0273-2289/99/77–79/0389/$12.75

Accumulation of Biopolymers in Activated Sludge Biomass

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Abstract

In this study, activated sludge bacteria from a conventional wastewater treatment process were induced to accumulate polyhydroxyalkanoates (PHAs) under different carbon-nitrogen (C:N) ratios. As the C:N ratio increased from 20 to 140, specific polymer yield increased to a maximum of 0.38 g of polymer/g of dry cell mass while specific growth yield decreased. The highest overall polymer production yield of 0.11 g of polymer/g of carbonaceous substrate consumed was achieved using a C:N ratio of 100. Moreover, the composition of polymer accumulated was dependent on the valeric acid content in the feed. Copolymer poly(3-hydroxybutyrate-co-3-hydroxyvalerate) [P(3HB-co-3HV)] was produced in the presence of valeric acid. The 3-hydroxyvalerate (3HV) mole fraction in the copolymer was linearly related to valeric content in the feed, which reached a maximum of 54% when valeric acid was used as sole carbon source. When the 3HV U in the polymer increased from 0–54 mol%, the melting temperature decreased from 178° to 99°C. Thus, the composition, and hence the mechanical properties, of the copolymer produced from activated sludge can be controlled by adjusting the mole fraction of valeric acid in the feed medium.

Index Entries: Activated sludge; carbon-nitrogen ratio; wastewater treatment; butyric-valeric acid ratio; poly(3-hydroxybutyrate-co-3-hydroxyvalerate).

Introduction

A wide variety of industrial and consumer plastic products have been increasingly regarded as a source of solid waste-management problems because of their nondegradable properties. In Hong Kong, 14 weight percents of the 10,000 tons of municipal solid wastes produced each day are plastic packaging materials and disposable products. Although plastics

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usage and plastics-waste generation is forecast to increase at 15%/yr over the next decade *(1,2)*, there has been considerable interest in developing environmentally-friendly materials to substitute for conventional plastics *(3,4)*. Polyhydroxyalkanoates (PHAs) have been recognized as better candidates for biodegradable plastics owing to their similar material properties to conventional plastics and their complete biodegradability. Approximately 300 different isolated microorganisms synthesize and accumulate PHAs as carbon and energy storage materials under the condition of limiting nutrients in the presence of excess carbon source. Among the various members of PHAs, only three kinds of PHAs, poly(3-hydroxybutyrate) [P(3HB)], poly(3-hydroxybutyrate-co-3-hydroxyvalerate) [P(3HB-Co-3HV)], and poly(3-hydroxyhexanoate-co-3-hydroxyoctanoate) [P(3HHx-co-3HO)], have been produced to a relatively high concentration with high productivity *(5)*.

Although, PHAs have advantages over the conventional plastics, widespread application of PHAs is hampered by high production cost. The current cost of PHAs is ~10 times higher than that of conventional plastics *(3)*. Substantial reduction in production cost is necessary before widespread applications in packaging and disposable products are possible. In the last several years, fermentation strategies for the production of PHAs with high productivity have been relatively well developed to bring down the overall cost. Recently, much effort has been devoted to isolating bacterial strains that can produce PHAs from cheap carbon sources.

Recently, many recombinant strains for economical production of PHA have been developed by cloning the PHA synthase genes from many microorganisms, including *Alcaligenes eutrophus*, *Ectothiorhodospira shaposhnikovii*, *Pseudomonas aeruginosa*, and *Pseudomonas mendocina (5)*. Recombinant *Escherichia coli* has been studied most extensively. The accumulation of P(3HB) in recombinant *Escherichia coli* could reach 80–90% of dry cell weight at the end of cultivation. However, the problem of the high oxygen demand during the high cell-density culture of recombinant *E. coli* needs to be solved to allow this process to be an economical alternative.

