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Production of Polyhydroxybutyrate by *Bacillus* Species Isolated from Municipal Activated Sludge

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Abstract

Plastic wastes are considered to be severe environmental contaminants causing waste disposal problems. Widespread use of biodegradable plastics is one of the solutions, but it is limited by high production cost. Biologic wastewater treatment generates large quantities of biomass as activated sludge. Only a few reports focus on the potential of utilizing resident *Bacillus* species from activated sludge in polyhydroxbutyrate (PHB) production as well as the production of PHB from food wastes. They have attractive properties such as short generation time, absence of endotoxins, and secretion of both amylases and proteinases that can well utilize food wastes for nutrients, which can further reduce the cost of production of polyhydroxyalkanoates (PHAs). Two PHA-producing strains, HF-1 and HF-2, were isolated from activated sludge. HF-1 outperformed HF-2 in terms of growth and PHB production in hydrolyzed soy and malt wastes. The isolated bacteria was characterized by DNA sequence alignment. Cell extracts of HF-1 were also compared to *Bacillus megaterium* cell extracts on sodium dodecyl sulfate polyacrylamide gel electrophoresis. The biopolymers accumulated were analyzed by gas chromatography, nuclear magnetic resonance, and Fourier transform infrared methods.

Index Entries: Polyhydroxybutyrates; malt waste; soy waste; *Bacillus*; activated sludge; Fourier transform infrared; inclusion body; nuclear magnetic resonance.

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Introduction

Plastics have become an integral part of our lives, but the generation of plastic wastes has increased dramatically. In 1986–1998, about 15% of total domestic wastes or commercial and industrial wastes in Hong Kong were plastics *(1)*. The petroleum-derived plastics are not easily degraded by microorganisms. Plastic wastes are therefore considered to be severe environmental contaminants causing waste disposal problems.

Polyhydroxyalkanoates (PHAs) are polyesters of hydroxyalkanoates synthesized by numerous bacteria as intracellular carbon and energy storage compounds and accumulated as granules in the cytoplasm *(2)*, usually when essential nutrients such as N or P are limited in the presence of an excess carbon source *(3)*. The first PHA discovered was polyhydroxybutyrate (PHB), which is the most abundant form of PHA in nature.

By comparing the cost of BIOPOL™ (P[3HB-co-3HV] produced from fermentation by *Alcaligenes eutrophus*) (US$16/kg) with that of polypropylene (less than US$1/kg), the conventional plastics *(3)*, one can see that the cost of PHAs is much higher. The carbon source should be inexpensive because it is the major contributor to the total substrate cost (up to 50% of the total operating cost) *(4)*, and, thus, an expensive carbon source is not practical in large-scale industrial production. Several studies have investigated the use of low-cost substrate for PHA production, such as xylose *(4)*, molasses *(5)*, and malt waste and soy waste *(6)*.

Biologic wastewater treatment is the largest application of microorganisms in the service sector and generates large quantities of biomass as activated sludge. Our investigations showed the presence of different types of biopolymers in the activated sludge and the yield obtained was 6.5 g/L *(7)*. Only a few *Bacillus* strains were examined for their ability to accumulate PHAs, which naturally occur in biodegradable, biocompatible, and microbial thermoplastic. *Bacillus* strains have the attractive properties of short generation time, absence of endotoxins, and presence of both amylase and proteinase secretion that can well utilize food wastes. In this article, the potential of resident *Bacillus* species from activated sludge in PHB production as well as the production of PHB from food wastes are reported.

Materials and Methods

Activated Sludge

Activated sludge was collected from the Sha Tin Wastewater Sewage Treatment Plant in Hong Kong.

Microorganisms

Bacillus megaterium ATCC strain 11561 was kindly provided by Prof. Maura C. Cannon of University of Massachusetts. PHAs producing bacteria screened from activated sludge were named HF-1 and HF-2. The isolated strains were identified by Microbial ID (Newark, DE).

