Sequential Feeding of Glucose and Valerate in a Fed-Batch Culture of Ralstonia eutropha for Production of Poly(hydroxybutyrate-co-hydroxyvalerate) with High 3-Hydroxyvalerate Fraction

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Several important properties of poly(3-hydroxybutyric-co-3-hydroxyvaleric acids) (P(3HB-co-3HV)) depend mainly on the HV unit fraction of the copolymer. Sequential and simultaneous feeding of glucose and valerate were employed to produce P(3HB-co-3HV) in a fed-batch culture of Ralstonia eutropha, and the effects of feeding models on the cell growth, 3HV unit fraction, and copolymer productivity have been investigated. The sequential feeding of glucose and then valerate resulted in a cell density of 110.2 g/L, 3HV unit fraction of 62.7 mol %, and copolymer productivity of 0.56 g/(L·h), while the latter simultaneous feeding strategy never achieved the 3HV fraction of P(3HB-co-3HV) higher than 50%. A nuclear magnetic resonance study confirmed that the production of random copolymer P(3HB-co-3HV) with high 3HV unit fraction was possible even with sequential feeding of glucose and valerate.

Introduction

Poly(hydroxykanoates) (PHAs), represented mostly by poly(3-hydroxybutyric acid) (PHB) and poly(3-hydroxybutyric-co-3-hydroxyvaleric acids) [P(3HB-co-3HV)], are accumulated as intracellular polymers by a variety of microorganisms under certain nutrient limitation conditions (1–3). For commodity materials, the copolymer P(3HB-co-3HV) is more valuable than the homopolymer PHB because P(3HB-co-3HV) has more useful properties, particularly in terms of melting point, crystal growth rate, plasticity, and biodegradability (4–8).

P(3HB-co-3HV) has been produced in the cultivations of Ralstonia eutropha (R. eutropha) (5, 9–11), recombinant Escherichia coli (E. coli) (12, 13), Paracoccus denitrificans and Methylobacterium extorquens (14), Azotobacter vineanidii (A. vineanidii) UWD (15), A. latus (16), and Alcaligenes Sp. SH-69 (17) with several different carbon sources such as glucose and propionate, glucose and valerate, methanol and n-amyl alcohol, sucrose and valerate, butyrate and valerate, or only glucose, propional, and valerate, etc. Among them, R. eutropha, one of the most efficient producers for P(3HB-co-3HV) production, requires odd-numbered carbon substrates such as propionic acid, valeric acid, or propanol to produce the 3HV monomer (5). A number of studies have been carried out on the production of P(3HB-co-3HV) by fed-batch culture of R. eutropha from glucose and propionic acid because propionic acid is cheap. However, in most cases the 3HV unit fraction in copolymer is in the range of 5–20 mol %. The highest one was 29.8 mol % as reported by Du et al. (18), but the productivity was only 0.47 g/(L·h). On the other hand, valerate, as compared to propionic acid, showed the superior convertibility to 3HV units (19). A successful production of P(3HB-co-3HV) in high 3HV unit fraction (20 mol %) and high productivity (1.8 g/(L·h)) from glucose and valerate was demonstrated by Lee et al. (20). P(3HB-co-3HV) with 3HV unit fraction of more than 50 mol % already has been obtained in flask or fed-batch culture of R. eutropha but with a very low productivity (5, 21). It was believed that the variation of the 3HV fraction in copolymer would lead to a wide variety of thermomechanical properties (19). But, up to now, there are only a few reports on the production of P(3HB-co-3HV) with high 3HV unit fraction in relatively high productivity.

In this paper, we demonstrate the production of P(3HB-co-3HV) with high 3HV unit fraction by fed-batch culture of R. eutropha with glucose and valerate. Several feeding strategies of valerate were carried out to eliminate the inhibition of valerate on cell growth. The effects of valerate feeding modes on productivity of copolymer, 3HV unit fraction, and cell density were analyzed. The structure characteristics of copolymer were confirmed by its NMR spectra.

Materials and Methods

Microorganism and Medium. R. eutropha (formerly known as Alcaligenes eutrophus) NCIMB 11599 was used throughout this study. All medium compositions were described in detail at our previous work (22).

Fed-Batch Culture Conditions. Seed culture was prepared in a 500 mL flask containing 150 mL of medium by incubating at 30 °C for about 30 h. All fed-batch cultures were carried out in a 5 L jar fermentor with...
initial volume of 1.5 L at 30 °C. The pH was controlled at pH 6.7 with a 2 N HCl solution and a 28% NH4OH solution. The dissolved oxygen concentration was maintained at 20% of air saturation by automatically increasing the agitation speed up to 900 rpm and supplying pure oxygen if necessary. The valeric acid was fed separately, or together with glucose at a certain ratio of valeric acid to glucose.

