0273-2289/04/114/0361–0372/$25.00

Construction of Recombinant *Escherichia coli* Strains for Production of Poly-(3-hydroxybutyrate-*co*-3-hydroxyvalerate)

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Abstract

Plastic wastes constitute a worldwide environmental problem, and the demand for biodegradable plastics has become high. One of the most important characteristics of microbial polyesters is that they are thermoplastic with environmentally degradable properties. In this study, pUC19/PHA was cloned and transformed into three different *Escherichia coli* strains. Among the three strains that were successfully expressed in the production of polyhydroxyalkanoates (PHA), *E. coli* HMS174 had the highest yield in the production of poly-(3-hydroxybutyrate-co-3-hydroxyvalerate) (P[HB-HV]). The cell dry weight and PHA content of recombinant HMS174 reached as high as 10.27 g/L and 43% (w/w), respectively, in fed-batch fermentor culture. The copolymer of PHA, P(HB-HV), was found in the cells, and the biopolymers accumulated were identified and analyzed by gas chromatography, proton nuclear magnetic resonance spectroscopy, and differential scanning calorimetry. We demonstrated clearly that the *E. coli* host for PHA production has to be carefully selected to obtain a high yield. The results obtained indicated that a superior *E. coli* with high PHA production can be constructed with a desirable ratio of P(HB-HV), which has potential applications in industry and medicine.

Index Entries: *Escherichia coli*; polyhydroxyalkanoates; fed-batch fermentation; nuclear magnetic resonance; differential scanning calorimetry.

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Table 1
Thermal and Mechanical Properties of Different Polymer Samples *(6,7)*[a]

Sample	T_m (°C)	T_g (°C)	Tensile strength (Mpa)	Elongation to break (%)
P(3HB)	177	4	43	5
P(HB-HV) 10% HV	150	ND	25	20
P(HB-HV) 20% HV	135	ND	20	100

[a]ND, not determined; T_g, glass transition temperature; T_m, melting temperature

Introduction

Plastics have become integral to our lives, and the generation of plastic wastes has increased dramatically. From 1986 to 1998, about 15% of the total domestic, commercial, and industrial waste in Hong Kong was plastic *(1)*. The most immediate advantage of making biodegradable plastics is to address the problems of litter and marine pollution resulting from plastics disposal, which are difficult to solve any other way.

Polyhydroxyalkanoate (PHA) is a polyester of hydroxyalkanoates synthesized by numerous bacteria as an intracellular carbon and energy storage compound and accumulated as granules in the cytoplasm of cells *(2)*. PHA has been attracting much attention because it is a biodegradable, biocompatible, microbial thermoplastic that is regarded as a potentially useful polyester to replace petroleum-derived thermoplastics *(3)*.

The copolymer poly-(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHB-co-PHV) produced by *A. eutrophus* has generated more interest than poly-(R)-3-hydroxybutyrate (PHB) homopolymer. Since these bacterial polyesters are biodegradable thermoplastics, their mechanical and physical properties have received much attention. PHB is a relatively stiff and brittle material because of its high crystallinity. However, the physiochemical and mechanical properties of [P(HB-HV)] vary widely and depend on the molar percentage of 3-hydroxyvalerate (HV) in the copolymer *(4,5)* as shown in Table 1. Propionic acid is converted by a synthetase to propionyl-CoA, and the biosynthetic β-ketothiolase catalyzes the condensation of propionyl-CoA with acetyl-CoA to 3-ketovaleryl-CoA by the acetoacetyl-CoA reductase. The hydroxyvaleryl moiety is finally covalently linked to the polyester by the PHA synthase *(6)*.

However, the price of PHB-co-PHV exceeds US $10/kg, which is much higher than the cost of conventional oil-derived plastic *(7)*. PHA yield is another major factor of the economic production of PHA. The bacterium *Escherichia coli* has proven to be a powerful microbial species in the microbial synthesis of bioproducts using molecular biology techniques.

