Article ID: 1001-3555(2009)04-0298-06

Effect of Organic Acids on Biosynthesis of Poly-3-Hydroxybutyrate of *Methylosinus Trichosporium* IMV3011

ZHANG Ying-xin[1,3], XIN Jia-ying[1,2*], SONG Hao[1], XIA Chun-gu[1*]

(1. *State Key Laboratory for Oxo Synthesis & Selective Oxidation*, *Lanzhou Institute of Chemical Physics*, *Chinese Academy of Sciences*, *Lanzhou* 730000, *China*;
2. *College of Food Engineering*, *Harbin University of Commerce*, *Harbin* 150076, *China*;
3. *Graduate University of Chinese Academy of Sciences*, *Beijing*, 100039, *China*)

Abstract: Methanotrophs using methane and methanol as their source of carbon and energy will be an optimal strain for poly-3-hydroxybutyrate (PHB) biosynthesis in less expensive production. Then the study was carried on to improve the important limiting factors for the application of synthesis of PHB in methanotrophs Methylosinus trichosporium IMV3011 (IMV3011). It has been found that malic acid, an important intermediate in TCA cycle, is a favorable organic acid for the synthesis of PHB intracellularly. When the cell was cultivated for 24 h under different condition, the addition of malic acid with appropriate concentration (5.7×10^{-4} g/L in nutrients sufficient condition in the first stage and 0.03g/L in nutrients deficiency condition in the second stage) would improve the mass of PHB in biomass to 55% in contrast to that with no addition (40%). Combined with the activity of enzymes performed in PHB cycle, it was concluded that the optimal way to produce PHB in high yield is to inhibit tricarboxylic acid (TCA) cycle to certain extent and not decrease the activities of key enzymes in synthesis of PHB.

Key words: Poly-3-hydroxybutyrate; Organic acid; Methanotrophs; TCA cycle; Enzyme activity

CLC number: O643.32 **Document code**: A

Although Poly-3-hydroxybutyrate (PHB) has many useful features (biodegradability, biocompatibility and thermoplasticity), its great cost hampers its commercial biosynthetic production. To make this process less expensive, readily available carbon sources and active PHB producer strains are sought[1]. Methanotrophs which in most cases use methane or methanol as their source of carbon and energy, play an important role in the global carbon cycle and bioremediation of soil and water systems.

As is known, low growth rates and low bioconversion rates are the most important limiting factors for the application of synthesis of PHB in methanotrophs. Many efforts have been made to grow *M. trichosporium* OB3b[2~6] and *M. capsulatus* Bath [7] with high MMO activity. Some essential factors of the culture such as the pH, temperature and the concentration were optimized in these studies. It has been reported that carbon dioxide can shorten the lag growth phase of *M. trichosporium* OB3b and increase the final cell concentration [2]. The growth of *M. album* BG8 can be significantly enhanced [8] by adding chloromethane, one of the co-metabolic compounds of methanotrophs. Xing et al. [9] had found the most significant effect of citrate on cell growth enhancement among organic acids such as malate, citrate, succinate and maleate. However, the specific growth rate was still very low.

Moreover, an optimal culture condition for second stage which can increase the PHB concentration to 0.16 g/L under nutrients deficiency condition was sug-

Received date: 2008-12-22; **revised date**: 2009-02-11.

Foundation item: New Century Excellent Talents in University (NCET-05-0356); theNational Natural Science Foundation of China (20625308).

Biography: Zhang Ying-xin,

* Tel: 0931-4968089, Fax: 0931-4968129, E-mail: cgxia@lzb.ac.cn; Tel: 0451-84867393, E-mail: xinjiaying@yahoo.com.cn.

gested. Citric acid was added as an inhibitor of tricarboxylic acid cycle (TCA), which was found favorable for the PHB accumulation. Although these organic acids with certain concentration have been reported to improve the growth of methanotrophs [9], the study in IMV3011 also show TCA cycle can be inhibited to some extent by the key intermediates with appropriate concentration. However, to our knowledge, there are few reports on this aspect of methanotrophs PHB biosynthesis intracellular up to now.

In this study, the effect of citric acid on the PHB accumulation was further studied. And the effects of more organic acids, important intermediates in TCA cycle, were studied to determine optimal conditions for PHB production combined with the activities of enzymes essential to PHB cycle.

1 Experiments and Methods

1.1 Microorganisms and culture medium

The methanotrophs used was the strain *Methylosinus trichosporium* IMV3011, which was obtained from the Russia Institute of Microbiology and Virology (Kiev. Ukraine). The experiments were carried out in mineral salt medium [10].

