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Continuous production of poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate): Effects of C/N ratio and dilution rate on HB/HV ratio

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Abstract−*Ralstonia eutropha* was cultivated in a continuous stirred fermenter with various C/N ratios (20, 30, and 40), dilution rates, and organic salt substrates (sodium propionate or sodium valerate) to explore the microbial growth and the poly(3HB-*co*-3HV) accumulation. When sodium propionate was used as the secondary carbon source, the HB/HV molar ratio at various C/N ratios and dilution rates did not change appreciably (approximately 90 : 10). The highest poly(3HB-*co*-3HV) content in biomass (41.8%) and poly(3HB-*co*-3HV) productivity (0.100 g/(L·h)) occurred under the condition with a C/N ratio of 20 and dilution rate of 0.06 h^{-1}. When sodium valerate was used as the secondary carbon source, the productivity of poly(3HB-*co*-3HV) increased with increasing dilution rate for the C/N ratio of 30 and 40. The average HB/HV molar ratio ranged from 48 : 52 to 78 : 32. The feeding of sodium valerate promoted the accumulation of HV better than feeding sodium propionate did. This study shows that a potential strategy of manipulating by both C/N ratio and dilution rate could be used to control the HV unit fraction in poly(3HB-*co*-3HV) in a continuous cultivation.

Key words: Poly(3HB-*co*-3HV), Continuous Cultivation, Dilution Rate, *Ralstonia eutropha*, C/N Ratio, Propionate, Valerate

INTRODUCTION

PHA (polyhydroxyalkanoate), a biodegradable thermoplastic, can be produced by various microorganisms under specific nutrient-limited conditions including nitrogen, phosphorus or oxygen [1-4]. Poly-hydroxybutyrate (PHB) is a highly crystallized polymer that is not easily molded. Conversely, poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) (poly(3HB-*co*-3HV)) has a lower melting point and better flexibility, and has many applications [5-7]. The physical properties of poly(3HB-*co*-3HV) are affected by the fraction of HV monomer in poly(3HB-*co*-3HV). The melting temperature (T_m) and glass transition temperature (T_g) of poly(3HB) were 180 °C and 4 °C, respectively, while those of poly(3HB-*co*-3HV) with 97 mol% of HV were 42 °C and –44 °C, respectively [8]. The fraction of HV in poly (3HB-*co*-3HV) can be adjusted by the feeding of propionate, valerate or other carbon sources [9-12]. The feeding strategies, constant feeding of acid [13] or sequential feeding of glucose and propionate/valerate in the fed-batch cultures [14,15], influence the HB/HV molar ratio in the poly(3HB-*co*-3HV). The maximum PHA production rate is dependent on the substrate and the operating conditions used [16,17].

Microbes metabolize glucose much faster and more efficiently than propionate/valerate. *Ralstonia eutropha* can produce HV if organic salts, such as sodium propionate or sodium valerate, are given during the second stage of the poly(3HB-*co*-3HV) synthesis. However, excessive propionate or valerate may be toxic to *R. eutropha* and hinder microbial growth and poly(3HB-*co*-3HV) accumulation [14]. It is difficult to produce a high HV fraction of poly(3HB-*co*-3HV) in the simultaneous feeding of glucose and propionate/valerate. To avoid a concentration spike and the inhibitory effect of acidic substrates on cell growth and poly(3HB-*co*-3HV) synthesis, the feeding rate of organic substrate must be properly controlled to produce appropriate HV fractions in poly(3HB-*co*-3HV). The continuous production of PHBV has the following advantages: (1) The HV content in PHBV could be easily controlled by C/N ratio and dilution rate, while the HV content in PHBV was changing in the batch fermentation. (2) The inhibition of propionate or valerate on the cell growth was relieved in the continuous culture due to the dilution of a constant amount of propionate or valerate, while the inhibition was severe in the batch fermentation due to the same amount of sodium propionate or valerate added initially. In this study, propionate or valerate was fed in a continuous mode to cultivate *R. eutropha* (ATCC 17699). The influences of the C/N ratio with different dilution rates and organic salt (propionate or valerate) on microbial growth and PHB production at 26 °C were explored. The intracellular poly(3HB-*co*-3HV) production rates for the two different organic substrates and three C/N ratios were compared.

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MATERIALS AND METHODS

1. Microorganism and Culture Medium

Ralstonia eutropha (ATCC 17699) used in this study was purchased from the Food Industry Research and Development Institute of Taiwan (Hsin-chu, Taiwan).