Owing to high production costs, a number of researchers have investigated the use of cheap carbon sources as fermentation substrates. Methanol is one of the cheap carbon sources available. However, it was demonstrated that a long time was required for a methylotroph to produce large amounts of P(3HB), resulting in low productivity *(6)*. Moreover, it was suggested that the high production cost for P(3HB) could be significantly decreased by using photosynthetic bacteria with hydrogen and carbon monoxide as inexpensive fermentation substrates *(7)*. However, high capital costs resulted owing to safety problems in using a flammable gas substrate. Many researchers have demonstrated that high PHA production cost can be possibly lowered by using unpurified, low-cost organic wastes from agriculture and food processing. *Azotobacter vinelandii* UWD, a new bacterial strain for PHA production, was reported to produce PHA without nutrient limitation and appears to be the best strain for PHA production from unpurified substrate *(8–10)*.

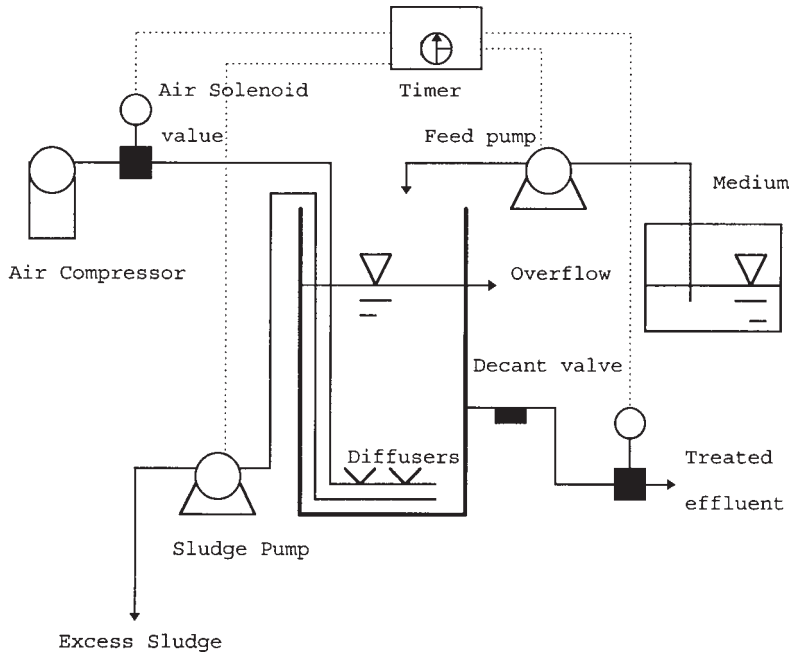

Fig. 1. A schematic diagram of a biological reactor.

To produce PHAs on a commercial scale, the cost of microbial growth substrates becomes a critical factor. Use of inexpensive biomass materials such as sludge bacteria would improve the economics of producing PHAs. In the studies by Chua et al. *(11,12)*, activated sludge bacteria in laboratory-scale wastewater treatment systems were induced, by controlling the carbon-nitrogen (C:N) ratio in the reactor liquor, to accumulate PHA-related storage polymers as a low-cost source of biodegradable plastics.

This paper describes a cost-effective technique that induces activated sludge bacteria in a conventional wastewater treatment process to produce PHAs. The thermal property of PHAs was also studied.

Materials and Methods

Production of Polymer Through Wastewater Treatment

Activated sludge was collected from municipal sewage-treatment plants. The activated sludge was cultivated in a laboratory-scale sequential batch reactor (SBR) of 12-L effective volume (Fig. 1). The wastewater was of a typical total organic carbon (TOC) of 2,500 mg/L, comprising of ketones and branched-chain carboxylic acids. The wastewater was supplemented with NH_4Cl at 0.48 g/L to result in a C:N mass ratio of 20, which is widely accepted as required for normal bacterial synthesis in activated sludge processes.

