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Improved tolerance of recombinant *Escherichia coli* to the toxicity of crude glycerol by overexpressing trehalose biosynthetic genes (*otsBA*) for the production of β-carotene

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HIGHLIGHTS

- *E. coli* cells were engineered to overexpress trehalose biosynthetic operon.
- Engineered cells were highly tolerant to toxicity of impurities in crude glycerol.
- Performances of engineered cells were not hampered even at 60 g/L of crude glycerol.

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ABSTRACT

This study aims to investigate whether overexpressing the trehalose biosynthetic gene, *otsBA* operon, in β-carotene-producing recombinant *Escherichia coli* protects cells from toxic impurities in crude glycerol. The concentrations of potassium and methanol in crude glycerol were too low to inhibit cell growth. Cell growth and production in control cell culture were inhibited significantly in the presence of a small amount of crude fatty acids. Peroxides were generated in the presence of crude fatty acids during autoclaving and, thus, the inhibitory effect of crude fatty acids was caused primarily by these peroxides. Engineered cells overexpressing *otsBA* tolerated crude fatty acids (⩽42 wet-g/L), methanol (⩽7.5 g/L), and *t*-BuOOH (⩽60 μM) in separate experiments and tolerated up to 60 g/L crude glycerol. These results demonstrate that overexpressing *otsBA* endowed cells with the capacity to tolerate the toxicity of crude glycerol for direct use.

1. Introduction

Biodiesel is a promising alternative and renewable fuel. The market for biodiesel is increasing; thus, its production is projected to reach 37 billion gallons by 2016 (Yang et al., 2012). Transesterification of vegetable oils (triglycerides) with methanol in the presence of a strong base such as potassium or sodium hydroxide produces biodiesel (fatty acid methyl esters) and glycerol as a co-product. Soap (fatty acid salts) is also generated either by neutralization of free fatty acids present in the oils as impurities by alkaline catalysts or by concurrent saponification of oils. Generally, 10 kg of glycerol is released for every 100 kg of biodiesel. This crude glycerol stream contains major impurities such as methanol, salts, and soap (Venkataramanan et al., 2012; Yang et al., 2012). The growing production of biodiesel has resulted in a worldwide surplus of glycerol and, thus, the price of crude glycerol has dropped significantly along with a simultaneous decrease in demand. Although refined glycerol can be obtained by purifying crude glycerol, this is not an economically feasible solution (Yazdani and Gonzalez, 2007). Therefore, biodiesel production will be promoted by reducing the cost if sustainable processes are developed to produce value-added products by utilizing crude glycerol directly.

Many studies have been conducted on the production of biofuels and chemicals through fermentation of *Clostridium* sp. (Chatzifragkou et al., 2010; Venkataramanan et al., 2012), yeast (Liang et al., 2010; Liu et al., 2011; Liu et al., 2012), fungi (Nitayavardhana and Khanal, 2011), and algae (Pyle et al., 2008) by utilizing crude glycerol. However, fermentation time is long with a prolonged lag time when crude glycerol is tested as the sole carbon source, leading to low productivity, and this problem has been attributed to the presence of impurities in the crude glycerol (Venkataramanan et al., 2012). In particular, impurities such as methanol and soap (fatty acid salts) are considered as major inhibitors to

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microbial growth, and they exist in significant concentrations in industrial feedstock (Pyle et al., 2008). Additionally, a novel finding from this study was that crude glycerol contained peroxides, which have a harmful effect on cell growth. Therefore, it is essential to develop a bacterial strain that can tolerate peroxides, as well as soap and methanol, to develop a novel fermentation process to utilize crude glycerol directly. It is essential to consider the advantages of trehalose, which is a disaccharide formed by a α,α-1,1-glucosidic bond between 2 α-glucose units. It has been demonstrated that overexpression of a trehalose biosynthetic gene (*otsBA*) in *Escherichia coli* increases trehalose level above that of the wild-type leading to increased osmotolerance of cells to salts and sugars, as well as to desiccation (Miller and Ingram, 2008; Purvis et al., 2005). Another report found that the accumulation of trehalose in yeast cells protects them from damage by reactive oxygen species, including hydrogen peroxides and organic peroxides (Alvarez-Peral et al., 2002; Nery et al., 2008). However, despite the great potential of trehalose, no attempt has been made to accumulate trehalose by overexpressing *otsBA* in *E. coli* to protect cells from the toxic impurities in crude glycerol. In a previous study, *E. coli* cells overexpressing the *otsBA* operon was constructed and these engineered *E. coli* cells accumulated high amount of trehalose (1.4 g/L) (Li et al., 2012). The other studies have also attempted to improve the microbial production of β-carotene in batch and fed-batch cultures of metabolically engineered *E. coli* in which foreign mevalonate pathway genes were introduced along with β-carotene biosynthetic genes (Nguyen et al., 2012; Yoon et al., 2009).

