

Production and Characterization of Poly(3-hydroxybutyric acid-*co*-3-hydroxyvaleric acid) from L-Valine by *Ralstonia eutropha*

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The biosynthesis of polyesters from different L-amino acids was investigated by wild-type *Ralstonia eutropha* (formerly *Alcaligenes eutrophus*). The accumulation and degradation of the polyesters was found to occur during the cultivation time from 6 h to 48 h, when *R. eutropha* was cultivated on nitrogen-poor medium containing 1% (w/v) of each L-amino acid as the sole carbon source by two-step batch fermentation. L-Valine was best among different L-amino acids used for the polyester productivity. The polyester content in dried cells produced from L-valine as the sole carbon source was up to 36 wt % during the cultivation time from 24 h to 36 h in a two-step fermentation and up to 27 wt % during 120 h of culture time in a one-step fermentation, respectively. The compositions and molecular weights of the polyesters produced from L-valine were ca. 90 mol% of 3-hydroxybutyric acid (3HB) and ca. 10 mol% of 3-hydroxyvaleric acid (3HV) units and up to 5×10^5–8×10^5 g mol^{-1}, respectively. As-biosynthesized P(3HB-*co*-ca. 10 mol% 3HV) was shown to have a random sequence distribution of 3HB and 3HV units by analysis of the ^{13}C NMR spectra and have a very narrow comonomer compositional distribution by the fractionation method with chloroform/heptane mixed solvent.

Most genera of bacteria biosynthesize poly(hydroxyalkanoic acid) (PHAs) as storage compounds and deposit these polyesters as insoluble granule inclusions in the cytoplasm.[1] This occurs mainly if bacteria are cultivated in the presence of an excess of a carbon source and the growth is limited by the lack of an essential nutrient, such as nitrogen, oxygen, sulfur, or phosphorus.[2] Since the discovery of poly(3-hydroxybutyric acid) [P(3HB)] in *Bacillus megaterium* by Lmoigne,[3] more than 150 differnt constituents of PHAs have been identified to date as different hydroxyalkanoic acids with 3–14 carbon atoms.[1,4] As these microbial polyesters are biodegradable thermoplastics and/or elastomers, they have attracted much attention as new environmentally compatible materials.[5,6] Poly(3-hydroxybutyric acid-*co*-3-hydroxyvaleric acid) [P(3HB-*co*-3HV)] has been a well-studied example of the many PHAs that can be produced by bacteria. In particular, the P(3HB-*co*-3HV)s with the compositions rich in 3HB content have been focused on since the commercially available materials are generally restricted to compositions between 0–30 mol% 3HV.[7,8] These copolyesters are substantially more flexible and can be melt-processed with less risk of thermal degradation.[9] Hence, the thermal and mechanical properties of P(3HB-*co*-3HV)s with up to 30 mol% 3HV compositions have been well studied. However, Yoshie et al.[10] reported recently that some bacterial P(3HB-*co*-3HV)s produced from the mixed carbon source of glucose and propionic acid or butyric acid and valeric acid were composed of mixtures of several random copolymers with different 3HV contents by using a solvent fractionation method. For example, the P(3HB-*co*-3HV) with 15.3 mol% 3HV composition was fractionated into five fractions ranging from 10.9 to 25.3 mol% 3HV, and even a copolyester with 6.5 mol% 3HV composition was fractionated into two fractions of 6.1 mol% and 15.7 mol% 3HV. Therefore, the physical and thermal properties of the as-biosynthesized P(3HB-*co*-3HV) from the mixed carbon substrates may be those of a blend of several pure copolyesters with different compositions. That is, be different from the "true" properties of pure copolymer with a narrow compositional distribution. However, no a random copolymer with a narrow compositional distribution may be produced, as long as the 3HV-unit related substrates such as propionic acid or valeric acid and the 3HB-unit substrates such as glucose or butyric acid were utilized as the mixed carbon sources. Unfortunately, thermally and mechanically good P(3HB-*co*-3HV) with ca. 10 mol% 3HV compositions has not been biosynthesized until now in the good productivity from single 3HV-unit related carbon source by wild-type *Ralstonia eutropha* (formerly *Alcaligenes eutrophus*). Whereas, the P(3HB-*co*-3HV)s with up to 8 mol% 3HV composition have been produced from single 3HV-unit unrelated carbon source such as fructose by a mutant of *Ralstonia eutropha* that is a spontaneous revertant to prototropy of an isoleucine-auxotropic mutant of wild-type *R. eutropha*, but the cell density was low with about 1.5 g L^{-1}.[11] Previously, Inoue et al. reported the biosynthesis of copolyesters containing 3HV unit in wild-type *R. eutropha* from medium containing 1 wt % of different L-amino acids as the sole carbon source. However, the yield of polyesters was very low, and the maximum contents of polyesters in dried cells were below 3.7 wt %.[12]

In this study, we investigated in detail the effects of cultivation time and concentration of L-amino acids on polyester production by wild-type *R. eutropha*, and found that the commercially available P(3HB-*co*-3HV)s with ca. 10 mol% 3HV compositions were biosynthesized at a comparatively high rate when L-valine, among different L-amino acids, was used as the single carbon source. Since L-valine is biosynthesized