The reactor was operated on a fill-and-draw cycle with a 2-h reaction time, a react-to-contact time ratio of 0.57, a batch-loading rate of 0.25 mg

COD/mg MLVSS-d, and an average organic reduction efficiency of 98.3%. During each cycle, the pumps for feeding, removing, and aeration were controlled by a programmable timer. When the reactor was operating under stable conditions, the nitrogen concentration in the wastewater was reduced to result in C:N ratio of 40, 60, 80, 100, 120, and 140, creating different degrees of nutrient deficiency. The SBR was stabilized at C:N ratio of 20 in between operations under each of the higher C:N ratios. Activated sludge samples of each C:N ratio were periodically taken from the reactor during the 2-h reaction time in a randomly selected operation cycle for analysis.

Variation of Copolymer Composition

Control of polymer composition was studied by carrying out six cycles of batch culture. Activated sludge was first cultivated with a wastewater of 1200 mg/L in terms of TOC in the SBR operated with 1.5-d hydraulic retention time (HRT) and 2-h aeration period. In each batch culture, 7–8 g polymer-free activated sludge were harvested and washed with distilled water to remove any residual nitrogenous matters and inoculated into an automatic jar fermenter of 3-L working volume (Bioengineering Model ALF, Ruti/Switzerland). The fermenter was fed with a nitrogen-free medium and operated at 300 rpm and 30°C for 48 h. The pH was automatically maintained at 7.0 by the addition of a sterilized 2 *M* NaOH solution.

The nitrogen-free medium contained butyric acid (C_4) and valeric acid (C_5) as carbon sources, and supplementary trace minerals and a growth factor as described by Chua et al. *(12)*. In separate batch cultures, the C_4 to C_5 weight ratios in the medium were respectively adjusted to 100:0, 80:20, 60:40, 40:60, 20:80, and 0:100 (g/g). The initial total concentration of fatty acids was adjusted to 1 g/L to avoid possible growth inhibition as reported by Kim et al. *(13)*. The fermenter was operated in a fed-batch mode by adding a total of 3 g of fatty acids into the fermenter once every 16 h. The culture broth was periodically sampled for analysis.

Analytical Methods

Activated sludge sample were analyzed for TOC, total kjeldahl nitrogen (TKN), dissolved oxygen (DO), pH, and dry cell mass. The TOC was conducted by TOC Autoanalyzer (ASTRO Model 2001). The TKN was determined using a Tecator Autoanalyzer KJELTEC AUTO 1030 ANALYZER). DO was measured by DO meter (YSI Model No. 55). The pH was monitored by pH meter (ORION Model EA 940). The dry cell mass was performed by mass liquor suspended solid (MLSS). The analytical techniques used in this study were performed according to Standard Methods *(14)*.

Extraction of the Polymer and Analysis of Polymer Composition

The activated sludge from the SBR and jar fermenter was centrifuged at 3000 rpm for 15 minutes and the intracellularly accumulated polymers were extracted by chloroform in accordance with the procedure by Lowell

Table 1
Reactor Operated Under C/N Ratio of 20

React time (h)	Residual TOC (mg/L)	Residual TKN (mg/L)	DO (mg/L)	pH	Dry cell mass (g)	Polymer accumulation (g)
0	274	12.6	4.7	6.67	19.9	0.01
0.25	229	9.3	4.8	6.56	20.4	0.02
0.50	176	7.9	4.0	6.60	20.7	0.05
0.75	135	5.3	1.3	6.90	20.8	0.07
1.00	129	4.7	0.7	6.64	20.9	0.09
1.25	78	4.0	0.4	6.91	21.3	0.09
1.50	46	3.0	0.3	6.50	21.5	0.10
1.75	41	2.8	0.2	6.58	21.5	0.10
2.00	25	2.0	0.2	6.81	21.7	0.10

et al. *(15)* and modified by Chua et al. *(11)*. The weight of the extracted polymers were measured to determine the productivity. The composition of the polymers extracted was analyzed by the gas chromatographic (GC) method described by Ho *(16)*.

Determination of Melting Temperature

The melting temperature of the extracted copolymers were determined in accordance with that described by Doi et al. *(17)* and Bluhm et al. *(18)*.

Results and Discussion

Effect of Different Values of C:N Ratio on Polymer Productivity During Wastewater Treatment

The SBR system was started up and operated for a period of 100 d to attain stable performance. It was consistently treating the wastewater with a TOC-reduction efficiency around 90%.