One of the major factors affecting the overall production cost is bioreactor productivity, which can be defined as the amount of PHA accumulated per unit volume per unit time. To increase PHA productivity, not

Table 2
E. coli Strains and Plasmids

	Relevant characteristics	Reference or source
E. coli strain		
XL1-Blue	*supE44 hsdR17 recA1 endA1 gyrA46 thi relA1 lac–*	Stratagene
HMS174	*recA1 hsdR rif* r	*E. coli* Genetic Stock Center at Yale University
DH5α	*supE44 ΔlacU169 (Φ80 lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Gibco
Plasmid		
pUC19	2.68 kb, Amp^r (80 μg/mL)	New England Biolabs
pKS-/PHA	8.2 kb, Amp^r (80 μg/mL)	Ref. *8*
pUC19/PHA	7.9 kb, Amp^r (80 μg/mL)	This study
pJM9131	8.55 kb, Kan^r (50 μg/mL), its own σ^{70} promoter	Dr. Douglas D. Dennies

only must the cultivation time be reduced, but the cell density and PHA (% dry cell mass) must be increased *(8)*. Fed-batch cultivation is a popular fermentation process design for improving yield and productivity.

In the present experiment, the yield of copolymer production was optimized on a molecular basis using a fermentation strategy.

Materials and Methods

Bacterial Strains and Plasmids

The bacterial strains and plasmids used are given in Table 2. Cells were maintained in 15% (v/v) glycerol stock at –80°C after growing in Luria-Bertan (LB)i medium (10 g/L of tryptone, 5 g/L of yeast extract, 10 g/L of NaCl) at 37°C overnight. Ampicillin (80 μg/mL) was added for *E. coli* strains with pKS-/PHA, pUC19, and pUC19/PHA, and 50 μg/mL of kanamycin was added for *E. coli* with pJM9131.

Construction of Recombinant E. coli *Strains*

Plasmids were isolated using the Wizard Plus SV Minipreps DNA Purification System (Promega, Madison, WI). Plasmid DNA was analyzed by electrophoresis in a 0.8% (w/v) agarose horizontal slab gel, and a 1-kb DNA ladder (Promega) was used as marker. DNA fragments were isolated from the agarose gel using the Concert Rapid Gel Extraction System (Pharmacia). Restriction enzymes and T4 DNA ligase (Pharmacia) were used according to the instructions provided by the supplier. A 5.2-kb *Eco*RI and *Hind*III restriction fragment comprising the entire *pha* operon was cut out from plasmid pJM9131, and these three genes form an operon in the order *phaC*, *phaA*, and *phaB* coding for PHA synthase, β-ketothiolase,

and reductase, respectively. The *pha* operon was used as an insert, and pUC19 carrying the *lac* promoter was employed as vector. The ligation product was transformed into *E. coli* cells XL1-Blue, DH5α, and HMS174 (recA1 hsdR; Rifr). Its capacity for PHA accumulation and composition was analyzed by gas chromatography (GC) and ^{1}H-nuclear magnetic resonance (NMR), respectively.

Medium and Cultivation

Preparation of Food Wastes Medium

Malt waste, mostly semisolids of spent barley and millet refuse, was obtained from Carlsberg Company, a Hong Kong beer brewery. Soy waste, chiefly semisolid cellular residues of soy beans, was collected from Vitasoy International Holdings Ltd, a Hong Kong soy milk company.

The malt and soy wastes were prepared with 80 g of waste that was hydrolyzed with 1 L of 0.6 *M* HCl, which broke down various oligosaccharides into simple sugars such as glucose. The mixture was allowed to reach 121°C and was incubated for 30 min at evaluated pressure (1 kgf/cm^2). The resultant mixture was neutralized by the NaOH pellet, centrifuged, and filtered. The hydrolyzed malt waste was diluted with a third volume of distilled water.

