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PRODUCTION OF POLY(3-HYDROXYBUTYRATE-*CO*-4-HYDROXYBUTYRATE) BY *PSEUDOMONAS ACIDOVORANS*

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Summary

Terpolyesters of 3-hydroxybutyrate (3HB), 4-hydroxybutyrate (4HB) and 3-hydroxyvarelate (3HV) were produced by *Pseudomonas acidovorans* in nitrogen-free culture solutions of 1,4-butanediol and pentanol. When 1,4-butanediol was used as the sole carbon source, a polyester with an unusually high 4HB fraction of 99 mol% was produced.

INTRODUCTION

A wide variety of bacteria accumulate optically active polymers of R-3-hydroxyalkanoic acids with a chain length ranging from 3 to 12 carbon atoms as an intracellular storage material of carbon and energy (Anderson and Dawes, 1990; Doi, 1990; Steinbüchel, 1991). In addition, 4-hydroxybutyric acid, 4-hydroxyvaleric acid and 5-hydroxyvaleric acid were found as constituents of polyesters accumulated by *Alcaligenes eutrophus* (Kunioka et al., 1988, 1989), *A.xylosoxidans* (Valentin et al., 1992) and *Rodococcus* sp. (Haywood et al., 1991). These bacterial poly(hydroxyalkanoates) (PHA) are biodegradable and biocompatible thermoplastics (Holmes, 1988; Doi, 1990), and the industrial scale production of PHA has begun (Byrom, 1987; Hanggi, 1990).

In previous papers (Kunioka et al., 1988, 1989), we reported that the copolyesters of R-3-hydroxybutyrate and 4-hydroxybutyrate, P(3HB-*co*-4HB), were produced by *A.eutrophus* when 4-hydroxybutyric acid, 1,4-butanediol or γ-butyrolactone was fed as the carbon source. The copolymer compositions ranged from 0 to 49 mol% 4HB, depending on the carbon source supplied. This paper reports that *Pseudomonas acidovorans* produces the P(3HB-*co*-4HB) with unusually high proportions of 4HB units (up to 99 mol%) in a nitrogen-free culture solution containing 1,4-butanediol. In addition, we reports that *P.acidovorans* produces a terpolyester of 3HB, 4HB and 3-hydroxyvalerate (3HV) from the mixed carbon substrates of 1,4-butanediol and pentanol.

MATERIALS AND METHODS

P.acidovorans (IFO 13582) was used as a strain in this study. Polyester synthesis was carried out by a two-step cultivation of *P.acidovorans*. The microorganism was first grown under aeration at 26°C in a nutrient-rich medium (100ml) containing 1g yeast extract, 1g polypeptone, 0.5g meat extract and 0.5g $(NH_4)_2SO_4$. The cells were harvested by centrifugation after 48h. Under these culture conditions accumulation of polyester in cells was not observed. To promote polyester synthesis about 0.3-0.4g (dry weight) quantities of the centrifuged cells were transferred into nitrogen-free mineral media (100ml, pH7.1) containing 1,4-butanediol and pentanol as carbon sources. The cells were incubated in these media for 48-96h at 26°C, harvested by centrifugation, washed with water, and finally lyophilized. Polyesters were extracted from the lyophilized cells with hot chloroform in a Soxhlet apparatus and purified by reprecipitation with hexane.

The ^1H nuclear magnetic resonance (NMR) analysis of polyesters was carried out on a JEOL FX-100 spectrometer. The 100-MHz ^1H NMR spectra were recorded at 27°C in a $CDCl_3$ solution of polyester (5 mg/ml) with 45° pulse (15μs), 5s pulse repetition, 1000-Hz spectral width, 8K data points, and 200 accumulations. The compositions of copolyesters were determined by integration of proton resonances in the ^1H NMR spectra (Doi et al., 1986, 1988). The ^{13}C NMR analysis of polyester samples was performed on a JEOL GX-500 spectrometer. The 125-MHz ^{13}C NMR spectra were recorded at 27°C in a $CDCl_3$ solution of polyester (25 mg/ml) with 45° pulse (10μs), 5s pulse repetition, 25000-Hz spectral width, 64K data points, and 15000 accumulations.