The residual TOC and TKN concentrations in the reactor liquor maintained a consistent depletion rate and the DO profile corresponded with the microbial activities in the activated sludge in the 2-h reaction time of the SBR operating cycle with a C:N ratio of 20 (Table 1). The pH of the reactor liquor was not controlled and fluctuated between 6.50 and 6.91. As the biomass increased, TOC, TKN, and DO decreased but polymer accumulation increased. This indicated that the biomass has the ability to convert organic pollution, at least in part, into bacterial reserve material, PHA. When the C:N ratio was increased to 40, 60, and 80, similar observations were made regarding the trends of residual TOC, DO, and pH. However, the residual TKN decreased to a consistent low level sooner then the residual TOC was depleted. When the C:N ratio was increased to 100, the TKN was almost depleted within the first 15 min, and the reactor entered a nitrogen-deficient condition (Table 2). When the C:N ratio was further

Table 2
Reactor Operated Under C/N Ratio of 100

React time (h)	Residual TOC (mg/L)	Residual TKN (mg/L)	DO (mg/L)	pH	Dry cell mass (g)	Polymer accumulation (g)
0	262	3.1	3.6	6.88	22.8	0.14
0.25	222	1.8	3.1	6.73	22.9	0.14
0.50	197	1.7	2.2	6.87	22.8	0.15
0.75	131	1.6	1.7	6.90	22.8	0.24
1.00	101	1.7	1.5	6.84	22.9	0.27
1.25	83	1.5	1.3	6.89	23.0	0.30
1.50	51	1.4	1.2	6.91	23.3	0.32
1.75	56	1.3	1.2	6.92	23.5	0.35
2.00	45	1.3	1.4	6.78	23.7	0.38

Table 3
Polymer Productivity Under Different C/N Ratios

C/N ratio	del X[a] (g)	del P[b] (g)	del S[c] (g)	$Y_{X/S}$ (g/g)	$Y_{P/X}$ (g/g)	$Y_{P/S}$ (g/g)	TOC removal eff. (%)
20	1.78	0.09	2.41	0.74	0.05	0.04	90.9
40	1.64	0.14	2.48	0.66	0.09	0.06	86.7
60	1.50	0.20	2.55	0.59	0.13	0.08	89.5
80	1.21	0.22	2.40	0.50	0.18	0.09	86.5
100	0.92	0.24	2.25	0.41	0.26	0.11	82.8
120	0.74	0.22	2.19	0.34	0.30	0.10	85.2
140	0.56	0.21	2.12	0.26	0.38	0.10	81.9

[a]del X, Net cell growth as measured in dry cell mass during the 2-h reaction time.
[b]del P, Net accumulation of intracellular polymers during the 2-h reaction time.
[c]del S, Net consumption of TOC during the 2-h reaction time.

increased to 120 and 140, the net cell growth decreased further and the polymer accumulation remained almost unchanged, between 0.21 and 0.22 g.

The effect of C:N ratios on TOC consumption, net cell growth, net polymer accumulation, and product yields during the 2-h reaction period are shown in Table 3. An increase in C:N ratio from 20 to 140 resulted in a decline in net cell growth, del X, from 1.78 to 0.56 g and therefore the specific growth yield, $Y_{X/S}$, from 0.74 to 0.26 g cell mass/g TOC. However, increasing the C:N ratio also resulted in an increase in specific polymer yield, $Y_{P/X}$, from 0.05 to 0.38 g polymer/g cell mass. Therefore, nitrogen seems to be important for normal growth of biomass in activated sludge but unfavorable for polymer accumulation. For this reason, it is suggested that cell-mass growth and polymer accumulation should be considered together to achieve a mutual benefit when the SBR was designed to operate. An intermittent nitrogen-feeding program must be established in order to optimize the polymer production without significantly affecting the normal treat-

