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Characteristics of Poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate) Production by *Ralstonia eutropha* NCIMB 11599 and ATCC 17699

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Abstract *Ralstonia eutropha* NCIMB 11599 and ATCC 17699 were grown, and their productions of poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate) [P(3HB-*co*-4HB)] compared. In flask cultures of *R. eutropha* NCIMB 11599, cell concentration, P(3HB-*co*-4HB) concentration and polymer content decreased considerably with increases in the γ-butyrolactone concentration, and the 4HB fraction was also very low (maximum 1.74 mol%). In fed-batch cultures of *R. eutropha* NCIMB 11599, glucose and γ-butyrolactone were fed as the carbon sources, under a phosphate limitation strategy. When glucose was fed as the sole carbon source, with its concentration controlled using an on-line glucose analyzer, 86% of the P(3HB) homopolymer was obtained from 201 g/L of cells. In a two-stage fed-batch culture, where the cell concentration was increased to 104 g/L, with glucose fed in the first step and constant feeding of γ-butyrolactone, at 6 g/h, in the second, final cell concentration at 67 h was 106 g/L, with a polymer content of 82%, while the 4HB fraction was only 0.7 mol%. When the same feeding strategy was applied to the fed-batch culture of *R. eutropha* ATCC 17699, where the cell concentration was increased to 42 g/L, by feeding fructose in the first step and γ-butyrolactone (1.5 g/h) in the second, the final cell concentration, polymer content and 4HB fraction at 74 h were 51 g/L, 35% and 32 mol%, respectively. In summary, *R. eutropha* ATCC 17699 was better than *R. eutropha* NCIMB 11599 in terms of P(3HB-*co*-4HB) production with various 4HB fractions.

Keywords: poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate), *Ralstonia eutropha,* fed-batch, fermentation

Poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate) [P(3HB-*co*-4HB)] was first obtained from *Ralstonia eutropha* in 1988 [1]. P(3HB-*co*-4HB) can be produced in a wide variety of polymeric materials, from hard crystalline plastics to very elastic rubber, by varying the monomer composition (4HB fraction) [2]. The *in vivo* degradation rate of P(3HB-*co*-4HB) is relatively high compared to those of other polyhydroxyalkanoates (PHAs), which can be controlled according to the 4HB fraction [3]. P(3HB-*co*-4HB) has recently been found to have desirable mechanical properties for applications in the medical and pharmaceutical field [4]. P(4HB) has been successfully applied in the tissue engineering of a trileaflet heart valve in a sheep model [3].

P(3HB-*co*-4HB), with a wide range of monomer compositions, was synthesized using two different carbon sources each for the generation of 3HB and 4HB monomers, respectively. To date, five wild-type bacteria, able to produce P(3HB-*co*-4HB), *i.e. R. eutropha* (formerly *Alcaligenes eutrophus*), *Alcaligenes latus*, *Comamonas acidovorans*, *Comamonas testosteronii* and *Hydrogenophaga pseudoflava*, have been identified [5]. Recombinant *Escherichia coli* was used to produce P(3HB-*co*-4HB), using glucose as the sole carbon source. Tepha Inc. (Cambridge, MA, USA) is currently producing P(4HB) homopolymer for medical applications using recombinant *E. coli* K12 [3]. However, detailed fermentation data on the production of P(4HB) are not currently available in the literature. Of these microorganisms, *R. eutropha* has been reported to produce P(3HB-*co*-4HB) with various 4HB fractions (0~100 mol%), and has been most extensively studied due to its ease of growth and ability to accumulate large amounts of polymers from simple carbon sources [6].

Recently, we reported a laboratory scale method for producing P(3HB-*co*-4HB) using *R. eutropha* ATCC 17699 [7]. Besides *R. eutropha* ATCC 17699, *R. eutropha* NCIMB 11599 is a glucose utilizing mutant that has been extensively used for the productions of P(3HB) and poly(3-hydroxybutyrate-co-3-hydroxyvalerate) [P(3HB-co-3HV)] in high cell density fed-batch cultures [8-12]. Fed-batch culture has been extensively employed to achieve high cell density culture, which is often necessary for high yield and productivity of the desired product

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Table 1. Production of P(3HB-*co*-4HB) from glucose and γ-butyrolactone by *R. eutropha* NCIMB 11599 in 72 h

Carbon sources (g/L)		Dry cell weight (g/L)	Polyester content (wt%)	Composition (mol%)	
Glucose	γ-Butyrolactone			3HB	4HB
20	0	7.61	86	100	0
16	4	7.71	86	100	0
12	8	5.49	77	99.6	0.41
8	12	3.78	78	99.5	0.47
4	16	1.70	67	99.4	0.62
0	20	0.91	59	98.3	1.74

[13-15]. In this study, the characteristics of P(3HB-*co*-4HB) production were compared by carrying out flask and fed-batch cultures of *R. eutropha* NCIMB 11599 and ATCC 17699.

