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Enhanced production of poly(3-hydroxybutyrate-co-3-hydroxyvalerate) by fed-batch cultures of *Pseudomonas* sp EL-2

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***Pseudomonas* sp EL-2 was cultivated to produce poly(3-hydroxybutyrate-co-3-hydroxyvalerate) [P(3HB-co-3HV)] from a structurally unrelated carbon source, glucose, by a fed-batch culture technique. Variation of the carbon to nitrogen (C/N) ratio of the medium produced optimal P(3HB-co-3HV) production at a C/N ratio of 95. Production of P(3HB-co-3HV) was favored by a dissolved oxygen tension of 40%. A maximum biomass concentration of 38 g L^{-1} containing 53% P(3HB-co-3HV) was achieved after 45 h of cultivation. This corresponds to a volumetric productivity of 0.84 g L^{-1} h^{-1}. The copolymer contained 7.5 mol% 3-hydroxyvalerate.** *Journal of Industrial Microbiology & Biotechnology* (2000) **24,** 36–40.

Keywords: P(3HB-co-3HV); copolymer; *Pseudomonas* sp EL-2; fed-batch culture

Introduction

Commonplace plastic objects such as bottles, wrappers, and food containers are highly resistant to degradation in the environment and persist as unsightly litter and can be dangerous to wildlife [8]. Therefore, there is a need to develop degradable polymers that have physical properties similar to those of synthetic plastics.

Polyhydroxyalkanoates (PHAs) are intracellular carbon reserve materials accumulated by a number of bacteria under certain unbalanced growth conditions [1]. Among them, poly-β-hydroxybutyrate (PHB) is a biodegradable thermoplastic polyester which can be used in similar ways to many conventional petrochemical-derived plastics currently in use [9]. However, PHB is a rather brittle material which is difficult to process since it decomposes at a temperature roughly 10°C above its melting point of 179°C [11]. Poly(3-hydroxybutyrate-co-3-hydroxyvalerate) [P(3HB-co-3HV)] shows several improvements, including a drop in melting point, reduction in average crystallinity, and increased flexibility and toughness [13].

Though most P(3HB-co-3HV) producers require propionic acid as cosubstrate, this compound is toxic, so that its concentration must be carefully controlled during fermentation [19]. Therefore, a bacterium producing P(3HB-co-3HV) from single carbon sources would solve such difficulties. Although papers have been published on P(3HB-co-3HV) production from glucose and propionic acid as carbon sources by some bacteria [1], little is known about production of P(3HB-co-3HV) by bacteria having the ability to produce P(3HB-co-3HV) from a structurally unrelated single carbon source except for *Alcaligenes* sp SH-69 [17]. Recently, we reported the synthesis of P(3HB-co-3HV) from glucose and other simple substrates as a structurally unrelated single carbon source by *Pseudomonas* sp EL-2 in the absence of precursor of the 3HV monomer [18,19]. The production of P(3HB-co-3HV) by this genus is a novel and interesting observation.

Fed-batch culture has been the most popular method to achieve a high cell density, which is often necessary for high productivity and yield of the desired product [10,21]. For this purpose, various techniques have been developed for PHB production including fed-batch culture of *Alcaligenes eutrophus* [10,12]. However, there is a lack of published information relating to fed-batch production of P(3HB-co-3HV) by *Pseudomonas* spp from a single carbon source. In order to optimize P(3HB-co-3HV) production in fed-batch culture, we have investigated the influence of C/N ratio on P(3HB-co-3HV) production and the possibility of cultivating *Pseudomonas* sp EL-2 at a high cell density in a fed-batch culture.

Materials and methods

Bacterial strain and media

Pseudomonas sp EL-2 was isolated from activated sludge [19]. Stock cultures were maintained on trypticase soy agar plates at 4°C with transfer every 14 days. The basal salts medium used for all fermentations consisted of 1% glucose, 0.2% $(NH_4)_2SO_4$, 0.45% $Na_2HPO_4 \cdot 7H_2O$, 0.15% KH_2PO_4, 0.02% $MgSO_4 \cdot 7H_2O$, 0.001% $FeSO_4 \cdot 7H_2O$, 0.002% $CaCl_2 \cdot 2H_2O$, and 2 ml trace element solution (H_3BO_3 300 mg, $CoCl_2 \cdot 6H_2O$ 200 mg, $ZnSO_4 \cdot 7H_2O$ 100 mg, $MnCl_2 \cdot 4H_2O$ 30 mg, Na_2MoO_4 30 mg, $NiCl_2 \cdot 6H_2O$ 20 mg, $CuSO_4 \cdot 4H_2O$ 10 mg per liter). The pH of the medium was adjusted to 7.0 with NaOH before sterilization.

