

Production of Poly(β -hydroxybutyrate-co- β -hydroxyvalerate) by Two-stage Fed-batch Fermentation of *Alcaligenes eutrophus*

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Production of poly(β -hydroxybutyrate-co- β -hydroxyvalerate)[poly(HB-co-HV)] from glucose and propionic acid was studied in a two-stage fed-batch fermentation using *Alcaligenes eutrophus* NCIMB 11599. When either glucose became sufficient or the feeding rate of propionic acid decreased, production of poly(HB-co-HV) increased but concomitantly resulted in a reduced fraction of HV. During the copolymer accumulation stage, the specific production rate of hydroxyvalerate (HV) increased up to 0.013 (g-HV/g-RCM/h) but it decreased as propionic acid was accumulated. Control of the propionic acid concentration in the medium, therefore, is considered to be one of the most important operating parameters for production of poly(HB-co-HV) with a higher HV fraction. A high titre of poly(HB-co-HV) (85.6 g/l) with HV fraction of 11.4 mol% could be obtained in 50 h by controlling the propionic acid concentration at 1 to 4 g/l.

The poly(β -hydroxybutyrate-co- β -hydroxyvalerate) [poly(HB-co-HV)] copolymer produced by *Alcaligenes eutrophus* has been of practical interest since it offers greater flexibility than the PHB homopolymer. It was obtained without difficulty by adding propionic acid to the culture of *A. eutrophus* (4, 7). Moreover, its hydroxyvalerate (HV) fraction could be controlled by changing propionic acid levels in the medium. Different HV fractions meant different degrees of flexibility of the copolymer obtained from the culture (5). Ballistreri (2) reported that increases in the HV fraction of poly(HB-co-HV) copolymer from 0 to 10 mol% decreased the melting point from 180 to 160°C, indicating that the processibility of the copolymer can be significantly improved. It was noted, however, that an HV fraction higher than 10 mol% is required for practical application of the copolymer.

Although much endeavor has been made toward the production of poly(HB-co-HV), the final titre of the product obtained was relatively low due to the toxicity of propionic acid (4, 8, 12). Recently, Kim *et al.* (9) reported a high yield fermentation system of poly(HB-co-HV) copolymer in which glucose concentration in the medium was controlled by using fee-

ding solutions with different ratios of glucose to propionic acid. It was noted that the product titre decreased from 117 to 64 g/l as the HV fraction increased from 4.3 to 14.3 mol%. The lower product titre with the higher HV fraction might be a result of the difficulty of controlling the propionic acid concentration of the medium. To obtain higher product titre with a high HV fraction, development of a feeding strategy that allows propionic acid concentrations to be controlled at a desired level should be considered. Kim *et al.* (8) developed a pH-stat feeding method to maintain propionic acid concentrations at a low level. However, the final concentration of the product obtained was too low to allow economic production of the copolymer. On the other hand, the concentration, not only of propionic acid, but also of glucose, in a culture broth can directly influence copolymer synthesis since it serves mostly as a substrate for composition of the HB unit of the copolymer.

In this study, therefore, a series of fed-batch fermentations were carried out to examine the effects of glucose and propionic acid on the accumulation of poly(HB-co-HV) in detail. Production of poly(HB-co-HV) with a high HV fraction was also attempted by controlling propionic acid concentrations in the culture broth during the copolymer accumulation stage.

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Key words: poly(β -hydroxybutyrate-co- β -hydroxyvalerate), poly(HB-co-HV), fermentation, *Alcaligenes eutrophus*

MATERIALS AND METHODS

Bacterial Strain

Alcaligenes eutrophus NCIMB 11599, one of the glucose utilizing mutants derived from *A. eutrophus* H-16, was used throughout the experiments.

Fed-batch Fermentation

A two-stage fermentation technique (11) was employed for enhanced production of the poly(HB-co-HV): Cells were first grown to the concentration of 60-70 g/l by feeding sufficient ammonium source, and then entered the copolymer accumulation stage which was stimulated by limiting the nitrogen source. Media used for fed-batch fermentation are shown in Table 1. Glucose concentration was maintained within the range of 10-20 g/l, unless otherwise specified, over the whole period of culture by controlling supply of the feed solution manually. Ammonium hydroxide solution (28%) was supplied as the nitrogen source at the amount required for pH control. In the copolymer accumulation stage, supply of ammonium hydroxide solution was replaced by 4N NaOH/KOH solution to induce nitrogen limiting conditions. During this stage, glucose and propionic acid were fed separately into the culture broth as described in the text. Fed-batch fermentations were carried out in a 5-liter jar fermentor (Korea Fermentor Co., Incheon, Korea) equipped with a dissolved oxygen (DO) analyzer and a pH controller. 200 ml of the seed culture, cultivated at 30°C for 24 h in shake flasks, was transferred to the fermentor containing 1.8 liter of the fermentation medium. The pH was controlled at 7.0. Dissolved oxygen concentration was maintained at above 10% of air saturation by controlling both the agi-

