Biotechnology and Bioprocess Engineering **2004**, 9: 196-200 ©KSBB

High Level Production of Supra Molecular Weight Poly(3-Hydroxybutyrate) by Metabolically Engineered *Escherichia coli*

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Abstract The supra molecular weight poly([R]-3-hydroxybutyrate) (PHB), having a molecular weight greater than 2 million Da, has recently been found to possess improved mechanical properties compared with the normal molecular weight PHB, which has a molecular weight of less than 1 million Da. However, applications for this PHB have been hampered due to the difficulty of its production. Reported here, is the development of a new metabolically engineered *Escherichia coli* strain and its fermentation for high level production of supra molecular weight PHB. Recombinant *E. coli* strains, harboring plasmids of different copy numbers containing the *Alcaligenes latus* PHB biosynthesis genes, were cultured and the molecular weights of the accumulated PHB were compared. When the recombinant *E. coli* XL1-Blue, harboring a medium-copy-number pJC2 containing the *A. latus* PHB biosynthesis genes, was cultivated by fed-batch culture at pH 6.0, supra molecular weight PHB could be produced at up to 89.8 g/L with a productivity of 2.07 g PHB/L-h. The molecular weight of PHB obtained under these conditions was as high as 22 MDa, exceeding by an order of magnitude the molecular weight of PHB typically produced in *Ralstonia eutropha* or recombinant *E. coli.*

Keywords: PHB, molecular weight, recombinant *E. coli*, fed-batch culture

INTRODUCTION

A wide range of bacteria are able to synthesize a polymer of (*R*)-3-hydroxybutyrate as an intracellular storage material [1-5]. Poly[(*R*)-3-hydroxybutyrate] (PHB) is accumulated as distinct granules inside the cytoplasm. The isolated polymer is a partially crystalline thermoplastic with biodegradable and biocompatible properties [6]. The metabolic pathway and enzymes involved in PHB synthesis have been studied extensively in *Ralstonia eutropha*. The genes for 3-ketothiolase (*phbA*), NADPH-linked acetoacetyl-CoA reductase (*phbB*) and PHB synthase (*phbC*), from *R. eutropha* and *Alcaligenes latus,* have been cloned and analyzed. They form an operon in the order of *phbC-phbA-phbB* [7,8]. Introduction of these three genes into *Escherichia coli* resulted in the accumulation of large quantities of PHB [3,8-10]. Recently, the time-dependent changes in the molecular weight of PHB, produced in a recombinant *E. coli* harboring the *R. eutropha* biosynthesis *phbCAB* genes, have been examined [11]. When the recombinant *E. coli* was cultured at pH 6.0, which is a suboptimal growth condition, PHB, having an extremely high molecular weight (20 MDa), could be obtained. This is an order of magnitude greater than the molecular weight of PHB typically produced in *R. eutropha* and recombinant *E. coli* [11]. The mechanical properties of this supra molecular weight PHB were markedly improved over normal molecular weight PHB [12,13]. For example, supra molecular weight PHB film has shown a significant increase of tensile strength and elongation-to-break up, 237 MPa and 112%, respectively. The supra molecular weight PHB homopolymer has also been suggested to be suitable for making a strong fiber with complete biodegradability [13,14]. Furthermore, it was found to be suitable as a drug carrier [15]. However, the actual application of supra molecular weight PHB, has been hampered due to the lack of a strategy for its efficient production.

In this paper, it is reported that PHB of various molecular weights, can be produced by employing metabolically engineered *E. coli* strains harboring the plasmids of different copy numbers containing the *A. latus phbCAB* genes. Also reported on, is the highly productive fed-batch cultivation strategy for the production of ultra-high-molecular weight PHB in high concentrations.

