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Microbial Production of Poly-β-Hydroxybutyric Acid from D-Xylose and Lactose by *Pseudomonas cepacia*

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Poly-β-hydroxybutyric acid (PHB) was produced from xylose and lactose by using *Pseudomonas cepacia*. Approximately 50% PHB (grams of PHB total/grams of biomass total) was produced. With a laser-based fluorescent probe, β-galactosidase activity was shown to be induced in *P. cepacia* cells grown on lactose but not in those grown on glucose or xylose. *P. cepacia* has the potential to produce biodegradable thermoplastics from hemicellulosic hydrolysates and cheese whey.

Because of disposal concerns, plastic production costs are expected to be increasingly influenced by environmental legislation (16). Thus, environmental pressures are forcing polymer manufacturers to consider biodegradable polymers as an alternative polymeric material. Poly-β-hydroxybutyric acid (PHB) and poly-β-hydroxyalkanoic acids, biodegradable thermoplastics, can be produced from a wide range of substrates by using bacteria (2). These biodegradable thermoplastics can be used as packaging material and can be utilized as drug delivery systems, since these polymers are immunologically inert (12).

Several factors influence the economics of biodegradable polymer production. Such factors include substrate cost (8, 9) and the ability to produce biodegradable polymers from inexpensive and renewable carbon sources, such as xylose and lactose (6). Recent estimates indicate that 435 million dry tons of agricultural and forestry residues containing xylose is produced per year (15). Lactose is generated in large volumes from cheese whey. About 28 billion pounds of liquid cheese whey is currently wasted in the United States (20).

We demonstrate in this report that PHB can be produced from xylose and lactose by using the wild-type microorganism *Pseudomonas cepacia* ATCC 17759 (American Type Culture Collection, Rockville, Md.). Moreover, by using a laser-based fluorescent probe, we found that β-galactosidase activity can be induced in *P. cepacia* cells grown on lactose but not in those grown on glucose or xylose. In addition, quantitative fermentation parameters for both xylose and lactose fermentations are reported. We demonstrate that *P. cepacia* can accumulate between 48 and 56% PHB, suggesting that *P. cepacia* is an attractive candidate for use in both bioremediation of industrial wastes and production of biodegradable plastics.

P. cepacia was maintained on nutrient agar (Difco) plates with glucose, xylose, or lactose (10 g/liter) as the carbohydrate source. Long-term stock cultures were maintained at 25°C and subcultured every 1 to 2 weeks. Seed cultures were prepared by inoculating 125 ml of defined medium (in a 300-ml Bellco flask) with a loop of cells from a 24- or 48-h-grown plate of *P. cepacia*. The defined medium consisted of the following ingredients (per liter): 30 g of glucose or xylose or 12 g of lactose, 10 g of $(NH_4)_2HPO_4$ or 2.0 g in nitrogen-limited cultures, 0.5 g of Na_2SO_4, 7.5 g of KH_2PO_4, and 25 ml of a trace element solution. The trace element solution consisted of the following ingredients (per liter): 0.5 g of citric acid, 2.5 g of KH_2PO_4, 0.5 g of $MgSO_4 \cdot 7H_2O$, 0.05 g of $CaCl_2 \cdot 2H_2O$, 35 mg of $FeSO_4 \cdot 7H_2O$, 7 mg of $MnSO_4 \cdot H_2O$, 11 mg of $ZnSO_4 \cdot 7H_2O$, 1 mg of $CuSO_4 \cdot 5H_2O$, 2 mg of $CoCl_2 \cdot 6H_2O$, 1.3 mg of $Na_2MoO_4 \cdot 2H_2O$, 2 mg of H_3BO_3, 0.35 mg of KI, and 0.5 mg of $Al_2(SO_4)_3$. The seed culture was incubated at 28°C and 250 rpm for 24 h (48 h for cells grown on lactose), and the cells were used to inoculate the nitrogen-rich phase of the fermentations.

