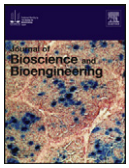

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Enhancement of retinal production by supplementing the surfactant Span 80 using metabolically engineered *Escherichia coli*

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The optimal temperature and pH for retinal production using metabolically engineered *Escherichia coli* in a 7-l fermentor were found to be 30°C and 7.0, respectively. The agitation speed was a critical factor for retinal production. The optimal agitation speed was 400 rpm (oxygen transfer coefficient, k_La, = 92 1/h) in batch culture and 600 rpm (k_La = 148 1/h) in a fed-batch culture of glycerol. Span 80 was selected as a surfactant for retinal production in metabolically engineered *E. coli* because Span 80 had proven the most effective for increased retinal production among the tested surfactants. Under the optimal conditions in the fed-batch culture with 5 g/l Span 80, the cell mass and the concentration, content, and productivity of retinal were 24.7 g/l, 600 mg/l, 24.3 mg/g-cells, and 18 mg l^{-1} h^{-1} after 33 h, respectively. They were 1.2-, 2.7-, 2.3-, and 2.7-fold higher than those in the fed-batch culture without Span 80, respectively. The concentration and productivity of retinal in this study were the highest ever reported. The hydrophilic portion of Span 80 (sorbitan) did not affect cell growth and retinal production, but the hydrophobic portion (oleic acid) stimulated cell growth. However, oleic acid plus sorbitan did not stimulate retinal production. Thus, Span 80, as a linked compound of oleic acid and sorbitan produced by esterification, proved to be an effective surfactant for the enhancement of retinal production.

[**Key words:** Retinal; *Escherichia coli*; Metabolically engineered cells; Surfactant; Span80; Fed-batch culture]

Retinal, the alcohol form of vitamin A, is an important material for vision, development, cell differentiation, cancer prevention, and skin protection. Retinal and its derivatives have been used in foods, cosmetics, pharmaceuticals, nutraceuticals, and animal feed additives, owing primarily to their anti-infective (1), anti-cancer (2), anti-oxidant, and anti-wrinkle functions (3). Retinal has been produced by chemical synthesis (4); however, this chemical process has some disadvantages, including complex purification steps and the formation of chemical wastes and undesired by-products. Thus, the enzymatic synthesis of retinal from carotenoids by β-carotene 15,15′-oxygenase (BCO) has recently attracted much attention (5,6).

Metabolic engineering of *Escherichia coli* has been suggested as an effective tool for retinal production, and has been applied to the production of various carotenoids, such as lycopene (7), β-carotene (8), canthaxanthin, astaxanthin (9), and zeaxanthin (10) by introducing foreign carotenoid biosynthetic genes into non-carotenogenic *E. coli*. Thus, the metabolically engineered strain of *E. coli*, which was prepared from the introduction of foreign BCO gene (11) into β-carotene producing *E. coli*, can produce retinal (Fig. 1).

Surfactants, which lower the surface tension between liquid and solid, are known to increase cell wall permeability, and to improve substrate uptake and product formation (12). Span 20, a non-ionic surfactant, has been shown to significantly enhance β-carotene production in *Blakeslea trispora* by inducing dispersed mycelia (13). The prevention of clump formation by the addition of Tween 80 has been shown to stimulate cell growth and lycopene production in metabolically engineered *E. coli* (14). As a result, surfactant supplementation may represent an effective tool for enhancement of carotenoid production in microorganisms, and may also be applicable to retinal production. However, the role of surfactant on the production of carotenoid or retinoid is currently not well elucidated.