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Table 1. PHB production in recombinant *E. coli* strains with different plasmids at 30°C and pH 6.9[a]

	pJC1	pJC2	pJC3	pJC4	pJC5
Cell concentration (g/L)	4.69 ± 0.03	5.21 ± 0.04	6.45 ± 0.04	6.71 ± 0.05	6.05 ± 0.04
PHB concentration (g/L)	1.83 ± 0.02	2.76 ± 0.01	4.64 ± 0.02	4.76 ± 0.03	4.54 ± 0.03
PHB content (wt%)	39 ± 3	53 ± 2	72 ± 3	71 ± 3	75 ± 2
Molecular mass (MDa)	0.87 ± 0.16	0.97 ± 0.17	0.89 ± 0.12	0.91 ± 0.11	0.90 ± 0.13

[a]Cultures were carried out in triplicate.

MATERIALS AND METHODS

Bacterial Strain and Plasmids

The *E. coli* strain used in this study was XL1-Blue (*supE44 hsdR17 recA1 endA1 gyrA96 thi- relA1 lacF'[proAB+ lacIq lacZΔM15 Tn10(tetr)]*). The plasmids pJC1, pJC2, pJC3, pJC4, and pJC5, harboring the *A. latus* PHB biosynthesis genes, have been previously described [8,9]. Briefly, pJC1 and pJC3 were pUC19 (New England Biolabs, Beverly, MA, USA) derivatives, containing the *A. latus* PHB biosynthesis genes. Plasmids pJC2 and pJC4 were pGEM-7Zf(+) (Promega, Madison, WI, USA) derivatives, containing the *A. latus* PHB biosynthesis genes and the *parB* locus. Plasmids pJC3 and pJC4 were constructed from pJC1 and pJC2, respectively, by removing 1 kb of unnecessary DNA fragment upstream of the promoter region of the *A. latus* PHB biosynthesis genes. Plasmid pJC5 is a pBluescript KS- (Stratagene, La Jolla, CA, USA) derivative, containing the *A. latus* PHB biosynthesis genes, the *parB* locus and the *E. coli ftsZ* gene [9].

Culture Conditions

The *E. coli* cells were maintained as a 20% (v/v) glycerol stock at -80°C, after growth in Luria-Bertani (LB) medium (10 g/L yeast extract, 5 g/L tryptone and 10 g/L NaCl).

Flask and fed-batch cultures were carried out in a chemically defined MR (pH 6.9) medium. The MR medium (pH 6.9), per liter, contains: 22 g KH_2PO_4, 3 g $(NH_4)_2HPO_4$, 0.7 g $MgSO_47H_2O$, 0.8 g citric acid and 5 mL of trace metal solution. The trace metal solution, per liter, contains: 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 $(NH_4)_6Mo_7O_{24}4H_2O$ and 0.02 g $Na_2B_4O_710H_2O$ [10]. Flask cultures were performed in 250 mL flasks containing 50 mL of medium. For the fed-batch cultures, seed cultures were prepared by growing cells in a shaking incubator overnight at 30°C and 250 rpm. Fed-batch cultures were carried out at 30°C in a 6.6 L jar fermentor (Bioflo 3000, New Brunswick Scientific Co., Edison, NJ, USA), initially containing 1.6 L of MR medium. Separately sterilized glucose and thiamine were supplemented into the MR medium to the final concentrations of 20 g/L and 10 mg/L, respectively.

Culture pH was controlled at 6.0 or 6.9 by the addition of 28% (v/v) ammonia water. The dissolved oxygen concentration (DOC) was maintained as desired by the automatic change of the agitation speed up to 1,000 rpm and by the pure oxygen percentage. The feeding solution during fed-batch culture per liter, contained: 700 g of glucose, 15 g of $MgSO_47H_2O$ and 250 mg of thiamine. The pH-stat feeding strategy was employed for fed-batch cultures. When the pH rose to a value greater than its setpoint (6.0 or 6.9), by an increment of 0.1, an appropriate volume of feeding solution was automatically added to increase the glucose concentration in the culture medium to 20 g/L.

