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Conversion of Food Industrial Wastes into Bioplastics

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ABSTRACT

The usage of plastics in packaging and disposable products, and the generation of plastic waste, have been increasing drastically. Broader usage of biodegradable plastics in packaging and disposable products as a solution to environmental problems would heavily depend on further reduction of costs and the discovery of novel biodegradable plastics with improved properties. In the authors' laboratories, various carbohydrates in the growth media, including sucrose, lactic acid, butyric acid, valeric acid, and various combinations of butyric and valeric acids, were utilized as the carbon (c) sources for the production of bioplastics by *Alcaligenes eutrophus*. As the first step in pursuit of eventual usage of industrial food wastewater as nutrients for microorganisms to synthesize bioplastics, the authors investigated the usage of malt wastes from a beer brewery plant as the C sources for the production of bioplastics by microorganisms. Specific polymer production yield by A. Latus DSM 1124 increased to 70% polymer/cell (g/g) and 32g/L cell dry wt, using malt wastes as the C source. The results of these experiments indicated that, with the use of different types of food wastes as the C source, different polyhydroxyalkanoate copolymers could be produced with distinct polymer properties.

Index Entries: Polyhydroxyalkanoate (PHA); polyhydroxybutyrate (PHB); polyhydroxyvalerate (PHV); malt waste; Soya waste; *Alcaligenes eutrophus; Alcaligenes latus.*

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INTRODUCTION

Recently, plastic usage has increased drastically *(1)*. Product-packaging plastic materials account for a large fraction of total solid-waste generation, and these are considered environmentally harmful because they are generally nonbiodegradable, or expensive for recycle usage. Of the 133 million t of solid wastes generated each year in the early 1980s, more than 30% by weight of these materials were plastics *(2)*. In Hong Kong, 9500 t of municipal solid wastes are delivered for disposal at incineration plants and landfills each day *(3)*. Approximately 11% (wet wt) of these wastes are plastics materials, including a high proportion of packaging materials and disposable products. Plastics usage and plastics-waste generation are forecast to increase at 15%/yr over the next decade *(4)*.

In the past decade, there has been much interest in the development and production of biodegradable plastics. Various biodegradable plastics have been produced by incorporating natural polymers into conventional plastics formulations, by chemical synthesis, or by microbial fermentation. In the search for a biodegradable plastic of natural or biological origin, a family of more than 40 polyhydroxyalkanoates (PHAs) and their related copolymers has been discovered, and has emerged as environmentally friendly materials. These polymers are completely biodegradable into carbon dioxide and water within a few months of burial *(5)*. Certain bacteria, such as *Escherichia coli, Clostridia* spp., and *Alcaligenes eutrophus*, produce polyhydroxybutyrate (PHB) as an intracellular metabolite and a back-up carbon (c) source when an unfavorable environment is encountered *(6)*. PHB can be synthesized intracellularly (by polymer synthase) from sugars and fatty acids by the condensation of D-3-hydroxybutyryl-CoA (catalyzed by acetoacetyl-CoA reductase), formed from acetyl-CoA via acetoacetyl-CoA (catalyzed by 3-ketothiolase) and butyryl-CoA, respectively. Microbial PHB is a biodegradable, biocompatible thermoplastic. PHB has not been commercially exploited widely because of its high price, compared with traditional thermoplastics *(7)*. Simple organic substrates, such as sucrose *(8)*, and glucose *(9)* or ethanol, propanol *(10)*, methane *(11,12)*, and so on, as C sources, and inorganic chemicals, such as ammonium or ammonia, as nitrogen (N) sources, are used in the production of PHB *(8,10)*. In a recent study, the use of microorganisms in activated sludge obtained from a wastwater treatment plant to synthesize PHAs was reported from this laboratory *(13)*. Broader usage of biodegradable plastics in packaging and disposable products as a solution to environmental problems would heavily depend on further reduction of costs and the discovery of novel biodegradable plastics with improved properties. In this study, malt refuse from a brewery was used as both C and N sources for cell growth.

MATERIALS AND METHODS

Microorganism

Alcaligenes eutrophus H16 (ATCC 17966) was purchased from American Type Culture Collection. *Alcaligenes latus* DSM 1122 and DSM 1124, gifts from George Chen of Tsinghua University, were maintained on nutrient agar slant at 4°C by monthly subculture. Mixed cultures from activated sludge were obtained from a municipal wastewater treatment plant at Shatin, Hong Kong.

