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Production of poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) by high cell density cultivation of recombinant *Escherichia coli*

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1. Introduction

Polyhydroxyalkanoates (PHAs) are polyesters, which are accumulated as energy and carbon storage materials by numerous microorganisms usually when a nutritional component such as N, P, S, O or Mg is limited in the presence of excess carbon source[1]. PHAs have been considered to be good biodegradable substitutes for petroleum-derived synthetic plastics because of their similar material properties to synthetic polymers and complete biodegradability after disposal. Recently, the PHA biosynthesis genes of *Aeromonas caviae*, which can produce a copolymer composed of 3-hydroxybutyrate and 3-hydroxyhexanoate [P(3HB-co-3HHx)], were cloned and characterized in detail[2]. This copolyner shows similar mechanical property to LDPE. PHA biosynthesis genes of *A. caviae* consisted of *orf1*, *phaC* and *phaJ* genes encoding phasin, PHA synthase, and (R)-specific enoyl-CoA hydratase, respectively. Also, Lee et al[3]. reported high level production of P(3HB-co-3HHx) from oleic acid by fed-batch culture of a newly isolated *Aeromonas hydrophila* strain[3]. There have been several reports on the high cell density cultivation of recombinant *E. coli* for the high level production of poly(3-hydroxybutyrate) and poly(3-hydroxybutyrate-co-3-hydroxyvalerate)[4,5]. Therefore, it was aimed to develop a metabolically engineered *E. coli* strain capable of efficiently producing P(3HB-co-3HHx).

In this study, we constructed several metabolically engineered recombinant *E. coli* strains harboring different plasmids containing novel artificial PHA operons consisting of the *Aeromonas* PHA biosynthesis related genes and *Ralstonia eutropha* reductase gene. We also developed a strategy for the high cell density fed-batch cultivation in order to achieve high level production of P(3HB-co-3HHx) from dodecanoic acid.

2. Materials and methods

Bacterial strain and plasmid. *Aeromonas salmonicida achromogenes* and *Aeromonas hydrophila* strains were isolated from the raw sewage samples. *E. coli* XL1-Blue was used as a host strain for general cloning purposes and *E. coli* LS5218 for the production of PHA. All the DNA fragments used in plasmid construction were obtained by PCR. Finally, prTrp3ACnJB and prTrp3ACnJBOF1 were constructed and used for the production of PHA. In prTrp3A-CnJB, the artificial PHA operon consisting of the *A. salmonicida achromogenes* $phaC_{Asa}$ gene, the *A. hydrophila* $phaJ_{Ah}$ gene and the *R. eutropha* $phbB_{Re}$ gene was expressed by the constitutive *rtrp* promoter. In prTrp3A-CnJBOF1, the *A. hydrophila orf1* gene was inserted in front of the *A.*

salmonicida achromogenes $phaC_{Asa}$ gene in prTrp3A-CnJB.

Culture conditions. Two wild type *Aeromonas* strains were cultured in Luria-Bertani (LB) medium at 30 °C. *E. coli* XL1-Blue was grown at 37 °C in LB medium. Recombinant *E. coli* LS5218 strains were cultivated in chemically defined MR medium supplemented with dodecanoic acid (Junsei Co., Tokyo, Japan) as a carbon source at 37 °C for the production of PHA. All flask cultures were carried out in a rotary shaker at 250 rpm. For the cultivation of recombinant *E. coli* strains, ampicillin (Ap, 50 mg/L) was added. The MR medium (pH 6.5) contains per liter: 6.67 g KH_2PO_4, 4 g $(NH_4)_2HPO_4$, 0.8 g $MgSO_47H_2O$, 0.8 g citric acid, and 5 mL trace metal solution. The trace metal solution contains per liter 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 g $CuSO_45H_2O$, 0.1 g $(NH_4)_6Mo_7O_244H_2O$, 0.02 g $Na_2B_4O_710H_2O$. Carbon source and $MgSO_47H_2O$ were separately sterilized and were added to give desired concentrations.

Fed-batch cultivations. Fermentation A and B were carried out in a 6.6 L jar fermentor (Bioflo 3000, New Brunswick Scientific Co., Edison, NJ) containing 1.6 L of MR medium containing 5 g/L of dodecanoic acid. Foaming was controlled by adding Antifoam A (Sigma Chemical Co., St. Louis, MO). Seed cultures were prepared in 0.4 L of MR medium for fermentation A and B by growing cells in a rotary shaker overnight at 37 °C and 250 rpm. The pH was controlled at 6.5 by the automatic addition of 28% (v/v) NH_4OH because foaming became severe when pH was higher than 6.5. In fermentation A and B, the DOC was controlled as desired by automatically controlling the agitation speed up to 1000 rpm and the pure oxygen percentage. In fermentation A, twelve feeding solutions were prepared in 20 mL aliquots containing 10, 10, 20, 20, 20, 30, 30, 30, 30, 30, 30, and 30 g dodecanoic acid, and were fed into the fermentor in the same order. All the feeding solutions contained 0.02 g citrate and 0.02 g $MgSO_47H_2O$ per g dodecanoic acid. In fermentation B, sixteen feeding solutions were prepared in 20 mL aliquots containing 10, 10, 20, 20, 25, 25, 30, 30, 35, 35, 35, 35, 35, 30, 30, and 30 g dodecanoic acid, and were fed into the fermentor in the same order. All the feeding solutions except the last four aliquots contained 0.02 g citrate and 0.02 g $MgSO_47H_2O$ per g dodecanoic acid.