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Culture conditions

Seed cultures were inoculated by picking an isolated colony from a plate and transferring cells by loop to a 250-ml Erlenmeyer flask containing 100 ml basal salts medium. Flasks were incubated for 24 h at 30°C and 180 rpm on a rotary shaker. Unless otherwise indicated, fermentations were initiated by inoculating medium with a 10% (v/v) inoculum of seed culture (optical density at 660 nm, 9.0–9.5).

The effect of glucose concentration on the growth of *Pseudomonas* sp EL-2 was studied in 250-ml flask cultures in duplicate using 100 ml working volumes. Cells activated in seed medium were inoculated in medium with 1–90 g L^{-1} glucose. To determine the effect of carbon/nitrogen (C/N) ratio on production of P(3HB-co-3HV), the initial concentration of $(NH_4)_2SO_4$ varied from 0 to 4 g L^{-1} in batch cultures on the basal salts medium containing 10 g L^{-1} of glucose. Shake flask cultures were incubated at 30°C and 180 rpm on a rotary shaker for 24 h.

All fermentor studies were performed using 5-L continuously stirred baffled fermentors (B Braun Co, Melsungen, Germany) equipped with three six-bladed disc-turbine impellers and pH, and pO_2 electrodes. The initial volume of the culture was 2 L. Temperature and pH were controlled at 30°C and 7.0, respectively. The pH was controlled with 2 N HCl solution and 2 N NaOH solution. The aeration rate was 2 vvm (volume of gas per volume of reactor per min) under all conditions. The initial rate of agitation was 300 rpm, and this was increased manually during the early stages of the fermentation to 1000 rpm to maintain dissolved oxygen (DO) at 40% relative to saturation.

Fed-batch fermentation

Fermentation was carried out in two phases: (a) a biomass production phase; (b) a P(3HB-co-3HV) production phase. The feeding method in fed-batch culture was intermittent. Cells were first grown to a concentration of 24 g L^{-1} by feeding sufficient nitrogen source, and then entered the P(3HB-co-3HV) production stage which was stimulated by limiting the nitrogen source. The concentration of glucose and $(NH_4)_2SO_4$ in the feeding medium for the growth stage was adjusted to 300 g L^{-1} and 60 g L^{-1} (C/N = 9.5), respectively. Those for P(3HB-co-3HV) production stage were adjusted to 300 g L^{-1} and 6 g L^{-1} (C/N ratio = 95), respectively. Before the carbon source in the culture broth became depleted, the concentrated glucose solutions were supplied to keep the concentration in the fermentor between 10 and 20 g L^{-1}.

Analytical methods

Cell growth was monitored by measuring the culture turbidity at 660 nm. Cell dry weight (DCW) was determined by washing the centrifuged cells once with distilled water and drying them to constant weight at 105°C. The P(3HB-co-3HV) content of the cells and its composition were determined by gas chromatography as described by Braunegg *et al* [3]. The identity of the individual monomer units were confirmed by 125 MHz ^{13}C-NMR (Varian Unity Plus, Palo Alto, CA, USA). The copolymers were extracted from the dried cells with hot chloroform in a Soxhlet apparatus and purified by reprecipitation with hexane. The melting

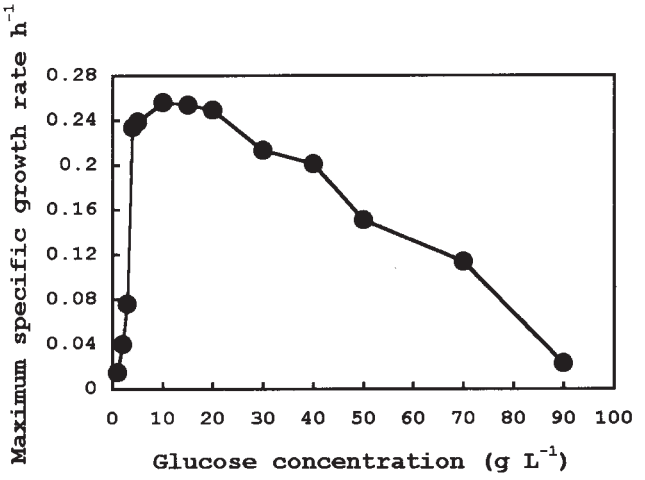

Figure 1 Effect of glucose concentration on the specific growth rate of *Pseudomonas* sp EL-2 grown on basal salts medium. The maximum specific growth rate was calculated by a linear regression method from the logarithm of optical density *vs* time during the early exponential growth phase.