R. eutropha NCIMB 11599 and ATCC 17699 (KCCM 11972) were the strains used in this study, which can use glucose and fructose, respectively, as the sole carbon sources. The medium for the seed and flask culture was (per L): KH_2PO_4, 1.5 g; $Na_2HPO_4 \cdot 12H_2O$, 9 g; $(NH_4)_2SO_4$, 1 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; and 1 mL of a trace element solution [8]. A total of 20 g/L of the carbon sources (glucose/fructose + γ-butyrolactone) was used, with variation of the glucose/fructose concentration depending on the concentration of γ-butyrolactone used. The initial medium for the fed-batch culture was (per L): glucose/fructose, 20 g; $(NH_4)_2SO_4$, 4 g; KH_2PO_4, 1.54~6.15 g; citric acid, 1.7 g; $MgSO_4 \cdot 7H_2O$, 1.2 g; and 10 mL of a trace element solution. Glucose/fructose and $MgSO_4 \cdot 7H_2O$ were autoclaved separately, and aseptically added to the medium. An 800 g glucose/fructose per L solution and a 99% γ-butyrolactone solution were used as feeding nutrient solution in the fed-batch culture.

Flask cultures were carried out to investigate the effects of the carbon sources concentrations on the cell growth and P(3HB-*co*-4HB) production by *R. eutropha* NCIMB 11599. Cells were grown under aeration at 30°C, on a shaking incubator, in 250 mL flasks containing 50 mL of mineral medium. Fed-batch cultures were carried out to produce high concentrations of cells and PHA. The seed culture was prepared in a 250 mL flask, on a reciprocal shaker, at 30°C for 1~2 days. One hundred mL of seed culture was used to inoculate the fermentor (2.5 L, Kobiotech, Incheon, Korea). The initial volume of the culture was 1 L. The pH was controlled at 6.8 with a 28% NH_4OH solution. The dissolved oxygen (DO) level was kept above 20% by increasing the agitation speed to 1,500 rpm. To allow polymer accumulation, phosphate limitation was applied when the cell concentration reached 42~130 g/L. At the same time, fructose or glucose feed solution (800 g/L) was replaced with γ-butyrolactone. Fructose was fed using the DO-stat method. When the fructose became exhausted, the DO content rose rapidly, with a total 20 g of carbon source supplied at that instant. Glucose was fed using on-line glucose analyzer (ProcessTRACE 1.21 MT, TRACE Analytics, Braunschweig, Germany). The glucose concentration in the culture broth was feedback controlled, with the set point of 20 g/L. In the second stage, after phosphate limitation, the γ-butyrolactone was constantly fed at 1.5~6 g/h.

The cell concentration was measured after vacuum drying the cells at room temperature for 48 h. The PHA and γ-butyrolactone concentrations were determined using gas chromatograph (HP 5890A, Hewlett Packard, USA), equipped with a Carbowax 20M capillary column and flame ionization detector (FID), with benzoic acid and octanoic acid, respectively, as the internal standards [16]. Standards were obtained from purified, solvent-extracted P(3HB-*co*-4HB), the composition of which was verified by 400 MHz ^1H nuclear magnetic resonance (NMR) spectroscopy. The phosphate concentration was determined by ion chromatography.

Initially, the characteristics of cell growth and P(3HB-*co*-4HB) production in the flask cultures of *R. eutropha* NCIMB 11599 were examined. The results of polymer production from the different carbon sources are shown in Table 1. The homopolymer of 3HB was produced when glucose was used as the sole carbon source. Cell concentration, and PHA concentration and content decreased considerably with increasing γ-butyrolactone concentration. This seemed to be due to the toxicity of the γ-butyrolactone in the culture medium. The mole fractions of the 4HB unit in the copolymer were very low, increasing from 0 to 1.74 mol% with increasing γ-butyrolactone concentration.

