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Full Length Research Paper

An experimental study on molecular weight of poly-3-hydroxybutyrate (PHB) accumulated in *Methylosinus trichosporium* IMV 3011

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Due to the effects of molecular weight (Mw) of a polymer on its properties, influencing factors on the Mw of poly-3-hydroxybutyrate (PHB) synthesized by methanotrophs were studied. Of the four kinds of methanotrophs, *Methylosinus trichosporium* IMV 3011 (IMV 3011) was found to be able to produce the PHB with the highest Mw value (1.2 M) under normal condition and then used for the other research on Mw of PHB. With methanol total concentration of 0.30 % (v/v), the Mw of PHB synthesized by the IMV 3011 can reach higher Mw (1.4 M) under the optimized cultivated condition. And with some essential substrates added, the Mw of PHB accumulated intracellular also changed. Moreover, through the study on the key enzymes activities in PHB metabolism under different cultivated condition, it was suggested that the Mw of PHB be controlled by the activity of key enzymes and the kind of microorganisms. This conclusion may also contribute to the synthesis of PHB with different Mw in an appropriate microorganism.

Key words: Molecular weight (Mw), poly-3-hydroxybutyrate, methanotrophs, methane, poly-3-hydroxybutyrate (PHB) synthetase.

INTRODUCTION

Poly-3-hydroxybutyrate (PHB) can be accumulated by various species of bacteria. Due to its biodegradability and its extraordinary properties, PHB has interesting potential applications in medicine, food packaging and agriculture (Ikada and Tsuji, 2000). Of the possible carbon sources, methane and methanol could be used as a suitable substrate for the production of PHB. PHB is an internal reducing-energy storage polymer that can be used as an alternative reducing-energy source by methanotrophs cultivated under starvation conditions. Figure 1 shows two types of methanotrophs, type I and II, which assimilate carbon via the ribulose monophosphate and the serine pathways, respectively.

$$8CH_4 + 12O_2 \rightarrow C_4H_6O_2 \text{ (PHB monomer)} + 4CO_2 + 12ATP + FPH_2 \quad (1)$$

The overall equation for organisms using the serine cycle is: a theoretical yield of PHB of 67% (86 g/128 g of CH_4) was given (Anderson and Dawes, 1990). And we have previously reported on the ability of *Methylosinus trichosporium* IMV 3011 (IMV 3011) to accumulate PHB (Zhang et al., 2008), with yield above 35% using methane and methanol as carbon source.

Although, considerable attention has been devoted to the fermentation of PHB producers, many studies have focused predominantly on the PHB yield and production rate of the cells, possibly because of the currently high production costs of PHB. While numerous works on the

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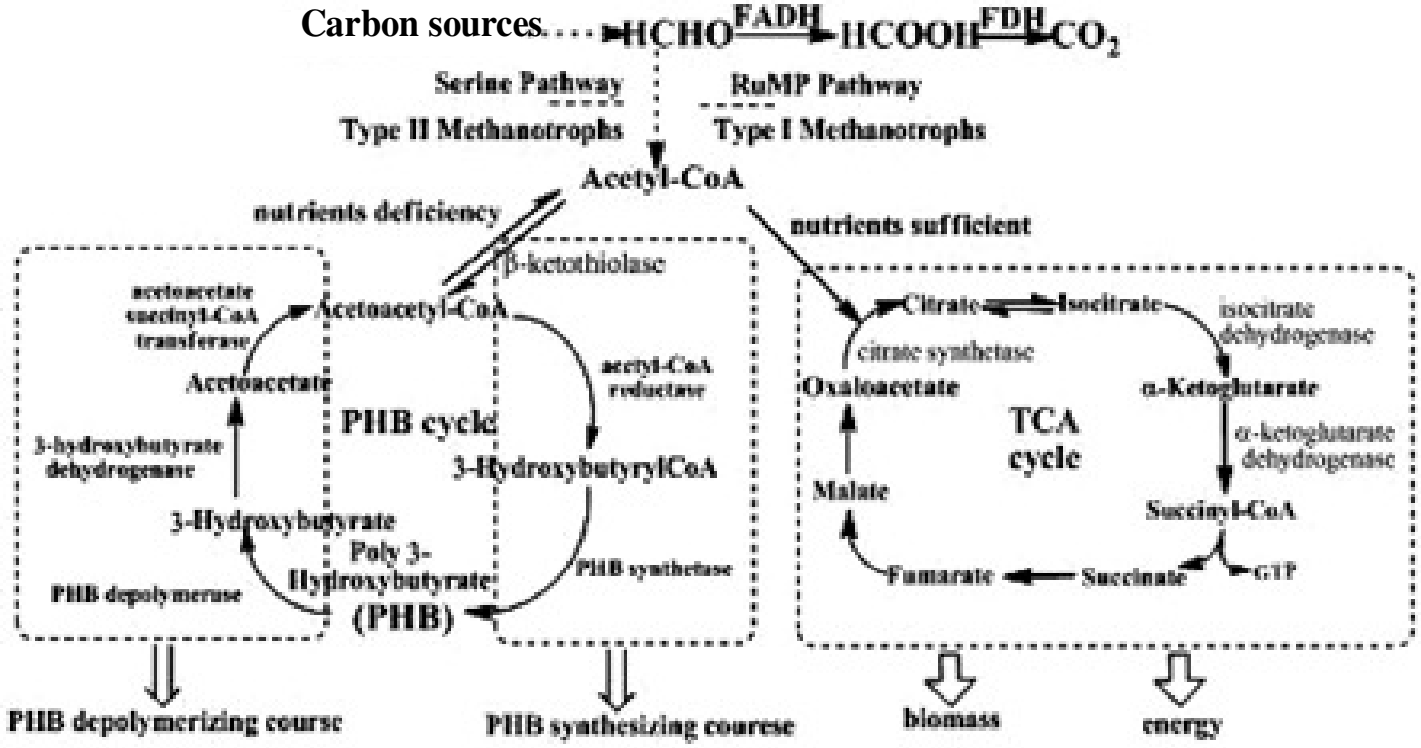