This study aims to investigate whether overexpressing *otsBA* in β-carotene-producing recombinant *E. coli* could increase the tolerance of these engineered cells to the toxicity of crude glycerol and thus utilize it directly for producing β-carotene. A β-carotene producing recombinant *E. coli* was engineered to overexpress *otsBA*. Engineered cells were cultured in synthetic medium supplemented with pure glycerol and different concentrations of methanol, crude fatty acids, or peroxides to investigate the individual effects of these impurities. The performance of the engineered cells was evaluated in medium supplemented with different concentrations of crude glycerol compared to that of control cells. This is the first report on the role of trehalose for protecting *E. coli* against toxic impurities in crude glycerol.

2. Methods

2.1. Chemicals

Crude glycerol was obtained from M Energy Co. Ltd. (Pyeongtaek, Korea). Yeast extract and tryptone were purchased from Difco (Detroit, MI, USA). IPTG, antibiotics, alcohol oxidase, and other chemicals were purchased from Sigma–Aldrich (St. Louis, MO, USA). All enzymes were purchased from Takara Bio (Shiga, Japan). Fatty acids were isolated from crude glycerol following a procedure described previously (Pyle et al., 2008); the crude glycerol was mixed with distilled water at a ratio of 1:4 (v/v), and the pH of this solution was adjusted to 3 using hydrochloric acid (37%) to convert the soap into free fatty acids. The fatty acid precipitate was separated by centrifugation and then harvested by vacuum filtration on Advantec No. 2 filter paper (Advantec Tokyo Roshi Kaisha, Ltd., Tokyo, Japan). The recovered fatty acid cake was spiked back into the medium at the desired amount, if necessary. Methanol and tert-butyl hydroperoxide were sterilized by passing them through a 0.2 μm disposable syringe filter (DISMIC-13cp, Advantec Tokyo Roshi Kaisha, Ltd.) and then spiked into sterilized medium at different concentrations to investigate their individual effects.

2.2. Bacterial strain and culture conditions

β-Carotene-producing recombinant *E. coli* cells that did or did not overexpress *otsBA* were used in this study. Control cells were *E. coli* DH5α containing the pT-DHB plasmid bearing the β-carotene biosynthetic genes and the pS-NA plasmid bearing the genes encoding all MVA pathway enzymes (Yoon et al., 2009). Engineered cells had the additional pB-*otsBA* plasmid for trehalose biosynthesis, as well as pT-DHB and pS-NA. The blank pBBR1MCS-2 plasmid was introduced into control cells for comparison. Detailed information on the vectors is shown in Table 1.

All flask cultures were performed in 500 mL baffled flasks using a shaking incubator (Jeio Tech, Seoul, Korea) at 190 rpm. Glycerol stocks of recombinant cells were inoculated into 50 mL of Luria–Bertani broth supplemented with filter-sterilized antibiotics (100 mg/L ampicillin, 50 mg/L chloramphenicol, and 50 mg/L kanamycin), and cultured at 37 °C. When the optical density of the culture broth reached 0.6 at 600 nm (OD_{600}), 5 mL of seed culture was transferred to 50 mL of defined R medium (Lee and Chang, 1993) supplemented with pure or crude glycerol at different concentrations, as well as with antibiotics. R medium composition was 13.5 g K_2HPO_4, 4 g $(NH_4)_2HPO_4$, 1.4 g $MgSO_47H_2O$, 1.7 g citric acid, 0.3 g thiamine, and 10 mL trace element solution per 1 L. The composition of trace elements in solution is as follows (per L of 5 M HCl): 10 g $FeSO_47H_2O$, 2 g $CaCl_2$, 2.2 g $ZnSO_47H_2O$, 0.5 g $MnSO_44H_2O$, 1.0 g $CuSO_45H_2O$, 0.1 g $(NH_4)_6Mo_7O_244H_2O$, and 0.02 g $Na_2B_4O_710H_2O$. The main culture was carried out at 25 °C, and IPTG was added to a final concentration of 0.5 mM when the culture reached an OD_{600} = 5. Cell growth and β-carotene production in cultures were examined after 5 days under different culture conditions.

2.3. Plasmid construction for overexpressing the otsBA operon

We previously synthesized the *otsBA* operon from *E. coli* MG 1655 genomic DNA by polymerase chain reaction (PCR) and then cloned it into the pTrc99A plasmid (Amersham Bioscience, Piscataway, NJ, USA) (Li et al., 2012). The *otsBA* fragment was excised from the pTrc99A-*otsBA* plasmid with *EcoRI* and *XbaI* and then ligated into pBBR1MCS-2 (Addgene, Cambridge, MA, USA) at the same sites. The newly prepared pBBR1MCS-2 containing *otsBA*

Table 1
Strains and plasmid used in this study.

