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NOTE

High Cell Density Cultivation of *Pseudomonas putida* BM01 Using Glucose

KIM, GUK JIN, IN YOUNG LEE*, DAE KEON CHOI, SUNG CHUL YOON[1], AND YOUNG HOON PARK

Bioprocess Technology Research Group, Korea Research Institute of Bioscience and Biotechnology, P.O. Box 115, Yusong, Taejon 305-600, Korea
[1]Department of Microbiology, Gyeongsang National University, Chinju 660-701, Korea

Pseudomonas putida BM01 was grown efficiently on glucose as the sole carbon source with a supply of a nitrogen source in pH-stat mode using a low setpoint limit. A final cell concentration of 100 g/l was obtained in 30 h of fed-batch cultivation by controlling glucose concentration within the range of 5-20 g/l and maintaining dissolved oxygen tension above 10% saturation using pure oxygen. This high cell density culture technique is believed highly useful for the production of poly(3-hydroxyalkanoates) by this strain.

High cell density cultivation (HCDC) is often necessary for mass production of fermentative products. However, little has been reported on the HCDC of *Pseudomonas* strains. *Pseudomonas* strains have been attracting a lot of attention because they produce many different types of poly(3-hydroxyalkanoate) (PHA) copolymers (1, 3, 8, 10). Since PHAs are accumulated in the form of cellular granules, HCDC would be a prerequisite for a high productivity of PHAs. For the cultivation of *Pseudomonas* strains, a number of organic compounds such as alkanes, aliphatic alcohols, carboxylic acids, and aromatic compounds can be used as carbon sources. Preusting *et al.* (7) reported that *P. oleovorans* grew to a biomass concentration of 35.0 g/l by using n-octane in a two-liquid phase fed-batch system. Recently, Huijberts and Eggink (4) reported that they cultivated *P. putida* to a final cell density of 60 g/l using oleic acid as a carbon source. Despite their efforts, however, the final cell concentrations were still relatively low. Moreover, one thing that should be considered for HCDC is the use of inexpensive substrates. Glucose, which can be also used for cell growth, seems to be the substrate of choice to achieve an HCDC with economic feasibility. In the present study, optimal conditions for HCDC of *Pseudomonas putida* BM01 were achieved by using glucose as the sole carbon source.

Pseudomonas putida BM01 isolated by Yoon *et al.* (10) was used throughout the experiments. The fermentation medium contained (per liter): 20 g glucose, 3.0 g $(NH_4)_2SO_4$, 3.32 g $Na_2HPO_4 \cdot 12H_2O$, 0.83 g KH_2PO_4, 0.2 g $MgSO_4 \cdot 7H_2O$, 20 mg $FeSO_4 \cdot 7H_2O$, 10 mg $CaCl_2$, and 1 ml of a trace element solution (0.3 g H_3BO_3, 0.2 g $CoCl_2 \cdot 6H_2O$, 0.1 g $ZnSO_4 \cdot 7H_2O$, 30 mg $MnCl_2 \cdot 4H_2O$, 30 mg $Na_2MoO_4 \cdot 2H_2O$, 20 mg $NiCl_2 \cdot 6H_2O$, 10 mg $CuSO_4 \cdot 5H_2O$ per liter of 0.1 N HCl). The feed solution for fed-batch operations was prepared by dissolving 700 g glucose, 16.6 g $Na_2HPO_4 \cdot 12H_2O$, 4.15 g KH_2PO_4, 1.0 g $MgSO_4 \cdot 7H_2O$, 100 mg $FeSO_4 \cdot 7H_2O$, 50 mg $CaCl_2$, 2.0 g Na-citrate, and 5 ml of the trace element solution in 1 liter of 0.05 N HCl. Ammonium hydroxide solution (28%), supplied for pH control, was used as the nitrogen source in fed-batch operations. Fermentations were carried out in a 5-liter jar fermentor (Korea Fermentor Co., Incheon, Korea) equipped with a DO analyzer and a pH controller. Two hundred ml of seed culture, cultivated at 30°C for 12 h in shake flasks, was transferred to the fermentor containing 1.8 liter of the fermentation medium. The pH was controlled at 7.0. Pure oxygen was supplied with air to maintain the dissolved oxygen tension at 10-40% saturation.

Cell growth was monitored by measuring the optical density of the culture broth at 600 nm by using a Bioscreen (Labsystems, Helsinki, Finland). The cell concentration was also determined by measuring the dry cell weight. Glucose concentration was determined by using a glucose analyzer (YSI, Ohio, USA) and ammonia concentration was determined by the indophenol method (9). PHA concentration was determined by using a gas chromatograph (Hewlett Packard, Avondale, USA) with benzoic acid as an internal standard (2).

