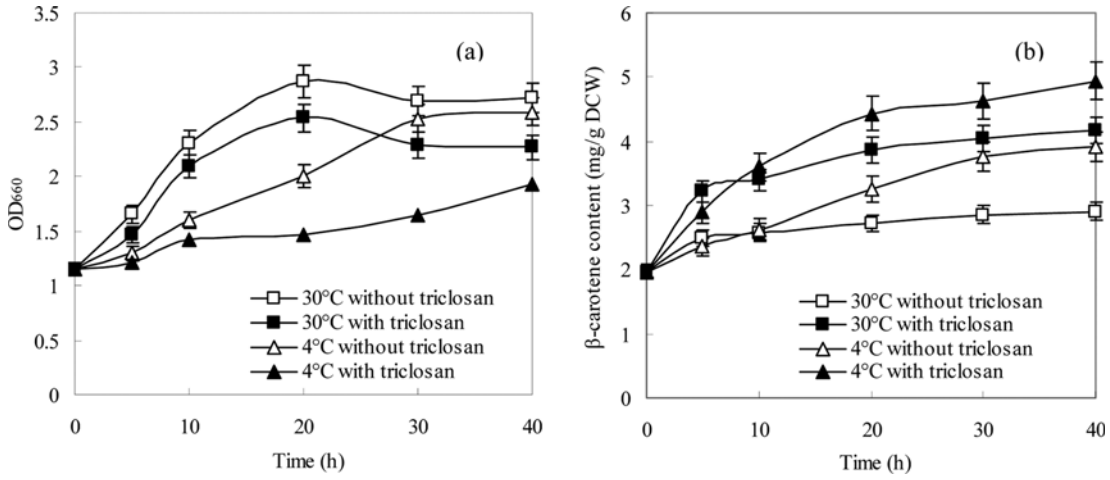

Fig. 5. Effect of 30 mg/L triclosan addition on cell growth (a) and β-carotene production (b) at 30 °C and 4 °C.

increment of β-carotene content compared to that of 30 °C due to the accumulation of more fatty acid at low temperature. To verify the hypothesis, 30 mg/L triclosan, an inhibitor of fatty acid synthesis, was introduced into the medium with downshifting temperature. Triclosan is a broad-spectrum antibacterial agent that inhibits fatty acid synthesis at the enoyl-acyl carrier protein reductase (FabI) step, which can efficiently reduce the content of fatty acids in cells [21,22]. The added concentrations were referenced from the previous study [20]. The results showed that addition of triclosan inhibited the cell growth and the inhibition was more pronounced at 4 °C, which might be due to limited availability of UFAs for yeast cells to resist cold stress. However, triclosan treatment significantly stimulated β-carotene biosynthesis irrespective of temperature (Fig. 5). The highest value (4.94 mg/g DCW) was obtained at 4 °C, which was 28.3% higher than that of the control without triclosan. This value was also 18.8% higher than that of 30 °C. This implied that more carbon sources are transferred to carotenoids pathway under the circumstance of blocking fatty acid synthesis at low temperature. This conclusion was further confirmed by the observation that 17.8% increment of ergosterol content compared to that of 30 °C (data not shown). In addition, we compared the transcriptions of three key genes, *HMG1*, *ERG19* and *ERG20*, at both temperatures (Fig. 6). The increased expression ratios (4 °C *vs* 30 °C) of *HMG1* (1.52 folds), *ERG19* (2.81 folds) and *ERG20* (2.28 folds) suggested that higher expressions of key mevalonate pathway genes contribute to more β-carotene accumulation at low temperature (4 °C). Note that further increasing triclosan concentration to 60 mg/L can result in significant inhibition of cell growth, which negatively influences β-carotene biosynthesis (data not shown).

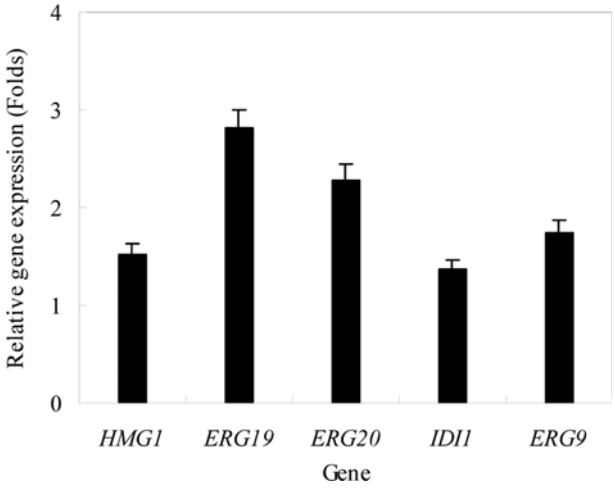

Fig. 6. Effect of 30 mg/L triclosan addition on relative genes expressions in mevalonate pathway (4 °C *vs* 30 °C) at 40 h.

