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Optimum Fermentation Conditions for Microbial Polyester Synthesized by *Delftia Aacidovorans*

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Delftia acidovorans was grown in fed-batch and chemostat cultures with various carbon sources. In the fed-batch cultures, the synthesis rate of the polymer was significantly controlled by the pH value, concentration of the carbon sources, temperature, density of the cell, incubation time, etc. The optimum pH value is 7.0, the mixed carbon source is 10 g, and the cultivation time is 72 hours by *D. acidovorans* in this study. The composition and properties of the poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate) [P(3HB-*co*-4HB)] were studied using differential scanning calorimetry, gel permeation chromatography (GPC) and proton nuclear magnetic resonance spectroscopy (^{1}H-NMR). The maximum 4HB unit composition of P(3HB-*co*-4HB) obtained by the fed-batch culture with a cell concentration of ca. 2.5 g/*l* reached 94 mol% though the P(3HB-*co*-4HB) content was always low, i.e., ca. 13 wt%.

Key words: Fermentation, Microbial poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate), Biodegradation

1. Introduction

Biodegradable polymer development is one of the biotechnology research areas, not only is it one of the green manufacturing processes, but also degradable by microorganisms in the environment. Therefore, such manufactured synthetic products do not need further processes after use and can be decomposed into carbon dioxide and water by microorganisms. Either for environmental protection or for medical applications, it is an extremely good material [1-3].

Microbial poly(hydroxyalkanoic acids) (PHAs) are very similar to the function of saccharine compounds. They are also produced by microorganisms like a typical carbon substrate and energy source in nature, biodegradable and biocompatible thermoplastic polymers. A number of microorganisms such as bacteria and fungi can excrete enzymatic PHB depolymerase to hydrolyze the PHAs into low molecular weight materials which are water-soluble in the environment. Microorganisms utilize the resulting products and store them as nutrients within the cells, and eventually convert them into water and carbon dioxide via the cyclic metabolic processes [4-7].

In recent years in the tissue engineering field, it has become fashionable to use biodegradable polymers to manufacture 3D scaffolds for cell growth and division into needed tissues to be used for body transplants and other applications. Because the product after decomposition is harmless to humans, there is no side effect if transplanted into the human body, therefore, research now using microorganisms to synthesize biodegradable polymers is an upcoming trend [8-11].

Microorganism-synthesized polymers are produced using organic acids, sugars and alcohol as starting materials. Currently, these microorganisms have synthesized polymers that have included P(3HB), P(3HB-*co*-3HV), P(3HB-*co*-3HP), P(3HB-*co*-4HB), and so on. [12-15]. Due to the specificity of the microorganism's matrix, different microorganism types and culture conditions, such as pH, temperature, microorganism concentration, and carbon source, etc, the produced biodegradable polymer varies [16-19].

In this study, we observed the influence of the carbon source concentration, pH and incubation time on the biosynthesis of the P(3HB-*co*-4HB) polymer. In addition, the production of P(3HB-*co*-4HB) by *D. acidovorans* from mixed carbon sources of *n*-butyric acid and 1,4-butanediol, which are relatively low cost materials, is reported. Moreover, we also discuss the composition of P(3HB-*co*-4HB) during fermentation by controlling of the mixed carbon sources.

2. Experimental procedures

2.1 Biosynthesis of poly(hydroxyalkanoates)

The fermenter (MDL-1000, Marubishi Bioengineering Co.) was used to synthesize the biodegradable polymer. In order to accurately control the optimum fermentation condition, the internal built-in processing software (Labo-Coontroller, MDL-6C) of the fermenter system were utilized. The microorganism, *D. acidovorans* (IFO13582), was used in this study. The poly(hydroxyalkanoates) synthesis was carried out by the two-step fermentation of *D. acidovorans*. The

microorganism was first grown at 26 °C in a nutrient-rich medium (pH 7.0, 1 liter) containing 10 g polypeptone, 10 g yeast extract, 5 g meat extract and 5 g $(NH_4)_2SO_4$. The cells were harvested after 48 hours and rinsed with water. Under these culture conditions, no accumulation of polyesters in the cells was observed.