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by the cultivation of *Hydrogenomas* H16[13] or *Aerobacter aerogenes*[14] on sucrose or glucose with urea as the nitrogen source, it is a renewable resource, and then may be a candidate for the carbon and/or nitrogen sources for the microorganism. Recent remarkable progress in fermentation and organic synthetic technology has enabled us to obtain optically active L-amino acids at low prices. Therefore, various L-amino acids, including L-valine, are attractive raw materials as renewable resources, and the different amino acid-based polymers that are not only biocompatible materials but also functional chemical materials have recently been synthesized by Endo et al.[15]

Experimental

Culture Method. *Ralstonia eutropha* H16 (formerly *Alcaligenes eutrophus*, ATCC 17699) was used in this study. Polyester synthesis from different L-amino acids was carried out by a two-step batch cultivation of *R. eutropha* H16, in which the cells were first grown in nutrient-rich medium and next transfered into a nitrogen-poor mineral medium containing L-amino acids as the sole carbon source. Polyester synthesis from L-valine was also carried out by a one-step batch cultivation, in which *R. eutropha* H16 was inoculated into a mineral medium containing L-valine alone.

In the two-step batch fermentation, *R. eutropha* H16 cells were first grown under aerobic conditions at 30 °C and pH 7.0 for 24 h on a reciprocal shaker in a 500-mL Sakaguchi flask containing 100 mL of nutrient-rich medium containing 1 g of yeast extract, 1 g of polypeptone, 0.5 g of meat extract, and 0.5 g of $(NH_4)_2SO_4$. The cells were harvested by centrifugation at 5000 g for 15 min. Under these culture conditions, the accumulation of polyester in the cells was not observed. About 0.31 g quantities of the centrifuged cell of the seed culture were transferred into 100 mL of mineral medium (pH 7.2) containing 0.102 g of KH_2PO_4, 1.11 g of $Na_2HPO_4 \cdot 12H_2O$, 0.02 g of $MgSO_4$ and 1 mL of a microelement solution. The microelement solution contained 9.7 g of $FeCl_3$, 7.8 g of $CaCl_2$, 0.156 g of $CuSO_4 \cdot 5H_2O$, 0.119 g of $CoCl_2$, 0.118 g of $NiCl_2 \cdot 6H_2O$ and 0.062 g of $CrCl_2$ (per cubic decimeter of 0.1 M HCl (1 M = 1 mol dm^{-3})). 10 g dm^{-3} of each L-amino acid was added to the nitrogen-free mineral medium mentioned above and the pH value of the media was adjusted to 7.2 by addition of dilute sodium hydroxide or sulfuric acid solution. After the L-valine containing mineral medium was sterilized for 10 h under ultraviolet light, the cells were aerobially cultivated in this medium for 3–48 h at 30 °C and 120 rpm, harvested by centrifugation, and finally lyophilized. Polyesters were extracted from the lyophilized cells with hot chloroform in a Soxhlet apparatus and purified by reprecipitation with hexane.

In the one-step batch fermentation, *R. eutropha* H16 was inoculated into a 100 mL of mineral medium (pH 7.2) containing 0.102 g of KH_2PO_4, 1.11 g of $Na_2HPO_4 \cdot 12H_2O$, 0.02 g of $MgSO_4$ and 1 mL of a microelement solution. The prescribed concentrations of L-valine as the sole carbon source was added to the above mineral medium. The cells were aerobically cultivated for the prescribed periods at 30 °C and pH 7.2 on a reciprocal shaker in a 500-mL Sakaguchi flask. The isolation and purification were the same as the two-step cultivation method.

Fractionation Procedure. As-produced copolyesters were treated as following: 100 mg of original sample was dissolved in 20 mL of chloroform, and about 20 mL of heptane was added dropwise to this solution with stirring until becoming turbid. After refluxing for 1 h, the mixed solution was allowed to stand overnight at room temperature. The precipitated polyester and soluble polyester were separated in the usual way and weighed.

Analytical Procedure. The compositions and sequence distributions of copolymers were determined by analyses of ^{1}H and ^{13}C NMR spectra. ^{1}H NMR and ^{13}C NMR analyses were carried out on a JEOL GX-270 spectrometer. The 270-MHz ^{1}H NMR spectra were recorded at 21.3 °C in a $CDCl_3$ solution of polyester (4 mg mL^{-1}) with a 4.9 μs pulse width (45° pulse angle), 16000 data points, and 32 accumulations. The ^{1}H noise decoupled 67.5-MHz ^{13}C NMR spectra were recorded at 21.3 °C in a $CDCl_3$ solution of polyester (20 mg mL^{-1}) with a 4.0 μs pulse width (45° pulse angle), 5 s pulse repetition, 20000 Hz spectral width, 32000 date points, and 15000 accumulations. Tetramethylsilane (Me_4Si, $\delta = 0$) was used as an internal chemical shift standard.

Molecular weights were determined by gel permeation chromatography (GPC) using polystyrene calibration on a JASCO 807-IT equipped with a TOSOH TSK-GEL G4000HXL column at 25 °C in $CHCl_3$. The sample concentration was 1.0 mg mL^{-1} and the flow rate of chloroform was 1 mL min^{-1}.