In the present study, the effect of surfactant on retinal production in metabolically engineered *E. coli* was investigated, and Span 80 was selected as an effective detergent. The enhancement of retinal production in fed-batch cultures was carried out via supplementation with Span 80. The contributions of hydrophobic portion (oleic acid) and/or hydrophilic portion (sorbitan) of Span 80 for cell mass and retinal production were also investigated.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media A metabolically engineered strain of *E. coli* K12 harboring the pT-DHBSR plasmid containing the genes encoding for the enzymes of the retinal biosynthetic pathway, as well as the pS-NA plasmid containing the genes encoding for the enzymes of the entire mevalonate pathway. The pT-DHBSR plasmid was constructed from the cloning of the *blh* gene from the uncultured marine bacterium 66A03 (11) into the pT-DHB plasmid which was constructed from the cloning of *dxs* from *E. coli*, *ipiHP1* from *Haematococcus pluvialis*, *crtE*, *crtB*, and *crtI* from *Pantoea agglomerans*, and *crtY* from *P. ananatis* into pTrc99A (Amersham Biosciences, Piscataway, NJ, USA). The pS-NA plasmid was constructed from the cloning of *mvaE* (the bifunctional gene of *mvaA* and *phaA*) and *mvaS* from *Enterococcus faecalis*, *mvaK1*, *mvaK2*, and *mvaD* from *Streptococcus pneumoniae*, and *idi* from *E. coli* into pSTV28

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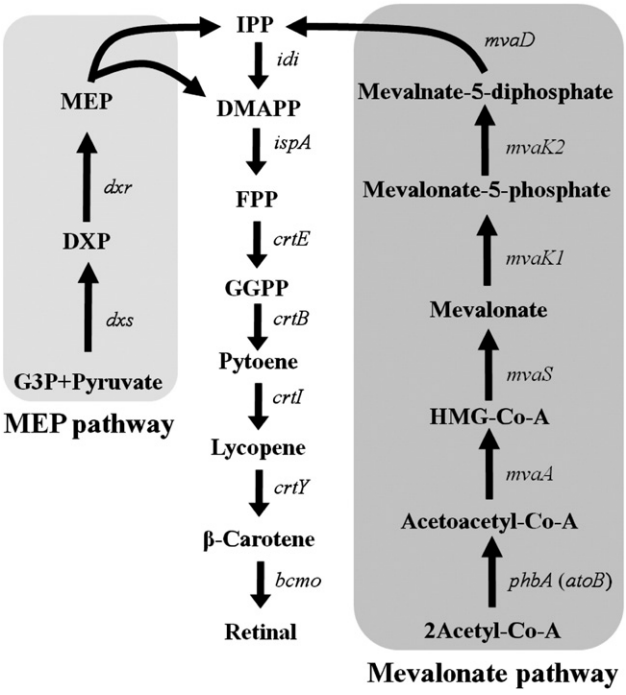

FIG. 1. Biosynthetic pathway of retinal in *Escherichia coli* from a foreign mevalonate pathway and a native 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway. Gene names and its encoded enzymes are as follows: *phaA*, acetyl-CoA acetyltransferase; *mvaA*, HMG-CoA reductase, *mvaS*, HMG-CoA synthase; *mvaK1*, mevalonate kinase; *mvaK2*, phosphomevalonate kinase; *mvaD*, mevalonate 5-diphosphate decarboxylase; *dxs*, DXP synthase; *dxr*, DXP reductoisomerase; *idi*, IPP isomerase; *ispA*, FPP synthase; *crtE*, GGPP synthase; *crtB*, phytoene synthase; *crtI*, phytoene desaturase; *crtY*, lycopene cyclase; *bcmo*, β-carotene oxygenase. Pathway intermediates: G3P, D-glyceraldehyde-3-phosphate; DXP, 1-deoxy-D-xyluose 5-phosphate; MEP, 2-C-methyl-D-erythritol-4-phosphate; IPP, isopentenyl diphosphate; DMAPP, dimethylallyl diphosphate; and FPP, farnesyl diphosphate; GGPP, geranylgeranyl diphosphate.

