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Culture Conditions Affecting the Molecular Weight Distribution of Poly(3-Hydroxybutyrate-*co*-3-Hydroxyvalerate) Synthesized by *Alcaligenes* sp. SH-69

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The weight average molecular weight of poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) synthesized by *Alcaligenes* sp. SH-69 was altered between 3.2×10^5 and 1.1×10^6 depending upon various culture conditions. It appeared that culture conditions favorable for the efficient production of copolyesters promoted the formation of higher molecular weight copolyesters. Polydispersity indices of isolated copolyesters were in the range of 1.5 to 2.5.

Key words: poly-β-hydroxyalkanoates, molecular weight, *Alcaligenes* sp. SH-69, copolyester, culture conditions

Bacterial polyesters classified as poly-β-hydroxyalkanoates (PHAs) have been the focus of extensive research because of their potential application as biodegradable and biocompatible thermoplastics (11, 12). Since certain copolyesters of 3-hydroxybutyrate (3HB) and other hydroxyalkanoate monomer units have more reliable thermomechanical properties than poly-β-hydroxybutyrate (PHB) homopolyester (2), interest in the microbial production of copolyesters has increased considerably. Of these copolyesters, a copolyester consisting of 3HB and 3-hydroxyvalerate [poly(3HB-*co*-3HV)] has been of the greatest commercial interest and it is being produced industrially using a strain of *Alcaligenes eutrophus* by Zeneca Bioproducts, UK, under the trade name "Biopol".

Alcaligenes sp. SH-69 has been shown to be an exceptional strain that produces poly(3HB-*co*-3HV) from single carbon sources such as glucose, sucrose, and sorbitol (8, 9). This organism is potentially useful for commercial production of poly(3HB-*co*-3HV) since price reduction can be achieved not only by reducing the substrate costs but also by facilitating fermentation process (13, 14). Particularly, it has been reported that the molar fraction of 3HV in the copolyester produced by this organism can be enhanced to a maximum level of 53.0 mol% by addition of amino acids, such as threonine, isoleucine, and valine, into glucose medium (18).

Control of molecular weights of PHA is most crucial for practical application because physical properties and biodegradability of the polyesters are dependent on their molecular weights (10, 15). Since biosynthesis and accumulation of PHAs are affected by culture conditions, it seems likely that culture conditions would be important in determining molecular weights of PHAs. However, there have been only a few published papers reporting the control of its polymerization degree under different environmental conditions (5, 16, 17). In this study, we have investigated culture conditions that affect molecular weights of poly(3HB-*co*-3HV) copolyesters produced by *Alcaligenes* sp. SH-69.

Alcaligenes sp. SH-69 was grown aerobically in the 300 ml Erlenmeyer flasks containing 50 ml of basal medium as described previously (14, 18). The medium was inoculated with a 5% (v/v) inoculum of an overnight culture in the same medium, except that it contained 1% glucose and 0.2% $(NH_4)_2SO_4$. In the cases of amino acid addition experiments, sterilized amino acids with varying concentrations were added to the culture medium as described elsewhere in the text. After the flasks were inoculated, they were incubated at 37°C and 180 rpm for 24 h. All fermentor experiments were conducted in a 2.5 L jar fermentor (Korea Fermentor Co., Korea) with a working volume of 1 L. The culture medium used was

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the same as that used in the shake flask experiments. The medium was inoculated with a 10% (v/v) inoculum and the culture was grown at 37°C until the carbon source was depleted. The pH was automatically controlled at optimal value, 7.0±0.1. The air flow rate was 1.0 vvm and agitation speed was 420 rpm. Stock cultures were maintained at 4°C by periodical transfer on a solid medium (yeast extract 1%, peptone 1%, meat extract 1%, and agar 1.8%).