1.2 Cultivation conditions

Normal conditions: Under routine cultivation conditions, liquid cultures were grown in aliquots 50 mL of the mineral salt medium in 250 mL shake-flasks. Shake-flasks were stoppered with rubber seal and gassed with methane: air (1:1, v/v) gas mixture replenished every 12 h. The cultivation of cells was carried out at 30 ℃ for about 72-96 h[11].

Culture with methanol: It took 48 ~ 72 h for the culture to start growth in methanol vapor. The grown culture under methanol vapor was adapted to grow on liquid methanol by the serial transfer of the culture into the medium (from 0.1% up to 4%)[10, 12].

All cultivations were performed in a sterile manner in two stages [13]. Citric acid, malic acid, succinic acid, malonic acid, α-ketoglutaric acid, important intermediates of TCA, was added to the culture with different concentration for the study of synthesis of PHB intracellular.

1.3 Analysis

1.3.1 Biomass concentration　Absorbance was measured at 660 nm (HP 8453, 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 ℃ to a constant mass.

1.3.2 Poly- 3-hydroxybutyrate

Recovery of PHB [14]

After centrifugation or separation, the biomass was freeze dried. Lipids and color substances were then removed by extraction with methanol (80%, 1.5 h, 50 ℃). In the second step, PHB was extracted from the biomass with chloroform (1.5 h, 70 ℃), the dissolved PHB was precipitated with methanol. PHB was washed twice with methanol, separated by filtration.

Analysis of PHB content

PHB content was determined by gas chromatography [15]. About 40 mg of dried biomass powder was suspended in 4 mL of chloroform, 4 mL of methanol containing vitriolic acid (15%) and 20 mg of benzoic acid, and the mixture reacted at 100 ℃ for 4 h. After cooled, 4 mL of distilled water was added. The heavier phase was directly analyzed on gas chromatography (Agilent 6820 system, U. S. A, with an FID, a capillary column: 0.23 mm 30 m; stationary phase, SE-54). Pure poly-3-hydroxybutyric acid was used as standard sample.

1.3.3 Analysis of Enzyme activity　The supernatant was prepared with the method [1] and then used as cell-free extract for assaying enzyme activities in a 1-cm constant-temperature cell at 30 ℃ with a Hewlett Packard 8 453 spectrophotometer.

β-Ketothiolase (*EC* 2.3.1.9) *activity* [16~18]: The reaction mixture (1 mL) contained 100 mmol/L Tris-HCl pH 8.3, 25 mmol/L $MgCl_2$, 100 μmol/L acetoacetyl CoA, and 100 μmol/L CoA. The reaction was assayed spectrophotometrically at D303.

Acetoacetyl-CoA reductase (EC 1.1.1.36) activity [19]: The reaction mixture contained 100 mmol/L potassium phosphate buffer pH 5.5, 1.5 mL; 0.25

mmol/L $MgCl_2 6H_2O$, 0.12 mL; 12.5 mmol/L dithiothreitol(DTT), 0.1 mL; 6.0 mmol/L NADH, 0.1 mL; 18 mmol/L acetoacetyl-CoA, 10 μL; 0.1 mL of the extract, and water to adjust the volume to 2.5 mL. The reaction was assayed spectrophotometrically at D340.

PHB synthetase (EC 2.3.1) activity [20]: The reaction mixture contained 25 mmol/L Tris-HCl-buffer containing 1 mmol/L, 5,5′-dithiobis- 2-nitrobenzoic acid (DTNB), 30 mmol/L D-3-hydroxybutyryl-CoA, and the extract. The reaction was assayed spectrophotometrically at D412.

PHB depolymerase activity[1]: Mixture of 50 mmol/L Tris HCl pH 8.0, 1 mmol/L $CaCl_2$, and 150 μg PHB/ mL extract. The rate of D650 decrease was recorded and calculated molar absorption for PHB was taken to be 0.00494 μL ng^{-1} cm^{-1}.

Protein was assayed by the coomassie brilliant blue G-250 method. The mean results of three independent analyses are shown in the table.

