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Efficient production of lycopene in *Saccharomyces cerevisiae* by expression of synthetic *crt* genes from a plasmid harboring the *ADH2* promoter

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ABSTRACT

Lycopene is an effective antioxidant proposed as a possible treatment for some cancers and other degenerative human conditions. This study aims at generation of a yeast strain (*Saccharomyces cerevisiae*) of efficient productivity of lycopene by overexpressing synthetic genes derived from *crtE*, *crtB* and *crtI* genes of *Erwinia uredovora*. These synthetic genes were constructed in accordance with the preferred codon usage in *S. cerevisiae* but with no changes in amino acid sequences of the gene products. *S. cerevisiae* cells were transformed with these synthetic *crt* genes, whose expression was regulated by the *ADH2* promoter, which is de-repressed upon glucose depletion. The RT-PCR and Western blotting analyses indicated that the synthetic *crt* genes were efficiently transcribed and translated in *crt*-transformed *S. cerevisiae* cells. The highest level of lycopene in one of the transformed lines was 3.3 mg lycopene/g dry cell weight, which is higher than the previously reported levels of lycopene in other microorganisms transformed with the three genes. These results suggest the excellence of using the synthetic *crt* genes and the *ADH2* promoter in generation of recombinant *S. cerevisiae* that produces a high level of lycopene. The level of ergosterol was reversely correlated to that of lycopene in *crt*-transformed *S. cerevisiae* cells, suggesting that two pathways for lycopene and ergosterol syntheses compete for the use of farnesyl diphosphate.

1. Introduction

Lycopene is a polyenic chromophore with 11 double bonds and is used as a color ingredient in many food formulations (Richelle et al., 2002). It is, together with several other carotenoids, an important nutrient since it protects human against cancers (Olson, 1986; Astorg, 1997). A great interest has recently been focused on lycopene due to its preventive activity against several pathologies, such as cardiovascular disease (Rao, 2002), hepatic fibrogenesis (Kitade et al., 2002), solar light-induced erythema (Stahl and Sies, 2002), human papillomavirus persistence (Sedjo et al., 2002) and some types of cancer, such as prostate, gastrointestinal, and epithelial (Clinton, 1998;

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Shi and Le Maguer, 2000). Lycopene plays a role in lung function (Schunemann et al., 2002), as well as in fetal growth (Sharma et al., 2003), besides the synergistic action with other bioactive compounds present in fruits and vegetables (Rao and Agarwal, 2000; Sharoni et al., 2002; Lee et al., 2004).

The instability of lycopene during processes of extraction, handling, and elimination of organic solvents from tomatoes makes the production an extremely delicate task, often requiring successive and complex procedures. This task can be much easier if overproduction of lycopene takes place in microorganisms, such as bacteria and yeasts (Yamano et al., 1994; Kang et al., 2005). A number of reports have described its production in recombinant microorganisms (Misawa and Shimada, 1997; Farmer and Liao, 2000, 2001; Jones et al., 2000; Schmidt-Dannert et al., 2000; Kim and Keasling, 2001; Sandmann, 2003; Lee et al., 2004).

Escherichia coli is regarded as the organism of choice in genetic engineering due to the wealth of genetic information and its high growth rate. However, the production of lycopene on industrial scale in *E. coli* is unlikely because *E. coli* is prone to growth stability problems in which plasmids can be cured during the course of cultivation (Wang et al., 2012). Considerable progress has been made in expressing all of the genes necessary to synthesize lycopene in *E. coli* (Ruther et al., 1997). The coexpression in *E. coli* cells of three exogenous genes, *crtE* for geranylgeranyl diphosphate (GGPP) synthase, *crtB* for phytoene synthase, and *crtI* for phytoene desaturase, is sufficient for the conversion of isopentenyl pyrophosphate (IPP) and farnesyl diphosphate (FPP) to lycopene (Lotan and Hirschberg, 1995). The edible yeast *Candida utilis* was also utilized as a safe organism for the cheap large-scale production of glutathione and RNA (Boze et al., 1992; Ichii et al., 1993, respectively). This yeast accumulates a large amount of ergosterol in the cell during stationary phase, thus, has the potential to produce large amounts of carotenoids by redirecting the carbon flux for the ergosterol biosynthesis into the carotenoid synthesis pathway via FPP. This approach resulted in the production of lycopene at 0.8 mg/g cell dry weight by expressing the three native genes *crtE*, *crtB* and *crtI* derived from *Erwinia uredovora* (Miura et al., 1998b). The same team has further conducted *de novo* biosynthesis of lycopene in *C. utilis* by using the three genes after being modified to match the codon usage of the *C. utilis* glyceraldehyde-3-phosphate dehydrogenase (GAP) gene; expressed in *C. utilis* at high levels (Miura et al., 1998a). This approach resulted in the increase of lycopene production up to 1.1 mg/g dry cell weight.

Alternatively, *Saccharomyces cerevisiae* is an attractive organism to express *crt* genes for lycopene synthesis as compared to *E. coli* and *C. utilis*. It has several properties, which make it a particularly attractive host for the expression of recombinant genes. It is classified as a GRAS (generally regarded as safe) organism. This feature makes it usable for bread making. The yeast cell wall does not contain any toxic components (such as pyrogens in the case of *E. coli*). *S. cerevisiae* is an easy organism to cultivate with no expensive equipment or media required. In *S. cerevisiae*, FPP is naturally synthesized by FPP synthase through the sequential head-to-tail condensations of dimethylallyl diphosphate (DMAPP) and geranyl diphosphate (GPP) with IPP (Fig. 1). FPP synthase is a common enzyme in the early stages of the biosynthesis of isoprenoids, such as sterols and triterpenes. Therefore, it may be feasible that the three genes for lycopene biosynthesis from *E. uredovora* are capable of producing carotenoids, e.g., lycopene in *S. cerevisiae*, which does not naturally synthesize carotenoids. This new pathway is alternative to the naturally occurring sterol pathway (Fig. 1). This can be done by switching the conversion of FPP in sterol biosynthetic pathway for the production of ergosterol to the isoprenyl diphosphate (IPP) pathway for the biosynthesis of lycopene. Synthesis of lycopene in *S. cerevisiae* cells can simply be detected by the color of colonies on agar plates.

The aim of the present study was the generation of recombinant *S. cerevisiae* cell line that synthesizes lycopene at a high level. We constructed three synthetic *crtE*, *crtB* and *crtI* genes based on the respective genes of *E. uredovora* (Misawa et al., 1990) and according to the preferred codon usage of *S. cerevisiae*. In the transformed *S. cerevisiae* cells, the three synthetic *crt* genes were efficiently expressed under control of the *ADH2* promoter and lycopene was synthesized at a high level.