Analytical Procedure

Cell growth was monitored by measuring the absorbance at 600 nm (OD_{600}) (DU® Series 600 Spectrophotometer, Beckman, Fullerton, CA, USA). Cell concentration, defined as dry cell weight (DCW) per liter of culture broth, was determined by weighing dry cells as described previously [8]. PHB concentration was determined by gas chromatography (HP5890, Hewlett-Packard, Wilmington, DE, USA) using n-benzoic acid as an internal standard [16]. The PHB content (wt%), was defined as, the percentage of the ratio of PHB concentration to cell concentration.

PHB was purified by the chloroform extraction method [17]. Cells were collected by centrifugation and were washed with hot acetone. After drying, the cells were mixed with 50 volumes of chloroform for 48 h at 30°C. Clear PHB solution was recovered by centrifugation, which was followed by polishing filtration. Finally, pure PHB was obtained by non-solvent precipitation with MeOH and filtration. The number average molecular weight (Mn) of the PHB sample was determined by gel permeation chromatography (GPC). The GPC was equipped with three serially connected columns having pore sizes of 10^3, 10^4, and 10^5 Å. Shodex SM-105 polystyrene (Showa Denko K. K., Tokyo, Japan) and chloroform were used as a molecular weight standard and mobile phase, respectively. The activity of PHB synthase was measured by the method of Gerngross and Martin [18].

RESULTS AND DISCUSSION

Molecular Weight of PHB Produced in Various Recombinant *E. coli* Strains

Five recombinant *E. coli* strains, harboring different plasmids, were cultured at 30°C and pH 6.9 in MR media containing 20 g/L glucose. Table 1 shows the final cell

Table 2. PHB production in recombinant *E. coli* strains with different plasmids at 30°C and pH 6.0[a]

	pJC1	pJC2	pJC3	pJC4	pJC5
Cell concentration (g/L)	4.19 ± 0.03	5.02 ± 0.03	6.15 ± 0.04	6.31 ± 0.03	5.53 ± 0.02
PHB concentration (g/L)	1.55 ± 0.01	2.76 ± 0.01	4.31 ± 0.02	4.71 ± 0.03	3.93 ± 0.01
PHB content (wt%)	37 ± 2	55 ± 2	70 ± 2	73 ± 3	71 ± 1
Molecular mass (MDa)	13 ± 4	22 ± 3	18 ± 3	19 ± 2	19 ± 3

[a]Cultures were carried out in triplicate.

Table 3. PHB production in recombinant *E. coli* strains with different plasmids at 22°C and pH 6.9[a]

	pJC1	pJC2	pJC3	pJC4	pJC5
Cell concentration (g/L)	2.13 ± 0.04	3.59 ± 0.04	4.12 ± 0.03	4.53 ± 0.04	3.91 ± 0.04
PHB concentration (g/L)	0.38 ± 0.02	0.75 ± 0.03	1.69 ± 0.04	1.77 ± 0.02	1.60 ± 0.02
PHB content (wt%)	18 ± 2	21 ± 3	41 ± 5	39 ± 4	41 ± 5
Molecular mass (MDa)	0.94 ± 0.16	1.30 ± 0.1	1.05 ± 0.10	1.10 ± 0.14	1.10 ± 0.12

[a]Cultures were carried out in triplicate.

concentration, PHB concentration, PHB content and molecular weight of the PHB obtained. The cell and PHB concentrations obtained with XL1-Blue harboring pJC3, pJC4 or pJC5 were higher than those obtained with XL1-Blue harboring pJC1 or pJC2. The highest cell and PHB concentrations of 6.71 and 4.76 g/L, respectively, were obtained with XL1-Blue (pJC4). Previously, we reported that fed-batch culture of recombinant *E. coli* XL1-Blue (pJC4) resulted in the highest productivity at 4.64 g PHB/L-h [8]. However, the molecular weight of PHB obtained with XL1-Blue (pJC2) was the highest among these recombinant *E. coli* strains.