Figure 1. Proposed pathway of metabolism of methanotrophs: the detailed pathway of metabolism of CH_4 and CH_3OH, TCA cycle, PHB cycle in methanotrophs. FADH: Formaldehyde dehydrogenase; FDH: formate dehydrogenase.

mass production of PHB and the control of copolymer composition have been conducted (Bitar and Underhill, 1990; Lee et al., 1992; Rhee et al., 1993), these studies on the PHB yield offer little insight into the nature or the properties of the PHB polymer. As the crystallinity, mechanical strength, biodegradability are decided by the molecular weight (Mw), the average value and the Mw distribution of the PHB are perhaps the most important properties for the commercial suitability of a given polymer. Yet works on the Mw of PHB that was accumulated in microorganisms have been scarce.

To satisfy diverse commercial demands, controlling the PHB Mw is required (Sung and Young, 1995). Therefore, the accumulation of the PHB Mw in diverse microorganisms has been widely studied. For example, Suzuki et al. (1988) reported the control of Mw of PHB accumulated by *Protornonas extorguens* using methanol. They have hypothesized, without supporting data, that the extracellular concentration of the carbon source influences the intracellular concentration of 3-hydroxybutyryl CoA. However, experiments on the PHB accumulated by *Alcaligenes eutrophus*, another microorganism from the same methylotrophs family, gives different results (Taidi et al., 1994).

The effects of many other factors on the PHB Mw in different kinds of microorganism have also been studied. Daniel et al. (1992) investigated the effect of NH_4^+, Mg^{2+} and PO_4^{3-} on the Mw of PHB accumulated by *Pseudomonas* 135, while Shimizu et al. (1993) examined the effect of butyric acid concentration on the distribution of the PHB Mw, as well as the effects of pH conditions on Mw of accumulated by *A. eutrophus.* Similar effects of the pH conditions on Mw accumulation in *Aureobasidium pullulan* was further proved by Lee and Yoo (1993). Then, Myshkina et al. (2008) widely studied the effect of growth conditions on Mw of PHB produced by *Azotobacter chroococcum* 7B and got PHB with different Mw by controlling factors such as pH, temperature, carbon source, etc. Moreover, the studies of Zhang et al. (1994) showed that even the cloned pathway for the PHB synthesis from the same strains had been introduced into sucrose-utilizing strains of *Escherichia coli, Klebsiella aerogenes and Klebsiella oxytoca*, the Mw of the PHB polymer made in the *K. aerogenes* 2688 and the *E. coli* JMU213 was much higher, up to 10^7, than that obtained from *A. latus* and *A. eutrophus*. Also, the recovery methods might also affect the PHB Mw value (Hahn et al., 1995).

In addition, the series of reaction involving the conversion of acetyl CoA into PHB, its depolymerization and oxidation back to acetoacetyl CoA, has been confirmed (Kawaguchi and Doi, 1992). Later, Sung and Young (1995) suggested that the PHB Mw was mainly determined in the early cultivation stage and that the

difference of Mw could be explained by the number of polymer chains.

However, it still need further study on obtaining PHB with Mw in certain range for application. Then, because of the ability of methanotrophs to synthesize PHB from cheap carbon sources, it will be of practical interest to determine if the PHB Mw from methanotrophs would be affected by the nature of the growth substrate or other factors.

In this study, we develop an efficient process to produce high yield of PHB with Mw (above 10^6) in methanotrophs (IMV 3011) with an aim to find the key factors influencing the Mw. We then investigate the temporal changes in the Mw of PHB polymers and the dependence of the Mw on cultivation conditions. The dependence is further explained on the basis of the mechanism of biological polymerization.

MATERIALS AND METHODS

Microorganisms and culture medium

The methanotrophs used

M. trichosporium IMV 3011 (IMV 3011) was obtained from the Russia Institute of Microbiology and Virology (Kiev. Ukraine).The experiments were carried out in the mineral salt medium as previously reported (Zhang et al., 2008);

M. trichosporium OB3b (OB3b) was from The Institute of Biochemical Engineering, Tsinghua University, Beijing, the basal medium employed for OB3b strain maintenance was a modified Higgins' nitrate minimal salts (NMS) (Park et al., 1991);

Methylococcus capsulatus HD6T (HD6T) was isolated from a soil sample around a natural gas well of Daqing, Heilongjiang Province.

Methylomonas sp. GYJ3 (GYJ3) was isolated from soil samples from the oil fields of Yumen, Gansu Province and cultivated as described by Shen et al. (1997). GYJ3 and HD6T were cultivated in the same culture as IMV 3011. All the experiments were carried out in triplicate. The reported values are an average of three runs with a variation of 2 to 5%.

Cultivation conditions (Zhang et al., 2008)

Normal conditions: Under routine cultivated conditions, liquid cultures were grown in aliquots 100 ml of the mineral salt medium in 250 ml shake-flasks. Shake-flasks were stopped with rubber seal and gassed with methane/air (1/1, v/v) gas mixture. The gas phase was replenished every 12 h with the same gas mixture. The cultivation of cells was carried out at 30°C for about 96 to 120 h. Moreover, cells used for the compare experiments in each group were first prepared under the same cultivated condition.

Culture with methanol and methane as carbon source

Direct addition of methanol at a concentration of 0.10 % (v/v) was found to be toxic for the growth of strain IMV 3011. It took 72 h for the culture to start growth with methanol vapor as carbon source. The growth culture under methanol vapor was adapted to grow on liquid methanol by the serial transfer of the culture into the medium with gradually increased concentration of methanol (from 0.10 % (v/v)/per day up to 2.0% (v/v)/per day).

