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Conversion of Industrial Food Wastes by *Alcaligenes Latus* into Polyhydroxyalkanoates

Peter H. Yu,*[,1] Hong Chua,[2] Ai-Ling Huang,[1] and Kwok-Ping Ho[1]

[1]Union Laboratory of Asymmetric Synthesis and Department of Applied Biology and Chemical Technology, E-mail: bcpyu@hkpucc.polyu.edu.hk; [2]Department of Civil and Structural Engineering, Hong Kong Polytechnic University, Hong Kong, China

Abstract

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. As the first step in our pursuit of eventual usage of industrial food wastewater as nutrients for microorganisms to synthesise environmental-friendly bioplastics, we investigated the usage of soya wastes from a soya milk dairy, and malt wastes from a beer brewery plant as the carbon sources for the production of polyhydroxyalkanoates (PHA) by selected strain of microorganism. Bench experiments showed that *Alcaligenes latus* DSM 1124 used the nutrients from malt and soya wastes to biosynthesise PHAs. The final dried cell mass and specific polymer production of *A. latus* DSM 1124 were 32g/L and 70% polymer/cells (g/g), 18.42 g/L and 32.57% polymer/cell (g/g), and 28 g/L and 36% polymer/cells (g/g), from malt waste, soya waste, and from sucrose, respectively. These results suggest that many types of food wastes might be used as the carbon source for the production of PHA.

Index Entries: Polyhydroxyalkanoate (PHA); polyhydroxybutyrate (PHB); malt; soya waste; Alcaligenes; *Alcaligenes latus*.

Introduction

The recent problems concerning the environment and solid waste management have created much interest in the development of biodegradable plastics. Various biodegradable plastics have been produced by

*Author to whom all correspondence and reprint requests should be addressed.

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 have emerged as environmentally-friendly materials. These polymers may retain the desired material properties of conventional synthetic plastics *(1)*, and they are completely biodegradable into carbon dioxide and water within a few months of burial *(2)*. One member of the PHAs, polyhydroxybutyrate (PHB), is a biodegradable, biocompatible thermoplastic with desirable properties such as moisture resistance, piezoelectricity, and optical purity *(3)*. Current applications of PHB for medical and industrial usage include surgical suture, surgical swabs, wound dressing, lubricant for surgeons gloves, and cosmetic and drug containers, and disposable items such as diapers or feminine hygiene products *(4)*. Certain bacteria, such as *Escherichia coli, Clostridia spp.*, and *Alcaligenes eutrophus*, produce PHB as an intracellular metabolite and a back-up carbon source when an unfavorable environment is encountered *(5)*. PHB has not been widely commercially exploited because of its high price compared with traditional thermoplastics *(6)*. Simple organic substrates such as sucrose *(7)*, glucose *(8)* or ethanol, propanol *(9)*, methane *(10)*, and so forth as carbon sources, and inorganic chemicals such as ammonium or ammonia as nitrogen sources are used in the production of PHB *(7,9)*. Recent investigation has shown that copolymers of PHA, such as poly-[3-hydroxybutyrate-copolymer-3-hydroxyvalerate], or expressed as P(3HB-co-3HV), has superior mechanical properties to PHB because it is more flexible and tougher, which extends its versatility in applications such as the manufacture of bottles and films *(11)*. Current commercial production of PHB using sugar as carbon source is not cost-effective. Broader usage of biodegradable plastics in packaging and disposable products as a solution to environmental problems would heavily depend on further reduction of costs *(12,13)*, improving PHB yield from the carbon source *(14)*, and the discovery of novel biodegradable plastics with improved properties. In this study, microbial production of polyhydroxyalkanoates using malt refuse from brewery, wastes from confectionery and ice cream factories, and soya waste from soya milk dairy as carbon and nitrogen sources are reported.

Methods

Microorganism

Alcaligenes latus DSM 1124, a gift from Professor (Dr.) George Chen of Tsinghua University, was maintained on nutrient agar slant at 4°C by monthly subculture.