2. Materials and methods

2.1. Strain and growth conditions

Saccharomyces cerevisiae DSY-5 strain (MATalpha leu2 trp1 ura3-52 his3::GAL1–GAL4 pep4 prb1-1122 yeast culture) was obtained from Dualsystems Biotech AG (Schlieren, Switzerland). Master plates of *S. cerevisiae* were prepared and stored at 4 °C for up to 1 month following standard procedures (Sherman et al., 1982). *S. cerevisiae* cell culture was prepared by inoculating single colonies from the master plate of *S. cerevisiae* to 1 ml standard YPD (yeast peptone dextrose) medium (Sambrook et al., 1989) in Eppendorf tubes, then left overnight at 30 °C with shaking (200–250 rpm) following the manufacturer's manual (Dualsystems Biotech AG). Cells were collected by centrifugation at 2500×*g* for 5 min. Pelleted cells were resuspended in 2 ml freezing mixture (YPD medium containing 20% glycerol) and stored at −80 °C.

2.2. Construction of synthetic crtE, crtB, and crtI genes

Synthetic *crtE*, *crtB* and *crtI* genes with optimized codons were constructed by Bioneer Corporation (Daejeon, Republic of Korea; Acc. Nos. KC954271, KC954270 and KC954272, respectively) in reference to the corresponding native genes from *E. uredovora* and the codon preference of *S. cerevisiae*. Table S1 shows the replacement of nucleotides between the native and synthetic genes. This modification resulted in the reduction of GC contents from ~55% to ~35% in each of the three synthetic genes (Fig. S1).

2.3. Construction of recombinant shuttle vectors

The pKS1 shuttle vector of 6648 bp (KickStart™ Dualsystem Biotech AG (Cat. No. P03305, Schlieren, Switzer-

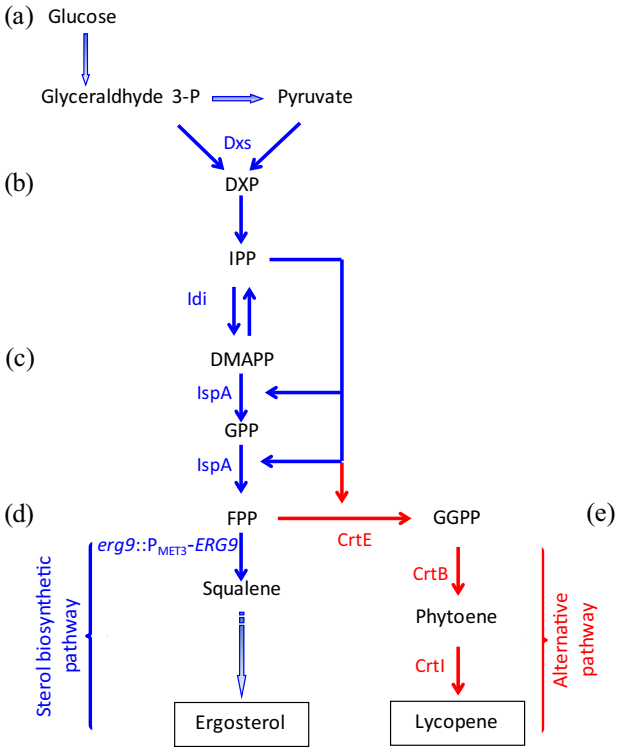

Fig. 1. Possible pathway for ergosterol and lycopene synthesis in *crtEBI*-transformed. *S. cerevisiae*. (a) Glycolysis pathway for glyceraldehyde-3-phosphate and pyruvate synthesis. (b) MEP (2-C-methyl-D-erythritol-4-phosphate) pathway for IPP and DMAPP synthesis. (c) Isoprenyl diphosphate pathway for FPP production. (d) Sterol biosynthetic pathway for ergosterol production. (e) Alternative pathway for lycopene synthesis composed of CrtE, CrtB, and CrtI. The latter pathway, indicated by red color, takes place in non-carotenogenic *S. cerevisiae* upon the introduction of the three *crt* genes and competes with the sterol biosynthetic pathway. Abbreviations: DXP, 1-deoxy-D-xylulose-5-phosphate; IPP, isopentenyl pyrophosphate; DMAPP, dimethylallyl diphosphate; GPP, geranyl diphosphate; FPP, farnesyl diphosphate; GGPP, geranylgeranyl diphosphate. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

land) was utilized for transformation of *S. cerevisiae* cells with the three *crt* genes (Fig. S2). This plasmid dual system included elements for selection and propagation of the plasmid in *S. cerevisiae* and *bacteria* as indicated in the manufacturer's manual. The plasmid harbors the glucose depletion inducible *ADH2* promoter, the *CYC1* terminator, and the *kan*^{r} (for neomycin resistance) gene needed to degrade the aminoglycoside antibiotic G418 (Sigma–Aldrich, Buchs SG, Switzerland, cat. No. A1720). Each of the synthetic and native genes was inserted into the *Sma*I-*Eco*RI site of the pKS1 shuttle vector to generate pKS1-*crtE*, pKS1-*crtB* and pKS1-*crtI* vectors (Fig. S2). Original construct (pKS1) as well as those with native *crt* genes, isolated by PCR from *E. uredovora* (Misawa et al., 1990), were used as controls.

2.4. Transformation of S. cerevisiae with pKS1 plasmid harboring a crt gene

S. cerevisiae cells were transformed with the pKS1 or pKS1 plasmid harboring native- or synthetic-*crt* genes following protocol in the manufacturer's manual (KickStart™ Dualsystem protein expression kit protocol, Schlieren, Switzerland). A 5-ml aliquot of liquid cultures of *S. cerevisiae* was centrifuged at 2500×*g* and pelleted cells were resuspended in 2.5 ml water. Then, 100 μl of resuspended cells were vortexed in 300 μl PEG/LiOAc master mixture that contained either of the pKS1, pKS1-*crtE*, pKS1-*crtB* or pKS1-*crtI* (1.5 μg). Mixtures of cultures with individual pKS1 vectors were incubated at 42 °C for 45 min, and then centrifuged at 700×*g*. Transformed *S. cerevisiae* cells were originally selected in standard growth conditions (30 °C with YPD medium adjusted at pH 7.0 and supplemented with 2.0% glucose and 40 μg/ml G418) following the manufacturer's manual. Transformed cells with pKS1 (control) and those with individual pKS1-*crtE*, pKS1-*crtB* or pKS1-*crtI* vector, which showed robust growth on YPD plates, were recovered and stored as glycerol stocks at −80 °C. We also tested the growth behavior of *S. cerevisiae* DSY-5 cells in the presence of G418 (0, 20, 40, 60, 80 or 100 μg/ml) in the medium to detect the concentration inhibiting the growth of *S. cerevisiae* non-transformed cells in the present study.