*Corresponding author
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Cell growth of the bacterial strain was examined on the Bioscreen with various carbon sources: acetic acid, propionic acid, butyric acid, valeric acid, octanoic acid, oleic acid, and glucose (Fig. 1). Octanoic acid was found to be the most efficient carbon source for cell growth, while the other caboxylic acids yielded poor cell growth. Glucose also showed a considerably high cell growth. And, glucose had no significant inhibitory effect at its concentration of below 40 g/l (data not shown). However, it was noted that the specific growth rate of the cell decreased from 0.45 to 0.20 h^{-1} as the initial concentration of octanoic acid increased from 0.5 to 10.0 g/l (Fig. 2). This indicates that octanoic acid inhibited the cell growth. Another point that should be mentioned concerns the change of culture pH. When glucose was used as the carbon source, pH decreased rapidly from 6.7 to 3.9 in the early growth phase, but this was not the case with octanoic acid (Fig. 3). These observation indicates that an appropriate control strategy is required for HCDC in fed-batch cultivation with these substrates. It should involve pH and/or substrate feed control. Since control of a culture pH is one of the easiest methods known so far, HCDC of *P. putida* BM01 was tried with glucose as the sole carbon source with pH control.

Previously, Lee *et al.* (6) reported a high cell concentration (184 g/l) of *A. eutrophus* which was obtained by supplying ammonium hydroxide solution (28%) as the sole nitrogen source. It was continuously fed by pH-stat mode using a low setpoint limit of 7.0. Therefore, in the present study, a similar feeding strategy was employed for fed-batch cultivations of *P. putida* with ammonium hydroxide solution. The feed solution containing glucose was also fed in such a manner that glucose concentration in the culture broth was maintained within the range of 5-20 g/l, based on measured values by glucose analyzer. This method made it possible to maintain ammonium concentration within the range of 0.5-1.5 g/l, allowing the cells to grow quite efficiently. Fig. 4a shows the time course of fed-batch culture with feeding ammonium hydroxide and glucose solutions. The final cell concentration was 61.2 g/l. However, accumulation of PHA, having the monomer unit of 3-hydroxyhexanoate, 3-hydroxyoctanoate, and 3-hydroxydecanoate, was poor. It was also noted that the limitation of dissolved oxygen restricted the cell growth after 15 h cultivation. Preusting *et al.* (7) and Huijberts and Eggink (4) also reported that dissolved oxygen concentration was one of the most important fermentation parameters for HCDC of *Pseudomonas* strains. By using the same operating strategy as in Fig. 4a along with the supply of

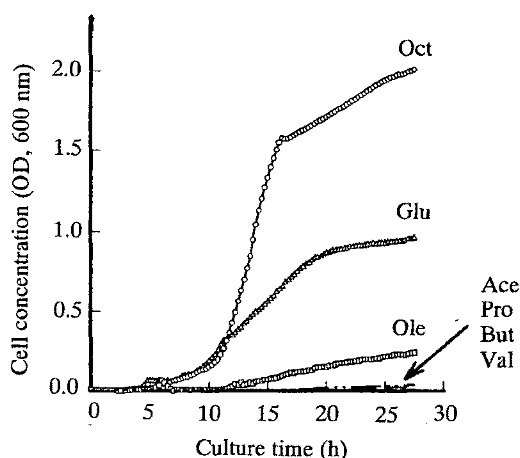

Fig. 1. Effect of carbon source on cell growth.
Initial concentrations of the carbon sources were 5.0 g/l. Abbreviations: Ace, acetic acid; Pro, propionic acid; But, butyric acid; Val, valeric acid; Oct, octanoic acid; Ole, oleic acid; Glu, glucose.

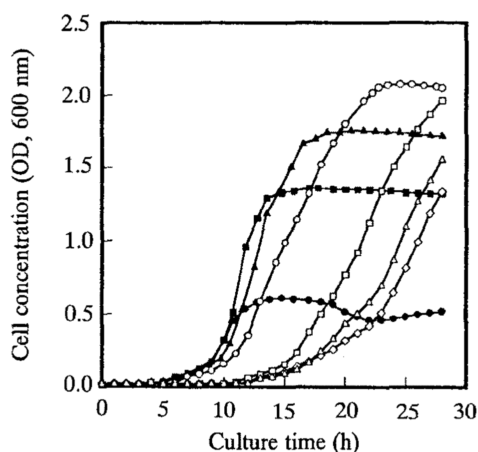

Fig. 2. Effect of octanoic acid concentration on cell growth.
Octanoate concentration (g/l): 0.5, (●); 1.5, (■); 3.0, (▲); 5.0, (○) 6.5, (□), 8.0, (△); 10.0, (◇).

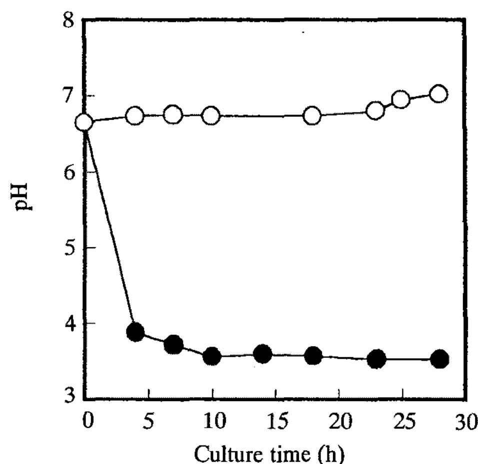

Fig. 3. pH changes during the cultivation.
Cells were grown on octanoic acid (○) and glucose (●) in 500 ml flask containing 100 ml of the medium in shaking incubator at 30°C.

