Biotechnol. Bioprocess Eng. **1996**, 1, 51-53

High Cell Density Cultivation of *Pseudomonas oleovorans* for the Production of Poly(3-Hydroxyalkanoates)

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Fed-batch culture of *Pseudomonas oleovorans* was carried out for the production of medium-chain-length polyhydroxyalkanoates (MCL-PHAs) using octanoate as a carbon source. Octanoate and the salt solution containing ammonium sulfate and magnesium sulfate were intermittently fed in the course of fermentation. Cell mass and PHA concentrations of 42.8 and 16.8 g/L, respectively, could be obtained in 40 h. The PHA content and the PHA productivity were 39.2% and 0.42 g PHA/L-h, respectively. The yields of cell mass and PHA were 0.71 g dry cell mass/g octanoate and 0.28 g PHA/g octanoate, respectively. Therefore, octanoate can be used for the production of MCL-PHAs to a high concentration with high productvity.

Key words: polyhydroxyalkanoate, PHA, *Pseudomonas oleovorans*, fed-batch, high cell density culture, octanoate

INTRODUCTION

Poly(3-hydroxyalkanoates) [PHAs] are optically active polyesters of 3-hydroxyalkanoates (3HAs) which are accumulated by a wide range of both Gram-negative and Gram-positive bacteria as a storage compound [1,6]. PHAs are usually formed as intracellular inclusion bodies under nutrient limiting conditions in the presence of excess carbon source [1,2,5,6], and have been drawing much attention as a candidate for biodegradable plastic material. PHAs can be divided into two groups depending on the number of carbon atoms in the monomer units: short chain length-PHAs (SCL-PHAs) for C3 to C5 and medium chain length-PHAs (MCL-PHAs) for C6 to C14 [3]. The polymer properties of PHAs can vary, from highly crystalline to rubbery, depending on the incorporated monomer units. Poly(3-hydroxybutyrate) [P(3HB)] and poly(3-hydroxybutyrate-co-3-hydroxyvalerate) [P(3HB-co-3HV)] are the members of PHAs that have been studied most extensively, and currently produced in an industrial scale by Monsanto (St. Louis, USA). The strategies for the production of SCL-PHAs have been well developed by employing several bacteria such as *Alcaligenes eutrophus, Alcaligenes latus, Azotobacter vinelandii, methylotrophs*, and recombinant *Escherichia coli* [4-7].

PHAs consisted of various MCL-3HAs were first detected in cells of *Pseudomonas oleovorans* ATCC 29347 grown on octane [8]. PHAs containing six different types of monomer units (C6 to C11) were found in *P. oleovorans* grown on C6-C10 alkanoates [9]. Poly(3-hydroxyhexanoate-co-3-hydroxyoctanoate) [P(3HHx-co-3HO)] is synthesized when *P. oleovorans* was grown on octane, octanoate, or octanol. P(3HHx-co-3HO) and other MCL-PHAs are semicrystalline elastomers with a low melting point and high elongation to break, which can be used as a biodegradable rubber [10,11]. Production of P(3HHx-co-3HO) to a high concentration by fed-batch and continuous culture of *P. oleovorans* from octane has been studied. The steady state cell concentration and polymer productivity of 11.6 g/L and 0.58 g PHA/L-h, respectively, were obtained in continuous cultivation [12]. Recently, P(3HHx-co-3HO) concentration and productivity of 12.1 g/L and 0.32 g PHA/L-h, respectively, were obtained by fed-batch culture of *P. oleovorans* using octane as a carbon source [13]. However, much oxygen has to be supplied to achieve high cell density since octane has lower oxygen content than octanoate or octanol. This is why a specially designed fermentor having high oxygen transfer capability had to be used for the high cell density culture of *P. oleovorans* from octane [13]. Furthermore, octane is highly flammable and may be dangerous when pure oxygen is supplied. In this study I report high cell density cultivation of *P. oleovorans* for the production of MCL-PHAs using octanoate as a carbon source.

The strain used was *P. oleovorans* ATCC 29347. Cells were routinely grown in nutrient broth (Difco Laboratories, Detroit, MI) at 30°C and maintained as a 20% (v/v) glycerol stock at -80°C. Flask cultures were carried out in a 250 mL flask containing 50 mL of R medium [14] supplemented with varying concentrations (10-50 mM) of octanoate (Junsei Chemical Co., Japan) at 30°C and 250 rpm. The initial pH of the medium was adjusted to 7.0 with 10 N NaOH solution. Cell mass concentration, defined as dry cell weight per liter of culture broth, was determined by weighing dry cells prepared as follows. Culture broth of 2-20 mL was centrifuged in a preweighed tube, washed twice with distilled water, and dried in a vacuum oven (80°C) to a constant weight. The PHA concentration was determined by gas chromatography (Varian 3300, Palo Alto, CA) with n-bu-

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tyrate as an internal standard [15]. The PHA standards were prepared by solvent extraction [16], and their composition was verified by nuclear magnetic resonance spectroscopy. The concentration of octanoate was determined by gas chromatography as described elsewhere [17]. The PHA content is defined as the percentage of the ratio of PHA to dry cell weight.