2.5. Transformation of S. cerevisiae with pKS1 plasmid harboring crt genes in three-step manner

S. cerevisiae cells were transformed with the three pKS1 plasmids each bearing one of the three synthetic *crtE*, *crtB* and *crtI* genes in three steps. At the first step, wild-type cells in liquid YPD medium were transformed with the pKS1-*crtI* vector. Resultant *crtI*-transformed cells were visually

selected, as red-colored colonies, on solid culture plates supplemented with 0.2 mg/ml phytoene (HanDan YuShuo Chemical Co., Handan, China, cat. No. 13920-14-4), the substrate of CrtI (phytoene desaturase). At the second step, *crtI*-transformed cells were transformed with the pKS1-*crtB* vector. Resultant *crtI*/*crtB*-transformed cells were visually selected on solid culture plates of YPD medium supplemented with 0.2 mg/ml geranylgeranyl pyrophosphate (GGPP; HanDan YuShuo Chemical Co., Handan, China, cat. No. 6699-20-3), the substrate of CrtB (phytoene synthase). At the last step, *crtB*/*crtI*-transformed cells were transformed with the pKS1-*crtE* construct and resultant *crtE*/*crtB*/*crtI*-transformed cells (hereafter termed *crtEBI*-transformed cells or *crt*-transformed cells) were visually selected on solid culture plates of YPD medium with no supplementation of substrate. G418 was included in the medium during the three-step transformation at 40 mg/ml; the concentration inhibiting the growth of *S. cerevisiae* non-transformed cells. Then, we obtained six lines of *crtEBI*-transformed *S. cerevisiae* cells. These lines were further subjected to different concentrations of G418 (0, 20, 40, 60, 80 and 100 μg/ml) of G418 (Fig. S3) to detect the concentration to be used in selection of transformed cells in further experiments. This experiment showed that 40 μg/ml G418 in the medium was sufficient to inhibit the growth of non-transformed *S. cerevisiae* cells. Control transformants of *S. cerevisiae* with pKS1 and individual pKS1-*crt* vectors with optimized codons were resistant to 40, 60, 80 and 100 μg/ml G418 (Fig. S4). Six lines of *crtEBI*-transformed *S. cerevisiae* cells grew well in the presence of 80 μg/ml G418 (Fig. S5). Among the six lines, Line 2 cells showed the highest rate whereas Lines 3 and 6 cells showed the lowest rate of growth. However, the difference in growth rate among the six lines was not statistically significant. In the following experiments, *crtEBI*-transformed *S. cerevisiae* cells were grown in the presence of 80 μg/ml G418.

2.6. RT-PCR analysis

Transcript levels of synthetic *crtE*, *crtB* and *crtI* genes in single randomly selected events of *crtEBI*-transformed *S. cerevisiae* cells were analyzed by RT-PCR. RNAs were extracted with QIAzol® (Qiagen, Düsseldorf, Germany) from *S. cerevisiae* cells that had been transformed with pKS1 (negative control) and pKS1-*crtEBI* vectors. Then, RNAs were treated with RNase-free DNase (Promega, Madison, WI, USA). First-strand cDNA was synthesized using 1 μg of total RNA, 0.5 μg forward and reverse primers of each gene (Table S2) and Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA) to a final volume of 20 μl. RT-PCR was performed in a 20-μl reaction mixture using 1 μl cDNA, 1× PCR buffer (with 1.5 mM $MgCl_2$), 200 μM dNTPs, 200 nM of each gene-specific forward and reverse primers and 0.2 U of Taq DNA polymerase (Promega, Madison, WI, USA). Primers were designed using the Universal Probe Library Assay Design Center (Roche, www.roche-applied-science.com, 2012). To ensure the complete absence of DNA contamination, PCR for the original RNA samples was run and results were negative (data not shown). Each cycle consisted of denaturation at 94 °C for 15 s, annealing at 55 °C for 30 s, and extension at 72 °C for 45 s. Number of cycles was 40 for amplifying fragments of all genes. Amplicons were run on a 1.2% agarose gel stained with ethidium bromide, and visualized using the Gel Doc XR (Bio-Rad Laboratories; Hercules, CA, USA). The yeast *actin* gene (L00026.1) was used as a reference during RT-PCR with the forward (5′-GAAATGCA AACCGCTGCTCA-3′) and reverse (5′-TACCGGCAGATTCCAA ACCC-3′) primers to amplify 145 nt.

2.7. Western blotting analysis

Translates of synthetic *crtE*, *crtB* and *crtI* genes, namely, CrtE, CrtB, and CrtI, in randomly selected events of *crtEBI*-transformed *S. cerevisiae* cells were analyzed by Western blotting. Total proteins were extracted from *S. cerevisiae* cells according to Kushnirov (2000). Protein content was estimated using a BCA protein assay kit (Pierce, Rockford, IL, USA) with BSA as a standard. SDS–PAGE (12% acrylamide gel) was performed according to Laemmli (1970). Three kinds of antibody against CrtE, CrtB, and CrtI, respectively, were prepared utilizing the Peptide Supplied Polyclonal Antibody Package (Rabbit) (GenScript USA Inc., Piscataway, NJ, USA, cat. No. SC1015) and used after dilution of 1:25,000. Western blotting was performed according to the manufacturer's instructions (Promega Protocols and Applications Guide, 1991; Promega Inc., Madison, WI, USA). Nitrocellulose membranes were incubated in anti-CrtE, anti-CrtB and anti-CrtI antibodies for 30 min followed by incubation in goat anti-rabbit IgG alkaline phosphatase conjugate (Promega, Madison, WI, USA). Crt proteins were detected using 4-nitroblue-tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) substrates (Promega Inc.).

2.8. Quantification of lycopene and ergosterol in crtEBI-transformed cells

S. cerevisiae cells were harvested by centrifugation at 3000×*g* for 10 min, washed with distilled water, and lyophilized. Samples were kept at −80 °C until use. For extraction of lycopene, 100 mg of the lyophilized cells were suspended in 2 ml of 0.9 M sorbitol solution containing 0.3 mg/ml of Zymolyase 100T (Sigma Chemical Co., St. Louis, Mo, USA, cat. No. L5263) and incubated for 2 h at 37 °C. Glass beads (diameter 0.4 mm, Sigma Chemical Co.) and 10 ml of acetone were added and the cell suspension was vortexed to pulverize the cells. After the acetone fraction was collected, lycopene was extracted by adding 10 ml of petroleum ether. This procedure was repeated twice. The resultant petroleum ether fractions were combined, evaporated to dryness and dissolved in chloroform–methanol (9:1, by volume).