	Description	Sources
Strains		
E.coli DH5α	F^-, φ80d*lacZ*ΔM15, Δ(*lacZYA-argF*)U169, *deoR*, *recA*1 *endA*1, *hsdR*17($r_k^-m_k^+$), *pho*A, *sup*E44, λ^-, *thi*-1, *gyrA*96, *relA*1	
Plasmids		
pSTV28	P_{lac} expression vector, pACYC184 origin, *lacZ*, Cm^r	Takara
pTrc99A	P_{trc} expression vector, pBR322 origin, $lacI^q$, Amp^r	Amersham Bioscience
pBBR1MCS-2	P_{lac} expression vector, pBR328 origin, *lacZ*, Kan^r	Addgene
pS-NA	pSTV containing *mvaE* and *mvaS* of *E. faecalis*, *mvaK1*, *mvaK2*, and *mvaD* of *S. pneumonia*, and *idi* of *E. coli*	Yoon et al. (2009)
pT-DHB	pTrc99A containing *crtE*, *crtB*, and *crtI* of *P. agglomerans*, *ipiHP1* of *H. pluvialis*, *crtY* of *ananatis*, and *dxs* of *E. coli*	Yoon et al. (2009)
pB-*otsBA*	pBBR1MCS-2 containing *otsBA*	This study

(pB-*otsBA*) was used to transform β-carotene-producing *E. coli* DH5α that harbored pT-DHB and pS-NA.

2.4. Analytical methods

The OD_{600} was measured with a UV/Vis spectrophotometer (Hewlett–Packard, Palo Alto, CA, USA) to monitor cell growth. Medium containing crude glycerol was turbid due to the presence of a high amount of soap, thus, it was essential to remove it for an accurate measurement of cell growth as follows; 1 mL of culture broth was centrifuged at 10,000*g* for 10 min at 4 °C. The supernatant was taken for later analysis, and the soap layer on the wall of the tube was carefully wiped with a clean cotton swab. One mL of distilled water was added to the tube containing the cell pellet only and vortexed. The OD_{600} of this solution was measured to estimate cell growth. β-Carotene content of the cells was measured using a UV/Vis spectrophotometer; 1 mL of culture broth was taken, appropriately diluted, and the orange-colored cell pellet was harvested by centrifugation at 10,000*g* for 10 min. After washing once with distilled water, the cell pellet was resuspended in 1 mL of acetone and incubated at 55 °C for 15 min in the dark. Next, the nearly colorless cell pellet was removed by centrifugation (10,000*g* for 10 min), and the amount of β-carotene in the acetone extract was determined by measuring absorbance at 454 nm. Authentic β-carotene was used as the standard. The concentration of residual glycerol was determined by UV/Vis spectrophotometer using a commercial kit supplied by Megazyme (Wicklow, Ireland).

Fatty acids in the crude glycerol were converted into their methyl esters according to the protocol developed by Folch et al. (1957) and then analyzed by gas chromatography. A 6.5–7 g aliquot of crude glycerol was dissolved in 20 mL of extraction solvent (chloroform:methanol = 2:1). After a 1-h incubation at room temperature, small particulates were removed by passing the solution through filter paper (Hyundai Mirco No 51, Seoul, Korea). Next, NaCl was added to the filtrate (0.22% of NaCl) to facilitate phase separation, followed by centrifugation (10,000*g* at 4 °C for 10 min); the bottom organic phase was harvested and dried under N_2. This pellet containing crude fatty acids was redissolved in 1 mL of methylene chloride (including 2.76 mg/L heptadecanoic acid as an internal standard). One mL of 14% BF_3-methanol solution was added to this solution and the methylation reaction was carried out at 90 °C for 10 min. The methyl ester mixtures resulting from the reaction were extracted with 1 mL hexane. After removing the trace amount of water with Na_2SO_4, the hexane extract was directly analyzed by gas chromatography (M600D, YongLin, Seoul, Korea). The system was equipped with a flame-ionization detector and used a fused silica capillary column (100 m × 0.25 mm i.d. × 0.2 μm film thickness, SP™-2560, Supelco, Bellefonte, PA, USA). Nitrogen was used as the carrier gas at a flow rate of 2.7 mL/min. The oven temperature was initially held at 220 °C for 2 min, raised to 240 °C at a rate of 2 °C/min, and held for 20 min. The injector temperature was maintained at 250 °C, and detector temperature was held at 180 °C for 6 min, then increased to 250 °C at 5 °C/min. Injection volume was 1 μL with a split ratio of 10:1. Fatty acids were identified by comparing the retention times with those of standard fatty acids (Supelco 37 Component FAME Mix) and quantified by comparing their peak area with that of the internal standard.