Biopolymer Purification. Copolymer, P(3HB-co-3HV), was extracted from the lyophilized cells with chloroform containing 2% (v/v) H2SO4 and precipitated in ice-cold methanol (10-fold excess) after removing the cell residuals. The precipitated polymer was redissolved in ice-cold methanol (10-fold excess) after removing the cell residuals. Finally, copolymers were air-dried and stored at 4 °C.

Analytical Procedures. Cell growth was monitored by measuring the optical density at 600 nm with a spectrophotometer (Beckman). The methods used to determine the dry cell weight (DCW), valeric acid concentration, glucose concentration, P(3HB-co-3HV) concentration, and 3HV unit fraction were described previously (11). The residual cell weight (RCW) was defined as the cell concentration minus P(3HB-co-3HV) concentration. The 3HV unit fraction (mol %) was defined as the mole percentage of 3HV units in P(3HB-co-3HV).

The 1H and 13C NMR analyses of copolymer samples were carried out with a Bruker AMX FT 500 MHz NMR spectrometer. The 500 MHz 1H NMR and the 127 MHz 13C NMR spectra were recorded at 27 °C in a CDCl3 solution of the copolymer (15 g/L).

Results

Figure 1 shows the consumption rates of valeric acid at an initial glucose concentration of 7.3 g/L in flask cultures of R. eutropha. Valeric acid and glucose were hardly consumed during the 94 h fermentation when the initial valeric acid concentrations were 2.06 and 3.51 g/L, respectively. The average valeric acid and glucose consumption rates were 0.021 and 0.096 g/(L h), respectively. At a higher valeric acid concentration of 1.20 g/L these consumption rates decreased to 0.017 and 0.055 g/(L h), respectively, showing that the valeric acid consumption decrease (42.7%) is more sensitive than glucose consumption (19.0%). This result was used to determine the feeding rate of valeric acid in fed-batch cultures.

Two feeding models can be considered for feeding valeric acid in fed-batch cultures. To avoid the inhibitory effect of valeric acid on cell growth and P(3HB-co-3HV) synthesis, valeric acid was fed on a basis of the consumed glucose in a proportional rate examined in flask cultures. As shown in Figure 2, the valeric acid concentration was successfully maintained below 10 g/L. The final DCW, RCW, PHB-co-HV concentration, and HV fraction reached 165.6, 64.0, 101.6 g/L, and 24.8 mol %, respectively. This resulted in a PHB-co-HV productivity of 2.28 g/(L h).

The second model used for feeding valeric acid was pH stat. Figure 3 shows the time course of the fed-batch culture of R. eutropha, where the valeric acid was fed by pH stat when the DCW reached 100 g/L and glucose feeding ceased. At the beginning of valeric acid feeding, a certain amount of valeric acid was continuously fed in a short time for increasing valeric acid concentration to about 3 g/L. A rapid increase in the HV fraction of copolymer at the beginning of the valeric acid feeding was found. P(3HB-co-3HV) with a high HV fraction, 62.7 mol %, was synthesized at a productivity of 0.56 g/(L h).

Although the productivity is much lower than that obtained in the proportional feeding, it is the highest one in productions of P(3HB-co-3HV) with a high HV fraction by fed-batch cultures (as shown in Table 1). The final DCW, RCW, and P(3HB-co-3HV) concentration were 110.2, 72.3, and 37.8 g/L, respectively. This demonstrated a successful way to synthesize the P(3HB-co-3HV) with high HV fraction for expanding the properties of biopolymers.

To confirm the composition and distribution of the monomers in this copolymer, the 1H and 13C NMR spectra were recorded. The 500 MHz 1H NMR spectrum of the polymer in chloroform indicates that it contains two monomeric units: 3HB and 3HV (Figure 4). Based on the peak areas of the CH3(1) proton resonance and the CH3(5) proton resonance, the mole fraction of 3HV unit was determined as 64.2 mol %, which is very close to the
units such as 3HV-3HV, 3HV-3HB, and 3HB-3HB. There was about 30.3% 3HV-3HB diad sequences existing in the copolymer. This means that the copolymer is in random form.

**Discussion**

Several feeding strategies have been used to add organic acids for producing P(3HB-co-3HV) in fed-batch cultures of microorganisms because the high concentration of organic acids in culture broths inhibit cell growth and product formation. Previously, P(3HB-co-3HV) with an HV fraction of 20.5 mol % and productivity of 1.8 g/(L-h) has been obtained in the fed-batch culture of R. eutropha from glucose and valerate, whereas the detailed feeding method of valerate was not reported (20). It is the highest productivity of P(3HB-co-3HV) reported up to now. Propionic acid can be fed together with glucose by using an on-line glucose analyzer (9). The HV fraction in the copolymer varied from 4.3 to 14.3 mol % depending on the ratio of propionic acid to glucose in the feeding solution. Propionic acid accumulated in the broth when the ratio of propionic acid to glucose was high. Choi and Lee (12) employed the pH-stat method to feed glucose and propionic acid for the production of P(3HB-co-3HV) in the fed-batch culture of recombinant E. coli. The HV fraction in copolymer increased with the ratio of propionic acid to glucose, and the highest HV fraction was 15.3 mol % in a productivity of 1.73 g/(L-h) at a final propionic acid concentration of 11 g/L. The above two results show that there is some limitation in increasing the HV fraction in the copolymer with mixed feeding of glucose and propionic acid.