For extraction of ergosterol, 150 mg of the lyophilized cells were suspended in 3 ml of 0.1 M NaCl. Then, 10 ml of pyrogallol solution (1.0%, w:v in ethanol), 2 ml of 1 M KOH, and 2 g of KOH were added. The mixture was incubated for 30 min at 70 °C. A total of 19 ml of NaCl solution (1.0%, w:v) and 15 ml of hexane–ethyl acetate (9:1, v:v) were added to this suspension, and the hexane–ethyl acetate layer was collected. This procedure was repeated twice. Resultant fractions of hexane–ethyl acetate were combined, evaporated and dissolved in chloroform. The

extracted lycopene and ergosterol were combined and subjected to high-performance liquid chromatography (HPLC) as described by Miura et al. (1998b). Multiple comparisons were performed for lycopene and ergosterol levels based on the least significant differences, or LSD, at 5%.

2.9. Determination of biomass and glucose concentration in culture medium

Biomass of *S. cerevisiae* cells was determined by means of turbidity, which was monitored spectrophotometrically at 546 nm every 5 h in three replicates. A glucose assay kit (GAGO-20, Sigma) was used to determine the glucose concentration in the culture medium. After growth for designated periods, the culture was centrifuged at 2500×*g* for 5 min, and the resultant supernatant was subjected to the determination of glucose concentration.

3. Results

In order to generate a recombinant *S. cerevisiae*, which would synthesize lycopene at a high level, we constructed three synthetic *crt* genes and expressed them in this organism under control of the *ADH2* promoter, which was activated upon depletion of glucose in the culture medium (see details in Section 2). The resultant transformants of *S. cerevisiae* synthesized lycopene at a level of 3.3 mg/g dry cell level, which was much higher than the previous reported level of lycopene in recombinant *S. cerevisiae* in previous studies where native *crt* genes but no glucose depletion-inducible promoter was used.

3.1. Expression of crt genes in crtEBI-transformed cells

Randomly selected *crtEBI*-transformed *S. cerevisiae* cells were subjected to molecular analysis of the expression of the three synthetic *crt* genes. *S. cerevisiae* cells transformed with the pKS1 vector only were served as controls. The RT-PCR analysis (Fig. 2) revealed that the three synthetic *crtE*, *crtB* and *crtI* genes were transcribed. Amplicons of the three synthetic genes were recovered at the expected sizes, suggesting that the *crt* genes were transcribed in *crtEBI*-transformed cells. The RT-PCR analysis of transcripts from control (pKS1-transformed) cells showed no amplicons on the gel.

Western blotting analysis (Fig. 3) indicated that CrtE, CrtB, and CrtI were detected in the *crtEBI*-transformed *S. cerevisiae* cells. These results suggested that transcripts of the three synthetic genes were translated in *S. cerevisiae*. In *crtE*, *crtB* or *crtI*(native)-transformed *S. cerevisiae* cells, RT-PCR analysis detected the transcripts of *crt* genes, while results of Western blotting analysis for the corresponding translates, namely, Crt proteins were negative). We hypothesized that this was due to the lack of many codons in the transcripts that are necessary for the translation in *S cerevisiae*.

3.2. Lycopene synthesis in crtEBI-transformed S. cerevisiae

Lycopene and ergosterol levels were determined in six lines of *crtEBI*-transformed *S. cerevisiae* cells and other various kinds of transformed *S. cerevisiae* cell (Table 1). *S. cerevisiae* transformed with only one of the synthetic *crt* genes did not synthesize lycopene. Only the six lines of *crtEBI*(synthetic)-transformed *S. cerevisiae* cells were active in the synthesis of lycopene. Among the six lines, Line 2 cells yielded the highest (1305 μg/g dry cell weight) level of lycopene, while Lines 3 and 6 yielded the lowest (635 and 595 μg/g dry cell weight, respectively). By contrast, the ergosterol level was the highest (2450 μg/g dry cell weight) in the control *S. cerevisiae* cells (transformed with the pKS1 vector only). Among the six lines of *crtEBI*-transformed *S. cerevisiae* cells, the lowest level (960 μg/g dry cell weight) of ergosterol was found in Line 2, while the highest was found in Lines 3 and 6 (1680 and 1600 μg/g dry cell weight, respectively). The comparison of the levels of lycopene with the growth profile (Fig. S5) suggested that they were positively correlated. Fig. 4 show that the levels of lycopene and ergosterol were reversely correlated among six lines of *crtEBI*-transformed *S. cerevisiae* cells. This might suggest that they competed to each other during their synthesis, as predicted in Fig. 1. Based on results of these experiments, Line 2 of *crtEBI*-transformed *S. cerevisiae* was used in further experiments.

3.3. Growth of crtEBI-transformed cells depended on glucose concentration

The growth profile of *crtEBI*-transformed *S. cerevisiae* cells of Line 2 was examined under standard conditions

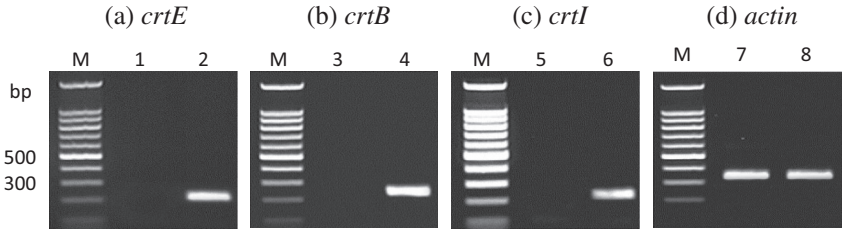

Fig. 2. Analysis by RT-PCR of *crtE*, *crtB* and *crtI* transcripts in *crtEBI*-transformed *S. cerevisiae* (Line 2). Transformed cells were grown in YPD medium supplemented with 80 μg/ml G418 at 30 °C, pH 7.0, and 2% glucose for 45 h. RNAs were extracted from these cells and purified as described in Section 2. Primers used for RT-PCR (Table S2) were specific to: (a) the *crtE* gene to generate amplicons of 222 bp; (b) the *crtB* gene to generate amplicons of 217 bp; (c) the *crtI* gene to generate amplicons of 219 bp; and (d) the *actin* gene to generate amplicons of 337 bp. Odd-numbered lanes, control cells that were transformed with the pKS1 plasmid; even-numbered lanes, *crtEBI*-transformed cells. Primers specific to the *actin* gene (Table S2) were used to demonstrate the transcription activity in *S. cerevisiae*. Bands of RT-PCR were visualized using Gel Works 1D advanced gel documentation system (UVP, UK). M refers to the 100-bp ladder (Bioron, Ludwigshafen, Germany).