Table 1. Fermentation media for two-stage fed-batch operation.

| Component | Media (g/l) | | |
|--|--------------|---------------------------------------|-------------------------|
| | Basal medium | First-stage feed stock ^(a) | Second-stage feed stock |
| Glucose | 20.0 | 700 | 700 |
| Propionate | - | - | ^(b) |
| (NH ₄) ₂ SO ₄ | 3.0 | 0 | 25.0 |
| Na ₂ HPO ₄ ·12H ₂ O | 3.32 | 33.2 | 8.3 |
| KH ₂ PO ₄ | 0.83 | 8.3 | 2.1 |
| MgSO ₄ ·7H ₂ O | 0.2 | 8.0 | 4.0 |
| FeSO ₄ ·7H ₂ O | 0.02 | 0.2 | 0.1 |
| CaCl ₂ ·2H ₂ O | 0.01 | 0.1 | 0.05 |
| Trace element ^(c) | 2 ml | 20 ml | 10 ml |
| Na-citrate | - | 2.5 | 2.5 |

(a); In the first stage, ammonia water (28%) was supplied as the nitrogen source along with control of pH, (b); Supply of propionate in the second stage was described in the text, (c); Trace element composition (per liter): 0.3 g H₃BO₃, 0.2 g CoCl₂·6H₂O, 0.1 g ZnSO₄·7H₂O, 30 mg MnCl₂·4H₂O, 30 mg Na₂MoO₄·2H₂O, 20 mg NiCl₂·6H₂O, 10 mg CuSO₄·5H₂O.

tation speed and the aeration rate up to 1000 rpm and 2.0 vvm, respectively.

Analytical Methods

Cell growth was monitored by measuring the optical density of the culture broth at 600 nm. The cell concentration was also determined by measuring the dry cell weight. Glucose concentration was determined by a glucose analyzer (YSI, Ohio, USA). Phosphate was measured according to the procedure of Bartlett (11) and ammonia by the indophenol method (13). Poly(HB-co-HV) concentration was determined by gas chromatography (Hewlett Packard, Avondale, USA) with benzoic acid as an internal standard (3). Propionic acid was analyzed with a HPLC (TOSHO CCPE, TOSHO, Japan) with a TSK-GEL column (4.6×150 mm, TOSHO, Japan). NH₄H₂PO₄ (5 mM), adjusted to pH 2.4 with phosphoric acid, was used as an elution buffer at a flow rate of 1.0 ml/min.

RESULTS AND DISCUSSION

Effect of Inorganic Salt on Poly(HB-co-HV) Accumulation

The effect of inorganic salts on poly(HB-co-HV) accumulation was studied in flask cultures. It was observed that poly(HB-co-HV) accumulation was enhanced in the presence of (NH₄)₂SO₄ and MgSO₄·7H₂O (Fig. 1). It is of particular interest to note that the HV

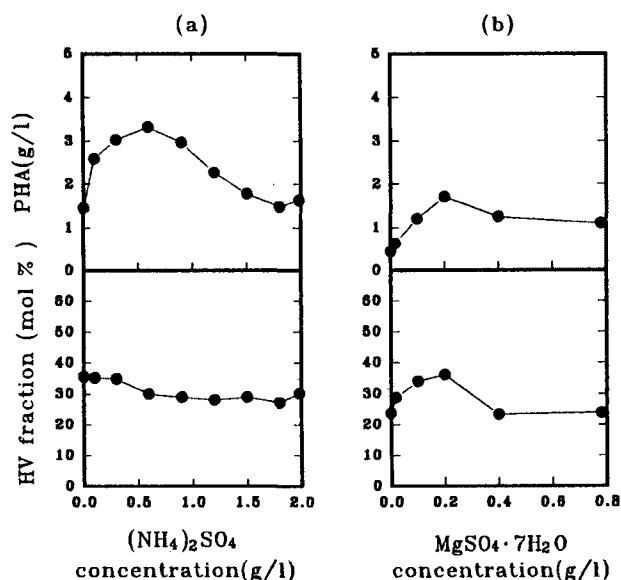


Fig. 1. Effect of inorganic salt on poly(HB-co-HV) accumulation and HV content of the copolymer.

Two-stage culture technique was employed in flask cultures. The cells were first grown at 30°C for 29 h in LB medium, then washed cells were transferred to the minimal medium containing different concentration of nutrient.

fraction (the ratio of the amount of hydroxyvalerate to total copolymer) in the copolymer reached the highest level when 0.2 g/l of MgSO₄·7H₂O was added. After a series of flask cultures with various sources of inorganic salts, media for fed-batch operations shown in Table 1 were designed and used throughout the study.