Media

Preparation of Liquid Seed Medium for Fermentation

The composition of the liquid seed medium was 4 g maltose, 0.2 g K_2HPO_4, 0.4 g $(NH_4)_2SO_4$, 0.02 g $MgSO_4 \cdot 7H_2O$, 0.01 g citrate-Fe(III), 0.01 g yeast extract, 0.01 g meat peptone, and 200 mL tap water. The pH of the media was 7.0. After autoclaving and inoculating, the liquid seed media was incubated at 4*g*, 35°C of shaker (Forma Scientific Model 4518 Table Top Incubator Orbital Shaker) for 24 h.

Preparation of Fermentation Medium

Malt waste, mostly semisolids of spent barley and millet refuse, was obtained from a local beer brewery. Soya waste, chiefly semisolid cellular residues of soya beans (imported from the United States), was collected from a local soya milk company. The ratio of the C and N contents of the malt and soya wastes were 7:1, and 8:1, respectively, as determined by total organic carbon (TOC) and total Kjeldahl nitrogen (TKN) methods. About 300 g dry milled waste was hydrolyzed with 2500 mL 1 *N* HCl at 100°C for 9 h, and then the slurry was centrifuged. The malt filtrate was neutralized with NaOH to pH 7.0. In addition, 6 g K_2HPO_4, 2 g Citrate-Fe(III), and 1 g $MgSO_4 \cdot 7H_2O$ were added to the malt filtrate. Finally, the total volume of the fermentation medium was 2.4 L. The above solution was transferred to the vessel of a 5-L bioengineering fermenter. Sterilization was conducted at 121°C for 30 min.

Preparation of Nitrogen-Limited Medium

100 g sucrose, 2 g K_2HPO_4, 3g Citrate-Fe(III), and 1 g $MgSO_4 \cdot 7H_2O$ were dissolved in 300 mL water, and autoclaved for 20 min at 121°C. 100 g glucose, 2 g K_2HPO_4, 2 g $(NH_4)_2SO_4$, and 1 g $MgSO_4 \cdot 7H_2O$ were dissolved in 300 mL water, and autoclaved for 20 min at 121°C.

Fermentation

The fermentation was carried out in the computer-controlled bioengineering fermenter with the growth conditions set at DO = 20, T = 35°C,

and pH = 7.0. The Antifoam 289 (purchased from Sigma, St. Louis, MO) was used, and 10 *N* NaOH was employed to adjust the pH. At each hour, 20 mL of sample were pumped out for cell dried weight, PHB, TOC, TKN analysis. Although high-density cells were obtained, the N-limited medium was fed to fermenter to increase C/N, in order to promote PHB formation in the cells.

Extraction and Precipitation of Biopolymers

After fermentation, the fermentation broth was concentrated by centrifugation at 42,110*g* for 25 min, washed twice, and freeze-dried. Then, 8 g of cell powder was treated with 100 mL chloroform and 100 mL of 30% sodium hypochlorite, and the mixture was agitated in a shaker at 4*g* at 30°C for 150 min. After the treatment, the dispersion was centrifuged at 12,282*g* for 10 min. The three separate phases were obtained. The upper phase was the hypochlorite solution, the middle phase contained non-PHB cell material (NPCM) and undisrupted cells, and the bottom phase was the chloroform layer, containing PHB. First, the hypochlorite solution phase was removed with a pipet, and then the chloroform phase was obtained by filtration *(14,15)*, and further concentrated by a setup of distillation to a final volume of 20 mL. Then, the PHB material was precipitated by mixing methanol with the concentrated chloroform (methanol:chloroform, 9:1). Finally, the white precipitate was filtered by simple filtration, and then dried.

Analytical Methods

CDW Analysis

5 mL fermentation broth was centrifuged, washed with dH_2O, and then dried at 105°C for 2 h.

PHB Analysis

5 mL fermentation broth was centrifuged and washed with dH_2O. Afterward, it was mixed and vortexed with 2 mL 10% NaClO for 2 min, and then 5 mL dH_2O was added, and it was centrifuged immediately. Then, the biopolymers were washed with dH_2O and dried at 105°C for 2 h.

TOC Analysis

Adequate diluted fermentation broth supernatant was taken to measure TOC with an Astro 2000 TOC Analyzer. A 100-mg C/L sucrose solution was used as a standard. The procedure of analysis was according to APHA (4500-Norg) *(16)*.

