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Microbial Synthesis and Characterization of Physiochemical Properties of Polyhydroxyalkanoates (PHAs) Produced by Bacteria Isolated from Activated Sludge Obtained from the Municipal Wastewater Works in Hong Kong

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Abstract

The first objective of this study was the measurement of physical properties of P(3HB-co-3HV) copolymers with different (hydroxybutyrate) HB to (hydroxyvalerate) HV ratios produced by *Bacillus cereus* (TRY2) isolated from activated sludge. The 3HV PHBV copolymers were 0.05, 22.6, 39.2, 54.1, and 69.1 mol%, respectively. The second objective was to study possible wastewater treatment and production of PHAs at the same time by *B. cereus* (TRY2) and *Pseudomonas* spp. (TOB17) (both were isolated from activated sludge), recombinant *Bacillus DH5α*, and a combination of the above three bacteria. The results were satisfactory; the maximum COD and TOC of the sewage sludge reduced were 53.5% and 67.5%, respectively.

Index Entries: Activated sludge; *Bacillus cereus*; PHA; *Pseudomonas* spp.; wastewater treatment.

Introduction

Polyhydroxyalkanotes (PHAs) have been studied and investigated for hundreds of years. Compare to the production by pure culture, PHA

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production by activated sludge from wastewater treatment plants requires less expensive substrates (raw materials present in the influent) and simpler processes (no sterilization of reactor and less process control) thus allowing for significantly reduced operating costs. Therefore, PHA production by activated sludge is an attractive alternative to the use of pure cultures. The thermal and physical properties of PHAs have been studied for more than 20 yr. Studies report *(1,2)* that PHB properties (e.g., thermoplastic process ability and water resistance) are similar to thermal plastics; however, brittleness, low extension to break, and lack of flexibility limit PHB application. To overcome these problems, the physical and thermal properties of PHA copolymer [especially polyhydroxybutyrate-valerate (PHBV)] were widely studied *(3)*. Byrom *(4)* reports that the copolymer P(3HB-co-3HV) had two major advantages over the polyhydroxybutyrate (PHB) biopolymer, i.e., the melting point and the level of crystallinity of P(3HB-co-3HV) was lower than that of PHB. Moreover, copolymer of PHBV with higher HV% tends to be softer and tougher and materials with lower HV% tend to be harder and more brittle.

Materials and Methods

Thermogravimetric Analysis

Thermogravimetric analysis (TgA) was performed with a Perkin-Elmer TG7 thermogravimetry analyzer. PHA sample (5–15 mg) was weighed and transferred to the sample pan of the TG7 analyzer. The starting temperature was 30°C and the sample was heated from 30°C to 400°C at the rate of 10°C per min.

Differential Scanning Calorimetry Analysis

The thermal behavior of PHA samples by differential scanning calorimetry (DSC) analysis was performed was a Perkin Elmer 1020 Series DSC7 Thermal Analysis System (Perkin Elmer, Germany). PHA sample (5–10 mg) was weighed and packed evenly on the aluminium pan and then transferred to the DSC analyzer. The sample was heated from −50°C to 200°C at 10°C per min for melting and crystallization temperature measurement. The glass transition temperature (T_g) was measured by heating the sample from −50°C to 200°C at 10°C per min.

Water Resistance Testing

The PHA film sample was placed inside an oven at 50°C for 24 h to remove water from the PHA film sample, and weighed. Then it was soaked in water at 25°C and 60°C for 24 h. It was re-weighed to determine the degree of water absorption. Then it was put inside the oven at 50°C for 24 h for complete evaporation of water and was weighed again to measure the degree of material deformation.

Tensile Testing

The tensile strength measurement was performed by the Texture Analyzer TA500. The thickness of the PHA film was measured with a micrometer. The measurement was started by setting the pulling speed of the texture analyzer to 5 mm/min and was stopped just after the PHA film failed.

PHA Production Potential in Activated Sludge by Shake-Flask Studies

Bacillus cereus TRY2 and *Pseudomonas spp.* (both were isolated from activated sludge of Tai Po wastewater treatment plant, HK), recombinant *Bacillus DH5*α and mixed culture of the above three bacteria were also used in the shake-flask experiments. Four different cultural media were used in the shake-flask comparison experiments. The first one was complex growth medium that included glucose (2 g/L), peptone (2 g/L), yeast extract (2 g/L), sodium acetate (4 g/L), potassium dihydrogen phosphate (0.44 g/L), hydrated magnesium sulfate (0.5 g/L), ammonium sulfate (0.5 g/L), and filter-sterilized vitamin solution (10 mL/L), which consists of biotin (0.08 g/L), inositol (5 g/L), thiamine (5 g/L), calcium pantothenate (2 g/L), and pyridoxine hydrochloride (2.25 g/L). The second medium was synthetic wastewater that was prepared according to *(5)*. The third and fourth media were actual activated sludge collected from the Tai Po and Yuen Long wastewater treatment works, HK, respectively, together with hydrated magnesium sulfate (89 mg/L) and dipotassium hydrogen phosphate (60 mg/L). Cultures were incubated at 30°C, 250 rpm, pH 7.0–7.2 and 24 h, respectively.