All cultivations were performed in a sterile manner in two stages

Stage one: Shake flask studies were conducted at 30°C in sealed 250 ml flasks. Each flask contained 100 ml of liquid medium under a CH_4/air gas mixture (1:1 v/v). To keep the constant methanol concentration, the concentration of methanol (v/v/day) added in the liquid medium every day (for 3 days) was 0.10, 0.20, 0.30, 0.40, 0.50, 1.0 and 2.0%. In stage one; the cultivation usually took 144 h.

Stage two: Cell pellets were harvested by centrifugation from stage one in a sterile manner and cultivated in the nutrients deficiency (nutrients imbalanced) medium with the compositions (g/l) as reported (Zhang et al., 2008). In stage two, the cultivation usually took 120 h.

Culture with other carbon sources

Glucose, formate acid, 3-hydroxybutyrate and ethanol were separately added to the culture medium as carbon sources to accumulate PHB. The Mw of PHB accumulated by adding these substrates were studied compared with the Mw of PHB accumulated by methanol (Figure 2). The strain of IMV 3011 was domesticated by adding minim of these compounds. Then these substrates were added to the culture in the same mol of C as methanol to survive the cell growth under a CH_4/air gas mixture (1:1 v/v). After two-stage procedure, the cell were harvested and used to recover the PHB in it.

Culture medium with some substrates favorable to accumulation of PHB

Citric acid, α-ketoglutaric acid, succinic acid, malic acid and malonic acid, these are important intermediates in TCA and other substrates like ammonium acetate, sodium formate added to the culture medium separately at appropriate concentrations can promote PHB cycle and then contribute to the accumulation of PHB in the cell. These substrates with certain concentrations, which have great effects on the PHB accumulation in the cell in different cultivated stage, were added to the culture at initial of the cultivation course for the study of Mw of PHB synthesized in the cell under a CH_4/air gas mixture (1:1 v/v).

Analysis

Biomass concentration

Absorbance was measured (HP 8453, on a 660 nm spectrophotometer; blank: mineral salt medium): The biomass concentration was evaluated using a calibration curve.

Dry weight: 100 ml of the cell suspension was centrifugated at 9000 rpm for 10 min and the sediment was dried at 105°C to a constant mass.

PHB recovery

After centrifugation, the biomass was separated and freeze dried. Lipids and color substances in the freeze-dried biomass were then removed by extraction with methanol (80% v/v, 1.5 h, 50°C). In the second step, PHB was extracted from the biomass with chloroform(1.5 h, 70°C), the non-PHB cell matter was removed by

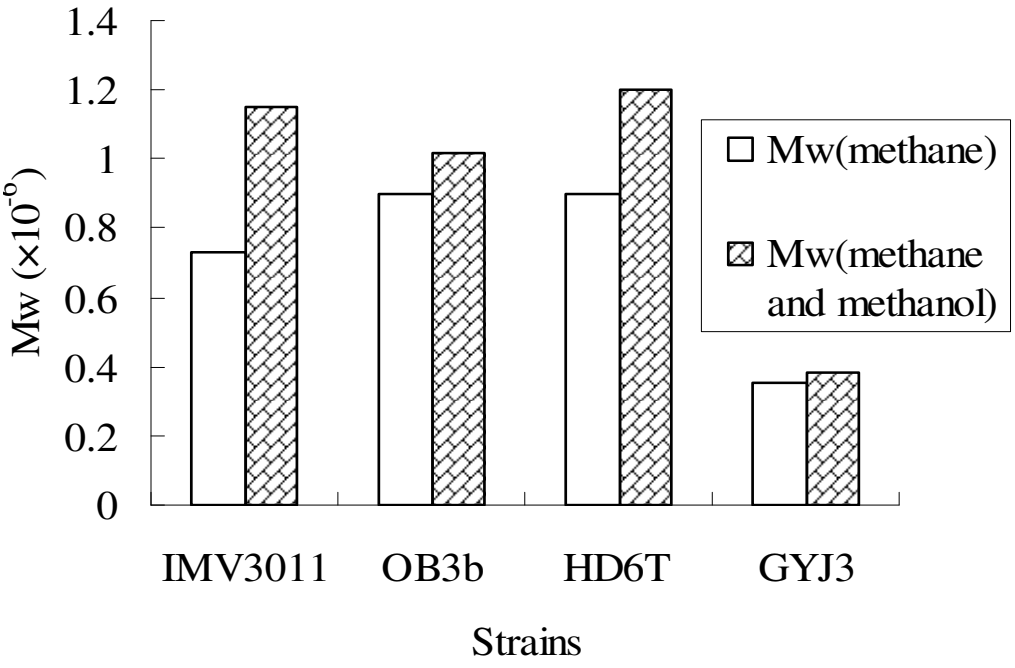

Figure 2. Mw of PHB accumulated by four kinds of strains with and without methanol: (a) methane (methane content of 50% and air content of 50%); (b) methane (methane content of 50% and air content of 50%) and methanol (0.10% (V/V)/d).

filtration and the dissolved PHB was precipitated with methanol. PHB was washed twice with methanol, separated by filtration and dried at 60°C for 2 h.

PHB analysis

PHB content (Wendlandt et al., 2001): PHB content was determined by gas chromatography. The heavier phase was directly analyzed on gas chromatography (Agilent 6820 system, U.S.A, with an FID, a capillary column: 0.25 mm×30 m; stationary phase, SE-54). Pure poly-3-hydroxybutyric acid was used as standard sample.