Effect of Glucose and Propionic Acid on Poly(HB-co-HV) Accumulation

Two-stage fed-batch fermentation technique is often recommended for increased production of polyhydroxyalkanoates (PHAs), in which the cell growth stage is followed by the PHA accumulation stage. Since this technique has been successfully used for the production of PHB (11), it was also employed for copolymer production in this study. Two sets of fed-batch operations were carried out to examine the effect of glucose and propionic acid on copolymer production: (1) Fixed feeding rates of propionic acid under different conditions of glucose concentration and (2) different feeding rate of propionic acid while maintaining glucose concentration at 10-20 g/l. Production of the copolymer increased from 32 to 54.6 g/l as glucose in the medium became more available, while the HV fraction in the copolymer decreased from 23.7 to 7.8 mol% (Fig. 2). It was also noted that copolymer production increased from 54.6 to 76.7 g/l as the feeding rate of propionic acid decreased, while the HV fraction in the copolymer was even further reduced from 7.8 to 3.6 mol% (Fig. 3). From these results, it can be seen that the microorganism utilizes glucose more favorably than propionic acid to accumulate the copolymer when both glucose and propionic acid are

present, resulting in a reduced fraction of HV. This is probably due to the fact, as reported by Haywood *et al.* (6), that 3-ketothiolase, the key enzyme in the PHB biosynthetic pathway, has higher affinity to acetyl-CoA than propionyl-CoA. It is also interesting to note that, although only propionic acid is available as the sole carbon source (Fig. 1-(a)), the HV fraction does not go higher than a certain level. This implies that propionic acid is also utilized to synthesize PHB and other cellular materials.

To examine the effect of propionic acid on the biosynthesis of HV in the copolymer, the specific production rate of HV from propionic acid, q_{HV}, was

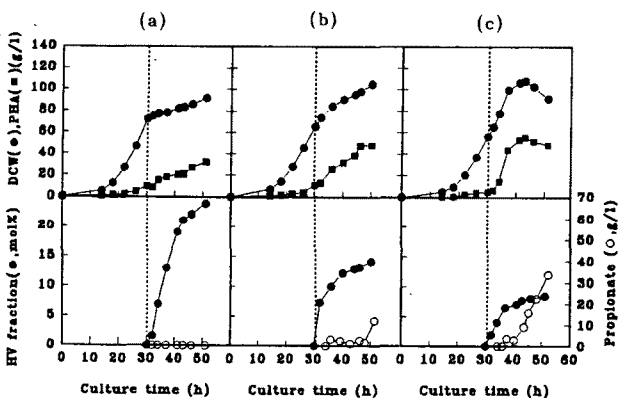


Fig. 2. Effect of glucose concentration at the second stage on accumulation of poly(HB-co-HV) in two-stage fed-batch operation. (a); without glucose feeding, (b); glucose limiting condition, (c); controlled glucose feeding to maintain relatively sufficient condition (10-20 g/l). In all cases, propionic acid feed solution (800 g/l) was fed into the jar fermentor at a flow rate of 12.0±0.5 ml/h. Dotted vertical lines indicate the onset of the second stage where propionic acid was supplied.

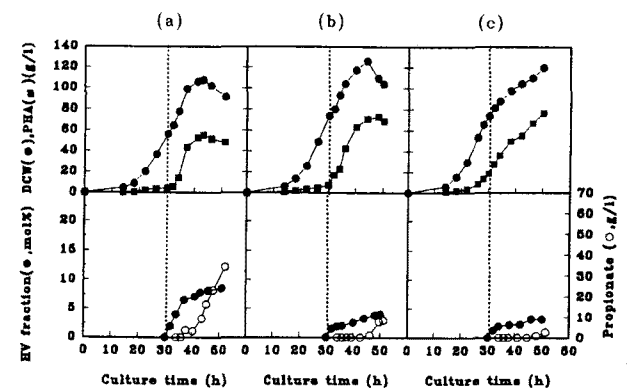


Fig. 3. Effect of propionic acid feeding rate at the second stage on accumulation of poly(HB-co-HV) in a two-stage fed-batch operation.

Propionic acid feed solution concentration: (a); 800 g/l, (b); 400 g/l, (c); 200 g/l. In all cases, feeding rate of propionate was 12.0±0.5 ml/h and glucose concentration was maintained within the range of 10-20 g/l. Dotted vertical lines indicate the onset of second stage where propionic acid was supplied.

