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Full Length Research Paper

Poly-3-hydroxybutyrate production from methanol by *Methylosinus trichosporium* IMV3011 in the non-sterilized fed-batch fermentation

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The methanotrophic bacteria have promising applications in the production of a biopolymer, poly-3-hydroxybutyrate (PHB). The fed-batch fermentation strategies were studied for PHB accumulation by methanol-grown *Methylosinus trichosporium* IMV3011 under the non-sterilized conditions. The biomass of 2.91 g dry wt/L and PHB accumulation of 47.6% (w/w) were reached in the fed-batch fermentation. In order to increase PHB content, some organic acids were added into the culture as inhibitors of tricarboxylic acid cycle (TCA). It was found that the malic acid should be optimum for PHB accumulation. The biomass and PHB yield would increase to 3.32 g dry wt/L and 58.5% (w/w), respectively by adding 0.2 g/L malic acid. Other key intermediates in the metabolism such as citric acid, succinic acid and acetyl-CoA, also influenced the cell growth and PHB accumulation to a certain extent. The PHB produced in the fermentation process was of steady quality with molecular weights up to 1.7 × 10^6 Da.

Key words: Biosynthesis, poly-3-hydroxybutyrate, methanotrophic bacteria, methanol, fed-batch fermentation.

INTRODUCTION

Polyhydroxyalkanoates (PHAs) have been intensively studied in the last two decades as possible substitutes for conventional polymers. Poly-3-hydroxybutyrate (PHB), the most representative one of polyhydroxyalkanoates (PHAs), could be produced by various species of bacteria as intracellular storage compounds (Chen, 2009; Steinbüchel and Lütke-Eversloh, 2003; Luengo et al., 2003). Due to the complete biodegradability, biocompatibility, optical purity and the similar properties with common plastics such as thermoplasticity and hydrophobicity, PHB could not only be an attractive substitute for the common polymers, but also had several useful potential applications, especially in medicine and agriculture. However, the manufacturing costs of PHB is still too high in comparison with polymers of petrochemical origin (Akiyama et al., 2003), which was a major obstacle for large-scale commercial exploitation. Since the cost of PHB production depended largely on the price of the substrate used for fermentation (Lee et al., 1999; Reddy et al., 2003), it was necessary to find suitable inexpensive carbon and nitrogen sources.

Methanotrophic bacteria could use methane or methanol with low concentration as carbon source for growing. Such low concentration of methane was hardly to be used in the chemical process. It had previously been reported that the methanotrophic bacteria had ability to accumulate PHB in the imbalance growth

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conditions (Khanna and Srivastava, 2005; Wendlandt et al., 2005). The two substrates of methane and methanol could be available in large volumes with the correspondingly lower price. Furthermore, most other kinds of microorganisms in cultivated circumstance could not grow by using methane or methanol as carbon source. The contaminated microorganisms in the culture medium would be sterilized or inhibited by existing of methanol. Therefore the utilization of methane or methanol as carbon source by methanotrophic bacteria would perhaps be exploited for the cost-effective commercial production of PHB in an open non-sterilized cultivated system. The fundamental PHB accumulated ability of methanotrophic bacteria had been researched in several reports (Wendlandt et al., 2001, 2005; Kim et al., 1999). Due to the characteristic of methanotrophic bacteria, cells growth could not reach at a high level just with methane as carbon source (Wendlandt et al., 2001; Shah et al., 1996), and the PHB yield was lower relatively to the microorganism strains which had been used in the commercial production of PHB.

In our previous research (Zhang et al., 2008), the appropriate cultivated condition in the flasks for *Methylosinus trichosporium* IMV3011 was determined. The high concentration of biomass and the high yield of PHB with high molecular weight were achieved by *M. trichosporium* IMV3011 using methane and methanol as co-carbon sources. The growth of *M. trichosporium* IMV3011 was significantly enhanced in comparison with the absence of methanol. Similar results were also reported in the previous study (Benstead et al., 1998). In this article, the concentration of biomass and PHB were very similar when co-carbon sources of methane and methanol or sole carbon source of methanol was used respectively. During the serial cells acclimation, methanol could be used as sole carbon source for PHB production in stead of co-carbon sources, so the batch fermentation and fed-batch fermentation of *M. trichosporium* IMV3011 were studied by using methanol as sole carbon source. Both the cells growth and PHB accumulation were improved greatly by the good fed-batch fermentation strategies. It was found that some key intermediates of tricarboxylic acid (TCA) cycle should be favorable for PHB accumulation in our studies. And the PHB production would be affected indirectly by controlling the TCA cycle in the metabolism.