Cell growth was monitored by measuring optical density at 660 nm. Dry cell weight (DCW) was measured by drying the harvested cells to constant weight at 105°C. Glucose concentration in the broth was determined using a glucose analysis kit (Sigma, USA), while ammonium ions were determined using an ion meter (Cole Parmer, USA) coupled with an ammonium electrode. PHA content and its composition were determined by gas chromatography as described by Braunegg *et al.* (4). The internal standard was benzoic acid, and the external standards were copolyesters (Biopol) of which 3HB and 3HV monomer contents were known. The amount of PHA was quantified by summing the amounts of 3HB and 3HV monomers detected.

PHA samples for molecular weight determination were extracted from the freeze-dried cells with hot chloroform and purified by precipitation with methyl alcohol. Weight average molecular weight (Mw), number average molecular weight (Mn), and polydispersity index (Mw/Mn) were determined by a gel permeation chromatography (GPC) system equipped with a Waters 590 programmable HPLC pump, a Waters 401 refractive index detector, a U 6K injector, Phenomennex GPC columns in series, and a guard column. Polystyrene standards (Sigma) with low polydispersity were used to establish a calibration curve. Chloroform was used as the eluent at a flow rate of 1.0 ml/min. Sample concentrations were 1 mg/ml and injection volumes were 100 µl. Chromatograms were recorded and molecular weights were calculated using a PC compatible equipped with Chromate Data Analysis Kit (Interface Co. Ltd., Korea).

To elucidate the effect of pH on the molecular weights of poly(3HB-*co*-3HV) produced by Alcaligenes sp. SH-69, batch fermentation experiments were carried out with varying pH (Fig. 1). The Mw of the isolated copolyesters ranged from 3.8×10^5 to 7.9×10^5. The copolyester which was synthesized at the optimal pH for the growth and PHA accumulation, *i.e.* pH 7.0 (14), had the highest Mw. The increase or decrease of pH from 7.0 resulted in a progressive decrease of Mw. The Mn also decreased but more rapidly than Mw, which resulted in a progressive increase in polydispersity indices from 1.5 to 2.5 at acidic or basic pHs. Even though clear correlation between PHA content and polymerization degree was not observed, the results indicated that the optimal pH is favorable for the production of poly(3HB-*co*-3HV) with higher molecular weight. Molecular weights of PHAs accumulated in cells grown at various temperatures from 25 to 40°C at pH 7.0 were also examined, however the Mw of the PHAs did not vary significantly and maintained in the range of 6.8×10^5~8.4×10^5.

Carbon source concentration has been proposed as one of the most important factors affecting the molecular weight of PHA. Suzuki *et al.* (16) have reported that lowering concentration of methanol, which is a carbon source in methylotrophs cultivation, *Protomonas (Methylobacterium) extorquens*, caused a production of

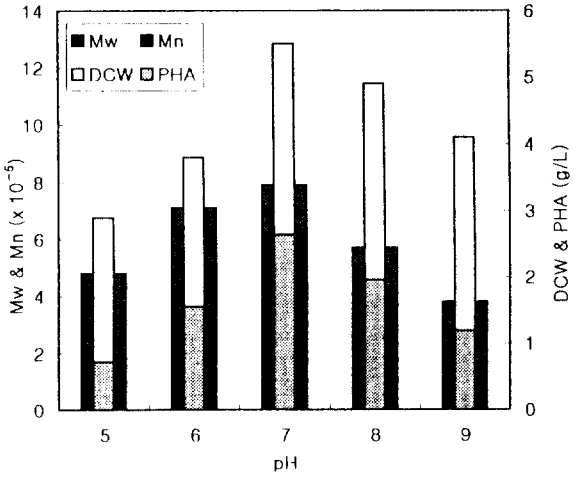

Fig. 1. Effect of pH on accumulation and molecular weight of poly(3HB-*co*-3HV) by *Alcaligenes* sp. SH-69 in jar-fermentor cultures. The molar fraction of 3HV in the copolyesters did not vary significantly and amounted to 5~7 mol%.