Fermentation

A glycerol stock of cells was used directly as an inoculum in all experiments. It was first inoculated into 5 mL of LB broth in universal bottles. One percent inoculum was added to a 1-L conical flask containing 200 mL of flask fermentation medium that was grown on a rotary shaker at 250 rpm and 37°C. Antibiotic ampicillin was added in a final concentration of 80 μg/mL.

Fed-batch fermentation was performed in a computer-controlled 15-L Biostat C fermentor (B. Braun Biotech, Melsungen, Germany) with 8 L of culture medium with the conditions of 20% dissolved oxygen that was controlled based on both airflow (1 to 2 vvm) and stir rate (300–1000 rpm). The temperature was maintained at 37°C, and the pH was maintained at 7.0 by 2 *M* NaOH only. The basic medium for fed-batch fermentation was the same as for the seed flask (medium A: 10 g/L of glucose, 7 g/L of KH_2PO_4, 1 g/L of $MgSO_4$, 1.5 g/L of citrate, 2 g/L of tryptone, 2 g/L of yeast extract, and 1 g/L of propionate; pH 7.0) except 15 g/L of glucose, 6 g/L of tryptone, and 1.5 g/L of propionate were used. Medium B (750 g/L of glucose and 15 g/L of $MgSO_4 \cdot 7H_2O$, and 75 g/L of propionate) was used as the fed medium.

The medium in the fermentor was inoculated with 1% (v/v) inoculum, and feeding was started when the glucose in the medium was completely consumed with a rise in pH since the medium's pH increased as a result of glucose depletion and the use of organic acid as the carbon source. When the pH rose, the base pump was turned off and the fed rate of the fed medium was controlled by an acid pump. When the glucose feed in, the

acid formation from cells was able to lower the pH and the pH rose again when the glucose was used up again.

In shake-flask fermentation, the food wastes were placed in a 1-L flask with 250 mL of waste medium. To maintain the lowest production cost, no other chemicals were added.

Extraction of Biopolymers

After fermentation, the culture was centrifuged at 14333*g* for 25 min at 4°C, washed with ddH_2O, and freeze dried. One gram of the freeze-dried cell powder was treated with a dispersion containing 15 mL of chloroform and 30% NaOCl solution. The mixture was incubated at 37°C with 250 rpm of agitation for 1.5 h, then centrifuged at 2610*g* for 15 min, resulting in three phases. The upper phase was a hypochlorite solution, the middle phase contained the non-PHA cell material and undisrupted cells, and the bottom phase was chloroform-containing PHA.

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

Analytical Methods

Analysis of Total Organic Carbon

The fermentation medium was analyzed with an Astro 2000 TOC Analyzer according to the APHA (4500-Norg) method *(9)*.

Analysis of Total Kjeldahl Nitrogen

The fermentation medium was analyzed with a Kjeltec Auto 1030 Analyzer according to the APHA (5310C) method *(9)*.

GC Analysis

Freeze-dried cells or extract polymer was added to 1 mL esterification solution (3 mL of 95–98% H_2SO_4, 0.29 g of benzoate, and 97 mL of methanol), and 1 mL of chloroform. The mixture was heated at 100°C for 4 h. ddH_2O (1 mL) was added to the cooled mixture, which was vortexed for phase separation. The lower organic phase portion (1 µL) was subjected to GC analysis, which was performed on a Hewlett Packard 5890 Series II gas chromatograph with a flame ionization detector using a Supelco (10% Carbowax 20 M with 80/100 mesh size Chromosorb WAW) packed column 6 ft in length. Nitrogen was chosen as the carrier gas at a flow rate of 20 mL/min. The working temperature of the column, injector, and the flame ionization detector was 135, 260, and 300°C, respectively. The temperature was kept stable for 10 min to determine both the content and composition of the polymer.