Media

Preparation of Growth Medium

Medium A consisted of a nutrient-rich medium LB broth (10 g/L of tryptone, 5 g/L of yeast extract, 10 g/L of NaCl) and 10 g/L of glucose.

Preparation of PHA Accumulation Medium

Medium B consisted of a medium for bioplastics accumulation containing 3.57 g/L of Na_2HPO_4, 0.25 g/L of $(NH_4)_2SO_2$, 1.50 g/L of KH_2PO_4, 0.20 g/L of $MgSO_4 \cdot 7H_2O$, and 20 g/L of glucose.

Preparation of Food Wastes Medium

Malt waste, mostly semisolids of spent barley and millet refuse, was obtained from Carlsberg, a beer brewery in Hong Kong. Soy waste, chiefly semisolid cellular residues of soy beans, was collected from Vitasoy International Holdings, a soy milk company in Hong Kong. The ratio of the C and N contents of the malt and soy wastes were 7:1 and 8:1, respectively, as determined by total organic carbon (TOC) *(8)* and total Kjeldahl nitrogen (TKN) *(8)* methods.

Two hundred grams of the waste was hydrolyzed with 1 L of 0.5 *M* HCl. The mixture was incubated at 90°C for 8 h. The resultant mixture was centrifuged at 14,333*g* for 20 min. The supernatant was filtered to remove debris and adjusted to pH 7.0 by adding NaOH. The medium was autoclaved for 30 min at 121°C and used as the substrate for the growth of bacteria.

Preparation of Trace Elements Solution

One hundred milliliters of water consisted of 0.60 g of $FeCl_3 \cdot H_2O$, 0.1 g of $CaCl_2 \cdot 2H_2O$, 0.03 g of H_3BO_3, 0.002 g of $CoCl_2 \cdot 6H_2O$, 0.010 g of $ZnSO_4 \cdot 7H_2O$, 0.003 g of $MnCl_2 \cdot 4H_2O$, 0.003 g of $Na_2MoO_4 \cdot 2H_2O$, 0.0024 g of $NiCl_2 \cdot 6H_2O$, and 0.001 g of $CuSO_4 \cdot 5H_2O$.

Screening of PHA-Producing Bacteria from Activated Sludge

Activated sludge (50 mL) was transferred into conical flasks. The flasks were placed in a water bath at 87°C for 8 min to kill all nonspore-forming cells *(9)*, leaving the surviving *Bacillus* spores that are heat resistant. The sludge sample was centrifuged at 2610*g* for 10 min. The supernatant was discarded, and the pellet was resuspended with 100 mL of sterile medium A and incubated for 18 h at 37°C with 250 rpm shaking to enrich the cell population. The culture from medium A (25 mL) was centrifuged at 2610*g* for 10 min. The supernatant was discarded. The pellet was resuspended in 100 mL of medium B with 0.1 mL of trace elements solution. The culture was incubated at 37°C for 16 h with 250 rpm shaking for PHB accumulation.

Serial dilutions of 10^{-1}–10^{-7} of cell culture from medium B were prepared (the culture was diluted with 0.9% NaCl solution). The culture from each dilution was streaked on production agar (medium B with 20 g/L of glucose, 1 mL/L of trace elements solution, and 1.5% agar) plates. The

plates were incubated for 16 h at 37°C. The cells from each single colony were subjected to Fourier transform infrared (FTIR) analysis.

The selected PHA-producing bacteria were cultivated and subjected to Gram staining, endospore staining, and microscopic morphology examination to select the potential PHA-producing *Bacillus* strains.

Fermentation

Glycerol stocks of all bacterial cells were used as inoculum and were prepared by cell culture with a final glycerol concentration of 15% (v/v) and stored at –80°C. It was first inoculated into 5 mL of 2 XYT medium (1.6 g of tryptone, 1.0 g of yeast extract, and 0.5 g of NaCl in 100 mL of distilled water) in universal bottles. A 1% inoculum was used in flask fermentations. Cultures were grown on a rotary shaker at 250 rpm at 37°C.

