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Enhancement of β-Carotene Production by Over-Expression of HMG-CoA Reductase Coupled with Addition of Ergosterol Biosynthesis Inhibitors in Recombinant *Saccharomyces cerevisiae*

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Abstract In this study, the synergistic effect of over-expressing the 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase gene and adding ergosterol synthesis inhibitor, ketoconazole, on β-carotene production in the recombinant *Saccharomyces cerevisiae* was investigated. The results showed that the over-expression of HMG-CoA reductase gene and adding 100 mg/l ketoconazole alone can result in 135.1 and 15.6% increment of β-carotene concentration compared with that of the control (2.05 mg/g dry weight of cells), respectively. However, the combination of overexpressing HMG-CoA reductase gene and adding ketoconazole can achieve a 206.8% increment of pigment content (6.29 mg/g dry weight of cells) compared with that of the control. Due to the fact that over-expression of the HMG-CoA reductase gene can simultaneously improve the flux of the sterol and carotenoid biosynthetic pathway, it can be concluded that under the circumstances of blocking sterol biosynthesis, increasing the activity of HMG-CoA reductase can result in more precursors FPP fluxing into carotenoid branch and obtain a high increment of β-carotene production. The results of this study collectively suggest that the combination of overexpressing HMG-CoA reductase gene and supplying ergosterol synthesis inhibitor is an effective strategy to improve the production of desirable isoprenoid compounds such as carotenoids.

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Introduction

β-Carotene, a tetraterpenoid containing eight isoprene units, is a known precursor of vitamin A and has been used in food and feed products. In addition, it is used as an antioxidant to reduce cellular or tissue damage and as coloring agent for food products, e.g., margarine, soft drinks, and baked goods [7]. β-Carotene is primarily produced by a wide range of microorganisms such as *Sphingomonas* sp. (bacteria), *Dunaliella bardawil* (algae), *Blakeslea trispora* (fungi), and *Rhodotorula* spp. (yeast) [2, 6, 15]. In recent years, the genetic microorganism producing heterogeneous β-carotene such as *E. coli*, *Saccharomyces cerevisiae*, and *Candida utilis* were constructed, and the production of pigment was investigated by metabolic engineering strategy [10, 12, 19, 22]. In contrast to other hosts, *S. cerevisiae* exhibits an efficient isoprenoid metabolism and is capable of accumulating large quantities of the triterpenoid ergosterol in the membranes. Furthermore, *S. cerevisiae* is regarded as GRAS organism, which is desirable for the production of β-carotene for pharmaceutical, nutritional, and feed applications [10].

It has been shown that sufficient supply of precursors in the mevalonate pathway is crucial for accumulation of carotenoid. Therefore, several metabolic engineering strategies had been applied to enhance pigment production, including (1) over-expression of key enzyme 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase in the mevalonate pathway, which can increase the supply of farnesyl diphosphate (FPP), a common compound that serves in the cell as a precursor of numerous metabolites such as sterols, dolichols, and ubiquinones [16, 19]; (2) down-regulating or inhibiting the squalene and ergosterol synthesis, such as addition of ergosterol biosynthesis inhibitors, which limits ergosterol accumulation and drives

more intermediate FPP into the carotenoid biosynthesis pathway [11, 17].

Besides carotenoids, over-expression of the HMG-CoA reductase gene is found to increase the flux of the sterol biosynthetic pathway as well [16]. Therefore, it is assumed that combining both metabolic strategies, namely, blocking ergosterol biosynthesis under the condition that over-expressing the HMG-CoA reductase can result in more precursor FPP fluxing to carotenoid branch and achieve a higher increment of carotenoid production compared with that of applying any single strategy alone. Therefore, the purpose of the study is to evaluate the effect of ergosterol synthesis inhibitor, ketoconazole, on β-carotene production of the recombinant *S. cerevisiae* SC004 strain and its derivative strain SC017 that over-expresses the catalytic domain of HMG-CoA reductase gene (*tHMG1*). In addition, ergosterol synthesis was also determined.