Media

1. 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, 200 mL tap water. The pH of the media was 7.0. The inoculated liquid seed media was incubated at 35°C in a shaker (Forma Scientific Model 4518 Table Top Incubator Orbital Shaker) at 200 rpm for 24 h.

2. Preparation of fermentation medium: A sample of 300 g dry milled malt or soya wastes was treated with 2500 mL 1 *N* HCl at 100°C for 9 h, and then centrifuged. The 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 filtrate. Finally, the total volume of the fermentation medium was 2.4 L. The aforementioned solution was transferred to the 3.7 L Bioengineering Fermentor. Sterilization was conducted at 121°C for 30 min.
3. Preparation of nitrogen-limited medium: 100 g sucrose, 2 g K_2HPO_4, 3 g Citrate-Fe(III), and 1 g $MgSO_4 \cdot 7H_2O$, were dissolved in 300 mL water, autoclaved for 20 min at 121°C.

Fermentation

The fermentation was carried out in the computer-controlled Bioengineering Fermentor with the growth conditions set at DO = 20%, T = 35°C, and pH 7.0. The fermentor was operated in a batch mode with 2.4 L hydrolyzed food wastes at the beginning of fermentation. At the intervals of every hour, samples of 20 mL were pumped out for cell dry weight (CDW), PHB, total organic carbon (TOC), and total kjeldahl nitrogen (TKN) analysis. At the polymer production phase, the reduction of carbohydrate concentration in the fermentation medium was monitored with the phenol-sulfuric acid method, and a fed-batch mode was operated with a nitrogen-free medium (with sucrose) fed continuously into the fermentor to maintain the carbohydrate concentration between 20–25 g/L and to obtain a higher C/N ratio in the medium for the promotion of PHB synthesis.

Extraction and Precipitation of Biopolymer

After fermentation, the biomass was concentrated by centrifugation at 8000 rpm for 25 min, washed twice, and freeze-dried. Then, 8 g of cells were treated with 100 mL chloroform and 100 mL of 30% sodium hypochlorite. The mixture was agitated in a shaker at 200 rpm, 30°C for 150 min. After the treatment, the dispersion was centrifuged at 4000 rpm for 10 min. The bottom phase was the chloroform layer containing PHB. First the hypochlorite solution phase was removed with a pipet, the chloroform phase was obtained by filtration and concentrated by distillation, and then the PHB material was precipitated by mixing methanol with the concentrated chloroform *(15,16)*.

Analytical Methods

1. CDW analysis: 5 mL fermentation broth was centrifuged, washed with distilled water, and then dried at 105°C for 2 h.

2. TOC analysis: Adequate diluted fermentation-broth supernatant was taken to measure TOC with an ASTRO 2000 TOC Analyzer. A 100 mg carbon/L sucrose solution was used as a standard. The procedure of analysis was according to APHA (4500-Norg) *(16)*.
3. TKN analysis: A 1 mL fermentation broth supernatant was analyzed with a Kjeltec Auto 1030 Analyzer. The method was according to APHA (5310C) *(17)*.
4. Melting point (MP) measurement: Melting point was performed by using a digital melting point apparatus (Electrothermal Co.). The temperature increase rate program was set at 10°C/min.
5. Gas chromatography analysis: Standard PHB was purchased from Fluka Co. Gas Chromatographic analysis was performed on a Varian Model 3700 Gas Chromatograph using a 1/8 inch diameter Chromosorb-WAW column with 80/100 in mesh size, and 6 ft in length (Supelco Co.) according to a method described by Jan et al. *(18)*.
6. ^{1}H nuclear magnetic resonance (NMR) analysis: The analysis was carried out on a Bruker DPX 400 spectrometer using a 5 mm $^{1}H/^{13}C$ dual probe. The NMR spectra were recorded at room temperature from a $CDCl_3$ solution of the extracted biopolymer with 30°pulse angle. Chemical shifts were referenced to the internal reference trimethylsilane (TMS) *(19)*.