Differential scanning calorimetry (DSC) data of polyesters were analyzed in the temperature range of −100 to 200 °C on a SEIKO SSC 5000 DSC 220 equipped with a cooling accessory under a nitrogen flow of 30 mL min^{-1}. Samples were heated from 0 to 200 °C at a rate of 20 °C min^{-1} under a nitrogen stream. The melting temperature (T_m) and the enthalpy of fusion (ΔH_m) were determined from the DSC endotherms. For measurement of the glass-transition temperature (T_g), the samples were maintained at 200 °C for 1 min, and then rapidly quenched at −100 °C, They were then heated from −100 °C to 200 °C at a heating rate of 20 °C min^{-1}. The T_m and T_g were taken as the midpoint of the heating capacity change.

The stress-strain curves of cast films of copolyester samples were obtained at 23 °C with a strain rate of 20 mm min^{-1} on a Shimadzu AG-5KNE Auto GRAPH tensile mashine. The mechanical tensile data were calculated from such curves on an average of three specimens.

Results and Discussion

Biosynthesis of the Polyesters from Different L-Amino Acids. Inoue et al. reported the biosynthesis of polyesters from different naturally occurring α-amino acids by wild-type *Ralstonia eutropha*, but the yields of polyesters in dried cells were very low, with 3.7 wt % or less.[12,16] However, an examination of cultivation time and concentration of amino acid has not been conducted for improving the yield of polyesters. We investigated in detail on the polyester production conditions from different L-amino acids by wild-type *R. eutropha*. The biosynthesis of polyesters was conducted in a nitrogen-poor medium containing 10 g dm^{-3} (1%, w/v) of each L-amino acid as the sole carbon source by two-step batch fermentation. In two-step batch fermentation, *R. eutropha* was first grown for 24 h in a nutrient-rich medium in which polyester was not accumulated within the cells. After that, the collected cells were transferred into a mineral medium containing an L-amino acid and incubated at 30 °C and pH 7.2. Figure 1 shows the time courses of dry cell weight and polyester production from different L-amino acids during the two-step batch cultivation of *R. eutropha*. During an initial cultivation time, the increase in rate of the dry cell weights obtained from different L-amino acids is larger than that of the polyester weights accumulated. This suggests that both cell growth and polyester accumulation

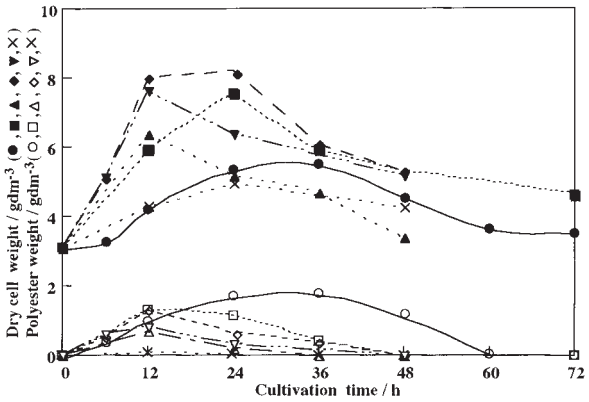

Fig. 1. Time courses of dry cell and polyester weights from 1% (w/v) of different L-amino acids by *R. eutropha* at 30 °C. L-Valine (●, ○), L-phenylalanine (■, □), L-glutamic acid (▼, ▽), L-leucine (◆, ◇), L-isoleucine (▲, △), L-threonine (×, ×).

occurred simultaneously. This may be reasonable since *R. eutropha* is cultivated under the presence of poor nitrogen generated from amino acid used as the sole carbon source. The maximal dry cell weights and accumulated polyester weights from a 1% (w/v) media of trial L-amino acids were observed in a comparatively short cultivation time between 6 h and 36 h, and the accumulated polyesters were observed to be almost degradated in vivo after 48 h of incubation, except for L-valine. The previous report of Inoue et al.[16] on the production of polyesters from different L-amino acids in *R. eutropha* was only a result of the 48 h culture time. Hence, the polyesters accumulated in cells during 48 h of culture time may apparently result in low yields because of comparatively rapid degradation in vivo. Table 1 shows the production of polyesters from a mineral medium containing 1% (w/v) of different L-amino acids as the sole carbon source at culture times of 12 h and 24 h, respectively. The polyester productivities from most of the L-amino acids used as a carbon source were higher at a culture time of 12 h rather than 24 h, and the essential amino acids, such as L-valine, L-phenylalanine, L-leucine, L-isoleucine, and L-methionine, among different L-amino acids, were comparatively effective for polyester accumulation. In paticular, the polyester content in a dried cell was as large as 32 wt % at the culture time of 24 h, when 1% (w/v) of L-valine as a sole carbon source was fed to the wild-type *Ralstonia eutropha*. The polyesters obtained from L-amino acids were 3-hydroxybutyric acid (3HB) homopolymer [P(3HB)], or copolymers consisting of 3HB units and a minor amount of 3-hydroxyvaleric acid (3HV) by analysis of ^{1}H NMR spectra.