Every liter of the basal medium contained Na_2HPO_4, 3.6 g; KH_2PO_4, 1.5 g; $(NH_4)_2SO_4$, 1.2 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; FeS, 0.06 g; $CaCl_2$, 0.01 g and 5.0 mL trace mineral solution. Every liter of the trace mineral solution contained Na_2EDTA, 6.0 g; $FeCl_3 \cdot 6H_2O$, 0.29 g; H_3BO_3, 6.84 g; $MnCl_2 \cdot 4H_2O$, 0.86 g; $ZnCl_2$, 0.06 g; $CoCl_2 \cdot 6H_2O$, 0.026 g and $CuSO_4 \cdot 5H_2O$, 0.002 g.

2. Experimental Designs

The inoculum was prepared in an Erlenmeyer flask containing a basal medium supplemented with 2% (w/v) glucose and 0.2% (w/v)

yeast extract at 26 ºC for 48 h. The culture of *R. eutropha* was inoculated in a 3-L fermenter with a working volume of 2 L at 26 ºC. When the microbial growth of culture reached its stationary phase, the operation was switched into a continuous mode, i.e., the medium was introduced into the fermenter, and the culture broth was drawn from the fermenter at the same rate. The dissolved oxygen (DO) concentration was monitored with a DO electrode (InPro 6000-T96, Mettler Toledo, Switzerland) throughout the duration of cultivation. The DO concentration was maintained at least 20% above the level of air saturation by dynamically adjusting the aeration rate and the agitation speed in response to the oxygen level. The culture medium was controlled at pH 7.0 with a pH controller by adding either 2N H_2SO_4 or 2N NaOH solution whenever necessary. Samples were taken at certain time intervals to monitor the biomass, nitrogen, glucose, PHB and poly(3HB-*co*-3HV) concentrations in the culture broth.

3. Analytical Methods

3-1. Biomass

Initially, the biomass in the culture broth was minimal and could be measured with a spectrophotometer (U-2000, Hitachi, Japan) set at 510 nm. When the absorbance value increased above 1.0 (corresponding to a biomass of 0.32 g/L), the gravimetric method was used to measure the biomass. A 20 mL culture sample of cell suspension was centrifuged and washed twice with distilled water. The sediment (microbial cells) was then freeze-dried until a constant mass was reached. The residual biomass is defined as the difference between the total biomass and mass of poly(3HB-*co*-3HV).

3-2. Glucose, Sodium Propionate and Sodium Valerate

Glucose, sodium propionate and sodium valerate concentrations were measured by HPLC (Intelligent HPLC pump, PU-980, Jasco, Japan; Intelligent UV/Vis detector, UV-975, Jasco, Japan; Refractive index detector, RID-6A, Shimadzu, Japan) using a Bio-Rad Aminex HPX-87H column. The aqueous solution of 0.008 N H_2SO_4 was used as a mobile phase with a constant flow rate of 0.6 mL/min.

3-3. Nitrogen

$(NH_4)_2SO_4$ was used as the nitrogen source, and its nitrogen concentration was measured by the micro-Kjeldahl method.

3-4. Poly(3HB-co-3HV)

The poly(3HB-*co*-3HV) content was measured with a gas chromatographer (G3000, Hitachi, Japan) using a capillary column (Supelcowax 10, 30 m×0.53 mm ID, Supel Co., Bellefonte, PA, U.S.). Initial column temperature was 100 ºC, which was held constant for 5 min. Then, the temperature was increased to 180 ºC at a rate of 8 ºC/min for 5 min. The detector (FID) temperature was 260 ºC. Nitrogen was used as a carrier gas with a constant flow rate of 5 mL/min. Methylation of hydroxybutyrate and hydroxyvalerate was prepared according to the Riis and Mai method [18].

RESULTS AND DISCUSSION

To avoid a high concentration spike and inhibitory effect of organic acidic substrates on the microbial growth, *Ralstonia eutropha* was cultivated in a continuous stirred fermenter at 26 ºC and pH 7 with glucose as the primary carbon source and propionate/valerate as the secondary carbon source. The cultivation was carried out under a high concentration of nitrogen (2.5 g/L of $(NH_4)_2SO_4$) in a batch system to promote the microbial growth (balanced growth conditions) and in nitrogen limited conditions (1.0 g/L of $(NH_4)_2SO_4$) with continuous feeding to enhance polymerization of poly(3HB-*co*-3HV) (unbalanced growth conditions). Three factors, the C/N ratio, the dilution rate, and the organic salt in the feeding substrates, were used to explore the microbial growth and the dynamic responses of HB and HV in poly(3HB-*co*-3HV) production. The organic salt (sodium propionate or sodium valerate) was fixed at 5.0 g/L, and the C/N mass ratio was varied from 20 to 40 by adjusting the concentration of glucose.