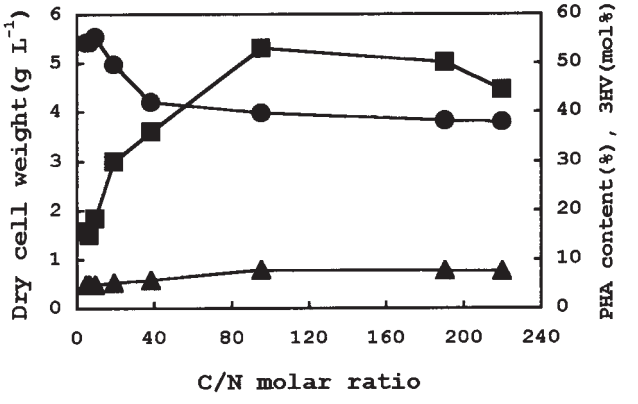

Figure 2 Effect of C/N ratio on PHA production by *Pseudomonas* sp EL-2. ●, Dry cell weight; ■, PHA content; ▲, 3HV.

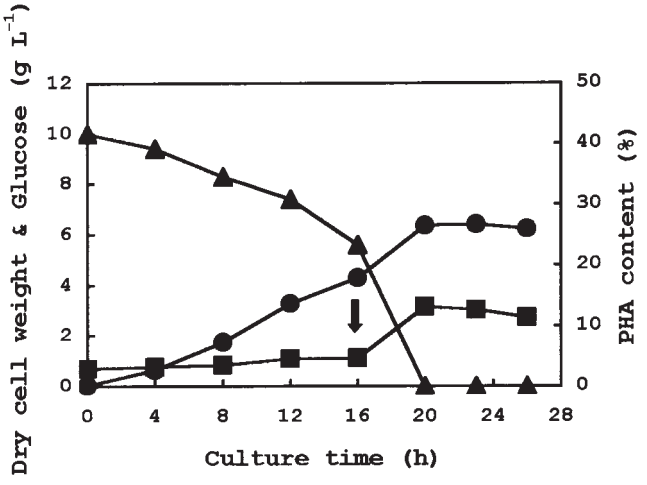

Figure 3 Time course of batch culture of *Pseudomonas* sp EL-2 with glucose as the substrate. The arrow indicates the time at which the nitrogen source was exhausted. After this point, the cells continued to produce P(3HB-co-3HV). ●, Dry cell weight; ■, PHA content; ▲, glucose concentration.

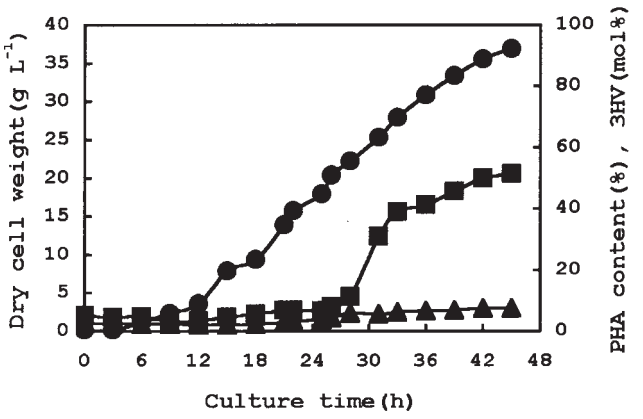

Figure 4 Time course of a fed-batch culture of *Pseudomonas* sp EL-2. ●, Dry cell weight; ■, PHA content; ▲, 3HV.

temperature (T_m) was measured using a Perkin-Elmer differential scanning calorimeter (DSC). The concentration of glucose and of ammonium ions was determined by the dinitrosalicylic acid method [5] and the indophenol blue method [23], respectively.

All treatments were done in duplicate or triplicate cultures and the means of the results of duplicate assays were compared. The variation between replicates in all analytical determinations was less than 5%.

