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PRODUCTION OF MEDIUM CHAIN LENGTH POLYHYDROXYALKANOATES FROM OLEIC ACID USING *Pseudomonas putida* PGA1 BY FED BATCH CULTURE

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Abstract

Bacterial polyhydroxyalkanoates (PHAs) are a class of polymers currently receiving much attention because of their potential as renewable and biodegradable plastics. A wide variety of bacteria has been reported to produce PHAs including Pseudomonas strains. These strains are known as versatile medium chain length PHAs (PHAs-mcl) producers using fatty acids as carbon source. Oleic acid was used to produce PHAs-mcl using *Pseudomonas putida* PGA 1 by continuous feeding of both nitrogen and carbon source, in a fed batch culture. During cell growth, PHAs also accumulated, indicating that PHA production in this organism is growth associated. Residual cell increased until the nitrogen source was depleted. At the end of fermentation, final cell concentration, PHA content, and productivity were 30.2 g/L, 44.8 % of cell dry weight, and 0.188 g/l/h, respectively.

Keywords: Biodegradable plastics, medium-chain-length polyhydroxyalkanoates (PHAs-mcl), oleic acid, Pseudomonas putida PGA 1, fed batch fermentation

1. Introduction

Polyhydroxyalkanoates (PHAs) are intracellular carbon and energy materials that are accumulated by various microorganisms under certain unbalanced growth conditions [1-3]. PHAs have attracted much industrial attention to be used in a wide range of consumer products, agriculture, marine, and medical application. These polyesters have been divided into 1) short chain length PHAs (PHAs-scl) consisting 3 to 5 carbon atoms, and 2) medium chain length PHAs (PHAs-mcl) consisting 6 to 14 carbon atoms. PHAs-mcl are biodegradable elastomers with low crystallinity and low glass transition temperature. Therefore, PHAs-mcl has attracted interest because they have flexible properties for wide range of applications that cannot be obtained with PHAs-scl [3-4].

Pseudomonas strains such *as Pseudomonas putida, Pseudomonas aeruginosa, Pseudomonas oleovoran* are known as versatile PHAs-mcl producers. *P.putida* was reported to produce medium-chain-length polyhydroxyalkanoates by high-cell-density cultivation under phosphorus limitation [5]. The other strain, *P. putida* PGA1, has been studied for the production of PHAs-mcl using saponified palm kernel oil (SCPKO) as a carbon source [6]. This experiment was conducted in Erlenmeyer flasks, using rotary shakers. In order to scale-up the process, further studies in bioreactor are needed to establish the kinetics of PHA fermentation using oleic acid as a model carbon source and later extended to saponified vegetable oils. In this paper, we report the production of PHAs in a fermenter using continuous feeding of both nitrogen and oleic acid in a fed batch culture.

2. Methods

Bacterial Strain. *P. putida* PGA1 was kindly given by Professor G. Eggink from the Agrotechnological research Institute, Wageningen, The Netherlands.

Oleic Acid. Oleic acid from two different local companies was used in the experiments. Oleic acid A was from Southern Acids (M) Ltd, Klang and oleic acid B was from Palm-Oleo Sdn. Bhd, Selangor. Oleic acid A was yellowish whereas Oleic acid B was slightly yellowish.

Flask Experiments for Seed Culture. Flask experiments were conducted in several 1000-ml Erlenmeyer flasks containing 300 ml of modified-R medium plus 1 g/L or 3 g/L of oleic acid. Composition of the modified medium is: 22 g/l KH_2PO_4, 3.0 g/L $(NH_4)_2HPO_4$, 1.4 g/L $MgSO_4.7H_2O$, 0.8 g/L citric acid, and 10 mL/L trace metal solution. The trace metal solution contained per liter of 5 *M* HCl: 10.0 g $FeSO_4.7H_2O$; 2.0 g $CaCl_2$; 2.2 g $ZnSO_4.7H_2O$; 0.5 g $MnSO_4.4H_2O$; 1.0 g $CuSO_4.5H_2O$; 0.1 g $(NH_4)Mo_7O_{24}.4H2O$; and 0.02 g $Na_2B_4O_7.10H_2O$. This medium was inoculated by 0.5 % (v/v) of overnight grown cells (20 h). The flasks were then incubated at 30°C, 240 rpm, and the cells were harvested at several time intervals, from 5 to 24 h, for determination of cell dry weight. The supernatants were used for the determination nitrogen concentration in it.

Fed-Batch Culture. Seed for the feed batch culture was prepared in flasks containing modified R medium plus 3 g/L of oleic acid by incubating for 20 h. The seed cultures were transferred into a 5-L fermenter, initially containing 2.7 L of modified R medium in which the initial KH_2PO_4 concentration was 7.5 g/L but with out nitrogen source. Initial concentration of carbon source was 6 g/L of oleic acid. Temperature and pH were controlled at 30 °C and 7.0, respectively and foaming was controlled by the addition of antifoam (silicone antifoaming agent, BDH). Dissolved oxygen concentration was maintained higher than 10 % of air saturation by controlling agitation speed and by oxygen enrichment. Aeration rate was between 1 - 2 liter/min and maximum agitation speed was set at 650 rpm. During fermentation, 440 g of oleic acid and 60 g of nitrogen source, $(NH_4)_2SO_4$, were fed continuously to the fermenter starting from 3h – 45 h and 2 h – 24 h, respectively. The nitrogen source for feeding was prepared by dissolving the $(NH_4)_2SO_4$ in 200 ml water, to which 1 gram of $MgSO_47H_2O$ was also added.