1. Effect of Propionate on Microbial Growth and Poly(3HB-co-3HV) Production

The effect of altering the dilution rate and C/N mass ratio (20, 30, or 40) on the biomass and the poly(3HB-*co*-3HV) composition was investigated. Fig. 1 shows the time courses of the biomass, residual biomass, HB, HV and poly(3HB-*co*-3HV) produced by *R. eutropha*. The microbes were first cultivated in a batch system by

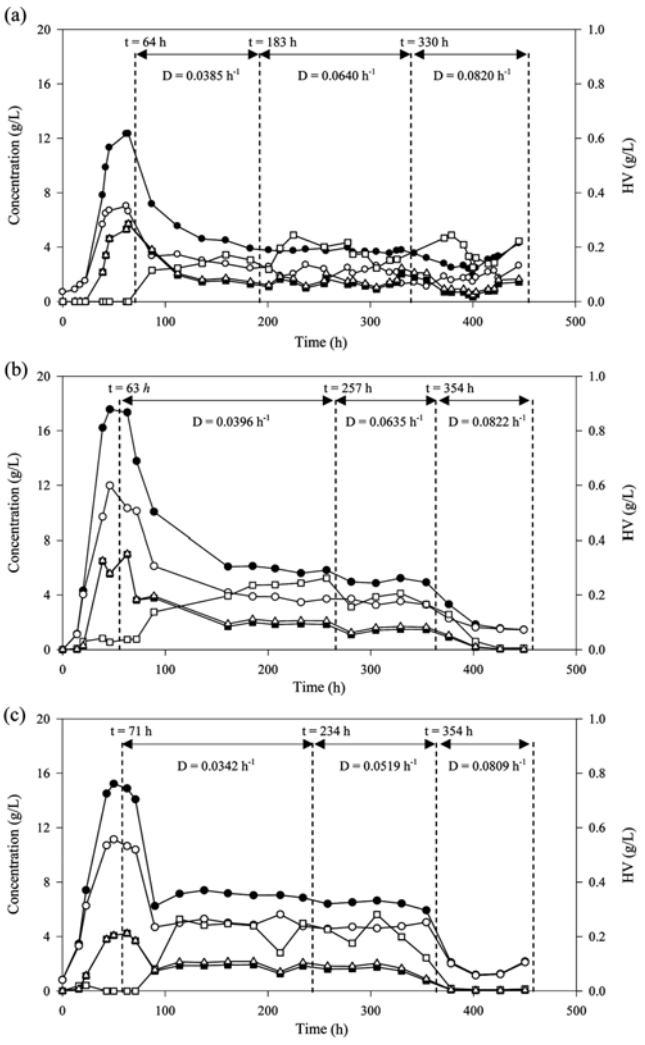

Fig. 1. Time courses of biomass, residual biomass, HB, HV, and poly(3HB-*co*-3HV) for a continuous cultivation *R. eutropha* under various dilution rates and a concentration of 5 (g/L) of sodium propionate in the feed at (a) C/N=20, (b) C/N=30 and (c) C/N=40. (●) biomass, (○) residual biomass, (■) HB, (□) HV, and (△) poly(3HB-*co*-3HV).

using glucose as the only carbon source for the cell growth. When the microbial growth reached the stationary phase, a lower dilution rate of the continuous feed was set to start with; then the rate was subsequently raised after a new steady state was reached.