Comparing with the results [7] of the one-step cultivation of *R. eutropha* ATCC 17699 using fructose and γ-butyrolactone as the carbon sources, the PHA contents produced by the *R. eutropha* NCIMB 11599 cells (59~86%) were higher than those by the ATCC 17699 cells (11~60%), while 4HB fractions were much lower in the NCIMB 11599 (maximum 1.74 mol%) than ATCC 17699 cells (maximum 27.4 mol%). These results suggest that γ-butyrolactone is a highly toxic substrate for *R. eutropha* NCIMB 11599 cells, and their efficiencies for 4HB incorporation into PHA polymers are very low.

In the fed-batch culture of *R. eutropha* NCIMB 11599, glucose and γ-butyrolactone were fed as the carbon sources, under a phosphate limitation strategy. These cultures were carried out as higher PHA concentrations and productivities have been reported under phosphate limitation than under nitrogen limitation [11,12]. Fig. 1A

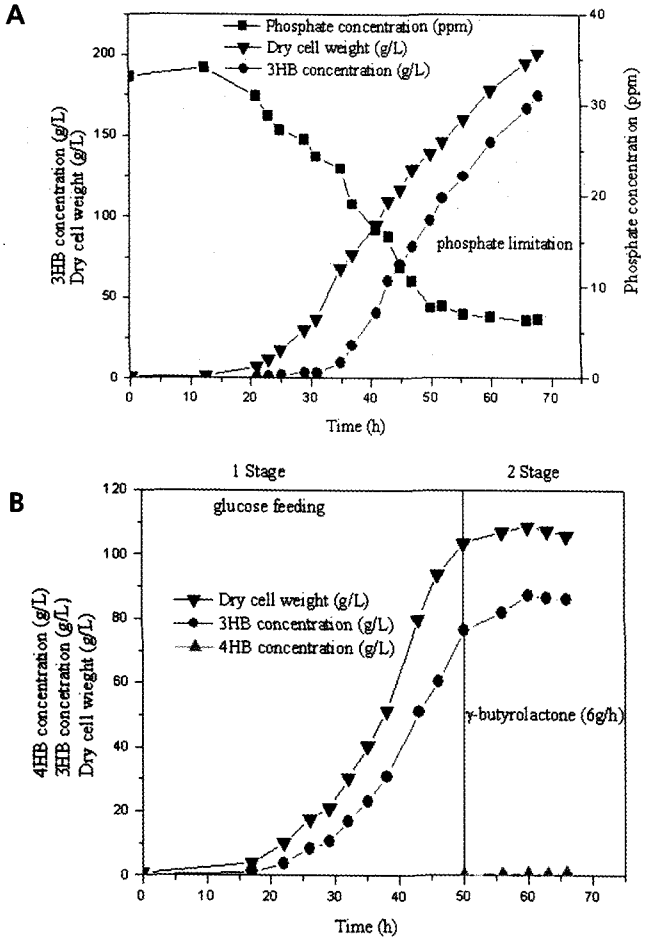

Fig. 1. Fed-batch culture of *R. eutropha* NCIMB 11599. (A) Production of P(3HB). Initial KH_2PO_4 concentration = 6.15 g/L. (B) Production of P(3HB-*co*-4HB). Initial KH_2PO_4 concentration = 4.23 g/L.