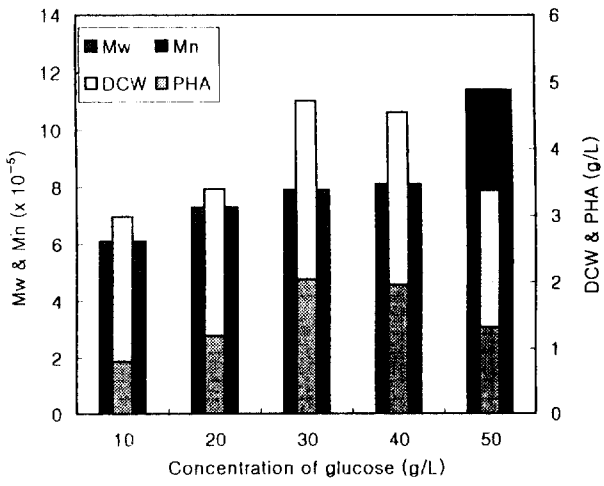

Fig. 2. Effect of glucose concentration on accumulation and molecular weight of poly(3HB-*co*-3HV) by *Alcaligenes* sp. SH-69.

PHB with a high molecular weight. Similarly, Mw of PHB or poly(3HB-*co*-3HV) produced by other bacteria such as *Alcaligenes eutrophus* (1, 15) and *Azotobacter vinelandii* UWD (5) decreased with increasing carbon substrate concentration. In this study, the molecular weight of the copolyesters accumulated by *Alcaligenes* sp. SH-69 was affected significantly by the concentration of carbon source (glucose) supplied (Fig. 2). Over the range of glucose concentrations (10~50 g/L), however, the Mw and Mn increased with increasing the initial concentration of glucose in the medium. In a previous study (14), it was reported that the maximum specific growth rate and maximum biomass yield of this organism was relatively high when the initial glucose concentration was between 5 and 20 g/L. By contrast, relatively high PHA content (% DCW) was observed in this study when glucose concentration exceeded 30 g/L, even though the molar fraction of 3HV in the copolyesters did not vary significantly and amounted to 4~6 mol%. These results indicate that the polymerization degree of PHA is dependent upon PHA content rather than specicific growth rate, which are different from results of earlier studies (1, 5, 15, 16). Meanwhile, molecular weights of copolyesters produced by *Alcaligenes* sp. SH-69 grown with glucose (20 g/L) and either ammonium sulfate, ammonium hydroxide, urea or yeast extract as a nitrogen source were not significantly different. In all cases, Mw values were in the range of 6.1×10^5~7.9×10^5.

It was known that the increase of glucose to ammonium (C/N) ratio in the culture medium could enhance the PHA content in *Alcaligenes* sp. SH-69 (14). To investigate the correlation between C/N ratio and polymerization degree, four fed-batch cultures were carried out in which ammonium sulfate feed solution was added simultaneously with glucose to keep C/N ratios (mol glucose/mol ammonium) at 5, 15, 29, or 50 as described previously (14). As shown in Table 1, the increase of C/N ratio resulted in a gradual increase of PHA content from 17.7 to 46 %DCW and Mw from 6.7×10^5 to 1.1×10^6 without any considerable variations in polydispersity indices (from 1.4 to 1.8).

Recently Yoon *et al.* (18) reported that addition of the amino acids, such as threonine, isoleucine, and valine, into culture medium greatly increased the molar fraction of 3HV in poly(3HB-*co*-3HV) produced by *Alcaligenes* sp. SH-69. In the present study, shake flask experiments were carried out using basal media containing various concentrations of threonine, isoleucine, and valine to investigate how amino acid concentration affect the molecular weight of the copolyester (Table 2). The molar fractions of 3HV in the copolyesters produced significantly increased with increasing concentrations of amino acids

Table 1. Experimental results for the production of poly(3HB-*co*-3HV) with various C/N ratios during the PHA accumulation sage fed-batch culure of *Alcaligenes* sp. SH-69. When the cell mass conentration reached about 35 g/L at 20 h, ammonium feed solution was added simultaneously with glucose to maintain the C/N ratio for a period of 20 hours