Fig. 1(a) shows the time courses of the biomass, residual biomass, HB, HV and poly(3HB-*co*-3HV) under a continuous cultivation with a C/N ratio of 20. The microbes were first cultivated in the batch mode, with little PHB accumulation relative to the microbial growth. The nitrogen source was completely exhausted at t=47 h, and the nitrogen concentration reduced to zero during the continuous cultivation (data not shown), so the cultivation quickly entered a nitrogen-limited period in which PHB accumulated rapidly. This indicated that the limitation of nitrogen coincided with the accumulation of PHB during cultivation [19]. The continuous feeding mode was set to start at t=64 h with a dilution rate D=0.0385 h^{-1}, and meanwhile the biomass and the PHB were 12.32 and 5.71 g/L, respectively. When t=136 h, the system reached a steady state having a biomass of 4.62 g/L, a poly(3HB-*co*-3HV) of 1.71 g/L, and an HB/HV molar ratio of 93 : 7. The microbes began to accumulate intracellular HV only when propionate had been fed into the fermenter. The biomass and the poly(3HB-*co*-3HV) continued to be produced as the system reached a new steady state. Appropriate regulation of residual propionate concentration in the medium is essential to the biosynthesis of HV and to relieve the inhibition of cell growth [13]. Next, the dilution rate was raised to 0.0640 h^{-1} at t=183 h, and a new steady state was reached at t=201 h with a decreased biomass and a decreased poly(3HB-*co*-3HV) (3.74 g/L and 1.56 g/L, respectively), and an HB/HV molar ratio of 90 : 10. When t=330 h, the dilution rate was increased to 0.0820 h^{-1}, and the system reached a new steady state at t=372 h. Both the biomass and the poly(3HB-*co*-3HV) declined to 2.94 and 1.16 g/L, respectively, and the HB/HV molar ratio was 84 : 16. With a C/N ratio of 20, as the dilution rate increased, both the biomass and the poly(3HB-*co*-3HV) decreased, while the molar ratio of HV in poly(3HB-*co*-3HV) increased. For such a high dilution rate, the residence time of nutrients and microbes in the fermenter was not long enough for cell growth and poly(3HB-*co*-3HV) accumulation to complete.

Fig. 1(b) shows the time courses of the biomass, residual biomass, HB, HV and poly(3HB-*co*-3HV) under a continuous cultivation with a C/N ratio of 30. The microbes were first cultivated in a batch mode, and the nitrogen substrate in the medium was exhausted completely at t=49 h. The continuous feeding mode was to start at t=63 h (having a biomass of 17.35 g/L, a PHB of 7.02 g/L and a PHB content of 40.5%) with a dilution rate D=0.0396 h^{-1}. The system reached a steady state with a biomass of 5.92 g/L, a poly(3HB-*co*-3HV) of 2.09 g/L, and an HB/HV molar ratio of 91 : 9. When t=257 h, the dilution rate was raised to 0.0635 h^{-1}, and both the biomass (5.00 g/L) and the poly(3HB-*co*-3HV) (1.55 g/L) declined; the molar ratio of HB/HV was 90 : 10. When the dilution rate was raised to 0.0822 h^{-1} at t=354 h, the system reached a new steady state at t=402 h. Both the biomass and the poly(3HB-*co*-3HV) decreased significantly, 2.05 and 0.33 g/L, respectively, and the HB/HV molar ratio was 90 : 10. Using similar dilution rates, the poly(3HB-*co*-3HV) production was higher for the C/N ratio of 30 than for the C/N ratio of 20, but the molar ratio of HB/HV was not appreciably different. The cultivation at C/N ratio 30 supplied with a higher amount of glucose for the microbial growth yielded higher poly(3HB-*co*-3HV) production than that at C/N ratio 20.

For a C/N ratio of 40, as in Fig. 1(c), the continuous feeding mode was to begin at t=71 h with a dilution rate of 0.0342 h^{-1}, and the biomass and the PHB were 14.08 g/L and 3.69 g/L, respectively. At t=114 h, the system reached a steady state, with a biomass of 6.98 g/L, a poly(3HB-*co*-3HV) of 1.97 g/L, and an HB/HV molar ratio of 91 : 9. Next, at t=234 h, the dilution rate was raised to 0.0519 h^{-1}, and a new steady state was observed at t=258 h with a biomass of 6.38 g/L, a poly(3HB-*co*-3HV) of 1.65 g/L, and an HB/HV molar ratio of 90 : 10. When the dilution rate was further increased to 0.0809 h^{-1} at t=354 h, the system reached a steady state with a biomass of 1.68 g/L, a poly(3HB-*co*-3HV) of 0.057 g/L, and an HB/HV molar ratio of 90 : 10.

