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Important Role of Catalase in the Production of β-carotene by Recombinant *Saccharomyces cerevisiae* under H_2O_2 Stress

Guo-liang Yan · Heng-yu Liang · Zhi-qun Wang · Xiao-fan Yang · Dan Liu · Jin-fu Liu · Chang-qing Duan

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Abstract The effect of H_2O_2 supplement on cell growth and β-carotene productions in recombinant *Saccharomyces cerevisiae* CFW-01 and CFW-01 *ctt1* deficiency in cytosolic catalase were investigated in shaking flasks. The results showed that supplement of H_2O_2 (0.5 and 1.0 mM) can significantly stimulate the β-carotene production. However, β-carotene levels of CFW-01 *ctt1*Δ under 0.5 and 1 mM H_2O_2 were 16.7 and 36.7% lower than those of CFW-01, respectively. Although lacking cytosolic catalase, no significant differences in cell growth were observed between CFW-01 *ctt1*Δ and CFW-01 under the same level of H_2O_2 stress. These results suggest that β-carotene can act as an antioxidant to protect the recombinant yeast from H_2O_2 oxidative damage in the absence of cytosolic catalase. However, catalase still plays an important role in the production of β-carotene under H_2O_2 stress. If catalase can not timely decompose H_2O_2, the free radicals such as OH· derived from H_2O_2 can result in decrease of β-carotene concentration. Therefore, in the production of β-carotene by H_2O_2 stress, not only the level of oxidative stress, but also the activities of catalase in cells should be considered.

Heng-yu Liang has equally contributed to this work

G. Yan · H. Liang · Z. Wang · X. Yang · D. Liu · C. Duan (✉)
Center for Viticulture and Enology, College of Food Science and Nutritional Engineering, China Agricultural University, Beijing 100083, People's Republic of China
e-mail: chqduan@yahoo.com.cn

J. Liu
Department of Food Science, Tianjin Agricultural University, Tianjin 300384, People's Republic of China

Introduction

β-Carotene, a tetraterpenoid contains eight isoprene units is a known precursor of vitamin A and has been used in food and feed products [10]. It was proved that β-carotene as an antioxidant can reduce cellular or tissue damage initiated by reactive oxygen species (ROS) both in vitro and in vivo [12, 21]. Therefore, the methods that can induce oxidative stress were used to enhance the β-carotene production such as light-illumination [3], high O_2 gas [6], supplement of hydrogen peroxide (H_2O_2) [9] and butylated hydroxytoluene [16].

It is well known that antioxidative enzymes such as superoxide dismutases (SOD, EC 1.11.1.6) and catalases (EC 1.15.1.1) play important roles in preventing oxidative damage in aerobic organisms. Their activity levels are associated with the resistant ability of cell to oxidative stress [8, 22]. Therefore, it is interesting to investigate the relationship of β-carotene and antioxidative enzymes in response to oxidative stress. However, until now, few reports on it are available [16, 17]. In addition, considering the protective role of β-carotene in oxidative stress, it is inferred that decrease of antioxidative enzyme activity in cells might increase β-carotene production in oxidative stress. Up to now, the strains used to produce β-carotene were all wild-type strains. Therefore, it is difficult to evaluate the effect of discrepancy in antioxidative enzymes activities on β-carotene production under oxidative stress.

Genetic engineering of non-carotenogenic organisms for the production of existing carotenoids has been intensively explored [13, 23]. The food yeast *S. cerevisiae* is generally recognized as safe and has been used for the production of β-carotene [20]. It is known that the genes in *S. cerevisiae* are easily manipulated such as gene integration and deletion. Therefore, in this study, the recombinant *S. cerevisiae*

producing β-carotene was constructed. In order to investigate the effect of discrepancy in antioxidative enzymes level on β-carotene production under oxidative stress, the CTT1 gene encoding cytosolic catalase, the main catalase in *S. cerevisiae* was further deleted in the recombinant yeast. The effects of exogenous H_2O_2, a typical ROS, on cell growth and β-carotene productions in both recombinant yeasts were examined.

Materials and Methods

Yeast Strain and Culture Conditions

The wild-type strain FY1679-01B (*MAT*a; *ura3-52*), obtained from EUROSCARF, Frankfurt, Germany, was used to construct the β-carotene producing strain CFW-01, CFW-01 *ctt1*Δ, and FY1679-01B *ctt1*Δ deficiency in CTT1 gene. Yeast cells were cultured in 100 ml of YPD medium (2% glucose, 2% peptone, and 1% yeast extract) at 30°C with reciprocal shaking (200 rpm) in 300-ml shaking flask. For selection of yeast transformants, minimal synthetic defined medium was used [19].