MATERIALS AND METHODS

Microorganisms and culture medium

The methanotrophic bacteria used was *M. trichosporium* IMV3011, which was obtained from the Russia Institute of Microbiology and Virology (Kiev. Ukraine).

The experiments were carried out in a mineral salt medium with following compositions (g/L): KH_2PO_4(0.4), K_2HPO_4(0.4), Na_2HPO_4(0.74), $CaCl_2$(0.024), $MgSO_4 \cdot 7H_2O$(0.3), NH_4Cl(0.5), NaCl(0.3), KNO_3(1.6), EDTA(0.01), $MnSO_4 \cdot 5H_2O$(5.23×10^{-4}), $ZnSO_4$(2.4×10^{-4}), $NaMoO_4 \cdot 2H_2O$(2.88×10^{-4}), $CuSO_4 \cdot 5H_2O$ (4×10^{-4}), $FeSO_4 \cdot 7H_2O$(4×10^{-3}), $FeCl_3$ (1×10^{-3}).

All chemicals were of analytical grade.

Carbon source

Direct addition of high concentration methanol was found to be toxic for the growth of strain IMV3011. However, the strain could grow when methanol was supplied as vapor. It took 72 h for the strain to start growth in methanol vapor. The grown strain under methanol vapor was adapted to grow with liquid methanol by the serial transfer of strain into the culture medium with gradually increased concentration of methanol (from 0.1 to 4%, v/v) (Hou et al., 1978; Mehta et al., 1991). With the serial cells acclimation (Figure 1), the strain IMV3011 in the flasks could grow well in the culture medium with about 0.3% (v/v, about 2.4 g/L) total concentration of methanol (Zhang et al., 2008).

Preparation of inoculum

The inoculum was prepared in 250 mL flasks containing 100 mL of mineral salt medium, flasks were incubated at 34°C on a rotary shaker at 250 rpm. When the biomass concentration in the flasks reached at about 1.0 g/L (dry weight), the required amount of inoculum was transferred to fermenter.

Batch fermentation

The production of PHB in *M. trichosporium* IMV3011 was carried out in 5 L fermenter with 3 L culture medium. The concentration of inoculum was 10% (v/v). The pH was controlled at 7.0. The temperature was controlled at 34°C. The agitation speed was set to and maintained at 300 rpm through out the fermentation. Dissolved oxygen concentration was maintained at 50% saturation value by manually adjusting the air flow rate (or gas mixture flow rate: 50% air + 50% methane) in response to the change of cell respiration rate.

Fed-batch fermentation

The studies were carried out in 5 L fermenter. Initial volume of culture media taken was 2 L and the concentration of inoculum was 10% (v/v). Temperature was maintained at 34°C. The pH was controlled at 7.0. Dissolved oxygen concentration was maintained at 50% saturation value by manually adjusting the air flow rate at the fixed 300 rpm agitation speed. For avoiding the inhibition of methanol on cells growth, the initial concentration of methanol was controlled at 0.125% (v/v, about 1g/L). During the intermissive fed-batch process, the feeding culture medium without nitrogen source was added 5 times separately to make total amount equal to those added in the batch process. With the each feeding, the 0.1% (v/v the total volume of medium in the fermenter at that moment) methanol was also added into the reactor. Using this feeding strategy, the initial nitrogen source was exhausted quickly and carbon source was continuously supplied. The accumulation of PHB in the cells would be improved by the imbalance between carbon source and nitrogen source.