As observed in Fig. 1, the biomass varied with different C/N ratios even before the continuous mode was set to start. During the initial batch cultivation, the highest biomass occurred at a C/N ratio of 30. A C/N ratio of 20 might not supply a large enough source of carbon for the microbial growth and poly(3HB-*co*-3HV) synthesis. A C/N ratio of 40, with a much higher carbon source, might induce a high osmotic pressure that consequently inhibits the microbial growth [20]. During the continuous mode, the production patterns of HB, HV and poly(3HB-*co*-3HV) were similar for all three C/N ratios. For a given C/N ratio, the biomass and the poly(3HB-*co*-3HV) production were high at the low dilution rate (0.04 and 0.06 h^{-1}) that a longer residence time of nutrients allowed microbes to grow and accumulate poly(3HB-*co*-3HV) in the fermenter. However, the HB/HV molar ratios at various C/N ratios and dilution rates did not vary appreciably when propionate was fed as the secondary carbon source.

Table 1 shows the poly(3HB-*co*-3HV) production over the cultivation periods for various C/N ratios and dilution rates. The poly(3HB-*co*-3HV) content in biomass and the biomass production rate were high in the dilution rate of 0.04 and 0.06 h^{-1}. The highest poly

Table 1. Production of poly(3HB-*co*-3HV) by *R. eutropha* when cultivated with propionate at different C/N ratios and dilution rates

C/N ratio	20			30			40		
Dilution rate (h^{-1})	0.0385	0.0640	0.0820	0.0396	0.0635	0.0822	0.0342	0.0519	0.0809
Biomass production rate (g/(L·h))	0.178	0.239	0.241	0.234	0.318	0.169	0.240	0.330	0.140
Poly(3HB-*co*-3HV)/Biomass (%)	36.9	41.8	39.5	35.3	30.9	16.1	28.1	25.8	3.8
HV unit fraction (mol%)	8.77	13.5	19.8	11.5	11.7	12.1	11.7	12.2	10.5
HB yield ($g_{HB}/g_{glucose}$)	0.300	0.410	0.460	0.190	0.172	0.030	0.152	0.160	0.014
HV yield ($g_{HV}/g_{propionate}$)	0.029	0.042	0.046	0.047	0.036	0.008	0.046	0.040	0.005
Poly(3HB-*co*-3HV) productivity (g/(L·h))	0.066	0.100	0.095	0.083	0.098	0.027	0.067	0.087	0.005

(3HB-*co*-3HV) productivity was the dilution rate of 0.06 h^{-1} that indicated the dilution rate suitable for the microbial metabolism in growth and poly(3HB-*co*-3HV) biosynthesis. The efficiency of substrate utilization is different in that the yield of HB from glucose (0.46 $g_{HB}/g_{glucose}$) was higher than that of HV from propionate (0.046 $g_{HB}/g_{propionate}$) for *Ralstonia eutropha* cultivated under a C/N ratio of 20 with dilution rate of 0.0820 h^{-1}.

2. Effect of Valerate on Microbial Growth and Poly(3HB-co-3HV) Production

Fig. 2 shows the effect of the C/N ratio and the dilution rate on the biomass and the poly(3HB-*co*-3HV) composition under a continuous cultivation with sodium valerate as the secondary carbon source in the feed. With a C/N ratio of 20, Fig. 2(a), the nitrogen substrate in the medium was depleted at t=50 h, and subsequently the PHB accumulation increased significantly. The continuous feeding mode was set to start with a dilution rate D=0.0359 h^{-1} at t=70 h. Meanwhile, the biomass was 12.32 g/L and the PHB was 3.40 g/L.