Results and Discussion

In the first part of the experiment, a specific culture of *A. latus* DSM 1124 was selected to ferment sugar into bioplastics. The fermentation was done at 35°C for 36 h with fed-batch and dissolve oxygen (DO) controlled between 20 and 40% in a 3.7 L Bioengineering Fermentor. Specific polymer-production yield by *A. latus* DSM 1124 increased to more than 36% polymer/cell (g/g) and 28 g/L cell dry weight with increasing C:N ratio using sucrose as the carbon source (Table 1).

In the second part of the experiment, the conversion of malt wastes (barley) obtained from a local beer brewery plant into bioplastics was investigated. Results of the experimental data displaying the changes in cell dry weight, the production of PHB polymers, and the weight percentage of polymer in the biomass during the course of fermentation are shown in Fig. 1A. The corresponding profiles of TOC, TKN, and carbon to nitrogen ratio (C:N) are shown in Fig. 1B. The polymers and cell mass began to accumulate in cells after 22 h of fermentation of malt wastes, and maximized after 50 h of fermentation. However, the ratio of C:N did not begin to rise until 30 h of fermentation. Specific polymer production yield by

Fig. 1. *(opposite page)* **(A)** The production of polymer by *Alcaligenes latus* using brewery malt wastes as a carbon source, showing cell growth and polymer accumulation. **(B)** The production of polymer by *Alcaligenes latus* using brewery malt wastes as a carbon source, showing residual TOC and TKN in medium during fermentation.

Table 1
Comparison of Fermentative Production of PHB
by Fermentor Using Three Different Carbon Sources by *Alcaligenes latus*

Substrate	Culture time (h)	Final cell conc. (g/L)	Final PHB conc. (g/L)	Final PHB content (%)	Cell productivity (g cell/L·h)	PHB productivity (g PHB/L·h)
Sucrose	69	28.02	10.16	36.26	0.41	0.15
Malt + sucrose	51	32.36	22.68	70.69	0.63	0.44
Soya + sucrose	51	18.42	6.00	32.57	0.36	0.11

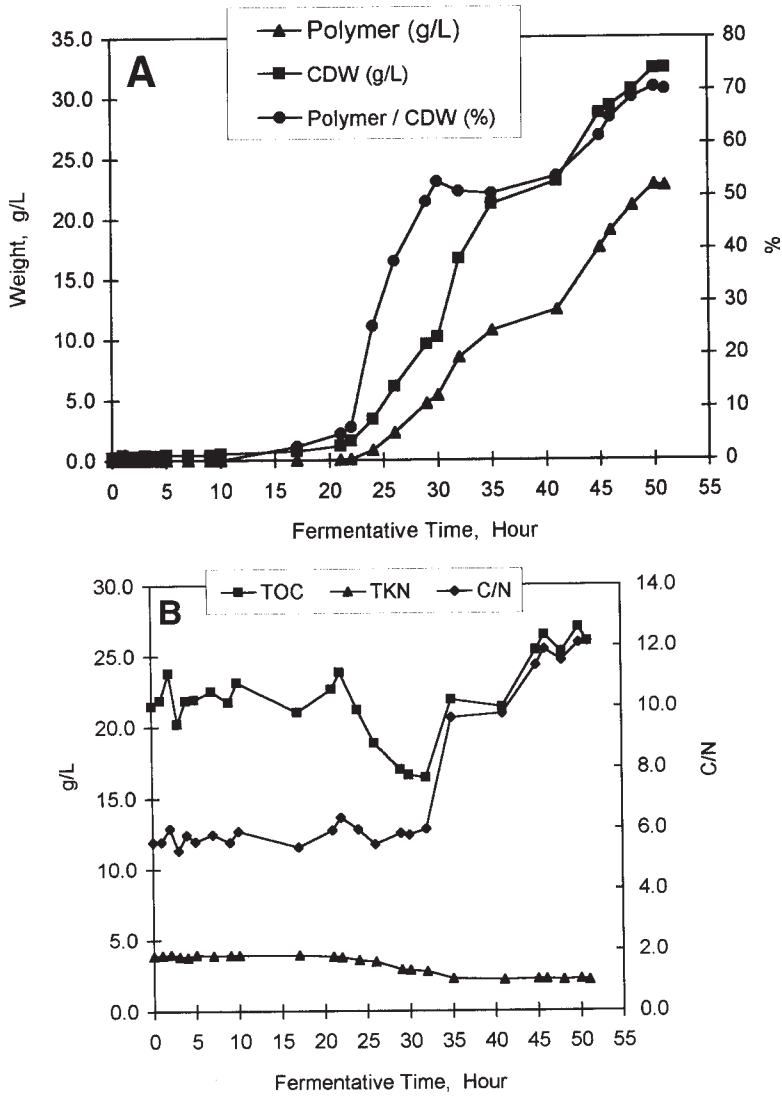

Fig. 1.