DISCUSSION

Manipulation of central metabolic pathways through metabolic engineering strategy has been widely applied to enhance carotenoid production in recombinant microbes [3,4,23]. Recently, the improvement of heterogeneous β-carotene production by optimizing the fermentation conditions has received more attention [6,7]. The results of thte present study indicated that exposure of recombinant *S. cerevisiae* to cold stress (4 °C) could result in 41.4% increment of β-carotene concentration compared to that of 30 °C. The enhancements were accompanied with the accumulation of fatty acid (mainly UFAs) and ergosterol, confirming that yeast cells require more UFAs and ergosterol syntheses to encounter cold stress (4 °C). Also, the expression levels of key mevalonate pathway genes including *HMG1*, *ERG20*, *IDI1*, *ERG9* and *OLE1* were induced by downshifting temperature. These genes control the flux of mevalonate pathway and over-expression of genes such as *HMG1* and *ERG20* can efficiently enhance isoprenois production in recombinant *S. cerevisiae*. Therefore, the increased β-carotene production by reducing temperature could be ascribed to the higher expressions of mevalonate pathway genes. The mevalonate pathway provides many functional compounds, including dolichols, ubiquinones, ergosterols and fatty acids. Their content would be rapidly regulated to response to the variation of environmental conditions through tight feedback control at multiple levels, including gene transcription, mRNA translation, enzyme activity and protein stability [17]. Beltran et al. found that the expression levels of *ERG20* and *IDI1* involved in sterol

metabolism were higher at 13 °C than those of 25 °C in wine yeast [24], which is consistent with our results. Recently, several authors confirmed that some harsh conditions such as low temperature, oxygen tension, osmotic and acid stress [25,26] can induce the expressions of mevalonate pathway genes (especially *HMG1*). On the other hand, these conditions have been proved to be capable of improving carotenoid synthesis in different wild microorganisms including *Dunaliella* sp, *Haematococcus pluvialis* and *Blakeslea trispora* [27-29]. Whether these conditions favor carotenoid formation involving the increased transcription levels of mevalonate pathway genes is worthwhile to investigate. It is well accepted that collaborative manipulation of multiple mevalonate pathway genes is more effective to improve heterologous isoprenoid production than single gene regulation because overexpression of one desired gene can easily lead to accumulation of toxic intermediates and decreased production of the desired final compound [30,31]. Our results implied that manipulating environmental conditions (such as reducing temperature) seems to be an alternative approach because it can simultaneously induce multiple key genes expressions of mevalonate pathway contributing to isoprenoid biosynthesis.

To further increase β-carotene content, we treated the cells with the inhibitor of fatty acid synthesis triclosan to expect to redirect more acetyl-CoA from fatty acid synthesis to carotenoid branch. The results showed that addition of optimal triclosan concentration could significantly enhance β-carotene concentration, which was consistent with the results of Miao et al. in astaxanthin biosynthesis by *P. rhodozyma* [20]. However, our results indicated that the stimulation was associated with the temperature. The highest β-carotene content was achieved at 4 °C, which was 18.8% higher than that of 30 °C with the same concentration of triclosan. Combining with the results of simultaneous increment of ergosterol contents and higher expression levels of *HMG1*, *ERG19* and *ERG20*, it can be concluded that more carbon source can be transferred from fatty acid syntheses to carotenoid and ergosterol pathways when blocking fatty acid synthesis at 4 °C condition compared to 30 °C.

CONCLUSIONS

The findings presented here indicate that exposure of yeast cells to low temperature (4 °C) can improve β-carotene biosynthesis of recombinant *Saccharomyces cerevisiae*, which involves the increased expression levels of several key mevalonate pathway genes. The combination of reducing temperature and adding fatty acid synthesis inhibitor is an effective strategy to improve the production of desirable isoprenoid compounds such as carotenoids.