Analysis

Methanol concentration

Methanol in the culture media was determined using a gas chromatograph (Agilent 6820 system, USA, with a flame ionization

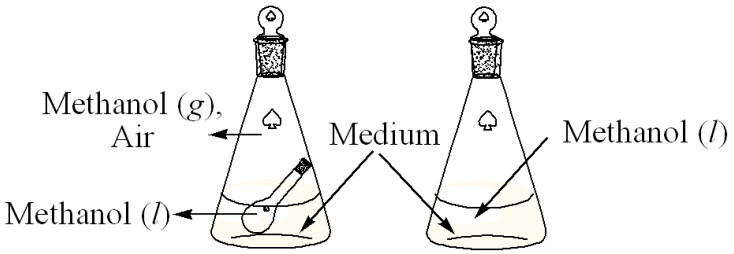

Figure 1. Cells cultivated with methanol gas and liquid methanol.

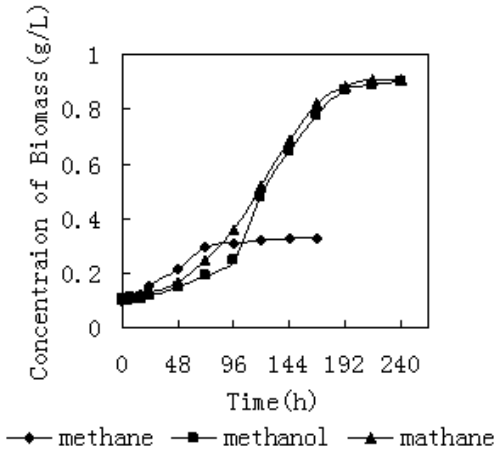

Figure 2. Growth curves of *M. trichosporium* IMV3011 under different carbon sources conditions.

detector and a capillary column 0.23 mm × 30 m, stationary phase, SE-54). Methanol analysis conditions were follows: injector/detector temperature, 250°C; column temperature, 160°C.

Biomass concentration

Absorbance was measured on a spectrophotometer (HP 8453, 660 nm) with mineral salt medium as the blank; the biomass concentration was evaluated using a calibration curve. Dry weight: 100 mL of the cell suspension was centrifuged at 9000 r/min for 10 min, and the sediment was dried at 105°C to a constant mass.

Poly-3-hydroxybutyrate recovery

After centrifugation, the biomass was freeze dried. Lipids and color substances 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 biomass was removed by filtration, and the dissolved PHB was precipitated with cool methanol. Poly-3-hydroxybutyrate was washed twice with methanol, separated by filtrating, and dried at 60°C for 2 h.

Poly-3-hydroxybutyrate analysis

Poly-3-hydroxybutyrate content: it was determined by gas chromatography (Riis and Mai, 1988). About 40 mg of dried biomass powder was suspended in 4 ml of chloroform, 4 ml of methanol containing vitriolic acid (15:85, v/v, vitriolic acid/methanol) and 20 mg of benzoic acid, and boiled at 100°C for 4 h. After cooled to room temperature, 4 ml of distilled water was added and the samples were shaken for 30 s. The heavier phase was directly analyzed on gas chromatograph (Agilent 6820 system with a flame ionization detector (FID) and a capillary column of 0.23 mm × 30 m; stationary phase, SE-54, USA). Pure PHB was used as standard sample.

Molecular weight: the molecular weight and its distribution was determined on a gel permeation chromatograph (GPC) system (Styragel HT 3, 5, 6E in series using HPLC (Waters 2695, USA)/RID (Waters 2414, USA)), relative to polystyrene standards (solvent, 0.1 mg/ml chloroform).

Nuclear Magnetic Resonance (NMR): The spectra were recorded on a Varian INOVA spectrometer in CDCl3 at 303 K (1H-NMR 400 MHz, 13C-NMR 100 MHz). It was established by using the ^{1}H and ^{13}C NMR spectra that *M. trichosporium* IMV3011 could synthesize a homopolymer consisting of 3-hydroxybutyrate monomers in the cells.

