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Effects of Culture Conditions on the Molecular Weight of Poly-hydroxybutyric acid (PHB) Produced by *Alcaligenes* sp. K-912

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The molecular weight of poly-hydroxybutyric acid (PHB) produced by *Alcaligenes* sp. K-912 is an important parameter characterizing the physical properties of the polymer. The effects of temperature and the levels of glucose, ammonium, phosphate and amino acids on the molecular weight of PHB were investigated. Molecular weight of PHB by temperature varied in the range of 380,000 to 550,000, 400,000 to 600,000 by glucose, 300,000 to 380,000 by phosphate, 400,000 to 1,000,000 by amino acids, respectively under the experimental conditions.

Poly-hydroxyalkanoates (PHAs) which are a member of the microbial and biodegradable thermoplastics family can be synthesized biologically by a wide range of bacteria. Certain PHA copolymers containing 3-hydroxybutyrate (3HB) and other hydroxy acid mononer units have more useful thermomechanical properties than do poly-3-hydroxybutyrate (PHB) homopolymer (1). At present, the PHA copolymer of the greatest commercial interest is the copolymer of 3-hydroxybutyrate and 3-hydroxyvalerate P(3HB-co-3HV) which is marketed under the trade name, "Biopol" (2).

While numerous works on the mass production of PHA and the control of copolymer composition have been conducted by many researchers, works on the molecular weight of PHB which is also an important parameter characterizing the physical properties of the polymer have been scarce. It is known that the molecular weight of a polymer affects its compatibility, glass transient temperature, permeability, solubility and viscosity (3).

Since the first paper on the molecular weight of PHB produced by *Protomonas extorquens* using methanol, published in 1988 by Yamane *et al.* (4), a few studies on the effect of culture conditions on the molecular weight of PHB have been performed (5, 6). In the present study, the effects of culture conditions on the molecular weight of PHB produced by *Alcaligenes* sp. K-912 using glucose were investigated.

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Key words: molecular weight, poly-hydroxybutyric acid, *Alcaligenes* sp., biodegradable polymer.

MATERIALS AND METHODS

Microorganism

Alcaligenes sp. K-912 which is a mutant of *Alcaligenes* sp. SH-69 (2) was used. Stock culture was maintained at 4°C by a periodical transfer on agar plates.

Media and Culture Conditions

The cells were first grown in the medium containing 10 g/L peptone, 10 g/L yeast extract, 5 g/L malt extract and 2.5 g/L NH_4Cl for 20 hours.

In the case of the flask culture, 10 mL of precultured solution was transferred to the 500 mL flask (200 mL working volume) containing 20 g/L glucose, 3 g/L yeast extract, 1 g/L NH_4Cl, 1.5 g/L KH_2PO_4, 3.2 g/L $Na_2HPO_4 \cdot 12H_2O$, 0.1 g/L $MgSO_4 \cdot 7H_2O$, 0.02 g/L $CaCl_2 \cdot 2H_2O$, 0.01 g/L $FeSO_4 \cdot 7H_2O$ and 3 mL trace elements (200 mg/L $ZnSO_4 \cdot 7H_2O$, 60 mg/L $MnCl_2 \cdot 4H_2O$, 600 mg/L H_3BO_3, 400 mg/L $CoCl_2 \cdot 6H_2O$, 40 mg/L $NiCl_2 \cdot 6H_2O$, 20 mg/L $CuSO_4 \cdot 4H_2O$, 60 mg/L $NaMoO_4 \cdot 2H_2O$). The cell was then cultivated at 36°C and 150 rpm in a shaking incubator.

Precultured solution (200 m*l*) was transferred to a 5L-jar fermenter (3L working volume) containing the same medium mentioned above and batch cultures were conducted at 250 rpm , 1 vvm and 36°C.

Analytical Methods

Dry cell weight was determined by measuring its optical density at 660 nm using a spectrophotometer (Kontron, Uvikon 930). Glucose concentration level was measured by the DNS method (8) and ammonium concentration by the Berthelot method (9). Its PHB content

was determined by the gas chromatographic method (Braunegg method) (10) using benzoic acid as a standard material. The molecular weight was determined by gel permeation chromatography at 40°C using G5000HXL column (TOSOH, TSK-GEL) connected to Waters 410 Differential Refractometer and 746 integrator. Chloroform was used as an eluent at a flow rate 1.0 of ml/min, and a sample concentration of 1.0 mg/mL was used. Polystyrene of a low polydispersity was used as a standard.