Yeast Genetic Manipulations

The integration vectors YIplac211 carrying the carotenoid biosynthesis genes including the gene *crtYB* (which encodes a bifunctional phytoene synthase and lycopene cyclase), *crtI* (phytoene desaturase) and *crtE* (heterologous GGPP synthase) from *Xanthophyllomyces dendrorhous* was kindly provided by Verwaal [20]. The vector was transformed into the FY1679-01B using the lithium acetate method [2], and the recombinant yeast CFW-01 was obtained. CTT1 deletion mutants CFW-01 *ctt1*Δ and FY1679-01B *ctt1*Δ were derived from CFW-01 and FY1679-01B, respectively. The deletion procedure was based on the method developed by Guldener et al. [4]. The specific primers contain the sequences homologous to the CTT1 genes (the forward primer: TCTCATGCCAATAAGATCAATCAGCTCAGC TTCACAAATGCAGCTGAAGCTTCGTACGC and the reverse primer: ATAGTGCTGCCTTAATTGGCACTTGC AATGGACCA AGTCTGCATAGGCCACTAGTGGAT). After transformation with plasmids, cells were plated on synthetic media containing G418 (200 μg/ml). Confirmation of successful integration was performed by diagnostic PCR and catalase activity determination.

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 300 ml flasks containing 100 ml medium and continuously agitated (200 rpm) at 30°C. Samples of 4 ml each were taken at the indicated time points and analyzed for catalase activity and β-carotene concentration.

Analytical Methods

Yeast growth was determined by the absorbance of OD_{660} and dry weight of biomass. Production of β-carotene was quantified by measuring the absorption of the acetone-extracted β-carotene at 450 nm [24]. Catalase activities were determined according to our previous study [22]. One unit of catalase activity is defined as the amount that decomposes 1 μmol H_2O_2/min. The amount of protein in the cell extract was measured using the Coomassie blue method [1].

Results

Comparison of Cell Growth and β-Carotene Production in Both Recombinant Strains

Figure 1 shows that the catalase activities of the four strains in YPD medium. The activities of CFW-01 *ctt1*Δ were only 42.8 and 62.4% of CFW-01 after cultured for 12 and 16 h, respectively. In the case of FY1679-01B *ctt1*Δ, the values were 47.8 and 59.5% of those of FY1679-01B, respectively. These results confirm that the CTT1 gene was successfully deleted in CFW-01 *ctt1*Δ and FY1679-01B *ctt1*Δ. The remained activities come from the peroxisomal catalase encoded by CTA1 gene [11]. There were not significant differences in catalase activities in FY1679-01B and CFW-01, suggesting that the introduction of

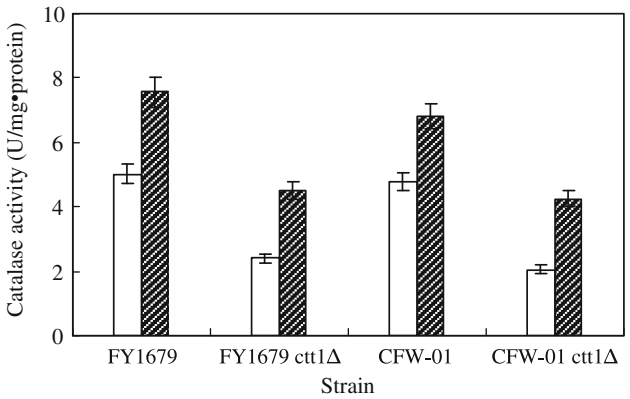

Fig. 1 Catalase activities of the four strains after 12 h (*open square*) and 16 h (*square with upper right to lower left fill*) cultivation in the batch culture. Values are the mean ± standard error of three experiments

β-carotene genes into yeast has little effect on catalase synthesis under normal condition.

The cell growths of four strains and β-carotene concentration in CFW-01 and CFW-01 *ctt1*Δ are showed in Fig. 2. The growths of wild-type strain FY1679-01B and FY1679-01B *ctt1*Δ were slower than those of CFW-01 and CFW-01 *ctt1*Δ. This could be due to the fact that the FY1679-01B and FY1679-01B *ctt1*Δ are uracil auxotroph. No significant differences in cell growth were found between CFW-01 and CFW-01 *ctt1*Δ, implying that the deletion of cytosolic catalase in recombinant *S. cerevisiae* has little effect on cell growth under non-oxidative condition. This is in agreement with the results of previous study [7]. All the four strains reached the late-exponential phase and stationary phase after 12 and 16 h cultivation, respectively.