Biosynthesis of the Polyesters from L-Valine in a Two-Step Fermentation. The culture condition of the biosynthesis from L-valine was examined in detail, because the productivity of the polyester from L-valine was effective and the polyesters obtained were P(3HB-*co*-3HV) with ca. 10 mol% 3HV compositions, which have been focused on as commercially available materials. Figure 2 shows the production of polyester, the weight of the dry cells, and residual biomass obtained by the cultivation of *R. eutropha* during 36 h from various amounts of L-valine in a two-step fermentation. The residual biomass is calculated as the difference between the dry cell weight and polyester content.[17] Both the dry cell and polyester weights increase with increasing concentration of L-valine up to 20 g dm^{-3}, and then remained at constant weights of 6 g dm^{-3} and 2 g dm^{-3}, respectively, beyond the L-valine concentration of 20 g dm^{-3}. In the 36 h culture, the change in the residual biomass for the L-valine concentrations was hardly observed, even if the L-valine concentration in the media was as high as 50 g dm^{-3}. This phenomenon was also similar to the case of the 24 h culture, but the residual biomass increased slightly with the L-valine concentrations above 48 h cultivation time (data not shown). These facts suggest that the wild-type

Table 1. Biosynthesis of Polyesters from Different L-Amino Acids (1%, w/v) for 12 h and 24 h at pH 7.2 and 30 °C by *R. eutropha* under Two-Step Fermentation

L-Amino acid	Cultivation time	Dry cell weight	Polyester content[a)]	Composition /mol%[b)]	
10 g dm^{-3}	h	g dm^{-3}	wt %	3HB	3HV
Val	12	4.19	23.75	90	10
	24	5.35	32.02	92	8
Phe	12	5.94	22.10	100	0
	24	7.55	14.90	100	0
Leu	12	8.00	16.12	100	0
	24	8.18	8.11	100	0
Ile	12	7.63	10.13	98	2
	24	6.37	5.32	97	3
Met	12	2.59	6.83	97	3
	24	2.47	3.81	99	1
Lys	12	2.17	1.90	100	0
	24	2.07	2.52	100	0
Trp	12	4.13	2.33	100	0
	24	6.52	0.11	—	—
Thr	12	4.35	2.90	99	1
	24	5.02	0.01	—	—
Tyr	12	5.91	7.92	100	0
	24	5.84	1.97	100	0
Gly	12	1.83	2.13	99	1
	24	2.56	0.93	—	—
Ser	12	4.37	2.47	100	0
	24	3.52	0.17	—	—
Ala	12	4.81	0.44	100	0
	24	4.20	0.19	—	—
Cys	12	10.20	0.42	100	0
	24	9.65	0.45	99	1
Pro	12	4.66	2.84	100	0
	24	6.32	0.11		
Gln	12	5.61	0.16	—	—
	24	4.47	0.00	—	—
Asn	12	4.46	0.00	—	—
	24	4.15	0.00	—	—
Glu	12	5.20	3.46	99	1
	24	4.69	0.09	—	—
Asp	12	4.46	0.00	—	—
	24	4.84	0.06	—	—
Arg	12	2.22	1.53	100	0
	24	2.40	1.00	100	0
His	12	5.50	0.29	99	1
	24	5.04	0.00	—	—

a) Polyester content in dry cell weights. b) Determined by ^{1}H NMR.

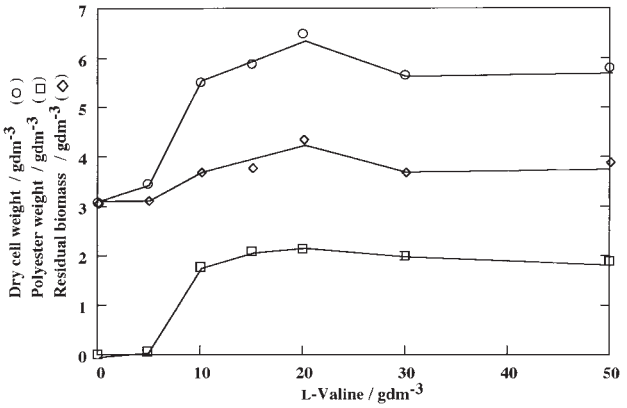

Fig. 2. Production of the polyester by the two-step fermentation of *R. eutropha* from different amounts of L-valine for 36 h at 30 °C. Dry cell weight (○), polyester weight (□), residual biomass (◇).

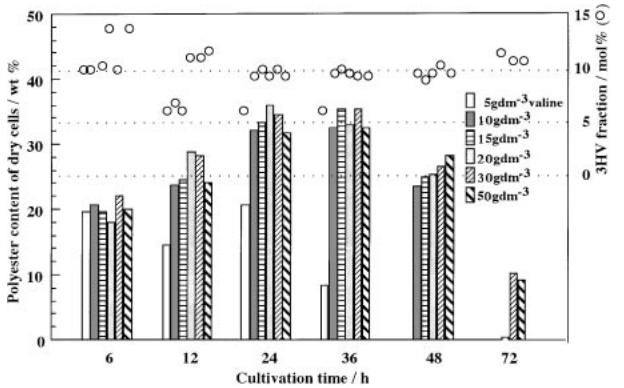

Fig. 3. Time courses of the polyester contents in dry cell weights and 3HV fractions of polyesters produced by the two-step fermentation of *R. eutropha* in the mineral medium containing different amounts of L-valine.