C/N ratio (mol/mol)	DCW (g/L)	PHA (g/L)	3HV (mol%)	Mw ($\times10^5$)	Mn ($\times10^5$)
5	82	14.7	6.7	6.7	3.7
15	73	25.2	5.4	8.8	5.9
29	66	29.7	4.0	10.4	7.4
50	58	26.7	4.4	11.2	6.6

Table 2. Effects of amino acid addition on accumulation and molecular weight of poly(3HB-*co*-3HV) produced by *Alcaligenes* sp. SH-69

Amino acid	(mM)	DCW (g/L)	PHA content (% DCW)	3HV (mol%)	Mw ($\times10^5$)	Mn ($\times10^5$)	Polydispersity (Mw/Mn)
-		4.5	43.2	6.5	7.9	4.9	1.6
Threonine	5	4.1	43.0	22.5	7.2	4.8	1.5
	10	3.8	38.9	46.4	6.9	4.1	1.7
	20	2.9	40.2	55.8	5.8	3.1	1.9
	30	1.7	24.5	50.5	4.8	2.2	2.2
Isoleucine	5	4.4	42.1	22.7	7.9	5.3	1.5
	10	3.5	37.6	33.0	7.0	4.7	1.5
	20	3.0	35.1	35.0	5.7	2.6	2.2
	30	2.0	31.4	32.2	5.9	2.6	2.3
Valine	5	4.8	45.7	10.3	9.2	5.1	1.8
	10	5.2	51.0	13.4	8.4	4.2	2.0
	20	4.8	48.2	20.5	8.5	4.7	1.8
	30	4.4	47.9	17.7	6.5	22.7	2.4

[a] No amino acid was added.

from 5 to 30 mM. However, supplementation with threonine or isoleucine decreased cell growth and PHA content, and, particularly, Mw of the copolyesters produced. At 30 mM threonine or 30 mM isoleucine, the Mw of the copolyesters was lower than 5.0×10^5. A decrease in Mw of poly(3HB-*co*-3HV) in response to 3HV mol% was also reported in a study producing poly(3HB-*co*-3HV) from *A. eutrophus* under experimental conditions in which valerate was used as a cosubstrate for the synthesis of 3HV monomer (7). On the other hand, the addition of valine in concentrations between 5 and 20 mM showed rather stimulatory effects on the cell growth and PHA accumulation, and the Mw values of the copolyesters produced under these conditions were relatively high when compared with that of the copolyester produced from a culture to which amino acid was not added. Thus, the addition of valine into culture medium led to increase in both 3HV mol% and molecular weight in poly(3HB-*co*-3HV). These results imply that the molecular weight of the copolyester increases under the circumstances favorable for the efficient production of the copolyester irrespective of its molar fractions of 3HB and 3HV.

The molecular weight of PHA might have both a positive and a negative effects on the practical applications of the biopolymer. For example, a PHA having Mw under 10^5 is preferable as a biodegradable carrier for long term medication dosage (10), whereas increase of Mw allows an improvement of the formation of biodegradable film (11). The present study shows that the Mw of poly (3HB-*co*-3HV) produced by *Alcaligenes* sp. SH-69 can be altered between 3.2×10^5 and 1.1×10^6 depending upon various culture conditions. It appeared that culture conditions favorable for the increase of PHA content promote the formation of higher molecular weight copolyesters. The values of Mw obtained in this study are considerably greater than the Mw of PHB homopolyester produced by *Pseudomonas* 135 (6) and *Protomonas (Methylobacterium) extorquens* (3, 16), but similar to poly(3HB-*co*-3HV) of *A. eutrophus* (7). The polydispersity indices of these copolyesters were in the range of 1.5~2.5, which are lower than the typical values (>3.0) of PHAs reported from other bacteria (1, 6), suggesting that size distribution of the copolyesters of *Alcaligenes* sp. SH-69 is narrower. Even though some culture conditions affecting the molecular weight of PHA have been confirmed in this study, the reasons for this behavior and the extent to which environmental manipulation may influence the molecular weight of polyester are not exactly clarified and need to be investigated.

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