Results and discussion

Effect of glucose concentration on cell growth

Figure 1 shows the growth rate of *Pseudomonas* sp EL-2 cultivated on different amounts of glucose. Growth was significantly affected at glucose concentrations above 20 g L^{-1} which were inhibitory, decreasing the growth rate and increasing the lag time. The optimal glucose concentration was 10 g L^{-1}. All subsequent fed-batch cultures were, therefore, carried out at this glucose concentration.

Effect of C/N ratio on P(3HB-co-3HV) production

In Figure 2 the relation between the C/N ratio and biomass and P(3HB-co-3HV) content are shown. The production of P(3HB-co-3HV) was the highest (52%) at a C/N ratio of 95, while cell growth was the greatest (5.7 g L^{-1}) at 9.5. This indicates that carbon utilization for cell mass build-up is facilitated by the presence of available nitrogen (low C/N molar ratio) in the culture medium. The mole fraction of 3HV monomer in the copolymer was 4.8–7.9 mol%. The 3HV fraction in the copolymer reached the highest level at a C/N ratio of 95. Higher C/N ratios than 95 did not increase the P(3HB-co-3HV) content of cells, which remained at 45% of the DCW. A possible explanation for this is that P(3HB-co-3HV) production is slightly inhibited by nitrogen deficiency. Although nitrogen limitation enhanced PHB production, complete nitrogen deficiency considerably damaged microbial activity including PHB synthesis [20]. This effect was observed by Ramsay *et al* [15] in continuous cultures with *Pseudomonas resinovorans* growing on octanoate in which the C/N ratio was varied. It was also reported that addition of a small amount of ammonia resulted in a more rapid increase in intracellular PHB content than was the case without ammonia feeding [21].

Effect of DO level on P(3HB-co-3HV) production

Two different levels of DO (20 and 40% of air saturation) were studied in batch cultures using the basal salts medium containing 20 g L^{-1} glucose. In these cultures an inoculum of 15% of the culture volume was used. The maximum DCW and P(3HB-co-3HV) content at 20% DO were 7 g L^{-1} and 18%, respectively. At 40% DO, a maximum cell concentration of 9 g L^{-1} and P(3HB-co-3HV) content of 23% were obtained. These results indicated that a high DO level is more beneficial to P(3HB-co-3HV) production than a low DO level. The DO level was maintained at 40% in subsequent fed-batch cultivations. On the other hand, when *Azotobacter vinelandii* UWD was cultivated in oxygen-limited condition, its PHB content per cell increased [14].

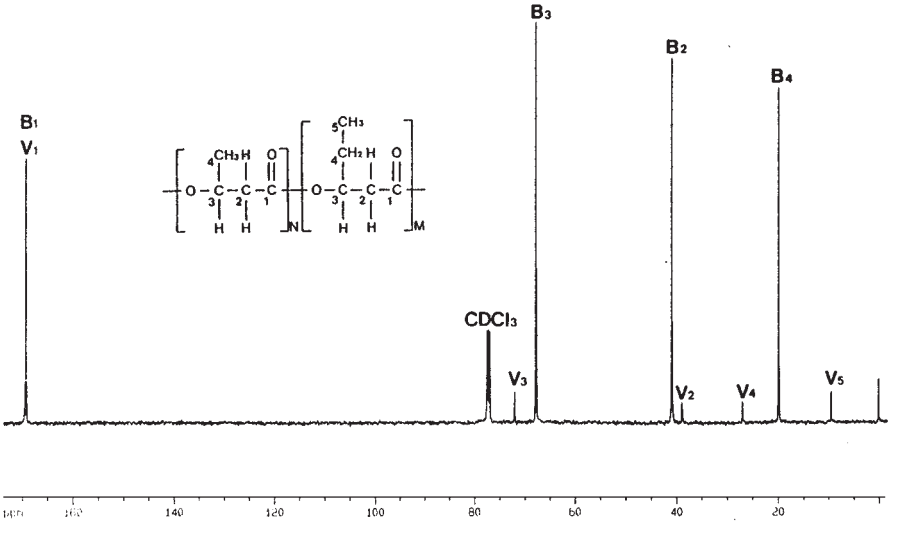

Figure 5 125 MHz ^{13}C-NMR spectrum of PHA isolated from *Pseudomonas* sp EL-2.