RESULTS AND DISCUSSION

Cell growth of *M. trichosporium* IMV3011 under different carbon sources conditions

From growth curves of *M. trichosporium* IMV3011 under different carbon sources conditions in Figure 2, the concentration of biomass was improved greatly when methanol was added into the culture, comparing with the cultivation by using methane as sole carbon source. And the helpful effect of methane on methanol utilization was unconspicuous and indistinct in our combined carbon source cultivation, which might be because that methanol would become the main carbon source for microorganism using when it was added into the culture. Thus, it was possible to use methanol as sole carbon source in the fermentation course. The mechanism of stimulative action of methanol might be due to two aspects: (1) the water-solubility of methanol was much better than methane, which would improve the utilization efficiency of carbon source in the cultivation for obtaining more biomass; (2) consumption of NADH was decreased by methanol introduction, and more NADH was provided to PHB accumulation.

The utilization efficiency of carbon source was very low because of the poor water-solubility of methane, which

Table 1. Key parameters in the batch fermentation of *M. trichosporium* IMV3011 under different carbon sources conditions.

Carbon sources	Methane	Methanol	Methane + Methanol
Lag phase (h)	13.3	85.5	62.4
$Biomass_{max}$ (g/L)	0.34	0.93	0.99
μ_{max} (h^{-1})	0.022	0.016	0.017
G (h)	13.7	18.8	17.7
PHB (%)	6.1	10.8	11.2

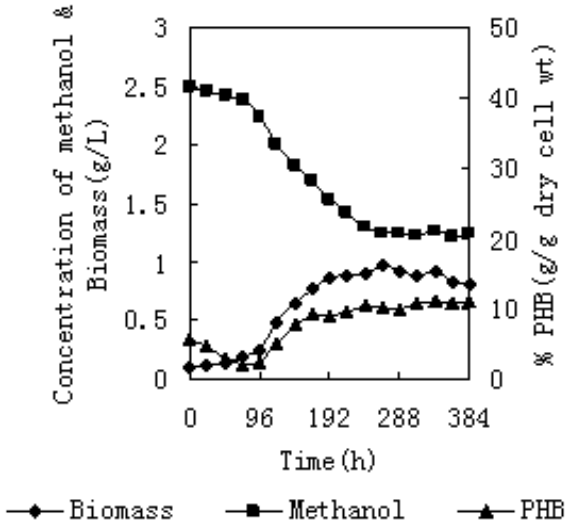

Figure 3. Time courses of biomass, PHB and substrate concentration in the batch fermentation.

might be the reason for the low values of cells growth and PHB accumulation (Table 1) with sole methane carbon source. Both biomass and PHB were improved when methanol was used as carbon source. But the lag phase of fermentation using methanol as carbon source would be prolonged comparing with the cultivation using methane. Methanotrophic bacteria used methane for growth and metabolism generally. Although *M. trichosporium* IMV3011 could use methanol as carbon source for obtaining better growing by cells acclimation, it need an acclimated period when it was inoculated into the culture medium with methanol, which might be the reason for a longer lag phase in cultivation using methanol.

Time-course of cell growth and PHB accumulation in the batch fermentation using methanol as sole carbon source

The time-course of cell growth and accumulation of PHB were presented in Figure 3. The growth of IMV3011 increased steadily with a lag phase of 85 h followed by the logarithmic phase and attained the stationary phase at 192 h. The maximum concentration of biomass could reach at 0.93 g/L. The concentration of PHB decreased in the lag phase and increased rapidly with the growth of IMV3011. The maximum accumulation was recorded at the stationary phase (11%, w/w of dry cells), after which a balance in the polymer level was observed. The probable reason of the initial decrease of PHB was that the cell inclusion had been used as carbon source for strain growth at the beginning.

The addition of high concentration methanol was inhibiting for the growth of strain. In the batch fermentation, the direct addition of 2.5 g/L methanol would not be benefit to the growth of IMV3011. The strain grew slowly and the lag phase was prolonged. And the only about 50% carbon source was be utilized.