Analysis by ^{1}H NMR

^{1}H NMR analysis was carried out on a Bruker DPX-400 spectrometer. ^{1}H NMR spectra were recorded at room temperature. Ten to 20 mg of

extracted biopolymer sample was put into the NMR tube, and 1 mL of deuterated chloroform ($CDCl_3$) solution was added to dissolve the sample. ^1H NMR spectra (400 MHz) were recorded, and tetramethylsilane was used as an internal reference *(10)*.

Analysis by Differential Scanning Calorimetry

Three to 4 mg of extracted biopolymers was encapsulated in aluminum pans for the measurements. Each sample was first annealed at 200°C for 3 min. The melting point was determined using a Mettler DSC 30 Thermal Analysis System. Dry nitrogen was used as the flow gas with a flow rate of 30 mL/min, calibrated with indium and mercury.

Results and Discussion

Construction of Recombinant Strains

A 5.2-kb *Eco*RI/*Hind*III restriction fragment comprising the entire *pha* operon obtained from plasmid pJM9131 was used as an insert, as well as plasmid pUC19 possessing *lac* promoter. The copy number of plasmid pJM9131 was lower than pUC19, and the effect of *lac* promoter on PHA production was investigated. Three *E. coli* strains were chosen. *E. coli* XL1-Blue and DH5α and typical *E. coli* expression host cells, which were recombinant-deficient strains, were extensively used. The HMS174 strain of *E. coli* was chosen because it contains a lactose utilization system and is recombinant deficient so that a plasmid containing lactose genetic regions will not recombine and make the construct unstable. The lactose utilization system present in *E. coli* HMS174 may be allowed better utilization of food wastes as a carbon source for the production of PHB *(11)*.

Cloning was confirmed by restriction digestions and agarose gel electrophoresis. It demonstrated that the newly cloned plasmid was successfully transformed into the *E. coli* XL1-Blue, HMS174, DH5α and demonstrated that the construction of the recombinant *E. coli* with plasmid pUC19 containing the entire *pha* operon was successful.

Comparison of PHA Production in Different Recombinant E. coli *Strains*

Comparison of the yield of PHA produced by the three new strains revealed that the *E. coli* HMS174 harboring plasmid pUC19/PHA accumulated the highest amount of PHA. The absorbance of the strains during flask cultivation at 600 nm is shown in Fig. 1.

Excess propionate is not taken up and metabolized by the cells, and the toxicity of propionate can inhibit cell growth. Figure 1 shows that the recombinant *E. coli* HMS174/PHA experienced better cell growth than the *E. coli* XL1-Blue/PHA and *E. coli* DH5α. It represents better resistance to the growth inhibition effect of the propionic acid. Hence, *E. coli* HMS174/PHA was chosen to be the target subjected to the high-cell-density fed-batch fermentation in the fermentor.

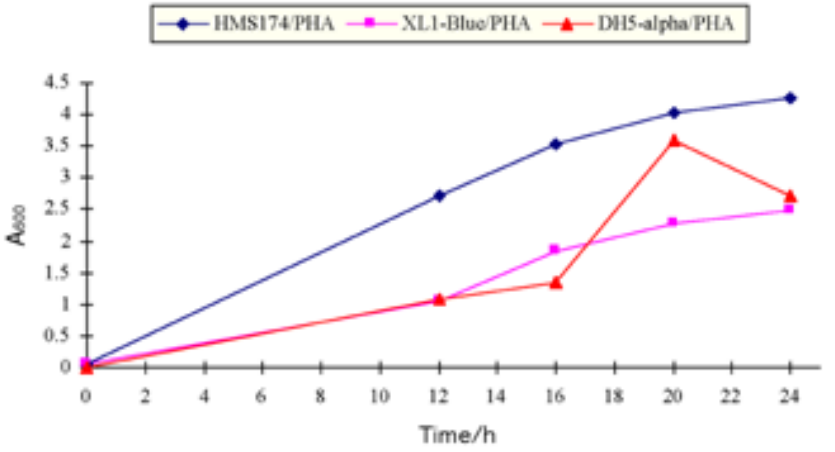

Fig. 1. Time profile of absorbance at 600 nm of *E. coli* strains during flask cultivation.