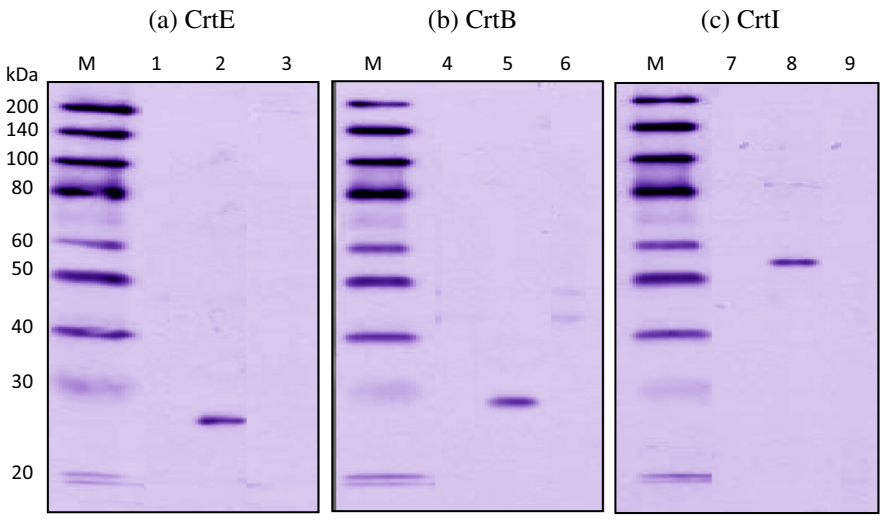

Fig. 3. Western-blotting analysis of CrtE, CrtB, and CrtI in *crtEBI*-transformed *S. cerevisiae* (Line 2). Transformed cells were grown as described in Fig. 2. Total proteins were extracted from transformed cells and applied to SDS-gel electrophoresis. Crt proteins were detected by primary antibodies specific to: (a) CrtE to generate a band at 28 kDa; (b) CrtB to generate a band at 27 kDa; and (c) CrtI to generate a band at 52 kDa. Lanes 1, 4 and 7, control cells that were transformed with the pKS1 plasmid; Lanes 2, 5 and 8, synthetic *crtEBI*-transformed cells; Lanes 3, 6 and 9, native *crtE, crtB and crtI*-transformed cells, respectively. M refers to the broad-range protein standard (Biotinylated Protein Ladder, Cell Signaling Technology, Danvers, MA 01923, USA).

Table 1
Levels of lycopene and ergosterol in *crt*-transformed *S. cerevisiae* cells. Transformed and control cells were grown at 30 °C and pH 7.0 for 45 h in YPD medium supplemented with 2.0% glucose and 80 μg/ml G418.

Strain	Plasmid	Lycopene	Ergosterol	Lycopene + ergosterol
		(μg/g dry cell weight)		
S. cerevisiae	pKS1 (control)	0*	2450 ± 110	2450
	pKS1-*crtE*	0*	2160 ± 95	2160
	pKS1-*crtB*	0*	2360 ± 135	2360
	pKS1-*crtI*	0*	2490 ± 124	2490
	pKS1-*crtEBI* Line 1	865 ± 12	1445 ± 95	2310
	pKS1-*crtEBI* Line 2	1305 ± 16	960 ± 80	2265
	pKS1-*crtEBI* Line 3	635 ± 10	1680 ± 75	2315
	pKS1-*crtEBI* Line 4	935 ± 11	1360 ± 85	2295
	pKS1-*crtEBI* Line 5	900 ± 12	1105 ± 70	2005
	pKS1-*crtEBI* Line 6	595 ± 8	1600 ± 75	2195

* Values not statistically analyzed.

(pH 7.0 and 30 °C), but at various concentrations (0.1%, 0.5%, 1% and 2%) of glucose (Fig. 5a). It was obvious that the biomass production increased with the increase in glucose concentration. The rate of growth and the biomass production of *crtEBI*-transformed *S. cerevisiae* were also examined at pH 6.0 and 35 °C (optimal conditions for lycopene synthesis; see later) in the presence of various concentrations of glucose (Fig. 5b). The results of this experiment were similar to that when *crtEBI*-transformed cells were grown at pH 7.0 and 30 °C (standard growth conditions). However, the maximum level of biomass production was higher in optimal than standard conditions at either concentration of glucose.

3.4. Synthesis of lycopene and ergosterol under various growth conditions

In order to find optimal conditions for the lycopene synthesis, we examined effects of various temperatures, pHs, and concentrations of glucose on the lycopene synthesis in Line 2 cells of *crtEBI*-transformed *S. cerevisiae* (Table 2). The results revealed that the highest level of lycopene (2380 μg/g dry cell weight) was found in the transformed cells grown under conditions of 35 °C, pH 6.0 and 0.5% glucose. It is notable that the glucose concentration of 2.0%, standard concentration in YPD medium and the optimal concentration for mass production, was rather inhibitory to the lycopene production, as compared to 0.5% glucose. We also investigated the effects of various temperatures, pHs, and glucose concentrations on the ergosterol production in *crtEBI*-transformed *S. cerevisiae* cells (Table 3). It is noted that under the conditions optimal to the lycopene production, transformed cells produced the lowest levels of ergosterol. The highest levels of ergosterol (~1.7 mg/g dry cell weight) were found in cells grown under conditions of pH 4.0, 20 °C and glucose concentration of 2.0% and 1.0%, which were conditions for the minimal levels of lycopene synthesis.

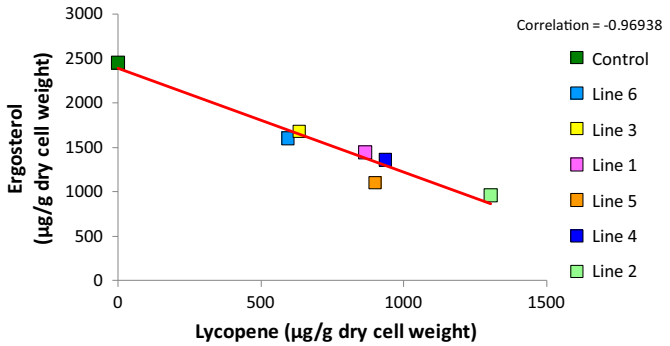

Fig. 4. Relationship between lycopene and ergosterol levels in *crtEBI*-transformed *S. cerevisiae* cells of six lines and control cells. This figure was deduced from the result in Table 1.