RESULTS AND DISCUSSION

Effect of Temperature

Like chemical reactions, microbial activity is sensitive to the temperature of its environment. To investigate the effect of the culture temperature on the molecular weight of PHB, flask cultures were performed. Temperatures were set at 25, 30 and 36°C, respectively. Consequently, the polymers with molecular weights ranging from 360,000 to 540,000 were obtained as shown in Fig. 1. The molecular weight of PHB was 540,000 at 25°C, 360,000 at 30°C and 420,000 at 36°C. Since the growth of the cell culture was most active at 36°C (7), all of the following experiments were conducted at 36 °C.

Effect of Glucose Concentration.

Carbon source concentration has been considered as one of the dominant factors affecting the molecular weight of PHB (4, 5) and other biopolymers (11). It was reported that the carbon source of a low concentration (methanol) had a favorable effect in obtaining a high molecular weight of PHB (4). Doi (5) suggested a mechanism for the PHB synthesis and reported also on the dependence of the molecular weight upon the type and concentration of the carbon source.

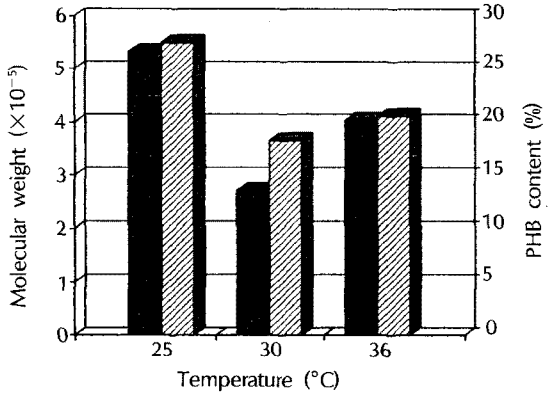

Fig. 1. Effect of temperature on the molecular weight of PHB in flask culture.
Molecular weight ▦; PHB content ▨.

From the flask cultures, it was found that the molecular weight of PHB was dependent upon the initial glucose concentration as shown in Fig. 2. At an initial glucose level of 10 g/L, the resulting molecular weight of PHB was 600,000, which was higher than those obtained at other glucose levels.

To investigate the change of molecular weight during the culture period, a batch culture using a fermenter was conducted with an initial glucose concentration of 20 g/L. The molecular weight of PHB was increased

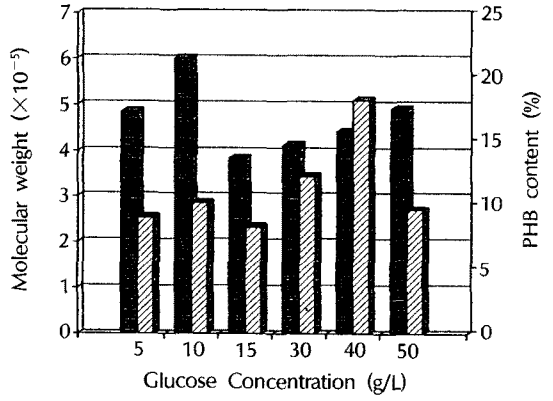

Fig. 2. Effect of initial glucose concentration on the molecular weight of PHB in flask culture.
Molecular weight ▦; PHB content ▨.

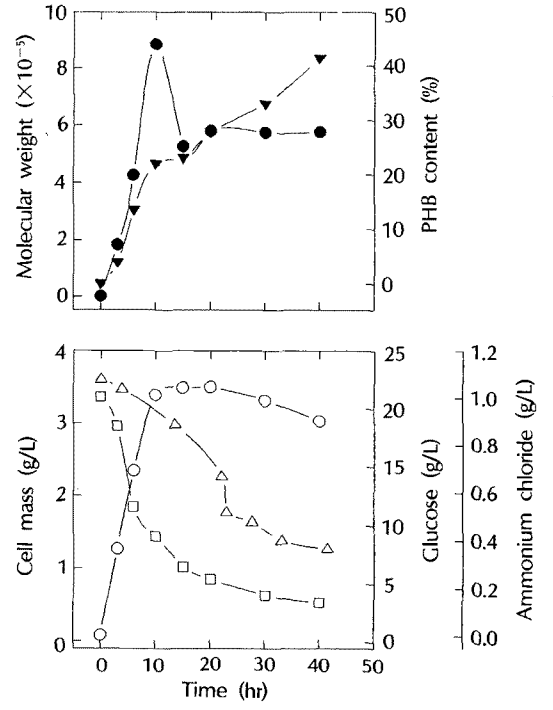

Fig. 3. Time courses of batch culture.
Molecular weight ●; PHB content ▼; Cell mass ○; Glucose □; NH_4Cl △.