Time-course of cell growth and PHB accumulation in the fed-batch fermentation using methanol as sole carbon source

To avoid the inhibiting effect of methanol, the carbon source should be added in batches. The initial concentration of methanol was controlled at 1g/L and the appropriate amount methanol (0.1% v/v the total volume of medium in the fermenter) was added 5 times separately in the whole fermentation process, which would shorten the lag phase of growth. The logarithmic phase attained at 76 h and the strain grew continuously. After 240 h, the maximum concentration of biomass would reach at 2.91g/L in the stationary phase. The maximum accumulation of PHB was reached at 47.6% (w/w of dry cells) at 312 h. The total concentration of methanol in the fed-batch fermentation was about 4.1g/L, and 94% carbon source was used in the process.

High PHB productivity could be achieved by several ways, including high cell density, high PHB concentration and short cultivation time. The results (Figure 4) showed that the concentration of biomass and PHB increased largely in the fed-batch fermentation comparing with the batch fermentation process. At the same time, the using ratio of carbon source was also improved by good fed-batch fermentation strategies. The lag phase was shortened from 85 h to 76 h and the logarithmic phase

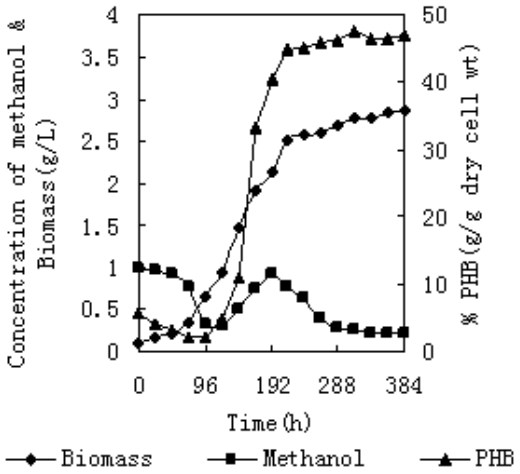

Figure 4. Time courses of biomass, PHB and substrate concentration in the fed-batch fermentation.