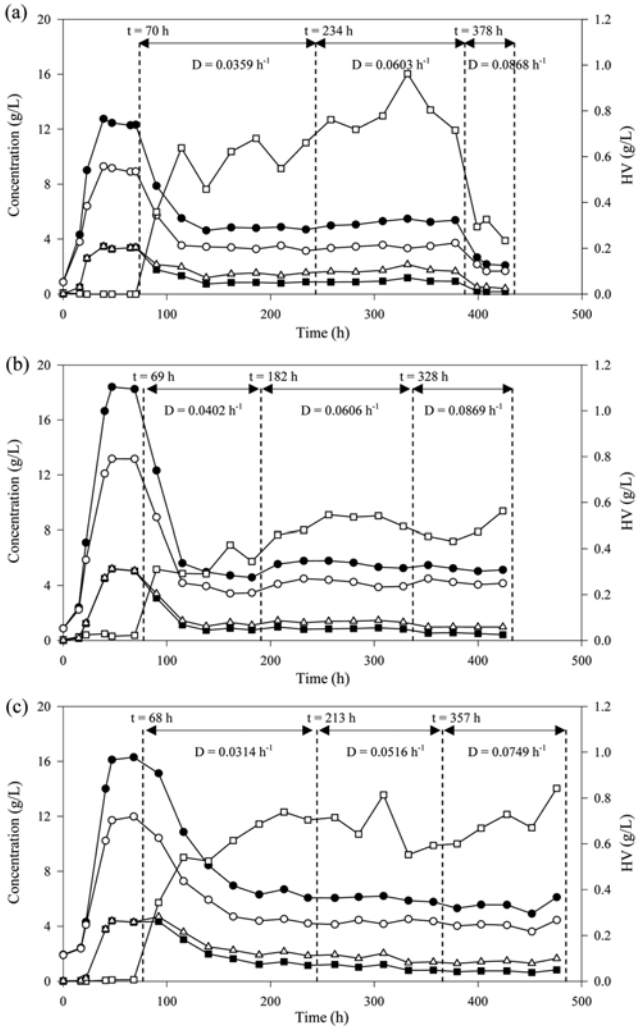

Fig. 2. Time courses of biomass, residual biomass, HB, HV, and poly(3HB-*co*-3HV) for a continuous cultivation *R. eutropha* under various dilution rates and a concentration of 5 g/L of sodium valerate in the feed at (a) C/N=20, (b) C/N= 30 and (c) C/N=40. (●) biomass, (○) residual biomass, (■) HB, (□) HV, and (△) poly(3HB-*co*-3HV).

At t=138 h, a steady state was established with a biomass of 4.77 g/L, a poly(3HB-*co*-3HV) of 1.41 g/L, and an HB/HV molar ratio of 65 : 35. At t=234 h, the dilution rate was raised to D=0.0603 h^{-1}; then a new steady state was reached at t=258 h with a biomass of 5.24 g/L, a poly(3HB-*co*-3HV) of 1.76 g/L, and an HB/HV molar ratio of 62 : 38. When the dilution rate was increased to 0.0868 h^{-1} at t=378 h, a new steady state was reached at t=399 h. Both the biomass (2.31 g/L) and the poly(3HB-*co*-3HV) production (0.48 g/L) decreased appreciably, which may be attributed to low supply of carbon sources and a short residence time of nutrients in the fermenter. The microbial growth was limited and consequently resulted in a decrease of poly(3HB-*co*-3HV) production. The HB/HV molar ratio in poly(3HB-*co*-3HV) was 48 : 52.

Fig. 2(b) shows the time courses of the biomass, HB, HV and poly(3HB-*co*-3HV) under continuous cultivation with a C/N ratio of 30. The microbes were first cultivated in a batch mode until the exponential phase was reached at t=69 h. Meanwhile, the biomass was 18.24 g/L and the PHB was 5.08 g/L. At the same time, the continuous feeding mode was activated with a dilution rate D=0.0402 h^{-1}. A new steady state was established at t=115 h with a biomass of 4.98 g/L, a poly(3HB-*co*-3HV) of 1.23 g/L, and an HB/HV molar ratio of 78 : 22. When t=182 h, the dilution rate was raised to 0.0606 h^{-1}, and the biomass (5.57 g/L) and the poly(3HB-*co*-3HV) (1.39 g/L) declined to a new steady state with an HB/HV molar ratio of 70 : 30. When the dilution rate was increased to 0.0869 h^{-1} at t=328 h, the system reached a new steady state at t=352 h. Both the biomass and the poly(3HB-*co*-3HV) decreased, 5.23 and 0.98 g/L, respectively, and the HB/HV molar ratio was 58 : 42. These results are similar to those of the cultivation of *Pseudomonas oleovorans* reported by Preusting et al. [21] and *R. eutropha* reported by Du et al. [6] and Yu et al. [22].

For a C/N ratio of 40 as shown in Fig. 2(c), the continuous feeding mode started at t=68 h with a dilution rate of 0.0314 h^{-1}. Meanwhile, the biomass was 16.3 g/L and the poly(3HB-*co*-3HV) was 4.31 g/L. When t=164 h, the system reached a steady state with a biomass of 6.65 g/L, a poly(3HB-*co*-3HV) of 2.11, and an HB/HV molar ratio of 74 : 26. When the dilution rate was increased to 0.0516 h^{-1} at t=213 h, the system reached a new steady state at t=236 h with a biomass of 6.02 g/L, a poly(3HB-*co*-3HV) of 1.71 g/L, and an HB/HV molar ratio of 67 : 33. After that, the dilution rate was raised to 0.0749 h^{-1} at t=357 h; a new steady state was observed at t=380 h with a biomass of 5.49 g/L, a poly(3HB-*co*-3HV) of 1.43 g/L, and an HB/HV molar ratio of 58 : 42.