To promote the polyester synthesis, about 2.5 g quantities of the rinsed cells were transferred into a mineral medium (pH 7.0) containing different carbon sources. The cells were cultivated in these media at 26 °C for 72 hours, harvested by centrifugation (4000 rpm, 15 min), rinsed with water and methanol and finally vacuum-dried at room temperature. The composition of the medium has been reported in a previous paper [12].

2.2 Extraction of poly(hydroxyalkanoates)

The polyester was extracted from the dried cells with hot chloroform and purified by precipitation with *n*-heptane. The polymers were then vacuum-dried for 48 hours. The polymer yield was measured using the following formula (1).

$$Y=(Wa / Wd) \times 100 \qquad (1)$$

where Y is the polymer content (wt%), Wa is the polymer weight (g) and Wd is the dried cell weight (g).

2.3 Composition of poly(hydroxyalkanoates)

The compositions of the poly(hydroxylalkanoate) samples were determined by analyzing the 500 MHz ^{1}H-NMR spectra, which were recorded using a JEOL α-500 spectrometer. The ^{1}H-NMR spectra were recorded at room temperature for a $CDCl_3$ solution of the poly(hydroxyalkanoate) (10 mg ml^{-1}) with a 4.7 μs pulse width, 5-s pulse repetition, 5000 Hz spectral width, 16K data point and 32 accumulations. Tetramethylsilane (Me_4Si, δ = 0) was used as the internal chemical shift standard.

2.4 Measurement of molecular weight (GPC)

Gel permeation chromatography (GPC) was carried out using an HLC-802A high-performance liquid chromatograph (Tosoh) at 38°C equipped with a series of four columns of TSK-gel and an RI-8 differential refractometer. Chloroform was used as the eluent at the flow rate of 0.1 ml min^{-1}, while the sample solution of 1 ml was used at a concentration of 1.0 g l^{-1}. Polystyrene standards with a narrow polydispersity were used to generate a calibration curve, and the apparent molecular weights were then calculated.

3. Results and discussion

3.1 PHA produced by organic carbon sources

The P(3HB) homopolymer is produced from short chain lengths of alkanoic acid with even carbon numbers. In contrast, a copolymer of 3-hydroxybutyrate and 3-hydroxyvalerate, P(3HB-*co*-3HV), is produced from alkanoic acids with odd carbon numbers by *A. eutropha* [2].

Table I lists the results of the P(3HA) production by *D. acidovorans* from alkanoic acid with different carbon numbers. Three kinds of polyesters are obtained from various organic carbon sources.

In this study, the P(3HB) homopolymer can be produced from alkanoic acid with even or odd carbon numbers. The P(3HB-*co*-4HB) and P(3HB-*co*-3HV) copolymers can be produced from alkanoic acid with odd and even carbon numbers, respectively.

Table I Production of polyester in *D. acidovorans* from various carbon sources in a flask for 72 h at 26°C.

Carbon Source (0.5 g/dL)	Polymer composition[a] 3HB	3HV	4HB
1,3-Butanediol	o	--	--
1,4-Butanediol	o	--	o
1,4-Pentanediol	o	o	--
1,5-Pentanediol	o	--	--
1,5-Hexanediol	o	--	--
1,6-Hexanediol	o	--	o
1-Hexanol	o	--	--
n-Butyric acid	o	--	--
Lauric acid	o	--	--
r-Butyrolactone	o	--	o
2-Methylhexanoic acid	o	--	--
2-Ethylhexanoic acid	o	--	--
4-Hydroxy-3-methyl-2-butaneone	o	--	--

a. Determined by ^{1}H-NMR.