Gas Chromatography Analysis

The gas chromatographic (GC) analysis has been developed for measuring the composition and content of PHA *(6)*. The content of PHA polymers can be calculated according to Guo-Cheng et al. Samples for gas chromatography were prepared according to Braunegg et al. *(6)*. The polymer analysis was performed on a Hewlett Packard 5890A Gas Chromatograph using a 25-m Ultra 2 (cross-linked 5% Ph. Me. Silicone) capillary column with a 0.33-µm-thick film. The working temperatures of the column, injector, and flame-ionization detector were 135°C, 260°C, and 300°C, respectively. The carrier gas was nitrogen and the flow rate was set at 20 mL/min.

Results and Discussion

Figure 1 shows that both the onset temperature (T_o), peak temperature (T_p), and final temperature (T_F) of first four different compositions of 3HV PHBV copolymer (0.05% to 54.1%) decreased gradually. The higher 3HV contents of PHBV copolymers decomposed much easier than those

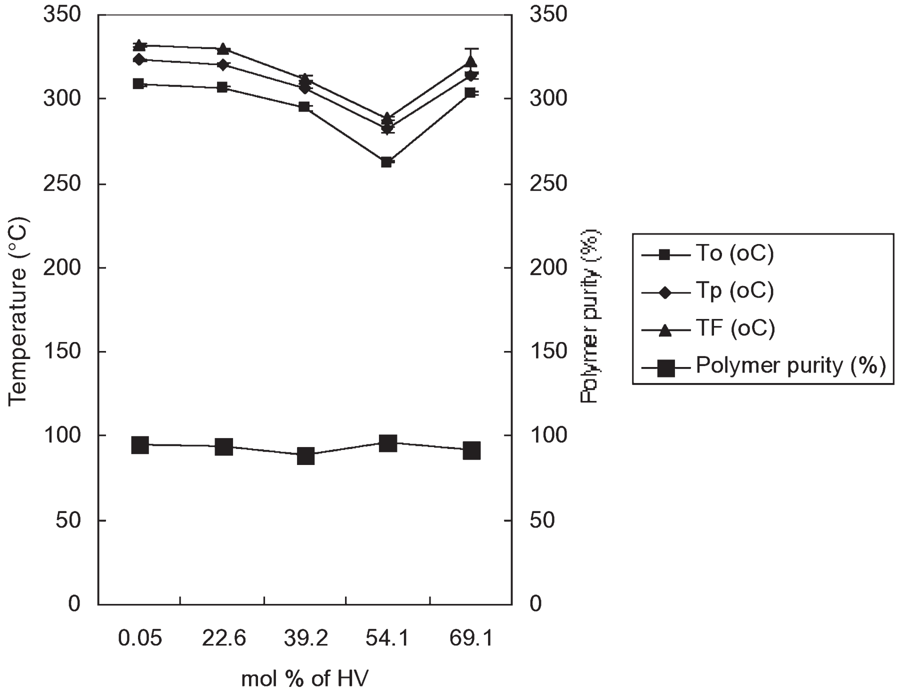

Fig. 1. Summary of thermogravimetric analysis (TgA) of five PHBV copolymers with different HV fraction (%).

with a lower HV content because the bulkiness (due to more side chains) of the PHBV with higher 3HV contents makes the polymers much easier to break. Nevertheless, when the content of 3HV PHBV copolymer increased from 54.1 to 69.1 mol%, all the results reversed. That meant higher contents of 3HV PHBV copolymers decomposed much easier. The thermal properties were less stable than PHBV copolymers with low HV contents except when the content of 3HV reached a high level, such as 69.1%. In this case, the trend is reversed. In differential scanning calorimetry (DSC) analysis, glass transition temperature (T_g) and melting temperature (T_m) were recorded with different PHBV copolymers having 3HV contents of 0.05, 22.6, 39.2, 54.1, and 69.1 mol%. As seen in Fig. 2, only the PHBV copolymer with 0.05 mol% 3HV had a positive glass transition temperature. The lowest working temperature of PHBV copolymers produced by *B. cereus* TRY2 with higher than 20 mol% of 3HV is under 0°C. According to Fig. 3, water resistance was better when the PHBV copolymer films were immersed in water at room temperature (25–30°C) than water at 60°C. One possible reason is that higher temperatures increase the rate of water diffusion. Referring to Fig. 4, the degree of soluble matter loss (%) was higher when the PHBV copolymer films were immersed in water at 60°C than in water at room temperature (25 to 30°C). The same explanation could be speculated.