Mw (Gerngross and Martin, 1995; Kusaka et al., 1997): The Mw and its distribution was determined at 40°C on a gel permeation chromatograph (GPC) system with Styragel HT 3, 5, 6E in series using HPLC (Waters 2695, U.S.A)/RID (Waters 2414, U.S.A), relative to polyethylene glycol (PEG) standards. A calibration curve was constructed with eight samples (number-average Mw = 1.2 $\times 10^3$-3.8 $\times 10^6$) of PEG standards with good polydispersity. Chloroform was used as eluent at a flow rate of 1.0 ml /min (40°C) and sample concentration of 1 mg/ml was applied.

NMR

The spectra were recorded on a Varian INOVA spectrometer in $CDCl_3$ at 303 K: ^{1}H-NMR: 400 MHz, ^{13}C-NMR: 100 MHz.

Analysis of enzyme activity

For enzymological study of the Mw of PHB biosynthesized by IMV 3011, cells were taken from those growths in nutrient-sufficient and nutrient-deficiency culture, respectively. They were centrifuged at 9000 rpm for 10 min, washed twice with 50 mM Tris-HCl buffer pH 7.4, resuspended in the same buffer and sonicated with a 100 W ultrasonicator at 20 kHz (80 × 10s at 4°C). Undestructed cells and their cell fragments were removed by centrifugation at 12000 rpm for 20 min and the supernatant was used as cell-free extract for assaying enzyme activities in a 1 cm constant-temperature cell at 30°C with a Hewlett Packard 8453 spectrophotometer (Doronina et al., 2008).

β-Ketothiolase (EC 2.3.1.9) activity (Volova et al., 2004): The reaction mixture (1.0 ml) contained 100 mM Tris HCl pH 8.3, 25 mM $MgCl_2$, 100 µM acetoacetyl CoA and 100 µM CoA. The mixture was first preincubated at 30°C for 2 min. Then the reaction was initiated by adding 0.050 ml the extract, assayed spectrophotometrically at *D*303nm and calculated with the molar absorption coefficient 17260 $M^{-1}cm^{-1}$.

Acetoacetyl-CoA reductase (EC 1.1.1.36) activity (Senior and Dawes, 1973): The reaction mixture contained 100 mM potassium-phosphate buffer pH 5.5, 1.5 ml; 25 mM $MgCl_2 \cdot 6H_2O$, 0.12 ml; 12.5 mM dithiothreitol (DTT), 0.10 ml; 6.0 mM NADH, 0.10 ml; 18 mM acetoacetyl-CoA, 10 µl; 0.10 ml of the extract and water to adjust the volume to 2.5 ml. The reaction was assayed spectrophotometrically at *D*340 nm.

PHB synthetase (EC 2.3.1) activity (Jossek et al., 1998): The reaction mixture contained 25 mM Tris-HCl-buffer containing 1.0 mM, 5,5'-dithiobis-2-nitrobenzoic acid (DTNB), 30 mM D-3-hydroxybutyryl-CoA and the extract. The reaction was assayed spectrophotometrically at *D*412 nm.

PHB depolymerase activity (Doronina et al., 2008): Mixture of 50 mM Tris HCl pH 8.0, 1.0 mM $CaCl_2$ and 150 µg PHB/ml extract. The rate of decrease at *D*650 nm was recorded and calculated molar

absorption for PHB was taken to be 0.0049 µl ng^{-1} cm^{-1}.

The amount of the enzyme digesting 1 µg of the polymer per min was taken to be unity. The other enzyme activities were expressed in terms of µmoles of the converted substrate or resulting product in min/mg protein (1U= µmol/min/mg protein). Protein was assayed by the Coomassie brilliant blue G-250 method. The mean results of three independent analyses are shown in the table. Typically data variation is less than 5%.

Chemicals

Most chemicals were of analytical grade obtained from sigma, U.S.A., Methane with a purity of 99.99% was commercially available.

RESULTS AND DISCUSSION

It is well known that PHB produced by methylotrophic bacteria is not appropriate for industrial application due to its lower Mw. In this study, methanotrophs can accumulate PHB with a higher weight-average Mw from methane and methanol in shake-flask than previously reported for methylotrophic bacteria (Suzuki et al., 1988; Taidi et al., 1994).

Mw of different kinds of methanotrophs

Many studies have shown significant difference in Mw of PHB synthesized by various PHB synthesis systems. In this study, the Mw of PHB obtained from four kinds of methanotrophs, OB3b, IMV 3011, HD6T, GYJ3, were compared.

Monooxygenation of methane might actually be profitable for the methanotrophic methanol metabolism, while part of the reducing equivalents from methanol metabolism could be used for the monooxidation of methane and surplus reducing equivalents could be converted into endogenous reducing power reserves such as PHB. Based on this conclusion, four strains cultivated with different carbon sources to synthesize PHB were studied. And because it may cause the strains contaminated with methanol as sole carbon source, the research was carried on with methane supplied. From the results, Mw up to 1.3 $\times 10^6$ can be produced by IMV 3011 with total methanol of 0.30% (v/v) under the methane and air gas mixture (1: 1, v/v). For PHB from the same strain, their Mw depends on the carbon sources. When cultivated with both methanol and methane, the PHB Mw was higher than that cultivated with methane only. Compared with some metabolically engineered bacteria like *E. coli* which could produce PHB with very high Mw (2.2×10^7), the Mw value of PHB accumulated by most kinds of methanotrophs was much lower. Still the Mw of PHB at about 10^6 also has extensive application in medical and so on. The earlier studies also showed that different strains may accumulate PHB with different Mw due to the difference in PHB synthesis system in the strains. Then the strain IMV 3011 was chosen for the study on accumulating PHB with higher content and higher Mw.