ACKNOWLEDGEMENTS

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REFERENCES

1. P. Palozza and N. I. Krinsky, *Methods Enzymol.*, **213**, 403 (1992).
2. S. H. Yoon, S. H. Lee, A. Das, H. K. Ryu, H. J. Jang, J. Y. Kim, D. K. Oh, J. D. Keasling and S. W. Kim, *J. Biotechnol.*, **140**, 218 (2009).
3. R. Verwaal, J. Wang, J. P. Meijnen, H. Visser, G. Sandmann, J. A. Berg and A. J. van Ooyen, *Appl. Environ. Microbiol.*, **73**, 4342 (2007).
4. H. Shimada, K. Kondo, P. D. Fraser, Y. Miura, T. Saito and N. Misawa, *Appl. Environ. Microbiol.*, **64**, 2676 (1998).
5. G. L. Yan, K. R. Wen and C. Q. Duan, *Curr. Microbiol.*, **64**, 159 (2012).
6. H. H. Luo, Y. Y. Niu, C. Q. Duan, H. J. Su and G. L. Yan, *Process. Biochem.*, **48**, 195 (2013).
7. L. H. Reyes, J. M. Gomez and K. C. Kao, *Metab. Eng.*, **21**, 26 (2014).
8. F. Shi, W. B. Zhan, Y. F. Li and X. Y. Wang, *World J. Microbiol. Biotechnol.*, **30**, 125 (2014).
9. J. Tronchoni, N. Rozès, A. Querol and J. M. Guillamón, *Int. J. Food Microbiol.*, **155**, 191 (2012).
10. T. Sakamoto and N. Murata, *Curr. Opin. Microbiol.*, **5**, 206 (2002).
11. G. L. Yan, H. Y. Liang, C. Q. Duan and B. Z. Han, *Curr. Microbiol.*, **64**, 152 (2012).
12. F. Shang, S. H. Wen, X. Wang and T. W. Tan, *J. Biotechnol.*, **122**, 285 (2006).
13. F. Z. Zhang, M. Ouellet, T. S. Batth, P. D. Adams, C. J. Petzold, A. Mukhopadhyay and J. D. Keasling, *Metab. Eng.*, **14**, 653 (2012).
14. S. N. Cao, X. W. Zhang, N. H. Ye, X. Fan, S. N. Mou, D. Xu, C. W. Liang, Y. T. Wang and W. Q. Wang, *Biochem. Biophys. Res. Commun.*, **424**, 118 (2012).
15. H. Du, N. Wu, Y. Chang, X. H. Li, J. H. Xiao and L. Z. Xiong, *Plant Mol. Biol.*, **83**, 475 (2013).
16. K. J. Livak and T. D. Schmittgen, *Methods*, **25**, 402 (2001).
17. J. Maury, M. A. Asadollahi, K. Moller, A. Clark and J. Nielsen, *Adv. Biochem. Eng. Biotechnol.*, **100**, 19 (2005).
18. K. Tokuhiro, M. Muramatsu, C. Ohto, T. Kawaguchi, S. Obata, N. Muramoto, M. Hirai, H. Takahashi, A. Kondo, E. Sakuradani and S. Shimizu, *Appl, Environ, Microbiol.*, **75**, 5536 (2009).
19. J. Panadero, C. Pallotti, S. Rodriguez-Vargas, F. Randez-Gil and J. A. Prieto, *J. Biol. Chem.*, **281**, 4638 (2006).
20. L. L. Miao, S. Chi, Y. C. Tang, Z. Su, T. Yin, G. Guan and Y. Li, *FEMS Yeast. Res.*, **11**, 192 (2011).
21. R. J. Heath, J. R. Rubin, D. R. Holland, E. Zhang, M. E. Snow and C. O. Rock, *J. Biol. Chem.*, **274**, 11110 (1999).
22. E. D. Lund, P. Soudant, F. L. E. Chu, E. Harvey, S. Bolton and A. Flowers, *Dis. Aquat. Org.*, **67**, 217 (2005).
23. W. P. Xie, M. Liu, X. M. Lv, W. Q. Lu, J. L. Gu and H. W. Yu, *Biotechnol. Bioeng.*, **111**, 125 (2014).
24. G. Beltran, M. Novol, V. Leberre, S. Sokol, D. Labourdette, J. Guillamon, A. Mas, J. Francois and N. Rozes, *FEMS Yeast. Res.*, **6**, 1167 (2006).
25. G. Nagy, A. Farkas, A. Csernetics, O. Bencsik, A. Szekeres, I. Nyilasi, C. Vágvölgyi and T. Papp, *BMC Microbiol.*, **14**, 93 (2014).
26. L. T. A. Van, Y. H. Lin, C. L. Miller, S. L. Karna, J. P. Chambers and J. Seshu, *PLoS ONE*, **7**(5), e38171. DOI:10.1371/journal.pone.0038171

(2012).
27. A. Celekli and G. Donmez, *World J. Microbiol. Biotechnol.*, **22**, 183 (2006).
28. M. Orosa, D. Franqueira, A. Cid and J. Abalde, *Bioresour. Technol.*, **96**, 373 (2005).
29. F. Xu, Q. P. Yuan and Y. Zhu, *Process. Biochem.*, **42**, 289 (2007).
30. V. G. Yadav, M. D. Mey, C. G. Lim, P. K. Ajikumar and G. Stephanopoulos, *Metab. Eng.*, **14**, 233 (2012).
31. J. D. Keasling, *Metab. Eng.*, **14**, 189 (2012).