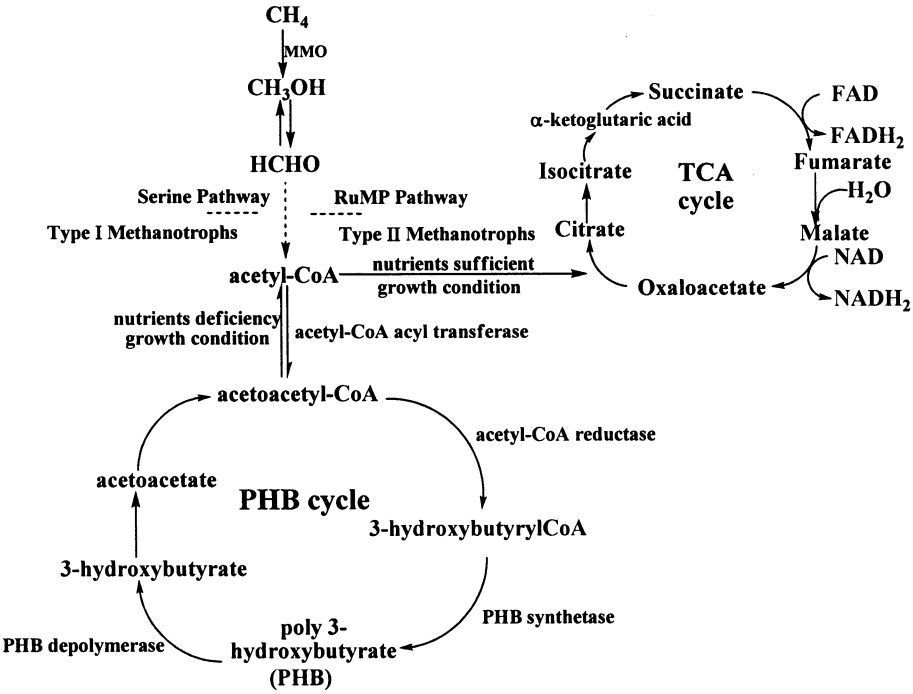
1.4 Chemicals

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

2 Results and Discussion

2.1 Effects of organic acids in TCA cycle on the PHB accumulation in IMV3011

The metabolism pathway in methanotrophs was shown in scheme 1. Generally, PHB is accumulated in



Scheme 1 Metabolism pathway of TCA and PHB cycle in methanotrophs

the cell when the growth stops [13]. Thus, the cultivation is 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 batch culture. In other words, harder TCA cycle proceeds, more carbon sources will probable be used for synthesis of PHB. The intermediates in TCA cycle would be essential factors.

Wide range of concentration of key intermediate in TCA was studied. In Table 1, it is shown that the appropriate concentrations of different organic acids and the accumulation of PHB under different condition. Each of the acids with excessive concentration will limit the growth of cells and then limit the accumulation of PHB. From the results, it is found that each acid had its own contribute to the growth and accumulation of PHB. In contrast to the results (0.56 g/L of X_{total},

Table 1 Effects of different organic acids on PHB accumulation in IMV 3011

	Citric acid		Malic acid		Succinic acid		Malonic acid		α-Ketoglutaric acid	
	a	b	a	b	a	b	a	b	a	b
Concentration(g/L)[c]	0.050	0.050	5.7×10^{-4}	0.030	0.010	1.2×10^{-5}	7.2×10^{-7}	0.050	5.0×10^{-5}	5.0×10^{-5}
X_{total}(g/L)	1.03	0.61	1.05	0.59	0.90	0.48	1.09	0.60	0.78	0.57
X_{PHB}/X_{total}(%)	18	37	17	55	18	45	17	38	20	36
X_{PHB}(g/L)	0.18	0.22	0.18	0.33	0.16	0.22	0.18	0.23	0.15	0.20

a: nutrients sufficient condition; b: nutrients deficiency condition;

c: appropriate concentration of organic acid added to different condition.

40% of X_{PHB}/X_{total} and 0.22 g/L of X_{PHB}) under condition with no addition, most of the addition can't help to improve the synthesis of PHB. Citric acid, malic acid and malonic acid were found to be most favorable for the cell growth under an appropriate concentration for PHB synthesis. Among all of the organic acids added to the culture, malic acid in certain concentration was found to be more favorable for the PHB synthesis than the others. The other organic acids can't be so favorable for the synthesis of PHB probable because they inhibit the TCA cycle or they have too low osmotic to enter into the cell to act on the synthesis of PHB.

It has also been reported that supplementation of organic acid at a certain cultivation time was able to enhance the cell growth to some extent [9]. For different acids, the optimal time of adding can also influence the accumulation of PHB intracellular. When malic acid with suitable concentration was added to the culture cultivated for 24h, it would do the best effect on the accumulation of PHB in the cell. The adding of substrates at incorrect time would lead to inaccurate results and wrong conclusions.