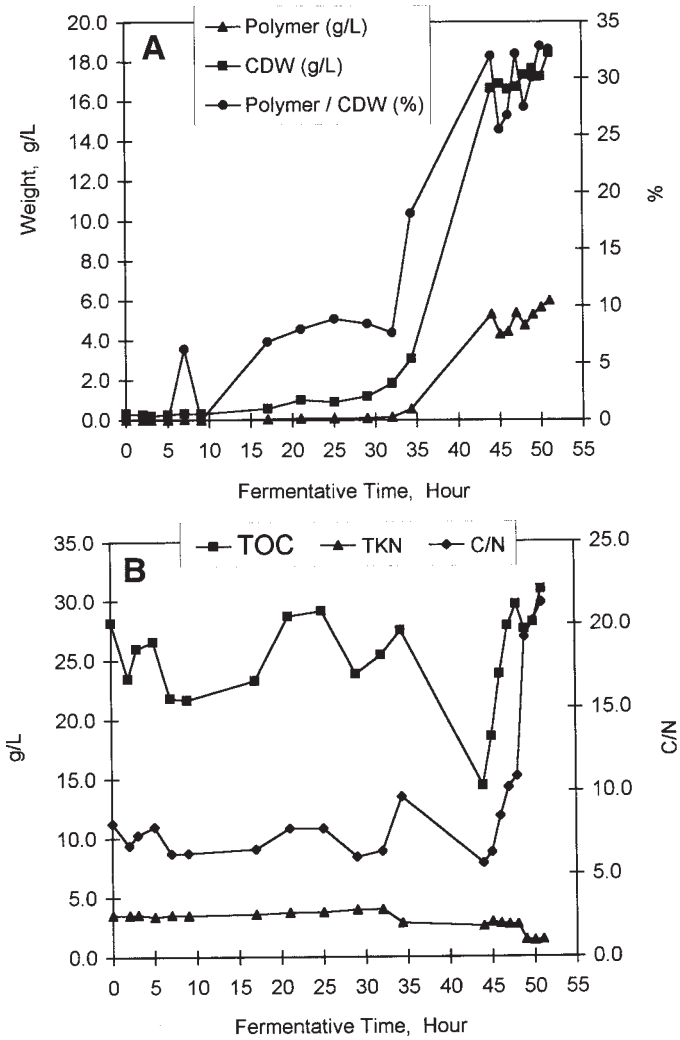

Fig. 2. **(A)** The production of polymer by *Alcaligenes latus* using soya wastes as a carbon source, showing cell growth and polymer accumulation. **(B)** The production of polymer by *Alcaligenes latus* using soya waste as a carbon source, showing residual TOC and TKN in medium during fermentation.

A. latus DSM 1124 increased to 70% polymer/cell (g/g) and 32 g/L cell dry weight using malt wastes as the carbon source during the nitrogen-limited stage of fermentation (Table 1).

In our further study of the ability of *A. latus* to use other types of industrial food wastes other than malt wastes, a soya waste from a local soya-milk manufacturer was used as a carbon source for the microorganisms. The results of the soya fermentation study are shown in Fig. 2A and Fig 2B. The final biomass and polymer concentrations were 18.4 g/L, and 6.00 g/L, respectively, or 32.6 % of the biomass dry weight was polymer; *see* Table 1). In this fermentation, sucrose was not added at the 44th h. The

beginning of rapid carbon consumption, cell growth, and polymer accumulation in the soya wastes experiment were not observed until the 35th h after the commencement of the fermentation, a 10-h delay when compared with the experiment of malt wastes. The results showed that *A. latus* could adapt itself to the fermentation condition of the brewery malt wastes sooner (with a shorter lag phase) than those of soya wastes in the synthesis of the biopolymers. Data comparing fermentative production of PHB by *A. latus* in fermentor using the three different carbon sources indicated that cell productivity and PHB productivity were highest when malt waste was used as the carbon source (Table 1).