The β-carotene concentrations increased with the cultivation time, and the maximum values of CFW-01 (2.57 mg/g dry weight) and CFW-01 *ctt1*Δ (2.16 mg/g dry weight) were obtained at 14 h, respectively. This suggests that the deletion of cytosolic catalase gene slightly decrease the β-carotene production.

Effects of H_2O_2 Addition on Cell Growth, β-Carotene Production, and Catalase Activities in Recombinant Yeasts

The effect of H_2O_2 addition on cell growth of the four strains and β-carotene productions and catalase activities in CFW-01 and CFW-01 *ctt1*Δ were further investigated. Considering the fact that initial addition of H_2O_2 can significantly inhibit cell growth, H_2O_2 (0.5, 1.0, and 2 mM) was added into the medium after 12 h cultivation.

As shown in Fig. 3, the addition of H_2O_2 inhibits the cell growth of four yeasts when compared to those of untreated cells (Fig. 2). The inhibitions degrees increased with the enhancement of H_2O_2 level. It should be noted that the inhibition degree in FY1679-01B *ctt1*Δ was evidently higher than those of FY1679-01B, confirming the result that the deletion of cytosolic catalase in *S. cerevisiae* can negatively influence the cell growth under H_2O_2 stress compared to that of wild-type strain [7]. However, no significant differences in cell growth were observed between CFW-01 and CFW-01 *ctt1*Δ. This suggests that β-carotene can substitute the cytosolic catalase as an

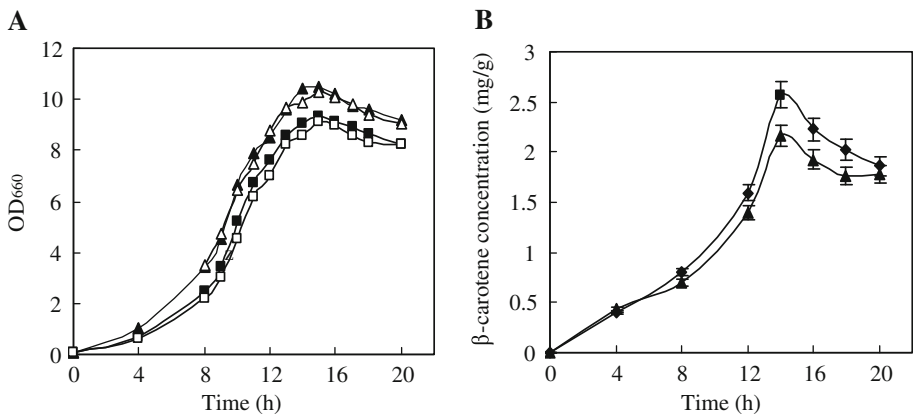

Fig. 2 Cell growth (**a**) and β-carotene concentration (**b**) in FY1679-01B (*filled square*), FY1679-01B *ctt1*Δ (*open square*), CFW-01 (*filled triangle*) and CFW-01 *ctt1*Δ (*open triangle*) in the batch culture. Values are the mean ± standard error of three experiments. In the case of cell growth, error bars are omitted in order to be clear