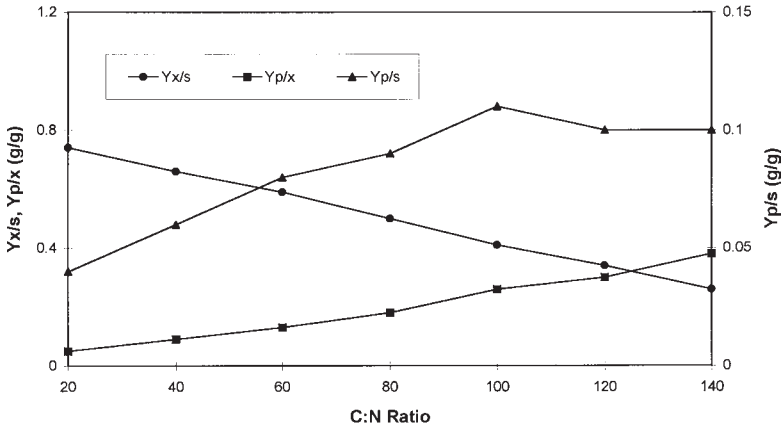

Fig. 2. Growth and polymer yields under different C:N ratios.

ment performance of the activated sludge process. The overall polymer production yield, $Y_{P/S}$, reached a maximum of 0.11 g polymer/g TOC under the C:N ratio of 100 (Fig. 2).

Changes in the C:N ratio above 20 did not significantly affect the efficiency of organic reduction by the SBR. The TOC removal efficiency remained above 80% for all the C:N ratios investigated (Table 3). These results are comparable to those found in earlier studies in our laboratory using a similar SBR system to treat a synthetic wastewater containing 4 g/L of glucose and food-industrial wastes *(11,12)*.

Accumulated polymeric materials were extracted from the microorganisms and analyzed by GC methods. They were determined to contain mainly monomers of 3-hydroxybutyric acid (3HB) and 3-hydroxyvaleric acid (3HV). The mole fractions of 3HB in the polymer varied from 46 to 100% for different batches of product extracted. Therefore, it is necessary to develop a strategy to control the composition of polymer accumulated in activated sludge for applications.

Control of Polymer Composition by Adjusting C_4 to C_5 Weight Ratio

Figure 3 shows the effect of changes in C_4 to C_5 weight ratio on the mole fraction of 3HV units in polymer produced by activated sludge, which was measured by GC. When butyric acid was utilized as the sole carbon source, only the homopolymer P(3HB), instead of P(3HB-co-3HV), was produced. The mole fraction of 3HV in the accumulated polymer increased proportionately with the valeric-acid concentration in medium. This may be owing to the fact that when butyric acid was the sole carbon source, propionyl-coA (precursor of 3HV unit) was not produced and so only the 3HB unit was formed, whereas when valeric acid was the sole carbon source, acetyl-coA and propionyl-coA were produced and available for formation of both 3HB and 3HV units. The maximum value of 3HV mole fraction in copolymer P(3HB-co-3HV) was restricted to 54 mol% because of relatively rapid

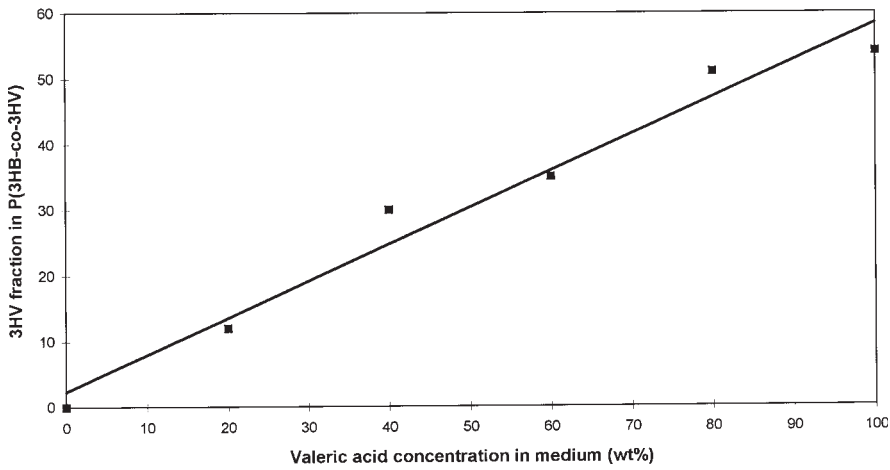

Fig. 3. Relationship between 3HV fraction in P(3HB-co-3HV) and valeric-acid concentration in the medium.