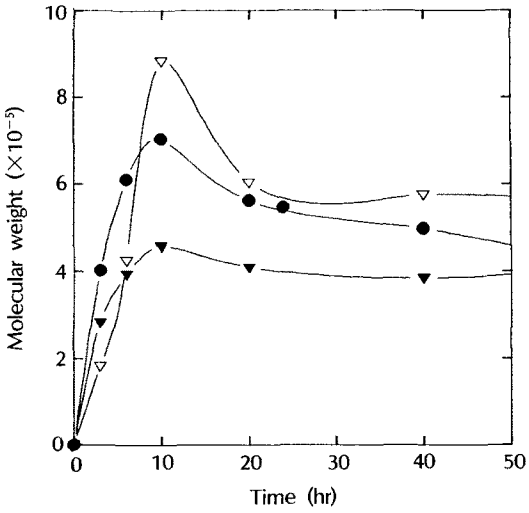

Fig. 4. Effect of initial glucose concentration on the molecular weight of PHB in batch culture.
10 g/L ●; 20 g/L ▽; 40 g/L ▼.

in the early cultivation period but then decreased thereafter, resulting in a convergent value as shown in Fig. 3. For all the following experiments using a fermenter, convergent values were taken to compare one another. As shown in Fig. 4, the glucose concentration proved to be very important in regulating the molecular weight of PHB. When the initial glucose concentration was 40 g/L, a polymer with the molecular weight of 400,000 was obtained. At 10 and 20 g/L of initial glucose conentration, polymers with molecular weights of 500,000 and 550,000 were obtained, respectively. These results suggest that there may be an optimal initial glucose concentration for obtaining the highest molecular weight of PHB.

A plausible explanation for the effect of glucose on the molecular weight of PHB was presented by Suzuki *et al.* (4); PHB precursor would be rich in high carbon source concentration and consequently result in a low molecular weight polymer. However, conformative interpretation of this theory has not yet been presented.

Effect of Ammonium Concentration

It has been reported that PHB synthesis is most active when nitrogen source is limited and glucose is in excess. As a result, many researchers have been studying the ammonium effect on PHB accumulation under a nitrogen source depletion (1, 7, 12, 13). It is also generally known that a complete nitrogen-deficiency causes damage to the microbial activity (14). This is because cell growth requires not only carbon source but nitrogen source which is an essential component of the cell body. Maintaining a suitable ammonium level is, therefore, very important for the mass production of PHB (15).

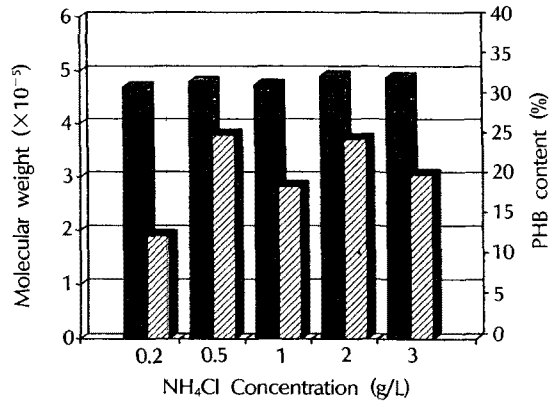

Fig. 5. Effect of initial ammonium concentration on the molecular weight of PHB in flask culture.
Molecular weight ▦; PHB content ▨.