Flask cultures were first carried out to find the effect of octanoate concentration on cell growth. Cells grew well in the medium containing 10 and 20 mM octanoate, but the growth was significantly inhibited at the concentrations higher than 30 mM. The cell mass concentrations obtained in 72 h were 0.8, 1.7, and 1.5 g/L in the media containing 10, 20, and 30 mM octanoate, respectively. No growth was observed when 50 mM octanoate was used. Fed-batch cultures were carried out in a jar fermentor (2.5 L, Korea Fermentor Company, Incheon, Korea) containing 0.9 L of R medium supplemented with 20 mM octanoate as the initial medium. Cells grown for 24 h in a 100 mL medium of the same nutrient composition were used as the inoculum. The pH was controlled at 7.0 using 6.73 M octanoate and 28% ammonia water as the acid and base, respectively. The dissolved oxygen level was maintained above 20% of air saturation by raising the agitation speed up to 1000 rpm and by using pure oxygen when required. Feeding solutions used were 2 M octanoate solution, the pH of which was adjusted to 7.0 with NaOH, and the salt solution consisting of 200 g/L $(NH_4)_2SO_4$ and 20 g/L $MgSO_4\ 7H_2O$. During the cultivation, cell growth was monitored by measuring the optical density at 600 nm of the cell suspension prepared as follows. Cells were collected by centrifugation from the known volume of culture broth, washed three times with distilled water, and resuspended in the same volume of 0.9% (wt/vol) NaCl solution. This was because the presence of octanoate interfered with the optical density measurement. The optical density was measured after appropriate dilution when the value was greater than 0.4. Nutrient feeding was carried out by intermittently adding 20 mL of 2 M octanoate and 7.5 mL of the salt solution after considering the amount of octanoate consumed to support the cell growth monitored by optical density measurement. The cell mass yield on octanoate was assumed to be 0.76 g dry cell mass/g octanoate [17].

The time profiles of cell mass, PHA and residual cell mass concentrations, and PHA content are shown in Fig. 1. The arrows indicate the points of feeding octanoate and the salt solution. Cells grew rapidly to reach 32 g/L in 26 h, then the growth slowed down. Cells started to synthesize PHA after 14 h of cultivation. The PHA content increased rapidly to 32.8% in 26 h, then increased slowly. Cell mass concentration, PHA concentration, and PHA content obtained in 40 h were 42.8 g/L, 16.8 g/L, and 39.2%, respectively. The PHA productivity was 0.42 g PHA/L-h. The octanoate concentration at the end of fermentation was as high as 6.8 g/L (equivalent to 47 mM), which suggests that the slow growth and PHA synthesis during the later part of fermentation was due to the accumulation of octanoate over the critical value (40 mM). The total amount of octanoate consumed was 68.6 g. Considering the volume change by nutrient feeding (the total increase of 467.5 mL) during the fed-batch culture and neglecting the volume change due to the sampling and the pH control, the yields of cell mass and PHA on octanoate are approximately 0.71 g dry cell mass/g octanoate and 0.28 g PHA/g octanoate, respectively. The cell mass yield was in good agreement with the assumed value of 0.76 g dry cell mass/g octanoate.

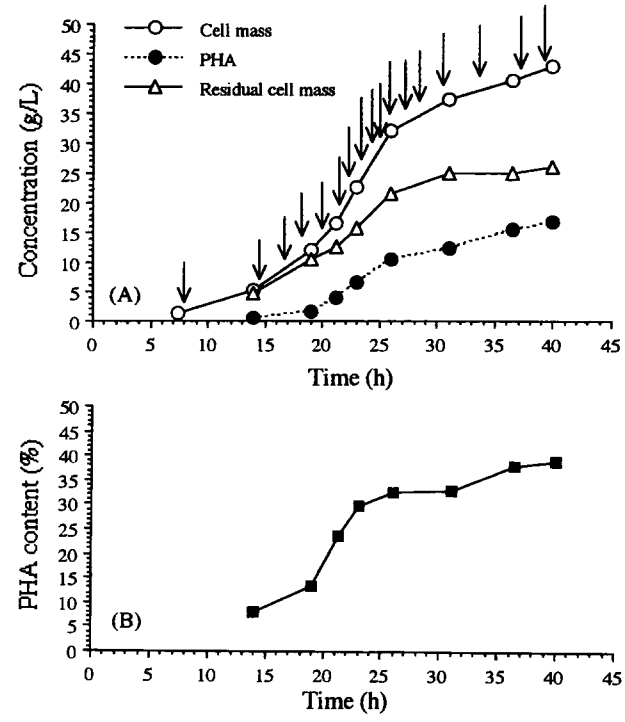

Fig. 1. The time profiles of (A) cell mass, PHA and residual cell mass concentrations, and (B) PHA content during the fed-batch culture of *Pseudomonas oleovorans* using octanoate as a carbon source. Nutrients were added intermittently as indicated by the arrows.

In this study I showed that MCL-PHAs can be produced to a high concentration by fed-batch culture of *P. oleovorans* using octanoate as a carbon source. Even though the use of octane for the production of MCL-PHAs has been reported [12,13], it is dangerous to use this highly flammable solvent in a large scale, especially with oxygen supply. There is no danger of explosion when using octanoate even with pure oxygen. I have also examined if octanol could be used as an alternative carbon source. Even though a relatively high concentration (13.8 g/L) of PHA could be obtained by fed-batch culture of *P. oleovorans* from octanol, it was difficult to separate cells from the broth. Cells were often stuck in the thick interface between octanol and aqueous layer even after a prolonged centrifugation. Therefore, octanoate seems to be a good substrate for the production of MCL-PHAs, the biodegradable rubber. Development of a better strategy of feeding octanoate may result in higher cell mass and PHA concentrations, and the enhanced PHA productivity.

Acknowledgements I would like to thank Prof. H.N. Chang and Prof. Y.K. Chang for their continued interest and support on this work. This work was supported by the MOST and LG Chemicals, Ltd.

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