Table 4
Production of P(3HB) and P(3HB-co-3HV) by Activated Sludge with Different Carbon Sources Ratios of Butyric and Valeric Acids

C_4 to C_5 (g/g)	Biomass weight (g/L)	TOC removal (%)	Polymer content (wt%)	$Y_{P/S}$[a] (g/g)	3HV fraction (mol%)	T_m (°C)
100:0	3.01	98.0	37.0	0.69	0	178
80:20	2.45	96.0	40.0	0.61	12	144
60:40	2.52	95.0	35.0	0.55	30	133
40:60	2.30	90.2	24.0	0.35	35	127
20:80	2.50	83.4	18.0	0.32	51	109
0:100	2.30	65.0	22.0	0.44	54	99

[a]$Y_{P/S}$, Polymer production yield (g polymer/g carbon), which was calculated as the polymer accumulated divided by the TOC consumed.

metabolism of propionyl-coA to succinyl-coA and then to acetyl-coA for producing energy in TCA cycle, not for HV monomer synthesis. These results also indicated that the 3HV mole fraction of the P(3HB-co-3HV) copolymer accumulated in activated sludge could be controlled by adjusting the valeric acid concentration in the medium.

Melting temperature (T_m) is one of the thermal properties for characterizing PHA polymers. The melting temperature of copolymer accumulated in activated sludge with different medium composition ranged from 99° to 178°C (Table 4). An increase of 3HV monomeric units in the P(3HB-co-3HV) resulted in a nearly proportionate decrease in polymer melting temperature which reached a minimum value of 99°C (Fig. 4). These results agreed with the study by Doi et al. *(17)* who observed the melting temperature for P(3HB-co-3HV) ranged from 96° to 160°C depending on the frac-

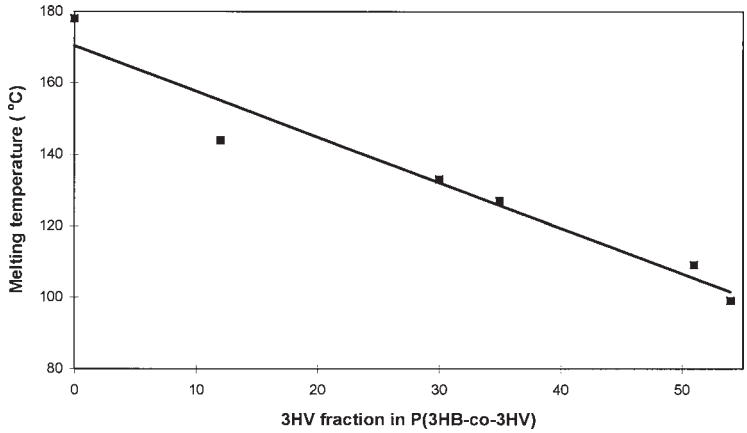

Fig. 4. Relationship between melting temperature and 3HV fraction in P(3HB-co-3HV).

tion of 3HB units. Therefore, the mechanical and physical properties of copolymer varied with 3HB to 3HV ratio (Fig. 5). A lower melting point seems to result in a loss of stiffness as well as poorer thermal resistance for processing, however, there is significant improvement in impact strength. This data appears to indicate that tailored high-value special property materials for specific applications may be obtained from activated sludge under controlled conditions.