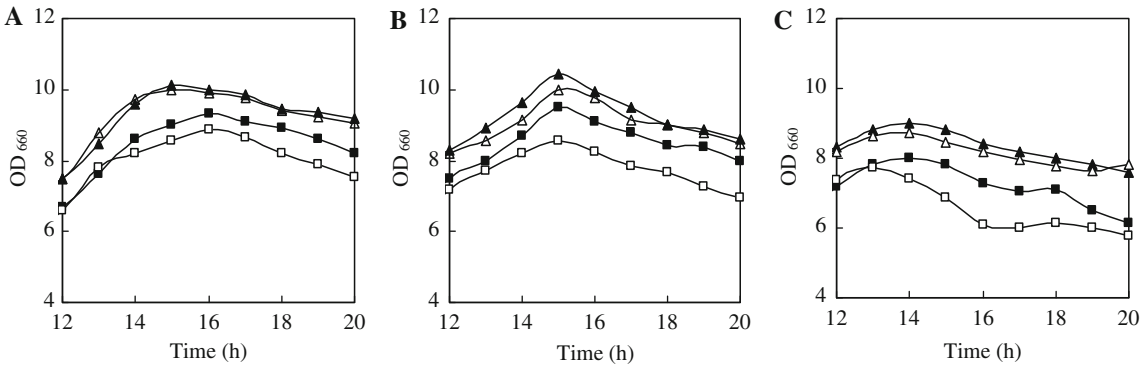

Fig. 3 Cell growth of FY1679-01B (*filled square*), FY1679-01B *ctt1*Δ (*open square*), CFW-01 (*filled triangle*) and CFW-01 *ctt1*Δ (*open triangle*) under different concentrations of H_2O_2, which were added into the medium at 12 h cultivation, respectively. **a**: 0.5 mM; **b**: 1 mM, and **c**: 2 mM. Values are the mean ± standard error of three experiments. The error bars were omitted in order to be clear

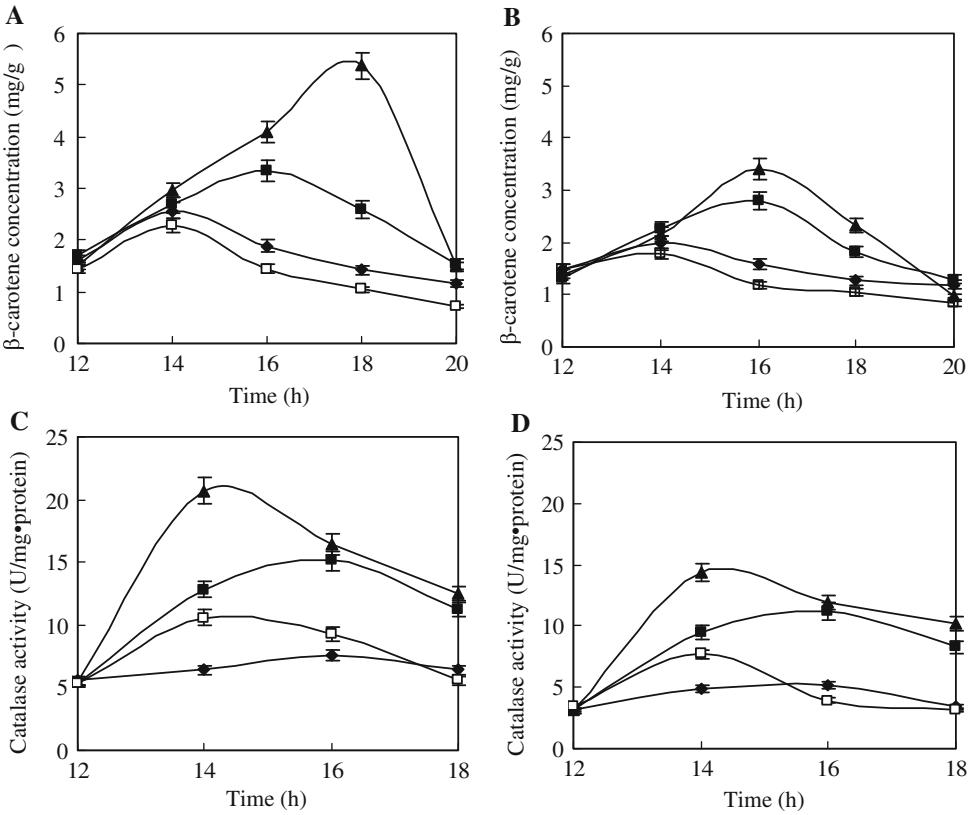

Fig. 4 β-Carotene concentration and catalase activities in CFW-01 (**a**, **c**) and CFW-01 *ctt1*Δ (**b**, **d**) under different concentrations of H_2O_2, which were added into the medium at 12 h cultivation, respectively. *Filled diamond* 0 mM; *filled square* 0.5 mM; *filled triangle* 1 mM, and *open square* 2 mM. Values are the mean ± standard error of three experiments

antioxidant to protect the cells from the H_2O_2 oxidative damage.

The productions of β-carotene were significantly stimulated by H_2O_2 addition (Fig. 4a, b). The pigment concentration of CFW-01 and CFW-01 *ctt1*Δ gradually increased after adding 1 mM H_2O_2 and reached the maximum values after 6 and 4 h of addition, which were 108.9 and 71.7% higher than those of un-treated cells, respectively. Different from the result of cell growth, the β-carotene levels of CFW-01 *ctt1*Δ under 0.5 and 1 mM H_2O_2 were 16.7 and 36.7% lower than those of CFW-01, respectively. When H_2O_2 concentration was further increased to 2 mM, remarkable decrease of β-carotene concentration in both recombinant yeasts were observed, and the values were even lower than those of un-treated cells, respectively.

