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The Microbial Production of Polyhydroxybutyrate from Methanol

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ABSTRACT

One hundred strains of methanol-utilizing bacteria were isolated. From them a methylotrophic strain 8502-3 was selected out for biodegradable plastics polyhydroxybutyrate (PHB) production on methanol. It was named *Hyphomicrobium zavarzinii* subsp. *chengduense* subsp. nov. The PHB production was performed with both fed batch and continuous fermentation. The high mol wt of PHB, ranging from 0.9×10^6 to 1.3×10^6 dalton, was obtained from methanol. PHB content in cells ranging from 40 to 59% was found with the highest productivity of 0.64 g PHB/L/h.

Index Entries: Biodegradable plastics; methanol fermentation; methylotroph; hyphomicrobium; polyhydroxybutyrate.

INTRODUCTION

Thermoplastic plastics polyhydroxybutyrate (PHB) possesses biodegradable, radiation-resistant, and piezoelectric properties, and may meet a wide range of requirements in surgery, medicine, agriculture, food industry, environment protection, and so on. The commercial product has been available by microbial fermentation. However, the cost of PHB product is rather high to restrict its practical application. In addition to the process of PHB extraction and purification, the raw material of fermentation is an important factor in influencing the cost. Cheap methanol can be

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used as a carbon source for the fermentation. Regretfully, the molecular weight of PHB produced from methanol is usually less than that from carbohydrate, even though 0.8×10^6 dalton has been reached (*1,2*). The purpose of this study is to try to find a suitable microorganism for the production of PHB with high molecular weight from methanol.

MATERALS AND METHODS

The Isolation and Purification of Methanol-Utilizing Bacteria

For the enrichment of methanol-utilizing bacteria, samples taken from various ecological environments in south China were incubated on mineral medium reported previously (*3*) containing methanol (0.3% v/v) as the sole carbon and energy source for microbial growth. For the isolation and purification, the common procedure with agar plates was used. Colonies grown up on the medium containing methanol (0.5% v/v) were picked out and incubated on aqueous medium (*4*).

The Selection of Strains for PHB Production

The preliminary selection of strains for PHB production, with regard to growth rate, cell concentration, PHB content in cells, and the sedimentation property of cells, was performed with shaken flasks. A few of strains selected through the preliminary screening underwent the testing in a 5-L fermentor to compare their growth ability and PHB productivity.

Fed Batch Fermentation

A 5-L fermentor or a 10-L fermentor (Model MD, B. E. Marubishi Co. Ltd., Japan) with automatic regulation of desolved oxygen, temperature, and pH value was used. The initial growth medium contained $(NH_4)_2SO_4$ (0.6 g/L), NaCl (0.5 g/L), EDTA (50 μg/L), phosphate (1.5 g/L), and methanol (4 mL/L). With the increasing of cell concentration, $(NH_4)_2SO_4$, phosphate, MeOH, and minor elements were fed at intervals to the fermentor to keep their concentrations changing around the initial level. The concentrations of ammonium and phosphate in the fermentation medium were analyzed at intervals by colormetry, and methanol concentration was determined by a Rank electrode (*5*).

Continuous Fermentation

Single-stage continuous fermentation was operated in a 10-L fermentor. For two-stage continuous fermentation, the effluent from the 10-L fermentor with normal medium was pumped into a 5-L fermentor with medium containing no ammonium.

Extraction and Purification of PHB and Analysis Methods

The extraction and purification of PHB within cells were performed by Law and Slepecky's method (*6*). The molecular weight of extracted PHB was determined by gel permeation chromatography (GPC) using standard polystyrene as reference. PHB content in cells was analyzed by spectrophotometry (*7*) and gas chromatography (*8*). Niclet MX-1E infrared spectrum analyzer (Niclet Co., USA) was used to detect infrared spectrum of the purified product and reference.

RESULTS AND DISCUSSION

Isolation and Selection of PHB-Production Bacteria

The enrichment was very successful. From the enrichment of almost every sample taken from the natural environment, methanol-utilizing bacteria could be obtained. One hundred strains grown best on methanol were taken for preliminary selection. Among them, 37 strains could accumulate PHB in cells more than 30% with the flask selection. Different growth conditions were tested for the 37 strains. Under improved conditions, seven strains could accumulate PHB to more than 40%.