TKN Analysis

A 1-mL fermentation broth supernatant was analyzed with a Kjeltec Auto 1030 Analyzer. The method was according to APHA (5310C) *(16)*.

Melting-Point (MP) Measurement

Melting point was performed by using an Electrothermal 9100 digital mp apparatus. The temperature increase rate program was set at 10°C/min.

Gas Chromatography Analysis

A 20 mg biopolymer was mixed with 1 mL chloroform and 1 mL esterification fluid (0.5 g benzoic acid, 8 mL 95–98% H_2SO_4, plus 242 mL methyl alcohol). The mixture was maintained at 100°C for 4 h. 1 mL water was added to the cooled mixture and vortexed. Standard PHB was purchased from Fluka (Buchs, Switzerland). Gas chromatographic analysis was performed on a Varian Model 3700 gas chromatograph, using a one-eight in diameter Chromosorb-WAW column with 80/100 in. mesh size, and 6 ft in length (from Supelco [Bellefonte, PA]). The recorder was a Shimadzu C-R5a Chromatopac. Nitrogen was the carrier gas, at a flow rate of 10 mL/min. The analysis started at 110°C for 3 min, whereupon the temperature was increased to 220°C at a rate of 8°C/min. After reaching 220°C, the temperature was maintained for 5 min before the analysis was terminated *(17)*.

^1H Nuclear Magnetic Resonance (^1H NMR)

The H^1NMR analysis was carried out on a Bruker DPX-400 spectrometer using a 5-mm $^1H/^{13}C$ dual probe. ^{1}HNMR spectra were recorded at room temperature from a $CDCl_3$ solution of the extracted biopolymers with 30-degree pulse angle. Chemical shifts were referenced to the internal reference Tetramethylsilane (TMS) *(18)*.

RESULTS

In the first part of the experiment, bioplastics were successfully biosynthesized by *A. eutrophus* H16, using organic acids as the C source. Varied concentration ratios of butyric acid and valeric acid were used as C substrates, i.e., 100% butyric acid and 0% valeric acid, mixture of 80% butyric acid and 20% valeric acid, mixture of 60% butyric acid and 40% valeric acid, mixture of 40% butyric acid and 60% valeric acid, mixture of 20% butyric acid and 80% butyric acid, and 0% butyric acid and 100% valeric acid. Analysis of the products of fermentation revealed the polymer yields and mps of the copolymers, as shown in Table 1. The polymer products may be harvested as described in Materials and Methods, and further purified by repeating the chloroform–methanol precipitation procedure. The isolated bioplastics flakes can be melted into an uniform liquid with appropriate plasticizers and additives, and molded into various types and shapes of plastic for product application, e.g., packaging films and surgical sultures and gloves.

Table 1
Polymer Yields of Bioplastics Synthesized by *Alcaligenes eutrophus* with Butyric and Valeric Acids

Sample	Carbon source C_4:C_5	Polymer yield (g/g)	Copolymers[a] mol ratio PHB:PHV	Melting point (°C)
1.	100:0	0.18	100:0	177.6
2.	80:20	0.41	88:12	144.0
3.	60:40	0.15	70:30	133.3
4.	40:60	0.10	65:35	127.1
5.	20:80	0.12	49:51	109.2
6.	0:100	0.06	46:54	99.2

[a] Copolymers: PHB, polyhydroxybutyrate; PHV, polyhydroxyvalerate.

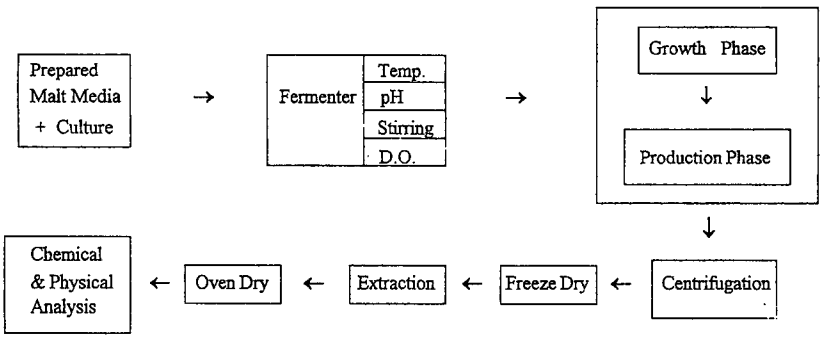

Fig. 1. Fermentation flow chart of bioplastics production.