Similar to β-carotene production, catalase activities were increased by H_2O_2 addition (Fig. 4c, d). The highest activities of both recombinant yeasts were found at 1 mM H_2O_2 after 2 h of addition. Due to the loss of cytosolic catalase, the activities in CFW-01 *ctt1*Δ were all lower than those of CFW-01. Further increment of H_2O_2 level to 2 mM also led to significant decrease of catalase activities, implying that the oxidative stress already overwhelm the protection by catalase and thus inactivate the antioxidative enzyme [18].

Discussion

It is well accepted that β-carotene can act as an antioxidant to protect the microorganism from the oxidative damage. These results showed that the introduction of β-carotene gene into the yeast deficiency in cytosolic catalase can increase the resistance of cell to H_2O_2 stress (Fig. 3), suggesting that β-carotene can substitute catalase to protect the yeast from H_2O_2 oxidative damage. Similar results were reported by Moore et al. [15], who found that although lacking Cu/ZnSOD, *Rhodotorula mucilaginosa* still show higher resistant to the growth-inhibitory effect of duroquinone (induce O_2^- stress) when compared to *S. cerevisiae*. It was also found that the addition of 10 μM β-carotene to medium with *R. mucilaginosa* treated by diphenylamine (an inhibitor of carotenoid) completely prevent the growth inhibition caused by duroquinone and hyperoxia.

It is reasonable to believe that the improving resistance of cells to oxidative stress by introduction of β-carotene genes has a good potential in yeast fermentation processes, because yeast cells are usually exposed to various stresses during fermentation including oxidative stress, ethanol stress, and high sugar concentration. High sugar concentration, ethanol stress, and high temperature are proved to be associated with the oxidative damage [8]. In view of the

high tolerance of *S. cerevisiae* to ethanol toxicity and high osmotic pressure, the recombinant *S. cerevisiae* is a good choice to produce carotenoid in large-scale production, in which some byproduct such as molasses and grape pomace could be used as substrates.

Considering the protective role of β-carotene in oxidative stress, it is inferred that decrease of antioxidative enzyme activity might increase β-carotene production under oxidative stress. Unexpected, our results showed that β-carotene concentration in CFW-01 *ctt1*Δ were all notably lower than those of CFW-01 under the same level of H_2O_2 stress (Fig. 4). Previous studies showed that the high oxidative stress induced by supplying H_2O_2 [5] and heavy metal (Ni) [14] indicated by the reduction of catalase activities, can cause significant decrease of β-carotene concentration. This was attributed to the conclusion that β-carotene is consumed or degraded by free radicals such as hydroxyl radical (OH·). Therefore, Nanou et al. [16] proposed that in the production of β-carotene by oxidative stress, the applied level of oxidative stress is very important. It is well known that the resistant ability of cells to oxidative stress is well associated with the level of antioxidative enzymes. Therefore, the levels of antioxidative enzymes in cells are also important for the production of β-carotene by oxidative stress. Based on these results, the low β-carotene concentration in CFW-01 *ctt1*Δ under H_2O_2 stress can be explained by the conclusion that due to loss of cytosolic catalase, H_2O_2 stress easily exceeds the protective ability of cells and thus more OH· are generated through the Fenton reaction, which result in decrease of β-carotene concentration. The further increment of H_2O_2 level to 2 mM resulting in fall of β-carotene concentration and catalase activities in both recombinant yeasts can also partly prove this conclusion.

Based on these results, it can be concluded that β-carotene can act as an antioxidant to protect the recombinant yeast from H_2O_2 oxidative damage in the absence of cytosolic catalase. However, catalase still plays an important role in the production of β-carotene under H_2O_2 stress. If catalase can not timely decompose the H_2O_2, the free radicals such as OH· derived from H_2O_2 can result in decrease of β-carotene concentration. Therefore, in the production of β-carotene by H_2O_2 stress, not only the level of oxidative stress, but also the activities of catalase in cells should be considered.