P. cepacia should only be able to grow on lactose if it is capable of producing β-galactosidase. By using a laser-based fluorescent probe for β-galactosidase, we found that β-galactosidase activity is induced in *P. cepacia* cells grown on lactose; these cells produced a relative fluorescence intensity 14.4 times higher than that of the control. In contrast, *P. cepacia* cells grown on xylose or glucose did not show β-galactosidase activity, producing a relative fluorescence intensity identical to that of the control. β-Galactosidase activity measurements were performed by using a slightly modified form of the procedure of Basile et al. (5).

Fermentations were performed in two stages. In the first phase, growth was promoted by using excess $(NH_4)_2HPO_4$ (10 g/liter) and PHB production was promoted in the second phase by limiting $(NH_4)_2HPO_4$ (1.1 g/liter). Cells from the seed culture were inoculated (10% [vol/vol]) into 500 ml of defined medium in a 2,000-ml Erlenmeyer flask and incubated at 28 to 30°C and 250 rpm for approximately 24 h (48 h for cells grown on lactose). The initial concentration of xylose or glucose, in both phases of the fermentations, was 30 g/liter. When lactose was used, the concentration was 12 g/liter. The initial pH in the shaker flasks was 7.0. After the cell density reached 6 to 8 g/liter (dry cell weight), the cells were centrifuged at 10,000 rpm (15,300 × *g*) and used to inoculate the nitrogen-limited phase of the fermentation. Cells from the growth phase of the fermentation were resuspended in 1,250 ml of defined medium [nitrogen limited, 2.0 g of $(NH_4)_2HPO_4$ per liter] in 2,800-ml Fernbach flasks. After reinoculation, the average initial pH was 6.3. The cells were incubated at 28 to 30°C and 250 rpm for 48 h (longer for cells grown on lactose). Fermentations were performed in duplicate and were repeated to verify the precision of the experiments. Error bars for each datum point in the figures were calculated from the mean and standard deviation of the duplicate reactors in each experiment. In some cases, the error bars were smaller than the symbols used to represent the data. The kinetic parameters reported in Table 1 were calcu-

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TABLE 1. Fermentation parameters for PHB production by *P. cepacia*

Substrate	μ_{max}[a] (1/h)	Yx/s[b] (g/g)	% PHB_{max}[c] (g/g)	$Y_{PHB,\ max}$[d] (g/g)	$q_{PHB,\ max}$[e] (g/g/h)
Glucose	0.32 ± 0.19	0.38 ± 0.13	46.6 ± 4.80	0.145 ± 0.09	0.017 ± 0.008
Xylose	0.34 ± 0.19	0.44 ± 0.08	48.8 ± 42.2	0.110 ± 0.05	0.018 ± 0.005
Lactose	0.10 ± 0.01	0.41 ± 0.06	56.0 ± 0.17	0.147 ± 0.08	0.016 ± 0.001

[a] μ_{max}, maximum specific growth rate.
[b] Yx/s, biomass yield (grams of total dry biomass produced per gram of substrate consumed).
[c] Maximum percentage of PHB accumulated in cells ([grams of PHB/gram of biomass] × 100).
[d] $Y_{PHB,\ max}$, PHB yield (grams of PHB produced per gram of substrate consumed).
[e] $q_{PHB,\ max}$, maximum specific rate of PHB production (grams of PHB produced per gram of dry biomass produced per hour).

lated from the mean of the individual parameters from two to four separate experiments. The specific rates were calculated as described by Roberts et al. (19) and Bailey and Ollis (3) and based on total dry cell biomass.

The maximum specific growth rates of cells grown on xylose and those grown on glucose were similar, but that of cells grown on lactose was significantly lower (Table 1). For each substrate, the maximum specific growth rate occurred during the first phase of the *P. cepacia* fermentations, while cell growth significantly declined in the second phase of the fermentations (Fig. 1, 2, and 3). Although the growth rate was lower on lactose, the biomass yield (grams of total dry biomass produced per gram of substrate consumed) was approximately the same (Table 1) as that of xylose- and glucose-grown cells. Xylose, glucose, and lactose concentrations were determined by the high-pressure liquid chromatography method of Kastner et al. (14).