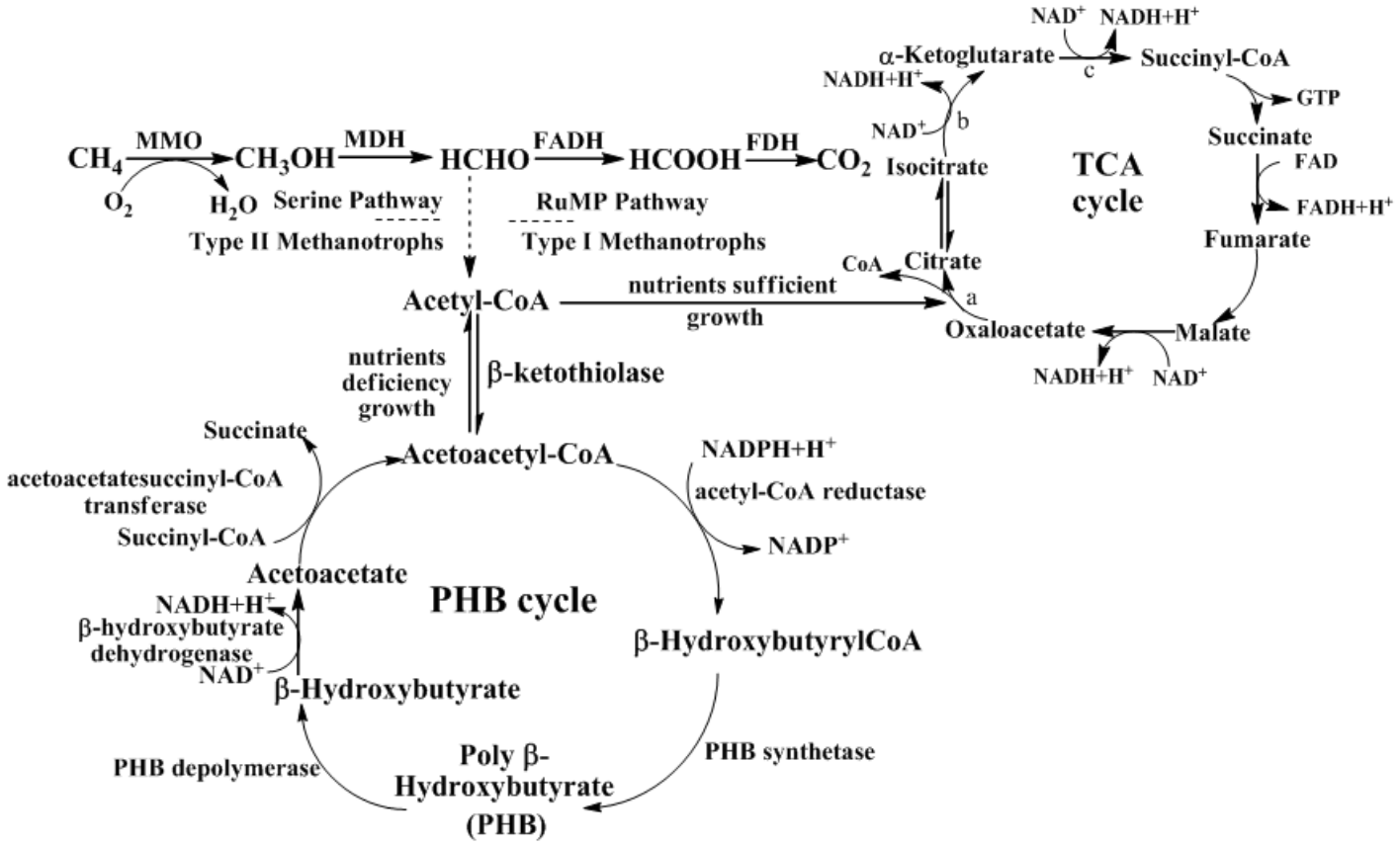

Figure 5. Metabolism pathways of TCA cycle and PHB cycle in the Methanotrophic bacteria.

was prolonged to 240 h, which made the cells grow sufficiently. The maximum specific growth rate was improved from 0.016 to 0.021h^{-1} by the fed-batch fermentation strategies with the shortening of generation time from 18.8 to 14.3 h.

Effects of organic acids on the PHB accumulation in the fermentation of IMV3011

The metabolism pathway in methanotrophic bacteria was shown in Figure 5. When all nutriments were sufficient in

Table 2. Effects of different organic acids on cell growth and PHB yield.

organic acids concentration (g/L)	$X_{dry\ cell}$ (g/L) [a]	% PHB (g/g dry cell) [b]	Adding time (h)
Citric acid 0.10	3.04	35.4	–
Citric acid 0.30	2.81	53.7	–
α-Ketoglutaric acid 0.01	2.91	46.8	–
α-Ketoglutaric acid 0.05	2.87	47.3	–
Succinic acid 0.05	2.89	42.1	72
Succinic acid 0.20	3.19	52.8	72
Malic acid 0.05	2.99	46.5	24
Malic acid 0.20	3.32	58.5	24
No addition	2.91	47.6	

a: The maximal dry weight of biomass at the stationary phase b: The maximal accumulation of PHB at the stationary phase –: no effect.

the cultivation, the metabolism would enter TCA cycle. But if the cultivation was under a nutrients deficiency condition, the PHB cycle would be a primary metabolism pathway instead of TCA cycle. Generally, the TCA cycle pathway was benefit to the cell growth, the carbon source was finally transformed into the cell mass and energy. So the cultivation of microorganism for PHB accumulation was generally performed in two stages: a continuous growth phase (nutrients sufficient) and a PHB accumulation phase (nutrients deficiency) under different concentrations of essential nutrients in the batch culture. In our study, all nutriments were sufficient in the beginning of the fed-batch fermentation. With the cells growing continuously, the nutriments were becoming insufficient. The methanol (carbon source) and other components except for nitrogen source were added into the fermentor, which would lead to the high ratio of C/N. PHB content could be greatly improved under deficiency of nitrogen source.