The three most promising strains were taken for further testing in a fermentor. It was found out that strain 8502-3 was desirable for PHB production with rapid growth, high cell concentration, high intracellular PHB content, and good settling property.

Strain 8502-3 could also accumulate PHB/PHV copolymer within cells using methanol as a main source of carbon and energy for its growth; however, propionate or valerate (0.1% v/v) were needed to be added into the medium (Fig. 1). Strain 8502-3 has been identified and named *Hyphomicrobium zavarzinii* subsp. *chengduence* subsp. nov. (*9*).

The Production of PHB

The PHB productivity of strain 8502-3 is shown in Tables 1 and 2. The fermentations were operated at 35°C and pH 7.2. In two-stage continuous fermentations, the highest PHB content in cells was 59.1% with productivity as high as 0.64 g PHB/L/h.

There was no automatic regulation equipment for ammonium and methanol in the fermentor used in the experiments. The consumption of ammonium and methanol by growing cells was so rapid that it was very difficult to regulate manually their concentrations in the fermentation medium. The overloading and exhaustion of methanol occurred frequently,

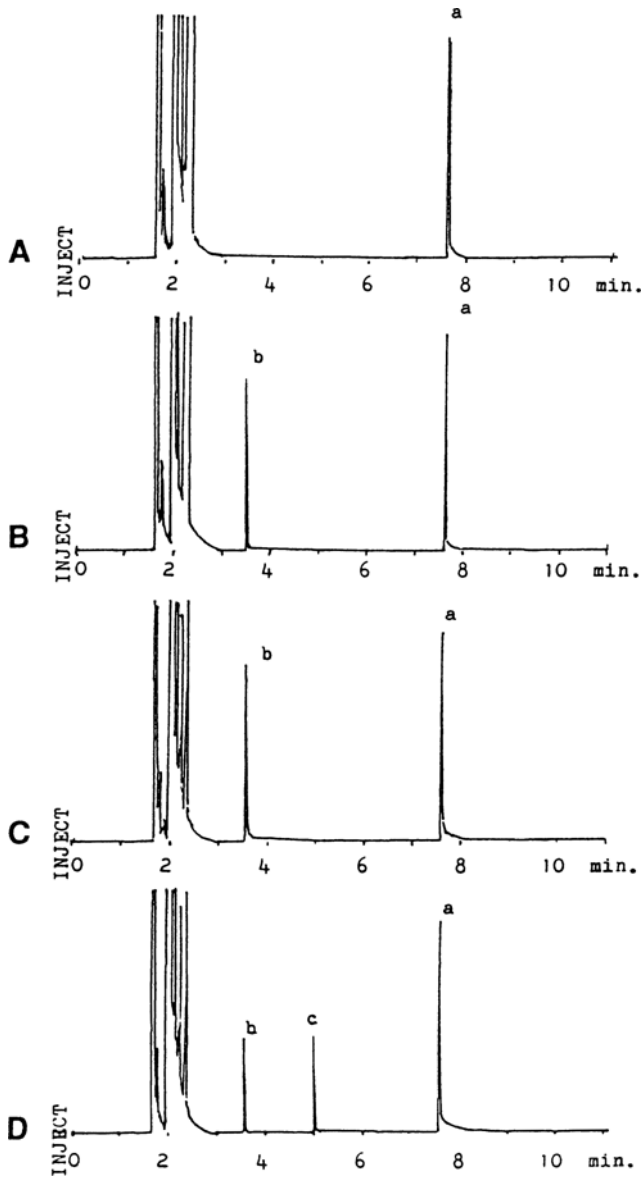

Fig. 1. Chromatograph of purified product by strain 8502-3 with gas chromatography (*8*) A: Benzoate as the standard for determination. B: Authorized hydroxybutyrate mixed with benzoate. C: The mixture of benzoate and purified PHB. D: The mixture of benzoate and purified PHB/PHV, which was produced by strain 8502-3 from methanol with propanoic acid added a—benzoate, b—hydroxybutyrate, c—hydroxyvalerate as checked with another reference.