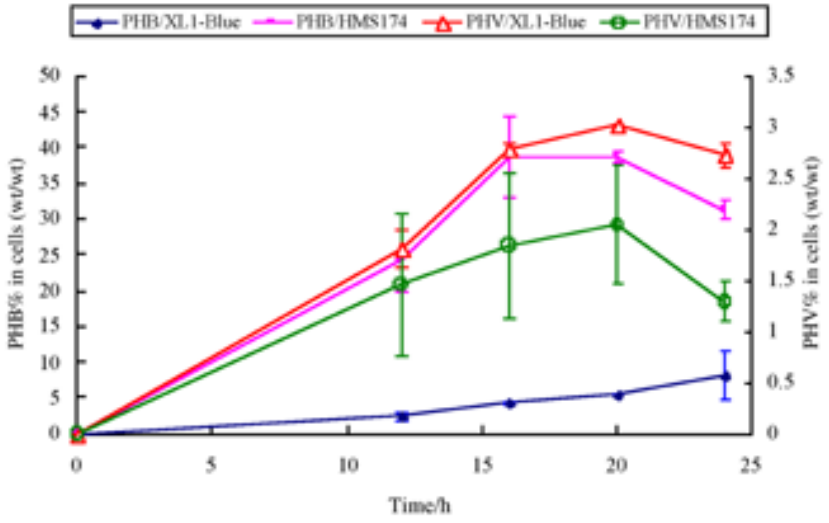

Fig. 2. Percentages of PHB and PHV in CDW of *E. coli* strains during flask cultivation.

The composition and percentage yield of biopolymer within the cells were analyzed by GC, and the GC spectra show that the biopolymer accumulated was P(HB-HV). The percentage yield of copolymer of dry cell weight (DCW) at different times is shown in Fig. 2. It shows that most biopolymer accumulated in the cells was PHB and its yield was much higher using *E. coli* HMS174/PHA compared with *E. coli* XL1-Blue/PHA. A higher PHV yield was also obtained using *E. coli* HMS174/PHA. The PHA percentage yield was as high as 41% (w/w) of its CDW in flask culture, which was higher than the recombinant *E. coli* previously cloned *(12)*.

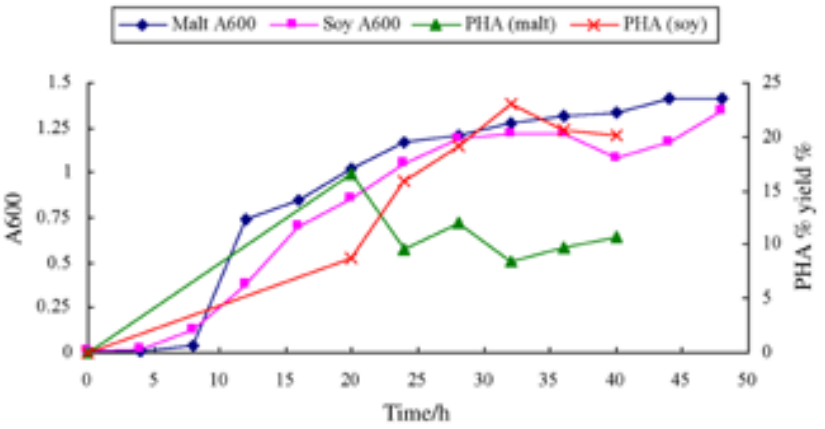

Fig. 3. Time profile of absorbance (A_{600}) of recombinant *E. coli* in malt and soy wastes.

There are two possible reasons for these differences in yield. First, the pUC19 might be a better expression vector for *pha* operon in this case, and therefore a higher amount of PHA accumulated. Second, the capacity of PHA synthesis in different *E. coli* strains was different because the metabolite may be different in *E. coli* HMS174 and *E. coli* XL1-Blue.