Materials and Methods

Yeast Strains and Medium

The wild-type strain SC0000 (*MAT*a; *ade2, arg4, leu2-3,112, trp1-289, ura3-52*), obtained from EUROSCARF, Frankfurt, Germany, was used to construct the β-carotene producing strain SC0001. For selection of yeast transformants, minimal synthetic defined medium was used [18].

Plasmid and Yeast Transformation

The integration vector YIplac211YB/I/E* carrying the carotenoid biosynthesis genes and vector YIplac204 *tHMG1* were kindly provided by Verwaal et al. [19]. The vector YIplac211YB/I/E* includes the genes *crtYB* (encodes a bifunctional phytoene synthase and lycopene cyclase), *crtI* (phytoene desaturase), and *crtE* (heterologous GGPP synthase) cloned from *X. dendrorhous*. The expression of three genes was driven by the *S. cerevisiae* GPD strong constitutive promoter and CYC1 terminator. The expression of HMG-CoA reductase gene in the YIplac204 *tHMG1* was under the control of *S. cerevisiae* TDH constitutive promoter and CYC1 terminator. To construct the control plasmid YIplac204 without HMG-CoA reductase gene, the plasmid YIplac204 *tHMG1* was digested with Pst I and a 3,545 bp fragment was obtained. The fragment was linked by T4 DNA ligase directionally and obtained the plasmid YIplac211. The integration vectors YIplac211YB/I/E* were linearized with Stu I (target into *ura3-52* locus) and transformed into the wild-type strain SC0000 to create strains SC0001 using the lithium acetate method [3]. To create the YB/I/E* + tHMG1 strain, the vector YIplac204 *tHMG1* was linearized with EcoR V to target integration into the *trp1-289* locus. The linearized vectors were transformed into SC0001 and obtained the strain SC017 (YB/I/E* + tHMG1). The control yeast SC004 was obtained by introduction of the linearized vector YIplac204 into yeast SC0001.

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 minimal synthetic defined medium and continuously agitated (180 rpm) at 30°C. Ketoconazole was first dissolved in ethanol and then added into the fermentation medium after 10-h cultivation. Final ethanol concentration did not exceed 0.25% (v/v). Samples were taken at the indicated time points and analyzed for the contents of β-carotene and ergosterol.

Analytical Methods

Yeast growth was determined by the dry weight of biomass. β-Carotene contents were determined according to our previous study [21]**.** Ergosterol contents were measured by the method described by Francis et al. [5].

Results

Effect of Over-Expression of *tHMG1* on Cell Growth, β-Carotene Production, and Ergosterol Contents in Recombinant Strains

First, the effect of over-expression of *tHMG1* on cell growth and β-carotene production in strains SC004 (YIplac211YB/I/E* + YIplac204) and SC017 (YIplac211YB/I/E* + YIplac204 *tHMG1*) were investigated (Fig. 1). As expected, the production of β-carotene was significantly increased by over-expression of *tHMG1*, which is in agreement with the results of several other studies [16, 19, 20]. The pigment was gradually accumulated with the biomass increment and the highest pigment concentration (4.82 mg/g dry weight of cells) obtained at 12 h was 135.1% higher than that of strain SC004. However, cell growth was markedly inhibited compared with that of strain SC004, which could be attributed to the metabolic burden induced by accumulation of β-carotene in cell membranes [8, 13]. As a result, the β-carotene yield of SC017 was increased by 81.4% compared with that of SC004.

The ergosterol contents of SC004 and SC017 were also determined (Fig. 2). Unlike β-carotene production, over-expression of *tHMG1* resulted in a slight stimulation of ergosterol synthesis. The ergosterol content of SC017 at 12 and 18 h was only 11.7 and 9.6% higher than those of SC004,