Table 2 shows the same set of data obtained by culturing these strains at 30°C and pH 6.0. Cell and PHB concentrations obtained were almost same as those obtained at pH 6.9. However, the molecular weights of PHB produced were much higher at pH 6.0. The highest molecular weight of 22 MDa was obtained with XL1-Blue (pJC2). Cell concentration, PHB concentration, PHB content and the molecular weight of PHB obtained, by culturing the recombinant *E. coli* strains at 22°C and pH 6.9, are shown in Table 3. At this lower cultivation temperature, the molecular weights of PHB produced in all recombinant *E. coli* strains increased a little, but the cell and PHB concentrations were considerably lower. From the *in vivo* and *in vitro* polymerization studies, the major factors regulating the molecular weight of PHB have been suggested to be the activity of PHB synthase [11,18-21]. As the activity of PHB synthase increased, the molecular weight of synthesized PHB decreased. An attempt was made to determine the activity of PHB synthase from recombinant *E. coli* strains, cultured under various conditions, but the activity was not detectable. A previous report suggested that the activity of PHB synthase in recombinant *E. coli* was much lower than that in other wild-type PHB producers, and is hardly detectable without the overexpression of PHB synthase [21]. Nonetheless, the finding in this study, that supra molecular weight PHB could be produced at a suboptimal pH, also supports the hypothesis, that a low activity of PHA synthase is beneficial for obtaining supra molecular weight PHB. To investigate the time-dependent changes of the molecular weight of PHB during fermentation and to examine the possibility of high-level production of supra molecular weight PHB, fed-batch cultures were subsequently carried out.

Production of Supra Molecular Weight PHB by Fed-batch Culture of Recombinant *E. coli* XL1-Blue (pJC2)

Initially, fed-batch culture of recombinant *E. coli* XL1-Blue (pJC2) was carried out at 30°C and pH 6.9. The time profiles of cell concentration, PHB concentration and the molecular weight of PHB are displayed in Fig. 1. Cell concentration, PHB concentration and PHB content, obtained in 45.5 h, were 186 g/L, 103.8 g/L and 55.8 wt%, respectively. The molecular weight of PHB rapidly decreased until 22 h, as PHB concentration increased, then it plateaued out towards the end of fermentation. The highest molecular weight of 6 MDa was obtained at a very early stage of growth. These results suggest that a chain transfer reaction is occurring during the accumulation of PHB.

Based on the flask culture results, fed-bath culture of recombinant *E. coli* XL1-Blue (pJC2) was carried out at pH 6.0 for the production of supra molecular weight PHB. As shown in Fig. 2, cell concentration, PHB concentration and PHB content, obtained in 43.3 h, were 171.2 g/L, 89.8 g/L and 52.4 wt%, respectively, resulting in a relatively high PHB productivity of 2.07 g PHB/L-h. The molecular weight of PHB steadily increased up to 22 MDa at 30 h and then reached a plateau. Koizumi and