Production of P(HB-HV) Using a Recombinant Strain of E. coli *DH5α (pUC19/PHA) in Different Food Wastes*

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) *(13)*. The ratios of the C and N contents of the hydrolyzed malt and soy wastes were 9:1 and 7.5:1, respectively, as determined by total organic carbon *(4)* and total Kjeldahl nitrogen (TKN) *(4)*.

We cultivated the *E. coli* DH5α (pUC19/PHA) in malt and soy wastes and compared it to PHA production to determine its ability to utilize food wastes. The absorbance at 600 nm of the recombinant *E. coli* in the food wastes is shown in Fig. 4.

The results in Fig. 3 show that the recombinant *E. coli* had better growth in 1:3 malt waste medium than in the soy waste medium. The efficient utilization of the food waste medium can greatly reduce the cost of PHA production. The PHA accumulation of the recombinant *E. coli* in malt and soy wastes medium was 16 and 23% of the CDW, respectively.

Production of P(HB-HV) Using Recombinant Strain of E. coli *HMS174 (pUC19/PHA) in a 15-L Fermentor*

Because the *E. coli* HMS174/PHA exhibited good growth in the presence of propionate and also had a high yield of P(HB-HV) accumulation in flask fermentation, *E. coli* HMS174/PHA was subjected to a fed-batch culture in which the cells were fed by glucose and propionic acid. The A_{600}

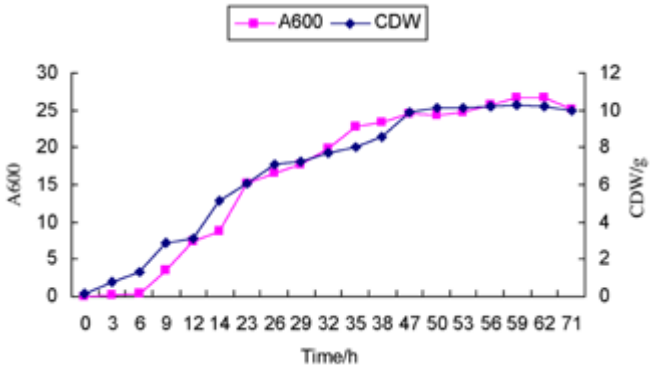

Fig. 4. Time profile of absorbance at 600 nm and CDW of *E. coli* HMS174 with plasmid pUC19/PHA during 15-L fermentor cultivation.

Table 3
Effect of Ampicillin on PHA Production
in *E. coli* HMS174 with Plasmid pUC19/CAB

	Culture Time (h)				
Strain	0	12	16	20	24
HMS 174 pUC19/CAB with ampicillin					
PHB% (w/w)	0	11.69	18.36	17.92	26.74
PHV% (w/w)	0	0.29	0.43	0.92	1.77
Total PHA% (w/w)	0	11.99	18.78	18.83	28.51
A600	0.022	1.99	3.08	3.98	5.19
HMS 174 pUC19/CAB without ampicillin					
PHB% (w/w)	0	12.26	16.78	24.48	29.80
PHV% (w/w)	0	0.30	0.61	0.69	0.91
Total PHA% (w/w)	0	12.56	17.40	25.17	30.71
A_{600}	0.018	1.88	2.93	4.08	4.69

and its CDW were recorded at different time points throughout the fermentation, and the A_{600} and CDW reached as high as 26.7 and 10.27 g/L, respectively, at 59 h of cultivation in the fed-batch culture. The results are shown in Fig. 4.

The use of antibiotics in large-scale fermentation is not desirable because of the high production cost. *E. coli* HMS174 with plasmid pUC19/CAB showed relatively low instability problems during PHA production in the absence of antibiotic selective pressure when in the flask culture (Table 3). The percentage yield of PHA in cell culture with or without ampicillin was similar. Therefore, it was desirable to subject this strain to a fermentor experiment.