Effects of different nutrients

By studying the effects of nutrients deficiency of the culture medium on the Mw of PHB accumulated in IMV 3011, it is found that the Mw of PHB produced by IMV 3011 was influenced by several factors besides the recovery methods of PHB. The results (Figure 3a, f) showed great difference with methylotrophic bacterium, *Pseudomonas* 135 (Daniel et al., 1992).

In IMV 3011, the PHB content of 32% was determined of the total cell mass on NH_4^+-limited medium, 27% on Mg^{2+}-limited medium, 24% on NO_3^--limited medium and 18% on HPO_4^{2-}-limited medium. The average relative Mw was also influenced by the nutrients deficiency in the culture medium, 1.0×10^6 in NH_4^+-limited culture medium, 1.1×10^6 in Mg^{2+}-limited medium, 1.3×10^6 in NO_3^--limited medium and 1.2×10^6 in HPO_4^{2-} -limited medium (Figure 3a, d). The influence of NO_3^- was the most dramatically, the higher concentration of nitrate was the lower Mw value the PHB has. There is also a small decrease in Mw with concentrations of HPO_4^{2-} increasing. But the adverse result was found when the concentration of NH_4^+ was altered. When the concentrations of NH_4^+ increased, the Mw of PHB became higher. And $MgSO_4$ did the same effects as NH_4^+. It was also found that the contributions of Cu^{2+} and Fe^{3+} to Mw of PHB were a little different from other substrates. At certain concentrations of the two ions, a special low or high Mw will be achieved (Figure 3e, f). It is probably because the two ions effect on the activity of methane monooxygenase (MMO) of the cell may help them controlling Mw indirectly. According to the explanation of the mechanism of PHB synthesis, it can be suggested that the Mw value of PHB may be influenced by the PHB synthetase to some extent. When the nutrients concentration was kept at a certain level, the PHB synthetase activity and concentration would achieve at an appropriate level for the high Mw of PHB accumulated in the strains. But the PHB content in the cell did not influence the Mw value of PHB much.

Effect of the two-stage cultivation

PHB Mw also was affected by the age of the culture (Chen and Page, 1994). The course of PHB Mw over time during the accumulation process is presented in Figure 4a. The number-average Mw of PHB decreased with time during the course of PHB accumulation and they were markedly dependent on the cultivation conditions of the IMV 3011, ranging from 0.9 $\times 10^6$ to 1.3 $\times 10^6$.

It showed that cells in the nutrients sufficient condition produced PHB Mw at about 1.2×10^6 (Figure 4a). By 48 h the Mw had dropped a little, giving the lowest Mw and