(Takara Bio, Shiga, Japan). The detailed procedures for the construction of the pT-DHB and pS-NA plasmids were consistent with those described in the previous report (15). The growth medium was 2YT medium (16 g/l tryptone, 10 g/l yeast extract, and 5 g/l NaCl) containing 10 g/l glycerol. The production medium consisted of 10 g/l glycerol, 11.2 g/l KH_2PO_4, 3 g/l $(NH_4)_2HPO_4$, 0.3 g/l NaCl, 1 g/l $MgSO_4.7H_2O$, trace elements (2.5 mg/l $CoCl_2 \cdot 6H_2O$, 15 mg/l $MnCl_2 \cdot 4H_2O$, 1.5 mg/l $CuCl_2 \cdot 2H_2O$, 3 mg/l H_3BO_3, 2.5 mg/l $Na_2MoO_4 \cdot 2H_2O$, 13 mg/l $Zn(CH_3COO)_2 \cdot 2H_2O$, and 12.5 mg/l Fe(III)citrate), and amino acids (1.1 g/l leucine, 0.7 g/l isoleucine, 2 g/l lysine, 3.3 g/l phenylalanine, 0.4 g/l valine, 1.5 g/l threonine, 3.3 g/l methionine, and 2.2 g/l glutamine). Antibiotics (50 mg/l ampicillin, and 25 mg/l chloramphenicol) were added to the growth and production media. In the fermentor cultures, 10 g/l glucose and 7.5 g/l L-arabinose were added initially to the culture media as auxiliary carbon sources (16).

Culture conditions A single colony of metabolically engineered *E. coli* was inoculated into a 20 ml-test tube containing 5 ml of the growth medium and cultivated at 37°C with agitation at 200 rpm for 12 h. In the flask culture, the seed culture of 2 ml was transferred to a 250-ml baffled flask containing 50 ml of the production medium, and cultivated at 30°C with agitation at 200 rpm for 40 h. In the fermentor culture, the seed culture of 4 ml was transferred to a 500-ml baffled flask containing 100 ml of the growth medium and cultivated at 37°C with agitation at 200 rpm for 5 h. This culture broth of 100 ml was subsequently transferred to a 7-l fermentor (Biotron, Buchenon, Korea) initially containing 2 l of the production medium, then cultivated at 30°C and pH 7.0. The aeration rate was fixed at 1.0 vvm. The agitation speed was 200–400 rpm during the fermentation and/or was adjusted to 500–800 rpm after 8 h to evaluate the effect of agitation speed on retinal production. The dissolved oxygen (DO) of 20% was maintained by gradually increasing the agitation speed from 500 to 1200 rpm after 8 h. In the fed-batch culture of glycerol, a feeding solution containing 900 g/l glycerol was added to the fermentor with a peristaltic pump at a flow rate ranging from 0.3 to 0.5 ml/min (Watson-Marlow 101 U/R, Wilminghton, MA, USA), and the glycerol concentration was maintained within 10 g/l during the fermentation. Total added glycerol in the fermentor was approximately 140–150 g/l.

Effects of surfactant and Span 80 components on retinal production The effects of surfactant type and concentration on cell mass and retinal production were evaluated using several surfactants, including Span 20, Span 40, Span 60, Span 80, Tween 20, Tween 40, Tween 80, Brij 58, and Triton X-100, by varying the surfactant concentrations from 0.5 to 20 g/L in the flask cultures. The effects of Span 80 and its components in on cell mass and retinal production were assessed by varying the molar concentrations of oleic acid (hydrophobic portion), sorbitan (hydrophilic portion), oleic acid plus sorbitan, and Span 80 (sorbitan monooleate) from 2.3 to 23.3 mM. Span 80 and oleic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA), and sorbitan was purchased from CSC (Yangsan, Korea).