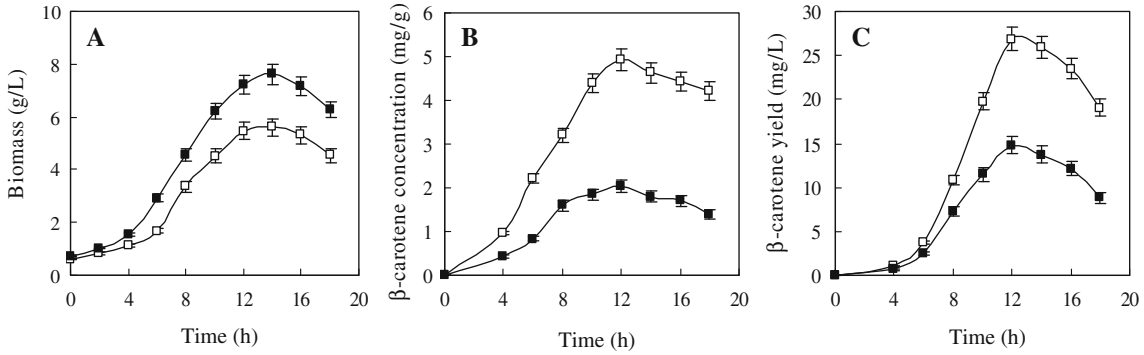

Fig. 1 Cell growth (**a**), β-carotene concentration (**b**), and β-carotene yield (**c**) of recombinant yeasts SC004 (*filled square*) and SC017 (*open square*). Values are the mean ± standard error of three experiments

respectively. This was consistent with the results of Polakowski et al. [14]. This is due to the fact that ergosterol biosynthesis was under tight transcriptional regulation in yeast. Besides HMG-CoA reductase, there are several other regulation steps [9, 14]. In comparison, the expression of carotenoid biosynthesis genes was under the control by the *S. cerevisiae* GPD strong constitutive promoter, which eliminates regulatory effects on targeted gene expression in yeast.

Effects of Adding Ketoconazole on Cell Growth, β-Carotene Production, and Ergosterol Synthesis in Recombinant Strains SC004 and SC017

Previous studies indicated that the addition of ergosterol synthesis inhibitor can increase carotenoid accumulation due to redirection of precursors such as FPP to carotenoid synthesis branch [11, 17]. In order to evaluate the synergistic effect of overexpressing the HMG-CoA reductase gene and adding ergosterol synthesis inhibitor on β-carotene production, the effect of adding ketoconazole on β-carotene production in strains SC004 and SC017 was compared. Ketoconazole is an imidazole derivative that inhibits the cytochrome P-450-dependent C14-demethylase, which is required in the conversion of lanosterol to ergosterol [1]. Considering the fact that initial addition of ketoconazole can significantly inhibit cell growth, 30 and 100 mg/l ketoconazole were added into the medium after cell reached the late-exponential phase (after cultivated for 10 h). As illustrated in Fig. 3, cell growth was slightly inhibited by ketoconazole addition and the inhibition level increased with the enhancement of concentration (Fig. 3a, c). The ketoconazole stimulated the β-carotene production in both recombinant yeasts, and the pigment content kept increasing after ketoconazole addition, especially at the concentration of 100 mg/l. The highest β-carotene concentration in the strain SC004 obtained at 16 h was 15.6% higher than that of the control without

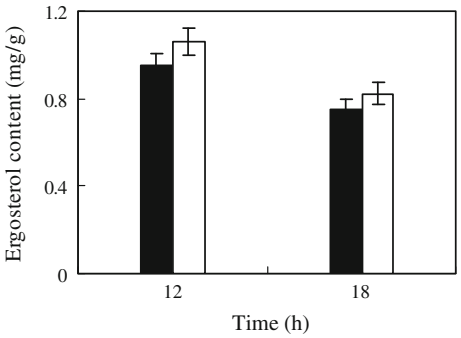

Fig. 2 Ergosterol contents of recombinant yeasts SC004 (*filled square*) and SC017 (*open square*). Values are the mean ± standard error of three experiments

ketoconazole (Fig. 3b). In the case of strain SC017, however, the highest β-carotene concentration (6.29 mg/g dry weight of cells) with 100 mg/l ketoconazole increased by 30.5% compared with that of the control (Fig. 3d). It should be mentioned that this value was 206.8% higher than that of SC004 without ketoconazole. These results suggest that combination of overexpressing HMG-CoA reductase gene and blocking ergosterol synthesis is more effective than anyone alone to increase β-carotene production.