Batch fermentation was carried out in the computer-controlled Bioengineering 3.7-L fermentor (Bioengineering, Switzerland) with growth conditions set at 37°C and pH 7.0. The pH was adjusted by adding 2 *M* HCl and 2 *M* NaOH. One hundred milliliters of seed culture of the target strain (4% of fermentation medium) was inoculated into 2.5 L of malt waste medium.

Extraction of Biopolymers

After fermentation, the culture was centrifuged at 14,333*g* for 25 min at 4°C, washed with distilled water, and freeze-dried. One gram of the freeze-dried cell powder was treated with a dispersion containing 15 mL each of chloroform and 30% NaOCl solution.

The mixture was incubated at 37°C with 250 rpm agitation for 1 h, and then centrifuged at 2610*g* for 15 min, which resulted in three phases. The upper phase was a hypochlorite solution, the middle phase contained the non-PHB cell material and undisrupted cells, and the bottom phase was chloroform-containing PHB.

The bottom chloroform layer was filtered and allowed to concentrate by evaporation to a final volume of 5 mL. Pure PHB was obtained by nonsolvent precipitation (chloroform:methanol at a ratio of 1:9). Finally, the white precipitate was dried and weighed.

Isolation of PHB Inclusion Bodies

The culture of *B. megaterium* 11561 and the HF-1 after fermentation were pelleted at 6000*g* in 4°C for 20 min and resuspended in 5 mL of 10 m*M* Tris-Cl, pH 8.0; 1 m*M* EDTA; 20 m*M* $MgSO_4$; and 0.25 *M* sucrose at 4°C. Lysozyme was added to a final concentration of 1.5 mg/mL and the solution was incubated at 37°C for 15 min and room temperature for 10 min. The cells were broken by sonication for 10 min.

Aliquots of 1 mL of lysate were loaded on sucrose step gradients in 5-mL ultracentrifuge tubes and consisted of 0.9 mL of each of the following sucrose concentrations: 2.0 *M*, 1.66 *M*, 1.33 *M*, 1.0 *M*, and 0.4 mL of 0.66 *M*

in TE (10 m*M*, Tris-Cl, pH 8.0; 1 m*M* EDTA). The tubes were centrifuged at 150,000*g* for 1 h at 10°C. The inclusion bodies, which banded about midtube, were collected, washed in 20 vol of TE, and pelleted at 20,000*g*. The sucrose gradient steps were repeated for further purification, and the purified inclusion bodies were stored in TE buffer at 4°C.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

Resuspended the pelleted inclusion bodies by TE with 2% sodium dodecyl sulfate (SDS). An equal volume of 2X sample buffer was added prior to boiling for 5 min, samples were centrifuged for 3 min to pellet the PHA, and the supernatant was loaded on an SDS 12% polyacrylamide gel. Coomassie Brilliant Blue R-250 staining and silver staining (Bio-Rad) were used after the gel electrophoresis for analysis.

DNA Sequencing

The DNA fragment from polymerase chain reaction (PCR) of HF-1 was sequenced from both ends, using designed primers based on the sequence of *pha* gene cluster from *B. megaterium*, following the protocol of an ABI Prism DNA Sequencing Kit (Perkin-Elmer). By using the dye-terminator chemistry, cycle sequencing, and an ABI Prism 310 sequencer (Perkin-Elmer). Sequence assembly was performed by using the software Advanced BLAST (National Center for Biotechnology Information).

Analytical Methods

TOC Analysis

Fermentation medium was analyzed with an Astro 2000 TOC Analyzer. The method was according to APHA (4500-Norg) *(8)*.

TKN Analysis

Fermentation medium was analyzed with a Kjeltec Auto 1030 Analyzer. The method was according to APHA (5310C) *(8)*.