Ralstonia eutropha may preferentially utilize L-valine as a carbon source for polyester accumulation rather than as a nitrogen source for cell growth during the compalatively short culture time from 24 h to 36 h. In contrast, other L-amino acids are almost only utilized for the cell growth of *R. eutropha* (Fig. 1). The results of polyester production while changing the amounts of L-valine and culture times together with the compositions of polyesters obtained are shown in Fig. 3. The optimal concentration of L-valine and culture time for polyester production were 10–30 g dm^{-3} (1–3% (w/v)) and 24–36 h, respectively, and the polyester content in dried cells under this condition was as high as 36 wt %. The polyesters obtained from L-valine were P(3HB-*co*-3HV), with compositions ranging from 7 mol% to 14 mol% 3HV. It is known that the amino acids are amphoteric electrolyts and change their charge state according to the pH value of the medium, and the solubility of amino acids becomes a minimum at the isoelectric point. The results of polyester production in 1% (w/v) L-valine in various pH values is shown in Table 2. The fermentation was carried out for 24 h at 30 °C. The highest dry cell wight and polyester weight were observed in the culture solution of pH 7.2 containing 1% (w/v) of L-valine, but the polyester contents in the dried cells were an almost constant 30 wt % in the pH range of 5.5–8.0. The polyester content in dried cells decreased slightly at pH 8.5. The 3HV composition of P(3HB-*co*-3HV) produced was ca. 9 mol%, independent of the pH variation in the culture solution. The production of polyester in *R. eutropha* from L-valine as the sole carbon source was slightly affected in a weakly acidic media of pH 5.5, weakly alkaline media of pH 8.0, and media of pH 6.0, which is isoelectric point of L-valine, respectively. Table 3 shows the effect of culture temperature on the polyester production from L-valine for 24 h at pH 7.2. The polyester contents in dried cells were constant, as high as 32 wt % at culture temperatures ranging from 25 to 35 °C, but decreased considerably at 40 °C.

Table 2. The Effect of pH Values on Polyester Production by *R. eutropha* from 1% (w/v) of L-Valine for 24 h at 30 °C

pH	Dry cell weight	Polyester weight	Polyester content[a)]	Composition /mol%[b)]	
	g dm^{-3}	g dm^{-3}	wt %	3HB	3HV
5.5	4.82	1.56	32.4	91	9
6.0	4.59	1.42	30.8	91	9
7.2	5.35	1.72	32.1	92	8
8.0	5.10	1.51	29.6	91	9
8.5	4.61	1.30	28.1	92	8

a) Polyester content in dry cell weights. b) Determined by ^{1}H NMR.

Table 3. The Effect of Cultivation Temperatures on Polyester Production by *R. eutropha* from 1% (w/v) of L-Valine for 24 h at pH 7.2

Temp.	Dry cell weight	Polyester weight	Polyester content[a)]	Composition /mol%[b)]	
°C	g dm^{-3}	g dm^{-3}	wt %	3HB	3HV
25	4.25	1.36	31.9	91	9
30	5.35	1.72	32.1	92	8
35	4.48	1.44	32.2	92	8
40	3.38	0.17	5.2	93	7

a) Polyester content in dry cell weights. b) Determined by ^{1}H NMR.

Biosynthesis of the Polyesters from L-Valine in a One-Step Fermentation. A two-step fermentation is an ineffective culture method because of the expensive nutrients for cell growth. Therefore, the production of P(3HB-*co*-3HV) in *R. eutropha* from L-valine as the sole carbon source was carried out by a one-step fermentation in which cell growth and polyester accumulation occur simultaneously in a mineral medium containing L-valine alone. Figure 4 shows the time courses of cell growth and polyester production for two different concentrations of L-valine, namely 1% (w/v) and 2% (w/v), during the one-step batch cultivation of *R. eutropha* at 30 °C and pH 7.2 under aerobic conditions. The times until cell growth began were for 24 h at 1% (w/v) and 48 h at 2% (w/v) of L-valine, respectively, and the maximal polyester weights were obtained at 96 h and 120 h in media containing 1% (w/v) and 2% (w/v) of L-valine, respectively. Figure 4 shows also the time courses of the polyester contents and components in

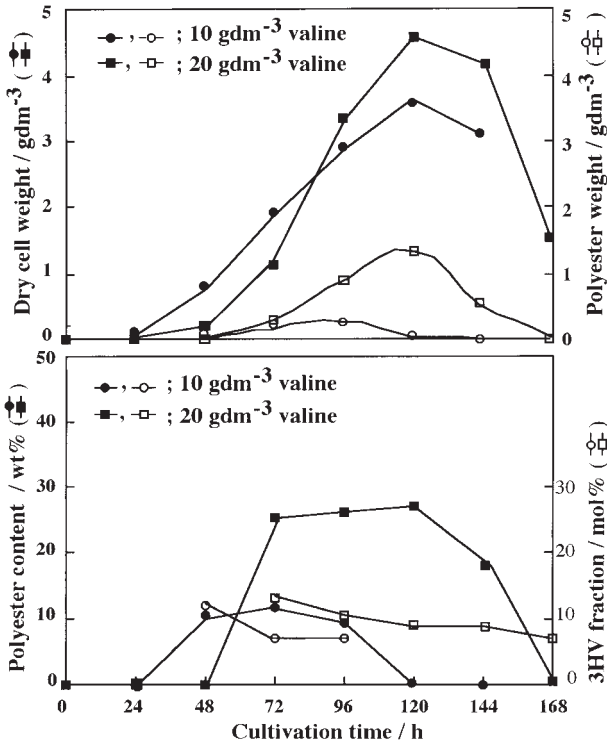

Fig. 4. Production of polyesyer from L-valine by one-step fermentation in *R. eutropha* at 30 °C. Dry cell weight and polyester content from 10 g dm^{-3} (-●-) or 20 g dm^{-3} (-■-) of L-valine; polyester weight and 3HV fraction from 10 g dm^{-3} (-○-) or 20 g dm^{-3} (-□-) of L-valine.

the one-step fermentation. The polyesters were accumulated up to 27 wt % of dry cell weights in *R. eutropha* from 2% (w/v) of L-valine during 120 h culture time. The 3HV fractions of polyesters were constant values of ca. 9 mol% at the culture time of the maximal polyester contents, similar to the results of the above-mentioned two-step fermentation, although they showed slightly high values during cell growth.