The substrate consumption rate was dependent on the carbon source. *P. cepacia* cells consumed lactose at a slower rate than xylose and glucose. The maximum specific lactose consumption rate was 0.2 g/g/h, compared with 0.49 g/g/h for xylose and 0.52 g/g/h for glucose. The specific substrate consumption rate (grams of substrate consumed per gram of dry biomass per hour) was calculated for each point after time zero, and the maximum value is reported.

P. cepacia cells began to accumulate PHB as the stationary

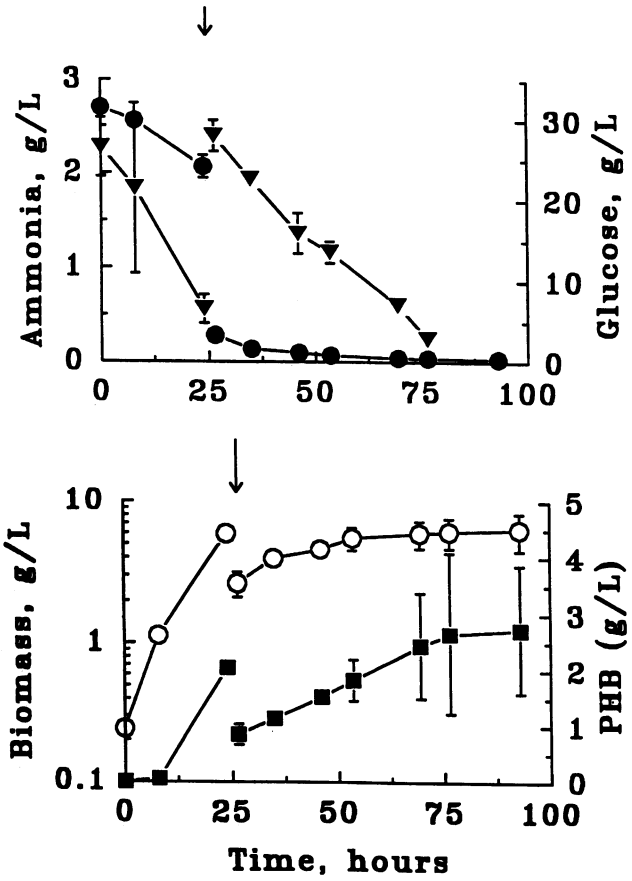

FIG. 1. Growth (dry biomass, ○) and production of PHB (■) on glucose (▼) under nitrogen-limited conditions (NH_3, ●). The arrow indicates the point at which the cells were reinoculated into fresh, nitrogen-limited medium.

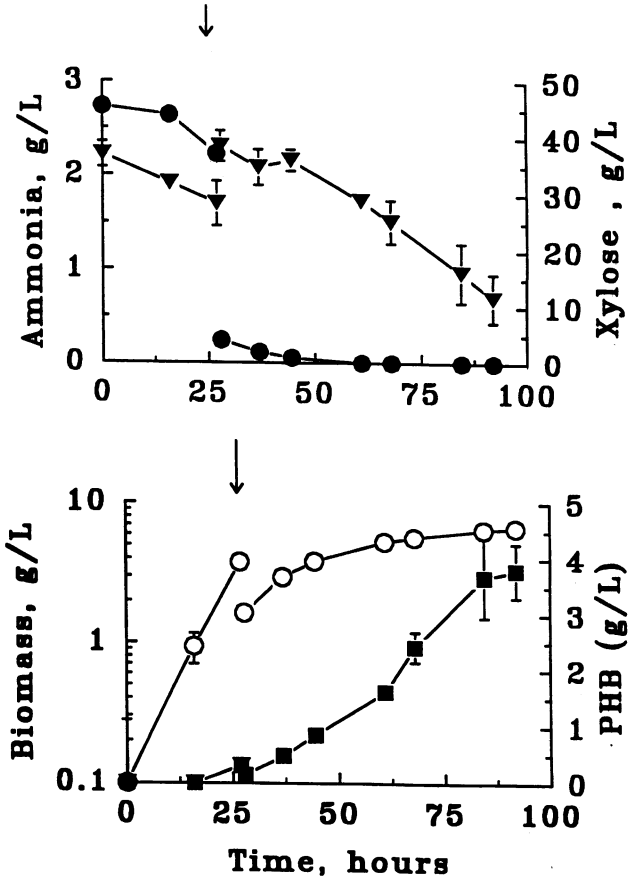

FIG. 2. Growth (dry biomass, ○) and production of PHB (■) on xylose (▼) under nitrogen-limited conditions (NH_3, ●). The arrow indicates the point at which the cells were reinoculated into fresh, nitrogen-limited medium.