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References

1. Bradford M (1976) A rapid and sensitive method of the quantification of microgram quantities of protein using the principle of protein-dye binding. Anal Biochem 72:248–255
2. Burke D, Dawson D, Stearns T (2000) Methods in yeast genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor
3. Georgiou DC, Tairis N, Polycratis A (2001) Production of β-carotene by *Sclerotinia sclerotiorum* and its role in sclerotium differentiation. Mycol Res 105:1110–1115
4. Guldener U, Heck S, Fielder T et al (1996) A new efficient gene disruption cassette for repeated use in budding yeast. Nucleic Acids Res 24:2519–2524
5. Huan XU, Yuan QP (2007) Catalase plays an anti-oxidant role in *Blakeslea trispora*. Liquor Mak 34:54–56
6. Iigusa H, Yoshida Y, Hasunuma K (2005) Oxygen and hydrogen peroxide enhance light-induced carotenoid synthesis in *Neurospora crassa*. FEBS Lett 579:4012–4016
7. Izawa S, Inoue Y, Kimura A (1996) Importance of catalase in the adaptive response to hydrogen peroxide: analysis of acatalasaemic *Saccharomyces cerevisiae*. Biochem J 320:61–67
8. Jamieson DJ (1998) Oxidative stress responses of the yeast *Saccharomyces cerevisiae*. Yeast 14:1511–1527
9. Jeong JC, Lee IY, Kim SW et al (1999) Stimulation of β-carotene synthesis by hydrogen peroxide in *Blakeslea trispora*. Biotech Lett 21:683–686
10. Kim SW, Sco WT, Park YH (1997) Enhanced synthesis of trisporic acids and β-carotene production in *Blakeslea trispora* by addition of non-ionic surfactant, Span 20. J Ferment Bioeng 84:330–332
11. Lushchak VI, Gospodaryov DV (2005) Catalases protect cellular proteins from oxidative modification in *Saccharomyces cerevisiae*. Cell Biol Int 29:187–192
12. Mathews-Roth MM (1984) Porphyrin photosensitization and carotenoid protection in mice; in vitro and in vivo studies. Photochem Photobiol 40:63–67
13. Misawa N, Shimada H (1998) Metabolic engineering for the production of carotenoids in non-carotenogenic bacteria and yeasts. J Biotech 59:169–181
14. Mishra S, Panjwani D, Mishra B et al (2010) Effect of excess nickle on induction of oxidative stress in *Zea mays* L. plants grown in solution culture. Int J Toxicol Pharm Res 2:10–16
15. Moore MM, Breedveld MW, Autor A (1989) The role of carotenoids in preventing oxidative damage in the pigmented yeast, *Rhodotorula mucilaginosa*. Arch Biochem Biophys 270: 419–431
16. Nanou K, Roukas T (2009) Oxidative stress response and morphological changes of *Blakeslea trispora* induced by butylated hydroxytoluene during carotene production. Appl Biochem Biotechnol 10.1007/s12010-009-8712-y
17. Nanou K, Roukas T, Kotzekidou P (2007) Role of hydrolytic enzymes and oxidative stress in autolysis and morphology of *Blakeslea trispora* during β-carotene production in submerged fermentation. Appl Microbiol Biotechnol 74:447–453
18. Piegeolet E, Corbisier PG (1990) Px, SOD and catalase inactivation by AO. Mech Age Dec 51:283–297
19. Verduyn C, Postma E, Scheffers WA et al (1992) Effect of benzoic acid on metabolic fluxes in yeast: a continuous-culture study on the regulation of respiration and alcoholic fermentation. Yeast 8:501–517
20. Verwaal R, Wang J, Meijnen JP et al (2007) High-level production of Beta-carotene in *Saccharomyces cerevisiae* by successive transformation with carotenogenic genes from *Xanthophyllomyces dendrorhous*. Appl Environ Microbiol 73:4342–4350

21. Weitberg AB, Weitzman SA, Clark EP et al (1985) Effects of antioxidants on oxidant-induced sister chromatid exchange formation. J Clin Invest 75:1835–1841
22. Yan GL, Hua ZZ, Du GC et al (2006) Adaptive response of *Bacillus* sp. F26 to hydrogen peroxide and menadione. Curr Microbiol 52:238–242
23. Yoon SH, Lee SH, Das A et al (2009) Combinatorial expression of bacterial whole mevalonate pathway for the production of β-carotene in *E. coli*. J Biotech 140:218–226
24. Yuan LZ, Rouviere PE, Robert A et al (2006) Chromosomal promoter replacement of the isoprenoid pathway for enhancing carotenoid production in *E. coli*. Metab Eng 8:79–90