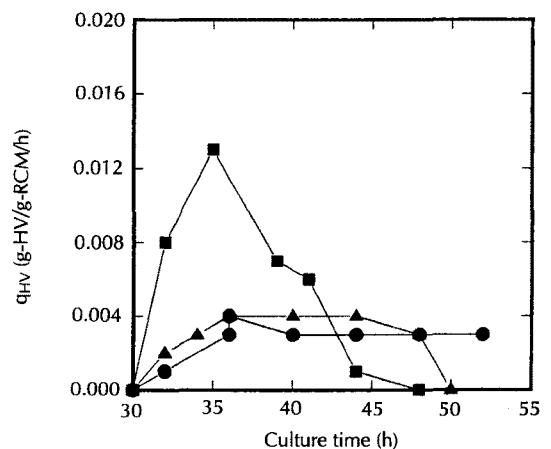


Fig. 4. Effect of propionic acid feeding rate at the second stage on specific production rate of hydroxyvalerate (HV) from propionic acid.

Symbol of (■), (▲), and (●) corresponds to culture condition of (a), (b), and (c) of Fig. 3. Details are described in Fig. 3.

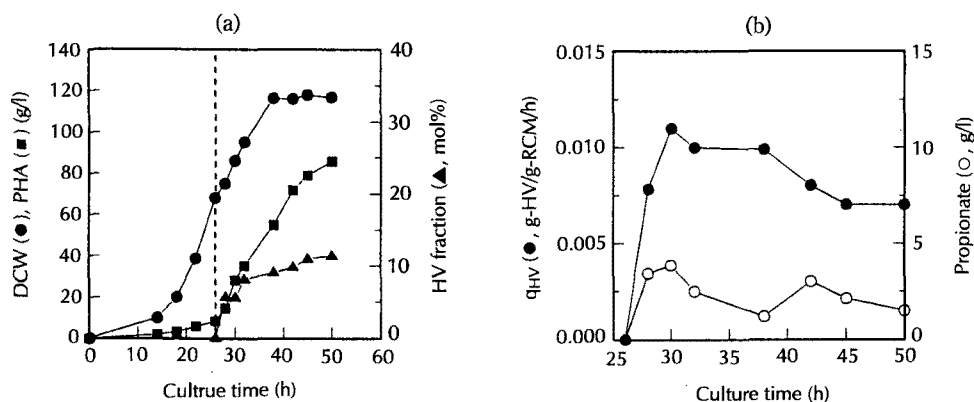


Fig. 5. Production of poly(HB-co-HV) by two-stage fed-batch cultivation of *Alcaligenes eutrophus* with control of propionic acid.

calculated from the results of Fig. 3. As shown in Fig. 4, qHV increased up to 0.013 (g-HV per g-residual cell mass per h) after onset of propionic acid addition, and then decreased sharply as the propionic acid was significantly accumulated. It was also noteworthy that when propionic acid was controlled under limited conditions, the qHV could be maintained at a constant level around 0.004. This also indicates that the formation of the HV fraction in the copolymer can be controlled to some extent by adjusting the feeding of propionic acid.

Production of Poly(HB-co-HV) by Controlled Feeding of Propionic Acid

Fed-batch cultures based on the optimized feeding strategy were conducted. From our previous study (10), it was found that poly(HB-co-HV) production was most favored at the propionic acid concentration of 2.0 g/l. During the copolymer production stage, therefore, the propionic acid concentration in the culture broth was maintained at around 2.0 g/l by controlling feed rate of the propionic acid solution. The feed solution containing inorganic salts was fed separately into the fermentor to maintain glucose concentration within the range of 10-20 g/l. A typical time course of the fermentation is described in Fig. 5. The copolymer was accumulated to a concentration as high as 85.6 g/l with an HV fraction of 11.4 mol% in 50 h. Specific production rates of HV from propionic acid, qHV, were maintained as high as 0.01 (g-HV/g-RCM/h) during the copolymer accumulation stage (Fig. 5-(b)), indicating that the control of propionic acid concentration successfully leads cellular activity in a direction which favors the accumulation of the copolymer.

In this paper, we reported production of poly(HB-co-HV) to a high concentration (85.6 g/l) with a high HV fraction (11.4 mol%) by fed-batch culture of *A.*

eutrophus with control of the concentrations of glucose and propionic acid. However, it was also found to be quite difficult to obtain a higher HV content in the present culture system. Although the production titre of poly(HB-co-HV) from the present study is high enough to consider an industrial scale application, a more elaborate study should be conducted to improve the HV fraction in the copolymer. Development of a new strain which can utilize propionic acid more effectively to make the HV fraction has therefore been started and the results will be reported soon.

Acknowledgement

The authors thank Sunhil Sugar Ltd. for supporting their work under the Highly Advanced National Project.

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(Received July 15, 1995)