3.2 Concentration of carbon source in biosynthesis

To evaluate the influence of the various carbon source concentrations on the biosynthesis of the P(3HB-*co*-4HB) polymer, the biosynthesis was carried out using a two-step fermentation (pH = 7.0) at 26 °C for 72 hours. Table II lists the results of the copolyester production by *D. acidovorans* for various concentrations of the carbon sources in the two-step fermentation for 72 hours at 26 °C. *D. acidovorans* produced the P(3HB-*co*-4HB) copolyesters from *n*-butyric and 1,4-butanediol as the mixed carbon sources. The 4HB fraction and polymer content of P(3HB-*co*-4HB) were observed by the changes in the mixed carbon source concentration of 5 g l^{-1}, 10 g l^{-1} and 15 g l^{-1}. P(3HB-*co*-4HB) was produced from 10 g l^{-1} of the mixed carbon sources, and the polyester contents in the dried cells were as high as 13 wt%, while the 4HB fraction of the polymer was 65 mol%. The 4HB fraction of the polymer was 65 mol% when 5 g l^{-1} of the mixed carbon source was used, which was the same as that of 10 g l^{-1} of the mixed carbon source, but the

polymer content was lower at about 9 mol%. When the polymer was produced from 15 g l^{-1} of the mixed carbon source in the biosynthesis, it was observed that both the 4HB fraction and polymer content were at their lowest

Table II Production of P(3HB-*co*-4HB) copolymer in *D. acidovorans* from various concentration of carbon sources by the two-step fermentation for 72 h at 26 °C.

Carbon sources (g/L)		Dry cell weight	Polyester Content	PHA composition[a] (mol%)		Molecular weight[b]	
Butyric acid	1,4-Butanediol	(g/L)	(wt%)	3HB	4HB	$Mn\times10^{-4}$	Mw/Mn
2	3	2.2	9	35	65	7.1	2.6
4	6	2.5	13	35	65	9.1	3.1
6	9	2.4	4	84	16	7.5	5.8

a. Determined by ^{1}H-NMR.
b. Determined by GPC.

Table III Production of P(3HB-*co*-4HB) copolymer in *D. acidovorans* from 1,4-butanediol as the sole carbon source under various pH conditions using the two-step fermentation for 72 h at 26 °C.

pH	Dry cell weight (g/L)	Polyester Content (wt%)	PHA composition[a] (mol%)		Molecular weight[b]	
			3HB	4HB	$Mn\times10^{-4}$	Mw/Mn
6.0	2.3	5	42	58	3.7	2.1
6.5	2.4	11	13	87	3.6	2.5
7.0	2.5	13	6	94	4.2	2.8
7.5	2.3	1	46	54	2.8	2.9

a. Determined by ^{1}H-NMR.
b. Determined by GPC.

values of 16mol% and 4wt%, respectively.

It is considered that microorganisms excrete extracellular PHB depolymerase to degrade the materials and then easily utilize it for incorporation with the carbon source into the intracellular sites. It is postulated that the microorganism could not excrete sufficient extracellular PHB depolymerase to degrade the materials when the concentration of the culture medium is too high. Consequently, the 4HB fraction and polymer content were decreased, because of not resolving it into material that as the carbon source is easy to absorb into the intracellular sites.

In conclusion, the optimum mixed carbon source of 10 g l^{-1} for obtaining a high polymer content by *D. acidovorans* was determined.

3.3 pH value of biosynthesis

To evaluate the influence of the change in pH during the biosynthesis of the P(3HB-*co*-4HB) polymer, the biosynthesis was carried out by the two-step fermentation with 10 g l^{-1} of 1,4-butanediol as the sole carbon source at 26 °C for 72 hours. Table III shows the results of the copolyester production by *D. acidovorans* from 1,4-butanediol as the sole carbon source at various pHs using the two-step fermentation for 72 hours at 26 °C. The highest contents of the copolymer in dried cells were observed in the pH 7.0 culture solution containing 10 g l^{-1} of 1,4-butanediol.