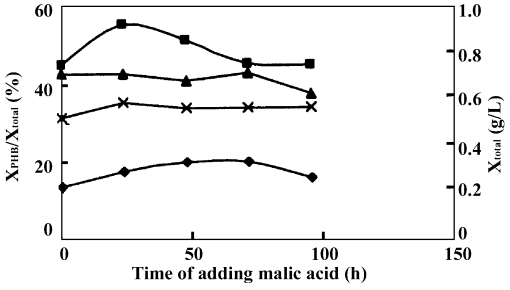


Fig. 1 Effect of the time of adding malic acid on growth and accumulation of PHB

◆ X_{PHB}/X_{total}(%): content of PHB in the cell cultivated in nutrients sufficient culture medium with malic acid (5.7×10^{-4} g/L) added at different time;

■ X_{PHB}/X_{total}(%): content of PHB in the cell cultivated in nutrients deficiency culture medium with malic acid (0.030g/L) added at different time;

▲ X_{total}, biomass of cell cultivated in nutrients sufficient culture medium with malic acid ($5.7\ 10^{-4}$ g/L) added at different time;

X_{total}, biomass of cell cultivated in nutrients deficiency culture medium with malic acid (0.030g/L) added at different time.

2.2 Effects of activities of key enzymes in PHB cycle on the PHB accumulation

The activities of enzymes in IMV3011 cell extracts in nutrients sufficient condition (0 ~ 17% PHB in the biomass) and in nutrients deficiency condition (12 ~ 27% PHB in the biomass) are shown in Table 2. At 96h of the first stage in nutrients sufficient and deficiency culture, we observed the activities of β-ketothiolase, acetoacetyl-CoA reductase, PHB synthetase, and PHB depolymerase (Table 2).

From the results, it was shown that the activities of different enzymes to accumulate PHB in IMV3011 were different under different cultivated conditions with different carbon sources. The activity of PHB synthetase was shown to do the greatest contribution to PHB synthesis. However, β-ketothiolase which is the key enzyme in PHB synthesis [17, 18] doesn't do much contribution. It is probable because that β-ketothiolase will be great important to the synthesis of PHB at the initiation of the course while it will be less important when it comes to the final of the course. Then the change of the activity of β-ketothiolase irregular was due to the PHB accumulation came to different stage when cultivated for 96h under different condition. But it is sure that the final PHB production will be decreased when any of the activities of these enzymes in the synthesis of PHB was especially low.

Table 2 Activities of key enzymes for synthesis of PHB in PHB cycle of IMV 3011 cells

	A		B		C	
	a	b	a	b	a	b
X_{total}/X_{PHB}, (%)	17.1	26.8	13.5	12.5	0	14.19
β-ketothiolase, (U)	0.012	0.023	0.021	0.0061	0.011	0.0019
acetoacetyl -CoA reductase, (U)	0.0036	0.0059	0.045	0.019	0.027	0.017
PHB synthetase, (U)	0.00077	0.0013	0.00030	0.0012	0.00099	0.0014
PHB depolymerase, (μg/min/mg protein)	2.86	9.85	5.30	10.6	0	4.89

A: carbon sources: gas with 50% methane, methanol; B: carbon sources: gas without methane, methanol;

C: carbon sources: gas with 50% methane, without methanol.

a: nutrients sufficient condition; b: nutrients deficiency condition.

Moreover, the activity of PHB depolymerase (2.86-10.6μg/min/mg protein) under different condition was higher than that in *M. trichosporium* OB3b (2μg/min/mg protein). And this maybe the main reason for the lower molecular weight (1.3×10^6) of PHB in IMV3011. It is also find that the activity of PHB depolymerase was dramatically higher under nutrients deficiency culture with higher PHB production; while the activity of the enzyme dropped to zero when no PHB was produced. It suggested that the PHB depolymerase would play an important role in supplying the cell energy by depolymerizing PHB when the cell need. Then it promotes the PHB cycle (Scheme 1) in the cell, and then the accumulation of PHB comes to decrease following with the finish of the cell cultivation under certain condition.

3 Conclusions

In this article, it has been found that malic acid is a favorable organic acid for the synthesis of PHB intracellular under different conditions in different cultivated stage. For its inhibition on TCA cycle and good effects on the activities of enzymes in synthesis of PHB, malic acid is an optimal addition for the synthesis process of PHB. Moreover, the time for adding the substrate should be taken into account. From the above, it has first been proved that the inhibition of TCA cycle will improve the synthesis ability of PHB in this strain. Meanwhile, it is better for the accumulation of PHB when the methods to inhibit the TCA cycle will be propitious to or sustain the activity of the enzymes that do a lot contributes to the accumulation of PHB. The study will help us to find more appropriate substrates and methods to improve the synthesis of PHB in methanotrophs.