In the second part of the experiment, a specific culture of *A. latus* DSM 1124 was selected to ferment sugar into bioplastics. Specific polymer production yield by *A. latus* DSM 1124 increased to more than 50% polymer/cell (g/g) and 30g/L cell dry wt, with increasing C/N ratio, using sucrose as the C source. The fermentation was done at 35°C for 36 h with fed-batch and dissolved oxygen controlled between 20 and 40 of calibrated units in a 5 L bioengineering fermenter.

A further experiment was conducted to investigate the conversion of malt wastes (barley), obtained from a local beer brewery, into bioplastics. A flow chart of the fermentation of the malt wastes and the isolation of the product PHAs is illustrated in Fig. 1. The results of the experiment are shown in Table 2. Specific polymer production yield by *A. latus* DSM 1124 increased to 70% polymer/cell (g/g) and 32g/L cell dry wt, using malt wastes as the C source. The data of cell growth and polymer accumulation, residual TOC and TKN in the medium during fermentation, and the changes in C/N ratio in the medium during the process of cell growth and polymer accumulation, are graphically presented in Figs. 2–4, respectively.

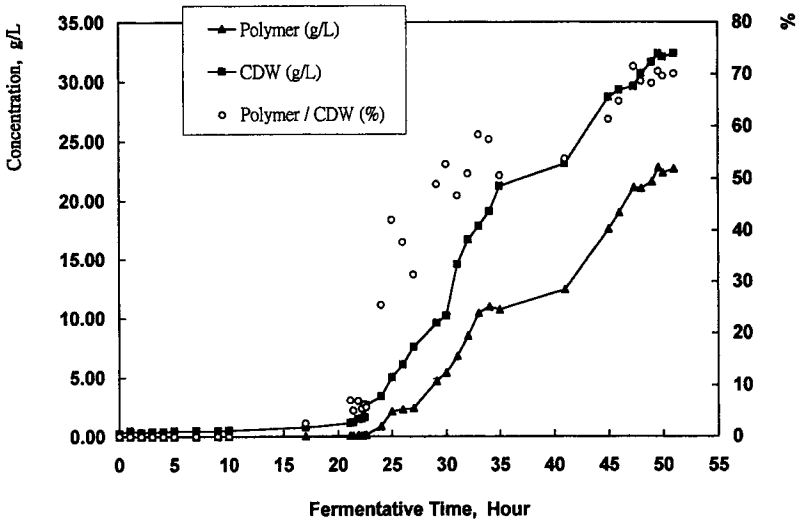

Fig. 2. Cell growth and polymer accumulation.

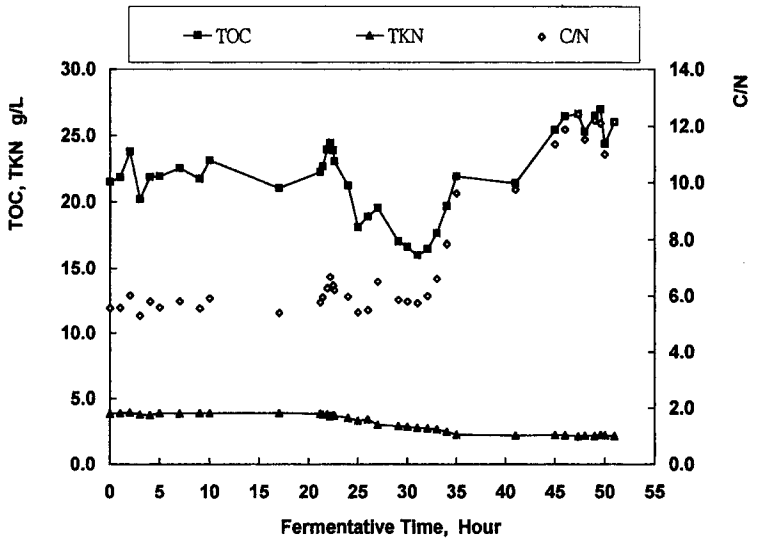

Fig. 3. Residual TOC and TKN in medium during fermentation.