The biomass, the residual biomass and the poly(3HB-*co*-3HV) production at the medium dilution rate were higher than those at the low and the high dilution rates for a C/N ratio of 20. A C/N ratio of 30 or 40 with a high dilution rate (0.08 h^{-1}) showed little to no effect on the biomass and the poly(3HB-*co*-3HV) production because the high concentration of carbon sources compensated for the short residence time in the fermenter. The operation condition, high C/N ratios of 30 and 40 with the high dilution rate (0.08 h^{-1}) in the continuous stirred fermenter, had the potential to produce poly(3HB-*co*-3HV) containing a high HV unit fraction of 42 mol%.

The average biomass production rates and poly(3HB-*co*-3HV) productivities over different cultivation periods for various C/N ratios are given in Table 2. The poly(3HB-*co*-3HV) content in biomass was high in a low dilution rate (0.03 or 0.06 h^{-1}). The HV content

Table 2. Production of poly(3HB-*co*-3HV) by *R. eutropha* when cultivated with valerate at different C/N ratios and dilution rates

C/N ratio	20			30			40		
Dilution rate (h^{-1})	0.0359	0.0603	0.0868	0.0402	0.0606	0.0869	0.0314	0.0516	0.0749
Biomass production rate (g/(L·h))	0.170	0.320	0.200	0.200	0.340	0.450	0.210	0.310	0.410
Poly(3HB-*co*-3HV)/Biomass (%)	29.7	33.5	20.7	24.5	24.9	18.8	31.7	28.4	26.0
HV unit fraction (mol%)	41.8	45.0	60.4	27.6	36.8	48.9	32.2	39.2	48.9
HB yield ($g_{HB}/g_{glucose}$)	0.192	0.243	0.174	0.145	0.139	0.132	0.294	0.176	0.148
HV yield ($g_{HV}/g_{valerate}$)	0.118	0.158	0.086	0.068	0.102	0.096	0.136	0.134	0.140
Poly(3HB-*co*-3HV) productivity (g/(L·h))	0.051	0.106	0.042	0.049	0.084	0.085	0.066	0.088	0.123

in poly(3HB-*co*-3HV) increased with a rising the C/N ratio at a high dilution rate (0.08 h^{-1}) in which a higher amount of valerate was supplied for the synthesis of HV. The HB yield reached a maximum, 0.294 g $_{HB}$/g $_{glucose}$, under a C/N ratio of 40 and a dilution rate of 0.0314 h^{-1}, while the HV yield (0.158 g $_{HV}$/g $_{glucose}$) was maximized under a C/N ratio of 20 and a dilution rate of 0.0603 h^{-1}/. The poly(3HB-*co*-3HV) productivity increased with rising dilution rate for the C/N ratios of 30 and 40.

3. Comparison of Poly(3HB-co-3HV) Production between Propionate and Valerate

As shown in Figs. 1 and 2, the biomass and poly(3HB-*co*-3HV) production decreased significantly at a high dilution rate (>0.08 h^{-1}) for all three C/N ratios fed with propionate and at a C/N ratio of 20 fed with valerate. This illustrates that the continuous feeding system might approach its washout point for such a high dilution rate [22]. However, no washout phenomena were observed at a C/N ratio of 30 or 40 when valerate was fed as the secondary carbon source. The higher the C/N ratio is in the feed, the higher the concentration of carbon available for the microbial growth and the poly (3HB-*co*-3HV) synthesis. The biomass production rates were comparable for the three C/N ratios fed with propionate and valerate, except for a C/N ratio of 30 or 40 with a higher dilution rate (>0.08 h^{-1}), as shown in Tables 1 and 2. The number of cells in culture broth is a determining factor for the poly(3HB-*co*-3HV) production. The HV content in poly(3HB-*co*-3HV) and the HV yield from valerate were higher than those from propionate. This indicated that *R. eutropha* utilized valerate better than propionate to synthesize HV. Some poly(3HB-*co*-3HV) might be degraded into acetyl-CoA that was used for the microbial growth and the HB biosynthesis when propionate was fed as the secondary carbon source [23]. A higher poly (3HB-*co*-3HV) productivity was obtained for a higher C/N ratio and a higher dilution rate for valerate. Valerate was better than propionate to promote HV accumulation. Valerate is much easier to convert into D-3-hydroxyvaleryl-CoA to produce HV catalyzed by 3-ketothiolase. However, four steps must be carried out to form an HV unit from propionate, compared to two steps needed from valerate [24].