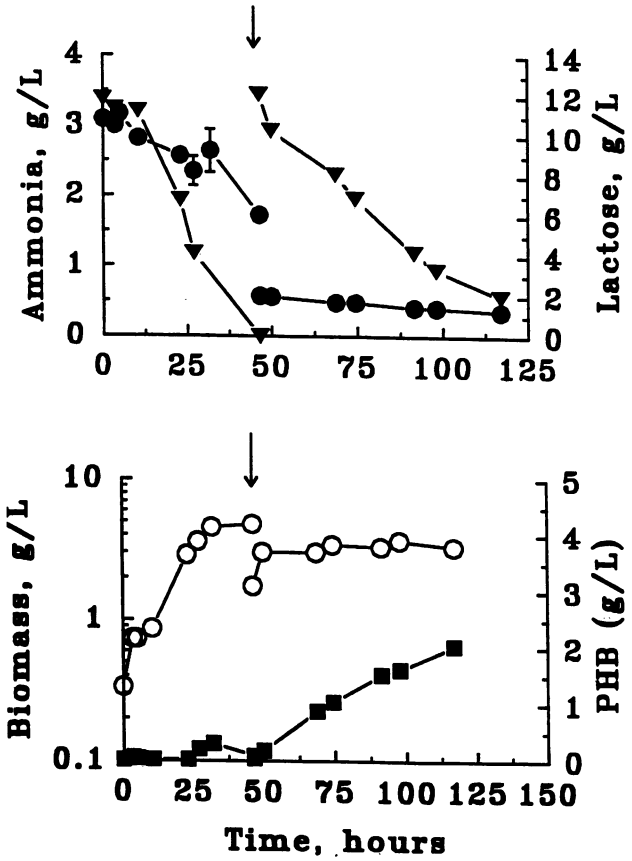

FIG. 3. Growth (dry biomass, ○) and production of PHB (■) on lactose (▼) under nitrogen-limited conditions (NH_3, ●). The arrow indicates the point at which the cells were reinoculated into fresh, nitrogen-limited medium.

growth phase was approached, and it continued to accumulate within the cells during the nitrogen-limited phase (Fig. 1, 2, and 3). *P. cepacia* cells grown on glucose, xylose, and lactose all accumulated similar percentages of PHB (Table 1). The maximum specific rates of PHB production (grams of PHB produced per gram of dry biomass produced per hour) from glucose, xylose, and lactose were similar (Table 1). PHB concentrations were determined by using the gas chromatographic method of Comeau et al. (10) with slight modifications. The volatile methyl esters obtained from the methanolysis reaction were identified by comparison of both their retention times and their mass spectra with those of standard methyl β-hydroxybutyrate by using a VG 70-SE mass spectrometer. PHB structure characterization was performed by ^1H nuclear magnetic resonance analysis with a Gemini 300 (300 MHz) spectrometer in deuterochloroform. The resulting PHB spectrum is identical to the ^1H nuclear magnetic resonance reported by Ishizaki and Tanaka (13).