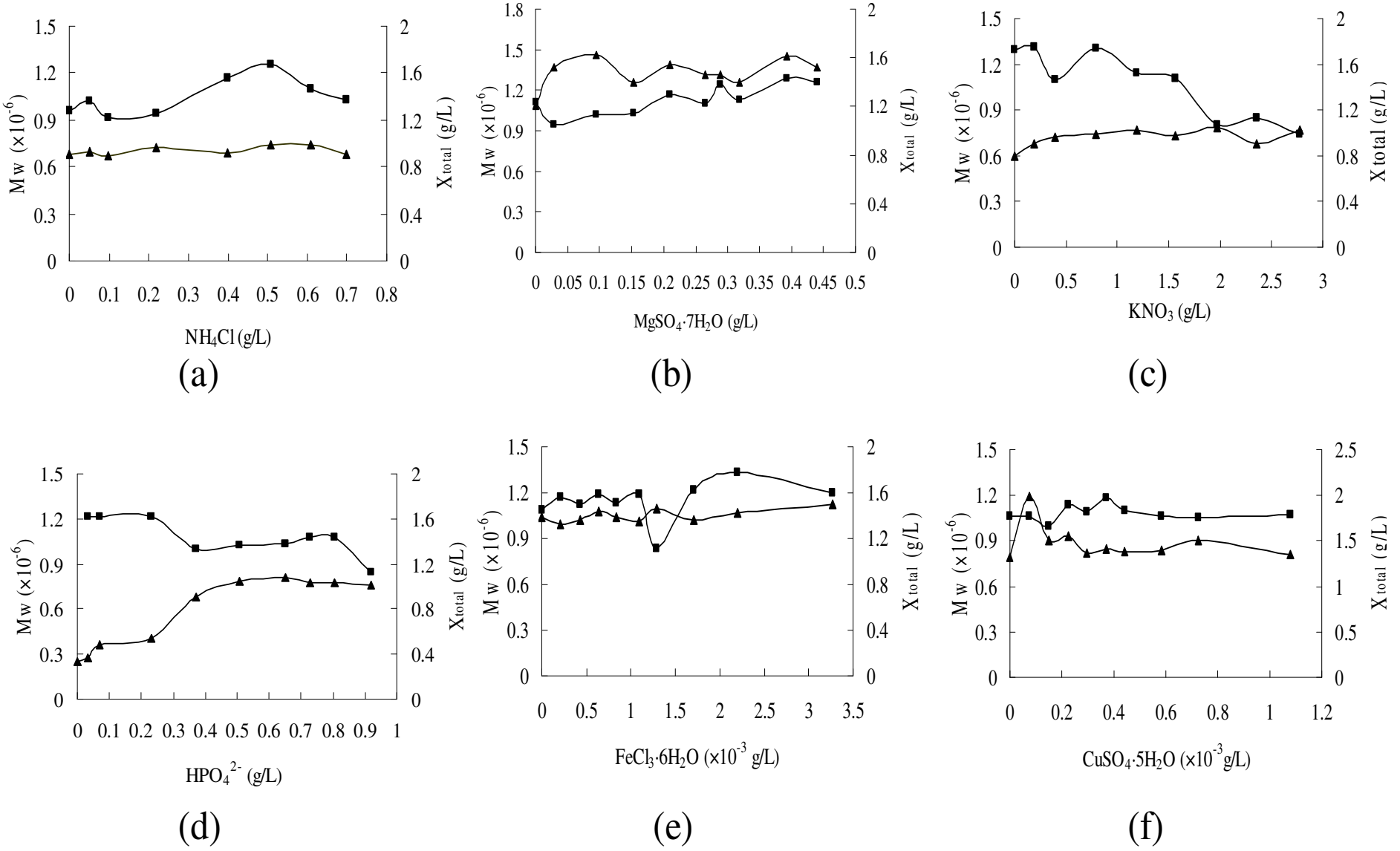

Figure 3. Effect of kinds of ions on Mw of PHB in IMV 3011:▲ X_{total}, biomass of cell cultivated in the culture medium with different concentration of the ions; ■ Mw of PHB obtained from cell cultivated in the culture medium with different concentration of the ions. Ions: (a) NH_4^+; (b) Mg^{2+}; (c) NO_3^-; (d) HPO_4^{2-}; (e) Fe^{3+}; (f) Cu^{2+}.

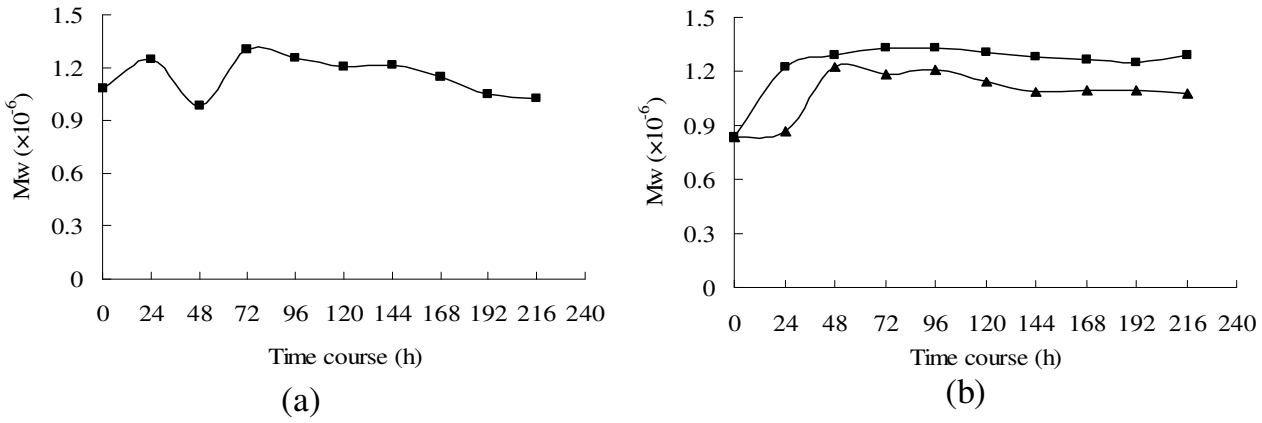

Figure 4. Change of Mw of PHB in the cell during the shake-flasks fermentation course: (a) ■ Mw of PHB obtained from cell during the fermentation course; (b) ▲ Mw of PHB obtained from cell cultivated in nutrients sufficient culture medium during the second fermentation stage; ■ Mw of PHB obtained from cell cultivated in nutrients deficiency culture medium during the second fermentation stage.

PHB content. And based on the same condition, Mw of PHB of the cell cultivated in nutrients sufficient culture medium and in nutrients deficiency culture medium were contrasted when the cell were transferred into second stage to cultivate during the two-stage cultivation. When coming into the second stage cultivation, the cells grown under nutrients deficiency condition produced polymer with higher Mw (1.3×10^6) than that of the cells grown in the nutrients sufficient condition (Figure 4b). And the PHB Mw of the cell cultivated in nutrients deficiency condition reached the top value more rapidly than that of the cell in cultivated in nutrients sufficient condition. From the result of the change of Mw of PHB during the growth course, it is found that the Mw was decreased sharply first with the decrease of the mass of PHB and then increased rapidly with the increase of the mass of PHB. Moreover, the cells cultivated in the nutrients deficiency condition could accumulate more PHB (50%) and keep the Mw of PHB at the same level for a longer time, although, with a little lower cell concentration. But the PHB content could keep at the high level for a longer time than the Mw value of PHB could do.

Effect of different substrates

From the study in the Mw of PHB produced by *M. extorquens* in shake-flask culture (Taidi et al., 1994), it has been found that the Mw of PHB was dependent on the initial concentration of methanol or sodium succinate, used as sole carbon sources. PHB with an Mw in the range of 0.2 to 1.7×10^6 could be produced by selection of the carbon source and its concentration. According to the report of Taidi et al. (1994), the Mw of PHB produced by *A. eutrophus* was high (1.1 to 1.6×10^6) and generally unaffected by the kind or concentration of the carbon source. But the use of glycerol as sole carbon source resulted in the accumulation of PHB with a markedly lower Mw (5.5 to 8.5×10^5) by this organism under similar conditions. Then different carbon sources and some other substrates were used for studying the factors in the Mw value of PHB.

Here, under the gas mixture with 50% methane and 50% air, methanol, glucose, formate acid, 3-hydroxybutyrate and ethanol were used respectively to estimate the influence of carbon sources on the Mw of PHB accumulated in IMV 3011. It was found that glucose, formate acid, 3-hydroxybutyrate or ethanol did not support the growth of IMV 3011with small amounts of PHB (2 to 9%, w/w) produced. While high production of PHB can be obtained with methanol as carbon source under nutrients sufficient condition. Moreover, the highest Mw values (1.5×10^6) could be obtained with low initial concentration of methanol. The Mw of the PHB decreased when cultivated in nitrogen-limited medium with methanol as the carbon source. The probable reason may be the poor growth condition could not keep PHB synthetase at an appropriate concentration with good activity.