Table 2
Production of PHB from Brewery Malt Wastes by *Alcaligenes Latus* DSM 1124

Time (h)	CDW (g/L)	Polymer (g/L)	TOC (g/L)	TKN (g/L)	C/N	Polymer/CDW (%)
0	0.26	0.00	21.45	3.86	5.56	0.00
1	0.46	0.00	21.84	3.92	5.58	0.00
2	0.34	0.00	23.81	3.96	6.02	0.00
3	0.42	0.00	20.20	3.82	5.29	0.00
4	0.38	0.00	21.85	3.77	5.80	0.00
5	0.48	0.00	21.90	3.92	5.59	0.00
7	0.46	0.00	22.52	3.87	5.81	0.00
9	0.44	0.00	21.72	3.91	5.55	0.00
10	0.54	0.00	23.14	3.91	5.92	0.00
17	0.78	0.02	21.02	3.90	5.39	2.56
21	1.20	0.06	22.66	3.81	5.95	5.00
22	1.60	0.10	23.86	3.75	6.36	6.25
24	3.38	0.86	21.20	3.55	5.98	25.44
26	6.10	2.30	18.86	3.43	5.49	37.70
29	9.60	4.70	17.02	2.90	5.86	48.96
30	10.20	5.38	16.62	2.87	5.80	52.75
32	16.68	8.50	16.46	2.75	5.99	50.96
35	21.24	10.74	21.90	2.27	9.63	50.56
41	23.10	12.42	21.38	2.19	9.76	53.77
45	28.70	17.60	25.42	2.24	11.36	61.32
46	29.28	18.98	26.46	2.23	11.89	64.82
48	30.66	21.04	25.30	2.20	11.53	68.62
50	32.34	22.80	27.00	2.23	12.10	70.50
51	32.36	22.68	26.02	2.14	12.15	70.09

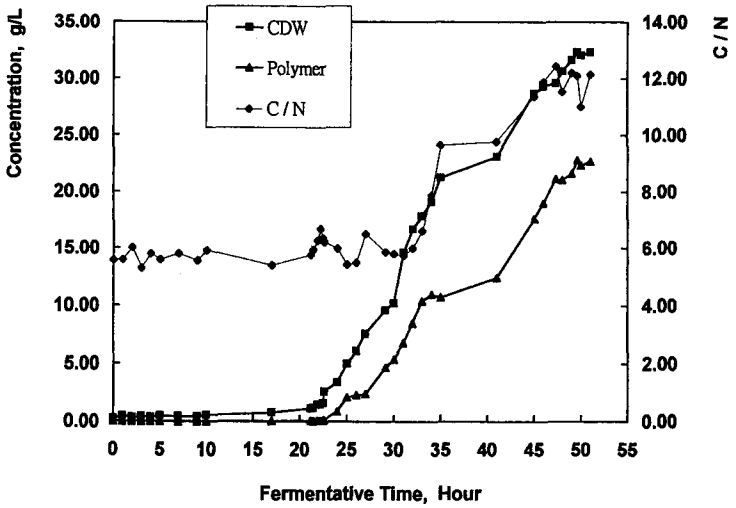

Fig. 4. Changes in C/N ratio during process of cell growth and polymer accumulation.