In other words, if the TCA cycle became harder, more carbon sources would probable be used for synthesis of PHB. So the indirect control of PHB cycle would be achieved by controlling TCA cycle. The key intermediates in TCA cycle would be essential factors for controlling the cycle. Citric acid, malic acid, succinic acid, ketoglutaric acid, important intermediates of TCA, was added to the culture with different concentration for the study of intracellular PHB synthesis.

It had also been reported that the supplementation of organic acid at a certain cultivation time would be able to enhance the cell growth of methanotrophic bacteria to some extent (Xing et al., 2006). However, the acids with excessive concentration would limit cell growth. The citric acid inhibiting effect for TCA cycle in the flasks had been reported in our previously studies (Zhang et al., 2008). The citric acid played an important role in the TCA cycle. When adding 0.10 g/L citric acid to the culture, much more biomass could be obtained by cultivating at nutrient balanced stage. However, adding citric acid with 0.30 g/L as inhibitor for the TCA cycle, the high concentration PHB content could be obtained.

In the fed-batch fermentation of IMV3011, the similar inhibiting effect of high concentration organic acid was also observed. Different concentration of key intermediate in TCA was studied. The appropriate adding concentrations of different organic acids and PHB accumulation under different condition were shown in Table 2. From the results, it was found that most organic acids should have their own contribute to the cells growth and PHB accumulation. In contrast to the results of no addition, most of the additions could help to improve PHB synthesis. Malic acid and succinic acid were found to be favorable for both the cell growth and PHB synthesis under an appropriate concentration. Citric acid with low concentration could improve the cell growth, and excessive citric acid could only increase the PHB concentration but biomass. Among all of the organic acids, malic acid with 0.2 g /L was found to be more favorable for the PHB synthesis than others, 58.5% PHB could be obtained under the condition. However α-ketoglutaric acid could not be so favorable for the cell growth and PHB synthesis, which would be probable because that α-ketoglutaric acid had too low osmotic to enter into the cell and act on the metabolism process.

Citrate was the first key intermediate in the TCA cycle. Excessive citric acid would inhibit the activity of citric acid synthetase and decrease the conversion of citrate, which could inhibit the acetyl-CoA from entering into TCA cycle. The TCA cycle were restrained, more acetyl-CoA would enter PHB cycle for synthesizing more PHB.

Malic acid was the substrate of the last reaction of TCA cycle and NADH would be produced in the step. NADH would improve the metabolism of cells very well. The results showed that the low concentration of NADH would be produced by addition low concentration of malic acid, which would not have good effect on the TCA cycle. But when the high concentration of malic acid was added into the system, the concentration of NADH accumulated was correspondingly high. Excessive NADH would probably inhibit the activity of key enzyme in the TCA cycle and

Table 3. Effect of sodium acetate on cell growth and PHB yield.

Concentration (g/L)	$X_{dry\ cell}$ [a] (g/L)	% PHB [b] (g/g dry cell)
0	2.91	47.6
0.05	3.16	50.8
0.10	3.35	56.0
0.30	3.31	57.1

a: The maximal dry weight of cell mass at the stationary phase b: The maximal accumulation of PHB at the stationary phase.

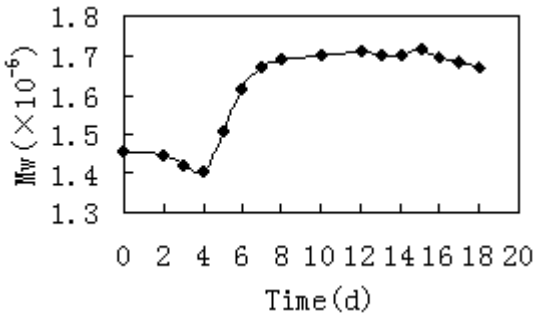

Figure 6. Change of molecular weight during the fed-batch fermentation course.

make more acetyl-CoA enter into the PHB cycle. The reason for improving PHB accumulation by adding succinic acid might be similar with malic acid.

The adding of organic acid at different time would lead to different results or wrong conclusions. The optimal adding time could improve PHB accumulation. When malic acid with suitable concentration was added to the culture cultivated for 24 h, and succinic acid was 72 h, the organic acids would do the best effects on the PHB accumulation. But the adding time of citric acid had not distinct effect on PHB accumulation.