Gas Chromatography Analysis

One milliliter of esterification solution (3 mL of 95–98% H_2SO_4, 0.29 g of benzoate, and 97 mL of methanol), freeze-dried cells, and 1 mL of chloroform were heated at 100°C for 4 h; and distilled water was added and vortexed to enhance phase separation. One milliliter of distilled water was added to the cooled mixture, which was vortexed for phase separation. A 1-µL portion of the lower organic phase was subjected to gas chromatography (GC) analysis. GC analysis was performed on a 5890 Series II Gas Chromatograph (Hewlett Packard), using an Ultra 2 (crosslinked 5% Ph Me silicone) Capillary Column 0.2 mm in diameter and 25 m long (Hewlett Packard).

Nitrogen was chosen as the carrier gas. Analysis was started at 70°C for 3 min and was increased to 120°C at a rate of 10°C/min. After reaching 120°C, the temperature was kept stable for 15 min to remove all nonvolatile component.

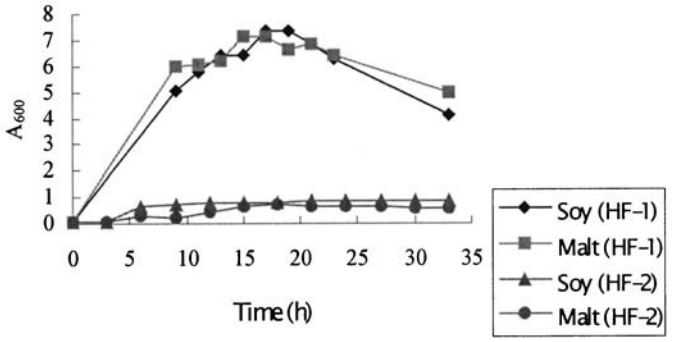

Fig. 1. Cell growth of HF-1 and HF-2 during flask fermentation in malt and soy wastes.

Analysis by FTIR Spectroscopy

Two to five milliliters of the cell culture was centrifuged at 2610*g* for 15 min. An appropriate amount of cells was transferred on an IR window (ZnSe Disc; Spectratech) and dried on it. A mirror was used to give the reflected IR to the horizontal laid window. With a scan of 32, resolution of 16, and autogain, spectra were recorded at wave numbers (cm^{-1}) from 400 to 4000 using a Mangna-IR spectrometer 750 (Nicolet).

Analysis by ^{1}H Nuclear Magnetic Resonance

^{1}H nuclear magnetic resonance (NMR) analysis was carried out on a DPX-400 Spectrometer (Bruker). ^{1}H NMR spectra was recorded at room temperature from a deuteriated chloroform ($CDCl_3$) solution of the extracted biopolymers. The 400 MHz ^{1}H NMR spectra were recorded.

Results and Discussion

Screening PHA-Producing Strains from Activated Sludge

In the first part of the experiment, two microbial strains (HF-1 and HF-2) were successfully screened from the activated sludge. The potential of the strains for the conversion of food wastes into bioplastic was investigated. The data of cell growth during the fermentation in malt and soy wastes are shown in Fig. 1. The OD_{600} of HF-2 was maintained at a low level throughout the fermentation in both malt waste and soy waste as media when compared with strain HF-1. This might be because the composition of hydrolyzed food wastes was not suitable for the growth of strain HF-2.

Comparison of the yield of PHB produced by the two strains from the results of the flask experiment shows that HF-1 accumulated the higher amount of PHB recovery (19.22% of its cell dry wt) than HF-2 (10.18% of its cell dry wt). Thus, further investigation was mainly concentrated on strain HF-1.