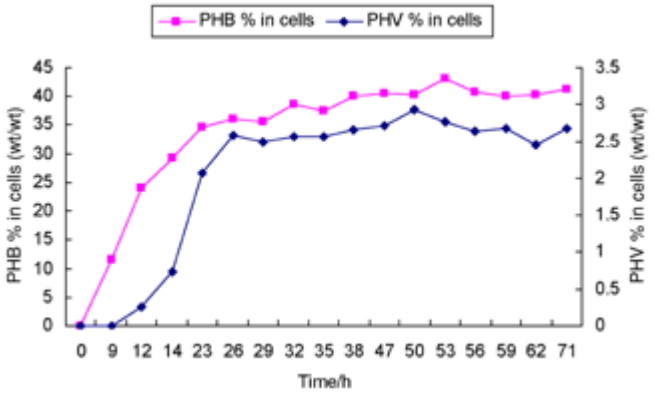

Fig. 5. Percentages of PHB and PHV in CDW of *E. coli* HMS174 with plasmid pUC19/PHA during 15-L fermentor cultivation.

A fed-batch culture was grown in which the cells were fed glucose and propionic acid. The PHA content of the culture was analyzed by GC, and the percentage yield of PHB and PHV in the cells is shown in Fig. 5. The cell concentration increased to 10 g/L CDW when 1% inoculation was used, and the PHB content was maintained at about 40% of CDW throughout the culture and reached as high as 43.2%. However, the PHV content was maintained at about 2% of CDW.

Physical Properties of Extracted Biopolymer from Fermentor

The composition of the hydroxyalkanoates units in extracted products were determined by analyzing the NMR spectra. The ^{1}H-NMR spectrum (Fig. 6) shows the presence of five groups of characteristic signals of the P(HB-HV). The signals with different chemical shifts are attributed to the different groups coupled to different numbers of protons and are labeled in Fig. 6. The spectra showed the presence of CH-, CH_2-, and CH_3- groups in the molecule as labeled in Fig. 6, but no -COOH and -OH groups. Thus, the compound should be a polymer, not a monomer. The magnitudes of the CH_3- and CH_2- groups of PHV in Fig. 6 are lower than those of the PHB that represented the lower percentage of PHV content in the biopolymer when compared with PHB content.

The melting temperature (T_m) of the biopolymer was determined from differential scanning calorimetry thermograms. The T_m value of the P (3HB) homopolymer was about 177°C and P (3HB) with 10% P(3HV) was about 150°C (Table 1), but the T_m values of biopolymer extracted from *E. coli* HMS174 were about 166°C. The T_m of the sample was lower than that of the P (3HB) homopolymer, but higher than that of the P (3HB) with 10% P (3HV), because the P (3HV) content in the sample is only about 4.5% of the biopolymer produced. The PHV content in extracted biopolymer was low, the flexibility of extracted biopolymer was low, and different fermentation conditions should be investigated.