The methanol concentration in the crude glycerol was measured using an enzyme-chemical assay (Gonchar et al., 2005); 0.2 mL of sample was added to 1.8 mL of the reagent containing 0.05% 3-methyl-2-benzothiazolione hydrazone and alcohol oxidase from *Pichia pastoris* with a final activity of 0.5 U/mL in 25 mM morpholinopropanesulfornic acid-KOH buffer (pH 7.0). After a 15-min incubation at 37 °C, 2 mL of 0.1% $FeCl_3$ in 30 mM HCl was added to this reaction mixture. A blue color developed after a 20-min incubation at 30 °C, and the OD_{670} of the mixture was measured using a UV/Vis spectrophotometer. The contents of potassium, sodium, and sulfur in the crude glycerol were determined using inductively coupled plasma atomic emission spectroscopy (JY Ultima2C, Jobin Yvon, France) at the Korea Basic Science Institute (Daejeon, Korea). Concentrations of hydrogen peroxide or organic peroxides were determined by colorimetric reaction using a PeroxiDetect™ Kit supplied by Sigma. In this method, peroxides convert Fe^{2+} to Fe^{3+} ions under acidic conditions and Fe^{3+} ions then form a colored adduct with xylenol orange. The purple color intensity was measured at 590 nm in a UV/Vis spectrophotometer; a 30% solution of hydrogen peroxide (H_2O_2) and a 70% solution of *tert*-butyl hydroperoxide were used for preparing the calibration curve.

3. Results and discussion

3.1. Characterization of crude glycerol

A crude glycerol analysis was performed. The crude glycerol was dark brown in color with a high pH and purity of 88%. It contained potassium ions (1.1%), methanol (14%), and fatty acid salts (17.4%) as major impurities. Crude fatty acids were recovered from the crude glycerol and analyzed. The crude fatty acids were palmitic acid (17.5%), stearic acid (4.7%), oleic acid (31.2%), linoleic acid (31.2%), and linolenic acid (4.9%) as major components whereas there were trace amounts of short- (<C14) or long (>C20) fatty acids. Biodiesel producers use potassium hydroxide as an alkali catalyst and excess methanol to drive the chemical transesterification to completion. The crude fatty acid salts are concurrently generated by neutralization of free fatty acids in oil by an alkaline catalyst or saponification of the oil through a side reaction (Pyle et al., 2008; Thompson and He, 2006) .

3.2. Cell growth and β-carotene production in a recombinant E. coli culture with and without otsBA overexpression

The β-carotene-producing recombinant cells were further transformed to overexpress *otsBA* and the performance of these engineered cells was examined by comparison with control cells in preliminary cultures using R-medium supplemented with 20 g/L pure glycerol. As shown in Fig. 1, both types of cells started growing with concomitant production of β-carotene after an 18 h lag time. Cell concentration reached an OD_{600} of 17, β-carotene production was 122 mg/L at 67 h, and then cell growth stopped even in the presence of residual glycerol in the control culture. In contrast, cell concentration reached an OD_{600} of 23 at 67 h in the engineered cell culture and then increased to a maximum of 28 at 90 h at which time glycerol was almost exhausted. β-Carotene concentration increased concomitantly with cell growth and was 147 mg/L at 67 h and then increased slightly. This result suggests that control cells may have become stressed as β-carotene accumulated inside cells, whereas the engineered cells tolerate this stress. β-Carotene is a hydrophobic compound and is more likely to remain attached to cellular membranes. Therefore, a perturbation of membrane structure by the accumulation of a high amount of β-carotene might cause membrane stress (Verwaal et al., 2010). Notably, trehalose contributed to preserve the integrity of the membrane and, thus, protected engineered cells from this stressed condition.

As mentioned above, crude glycerol contains toxic impurities. Therefore, the effects of these impurities on cell growth and β-carotene production should be examined before evaluating the feasibility of utilizing crude glycerol as a substrate for *E. coli* cultures.