During fermentation, the PHB content of the HF-1 cells was monitored by FTIR analysis, and the yield of PHB was estimated by the height ratio of

Table 1
Height Ratio of PHB Peak
to Protein Peak on FTIR Absorbance Spectra

Time (h)	Malt waste	Soy waste
9	0.676	0.641
11	0.720	0.693
13	0.738	0.606
15	0.740	0.488
17	0.720	0.420
19	0.700	0.286
21	0.556	0.321
23	0.556	0.216
33	0.520	0.047

The highest PHB accumulation in flask fermentation occurred at 15 h when malt waste was used as the medium, but at 11 h when soy waste was used. The spectra had a characteristic sharp PHA absorption band at a narrow range around 1726–1740 cm^{-1}, and the pattern is similar to that of previous studies *(10,11)*. This sharp absorption band is assigned to the stretching vibration for the ester carbonyl of the PHB.

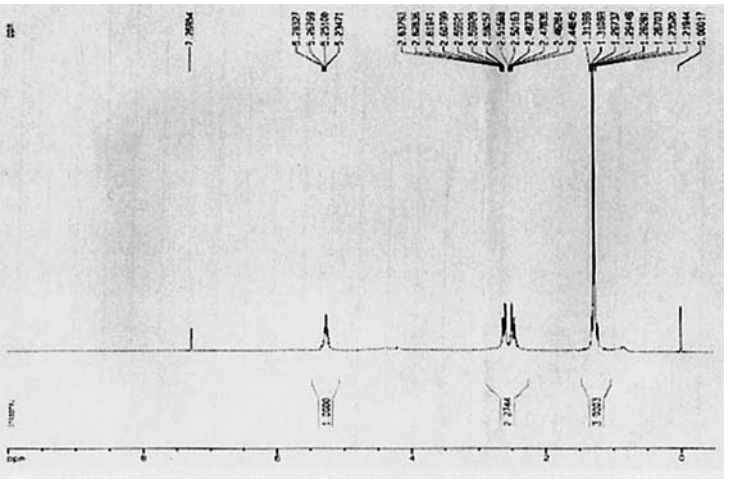

Fig. 2. NMR spectra of extracted biopolymer of HF-1.

PHB to protein, as shown in the absorbance spectra of FTIR and as listed in Table 1.

Data from ^{1}H NMR analysis of the extracted biopolymer produced by fermentation of HF-1 is displayed in Fig. 2.

The ^{1}H-NMR spectrum showed the presence of three groups of characteristic signals of the homopolymer PHB. The signal at about 1.26 ppm

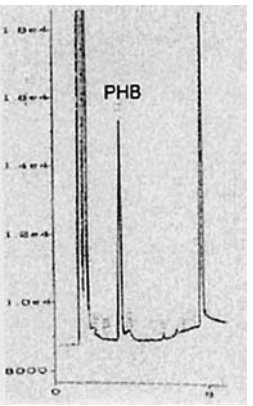

Fig. 3. GC spectrum of the HF-1 cells after fermentation.

was attributed to the methyl group coupled to one proton, the signal at about 2.52 ppm was attributed to a methylene group, and a multiplet at 5.25 ppm was attributed to a methyne group. The signal at about 7.27 ppm was owing to the chloroform. The spectra showed the CH–, CH_2– and CH_3– groups in the molecule, but no –COOH and –OH groups; thus, so the compound cannot be β-hydroxybutyrate and should be a polymer.

Identification of Screened Strain

The isolated strains were identified by Microbial ID. Identification was based on the fatty acids composition profiles of the bacteria and comparison with the profiles of the other bacteria in their library and expression of similarity by Similarity Index of how closely they matched the other known bacteria. Two strains that are the most similar to HF-1 are *Brevibacillus laterosporus* (0.745) and *Bacillus megaterium* (0.736).

Data from the GC analysis from freeze-dried cells of HF-1 after fermentation are shown in Fig. 3. The PHB peak was present at a retention time of 3.363 min but there was no PHV peak when compared to the spectra of the standard.

There were two choices of strains provided by Microbial ID; therefore further investigation was made. The DNA fragment after the PCR reaction was subjected to DNA sequencing in both forward and backward primers. The percentage identity was 92 and 94%, respectively, to those sequences of *B. megaterium*. This suggests that HF-1 was quite similar to *B. megaterium* 11561. The slightly unequal DNA sequencing might be owing to the sequencing error or the difference in subspecies strain.