Bacteria that produce PHB and polyhydroxyalkanoates generally produce polymers with relatively high molecular weights, making them suitable for processing. The PHB molecular weights reported for these experiments were lower than those reported for PHB produced from glucose and xylose by *P. pseudoflava* (6). The residence time of *P. cepacia* cells in the nitrogen-limited (second) phase of fermentation affects the molecular weight of the PHB. Cells which remained in the nitrogen-limited phase for approximately 48 h produced from xylose, lactose, and glucose PHB molecular weights of approximately 6×10^5, 6×10^5, and 5×10^5, respectively. Under similar conditions, PHB with a molecular weight of approximately 1×10^6 was produced by *P. pseudoflava* from xylose and glucose (6). The longer 72-h fermentation resulted in PHBs with lower molecular weights from xylose- and glucose-grown cells (Table 2). This same general trend of lower molecular weights with longer fermentation times was reported by Ballard et al. (4). The molecular weight of the PHB produced from lactose, however, was higher (approximately 873,000) than those of the PHBs produced from xylose and glucose. Also, the polydispersivity of the PHB produced from lactose was lower than that of the PHBs produced from glucose and xylose. The reason for these differences in molecular weight is unclear. Polymer molecular weights were determined by gel permeation chromatography. Molecular weights of the PHBs extracted from the cells were determined by comparing retention times to a polystyrene standard curve and then corrected for the solvent by using Mark-Houwink constants ($K = 1.18 \times 10^{-4}$ ml/g and $\alpha = 0.78$ for PHB and $K = 4.9 \times 10^{-3}$ ml/g and $\alpha = 0.794$ for polystyrene) (1, 7, 18) in the equation $[n] = KM^{\alpha}$, where $[n]$ is intrinsic viscosity, M is molecular weight, and K and α are the Mark-Houwink constants (11).

TABLE 2. Effect of fermentation time[a] on the molecular weights of the PHBs produced by *P. cepacia* from glucose, xylose, and lactose

Substrate	M_w[b] at fermentation time of:		M_n[c] at fermentation time of:		M_w/M_n ratio at fermentation time of:	
	48 h	72 h	48 h	72 h	48 h	72 h
Glucose	498,664	321,541	221,236	91,536	2.30	3.50
Xylose	595,086	265,061	250,855	68,490	2.47	3.96
Lactose	575,260	873,178	322,844	406,697	1.64	2.15

[a] Time from reinoculation into nitrogen-limited medium.
[b] M_w, weight-average molecular weight.
[c] M_n, number-average molecular weight.

All experiments were performed under suboptimal conditions with shaker flasks; thus, oxygen and pH were not controlled during either phase of the fermentations. Although pH did not significantly decline below its initial value of 7 in the first phase of the fermentations, it did decline, however, to 4.2 and 5.2 in the second phase of the glucose and xylose fermentations, respectively. The pH did not significantly decline at any time during the lactose fermentations.

P. cepacia is capable of producing poly-(β-hydroxybutyric-co-β-hydroxyvaleric) acid from mixtures of glucose and propionic acid (17). Thus, *P. cepacia* may be able to produce copolyesters from mixtures of xylose or lactose and organic acids, given its ability to grow and remain viable on carboxylic acids. Industrial PHB production requires a microorganism which accumulates large amounts of the polymer (9). The results reported here demonstrate that *P. cepacia* cells grown on xylose and lactose accumulated between 48 and 56% PHB (grams of PHB total/grams of biomass total). This is one of the highest reported PHB accumulation levels for cells grown on xylose and the first report of PHB production from lactose by a bacterium which was not genetically engineered to metabolize lactose. Moreover, our results suggest that production of PHB from hemicellulosic hydrolysates and cheese whey by *P. cepacia* may be possible, but further research is needed to determine if *P. cepacia* has the potential for commercial production of biodegradable polymers.