The dependence of HB/HV molar ratios on C/N ratio with propionate or valerate as the secondary carbon source is shown in Fig. 3. The average HB/HV molar ratio ranged from 48 : 52 to 78 : 22 with valerate. However, the average HB/HV molar ratio was approxi-

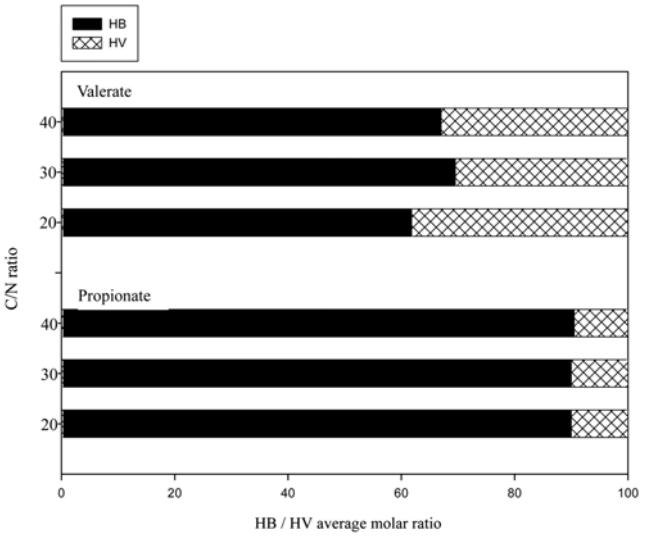

Fig. 3. HB/HV molar ratio in poly(3HB-*co*-3HV) for three C/N ratios and two organic salt substrates.

Table 3. Production of poly(3HB-*co*-3HV) by various microorganisms cultivated in the fed-batch or the continuous cultivation

Microorganisms	Cultivation	Carbon sources for HV biosynthesis	Culture/ residence time (h)	Biomass (g/L)	Poly(3HB-*co*-3HV) conc. (g/L)	HV unit fraction (mol%)	Poly(3HB-*co*-3HV) Productivity (g/(L·h))	References
Alcaligens eutrophus	Fed-batch	Valeric acid	66	9.8	6.4	59	0.097	26
Agrobacterium sp. SH-1	Fed-batch	Propionic acid	72	11.0	8.2	50	0.11	27
Ralstonia eutropha	Fed-batch	Valeric acid	66	110.2	37.2	62.7	0.56	14
R. eutropha DSM545	Fed-batch	Propionic acid	55	52.1	40.8	16.2	0.74	19
R. eutropha ATCC 17699	Continuous	Propionate	11	3.5	2.7	30	0.24	22
R. eutropha ATCC 17699	Continuous	Propionate	16	3.7	1.6	10	0.10	This study
R. eutropha ATCC 17699	Continuous	Valerate	13	5.5	1.6	42	0.12	This study

mately 90 : 10 with propionate, close to that obtained by Ruan et al. [25]. When propionate was fed, the HV fraction seemed to reach an upper fraction limit in the copolymer. Therefore, the cultivation with valerate can be used to produce a wider range of the HV fraction, and subsequently a variety of physical properties of poly(3HB-*co*-3HV), by controlling the C/N ratio and the dilution rate.

CONCLUSION

Experimental results show that the production of poly(3HB-*co*-3HV) and its HB/HV ratio were appreciably affected by the C/N ratio and the dilution rate. A rapid increase of the HV fraction in poly (3HB-*co*-3HV) was found at the beginning of feeding propionate or valerate. Valerate was better than propionate to promote the accumulation of HV. Different HV fractions in poly(3HB-*co*-3HV) could be obtained by controlling the C/N ratio and the dilution rate when valerate was fed in a continuous fermentation. Table 3 shows the comparison of poly(3HB-*co*-3HV) production by several microorganisms in fed-batch or continuous cultivations. The poly(3HB-*co*-3HV) productivity of *Ralstonia eutropha* is lower in the continuous cultivation than in the fed-batch cultivation. This study shows that a potential strategy of manipulating by both C/N ratio and dilution rate could be used to control the HV unit fraction in poly(3HB-*co*-3HV) in a continuous cultivation.

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