However, as valeric acid in the medium was increased, both TOC-removal efficiency and polymer production were adversely affected. The TOC-removal efficiency decreased from 98.0 to 65.0%, whereas the polymer content in dried biomass decreased from 40 to 18% (Table 4). The decrease in TOC removal efficiency reflected that the consumption rate of total fatty acid decreased as the valeric acid fraction in the medium was increased. The results were consistent with that reported by Ishihara et al. *(19)*, who observed that when butyric-acid concentration was maintained at a constant value while valeric concentration was increased, cell growth and fatty-acids consumption were substantially affected. The increase in valeric-acid content also exerted an inhibitory effect on polymer production, resulting in a decline in polymer/g TOC consumed. However, these observations were in contrast to that reported by Ishihara et al. *(19)* and Yamane *(20)* that the yield and polymer content remained unchanged despite the variation in valeric-acid concentration in the medium. The wide range of polymer-production yields between 0.69 and 0.32 (g/g), could be attributed to the complex microbial species in the ecosystem of the sludge, giving rise to widely varied metabolic pathways from fatty acid to polymer in different microbial species. On the other hand, Dave et al. *(21)* described that when activated sludge was incubated under nitrogen- and phosphorus-limiting conditions, selective overgrowth of *Bacillus* spp. from 5 to 80% (cell count) was observed. It is therefore believed that the variation of

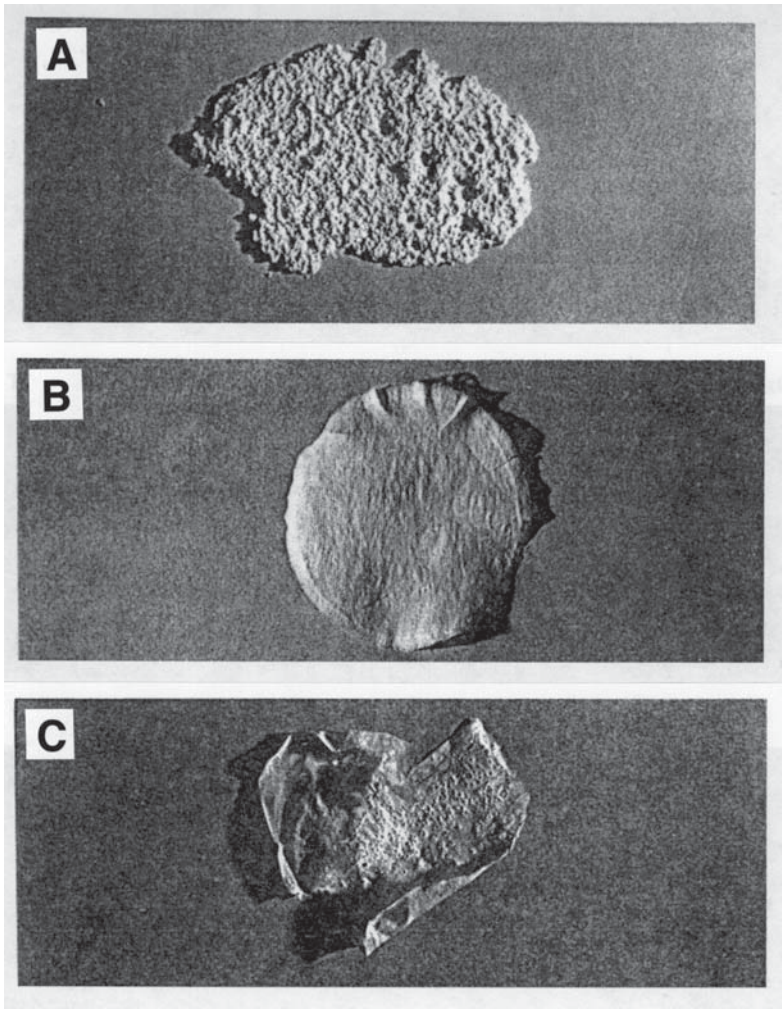

Fig. 5. The mechanical and physical properties of copolymer varied with 3HB to 3HV ratio. **(A)** The copolymer with higher 3HB mole fraction was more brittle. **(B)** The copolymer with intermediate 3HB mole fraction had an improvement in impact strength. **(C)** The copolymer with the highest fraction of 3HV was tougher and more flexible.

valeric-acid concentration in medium caused the changes in the balance of microbial species, and hence the prevailing metabolic pathways in activated sludge during the 48 h incubation. This, in turn, resulted in the wide ranging $Y_{P/S}$ values.