From the metabolism pathway in methanotrophs (Figure 1), it suggested that some of the intermediate substrates in TCA cycle could induce transport systems and catabolic enzymes to accumulate PHB. They may also contribute to synthesize PHB with higher Mw. Then citric acid, malic acid, succinic acid and malonic acid were taken for this study (Table 1). Also, the effect of another compound, ammonium acetate, which did contribute to the accumulation of PHB by synthesizing acetyl-CoA important for PHB cycle, was studied to compare the effects of the earlier mentioned factors. These substrates added in certain quantity maybe inhibit TCA cycle or promote PHB cycle to some extent. Then, these compounds with appropriate concentrations which would contribute to the PHB accumulation were added to the

Table 1. Effect of different substrates on Mw of PHB accumulated in IMV 3011.

Addition[a]	Concentration of the additions (g/l)[b]		Mw ($\times10^{-6}$)[c]	polydispersity[d]
	Nutrients sufficient stage	Nutrients deficiency stage		
Blank	0	0	1.3	1.44
Citric acid	0.10	0.30	1.0	1.46
α-Ketoglutaric acid	5.0×10^{-7}	5.0×10^{-7}	1.4	1.82
Succinic acid	0.013	1.2×10^{-4}	1.3	1.77
Malic acid	5.7×10^{-4}	0.030	1.2	1.91
Malonic acid	7.2×10^{-7}	0.050	0.64	1.39
Ammonium acetate	0.50	0.10	0.73	1.79
Sodium formate	1.7×10^{-4}	0	1.4	1.99

[a]Substrate added at an appropriate concentration in two-stage cultivation that can help to synthesize highest yield of PHB in cell; [b]concentrations of the additions added into the culture medium in different cultivated stage; [c]Mw ($\times10^{-6}$) of PHB recovered from the harvest cells cultivated in the culture medium with different concentration of addition; [d] polydispersity of PHB recovered.

culture. It was found that when α-ketoglutaric acid or sodium formate presented in the culture at certain concentration, which could promote the accumulation of PHB, IMV 3011 could produce PHB with an high Mw (1.4×10^6) (Table 1).

The results obtained suggest that the metabolic pathway for PHB polymerization is greatly affected by methanol concentration and slightly by other carbon sources. The PHB synthetase acts on the step of bonding 3-hydroxybutyryl-CoA and PHB fragment. The initial PHB fragment is composed from two molecules of 3-hydroxybutyryl-CoA. If the number of the PHB fragments is high in a cell, the polymerization degree of PHB will be small. On the other hand, highly polymerized PHB will be obtained by use of a few pieces of the precursor (Suzuki et al., 1988). In this connection the phenomenon obtained here that Mw of PHB was prominently varied around 0.30% (total amounts, v/v) of methanol which was the optimal concentration for the growth of the strain, will be explained by the following speculation. At concentration of methanol less than 0.30% (v/v), carbon source is limiting, then, the frequency for producing the PHB fragment as the precursor will be reduced. At higher concentrations, on the contrary, excessive molecules of 3-hydroxybutyryl-CoA form a number of precursors. Thus, PHB with Mw in the range 0.8 to 1.3×10^6 could be produced by selection of the carbon source and its concentration.

Polydispersity shows the distribution of polymer with different Mw in one compound. From polydispersity values shown in Table 1, it can be found that some were high while the Mw values were lower under different condition. When succinic acid was added, the PHB obtained with Mw 1.3×10^6 showed higher polydispersity value (means poor polydispersity) than that from the blank cultivated condition. Especially, when Malonic acid, Ammonium acetate added at certain concentration, the poor polydispersity caused the average-Mw decrease to 0.7×10^6 or so. It was probable because the condition inhibited the activity of the PHB synthetase and the concentration of the PHB synthetase and then, prevented some hydroxybutyrate molecules from polymerizing to high Mw polymer. Moreover, as the polydispersity decided the homogenization of a polymer, further study on this will be another interesting and valuable work.

It has been reported that PHB having higher Mw was obtained when *Alcaligenes* sp. K-912 was cultivated under high pH conditions. But in this study, the Mw was almost not affected by the pH. The influence of pH from 5 to 8 on the Mw of PHB accumulated in IMV 3011 was neglectable (Sung and Young, 1995).

Mechanism of the polymerization of PHB

Since the number of polymer chains was considered as a dominant factor in determining the Mw of PHB; the more the chains are, the less chances are given to propagate. And the polymerase was not enough for one polymer chain to propagate without excessive competition with other chains. In a previous paper (Kawaguchi and Doi, 1992; Kusaka et al., 1997), it has been suggested that a chain-transfer agent is generated in original strain *A. eutrophus* cells and in the recombinant *E. coli* cells during the course of PHB synthesis and reacts with a propagating PHB chain to regulate the chain length of PHB in the biological polymerization system of *D*-3-hydroxybutyryl-CoA with PHB synthetase. The generation of a chain-transfer agent may be induced by the formation of PHB. The addition of hydroxybutyryl CoA to PHB synthetase leads to formation of a granule-bound PHB synthetase and the Mw of the polymer can be controlled by the initial PHB synthetase concentration. Forms of the enzyme coexist and thus, complicate the determination of basic kinetic parameters. Moreover, the observation of Sim et al. (1997) that polymer Mw is

Table 2. Effects of activities of enzymes pertaining to PHB synthesis in different cultivated condition on Mw of PHB.