The carbon source feeding should depend on its consumption. Figure 1 shows that glucose and valeric acid were hardly consumed when the initial valeric acid concentration was higher than 2.06 g/L, but their maximal consumption ratio was about 0.4 when the initial valeric acid concentration was lower than 1.20 g/L. Based on the results in flask cultures, valeric acid and glucose were fed into the fermentor at a ratio of 0.4 with two pumps controlled by a glucose analyzer (Figure 2). The final P(3HB-co-3HV) containing 24.8 mol % 3HV unit was produced at the productivity of 2.28 g/(L-h). Both the HV fraction and the productivity are higher than those obtained in a similar experiment where the accumulation of valeric acid exceeded 10 g/L, but the cells grew in all processes (20). This means that the higher cell density can tolerate a higher valeric acid concentration. The same phenomenon was observed in the culture of propionic acid and glucose (11, 18). Because cells can consume glucose much faster than valeric acid, it appears difficult to produce P(3HB-co-3HV) with a high HV fraction in the simultaneous feeding of glucose and valeric acid (9, 12).

To produce P(3HB-co-3HV) with a higher HV fraction, another feeding strategy was employed. Several studies have shown that P(3HB-co-3HV) with a high HV fraction can be produced by feeding a single organic acid only. P(3HB-co-3HVs) containing 40 and 90 mol % HV units were synthesized in a flask culture of R. eutropha

**Table 1. Productions of P(3HB-co-3HV) with High HV Unit Fraction (More Than 50 mol %) by Fed-Batch Cultures of Several Microorganisms**

<table>
<thead>
<tr>
<th>microorganisms</th>
<th>carbon sources</th>
<th>culture time (h)</th>
<th>DCW (g/L)</th>
<th>P(HB-co-HV) conc (g/L)</th>
<th>3HV unit fraction (mol %)</th>
<th>productivity (g/(L-h))</th>
<th>ref</th>
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</thead>
<tbody>
<tr>
<td>Paracoccus denitrificans</td>
<td>methanol and n-amy1 alcohol</td>
<td>138</td>
<td>9.0</td>
<td>2.25</td>
<td>60</td>
<td>0.016</td>
<td>14</td>
</tr>
<tr>
<td>Paracoccus denitrificans</td>
<td>n-pentanol</td>
<td>30</td>
<td>6.8</td>
<td>1.08</td>
<td>99.6</td>
<td>0.036</td>
<td>8</td>
</tr>
<tr>
<td>Alcaligenes eutrophus</td>
<td>fructose and valeric acid</td>
<td>66</td>
<td>9.8</td>
<td>6.4</td>
<td>59</td>
<td>0.097</td>
<td>5</td>
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<tr>
<td>Agrobacterium sp. SH-1</td>
<td>propionic acid</td>
<td>72</td>
<td>11</td>
<td>8.2</td>
<td>50</td>
<td>0.11</td>
<td>23</td>
</tr>
<tr>
<td>Ralstonia eutropha</td>
<td>glucose and valeric acid</td>
<td>66</td>
<td>110.2</td>
<td>37.2</td>
<td>62.7</td>
<td>0.56</td>
<td>this work</td>
</tr>
</tbody>
</table>

Figure 3. Time profile of DCW, RCW, P(3HB-co-3HV) concentration, valeric acid concentration, and HV unit fraction in fed-batch culture of R. eutropha by feeding glucose and valerate in series: (a) (OD (600 nm), (b) DCW (g/L), and (c) RCW (g/L); (b) (●) P(3HB-co-3HV) concentration (g/L), (●) HV unit fraction (mol %), and (●) valerate concentration (g/L).