Analytical Methods. During fermentation, around 50 ml of culture broth was periodically removed for analysis. The culture samples were centrifuged to separate the culture supernatant and the cells. The supernatant was used for analysis of nitrogen and the cells were dried in oven at 105 °C, for cell dry weight determination. At the end of fermentation, the entire culture broth was centrifuged to collect all the cells. The cells were then used for PHA extraction, after washing with saline solution, followed by methanol and dried at 105 °C. Concentration of ammonium in the medium was determined by Berthelot reaction [7] and expressed as NH_3 concentration. PHA content of the samples was analyzed by gas chromatography (GC) [7]. Residual cell concentration is defined as cell concentration minus PHA concentration.

PHA Extraction. For the extraction of PHA, dried cells were suspended in chloroform (30 ml chloroform/1 gram cells) and refluxed for 4 h at 80 °C. The mixture was filtered through No.1 Wathman filter paper and the filtrate containing polymer was concentrated by evaporation. The polymer was precipitated in chilled methanol and dried in an oven at 40 °C.

3. Results and Discussion

Incubation Time of Seed Culture. Shake flask experiments were conducted to study the suitable incubation time for transferring the seed culture to the fermenter. Two oleic acids (oleic acid A and oleic acid B) were used at initial concentrations of 1 g/L and 3 g/L, to grow the inoculum. The results are shown in Figure 1 and Figure 2. They showed that cell growth

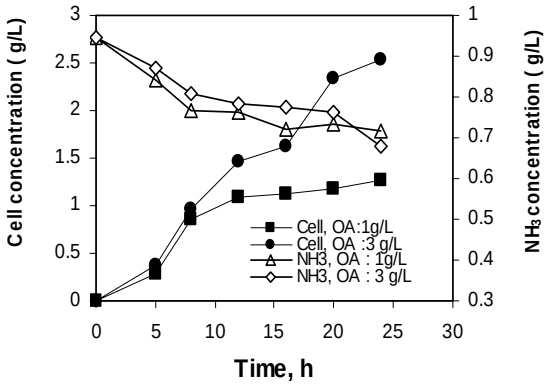


Figure 1. Time course of cell growth on 1 g/L and 3 g/L of oleic acid A in shake flask at 30 °C and 240 rpm (OA = oleic acid).

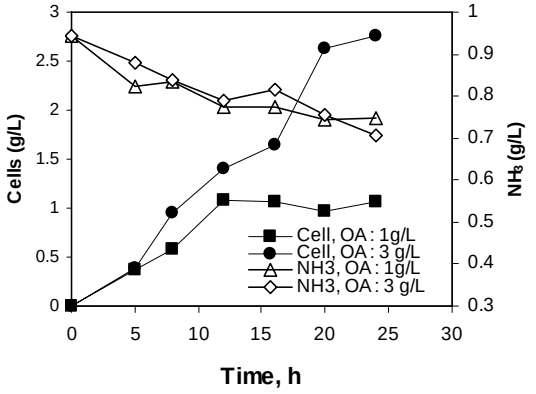


Figure 2. Time course of cell growth on 1 g/L and 3 g/L of oleic acid B in shake flask at 30 °C and 240 rpm (OA = oleic acid).

and nitrogen consumption from oleic acid A and oleic acid B were similar. It was suspected that there would be a difference in the cell growth since the color of oleic acid A was yellowish whereas, oleic acid B was lighter (slightly yellowish). The yellow color of oleic acid is mostly come from carotenoic compounds. Since the color different did not effect to the cell growth, it is suggested the carotenoic compound does not support or inhibit cell growth of *Pseudomonas putida*. It means when fatty acids from crude palm oil used as carbon sources, carotenoic compounds is not necessary to be isolated or split away from the fatty acids.