Effects of acetyl-CoA on the PHB accumulation in the fermentation of IMV3011

Acetyl-CoA was the key substrate of TCA cycle. It also could be transformed to acetoacetyl-CoA to enter the PHB cycle. So the concentration of acetyl-CoA and the ratio of entering TCA and PHB cycle were very important for PHB production.

Adding the high concentration of sodium acetate in the culture medium, the acetate would combine with dissociative CoA for abundant acetyl-CoA. Enough acetyl-CoA would be provided to the TCA cycle for cell growth. On the other hand, enough acetyl-CoA would relieve the inhibiting effect of dissociative CoA on β-ketothiolase and improve the production of acetoacetyl-CoA (Dawes and Senior, 1973; Oeding and Schlegel, 1973; Yamane, 1993). Furthermore, more acetoacetyl-CoA could promote the synthesis of β-hydroxybutyrate, the precursor of the PHB polymer.

The results (Table 3) showed that both cell growth and PHB accumulation would be increased by adding the certain concentration of sodium acetate. The optimum concentration of sodium acetate was 0.1 g/L. The cell growth and PHB accumulation would not increase obviously by adding higher concentration of sodium acetate. However, excessive acetate would inhibit the normal metabolism of IMV3011 when the adding concentration was above 0.3 g/L.

Molecular weight

The molecular weight and its distribution were determined by the GPC system. Figure 6 showed the change of molecular weight during the fed-batch fermentation process. The molecular weight reached the top and maintained the value for quite a long time. But after 216 h cultivation, the molecular weight began to decrease slowly, which could probably be attributed to the depolymerization of PHB with the senescence of cells. Moreover, the less decrease of M_w was founded in the initial stages of the logarithmic phase. The carbon source was consumed rapidly with the cells growth in the stage, so PHB in the cells would be depolymerized for using as carbon source. With the adding of methanol, the concentration and molecular weight of PHB increased again. The M_w in the fermentation (1.71×10^6 Da) was higher than the result (1.48×10^6 Da) in the flasks, and PHB from fed-batch fermentation was more steady (Zhang et al., 2008).

Conclusion

The growth of methanotrophic bacteria would be inhibited by methanol in some previous studies. But in this article, it was found that methanol should be a good carbon source for PHB accumulation and growth of *M. trichosporium* IMV3011 in stead of methane by a series of cells acclimation using methanol.

By the batch fermentation processes, the cell growth

and PHB formation were inhibited by high substrate concentrations. The fed-batch fermentation was very helpful for removing the substrate inhibition and improving the productivity. The carbon source concentration was maintained at the lower level by a reasonable feeding strategy in order to reduce the inhibiting effect of substrate. The C/N concentration played a significant role in overall PHB accumulation. Both the cell growth and PHB accumulation were improved under the high C/N condition by the fed-batch fermentation strategy. The maximal concentration of cells dry weight reached at 2.91 g/L and the PHB concentration was 47.6% in the fed-batch fermentation of IMV3011.

It was also found that some key intermediate in the metabolism, such as acetyl-CoA, citric acid, malic acid and so on, were helpful for further increasing of PHB production. Due to the inhibiting effects of high concentration organic acid on TCA cycle, the reaction activities in the PHB cycle would be increased on the contrary. In this article, 0.2 g/L malic acid was an optimal organic acid addition for intracellular PHB synthesis. The 3.32 g/L cells dry weight and 58.5% PHB could be obtained under the condition. It was proved firstly that the inhibition of TCA cycle should improve PHB synthesis ability of methanotrophic bacteria. Moreover, the study would help us to find more appropriate methods to improve PHB synthesis by this kind of microorganism under the open non-sterilized cultivated condition.

It was concluded that the molecular weight of intracellular PHB should be relative to the cell growth. The corresponding change of M_w would appear in various incubation stages of IMV3011. Further research work about PHB accumulation in methanotrophic bacteria IMV3011 was in progress.

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