The number-average molecular weights (*Mn*) of the copolyesters were in the range of 28,000-42,000, depending upon the fermentation conditions. As shown in Table III, the *Mn* value increased with an increase in the pH value of the culture solution. The molecular weight distributions of the copolyesters were unimodal and their polydispersities (*Mw*/*Mn*) were in the range of 2.1-2.9. The highest 4HB fraction and copolymer contents in the pH 7.0 culture solution containing 10 g l^{-1} of 1,4-butanediol of about 94 mol% and 13 wt%, respectively, were observed. When the pH value in the culture solution was higher than 7.0, the 4HB fraction and the polymer content significantly decreased to about 54 mol% and 1 wt%, respectively. The same results, i.e., the 4HB fraction of the polymer is 87 mol% and 58 mol% and the polymer content is 11 wt% and 5 wt% appear at the lower pH values (pH = 6.5, and 6.0), respectively.

In conclusion, the optimum pH value is 7.0 for obtaining the highest polymer content by *D. acidovorans*.

4. Conclusion

To obtain the highest polymer content for the two-step fermentation by *D. acidovorans,* the fermentation conditions were examined by changing the concentration of the carbon source, pH and incubation temperature. The optimized conditions experimentally determined are as follows:

pH	7.0
Incubation temperature (°C)	26
Concentration of carbon source (g l^{-1})	10

The highest polymer content was around 13% per dried cell weight.

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References

[1] Y. Saito and Y. Doi, *Int. J. Biol. Macromol.*, 16, 99-104 (1994).

[2] Y. Saito, S. Nakamura, M. Hiramatsu, and Y. Doi, *Polym. Int.*, 39, 169-174 (1996).

[3] H. Kimura, Y. Yoshida, and Y. Doi, *Biotechnol. Lett.*, 14, 445-450(1992).

[4] R.G. Lageveen, G. W. Huisman, H. Pseuting, P. Ketelaar, G. Eggink, and B. Witholt, *Appl. Environ. Microbiol.*, 54, 2924-2932 (1988).

[5] Y. Doi, Y. Kawaguchi, Y. Nakamura and M. Kunioka, *Appl. Environ. Microbiol.*, 55, 2932-2938 (1989).

[6] G. W. Huisman, O. DeLeeuw, G. Eggink, and B. Witholt, *Appl. Environ. Microbiol.*, 55, 1949-1954 (1989).

[7] Y. Doi, Microbial polyester, New York: VCH publishers, (1990).

[8] D. W. Hutmacher, *Biomaterials*, 24, 2529-2543 (2000)..

[9] S. Levenberg, N.F. Huang, E. Lavik, A.B. Rogers, J. Itskovitz-Eldor, and R. Langer, *PNAS*, 100, 12741-12746 (2003).

[10] Y.W. Wang, Q. Wu, and G.Q. Chen, *Biomaterials*, 24, 4621-4629(2003).

[11] Y.W. Wang, Q. Wu, and G.Q. Chen, *Biomaterials*, 25, 669-675 (2004).

[12] P.J. Senior, and E.A. Dawes, *J. Biochem.*, 125, 55-66(1971).

[13] M. Yokouchi, Y. Chatani, H. Tadokoro, K. Teranishi, and H. Tani, *Polymer*, 14, 267-272(1973).

[14] G. Renner, K. Pongratz, , and G. Braunegg, *Food Technol. Biotechnol.*, 34, 91-95(1995).

[15] N. Kamiya, Y. Yamamoto, Y. Inoue, R. Chûjô, and Y. Doi, *Macromolecules,* 22, 1676-1682 (1989).

[16] B. Taidi, A.J. Anderson, E.A. Dawes, and D. Byrom. *Appl. Microbiol. Biotechnol.*, 46, 451-463 (1994).

[17] Y. Doi, A. Segawa, and M. Kunioka, *Int. J. Biol. Micromol.*, 12, 106-111 (1990).

[18] H. Mitomo, W.-C. Hsieh, K. Nishiwaki, K. Kasuya, and Y. Doi, *Polymer*, 42, 3455-3461(2001).

[19] W.-C. Hsieh, H. Mitomo, K. Kasuya, and T. Komoto, *Journal of Polymers and the Environment*, 14, 79-87(2006).

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