Analytical procedures. Cell growth was monitored by measuring the absorbance at 600 nm (OD_{600}; DU Series 600 Spectrophotometer, Beckman, Fullerton, CA). PHA concentration was determined by gas chromatography (Donam Co., Seoul, Korea) equipped with a fused silica capillary column (Supelco SPB™-5, 30 m × 0.32 mm ID 0.25 μm film, Bellefonte, PA) using benzoic acid as an internal standard[3]. Cell concentration, defined as cell dry weight per liter of culture broth, was determined as previously described. The residual cell concentration was defined as the cell concentration minus PHA concentration. The PHA content (wt%) was defined as the percentage of the ratio of PHA concentration to cell concentration. The molar fractions of 3HB and 3HHx monomers were determined by ^1H and ^{13}C nuclear magnetic resonance (AMX 500, Bruker, Germany).

3. Results

PHA production in flask cultures. The recombinant *E. coli* LS5218 harboring prTrp3A-CnJB was cultured for 96h in a MR medium containing 5 g/L dodecanoic acid. The final P(3HB-co-3HHx) content and 3HHx fraction of 7 wt% and 5.9 mol%, respectively, were obtained in 96 h. The recombinant *E. coli* LS5218 harboring prTrp3A-CnJBOF1 produced P(3HB-co-3HHx) up to 10 wt% of DCW. The 3HHx fraction was 18.9 mol%.

PHA production in fed-batch cultures. The recombinant *E. coli* LS5218 harboring prTrp3A-CnJBOF1 was used for the fed-batch cultures. Time profiles of fed-batch cultures are shown in Figure 1. In fermentation A, the DOC was maintained at 40% of air saturation during the lag and initial growth phase and then the DOC was reduced to 10%. The final DCW, PHA concentration, P(3HB-co-3HHx) content and 3HHx fraction obtained in 36.5 h were 78.7 g/L, 18.1 g/L, 23 wt%, and 8.64mol%, respectively, which resulted in the PHA productivity of 0.49 g of PHA/L/h. After the DOC was reduced from 40% to 10% when DCW reached 50 g/L, P(3HB-co-3HHx) content increased by two fold (13 wt% to 23wt%), but cell growth was not inhibited properly. In fermentation B, the DOC was decreased stepwise. First, the DOC was maintained at 40% during the lag and initial growth phase, and then reduced to 10% earlier than the fermentation C during the active and late growth phase (ca. DCW of 40 g/L). During the stationary phase, the DOC was further reduced to 5%. When the DOC level was decreased to 5 %, cell growth was severely inhibited, and the PHA content and 3HHx fraction were increased. The final DCW, PHA concentration, PHA content and 3HHx fraction obtained in 40.8 h were 79 g/L, 21.5 g/L, 27.2 wt%, and 10.8 mol%, respectively, which resulted in the PHA productivity of 0.53 g of PHA/L/h.

4. References

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5. Acknowledgement

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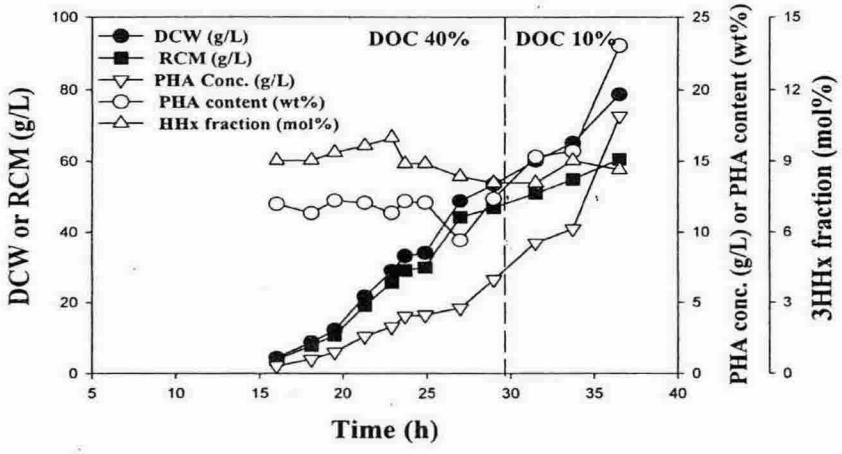

Figure 1a. Time profiles of dry cell weight (DCW, ●), residual cell concentration (RCM, ■), PHA content (○), PHA concentration (▽) and 3HHx fraction in polymer (△) during the fed-batch culture of *E. coli* LS5218 (prTrp3A-CnJBOF1) (fermentation A).

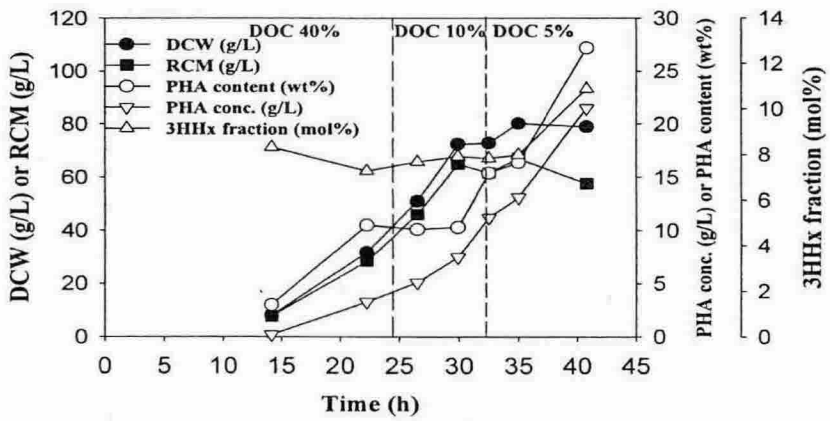

Figure 1b. Time profiles of dry cell weight (DCW, ●), residual cell concentration (RCM, ■), PHA content (○), PHA concentration (▽) and 3HHx fraction in polymer (△) during the fed-batch culture of *E. coli* LS5218 (prTrp3A-CnJBOF1) (fermentation B).