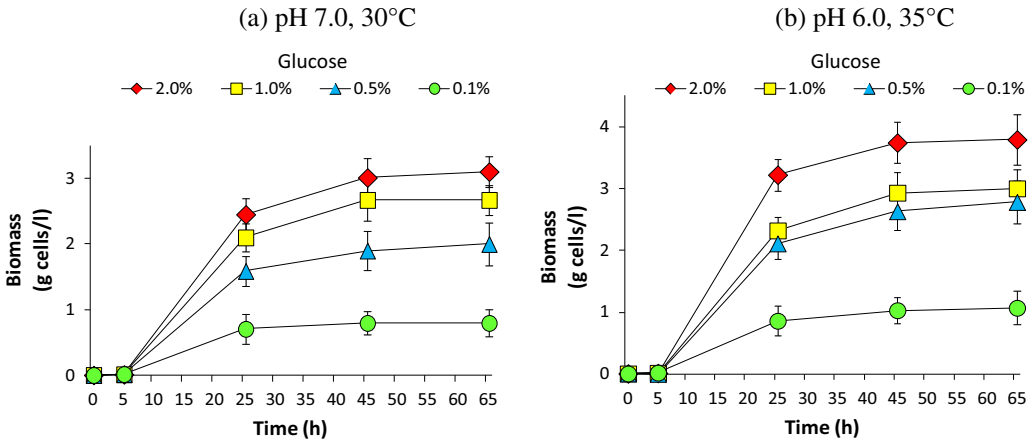

Fig. 5. Growth profile of *crtEBI*-transformed *S. cerevisiae* cells of Line 2. Cells were grown in YPD medium supplemented with 0.1%, 0.5%, 1% and 2.0% glucose under: (a) standard growth conditions (pH 7.0 and 30 °C): (b) optimal growth conditions (pH 6.0 and 35 °C).

Table 2
Lycopene levels in *crtEBI*-transformed *S. cerevisiae* Line 2 cells. Cells were grown for 45 h under various conditions of glucose concentration, temperature and pH. Measurement was performed three times and average numbers with standard deviations are presented. Rose, 2500~; blue, 2000–2499; green, 1500–1999; yellow, 1000–1499; white, ~999.

Glucose (%)	pH	Lycopene levels (µg/g dry cell weight) at various temperatures (°C)				
		20	25	30	35	40
0.1	4.0	990 ± 13	1103 ± 17	1289 ± 22	1802 ± 20	1772 ± 19
	5.0	877 ± 15	975 ± 20	1306 ± 28	1937 ± 22	1902 ± 22
	6.0	1098 ± 12	1123 ± 18	1444 ± 25	2303 ± 24	2002 ± 25
	7.0	995 ± 15	1000 ± 19	1305 ± 19	2001 ± 21	1881 ± 21
	8.0	903 ± 10	943 ± 18	998 ± 12	1603 ± 18	1408 ± 13
0.5	4.0	1022 ± 15	1205 ± 13	1433 ± 18	1997 ± 23	1731 ± 21
	5.0	1110 ± 13	1518 ± 16	1691 ± 24	2805 ± 27	2001 ± 29
	6.0	1492 ± 16	1977 ± 23	2792 ± 22	3280 ± 25	2341 ± 27
	7.0	1300 ± 20	1606 ± 20	1518 ± 23	2501 ± 27	2220 ± 26
	8.0	976 ± 14	1221 ± 21	1299 ± 17	1904 ± 24	1714 ± 16
1.0	4.0	788 ± 14	882 ± 16	1026 ± 16	1333 ± 17	1114 ± 13
	5.0	1002 ± 17	833 ± 10	1114 ± 11	1302 ± 15	1108 ± 17
	6.0	840 ± 10	999 ± 16	1159 ± 13	1666 ± 21	1210 ± 17
	7.0	801 ± 12	765 ± 11	904 ± 11	1501 ± 23	1006 ± 13
	8.0	809 ± 13	810 ± 11	713 ± 9	1400 ± 19	906 ± 14
2.0	4.0	918 ± 13	817 ± 13	1011 ± 20	1116 ± 25	1201 ± 21
	5.0	927 ± 15	902 ± 17	1209 ± 18	1313 ± 19	1332 ± 14
	6.0	1101 ± 11	940 ± 15	1331 ± 20	1743 ± 23	1701 ± 22
	7.0	969 ± 13	1008 ± 17	1317 ± 17	1530 ± 21	1401 ± 19
	8.0	873 ± 11	821 ± 11	977 ± 16	1213 ± 11	1206 ± 18

Table 3
Ergosterol levels of *crtEBI*-transformed *S. cerevisiae* Line 2 cells. Cells were grown for 45 h under various conditions of glucose concentration, temperature and pH. Measurement was performed three times and average numbers with standard deviations are presented. Boxes: rose, 1600∼; blue, 1400–1599; green, 1200–1399; yellow, 1000–1199; white, ∼999.

Glucose (%)	pH	Ergosterol levels (μg/g dry cell weight) at various temperatures (°C)				
		20	25	30	35	40
0.1	4.0	1571 ± 20	1417 ± 16	1386 ± 19	1208 ± 17	1375 ± 20
	5.0	1397 ± 18	1390 ± 13	1301 ± 19	1200 ± 17	1323 ± 18
	6.0	1211 ± 24	1133 ± 20	1114 ± 24	1006 ± 13	1100 ± 19
	7.0	1490 ± 18	1230 ± 14	1355 ± 17	1196 ± 14	1043 ± 18
	8.0	1561 ± 21	1416 ± 22	1527 ± 27	1114 ± 13	1303 ± 17
0.5	4.0	1402 ± 15	1339 ± 24	1257 ± 19	1002 ± 17	1314 ± 11
	5.0	1306 ± 21	1130 ± 19	1249 ± 15	840 ± 10	1159 ± 13
	6.0	1201 ± 23	1248 ± 26	1060 ± 22	921 ± 12	1004 ± 11
	7.0	1500 ± 19	1262 ± 18	1202 ± 17	839 ± 13	1313 ± 9
	8.0	1443 ± 17	1583 ± 22	1395 ± 21	988 ± 14	1426 ± 16
1.0	4.0	1735 ± 27	1569 ± 13	1502 ± 22	1077 ± 15	1533 ± 10
	5.0	1591 ± 20	1418 ± 13	1392 ± 25	1098 ± 12	1599 ± 16
	6.0	1309 ± 18	1227 ± 15	1281 ± 21	995 ± 15	1165 ± 11
	7.0	1431 ± 20	1291 ± 11	1208 ± 13	1203 ± 10	1210 ± 11
	8.0	1477 ± 16	1373 ± 11	1372 ± 19	1190 ± 13	1382 ± 16
2.0	4.0	1631 ± 29	1505 ± 27	1246 ± 28	1208 ± 17	1518 ± 16
	5.0	1441 ± 27	1380 ± 25	1244 ± 25	1217 ± 13	1477 ± 23
	6.0	1220 ± 26	1201 ± 27	1005 ± 19	1102 ± 17	1306 ± 20
	7.0	1514 ± 16	1404 ± 24	1098 ± 12	1240 ± 15	1221 ± 21
	8.0	1655 ± 21	1597 ± 23	1289 ± 22	1321 ± 11	1205 ± 13