Analytical methods The cell mass was determined using a linear calibration curve relating optical density at 600 nm and dry cell weight. The oxygen transfer coefficient (k_La) was calculated from the rate of DO concentration recovery from air after nitrogen degassing of the liquid phase (dynamic gassing-out method). Metabolically engineered *E. coli* cells were harvested by centrifugation at 13,000× *g* for 5 min at 4°C and washed once with water. The cell pellet was resuspensed in 1 ml of acetone and incubated at 55°C for 15 min with intermittent vortexing. The mixture was then centrifuged at 13,000 × *g* for 10 min, and the acetone supernatant was transferred to a new tube. The entire extraction process was conducted under reduced light conditions to prevent photobleaching and degradation. The concentration of retinal was determined using an HPLC system (Agilent 1100, Agilent, Santa Clara, CA, USA) equipped with a UV detector at 370 nm and a C18 column (YMC, Kyoto, Japan). The column was eluted with a 90:10 (v/v) mixture of acetonitrile and water as the mobile phase at a flow rate of 1.0 ml/min.

The reaction solution was extracted with an equal volume of ethyl acetate for determining oleic acid. The solvent was removed from the extract using a rotary evaporator. The obtained oleic acid was silylated with a 2:1 mixture of pyridine and *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (17). Silylated oleic acid in the organic phase was analyzed by a gas chromatograph (GC) (Agilent 6890 N) equipped with a flame ionization detector and a Supelco SPB-1 capillary column (Supelco, Bellefonte, PA, USA). The column temperature was increased from 150°C to 210°C for 15 min and then maintained at 210°C. The injector and detector temperatures were 260°C and 250°C, respectively. The concentrations of Span 80 and sorbitan were decided using the same HPLC system at 210 nm with an octadecylsilane (ODS) column (YMC). The column was eluted with a 70:30 (v/v) mixture of isopropyl alcohol and water as the mobile phase at a flow rate of 0.3 ml/min (18).

RESULTS

Optimization of culture conditions for retinal production In order to assess the effect of temperature on retinal production in metabolically engineered *E. coli* in batch cultures using a 7-l fermentor, the temperature was varied from 24°C to 33°C. The maximum specific retinal content and retinal concentration were noted at 30°C, whereas the maximum cell mass was detected at 27°C (data not shown). Batch cultures were carried out at pH levels ranging from 5.5 to 8.0, the specific retinal content and retinal concentration were maximal at pH 7.0 (data not shown). However, cell mass was maximal at pH 6.0.

The effect of agitation speed on cell growth and retinal production was evaluated in batch and fed-batch cultures. As the agitation speed increased, cell mass also increased. However, retinal production and specific retinal content were maximal at 400 rpm (oxygen transfer coefficient using water in the fermentor, $k_La, = 92$ 1/h) in batch culture (Fig. 2A) and 600 rpm ($k_La = 148$ 1/h) in fed-batch culture (Fig. 2B). The higher agitation speed in fed-batch culture for retinal production than that in batch culture may be attributable the 2.4-fold higher cell mass obtained in fed-batch culture. When the level of DO was maintained at 20%, the cell mass was the highest. However, the specific retinal content was the lowest. Thus, the optimum culture conditions for retinal production were a temperature of 30°C, a pH of 7.0, and an agitation speed of 600 rpm in fed-batch culture; these conditions were adopted for all subsequent experiments.

Selection of the surfactant for retinal production in metabolically engineered *E. coli* To select the most effective surfactant for retinal production in metabolically engineered *E. coli*, each non-ionic surfactant of Span 20, Span 40, Span 60, Span 80, Tween 20, Tween 40, Tween 80, Brij 58, or Triton X-100 was added, and its concentration was varied from 0.5 to 20 g/l into a separate flask. Surfactant supplementation was effective to increase cell mass (Table 1). In particular, significantly increases in cell mass were observed by supplementating the Span type surfactants. Among the surfactants tested, Span 80 was the most effective for increasing cell mass. The maximum cell mass was obtained by supplementation with 10 g/l Span 80. At this concentration, cell mass was 15.2 g/l, which was 2.7-fold higher than that obtained in the culture without Span 80. However, supplementation with the ionic surfactant SDS markedly inhibited cell growth (data not shown).