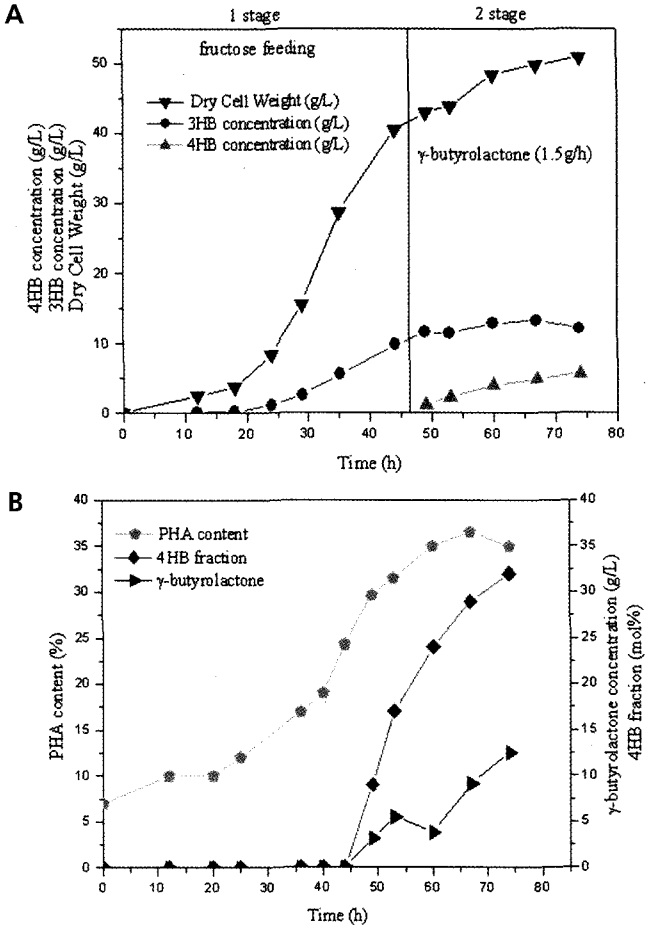

Fig. 2. Fed-batch culture of *R. eutropha* ATCC 17699. (A) Time courses of the cell and PHA concentrations. (B) Time courses of PHA content, 4HB mol fraction and γ-butyrolactone concentration. Initial KH_2PO_4 concentration = 1.54 g/L.

shows the result obtained when glucose was fed as the sole carbon source, using an on-line glucose analyzer, with an initial KH_2PO_4 concentration of 6.15 g/L. At 68 h, the cell concentration and P(3HB) content were 201 g/L and 86%, respectively. These results were similar to those obtained by Ryu *et al.* [11], with a corresponding initial phosphate concentration (221 g/L of cell concentration and 81% of polymer content). The P(3HB) homopolymer was produced as glucose was fed as the sole carbon source. After confirming that *R. eutropha* NCIMB 11599 cells could be grown to high density and with high polymer content, an attempt was made at P(3HB-*co*-4HB) production by supplying γ-butyrolactone as a 4HB precursor in the second step. Fig. 1B shows the result obtained with glucose feeding, using an on-line glucose analyzer, in the first step, with constant feeding (6 g/h) of γ-butyrolactone in the second. At 66 h, the cell concentration and PHA content were 106 g/L and 82%, respectively. The 4HB fraction was as low as 0.7 mol%, which was expected from the results of the flask culture. The yield of 4HB production from γ-butyrolactone was less than 0.01 (g 4HB/g γ-butyrolactone).

The same feeding strategy was applied to the fed-batch culture of *R. eutropha* ATCC 17699, with fructose and γ-butyrolactone as the carbon sources (Fig. 2). First, cells were grown to a concentration of 42 g/L by feeding fructose, using the DO-stat method. γ-Butyrolactone was constantly fed, at 1.5 g/h, in the second step. At 74 h, the cell concentration, PHA content and 4HB fraction were 51 g/L, 35%, and 32 mol%, respectively. The results obtained under phosphate limitation were similar to those obtained under nitrogen limitation (34 g/L of cell concentration, 42% of PHA content and 25 mol% of 4HB fraction) when γ-butyrolactone was constantly fed at 6 g/h in the second step [7]. The yield of 4HB production from γ-butyrolactone was 0.24 (g 4HB/g γ-butyrolactone), which was higher than that of *R. eutropha* NCIMB 11599. The γ-butyrolactone concentrations in the culture medium increased to 12.5 g/L after γ-butyrolactone feeding, suggesting that γ-butyrolactone was oversupplied in the second step, and the feeding strategy needed to be optimized.

In our previous study, *A. latus* and *C. acidovorans* were grown to evaluate their capabilities of P(3HB-*co*-4HB) production [17]. *A. latus* could be grown from a cheap carbon source, such as sucrose, and P(3HB-*co*-4HB), with 0~19 mol% 4HB fractions, could be obtained in both flask and batch cultures, but it was difficult to achieve high cell densities due to their high rates of oxygen uptake and PHAs decomposition to monomers on depletion of the carbon source. *C. acidovorans* was able to synthesize P(3HB-*co*-4HB) with high 4HB fractions of up to 100% in two-stage flask culture, but high cell densities could not be achieved.

From the above facts, it was found that *R. eutropha* ATCC 17699 was the best wild-type strain in terms of P(3HB-*co*-4HB) production with various 4HB fractions. NCIMB 11599 cells could be grown to higher cell densities and with high polymer contents using glucose as the carbon source, probably due to their lower specific growth rate compared to ATCC 17699 cells and; thus, their lower oxygen uptake rate. However, the 4HB fraction was very low as the γ-butyrolactone was highly toxic to the cells, with the efficiency of 4HB incorporation into PHA polymers being very low. By increasing the cell concentration in the first step, with optimizing of the γ-butyrolactone feeding in the second, it is expected that *R. eutropha* ATCC 17699 could be used to produce high concentrations of P(3HB-*co*-4HB) with various 4HB fractions.

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