From the results of the carbohydrates analysis, we found that the acid-hydrolyzed brewery malt wastes consisted of mainly maltose and lactose, and the acid-hydrolyzed soya milk wastes consisted of chiefly fructose, lactose, and maltose. However, there might be some other types of carbohydrates and proteins present in the wastes that made the differences in polymer yields and the optimized time needed for the polymer accumulation.

The results from gas chromatography (GC) and H^1-NMR analysis of bioplastics produced using malt wastes, and soya wastes, are shown in Fig. 3 and in Fig. 4, respectively. With the comparison of peak retention time of PHB standard and internal standard of benzoic acid, the results of GC clearly indicated that both types of samples of the biopolymers synthesized by *A. latus* using malt (Fig. 3) and soya wastes (Fig. 4) contained PHB. The H^1-NMR analysis of the sample biopolymers also established, with the chemical shifts of the sample peak positions, that the samples were monomer of PHB.

In our previous study, bioplastics were successfully biosynthesized by *A. eutrophus* H16 using different concentration ratios of butyric acid (100 to 0%) and valeric acid (0 to 100%) as the carbon source *(12)*. The extracted polymer products from the substrate of 100% butyric acid exhibited brittleness. In contrast, when using 100% valeric acid as substrate, the extracted polymer product showed more elasticity and was softer than using the other organic acid combinations. The results indicated that the higher the percentage of the ratio of butyrate to valerate, the higher the PHB content, polymer yield, and melting point of the product polymers. It was observed that the melting point of the polymers produced by the *A. latus* using the soya wastes (159°C) was 10°C lower than the PHB standard (170°C) and the polymers produced using the malt wastes (171°C). Thus, the specific type of the carbon source in the fermentation medium could make a difference in the product polymers synthesized by the culture.

The results of our fermentation study using the *A. latus* DSM 1124 showed that the carbon sources of sucrose, malt wastes, and soya wastes did make a difference in the production of PHB polymers under identical fermentation conditions. Not only was the difference in the quantity of polymers and cells produced, but also in the physical properties of the polymers produced, e.g., brittleness, softness, and so forth. In a previous