Table 1
PHB Production by Strain 8502-3 with Fed Fermentation

Experiments	1	2	3	4	5
Fermentor					
Total volume (L)	5	10	10	10	10
Working volume (L)	3	7	7	7	7
Growth medium					
Water[a]	d.w.	d.w.	t.w.	t.w.	t.w.
Ammonium salt	NH_4Cl	NH_4Cl	$(NH_4)_2SO_4$	$(NH_4)_2SO_4$	$(NH_4)_2SO_4$
Initial cell concentration (g/L)	0.23	0.40	0.33	0.40	0.52
Fermentation cycle (h)	54	49	42	54	40
Final cell concentration (g/L)	17.6	23.1	19.9	33.4	25.0
Maximum specific growth rate (/h)	0.120	0.100	0.092	0.095	0.112
Double time (h)	5.8	6.9	7.5	7.3	6.2
PHB content (g PHB/100 g dry cells)	40.5	43.0	-	-	-
Yield					
g Cells/g methanol consumed	0.31	0.31	0.31	0.29	0.35
g PHB/g methanol consumed	0.13	0.13	-	-	-

[a] d.w.: distilled water. t.w.: tap water.

Table 2
PHB Production by Strain 8502-3 with Continuous Fermentation

Experiments	1	2	3
Fermentor			
First stage			
Total volume (L)	10	10	10
Working volume (L)	5.9	5.5	5.5
Second stage			
Total volume (L)	-	5	5
Working volume (L)	-	1.0–1.5	1.0–1.5
Average dilution rate (h^{-1})[a]	0.042	0.051	0.046
Average cell concentration (g/L)			
First stage	23.3	23.3	24.7
Second stage	-	25.3	26.2
Cell productivity (g/L/h)	1.0	1.3	1.3
Average PHB content (g PHB/100 g dry cells)	39.4	39.6	47.0
PHB productivity (g PHB/L working vol/h)[a]	0.39	0.52	0.61
Yield			
g Cells/g methanol consumed	0.31	0.29	0.25
g PHB/g methanol consumed	0.12	0.12	0.16

[a]In the entire two-stage system for Experiment 2 and 3.

which might cause low methanol yield. It is possible that with automatic regulation of the concentrations of both ammonium and methanol, the yield of PHB from methanol might increase to 0.18 g PHB/g methanol consumed, which has been obtained in a batch fermentation without feeding additionally.

With flask cultures of strain 8502-3, more than 70% PHB content (g PHB/g dry cells) has been obtained. Consequently, the potential productivity of two-stage continuous fermentation with strain 8502-3 should be more than 0.9 g PHB/L working volume/h.

Characteristics of the Purified Product

It was ensured by both UV-spectrogram (*7*) and chromatography (*8*) that the acid hydrolysate of the purified product was the same as hydroxybutyrate. Infrared spectrum indicated no obvious difference from reference (Aldrich Chem. Co., Milwaukee, Wisconsin, USA) with both film and disk preparations for sample detection (Figs. 1,2, and 3).

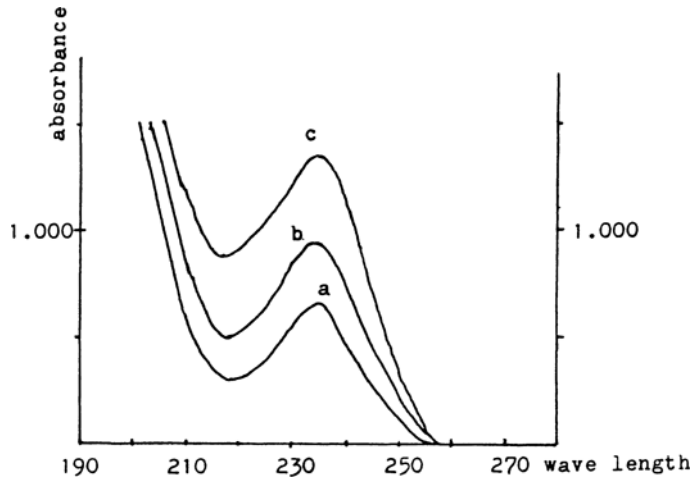

Fig. 2. UV Spectrogram of the acid hydrolysate of purified product by strain 8502-3 (*7*). a—Authorized hydroxybutyrate, b and c—purified cell product.