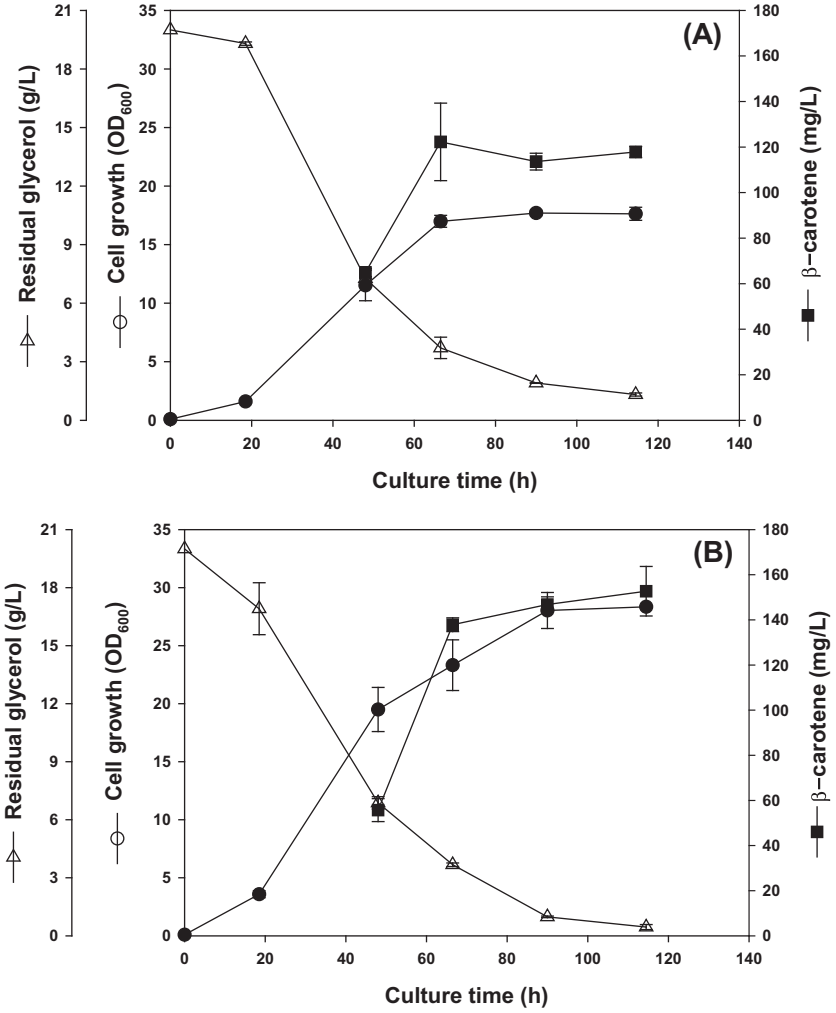

Fig. 1. Time profiles of cell growth and β-carotene production during culture of control (A) and engineered cells (B) in medium supplemented with 20 g/L pure glycerol.

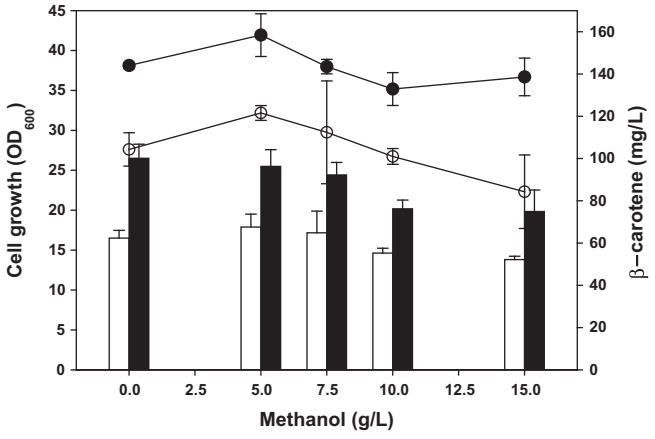

Fig. 2. Effect of methanol on cell growth (bar chart) and β-carotene production (symbols and line graph). Control (open symbols) and engineered cells (filled symbols) were cultured in medium supplemented with 20 g/L pure glycerol and different concentrations of methanol for 5 days.

3.3. Tolerance of E. coli cells to methanol toxicity

Most of the methanol (88%) remained in the medium after autoclaving (121 °C for 20 min). According to thermodynamic principle, the vapor pressure of methanol increases and, thus, its boiling point increases if pressurized; methanol evaporates at 90 °C at an autoclaving chamber pressure of 2.5 bar, whereas the boiling point of methanol is 65 °C at atmospheric pressure (Goodwin, 1987) . Additionally, the evaporation rate is lower under a saturated steam than that of dry air, which leads to incomplete evaporation of methanol. Therefore, it was essential to investigate the effect of methanol. Medium supplemented with 20 g/L pure glycerol was spiked with various concentrations of methanol (5–15 g/L). Cell growth and β-carotene production in the control and engineered cell cultures were not inhibited by the presence of methanol up to 7.5 g/L (Fig. 2), indicating that < 10 g/L methanol did not negatively influence the cell growth or production in the *E. coli* culture. The resistance of *E. coli* cells to methanol might be due to adaptive responses of membrane lipids to alcohols (Ingram, 1976); methanol has the least effect among the alcohols and, thus, the inhibitory effects of methanol on *E. coli* were prominently observed only at 1.0% (v/v) and higher.

3.4. Improved tolerance to crude fatty acid toxicity by overexpressing otsBA

Crude fatty acids were recovered from crude glycerol and different amounts (0.7–2.8 g in wet weight) were spiked back into the medium containing 20 g/L pure glycerol, which corresponded to the crude fatty acid concentrations in the medium of 14–57 wet-g/L. After 86 h of culture, cell growth and β-carotene production

of control cells decreased by 50% and 73%, respectively, in the presence of a small amount of crude fatty acids (14 wet-g/L) compared with the culture in the absence of crude fatty acids (Fig. 3). In contrast, they were not hampered even in the presence of a high amount of fatty acids (43 wet-g/L) in the engineered cell culture. This result clearly indicates that crude fatty acids significantly inhibit growth and production of *E. coli* cells but overexpressing *otsBA* endowed the cells with tolerance to the toxicity of these crude fatty acids.