PHA inclusion body–associated proteins were also analyzed by SDS-polyacrylamide gel electrophoresis to identify similarities between the strain HF-1 and *B. megaterium* (Fig. 4). Proteins that associated with PHA inclusion bodies were separated. There were at least 20 such proteins present in various quantities. The two most abundant proteins had

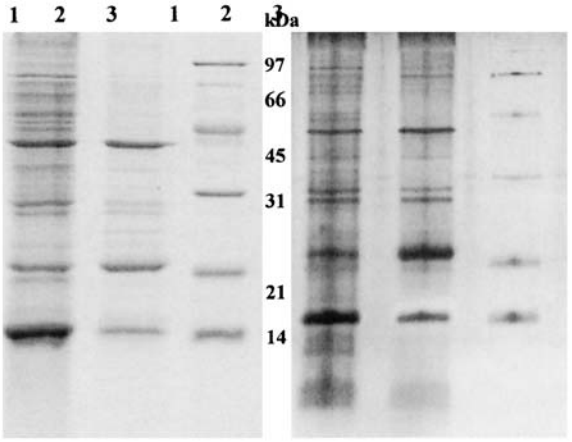

Fig. 4. PHA inclusion body–associated proteins. **(Left)** Coomassie blue staining; **(right)** silver staining. Lane 1, proteins from inclusion bodies of *B. megaterium* 11561; lane 2, proteins from inclusion bodies of HF-1; lane 3, molecular markers (14,400, 21,500, 31,000, 45,000, 66,200, 97,400 Daltons).

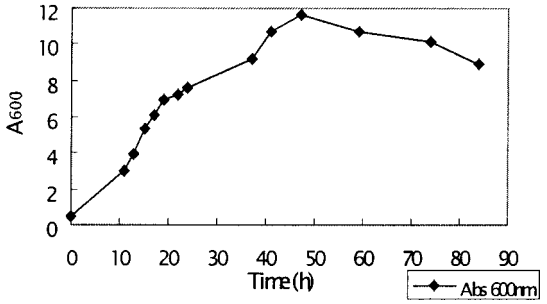

Fig. 5. Cell growth of HF-1 during fermentor fermentation in malt wastes.

molecular weights of approx 20 and 41 kDa and were found in both strains. The 14-kDa protein was lysozyme. The pattern of the visualized proteins bands after Coomassie blue and silver staining was similar in both strains excepts two bands. Thus, it could be suggested that HF-1 is closely related to *B. megaterium*.

Production of PHB from Malt Waste Using HF-1 in a 3-L Fermentor

The growth of HF-1 during fermentation is presented graphically in Fig. 5. According to the height ratio of PHB band to protein band on FTIR absorbance spectra (Table 2), the optimum PHB production time of HF-1 in fermentor fermentation with malt waste as medium was 15 h. The time was the same as for the flask experiment (Fig. 1), but the A_{600} was much higher. At 24 h, the PHB content had dropped dramatically owing to the consumption of the PHB by HF-1.

Table 2
Height Ratio of PHB Peak
to Protein Peak
on FTIR Absorbance Spectra

Time (h)	Malt waste
11	0.611
13	0.732
15	0.879
17	0.813
19	0.750
22	0.712
24	0.579

Conclusion

Two PHA-producing bacteria were successfully screened from municipal activated sludge. The results of the fermentation showed that the bacteria HF-1 well utilized food wastes for nutrients to produce biopolymer. The use of inexpensive carbon substrates to produce bioplastics would be beneficial in lowering the cost of PHA production. The potential of the screened bacteria on microbial biopolymer production was subjected to fermentor fermentation. The results showed that only PHB homopolymer could be produced. The biopolymer produced was characterized by FTIR, GC, NMR, and genetic analysis.

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