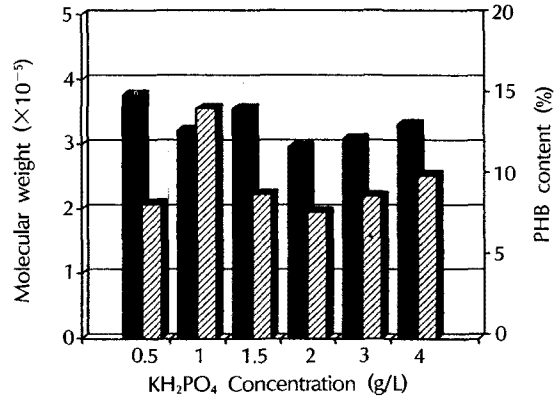

Fig. 6. Effect of initial phosphate concentration on the molecular weight of PHB in flask culture.
Molecular weight ▦; PHB content ▨.

Experiments were performed to investigate the effect of ammonium concentration on the molecular weight of PHB. As shown in Fig. 5, ammonium concentration changed the PHB content but did not significantly affect the molecular weight. The molecular weight of PHB was about 480,000 regardless of the ammonium level.

Effect of Phosphate Concentration

Granules and inclusions are often observed within the cells. Their natures differ in different organisms, but they usually function as a storage for energy or serve as structural building blocks (16). PHB, polyphosphate and glycogen are a few of the materials that are used as a storage. In addition, polyphosphate is known to be important in PHB accumulation (16). When polyphosphate is degraded under anaerobic condition, phosphate is evolved out of the cell to generate ATP, which in turn plays an important role in accumulating the PHB from the excessive carbon source. Thus, experiments were performed to elucidate the effect of phosphate concentration . As shown in Fig. 6, the effect of phosphate on PHB content was noticeable but the effect

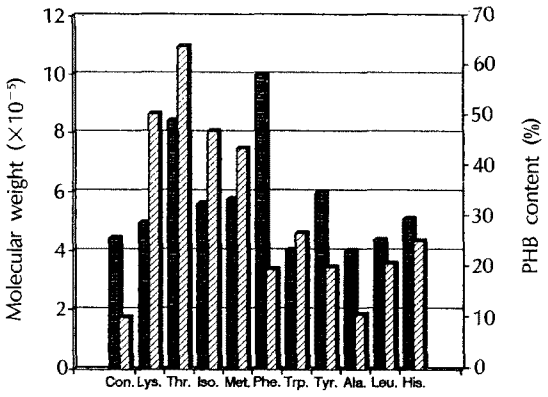

Fig. 7. Effect of amino acid on the molecular weight of PHB in flask culture.
Molecular weight ▦; PHB content ▨.

of phosphate on the molecular weight of PHB was not great. When the cell was cultivated with 0.5 g/L of potassium dihydrogen phosphate (KH_2PO_4), the molecular weight was 380,000, which was comparatively higher than those obtained at other levels.

Effect of Amino Acid

The carbon skeletons in amino acids can be completely oxidized or incorporated into glucose or fatty acids (18). In either case, the carbon skeletons in amino acids are first converted to pyruvate, acetyl-CoA, or particular intermediates of the citric acid cycle. Acetyl-CoA is a precursor in PHB formation. And propionyl-CoA, incorporated into acetyl-CoA forming copolymer P(HB-co-HV), can be converted from isoleucine, threonine or methionine through the amino acid catabolism. Therefore there may exist a relation between amino acid and PHB accumulation.

To investigate the effect of amino acid on the molecular weight of PHB, different amino acids (2 g/L) were added to each culture medium. As shown in Fig. 7 the results show that both the degree of polymerization and PHB content increased in comparison with the control when isoleucine, threonine, methionine, lysine and phenylalanine were added, respectively. Phenylalanine produced the highest molecular weight of PHB (1,000,000).

Except for phenylalanine, these are of the same amino acid family derived from oxaloacetate. Moreover, isoleucine, threonine and methionine are synthesized via homoserine from oxaloacetate. Oxaloacetate which is one of the main intermediates in TCA cycle is converted either to the amino acid mentioned above or to pyruvate and phosphoenolpyruvate. Therefore, when oxaloacetate-derived amino acids are affluent, oxaloacetate is converted to the intermediates of glycolysis which are precursors of PHB formation. These phenomena may cause the increase of PHB.

In this paper, the effects of several culture conditions on PHB biosynthesis are reported. It is still unknown in many cases why culture conditions affected the molecular weight of PHB, thus further investigation is needed. Nevertheless, the results of this study can provide an ideal culture condition for an effective PHB production.

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