The growth inhibitory effect of unsaturated fatty acids on many microorganisms is related to the concentration and degree of unsaturation of the fatty acids (Chatzifragkou et al., 2010; Kabara et al., 1972; Khulusi et al., 1995; Venkataramanan et al., 2012). It has also been proposed that the degree of growth inhibition is related to the incorporation of fatty acids into cytoplasmic membrane lipids of bacteria, which is accompanied by a diffusion barrier or membrane disruption (Khulusi et al., 1995). However, *E. coli* cells are not susceptible to long-chain fatty acids (C_{12}–C_{18}), although they are sensitive to medium-chain fatty acids (C_6–C_{10}) (Marounek et al., 2003; Fay and Farias, 1975). The lipopolysaccharide layer of *E. coli* cells protects them against inhibition by fatty acids (Sheu and Freese, 1973). Furthermore, *E. coli* cells can utilize exogenous long-chain fatty acids as a sole carbon and energy source through the fatty-acid beta-oxidation pathway (Zhang et al., 2006). In contrast, it is unknown why growth and production of control cells are inhibited in the presence of crude fatty acids composed of long-chain fatty acids ($\geqslant$96.7%). It is suspected that peroxides may exist in the crude fatty acids, which may cause inhibitory effects. Therefore, the following experiments were performed to demonstrate this notion.

3.5. Formation of peroxides from crude fatty acids and their toxicity to the cells

Crude and fatty acid-free crude glycerol solutions were prepared, in which the glycerol concentration was adjusted to 100 g/L by dilution with distilled water. After autoclaving the preparations at 121 °C for 20 min, peroxide generation was compared. It was observed that hydrogen peroxide (H_2O_2) and *tert*-butyl hydroperoxide (*t*-BuOOH) concentrations in crude glycerol were 41 μM and 94 μM, respectively whereas those were 6 μM and 22 μM, respectively, in the fatty acid-free preparation. This result indicates that higher amount of peroxides are generated in the presence of fatty acids than in their absence. As mentioned above, unsaturated fatty acids are abundant in crude glycerol. Peroxides are generated by autoxidation of unsaturated fatty acids. The hydrogen atoms in the fatty acids are removed and lipid alkyl radicals are produced in the initiation step. Heat accelerates free radical formation of fatty acids. The lipid alkyl radical reacts with oxygen and forms lipid peroxy radicals and another reactive radical. The lipid peroxy radical abstracts hydrogen from other lipid molecules and reacts with the hydrogen to form hydroperoxide and other lipid alkyl radicals (Choe and Min, 2006). Thus, it is suggested that fatty acids in crude glycerol are a major contributor to the formation of peroxides during autoclaving.

Accordingly, further experiments were performed to examine the effect of peroxides. *t*-BuOOH was tested, because it has higher toxicity and more was generated than H_2O_2 from crude glycerol. Compared to the culture in a medium without *t*-BuOOH, cells had a longer lag time and the cell concentration and β-carotene production were significantly lower in the presence of 30 μM *t*-BuOOH in the control cell culture, whereas those of engineered cells remained constant up to 60 μM *t*-BuOOH with a slight decrease in cell concentration at 90 μM *t*-BuOOH (Fig. 4). This result clearly shows that the growth of control cells was significantly inhibited by *t*-BuOOH from the early stage of culture leading to low yield of cells and β-carotene, whereas the engineered cells had better tolerance to *t*-BuOOH toxicity and, thus, their growth and β-carotene production were not significantly inhibited even in the presence of a high *t*-BuOOH concentration.

Exogenous peroxides can directly attack polyunsaturated fatty acids in cellular membranes and initiate lipid peroxidation, which results in the formation of membrane leaks, inactivation of membrane-bound proteins (Cabiscol et al., 2000; Yoon et al., 2002), as well as irreversible impairment of the respiratory function (Rodriguez et al., 1990). Although *E. coli* cells produce antioxidant enzymes to circumvent the harmful nature of peroxides, time is required for their induction (Cabiscol et al., 2000; Yoon et al., 2002) and cells are under severe oxidative stress when exposed to a greater quantity of exogenous peroxides than antioxidant enzyme capacity (Rodriguez et al., 1990). The experimental data on the toxicity of peroxides shown in this study are consistent with those of previous studies. In contrast, it has been reported that the accumulation of trehalose protects yeast cells and cellular proteins from damage by oxygen radicals during oxidative stress and is caused by hydrogen peroxides (Alvarez-Peral et al., 2002). In addition, trehalose protects *S. cerevisiae* from lipid peroxidation

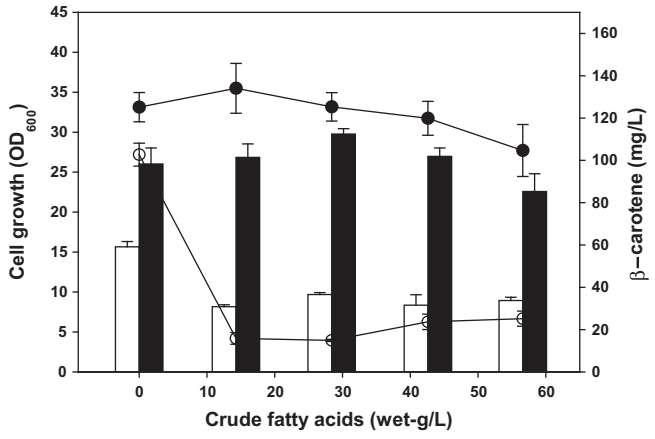

Fig. 3. Cell growth (bar chart) and β-carotene production (symbols and line graph) in the presence of crude fatty acids recovered from crude glycerol. Control (open symbols) and engineered cells (filled symbols) were cultured in medium supplemented with 20 g/L pure glycerol and different concentrations of crude fatty acids for 86 h.