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REFERENCES

1. **Akita, S., Y. Einaga, Y. Miyaki, and H. Fujita.** 1976. Solution properties of poly (D-β-hydroxybutyrate). 1. Biosynthesis and characterization. Macromolecules **9:**774–780.
2. **Anderson, A. J., and E. A. Dawes.** 1990. Occurrence, metabolism, metabolic role, and industrial uses of bacterial polyhydroxyalkanoates. Microbiol. Rev. **54:**450–472.
3. **Bailey, J. E., and D. F. Ollis.** 1986. Biochemical engineering fundamentals, 2nd ed., p. 397–398. McGraw-Hill Book Company, New York.
4. **Ballard, D. G. H., P. A. Holmes, and P. J. Senior.** 1987. Formation of polymers of β-hydroxybutyric acid in bacterial cells and a comparison of the morphology of growth with the formation of polyethylene in the solid state, p. 293–314. *In* M. Fontanille and A. Guyot (ed.), Recent advances in mechanistic and synthetic aspects of polymerization. Reidel (Kluwer) Publishing Co., Lancaster, United Kingdom.
5. **Basile, R., K. D. Hughes, P. E. Wisniowski, D. G. Gorenstein, F. E. Lytle, T. S. McCay-Buis, D. M. Huber, and B. C. Hemming.** 1993. Fast and sensitive laser-based enzymatic detection of the lactose operon in microorganisms. Anal. Biochem. **211:**55–60.
6. **Bertrand, J. L., B. A. Ramsay, J. A. Ramsay, and C. Chavarie.** 1990. Biosynthesis of poly-β-hydroxyalkanoates from pentoses by *Pseudomonas pseudoflava*. Appl. Environ. Microbiol. **56:**3133–3138.
7. **Brandrup, J. M., E. H. Immergut, and W. McDowell (ed.).** 1975. Polymer handbook, p. iv–17. John Wiley & Sons, Inc., New York.
8. **Byrom, D.** 1987. Polymer synthesis by microorganisms: technology and economics. Trends Biochem. Sci. **5:**246–250.
9. **Byrom, D.** 1990. Industrial production of copolymer from *Alcaligenes eutrophus*, p. 113–117. *In* E. A. Dawes (ed.), Novel biodegradable microbial polymers. Kluwer Academic Publishers, Dordrecht, The Netherlands.
10. **Comeau, Y., K. J. Hall, and W. K. Oldham.** 1988. Determination of poly-β-hydroxybutyrate and poly-β-hydroxyvalerate in activated sludge by gas-liquid chromatography. Appl. Environ. Microbiol. **54:**2325–2327.
11. **Crow, J. A.** 1993. Using calibration data as a diagnostic tool in GPC. LCGC **11:**668–671.
12. **Hanggi, U. J.** 1990. Pilot scale production of PHB with *Alcaligenes eutrophus*, p. 65–70. *In* E. A. Dawes (ed.), Novel biodegradable microbial polymers. Kluwer Academic Publishers, Dordrecht, The Netherlands.
13. **Ishizaki, A., and K. Tanaka.** 1991. Production of poly-β-hydroxybutyric acid from carbon dioxide by *Alcaligenes eutrophus* ATCC 17697. J. Ferment. Bioeng. **71:**254–257.
14. **Kastner, J. K., and R. S. Roberts.** 1990. Simultaneous fermentation of D-xylose and glucose by *Candida shehatae*. Biotechnol. Lett. **12:**57–60.
15. **Leeper, S. A., and G. F. Andrews.** 1991. A critical review and evaluation of bioproduction of organic chemicals. Appl. Biochem. Biotechnol. **28:**499–511.
16. **Lo, F., J. Petchonka, and J. Hanly.** 1993. Water soluble polymers: trend setters for the 21st century. Chem. Eng. Prog. **July:**55–58.
17. **Ramsay, B. A., K. Lomaliza, C. Chavarie, B. Dube, P. Bataille, and J. A. Ramsay.** 1990. Production of poly-(β-hydroxybutyric-co-β-hydroxyvaleric) acids. Appl. Environ. Microbiol. **56:**2093–2098.
18. **Ramsay, B. A., J. A. Ramsay, and D. G. Cooper.** 1989. Production of poly-β-hydroxyalkanoic acid by *Pseudomonas cepacia*. Appl. Environ. Microbiol. **55:**584–589.
19. **Roberts, R. S., H. W. Hsu, K. D. Lin, and T. J. Yang.** 1976. Amino acid metabolism of myeloma cells in culture. J. Cell Sci. **21:**609–615.
20. **Yang, S. T., H. Zhu, P. Lewis, and I. C. Tang.** 1992. Calcium magnesium acetate (CMA) production from whey permeate: process and economic analysis. Resour. Conserv. Recycl. **7:**181–200.