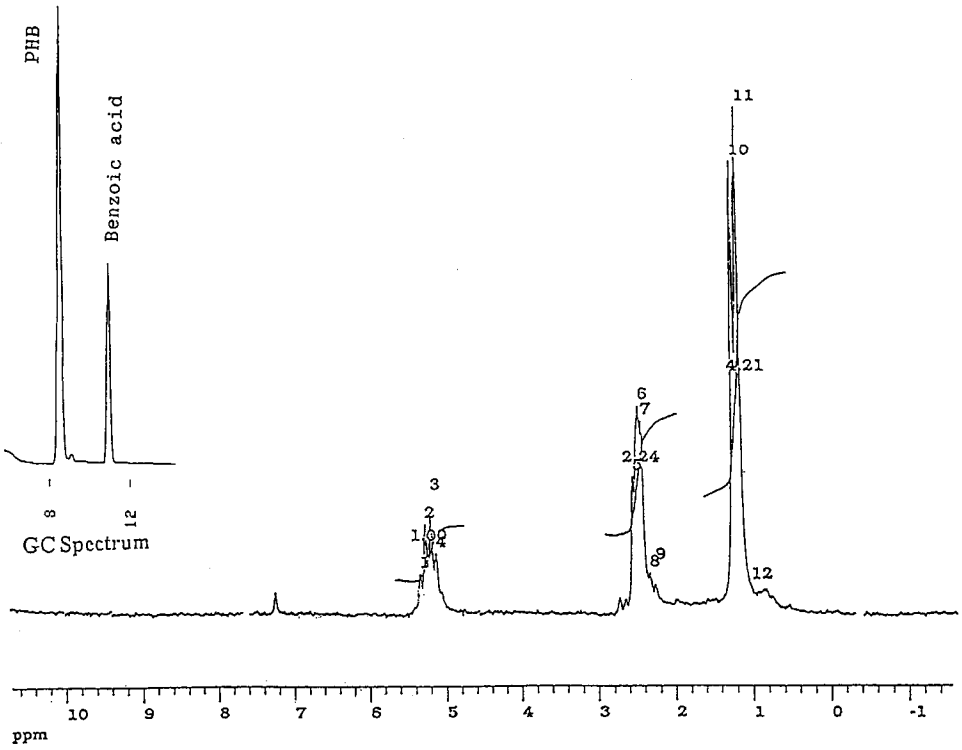

Fig. 5. H^1 NMR spectrum of PHB produced by *Alcaligenes eutrophus* using lactic acid as the sole carbon source.

Data from the gas chromatography and ^1H NMR analysis of the biopolymers produced by fermentation of lactic acid by *A. eutrophus*, and fermentation of malt wastes by *A. latus*, are displayed in Figs. 5 and 6, respectively.

DISCUSSION

The different properties of polymers produced by fermentation of various concentration ratios of butyric and valeric acids used as C substrates are shown in Table 1. The results indicated that the higher the % of the ratio of butyrate to valerate, the higher was the polymer yield, and the mp of the extracted polymer products was also shown to be higher. The texture of the biopolymers produced from substrate of 100% of butyric acid exhibited brittleness. On the contrary, the texture of extracted polymer product fermented from substrate of 100% valeric acid appeared to be more elastic and softer than using other substrates.

The results of fermentation of biopolymers by *A. latus*, using brewery malt wastes as a C source, are shown in Table 2. During fermentation, biopolymers began to accumulate in cells after 22 h of fermentation of malt wastes, and maximized production of the biopolymers after 50 h of fermentation. The results were accorded to the scheme of permitting cell