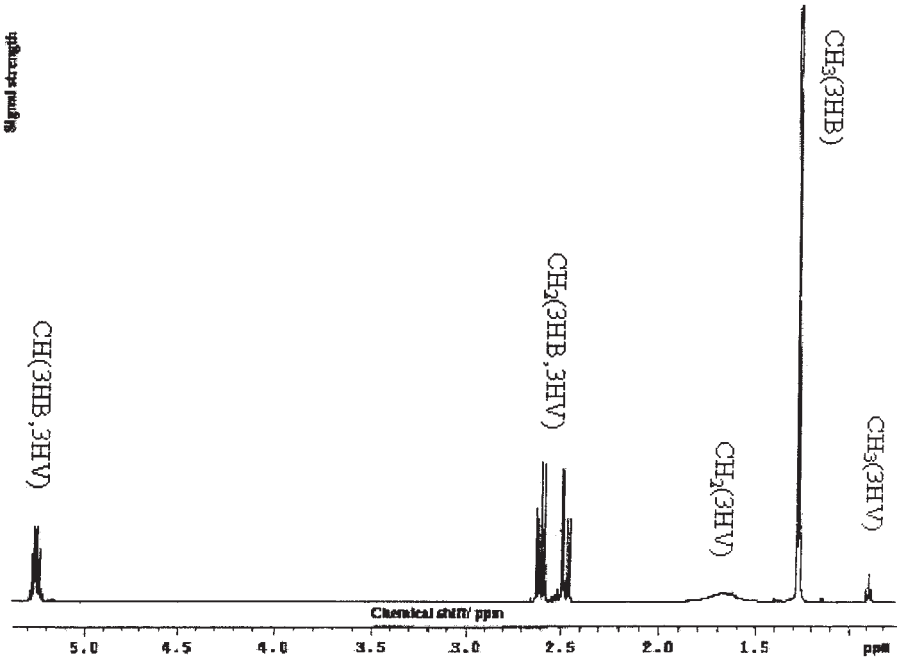

Fig. 6. ^{1}H NMR spectrum of biopolymer produced by *E. coli* HMS174 with plasmid pUC19/PHA in fermentation.

Conclusion

The construction of recombinant *E. coli* harboring *pha* operon from *R. eutrophus* was successful. The plasmids in XL1-Blue, HMS174, and DH5α strains were successfully expressed in the production of PHA, but *E. coli* HMS174 was superior for the production of [P(HB-HV)], whose levels could reach as high as 41% of its dry weight in shake-flask culture and could reach relatively high cell density. The CDW and PHA content of recombinant HMS174 could reach as high as 10.27 g/L and 43% (w/w), respectively, in fed-batch fermentor culture. The strains were also successfully expressed in hydrolyzed soy and malt wastes, which have the potential to lower the cost of production.

Acknowledgments

We wish to express our gratitude to the Hong Kong Polytechnic University and the University Grant Council of Hong Kong for their support (PolyU 5205/00M and PolyU 5272/01M) of this research. We also wish to thank the *E. coli* Genetic Stock Center at Yale University, which kindly contributed the *E. coli* HMS174; and Dr. Dennis Douglas (Department of Biology, James Madison University, Harrisonburg, VA), who kindly contributed the plasmid pJM9131.

References

1. Hong Kong Environmental Protection Department (1999), in *Environment Hong Kong 1999*, Hong Kong Government Press, China.
2. Lee, S. Y. (1996), *Biotechnol. Bioeng.* **49,** 1–14.
3. Chang, Y. K., Hahn, S. K., Kim, B. S., and Chang, H. N. (1994), *Biotechnol. Bioeng.* **44,** 256–261.
4. Doi, Y., Kitamura, S., and Abe, H. (1995), *Macromolecules* **23,** 4822–4828.
5. Porier, Y., Nawrath, C., and Somerville, C. (1995), *Biotechnology* **13,** 142–151.
6. Steinbuchel, A. (1991), in *Biomaterials: Novel Materials from Biological Sources*, Byrom, D., ed., Macmillan, New York, NY, pp 123–213.
7. Steinbuchel, A. (1995), in *Degradable Polymers: Recycling and Plastic Waste Management*, Albertsson, C. and Huang, J., eds., Marcel Dekker, New York, NY, pp. 61–68.
8. Yamane, T., Fukunaga, M., and Lee, Y. W. (1996), *Biotechnol. Bioeng.* **50,** 197–202.
9. Greenberg, A. E., Clesceri, L. S., and Eton, A. D. (1992), *Standard Methods for the Examination of Water and Wastewater*, 18th Ed., APHA, Washington, DC.
10. Yu, H. F., Chua, H., Huang, A. L., Lo, W. H., and Ho, K. P. (1999), *Water Sci Technol.* **40(1),** 365–370.
11. Dennis, D. (1990), US patent no. 5334520.
12. Slater., S., Gallaher, T., and Dennis, D. (1992), *Appl. Environ. Microbial.* **58(4),** 1089–1094.
13. Lee, S. Y. (1998). *Bioprocess Eng.* **18,** 397–399.