Fermentation

Figure 3 shows the time courses of cell growth and P(3HB-co-3HV) production during batch cultivation of *Pseudomonas* sp EL-2 in basal salts medium containing 10 g L^{-1} glucose. Nitrogen limitation occurred after about 16 h, corresponding to 0.16 g L^{-1} of P(3HB-co-3HV) in 4.21 g L^{-1} of DCW or about 3.8% by weight. After this point, the cells were no longer reproducing but continued to accumulate P(3HB-co-3HV). Glucose was exhausted after 24 h, resulting in a final production of 0.89 g L^{-1} of P(3HB-co3HV) in 6.8 g L^{-1} of DCW, corresponding to a P(3HB-co-3HV) content of 13% by weight. After glucose was exhausted, P(3HB-co-3HV) was gradually degraded with time, suggesting that the P(3HB-co-3HV) was utilized for energy generation under conditions of carbon starvation.

It was noted that the C/N ratio of the medium can greatly affect the P(3HB-co-3HV) production and cell growth (Figure 2). Therefore, a two-stage fed-batch cultivation was employed for enhanced production of P(3HB-co-3HV). Figure 4 shows the time course of P(3HB-co-3HV) production in fed-batch culture. Using the intermittent nutrient feeding method as described in Materials and methods, a high concentration of P(3HB-co-3HV) (20 g L^{-1}) with 3HV fraction of 7.5 mol% was obtained in 45 h when the total cell mass concentration reached 38 g L^{-1}. The P(3HB-co-3HV) content of the cells was 53% of the DCW and P(3HB-co-3HV) yield was 0.2 [g P(3HB-co-3HV) g^{-1} glucose]. Similar culture systems have been used to produce PHB to levels as high as 149 g L^{-1}, which were achieved partly because of the use of pure oxygen rather than air [21]. It is noteworthy that an overall P(3HB-co-3HV) productivity of 0.84 [g P(3HB-co-3HV) L^{-1} h^{-1}] was much higher than that from glucose and propionate (0.44 g L^{-1} h^{-1}) for *Alcaligenes eutrophus* DSM 545 [12]. The *Alcaligenes latus* process yielded a productivity of approximately 0.4 g L^{-1} h^{-1} on the basis of a weekly production of 1 ton PHB in a 15 m^3 fermentor [9]. However, in laboratory-scale fed-batch fermentations with *A. eutrophus* a productivity of 2.55 g L^{-1} h^{-1} was reported [10].

Figure 5 shows the 125 MHz ^{13}C-NMR spectrum of P(3HB-co-3HV) produced by *Pseudomonas* sp EL-2 grown on glucose. The indicated peak assignments are straightforward, with the chemical shifts for 3HB and 3HV units of the copolymer in close agreement with values previously reported by Doi *et al* [7].

Ballistreri *et al* [2] reported that increases in the 3HV fraction of copolymer from 0 to 10 mol% decreased the melting point from 180 to 160°C, indicating that the ability of the copolymer to be processed can be significantly improved. Therefore, the purified *Pseudomonas* sp EL-2 copolymer containing 7.5 mol% 3HV should exhibit a decrease in melting temperature. In a DSC experiment, we determined that the melting temperature of the copolymer was 166°C (data not shown). The melting temperature of the copolymer was in good agreement with previously reported values [6].

In the present fed-batch experiment the 3HV fraction amounted to 7.5 mol% of the copolymer. This can be compared with the results of Ramsay *et al* [16] who reported the production of 17 g L^{-1} of P(3HB-co-3HV) containing 5.0 mol% 3HV monomer by using glucose and propionic acid during the fed-batch culture of *A. eutrophus*. The production of P(3HB-co-3HV) by *Pseudomonas* sp EL-2 revealed that the molar fraction of 3HV monomer in copolymer was influenced by the carbon source used and growth conditions and ranged from 1.9 mol% to 49.3 mol% [19]. Even though copolymers with high 3HV mol% are not necessarily more useful than those with low 3HV mol%, alteration of 3HV in P(3HB-co-3HV) is desirable from an industrial viewpoint because it may offer the opportunity for production of different thermoplastics having various degrees of flexibility [22].

P(3HB-co-3HV) is already produced on a biotechnological scale for the manufacture of biodegradable bottles, and it is preferred to PHB since some mechanical properties of the copolymer, such as flexibility, are superior [4]. In contrast to the current process for the production of P(3HB-co-3HV) by *A. eutrophus* [1], propionate need not be provided as a cosubstrate in addition to glucose if *Pseudomonas* sp EL-2 is used. This would not only reduce the cost of the substrate but it would also allow easier control of the fermentation process.

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