Figure 4 shows the ergosterol contents in SC004 and SC017 with ketoconazole. It can be seen that the addition of ketoconazole inhibited ergosterol synthesis, which was consistent with the results of Sun et al. [17] and Miao et al. [11]. The ergosterol content in SC004 with 30 and 100 mg/l ketoconazole decreased by 14.4 and 22.9% after 4-h cultivation compared with that of the control, respectively (Fig. 4a). The value in strain SC017 was 13.7 and 26.5% lower, respectively (Fig. 4b). These results suggest that the improvement of β-carotene accumulation in both recombinant yeasts is due to the arrest of ergosterol biosynthesis by its synthesis inhibitor and redirection of intermediates (FPP) toward the branch of carotenoid synthesis.

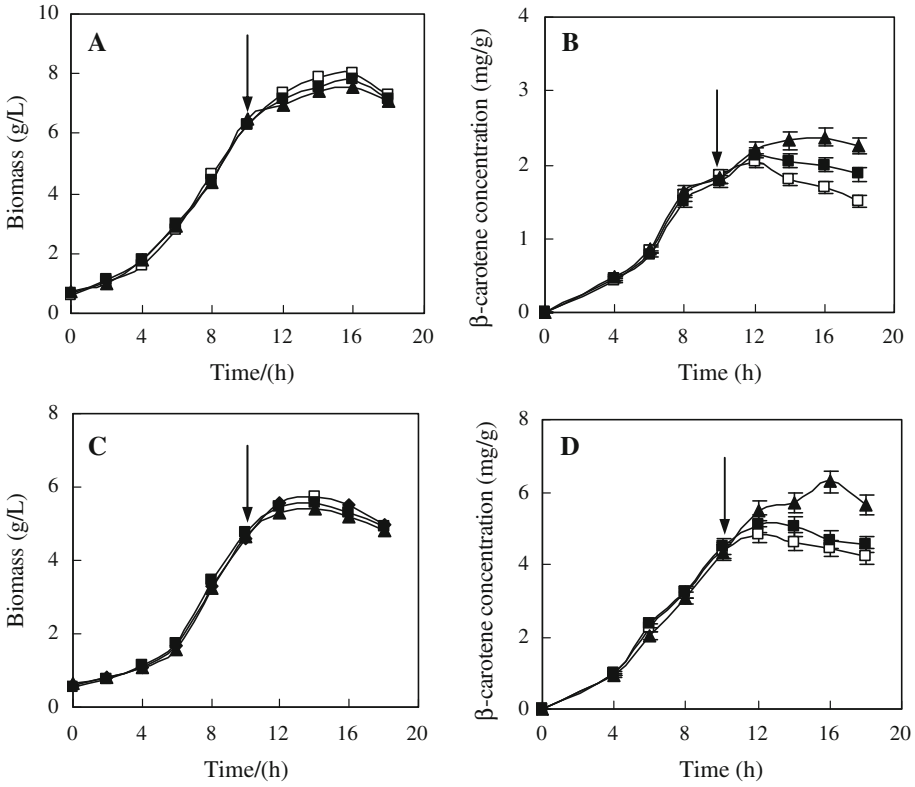

Fig. 3 Effect of 30 and 100 mM ketoconazole on cell growth of strain SC004 (**a**) and SC017 (**c**), and β-carotene concentration of strain SC004 (**b**) and SC017 (**d**): *open square* control; *filled square* 30 mM; *filled triangle* 100 mM. Values are the mean ± standard error of three experiments. The *arrows* indicate the time of ketoconazole addition