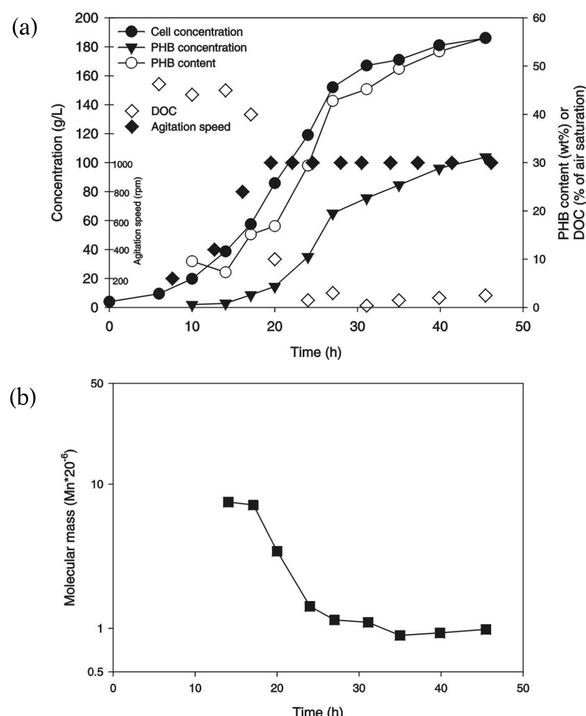

Fig. 1. Time profiles of (a) cell and PHB concentrations, PHB content, dissolved oxygen concentration and agitation speed, and (b) molecular weight of PHB (shown in log scale) during the fed-batch culture of XL1-Blue (pJC2) at pH 6.9 and 30°C.

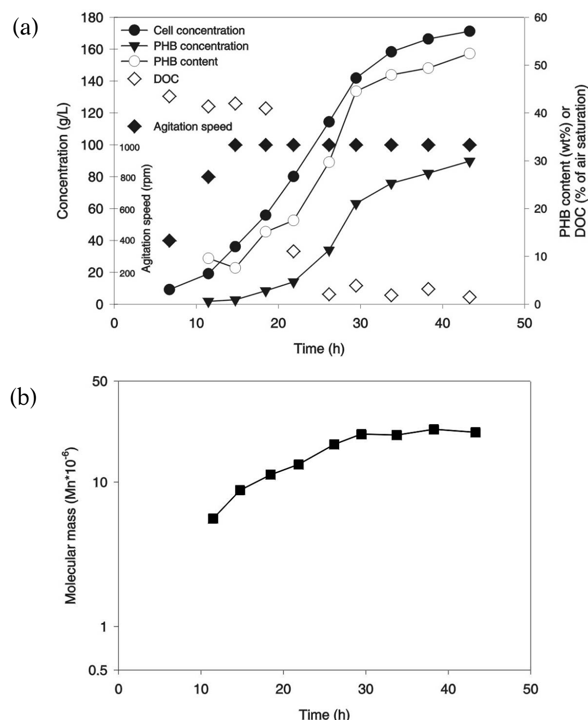

Fig. 2. Time profiles of (a) cell and PHB concentrations, PHB content, dissolved oxygen concentration and agitation speed, and (b) molecular weight of PHB (shown in log scale) during the fed-batch culture of XL1-Blue (pJC2) at pH 6.0 and 30°C.

Doi [20] suggested that a chain transfer agent might exist in *R. eutropha*, and might be responsible for the decrease of the molecular weight of PHB towards the end of cultivation. It was also suggested that this chain transfer agent might exist in *E. coli,* based on the finding that the molecular weight of PHB decreased during the fermentation [11]. However, production of extremely high molecular weight PHB was possible at pH 6.0, suggesting that this uncharacterized chain transfer agent did not affect polymerization at this pH.

PHB production can be a big metabolic burden to the *E. coli* cells. We have intensively investigated physiological changes occurring during the accumulation of PHB [4,8,9,10]. After considering these changes, we have applied DO limitation strategy for the production of PHB, which successfully allowed production of supra molecular weight PHB to high concentrations.

Until now, there have been several reports on the high level production of PHB and its copolymers [2,8,10,22], but no report on the production of novel supra molecular weight PHB. In this study, it is reported that the supra molecular weight PHB, which has much improved mechanical properties, could be produced to a high concentration of 89.8 g/L with a high PHB productivity of 2.07 g/L-h by a pH-stat fed-batch culture of metabolically engineered *E. coli* at pH 6.0. The strategies developed in this study should allow efficient production of supra molecular weight PHB for subsequent applications.

Acknowledgements This work was supported by a Korean Systems Biology Research Grant (M10309020000-03B5002-00000) of the Ministry of Science and Technology, Center for Ultramicrochemical Process Systems and by the BK21 project.

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[Received February 20, 2004; accepted June 3, 2004]