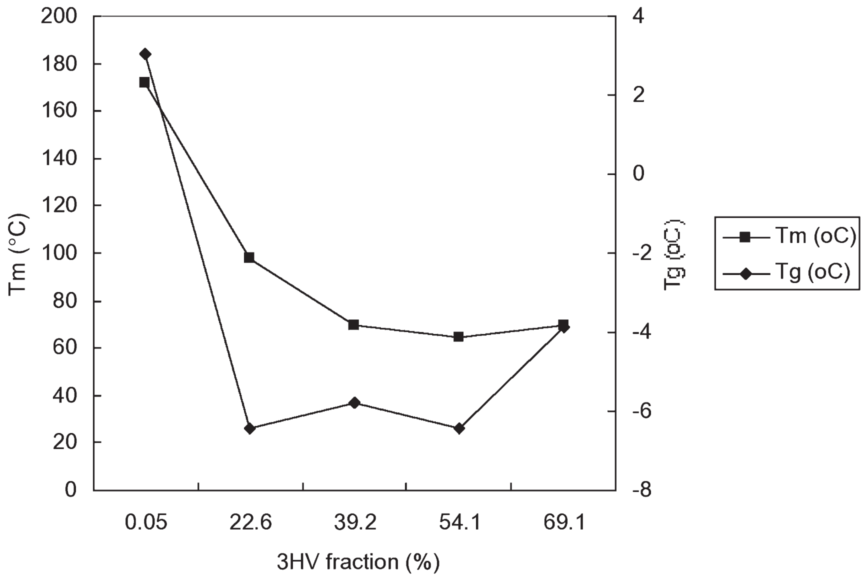

Fig. 2. Summary of differential scanning calorimetry (DSC) analysis of five different PHBV copolymers with different 3HV fraction.

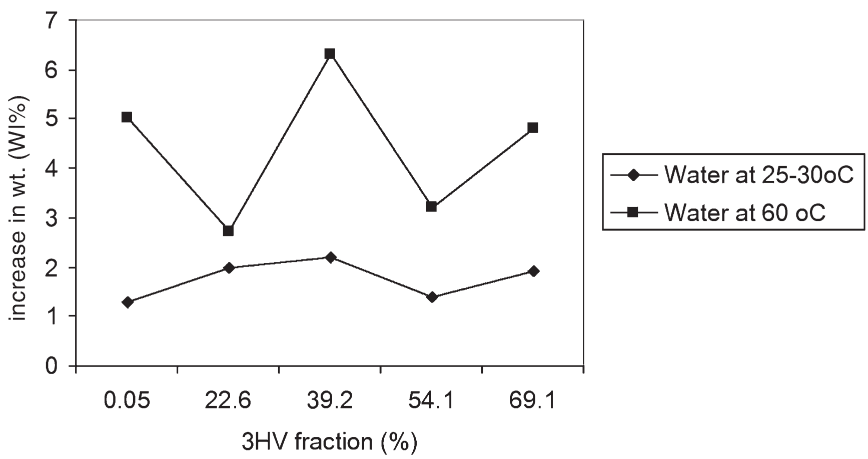

Fig. 3. Summary of increase in weight (%WI) of five different PHBV copolymer samples with different 3HV fraction (%) after immersion in water at different temperature (°C). % WI = percentage increase in weight of tested sample during immersion of water (degree of water resistance of the material tested).

The final physical property analysis of PHBV copolymers with different contents of 3HV was tensile strength and modulus analysis. According to Fig. 5, the tensile strength decreased from 5.19 N/mm^2 to 1.80 N/mm^2 gradually with increasing the content of 3HV in PHBV copolymer films from 0.05 to 69.1 mol%. The lower contents of 3HV in PHBV copolymers were more rigid and dense, hence more force was required to break or