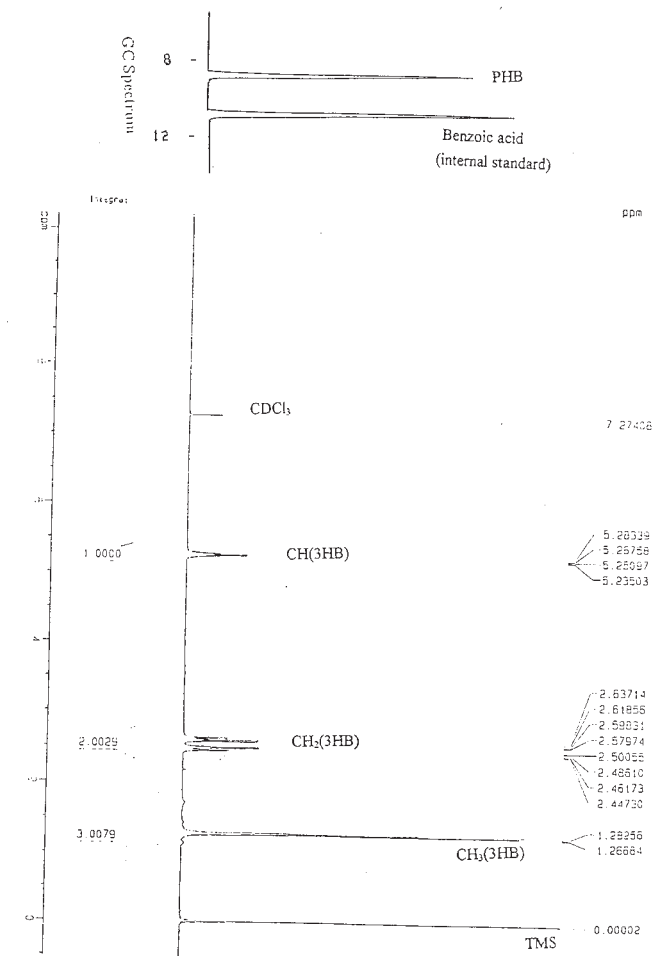

Fig. 3. GC spectrum and ^{1}H NMR spectrum of polymer produced by *Alcaligenes latus* DSM 1124 using brewery malt wastes as a carbon source.

study, we observed that the fermentation of glucose and fructose as carbon source by microorganisms from municipal activated sludge, and the fermentation of soymilk wastes as carbon source by *A. latus* DSM 1124, would produce PHB-PHV copolymers of distinctive copolymer ratio, endowing specific physical and thermoplastic properties typical of copolymers *(13,20,21)*. However, the fermentation of lactic acid by *A. eutrophus*, and the fermentation of malt wastes by *A. latus*, would produce 100% PHB as suggested by our data of GC and H^1-NMR *(21)*. Thus, the types of substrates used as the carbon source, and the types of microorganisms present, might affect the type of copolymers produced.

The results of this fermentation study showed it is feasible to produce polymers by *A. latus* using sucrose, malt, and soya wastes as carbon sources, but the rate and amount of polymers produced were dependent on the type of carbon source. Data from GC and H^1-NMR verified that the biopolymers

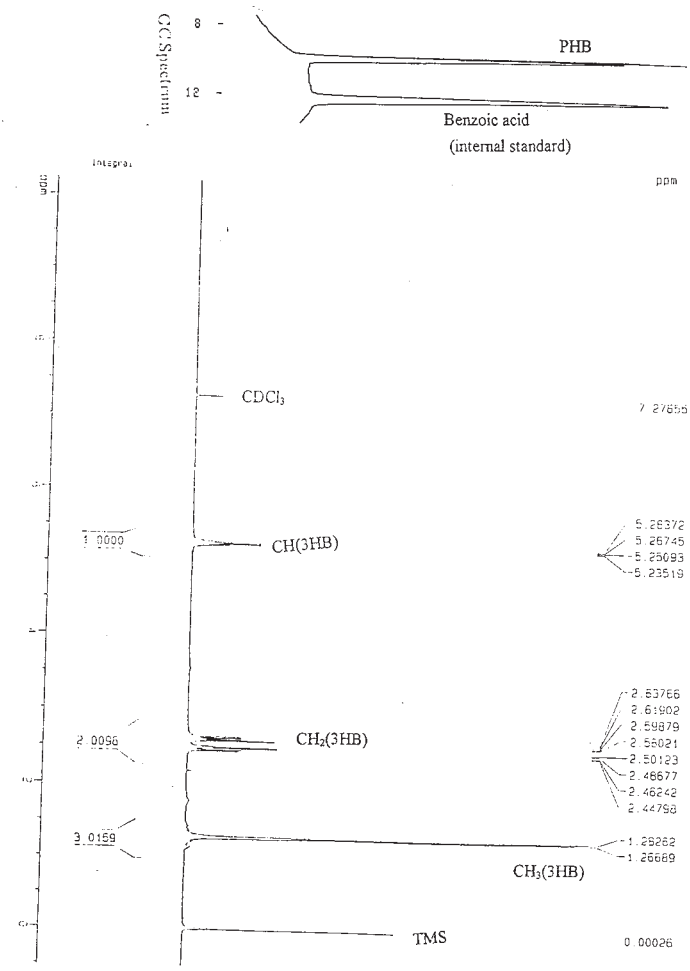

Fig. 4. GC spectrum and ^{1}H NMR spectrum of polymer produced by *Alcaligenes latus* DSM 1124 using soya wastes as a carbon source.

produced were PHB. The use of the food wastes as carbon source to generate bioplastics would tremendously reduce the cost of the production of bioplastics, and at the same time reduce waste production.

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