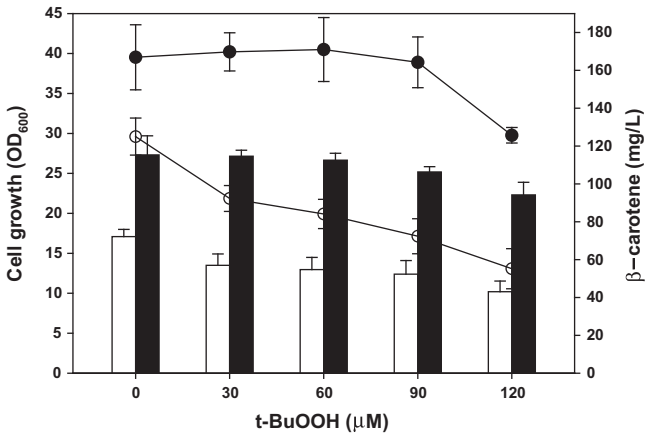

Fig. 4. Cell growth (bar chart) and β-carotene production (symbols and line graph) in the presence of *t*-BuOOH. Control (open symbols) and engineered cells (filled symbols) were cultured in medium supplemented with 20 g/L pure glycerol and different concentrations of *t*-BuOOH for 5 days.

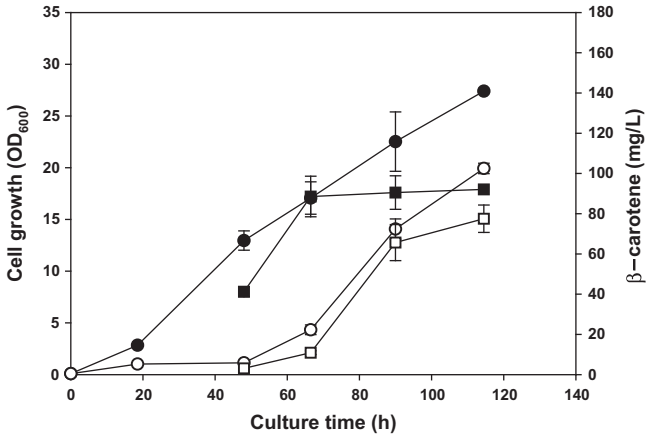

Fig. 5. Time profiles of cell growth (circles) and β-carotene production (squares) during culture of control (open symbols) and engineered cells (filled symbols) in medium supplemented with 20 g/L crude glycerol.

during oxidative stress (Herdeiro et al., 2006; Nery et al., 2008). Oku et al. (2003) proposed interactions between trehalose and unsaturated fatty acids; one trehalose molecule stoichiometrically interacts with one *cis*-olefin double bond of an unsaturated fatty acid, forming a stable complex and leading to significant reduction in oxidation levels.

In summary, peroxides were formed from crude fatty acids during autoclaving and these peroxides strongly inhibited the growth of *E. coli*. It is strongly suggested that overexpressing *otsBA* in *E. coli* cells promoted trehalose synthesis, which prevented cells from peroxidizing membrane lipids or impairing respiratory function by peroxides.

3.6. Cell growth and β-carotene production in cultures using medium supplemented with crude glycerol

Cultures were performed in medium supplemented with 20 g/L (glycerol basis) crude glycerol. The lag phase was prolonged in the control cell culture, leading to a long fermentation time (Fig. 5). Product titers and volumetric productivities were 2.8-fold and 1.7-fold lower, respectively, than those in cultures using pure glycerol. The prolonged fermentation time of crude glycerol due to its toxicity has been reported previously (Chatzifragkou et al., 2010; Venkataramanan et al., 2012).

In contrast, engineered cells grew with no lag phase. Although the cell growth rate was slightly lower, this result indicates that growth of engineered cells was not significantly inhibited in the presence of crude glycerol. β-Carotene production stopped after it reached 89 mg/L at 67 h, which was 61% of that obtained in pure glycerol. But, its volumetric productivity was 1.9-fold higher than that of control cells in cultures using crude glycerol. This result clearly shows that crude glycerol strongly inhibits cell growth and production, but overexpression of *otsBA* increases tolerance of *E. coli* cells to the toxicity of crude glycerol. Concentrations of toxic impurities increase when crude glycerol concentration increases. Therefore, cultures were performed to investigate the effect of different concentrations of crude glycerol, thereby determining the maximum concentration of crude glycerol that engineered cells can tolerate.

3.7. Effect of crude glycerol at different concentrations on cell growth and β-carotene production

Control and engineered cells were cultured in media supplemented with varying concentrations (20–80 g/L) of crude glycerol.