Fractionation and Properties of P(3HB-*co*-3HV) Biosynthesized from L-Valine. Yoshie et al. reported that the commercially available P(3HB-*co*-3HV)s with a range from 6 to 20 mol% 3HV biosythsized by *R. eutropha* from propionic acid and glucose as the mixed carbon sources were a blend of several pure copolyesters with a different composition.[10] This was investigated by a chloroform/heptane fractionating procedure. We examined whether the P(3HB-*co*-3HV)s with ca. 10 mol% 3HV obtained from L-valine as the sole carbon source also had broad compositional distribution. Results of the fractionation are shown in Table 4. The original bacterial samples with 8 mol% 3HV and 10 mol% 3HV were fractionated by precipitation from a 0.5% (w/v) chloroform solution with heptane according to the procedure of Yoshie et al.[10] The weight fractions precipitated first on addition of heptane (ca. 56% (v/v) to the amount of chloroform/heptane mixed solvent) were almost 100 wt % and the residues remaining in the solution with excess heptane were not recovered at all. The monomer compositions of fractionated samples were entirely similar to the as-biosynthesized P(3HB-*co*-3HV)s with 8 mol% 3HV and 10 mol% 3HV from L-valine, respectively. In addition, we studied the sequence distributions of 3HV and 3HB units in chloroform by analysis of ^{13}C NMR spectra. Figure 5 shows the 67.5-MHz ^{13}C NMR spectrum of the as-biosynthsized P(3HB-*co*-3HV) with 10 mol% 3HV obtained from L-valine, together with the chemical shift assignments, and Fig. 5 shows also the expanded spectra of each carbon resonance, which are split into multiplets owing to diad and/or 3HV-centered triad comonomer sequences. The assignments of these signals have been reported previously.[18] The profiles of ^{13}C NMR spectra of the fractionated sample were completely similar to the as-biosynthesized P(3HB-*co*-3HV). The mole fractions of 3HB and 3HV units, F_B and F_V, were determined using ^{1}H NMR spectra (data not shown). The diad sequence distributions of 3HB and 3HV units, F_{BB}, $F_{BV} + F_{VB}$, and F_{VV}, were determined from the well-resolved peaks of the carbonyl carbon (B_1 and V_1) and methine carbon (B_3) resonances, respectively. The 3HV-centered triad sequence distributions, F_{VVV}, F_{BVV}, F_{VVB}, and F_{BVB} were determined from the resolved peaks of the methylene carbon (V_4). The parameter D, which indicates whether the sample is a random copolymer or not, is defined as $D = F_{VV}F_{BB}/(F_{BV}F_{VB})$ by Chûjyô et al.[19] A statistically random copolymer has a D value close to 1. The experimental monomer sequence distributions and D values are also given in Table 4, compared with values calculated by using the Bernoullian statics applicable to a stastistically random copolymerization. As shown in Table 4, the observed se-

Table 4. Fraction and Sequence Distribution of P(3HB-*co*-3HV) Obtained from L-Valine

Heptane[a]	Polyester	Fraction weight	3HV[b]	Sequence distribution[c]									
% v/v		mg dm^{-3}	mol%	F_V	F_B	F_{VV}	F_{VB}	F_{BV}	F_{BB}	F_{VVV}	F_{BVV}	F_{BVB}	D[d]
	original		8	0.080	0.920	0.007	0.072	0.075	0.853	0.000	0.005	0.071	1.10
				(0.080	0.920	0.006	0.074	0.074	0.846	0.000	0.006	0.068)	
55	soluble	0	—										
	insoluble	100	8										
	original		10	0.100	0.900	0.012	0.091	0.092	0.805	0.001	0.010	0.080	1.15
				(0.100	0.900	0.010	0.090	0.090	0.810	0.001	0.009	0.081)	
57	soluble	0	—										
	insoluble	100	10										

a) Concentration of heptane in chloroform/heptane mixed solvent. b) Determined by ^{1}H NMR spectra. c) Determined by ^{13}C NMR spectra. Numbers in brackets were calculated by using Bernoullian statistics. d) $D = F_{VV}F_{BB}/F_{VB}F_{BV}$.