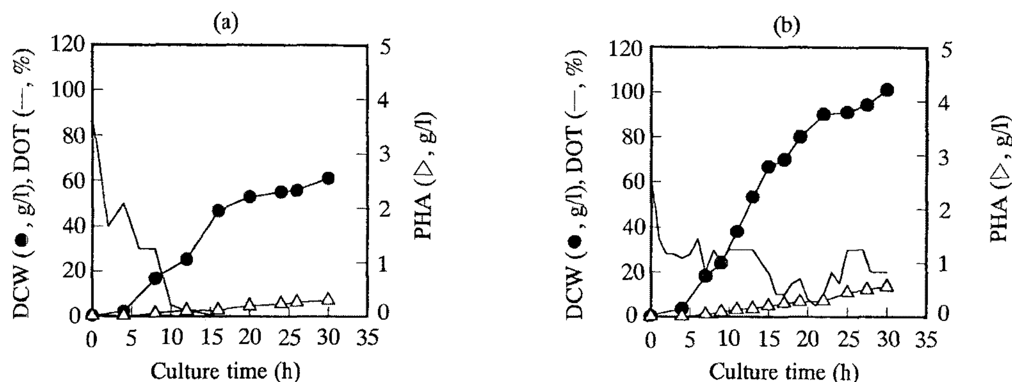

Fig. 4. Time courses of the cell concentration, PHA accumulation, and dissolved oxygen tension (DOT) during fed-batch cultivation of *P. putida* BM01.

(a) Fed-batch cultivation with air only, (b) Fed-batch cultivation by using pure oxygen (Pure oxygen was supplied together with air after 9 h cultivation). DCW is dry cell weight.

pure oxygen, a high cell concentration of 100 g/l could be obtained in 30 h fed-batch cultivation (Fig. 4b). However, it was also noted that PHA accumulation was only 0.6 g/l whereas this strain could accumulate PHA to its cellular content of 60% on octanoic acid as reported previously (10). Huijberts *et al.* (5) showed that *P. putida* KT2442 accumulated PHA with a cellular content of 16.9% by using glucose under ammonium-limited condition. In this regard, the lower accumulation of PHA in our experiments could be attributed to the carbon source used and the nutrient-sufficient condition. We further examined whether this HCDC technique could be used for the production of PHA. For this, a two-stage culture technique was employed. Cells were first grown on glucose in the first stage, and subjected to PHA accumulation stage by feeding octanoic acid instead of glucose. In the second stage, ammonium hydroxide solution (28%) supplied for pH control was replaced by 4 N sodium hydroxide solution to enhance PHA accumulation under the ammonium-limited condition. Fig. 5 shows the time courses of cell concentration, PHA accumulation, and PHA content during the two-stage fed-batch cultivation. In contrast to the little accumulation of PHA in the first stage, 11.2 g/l of PHA could be accumulated with its content of 23.5% (g PHA/g dry cell weight) in 18 h after switching the carbon source from glucose to octanoate. It is noteworthy that an overall PHA productivity of 0.37 (g PHA/l/h) was much higher than that reported by Preusting *et al.* (7), although the final PHA concentration (11.2 g/l) was at a similar level.

In this paper, we showed that *P. putida* BM01 could be grown to high concentration, over 100 g/l, by using glucose as the sole carbon source. In addition, pH-stat culture technique for feeding of the nitrogen source was found to be highly efficient for this glucose utilizing system. Efforts to produce PHA with a high yield are currently being made by using this HCDC technique in combination with an adequate PHA accumulation method.

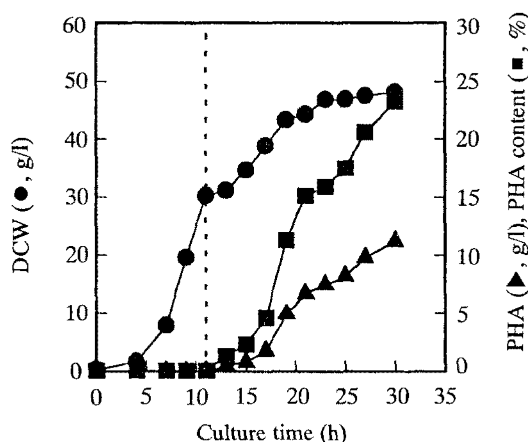

Fig. 5. Time courses of cell concentration, PHA accumulation, and PHB content in a two-stage fed-batch cultivation of *P. putida* BM01.

Dotted vertical line indicates the timing of switching mode from glucose to octanoic acid feeding. DCW is dry cell weight.

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