The melting point of the purified product was 179°C as determined by a microscopic melting-point detector (Model X4, The Third Optical Instrument Manufactory, China).

The molecular weight determined by GPC method is shown in Table 3. The molecular weight of PHB produced from methanol by strain 8502-3 is the highest in documents dealing with methanol-derivative PHB reported to date (*1,10,11*)—even higher than that of PHB from carbohydrate (*12,13,14*).

Methanol-utilizing strain 8502-3 is a desirable strain for PHB production with regard to its high productivity, its cheap fermentative substrate, and the high molecular weight of its product. However, its growth requires a high oxygen supply rate and, consequently, an effective fermentor with a K_La of at least 1800/h. A fermentor of this kind with a new design has been available commercially.

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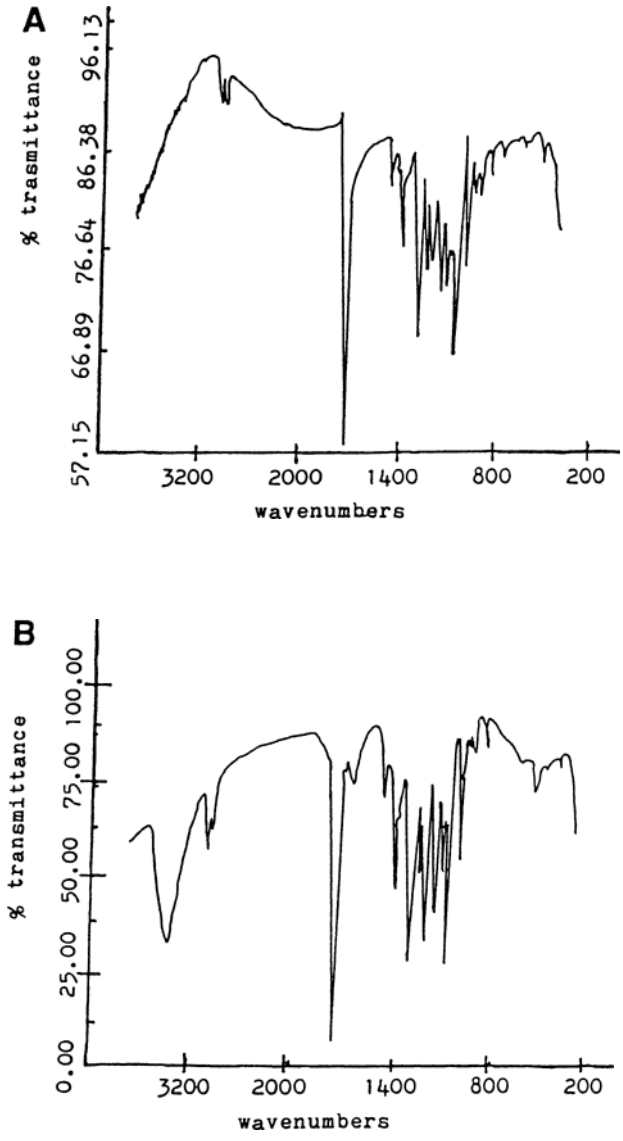

Fig. 3. Infrared spectrum of purified product by strain 8502-3. A: Reference from Aldrich Chm. Co. B: Purified cell product.

Table 3
Molecular Weight of PHB Produced by Strain 8502-3

Samples	M_w[a]	M_n[a]	α[a]
1	1.0×10^6	4.9×10^5	0.15
2	1.3×10^6	5.7×10^5	2.29
3	0.9×10^6	2.0×10^5	4.40

[a]M_w—Weight average molecular weight. M_n—Number average molecular weight. α—Distribution coefficient.

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