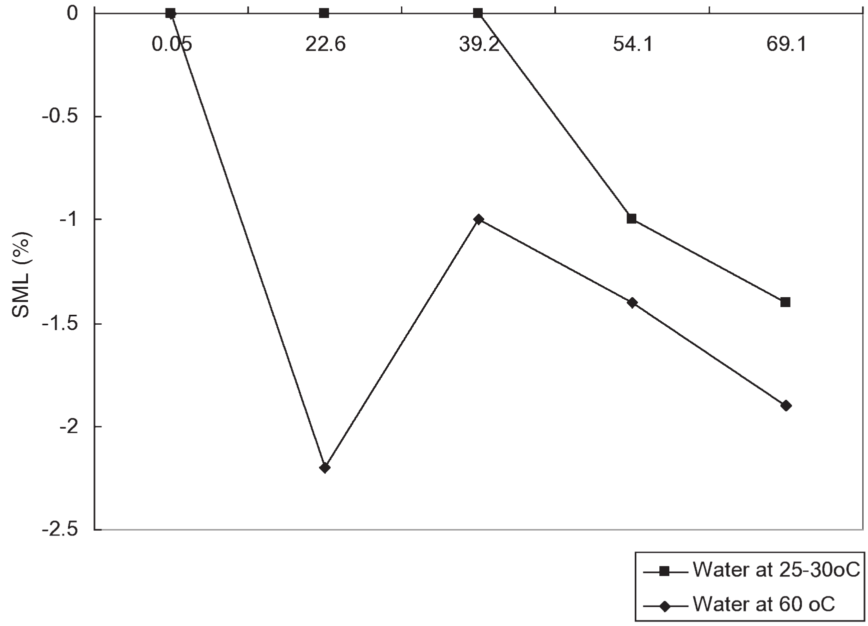

Fig. 4. Summary of soluble matter loss (% SML) of five different PHBV copolymer samples with different HV fraction (%) after immersion in water at different temperature (°C). % SML = percentage of soluble matter loss of tested sample during immersion of water (an indicator of the tested material deformation during immersion of water).

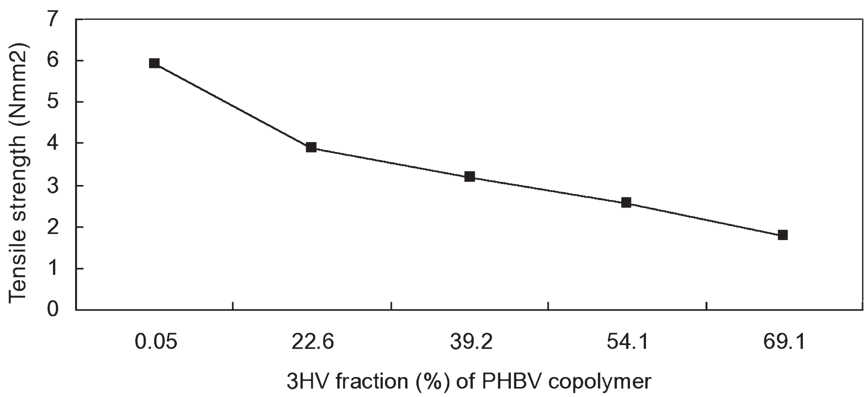

Fig. 5. Tensile strength (N/mm^2) analysis of PHBV copolymer samples with different HV fraction (%).

deform the polymers, meaning a higher tensile strength resulted. Regarding Fig. 6, the pattern was similar to that of tensile strength. Normally, plastics with higher initial modulus resist deformation but are brittle and tough, whereas those with lower initial modulus were easily deformed and more flexible. It might be concluded that PHBV copolymers with lower contents of 3HV were brittle and deformation was resisted and vice versa.

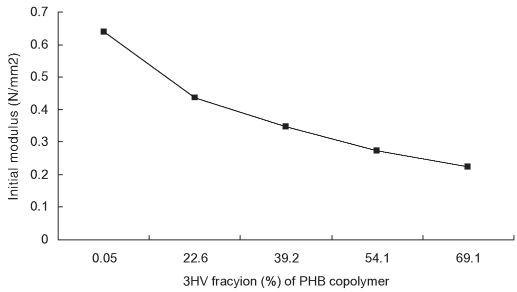

Fig. 6. Initial modulus (N/mm^2) analysis of PHBV copolymer samples with different HV fraction (%).