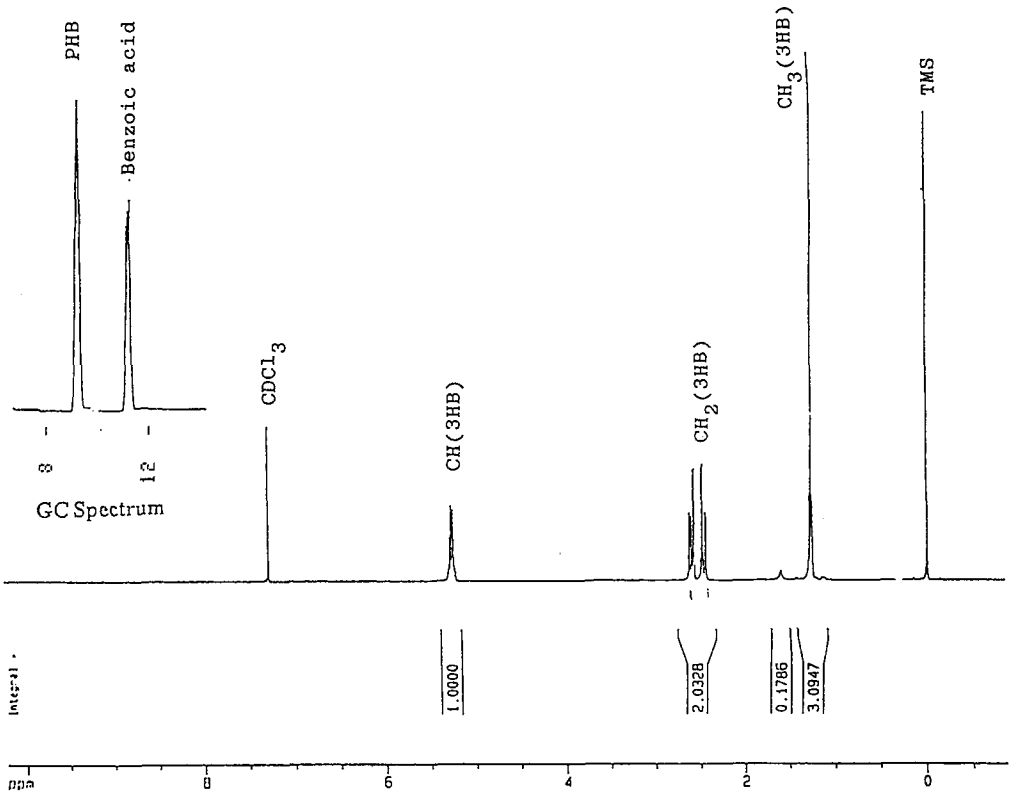

Fig. 6. H^1 NMR spectrum of PHB produced by *Alcaligenes Latus* DSM 1124 using malt wastes as the carbon source.

growth in the initial 22 h of fermentation, using malt wastes, and maximizing biopolymer production in the second part of the fermentation, when a N-limited medium was fed to the fermenter to increase the C/N ratio in the medium. The final biomass and polymer concentration were 32.36 and 22.68 g/L dry wt, respectively, successfully yielding a production of biopolymers of 70.1% dry wt of the biomass. Since the total C consumed during fermentation (140 g C was added to a 2 L vol of medium, and 60 g residual C remained at the end of fermentation) was 80 g/2 L, and the biopolymers produced was 22.68 g/L, the mass yield of bioplastics per mass of C source consumed was calculated to be 56%. The data in Figs. 3 and 4 show that there was a gradual decrease in residual TKN, but a gradual increase of the C/N ratio (from 6 to 12) after 30 of fermentation.

In an earlier study, it was shown that higher C/N ratio (deficiency of N in the medium) would promote the production of polymers by microorganisms *(13)*. However, the fermentation of lactic acid by *A. eutrophus*, and the fermentation of malt wastes by *A. latus*, would produce only PHB, as

Table 3
Copolymer Yields of Bioplastics Biosynthesized with Various Carbon Sources

Experiment	C source	Bacteria	Copolymers[a] mol ratio PHB:PHV
1	Butyric acid	*A. eutrophus*	100:0
2	Valeric Acid	*A. eutrophus*	0:100
3	Lactic acid	*A. eutrophus*	100:0
4	Glucose	*A. latus* DSM 1124	100:0
5	Malt waste	*A. latus* DSM 1124	100:0
6	Glucose	Activated sludge[b]	55:45
7	Fructose	Activated sludge[b]	20:80
8	Malt waste	*A. latus* DSM 1122	100:0
9	Malt waste	Activated sludge[b]	92:8
10	Soya waste	Activated sludge[b]	79:21

[a] Copolymers: PHB, polyhydroxybutyric acid; PHV, polyhydroxyvaleric acid.
[b] Activated sludge, from municipal wastewater treatment plant in Shatin, Hong Kong.

shown in the data of gas chromatography and ^{1}H NMR in Figs. 5 and 6. The shapes of the mass ion peaks looked different in the NMR spectra, because of the decoupling condition of the peaks in one spectrum (Fig. 6) during the process of analysis; the results of both spectra showed only one type of polymer was present, i.e., PHB.

In another study, the authors observed that the fermentation of glucose and fructose as C source by activated sludge, and the fermentation of soya wastes as C source by *A. latus* DSM 1124, would produce PHB–polyhydroxyvolerate (PHV) copolymers. The specific effects of the types of substrates and microorganisms on the copolymer composition (e.g., PHB–PHV) of biopolymers produced, as observed in the laboratory, are summarized in Table 3. Utilizing malt wastes as a C source, fermentation results showed that biopolymers of copolymer composition of 100% PHB, and 92% PHB and 8% PHV, could be synthesized by *A. latus* DSM 1124, and mixed cultures from activated sludge, respectively. On the other hand, mixed cultures from activated sludge could utilize soya waste to produce biopolymers of copolymer composition of 79% PHB and 21% PHV (endowed with physical and thermoplastics properties different than the other bioplastics produced). Thus, specific biopolymers with copolymers of desirable physical and mechanical properties (such as flexibility, tensile strength, and melting viscosity) can be formulated in fermentation by appropriate selection of substrates (or combination of substrates), and the type of PHA-producing microorganisms. The usage of less valuable food wastes as C source in fermentation would tremendously reduce the cost of the production of bioplastics and minimize waste production, and at the same time produce environmentally friendly bioplastics.

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