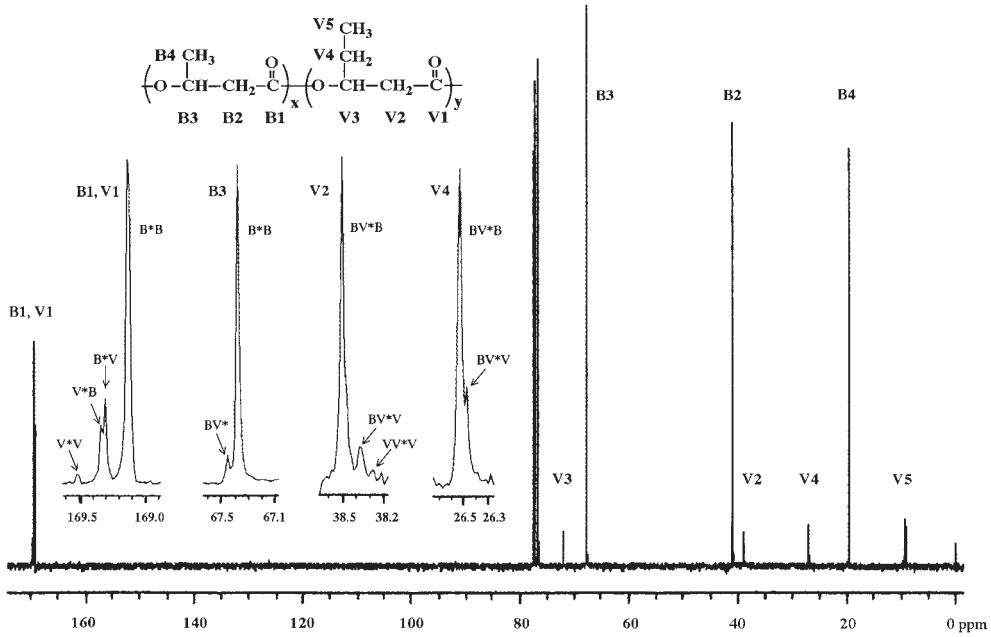

Fig. 5. 67.5-MHz ^{13}C NMR spectra of P(3HB-*co*-10 mol% 3HV) from L-valine, together with the chemical shift assignments and the expanded spectra for carbonyl and methylene carbon resonances.

Table 5. Properties of P(3HB-*co*-3HV) Produced from L-Valine by *R. eutropha*

Carbon source	L-Valine		Glucose + Propionic acid	Butyric acid + Pentanoic acid
Polymer	Sample 1[a)]	Sample 2[a)]	Sample 3[b)]	Sample 4[c)]
3HV content/mol%	10	9	11	9
Molecular weight[d)]/10^{-4} M_n	72	80	9	40
M_w/M_n[d)]	1.5	1.5	4.5	2.7
T_g[e)]/°C	3.3	2.6	2.0	2.5
T_m[e)]/°C	146	145	157	162
ΔH_m[e)]/J g^{-1}	76.2	71.3	—	66
Young modulus/GPa	n.d.[f)]	1.37	3	1.9
Tensile strength/MPa	n.d.	22	30	37
Elongation at break/%	n.d.	19	5	—

a) Sample 1 and Sample 2 were biosynthesized at culture times of 12 h and 36 h, respectively. b) Sample 3 showed the data described in Ref. 20. c) Sample 4 showed the data described in Ref. 21. d) Determined by GPC in chloroform. e) Determined by DSC at 20 °C min^{-1}. f) Not determined.

quence distributions for P(3HB-*co*-3HV) are in agreement with the calculated diad and triad values, and *D* values are close to 1. From these results, and the above-mentioned results of the fractionation, it is concluded that the sequence distributions of 3HB and 3HV units in the as-biosynthesized copolymer obtained from L-valine are statistically random and the copolymers are not a mixture of different random copolymers. Table 5 lists the thermal and mechanical properties of P(3HB-*co*-3HV) with ca. 10 mol% 3HV produced from L-valine, compared with those of the coplymers with a similar 3HV fraction biosynthesized from such the mixed carbon sources as propionic acid and glucose,[20] and butyric acid and valeric acid.[21] The melting point of the copolymer from L-valine was only one sharp peak, and was at a lower temperature than that from the mixed carbon substrates, but the enthalpy of fusion (ΔH_m) of the nonannealed sample was comparatively high. This suggests that P(3HB-*co*-3HV)s produced in *R. eutropha* from L-valine have a random composition distribution and are not a mixture of different random copolymers, and, moreover, have higher crystallinity than that of copolyesters produced from the mixed carbon sources. The number average molecular weights of the polyesters were up to 8×10^5 g mol^{-1}. Young's modulus and the tensile strength of P(3HB-*co*-9% 3HV) obtained from L-valine were slightly smaller than those from the mixed carbon substrates, while the elongation at break was about 4 times larger.

Figure 6 shows the schematic pathway of P(3HB-*co*-3HV) biosynthesis from L-valine in a wild-type *R. eutropha*, according to the proposed metabolism of branched-chain amino acids.[11] L-Valine is metabolized to 2-oxo-3-methylbutyric acid, which is oxidatively decarboxylated to 2-methylpropionyl-CoA by the branched-chain 2-keto acid dehydrogenase complex, and the latter is further metabolized via various other CoA to propionyl-CoA that is a precursor of 3HB and 3HV