All molecular weight data were obtained at 40°C by using a Shimadzu 6A GPC system and 6A refractive index detector with a Shodex K-80M column. Chloroform was used as eluent at a flow rate of 0.5 ml/min, and a sample concentration of 1.0 mg/ml was used. Polystyrene standards with a low polydispersity were used to make a calibration curve.

RESULTS AND DISCUSSION

Table 1 lists the results of the production of polyesters from 1,4-butanediol and pentanol by *P.acidovorans*. When 1,4-butandiol was used as the sole carbon source, the polyester content in dried cells increased to 18 wt% during the course of incubation from 48 to 96h, and the fraction of 4HB repeating units in P(3HB-*co*-4HB) copolymers increased from 93 to 99 mol% during incubation. Thus, *P.acidovorans* produced the P(3HB-*co*-4HB) copolymers with extremely high 4HB compositions of 93-99 mol% from 1,4-butanediol. When pentanol was used as the sole carbon source, a copolyester of 3HB and 3HV units was produced.

The terpolymers of 3HB, 4HB and 3HV were produced by *P.acidovorans* from the mixture of 1,4-butanediol and pentanol. The composition of terpolymers was markedly dependent on the fraction of 1,4-butanediol to pentanol. Fig 1 shows the 125-MHz ^{13}C NMR spectrum of P(5% 3HB-88% 4HB-7% 3HV) terpolymer, together with the chemical shift assignment for each carbon resonance. The carbonyl carbon resonances at 169-172 ppm are clearly resolved into eight peaks, arising from the different diad sequences of connecting the 3HB, 4HB and 3HV units (Doi et al., 1986, 1988). The eight peaks at 169.10, 169.27, 169.48, 170.03, 170.25, 171.86, 172.04, and 172.61 ppm are assigned to 3HB*-3HB, 3HB*-3HV and 3HV*-3HB, 3HV*-3HV, 3HB*-4HB, 3HV*-4HB, 4HB*-3HB, 4HB*-3HV and 4HB*-4HB sequences, respectively. The diad sequence distribution of 3HB, 4HB and 3HV units in the terpolymer was in good

Table 1. Production of polyesters from 1,4-butanediol and pentanol by *P.acidovorans* at 26°C.

Carbon source (g/l)		Incubation	Cell dry	Polyester	Polyester composition[b] (mol%)			Molecular weight	
$HO(CH_2)_4OH$	$CH_3(CH_2)_4OH$	time (h)	wt. (g/l)	content[a] (wt%)	3HB	4HB	3HV	$Mn \times 10^{-4}$	Mw/Mn
5	0	48	3.3	12	7	93	0	21.7	3.4
5	0	72	3.9	16	4	96	0	11.8	4.3
5	0	96	4.3	18	1	99	0	16.8	3.8
4.8	0.2	96	3.8	10	14	80	6	12.3	3.4
4	1	96	5.0	19	5	88	7	9.8	2.6
3	2	96	5.0	13	7	81	12	7.2	2.8
2.5	2.5	96	4.8	11	8	79	13	6.3	3.6
2	3	96	4.6	8	10	72	18	9.5	3.3
1	4	96	4.7	7	16	44	40	11.4	3.2
0	5	48	4.0	12	32	0	68	13.9	5.3

[a] Polyester content in dry cells

[b] Determined from ^{1}H-NMR spectra

agreement with the Bernoullian statistics applicable to a statistically random terpolymerization.

In conclusion, the use of 1,4-butandiol and pentanol as carbon sources for *P.acidovorans* is practical importance in the production of bacterial polyesters with high proportions of 4HB units.