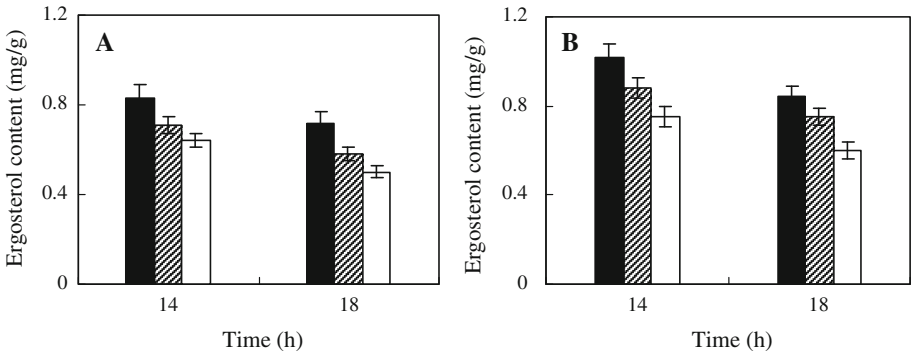

Fig. 4 Effect of 30 and 100 mM ketoconazole on ergosterol contents of recombinant yeasts SC004 (**a**) and SC017 (**b**): *filled square* 0 mM; *square with upper right to lower left fill* 30 mM; *open square* 100 mM. Values are the mean ± standard error of three experiments

Discussion

Manipulation of central metabolic pathways for sufficient supply of precursors has been shown to significantly enhance carotenoid production, including over-expression of key enzymes in the mevalonate pathway, HMG-CoA reductase gene [16, 19], and down-regulating or inhibition of squalene and ergosterol synthesis [11, 17]. With the aim to increase the content of β-carotene in recombinant yeast, the synergistic effect of overexpressing the HMG-CoA reductase gene and adding ergosterol synthesis inhibitor, ketoconazole, on β-carotene production in the recombinant *S. cerevisiae* was investigated herein. The results showed that the over-expression of HMG-CoA reductase gene alone resulted in 135.1% increment of β-carotene concentration compared with that of the control (2.05 mg/g dry weight of cells). The addition of 100 mg/l ketoconazole alone can increase the pigment content by 15.6% compared with that of the control due to limiting the accumulation of ergosterol. However, the combination of overexpressing the HMG-CoA reductase gene and adding ketoconazole can achieve a 206.8% increment of β-carotene concentration (6.29 mg/g dry weight of cells) when compared with that of the control. This value was also 30.5 and 165.4% higher than that of only overexpressing HMG-CoA reductase gene and that of adding ketoconazole alone, respectively. Previous studies found that over-expression of the HMG-CoA reductase gene can simultaneously improve the flux of the sterol and heterogeneous carotenoid biosynthetic pathway [16], as observed in this study (Figs. 1, 2), and the supplement of ergosterol synthesis inhibitor can increase the carotenoid accumulation due to

redirection of precursors to carotenoid synthesis branch [11, 17]. Therefore, it can be concluded that the high increment of β-carotene concentration in strain SC017 is due to more precursors FPP, resulted from over-expression of the HMG-CoA reductase gene, fluxing into the carotenoid pathway under the condition that ergosterol synthesis is blocked. These results suggest that combining the metabolic strategy of over-expression of the HMG-CoA reductase with blocking ergosterol biosynthesis is a feasible tool to improve β-carotene production in microorganism. In addition, overexpressing the HMG-CoA reductase gene is more effective than adding ergosterol biosynthesis inhibitor improving the carotenoid production.

In fact, the strategy of amplifying the rate-limiting reactions and minimizing the metabolic flux to biosynthetically related reaction increasing the targeted compound production in mevalonate pathway has been successfully applied. For example, in order to increase the CoQ_{10} in *E. coli*, Choi et al. [4] deleted the endogenous octaprenyl diphosphate synthase gene (*ispB*), which is responsible for transformation of CoQ_{10} to CoQ_8 and CoQ_9, and over-expressed the decaprenyl diphosphate synthase gene (*dps*) to enhance non-mevalonate pathway flux toward CoQ_{10} biosynthesis pathway. The final results showed that CoQ_{10} production was significantly enhanced by this combined strategy. Compared with gene disruption, adding ergosterol biosynthesis inhibitor is more convenient because its concentration can be easily controlled to avoid or decrease the negative influence on cell growth and metabolism. Considering the fact that numerous valuable isoprenoid compounds biosynthesis share the mevalonate pathway, it is believed that this combined strategy can be applied to increase the production of commercially desirable isoprenoid compounds.

In conclusion, the findings presented here suggest that the combination of overexpressing HMG-CoA reductase gene and supplying ergosterol synthesis inhibitors is an effective strategy to improve the production of desirable isoprenoid compounds such as carotenoids.

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