Enzyme	Condition A	Condition B	Condition C	Condition D
β-Ketothiolase ($\times10^2$, U)	2.33	0.61	0.19	3.68
Acetoacetyl-CoA reductase ($\times10^2$, U)	0.60	1.98	1.70	1.48
PHB Synthetase ($\times10^3$, U)	1.26	1.17	1.46	0.15
PHB Depolymerase (μg/min/mg protein)	9.85	10.6	4.89	2.74
Mw ($\times10^{-6}$)	1.1	1.3	1.4	0.69

Condition A: cell cultivated for 96 h in nutrients sufficient culture medium; Condition B: cell cultivated for 96 h in nutrients deficiency culture medium; Condition C: cell cultivated for 144 h in nutrients sufficient culture medium and then cultivated for 96 h in nutrients deficiency culture medium; Condition D: cell sonicated after cultivated for 144 h in nutrients sufficient culture medium and then cultivated for 96 h in nutrients deficiency culture medium.

inversely proportional to synthetase concentration suggests a mechanism in which each active enzyme unit is responsible for generation of one polymer chain. Furthermore, recent studies suggest that each polymer chain is covalently bound to the enzyme.

To find the effects of the key enzymes that may work for chain-transfer reaction on Mw of PHB accumulated in methanotrophs, the enzyme activities of methanotrophs IMV 3011 were studied. From Table 2, it was found that the activities of these enzymes in IMV 3011 (β-ketothiolase: 0.002 to 0.037U; acetoacetyl-CoA reductase: 0.006 to 0.019U; PHB synthetase: 0.001-0.015U) were much lower than those reported in OB3b (β-ketothiolase: 0.31 to 0.39U; acetoacetyl-CoA reductase: 0.50 to 0.52U; PHB synthetase: 0.025 to 0.077U) (Doronina et al., 2008). The results showed that Mw was affected more by PHB synthetase. But these enzymes were tied up in the microorganism: a mass of PHB with especially high Mw will be harvested when β-ketothiolase with low activity and PHB synthetase with high activity (Condition C in Table 2); while in Condition A, the reverse results were shown. Moreover, it is also shown in Table 2 that the activity of depolymerase (2.3 to 10.6 μg/min/mg protein) were much higher than that in OB3b (2.0 to 2.2 μg/min/mg protein). This may caused the Mw of PHB (1.5×10^6) synthesized in IMV 3011 lower than that in OB3b (2.5×10^6). As it is known, β-ketothiolase is the key enzyme for PHB synthesis. When β-ketothiolase and other enzymes are all in high activity, mass of 3-hydroxybutyrate are synthesized at first stage of the cultivation. When it came to accumulation stage of PHB, the Mw of PHB polymers decreased (Koizumi et al., 1995). Until the activity of β-ketothiolase decrease in certain condition after it has helped synthesizing mass of 3-hydroxybutyrate, PHB synthetase and other correlative factors will work for chain-transfer reaction. If the condition can be favourable for PHB synthetase, PHB with high Mw can be got. Moreover, with the inhibition of depolymerase in the chain-transfer reaction, higher Mw of PHB can be achieved. It can be concluded that the microorganism with lower activity of β-ketothiolase and depolymerase and higher activity of PHB synthetase would make an appropriate condition for the synthesis of high Mw of PHB. In addition, to synthesize high Mw of PHB in cells, good condition of cell, all enzyme system running well, is principal and essential.

It has been suggested that a chain-transfer reaction involving the propagating PHB chain on the active site of PHB synthetase does not take place in the *in vitro* polymerization system (Gerngross and Martin, 1995). So the cell extraction was studied, including the free-enzyme system. After sonicating, the Mw of PHB recovered from the cell did not caused much decrease. However, the Mw of PHB recovered from the extraction mixture of the sonicated cell (including fragments and cell-free extract) cultivated for another stage would be variable in a certain range (0.70 to 1.4×10^6). This is mainly because the system of enzymes for the synthesis of PHB would act as activated as they can with carbon sources and must not be inhibited by the microorganism. Then a wide range of Mw of PHB would be formed.

Therefore, the activity of the PHB synthetase was not the only factors that affect the Mw. In other words, in different microorganism, the Mw will be affected not only by the ability of the enzymes system for PHB synthesis and polymerization itself, but also by the circumstance the enzymes system which exist in it. All in all, in a certain microorganism, the Mw can be controlled in a certain range.

Conclusions

Methanotrophs has its own regulation which is different from other microorganism. Although, the four kinds of strains belonging to methanotrophs, can produce PHB with different Mw. Different PHB synthetases in different strains have their special ability to synthesize PHB with various Mw value. Even the same enzyme in different recombinants can also synthesize the PHB with different Mw. In this study, the same original strains under different condition or at the different time in cultivated course can also synthesized PHB with different Mw. Like IMV 3011, it could produce PHB with the Mw value (1.2×10^6) under normal condition. Furthermore, under the optimized cultivated condition, the Mw can reach higher value

(1.4×10^6) with methanol total concentration of 0.30 % (v/v). Moreover, while some compounds with appropriate concentrations can improve the accumulation of PHB in the cell, some of them were found to help improve the PHB synthetase concentration and express the activity better. Whereas, some others, like NH_4^+, do the reverse functions: the more (32%) PHB accumulated, the lower Mw (1.0×10^6) of PHB obtained. However, when the PHB content does not do much contributions to the Mw, factors that probably promote the activity expression of PHB depolymerase would be disadvantageous for the polymerization in the cell. Even more, the result of PHB Mw from IMV 3011 changed in 0.7 to 1.5×10^6 under different conditions, shows that certain microorganism has its own intrinsic ability to control the PHB accumulation and polymerization intracellular with Mw in a certain range.

This study shows that the results can be applied in the control of the Mw of PHB in certain conditions. However, to elucidate complete explanation on the Mw determination mechanism, further study must be performed to give exact description of how the conditions influence the polymerization course of PHB. There will be more applied value to find a way to synthesize different Mw of PHB by choosing an appropriate system.

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