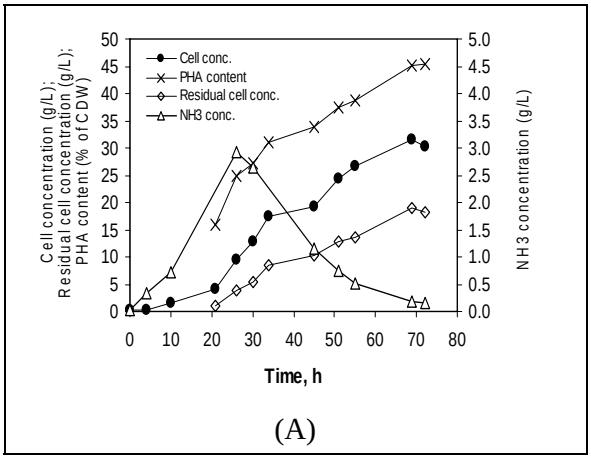
When 1 g/L and 3 g/L of oleic acid were used, maximum cell concentration reached was around 1 g/L after 12 h of incubation and 2.7 g/L after 24 h of incubation. These results show that oleic acid concentration of 1 – 3 g/L doest not inhibit the growth of *P.putida* PGA1. Figure 1 and Figure 2 indicate for an initial oleic acid concentration of 3 g/L, after 24 h of incubation, the cells entered the stationary phase of growth. An incubation time between 18 – 22 h is in the range of late exponential growth phase. Therefore, this range of cultivation is suggested to be a suitable incubation time for seed culture preparation. For further experiments, an incubation time of 20 h was selected for transfer of the seed culture to the fermenter.

Fed-Batch Culture. Fed-batch culture of *P.putida* PGA 1 was carried out in a 5-liter fermenter containing 3 liter of modified R medium. Oleic acid and $(NH_4)_2SO_4$, were fed continuously to the fermenter during fermentation. The results are shown in Figure 3. At the end of fermentation, final value of cell concentration, PHA concentration and PHA content reached were 30.2 g/L, 13.5 g/L and 44.8 % cell dry weight (CDW), respectively (Table 1). During fermentation, cell concentration, residual cell concentration, and PHA content increased with time. This indicates that PHA production in this organism is growth associated. This feature is an advantage in using *P.putida* for the production of PHA as with cell growth, PHA also accumulated. The residual cell concentration also increased until 69 h of cultivation. It is because nitrogen source is still available in the medium, until this time.

In the early part of cultivation, until 20 h, cell growth was very slow. It could be because lack of available nitrogen in the initial medium. Besides this, starting from 2 h of cultivation, some carbon source and cells flocculated and attached to the walls of the fermenter. It might be another reason that the cell concentration in the culture broth was very low during the first 20 h of cultivation.

Table 1. Summary of the Results at the End of Fed-Batch Fermentation

Carbon source	Total carbon source added (g)	Total NH_3 added (g)	Final cell dry weight (CDW), (g/L)	Final PHA concentration, (g/L)	Yield of PHA, (g PHA/ g carbon source)	PHA content, (CDW) (%)	Producti-vity, (g/l/h)	Final volume (L)	Total PHA per Batch (g)
Oleic acid	440.2	16.35	30.22	13.52	0.102	44.9	0.1878	3.3	44.77



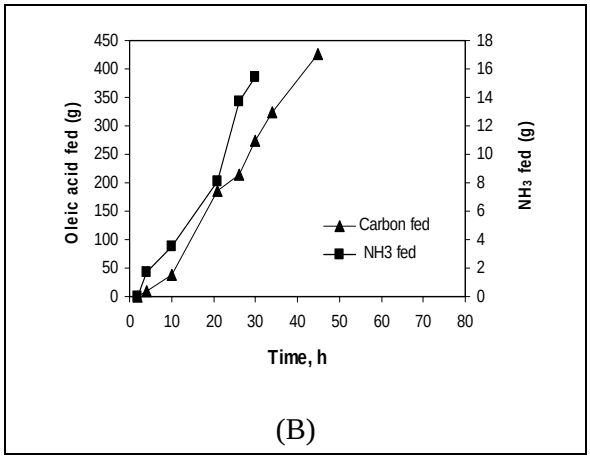


Figure 3. Time course of cell concentration, PHA content, and residual cell concentration (A); Amount of oleic acid and $(NH_4)_2SO_4$ as NH_3 fed to the fermenter (B).

Therefore, for further experiments, a certain amount of nitrogen will be supplied in initial medium. To overcome sticking of oleic acid and cells to the walls of the fermenter, $MgSO_4.7H_2O$ in initial medium will be reduced, since relatively high concentration of $MgSO_4 7H_2O$ in the modified R medium flocculated the oleic acid and these flocks got stuck to the walls of the fermenter due to agitation.

PHA extracted from the cells was sticky and slightly yellowish. Gas chromatography (GC) analysis showed that this PHAs gave similar monomer peak as obtained from oleic acid of previous experiments [6], consisting of C_6, C_8, C_{10}, C_{12}, and C_{14} (data not shown). PHA content of this result was lower than that of obtained from *Ralstonia eutropha* using soy been oil as a carbon source, i.e. 76 % CDW [8] and from *P. oleovorans* using octanoate as a carbon source, 1.e. 67 % CDW [9]. However, this result was higher compared than our previous result, i.e. 20 % CDW [6].

4. Conclusion

Oleic acid concentration of 1-3 g/L did not inhibit the growth of *P.putida* PGA1. The suitable incubation time to transfer inoculum to fermenter is between 18 – 22 h. Continuous feeding both carbon source and nitrogen source gave relatively high of PHA content (44.9 % CDW).

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