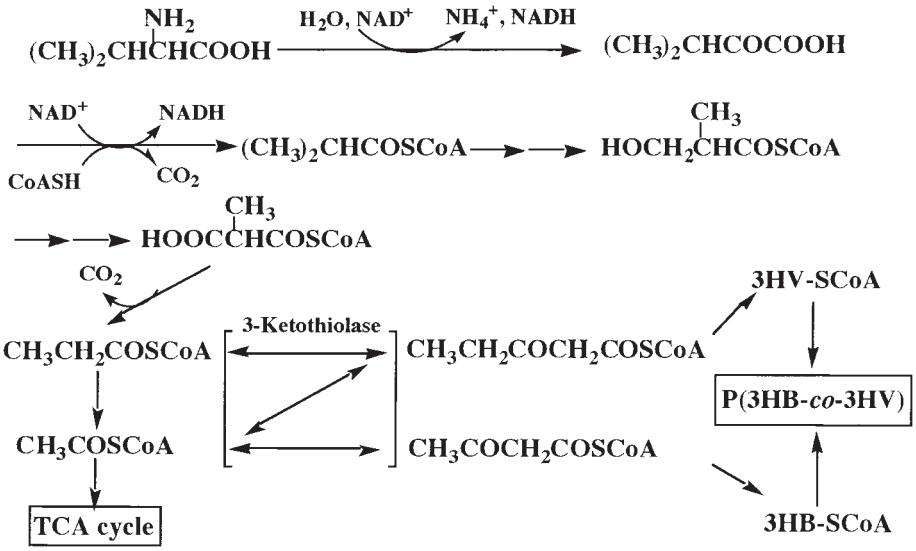

Fig. 6. Putative metabolic pathway of P(3HB-*co*-3HV) biosyntehsis from L-valine in wild-type *Ralstonia eutropha*.

monomeric units. In addition, the NH_4^+ liberated from amino acids may promote the in vivo degradation of polyesters accumulated in cells. This is suggested by the fact that the polyesters produced in *R. eutropha* from 1% (w/v) amino acids were perfectly degraded within 60 h of culture time, as shown in Fig. 1, while those from free-nitrogen carbon sources, such as fructose or butyric acid, were accumulated in cells over 100 h.[22]

In conclusion, commercially promising P(3HB-*co*-3HV)s with ca. 10 mol% 3HV compositions were produced up to 36 wt % of the dry cell matter in wild-type *Ralstonia eutropha* from L-valine as a single substrate. It was found that this biosynthesis occurred in a comparatively short cultivation time between 6 h and 48 h. The as-biosynthesized copolymers have a narrower comonomer compositional distribution, and a number-average molecular weight up to 8×10^5 g mol^{-1}.

References

1 K. Sudesh, H. Abe, and Y. Doi, *Prog. Polym. Sci.*, **25**, 1503 (2000).

2 A. J. Anderson and E. A. Dawes, *Microbiol. Rev.*, **54**, 450 (1990).

3 M. Lemoigne, *Bull. Soc. Chim. Biol.*, **8**, 770 (1926).

4 B. H. A. Rehm, T. A. Mitsky, and A. Steibüchel, *Appl. Environ. Microbiol.*, **67**, 3102 (2001).

5 P. A. Holmes, *Phys. Technol.*, **16**, 32 (1985).

6 N. D. Miller and D. F. Williams, *Biomaterials*, **8**, 129 (1987).

7 W. J. Orts, R. H. Marchessault, and T. L. Bluhm, *Macromolecules*, **24**, 6435 (1991).

8 M. Sanchez-Cuesta, J. Martinnez-Salazar, P. A. Baker, and P. J. Barham, *J. Mater. Sci.*, **27**, 5335 (1992).

9 R. Renstad, S. Karlsson, and A.-C. Albertsson, *Macromol. Symp.*, **127**, 241 (1998).

10 N. Yoshie, H. Menju, H. Saito, and Y. Inoue, *Macromolecules*, **28**, 6516 (1995).

11 A. Steibüchel and U. Pieper, *Appl. Microbiol. Biotechnol.*, **37**, 1 (1992).

12 K. Nakamura, Y. Gotô, N. Yoshie, Y. Inoue, and R. Chûjyô, *Int. J. Biol. Macromol.*, **14**, 117 (1992).

13 M. Reh and H. G. Schlegel, *Arch. Mikrobiol.*, **67**, 110 (1969).

14 A. K. Mukhopadhyay and S. K. Majumdar, *Zentralbl. Mikrobiol.*, **140**, 107 (1985).

15 F. Sanda and T. Endo, *Macromol. Chem. Phys.*, **200**, 2651 (1999).

16 M. Fujita, K. Nakamura, H. Kuroki, N. Yoshie, and Y. Inoue, *Int. J. Biol. Macromol.*, **15**, 253 (1993).

17 E. Heinzle and R. M. Lafferty, *Eur. J. Appl. Microbiol. Biotechnol.*, **11**, 8 (1980).

18 Y. Doi, M. Kunioka, Y. Nakamura, and K. Soga, *Macromolecules*, **20**, 2988 (1987).

19 N. Kamiya, Y. Yamamoto, Y. Inoue, R. Chûjyô, and Y. Doi, *Macromolecules*, **22**, 1676 (1989).

20 H. Mitomo, P. J. Barham, and A. Keller, *Polym. Commun.*, **29**, 112 (1988).

21 Y. Doi, "Microbial Polyesters," VCH Publishers, New York (1990).

22 Y. Kawaguchi and Y. Doi, *Macromolecules*, **25**, 2324 (1992).