Table 2
Cell growth and β-carotene production in the presence of different concentrations of pure or crude glycerol in control or engineered cell cultures.

Components	Concentration (g/L)	Control cells		Engineered cells	
		Cell growth (OD_{600})	β-Carotene (mg/L)	Cell growth (OD_{600})	β-Carotene (mg/L)
Pure glycerol	20	15.6 ± 0.9	104.7 ± 3.3	28.0 ± 1.8	175.5 ± 1.8
	40	15.9 ± 0.7	121.6 ± 6.7	26.5 ± 0.6	159.2 ± 5.5
	60	15.1 ± 1.2	92.4 ± 18.1	25.5 ± 2.4	149.6 ± 11.9
	80	13.1 ± 0.6	69.5 ± 26	23.3 ± 0.6	139.5 ± 22.3
Crude glycerol	20	17.2 ± 1.5	89.7 ± 13.5	31.3 ± 0.5	126.1 ± 17.3
	40	13.6 ± 0.9	66.9 ± 7.3	32.4 ± 1.0	150.9 ± 6.2
	60	10.2 ± 0.6	48.1 ± 9.1	30.7 ± 3.4	135.0 ± 11.9
	80	11.3 ± 0.8	52.8 ± 8.6	27.1 ± 0.7	110.4 ± 13.8

Cells were cultured in medium supplemented with different concentrations of pure or crude glycerol for 5 days.

Table 3
Concentration of impurities in medium supplemented with different concentrations of crude glycerol.

Crude glycerol (g/L)[a]	K^+ (mM)[b]	Fatty acids (wet-g/L)	Methanol (g/L)	H_2O_2 (μM)	*t*-BuOOH (μM)
20	160 ± 0.0	14.7 ± 0.6	2.9 ± 0.1	8.1 ± 2.6	18.8 ± 1.4
40	170 ± 0.0	29.3 ± 1.3	5.7 ± 0.3	16.2 ± 5.2	37.6 ± 2.8
60	173 ± 0.0	43.9 ± 1.9	8.6 ± 0.4	24.3 ± 7.8	56.4 ± 4.1
80	179 ± 0.0	58.6 ± 2.5	11.4 ± 0.6	32.4 ± 10.4	75.2 ± 5.5

[a] Concentrations of impurities in 100 g/L of crude glycerol were measured and then the calculations were performed.
[b] Basal medium contained 150 mM of K^+; thus, the true effect of crude glycerol increasing K^+ in medium was not significant.

As shown in Table 2, cell concentration and β-carotene production by control cells decreased steadily with increasing concentration of crude glycerol, whereas the concentration of engineered cells was almost the same up to 60 g/L. Notably, β-carotene production by engineered cells was the highest at 40 g/L and still higher at 60 g/L than that at 20 g/L. However, a slight decrease in cell concentration and β-carotene production of engineered cells was observed at 80 g/L. Cultures were performed in media supplemented with corresponding concentrations of pure glycerol for comparison. No significant differences in concentration of control cells were observed despite increasing the concentration up to 60 g/L (Table 3). By comparing cultures in media with pure and crude glycerol, it was certain that some impurities in the crude glycerol inhibited growth of control cells, but that the engineered cells tolerated these toxic materials.

As mentioned above, the major impurities in crude glycerol are fatty acid salts, methanol, and potassium ions. Based on these data, the quantity of these impurities was calculated in medium supplemented with varying concentrations of crude glycerol. As shown in Table 3, an increased amount of these impurities was observed as a higher amount of crude glycerol was added to the medium. The concentrations of crude fatty acids, methanol, and *t*-BuOOH in medium containing 60 g/L crude glycerol were 44 wet-g/L, 8.6 g/L, and 56.4 μM, respectively. Considering that engineered cells tolerated crude fatty acids (⩽42 wet-g/L), methanol (⩽7.5 g/L), and *t*-BuOOH (⩽60 μM) in separate experiments, it is understandable that engineered cells could tolerate up to 60 g/L crude glycerol. Higher potassium concentrations increase osmolarity, which may result in osmotic stress to *E. coli* and cause growth suppression. Medium supplemented with 20–80 g/L crude glycerol contained 160–179 mM potassium ions, respectively (Table 3). Considering

that basal synthetic R medium contains 154 mM potassium ions, the contribution of crude glycerol to increase potassium ion was small (4–19 mM). Accordingly, the effect of potassium caused by crude glycerol was not critical.

4. Conclusions

Potassium and methanol concentrations in crude glycerol were too low to inhibit cell growth. Rather, it was newly discovered that peroxides were generated in the presence of crude fatty acids during the autoclaving process and, thus, the inhibitory effect of crude glycerol was primarily caused by these peroxides. This is the first demonstration that overexpression of *otsBA* endows *E. coli* cells with the capacity to tolerate these toxic peroxides in crude glycerol, thereby crude glycerol can be utilized directly. This novel approach will strongly contribute to the development of a microbial process for producing value-added products or biofuels by utilizing crude glycerol directly without the requirement for further treatment.

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