In order to understand the mode of lycopene and ergosterol syntheses under various conditions of growth, we calculated the summation of lycopene and ergosterol (lycopene + ergosterol) levels (Table S3) and the percentage of lycopene to lycopene + ergosterol (Table S4). The results revealed that the levels of lycopene + ergosterol were highest under conditions of pH 6.0, 35 °C and 0.5% glucose, which were optimal to the lycopene production (Table 2), but were minimal to the ergosterol production (Table 3). These results suggest that the pathway to synthesize the precursors of ergosterol and lycopene syntheses was activated under conditions of pH 6.0, 35 °C and 0.5% glucose as compared to the standard conditions of pH 7.0, 35 °C and 2% glucose. The percentages of lycopene to lycopene + ergosterol levels (Table S4) were highest also under conditions of pH 6.0, 35 °C, and 0.5% glucose, which were optimal to the lycopene production (Table 2), but were minimal to the ergosterol production (Table 3). These results might suggest that the synthesis of lycopene in the alternative pathway (Fig. 1) was most activated under the conditions of pH 6.0, 35 °C, and 0.5% glucose, as compared with the synthesis of ergosterol in the sterol pathway. Overall, the high level of lycopene synthesis at the optimal conditions was achieved by two factors; namely, the activation of the pathway to synthesize the precursors to ergosterol and lycopene and the activation of lycopene-synthesizing alternative pathway (Fig. 1, red colored arrows).

3.5. Time-dependent profile of lycopene and ergosterol syntheses in crtEBI-transformed cells

We compared the time-dependent changes in lycopene and ergosterol levels under various conditions of growth in *crtEBI*-transformed *S. cerevisiae* cells (Fig. 6). It was remarkable that the level of ergosterol increased at early stages of growth in all cases of conditions examined. By contrast, the lycopene level started to increase at a later time; namely, 15, 20 and 35 h in the presence of 0.1%, 0.5% and 2.0% of the initial concentration of glucose, respectively (Fig. 6). Under standard conditions, pH 7.0 and 30 °C, in the presence of 0.1% or 0.5% glucose, the summation of lycopene and ergosterol reached almost the maximum level at 20 h and, then, continued to increase very slowly (Fig. 6a and b). In the presence of 2% glucose, the summation reached the maximum level at 20 h and maintained almost the same level up to 65 h (Fig. 6c). However, under the optimal conditions of growth, pH 6.0 and 35 °C, the summation level continued to increase after 20 h in the presence of 0.1% and 0.5% glucose (Fig. 6d and e). In the presence of 2% glucose, the summation reached the maximum level at 20 h and maintained almost the same level up to 65 h (Fig. 6f). A remarkable feature was the reverse relationship between changes in lycopene and ergosterol levels at late stages of the culture. In the presence of 0.1% and 0.5% glucose under standard conditions, the ergosterol level started to decrease and the lycopene level started to increase at 20 h (Fig. 6a and b). The changes in their levels were in a mirror image. Similar changes in ergosterol and lycopene levels were observed in all the other cases of growth conditions (Fig. 6c–f). Such a reverse relationship of the decrease in ergosterol level and the increase in lycopene level might suggest that after the onset of lycopene-synthesizing pathway (the alternative pathway; Fig. 1), a part of ergosterol was converted to lycopene via a reverse reaction of the sterol biosynthetic pathway (Fig. 1).

The advantage of using the *ADH2* promoter to drive *crt* genes in the present study was the regulation of *crt* gene expression, where the promoter is inactive until glucose was depleted in the medium. The onset of the lycopene synthesis upon the glucose depletion in *crtEBI*-transformed *S. cerevisiae* cells might be related to the transcriptional control of the expression of *crt* genes by the *ADH2* promoter, which is activated by depletion of glucose in the culture medium. We determined the concentration of glucose in the culture medium during the growth of *crtEBI*-transformed cells.