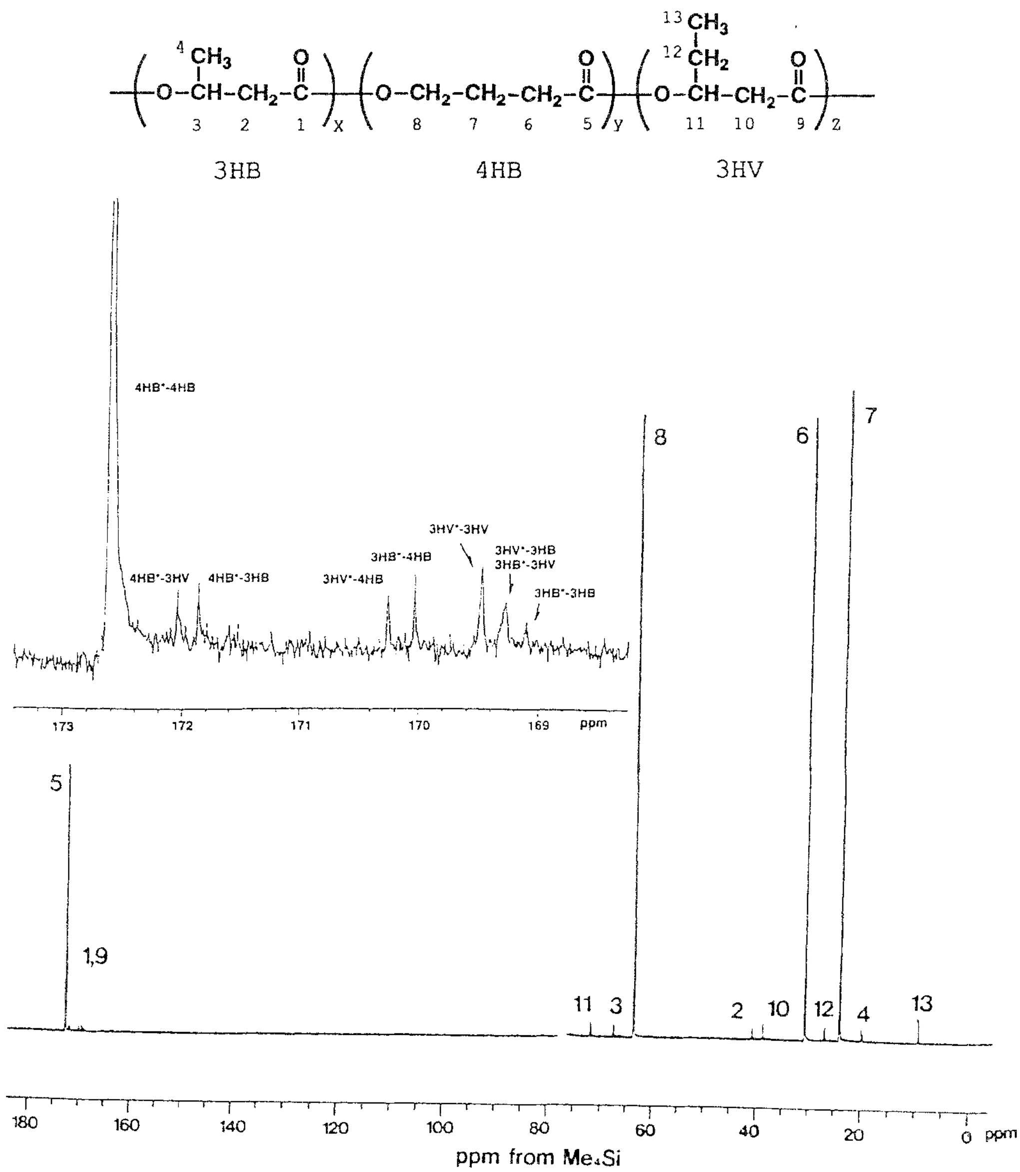

Figure 1. 125-MHz ^{13}C NMR spectrum of P(5%3HB-88%4HB-7%3HV) terpolymer in $CDCl_3$.

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