Figure 4. 500 MHz 'H NMR spectrum of P(3HB-co-3HV) with 62.7 mol % 3HV at 27 °C in chloroform. Chemical shifts are in parts per million downfield from tetramethylsilane (TMS). GC result (62.7 mol %). Figure 5 shows the 127 MHz 13C NMR spectrum of the polymer in chloroform and the expanded spectrum for carbonyl resonances. Clearly, the carbonyl resonances were resolved into three peaks, resulting from different diad sequences of 3HB and 3HV.
when propionic acid and valeric acid were used as the sole carbon source, respectively (5). In the fed-batch culture of R. eutropha, the P(3HB-co-3HV) concentration reached 37.1 g/L in 55.5 h when propionic acid was fed as the sole carbon source, but the 3HV fraction was not presented (10). When valeric acid was used as the sole carbon source, the 3HV fraction in P(3HB-co-3HV) could reach 67 mol %, but the P(3HB-co-3HV) concentration was very low (21). The highest 3HV fraction obtained in the production of P(3HB-co-3HV) was 99.6 mol % in a fed-batch culture of Paracoccus denitrificans by feeding n-pentanol only with a productivity of 0.036 g(L-h) (8). The main reason for their lower productivity is the inhibitory effect of n-pentanol on cell growth. But, we can see that feeding organic acid as the sole carbon source is one efficient way to increase the HV unit fraction in P(3HB-co-3HV).

A method of separately feeding glucose and valerate in series was employed: first glucose for cell growth and P(3HB) accumulation; then valerate for synthesizing the 3HV unit and increasing the 3HV unit fraction. When glucose feeding ceased, the valerate was fed by a modified pH-stat method based on an increase in the pH rate. At the beginning, valeric acid was fed continuously to make sure that the valeric acid concentration in the broth is about 3 g/L. In the whole process, the valeric acid concentration was successfully kept less than 5 g/L by the modified pH stat. A rapid increase in the 3HV fraction was observed at the beginning of the valeric acid feeding. At 66 h fermentation the HV fraction reached 62.7 mol % with a productivity of 0.56 g/(L-h). This is the highest productivity reported in the fed-batch cultures for P(3HB-co-3HV) productions (shown in Table 1).

Figure 5 also shows that the P(3HB-co-3HV) concentration just increased by 21% after valerate feeding began, but the 3HV unit fraction increased to 62.7 mol % from zero. It indicates that the degradation of PHB occurs in parallel with the synthesis of P(3HB-co-3HV) in R. eutropha cells in the culture medium containing valerate only as the carbon source. This showed the recycling characteristics between the 3HV unit and the 3HB unit, which were also found in the fed batch culture of R. eutropha when butyric acid and valeric acid were used as carbon sources (5). It sounds possible to achieve a much high productivity of P(3HB-co-3HV) with high 3HV fraction when the feeding of valeric acid is carried out after more P(3HB) is produced. On the basis of this idea, an experiment had been carried out, but it failed (data are not shown here), probably because of a decrease in the vitality and metabolic ability of cells with time. It took a very long time for the 3HV fraction to reach about 50 mol %.

The 3HV fraction in P(3HB-co-3HV) determined by GC was further confirmed by its 1H NMR spectrum. Table 2 shows the relative intensities for different diad sequences calculated from the peak areas. Similar to the random copolymer P(3HB-co-3HV) with 19 mol % 3HV (24), the experimental result of relative intensity for each diad sequence in this copolymer was very close to its theoretical value calculated on random distribution. This confirms that the copolymer produced in this study is a random copolymer rather than a block copolymer. Its properties can be found in the previous studies (5).

In conclusion, sequential feeding of glucose and valerate in a fed-batch culture of R. eutropha demonstrated an effective way for producing the random copolymer P(3HB-co-3HV) with high 3HV unit fraction, which could not be obtained in the simultaneous feeding of glucose and valerate. The mass production of P(3HB-co-3HV) with high 3HV unit fraction will extend the applications of PHAs. The recycling characteristics between 3HV and 3HB units in the fed-batch culture of R. eutropha showed

<table>
<thead>
<tr>
<th>13C chemical shifts (ppm)</th>
<th>sequences</th>
<th>rel intens (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>169.14</td>
<td>3HB-3HB</td>
<td>67</td>
</tr>
<tr>
<td>169.32</td>
<td>3HB-3HV</td>
<td>26</td>
</tr>
<tr>
<td>169.52</td>
<td>3HV-3HV</td>
<td>4</td>
</tr>
<tr>
<td>169.50</td>
<td>3HV-3HV</td>
<td>52</td>
</tr>
<tr>
<td>169.12</td>
<td>3HB-3HB</td>
<td>17</td>
</tr>
<tr>
<td>169.31</td>
<td>3HB-3HV</td>
<td>31</td>
</tr>
<tr>
<td>169.50</td>
<td>3HV-3HV</td>
<td>51</td>
</tr>
</tbody>
</table>

^a^ Calculated on the peak areas. ^b^ Calculated on the random distribution of 3HB and 3HV units.
that an amount of the 3HB unit was degraded as the 3HV unit was synthesized. So this method is not suitable for producing P(3HB-co-3HV) with a low 3HV unit fraction because valerate is much more expensive than propionic acid.

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References and Notes