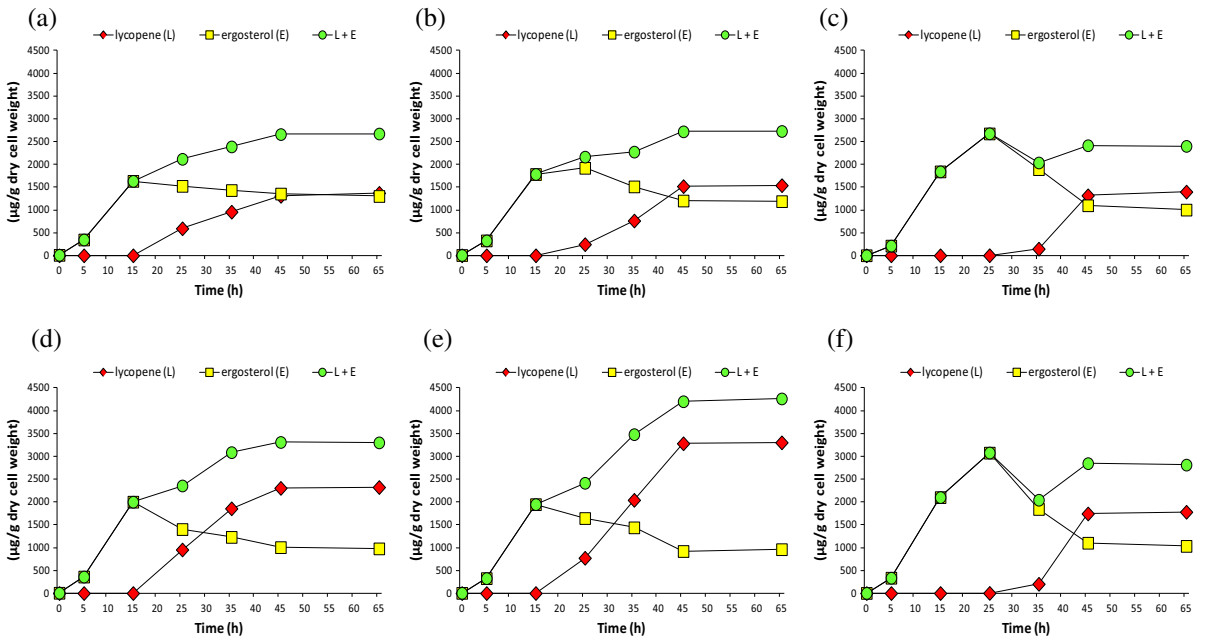

Fig. 6. Time-dependent changes in lycopene and ergosterol levels in *crtEBI*-transformed *S. cerevisiae* cells of Line 2 under various conditions of pH, temperature, and glucose concentration. Growth conditions were: (a) pH 7.0, 30 °C, and 0.1% glucose; (b) pH 7.0, 30 °C, and 0.5% glucose; (c) pH 7.0, 30 °C, and 2.0% glucose; (d) pH 6.0, 35 °C, and 0.1% glucose; (e) pH 6.0, 35 °C, and 0.5% glucose; (f) pH 6.0, 35 °C, and 2.0% glucose.

When the culture medium contained 0.5% glucose at inoculation (Fig. 6b and e), the glucose concentration decreased to 0.02% at 20 h, and was undetectable at 25 h, which was the time when the lycopene synthesis started. When the culture medium contained 2% glucose at the inoculation (Fig. 6c and f), the glucose concentration was decreased to 0.22% at 20 h, and was undetectable at 35 h when the lycopene synthesis started. These observations might suggest that the onset of lycopene synthesis was related to the depletion of glucose in the culture medium. This result was consistent to the de-repression of the *ADH2* promoter activity upon the deletion of glucose in the medium.

There was no significant difference in lycopene levels at late stages (45 and 65 h) when the *crtEBI*-transformed cells were grown under standard conditions in the presence of initial glucose concentrations of 0.1%, 0.5%, and 2%. By contrast, under optimal growth conditions, namely, pH 6.0 and 35 °C, the lycopene level at late stages of growth was the highest when the initial concentration of glucose was 0.5% (Fig. 6e). These results suggested that growth conditions for the maximum production of lycopene in the *crtEBI*-transformed *S. cerevisiae* would be; temperature, 35 °C; pH, 6.0; glucose concentration, 0.5%; and the period of incubation, 45 h.

4. Discussion

Previous studies have indicated that *C. utilis* transformed with native *crt* genes derived from a carotene-producing bacterium *E. uredovora* synthesizes lycopene and carotene (Miura et al., 1998a,b). In the present study, we transformed *S. cerevisiae* with the PKS1 plasmid that harbored synthetic *crt* genes, which were constructed according to the preferable codon usage of *S. cerevisiae*, for the lycopene production. According to our knowledge, this is the first time to use *S. cerevisiae* with synthetic *crt* genes for the successful recovery of lycopene and, under certain growth conditions, the level of lycopene in the transformed cells was at as high as 3.3 mg/g dry cell weight. These results prove utility of the synthetic genes as more efficient than the native genes in the production of lycopene.

Promoters previously used for the expression of *crt* genes in previous studies of *C. utilis* were *P14* and *P57*, *PMA*, *GAP* and *PGK* (Miura et al., 1998a,b). The highest lycopene level (1.1 mg/g dry cell weight) was reached when the *crtE* gene was driven by *GAP* promoter, the *crtB* gene was driven by *P14* promoter, and the *crtI* gene was driven by *PGK* promoter. In the present study, we employed the *ADH2* promoter of *S. cerevisiae* for the expression of the three synthetic *crt* genes in *S. cerevisiae*, and the high level of lycopene production was achieved in the *crtEBI*-transformed cells. With this promoter, the highest expression of *crt* genes and the most efficient production of lycopene were observed when the culture medium contained 0.5% glucose rather than 2% glucose, which was the glucose concentration in the standard YPD culture medium. The use of low concentration of glucose for the efficient production of lycopene might have a great advantage in the industrial production.

Previous studies demonstrated the advantage to use the *ADH2* promoter in heterologous expression of recombinant genes in *S. cerevisiae*. Price et al. (1990) utilized the *ADH2* promoter and indicated the catabolite repression (Gancedo, 1998) of several hundred-fold in the presence of 1% glucose. After 4 h, glucose was not detected and genes

encoding human cytokines were steadily expressed in *S. cerevisiae*. Lee and DaSilva (2005) also evaluated several growth factors influencing optimum activation of the *ADH2* promoter and reported the superior performance of the promoter relative to the commonly used inducible *CUP1* and *GAL1* promoters. They further studied the derepression of the *ADH2* promoter when yeast cells were cultivated in YPD containing 1% glucose and found that glucose was not detected at the early stationary phase (25 h), where biomass is high, and the expression level of the downstream gene (e.g., β-galactosidase) increased by 18-fold from the basal level. Afterwards, the *ADH2* promoter was fully induced and the expression level was increased steadily. However, there has been no report for the influence of 0.5% glucose. It was assumed that 0.5% glucose was depleted early enough to induce the steady expression of the three *crt* genes during the entire stationary phase, while maintaining high cell biomass. Lower concentrations of glucose might induce gene expression at the early log phase, when cell biomass was not high enough. On the other hand, higher glucose concentrations might result in higher biomass production, whereas the gene expression would take place at late stationary phase.

Optimization of the other growth conditions, e.g., pH of the medium and growth temperature, is important for maximizing growth rate of yeast cells, hence, its productivity. A number of reports have also considered growth conditions like temperature and pH of the medium as important factors affecting growth rate and biomass production of yeasts (Fleet and Heard, 1993; Charoenchai et al., 1998; Arroyo-López et al., 2009; Nadeem et al., 2013). These growth factors were also assessed in the present study for their influence on *crt* gene expression levels, consequently the production of lycopene in transformed *S. cerevisiae*.

5. Conclusion

The use of synthetic *crt* genes with codons preferred to *S. cerevisiae* cells is a successful approach for efficient overexpression of the foreign genes in this organism. The results of the present work also indicate that lycopene can be produced at high levels by the use of the *ADH2* promoter of *S. cerevisiae*. The advantage of the *ADH2* promoter relative to other commonly used promoters lies in its inducer-free characteristic, which allows uninterrupted bioreactor operations during industrial fermentation processes (Lee and DaSilva, 2005). Expression of the three synthetic *crt* genes takes place after glucose is consumed at the early stationary phase in which biomass is accumulated to a level enough to recover high levels of lycopene during mid and late stationary phase. The high expression levels in stationary phase are ideal for large-scale industrial applications of *S. cerevisiae*.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.plasmid.2014.03.001.

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