Table 1
Summary of Potential Wastewater Treatment and PHA Production by Three Different Bacterial Strains (and a Mix of Them) Growth in Different Medium

		Cultural medium			
Bacterial strain employed	Measurement	Complex medium	Synthetic medium	Yuen Long activated sludge	Tai Po activated sludge
Pseudomonas spp. TOB17	DW (g/L)	1.56	0.29	5.25	5.07
	PHA (mg/L)	149	5.2	12	54
	Reduced COD (%)	23.7	52.2	29.5	38.8
	Reduced TOC (%)	23.1	42.5	44.3	52.0
Recombinant *Bacillus DH5α*	DW (g/L)	2.70	0.17	5.13	3.45
	PHA (mg/L)	1123	0	4.1	11
	Reduced COD (%)	35.5	29.5	53.5	40.1
	Reduced TOC (%)	34.4	36.6	53.0	61.8
B. cereus TRY2	DW (g/L)	1.16	0.16	4.14	4.11
	PHA (mg/L)	206	0.6	4.1	10
	Reduced COD (%)	12.6	59.9	49.2	49.3
	Reduced TOC (%)	16.0	45.7	65.8	67.5
Mix of above three bacterial strains	DW (g/L)	1.30	0.14	4.92	3.84
	PHA (mg/L)	148	0.5	6.9	22
	Reduced COD (%)	16.7	17.1	17.3	15.3
	Reduced TOC (%)	27.1	44.1	53.2	58.5

Referring to Table 1, *Pseudomonas* spp. TOB17 produced more PHA when cultured with complex media because of the nutrient-rich chemical composition. The second observation was that the amount of PHA produced by bacterium cultured with Tai Po activated sludge was higher than that of Yuen Long activated sludge and synthetic wastewater. This might be because *Pseudomonas* spp. TOB17 was isolated from Tai PO activated sludge, which was the bacterium's original living environment to which it was better adaptated. Although the chemical composition of synthetic wastewater was similar to that of activated sludge, the biotic environment such as competition of nutrients between microorganisms and interdependent relationship between microorganisms was totally different from that of activated sludge, causing the poor growth and PHA production. According to the Table 1, the COD and TOC reduction (%) by *Pseudomonas* spp. TOB17 were lowest when the bacterium was cultured with complex media. Because it was a nutrient-rich medium with great excess of bacterial needs, the 24-h cultivation was short enough for nutrient utilization by the bacterium. According to Table 1, the concentration of PHA produced by another isolated bacterium *B. cereus* was higher in complex medium than the others and it was due to the same reason as described above. Moreover, *B. cereus* TRY2 was isolated from Tai Po activated sludge and had a better environment adaptation for growth and PHA production when cultured with Tai Po activated sludge than Yuen Long activated sludge. According to Table 1, it could easily be observed that when *B. cereus* TRY2 was cultured in complex medium, it had the lowest COD and TOC reduction (less than 20%). The reason is the same as explained when discussing the behavior of *Pseudomonas* spp. TOB17. When using recombinant *Bacillus DH5α* for investigation, the concentration of PHA produced was similar to the preliminary experiment, i.e., recombinant bacterium could produce much higher PHA than the isolated natural PHA makers. Nonetheless, the results of PHA production by recombinant *Bacillus DH5α* with synthetic wastewater, Yuen Long and Tai Po activated sludge were surprisingly similar to the results collected from *B. cereus* TRY2. It might be concluded that the nutrient content and living environment of activated sludge were not suitable for recombinant bacteria to grow and synthesize PHA. The results also showed that both the COD and TOC reduction of complex medium fermented with recombinant *Bacillus DH5α* were not as low as that fermented by *Pseudomonas* spp. TOB17 and *B. cereus* TRY2. One of the causes for high cell density and PHA production by the recombinant bacteria was the high efficiency of nutrient utilization, which accounts for the relatively high COD and TOC reduction. The objective for culturing *Pseudomonas* spp. TOB17, recombinant *Bacillus DH5α*, and *B. cereus* TRY2 together in the fermentation media was to increase the concentration of PHA. According to Table 1, the concentration of PHA and the COD and the TOC reductions were low. None of them produced more than 200 mg/L PHA. Moreover, none of the COD reductions could reach 20% in any media fermented with mixed bacteria. This might be because the inoculum employed for the mixed bacteria fermentation was higher than that of singe bacterium fermentation. Thus, the amount of oxygen required to degrade the organic compounds was large throughout the experiment causing a low COD reduction.

Conclusion

Different PHBV copolymers were subjected to physical and thermal properties analysis of thermogravimetric analysis, differential scanning calorimetry, water resistance testing, and tensile testing. After examining five different contents of 3HV PHBV copolymers by the above four analyses, the decomposition temperatures of T_o, T_p, and T_F (°C), polymer purity (%), glass transition temperature (°C), melting temperature (°C), degree of water resistance (%), amount of soluble matter loss (%), tensile strength (N/mm^2), and initial modulus (N/mm^2) were measured. The results showed that the maximum reduction of COD and TOC in the sewage sludge was 53.5% and 67.5%, respectively. The results of this project should encourage the government to solve part of the solid waste production and accumulation problem by bacterial fermentation of sewage sludge in wastewater treatment plants.

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