Conclusions

The novel technique for the synthesis of PHA from activated sludge is beneficial in two ways. On the one hand, excess sludge generated form activated sludge processes needs further treatment such as anaerobic

digestion and disposal by landfilling. This technique can reduce the quantity of excess sludge as the polymer portion in sludge is extracted, thereby reducing the costs of sludge treatment. On the other hand, large quantities of biomass harvested from activated sludge wastewater-treatment processes were induced to produce PHA at a low production cost. Furthermore, the composition and physical properties of the copolymers could be controlled by manipulation the carbon sources in the feed.

Acknowledgment

This project was supported by the Hong Kong Polytechnic University Research Grant V588 and supported in part by the Hong Kong Government Research Grant Council.

References

1. Chua, H., Yu, P. H. F., Xing, S., and Ho, L. Y. (1995), *J. Plast. Technol.* **18,** 132–148.
2. Hong Kong Government Industry Department (1991), in *Techno-Economic and Market Research Study of Hong Kong Plastic Industry*, vol. 1, 1990–1991, Hong Kong Government Press.
3. Chang, H. N. (1994), in *Better Living Through Innovative Biochemical Engineering*, Teo, W. K., ed., Singapore University Press, National University of Singapore, Singapore, pp. 24–30.
4. Chua, H., Yu, P. H. F., Xing, S., and Ho, L. Y. (1995), *Potential of Biodegradable Plastics as Environmentally-Friendly Substitutes for Conventional Plastics in Hong Kong*. Presented in 17th Symposium on Biotechnology for Fuels and Chemicals, May 1995, CO.
5. Sang Yup Lee (1996), *Biotechnol. Bioeng*. **49,** 1–14.
6. Suzuki, T., Yamane, T., and Shimizu, S. (1986), *Appl. Microbiol. Biotechnol.* **24,** 370–374.
7. Maness, P.-C. and Weaver, P. F. (1994), *Appl. Biochem. Biotech.* **45/46,** 395–407.
8. Page, W. J. (1989), *Appl. Microbiol. Biotechnol.* **31,** 329–333.
9. Page, W. J. (1992), *FEMS Microbiol. Rev.* **103,** 149–158.
10. Page, W. J. and Knosp, O. (1989), *Appl. Environ. Microbiol.* **55,** 1334–1339.
11. Chua, H., Yu, P. H. F., and Ho, L. Y. (1997), *Appl. Biochem. Biotechnol*. **63,** 627–635.
12. Chua, H., Yu, P. H. F., and Ho, L. Y. (1997), *J. IES Chem. Eng*. **37(2),** 9–13.
13. Kim, G. J., Yun, K. Y., Bae, K. S., and Rhee, Y. H. (1992), *Biotechnol. Lett.* **14,** 27–32.
14. American Public Health Association (1995), *Standard Methods for the Examination of Water and Wastewater*, 19th ed., Washington, DC.
15. Lowell, L. W. and Edwin, N. D. (1972), *Env. Sci. Technol.* **6(2),** 161–164.
16. Ho, L. Y. (1997), *Synthesis of Environmentally Friendly Materials*, Master Thesis, The Hong Kong Polytechnic University, Hung Hom, Kowloon, Hong Kong, China.
17. Doi, Y., Segawa, A., and Kunioka, M. (1990), *Intl. J. Biol. Macromol.* **12,** 106–111.
18. Bluhm, T. and Marchessualt, R. H. (1988), *Can. Chem. News*. **40,** 25–26.
19. Ishihara, Y., Shimizu, H., and Shioya, S. (1996), *J. Ferment. Bioeng.* **81,** 422–428.
20. Yamane, T. (1993), *Biotechnol. Bioeng*. **41,** 165–170.
21. Dave, H., Ramakrishna, C., and Desai, J. D. (1996), *Indian J. Exp. Biol.* **34,** 216–219.