Acknowledgements The authors acknowledge the financial support of the work by the Program for New Century Excellent Talents in University (NCET-05-0356), the National Natural Science Foundation of China (20625308), and the Chinese Academy of Sciences.

References:

[1] Doronina N V, Ezhov V A, Trotsenko Yu. A. *Appl. Biochem. Microbiol.* [J], 2008, **44**(2): 182 ~ 185

[2] Park S, Hanna M L, Taylor R T, *et al. Biotechnol. Bioeng.* [J], 1991, **38**(4): 423 ~ 433

[3] Park S, Hanna M L, Taylor R T, *et al. Biotechnol. Bioeng.* [J], 1992, **40**(1): 151 ~ 157

[4] Shah N N, Park S, Taylor R T, *et al. Biotechnol. Bioeng.* [J], 1992, **40**(6): 705 ~ 712

[5] Shah N N, Hanna M L, Jackson K J, *et al. Biotechnol. Bioeng.* [J], 1995, **45**(3): 229 ~ 238

[6] Shah N N, Hanna M L, Taylor R T. *Biotechnol. Bioeng.* [J], 1996, **49**(2): 161 ~ 171

[7] Yu S S F, Chen K H C, Tseng M Y H, *et al. J. Bacteriol.* [J], 2003, **185**(20): 5 915 ~ 5 924

[8] Han J I, Semrau J D. *FEMS Microbiol. Lett.* [J] 2000, **187**(1): 77 ~ 81

[9] Xing X-H, Wu H, Luo M-F, *et al. Biochemical Engineering Journal*[J], 2006, **31**(2): 113 ~ 117

[10] Mehta P K, Mishra S, Ghose T K. *Biotechnol Bioeng.* [J], 1991, **37**(6): 551 ~ 556

[11] Xin J-Y, Zhang Y-X, Zhang S, *et al. J. Basic Microbiol.* [J], 2007, **47**(5): 426 ~ 435

[12] Hou C-T, Laskin A I, Patel R N. *Appl. Environ. Microbiol.* [J], 1979, **37**(5): 800 ~ 804

[13] Zhang Y-X, Xin J-Y, Chen L-L, *et al. J. Nat. Gas*

Chem. [J], 2008, **17**(1): 103 ~ 109

[14] Wendlandt K -D, Jechorek M, Helm J, *et al. J. Biotechnol.* [J], 2001, **86**(2): 127 ~ 133

[15] Benstead J, King G M, Williams H G. *Appl. Environ. Microbiol.* [J], 1998, **64**(3): 1 091 ~ 1 098

[16] Volova T G, Kalacheva G S, Gorbunova O V, *et al. Appl. Biochem. Microbiol.* [J], 2004, **40**(2): 170 ~ 177

[17] Berndt H, Schlegel H G. *Arch. Microbiol.* [J], 1975, **103**(1): 21 ~ 30

[18] Oh D-H, Chung C-W, Kim J Y, *et al. J. Microbiol.* [J], 1997, **35**(4): 360 ~ 364

[19] Senior P J, Dawes E A. *Biochem. J.* [J], 1973, **134**(1): 225 ~ 238

[20] Jossek R, Reichelt R, Steinb chel A. *Appl. Microbiol. Biotechnol.* [J], 1998, **49**(3): 258 ~ 266

有机酸对甲烷利用菌 *Methylosinus trichosporium* IMV3011 生物合成聚-3-羟基丁酸酯的影响

张颖鑫[1,3]，辛嘉英[1,2*]，宋昊[1]，夏春谷[1*]

（1. 中国科学院兰州化学物理研究所　羰基合成与选择氧化国家重点实验室，甘肃 兰州 730000；
2. 哈尔滨商业大学　食品科学与工程省重点实验室，黑龙江 哈尔滨 150076；
3. 中国科学院研究生院，北京 10039）

摘　要：为了降低聚-3-羟基丁酸酯（PHB）的生产成本，利用甲烷和甲醇生长的甲烷利用菌是具有研究价值的一类菌种. 文章针对如何克服甲烷利用菌 IMV3011 积累 PHB 过程中受到制约的因素进行了考察，以改善此菌种生产 PHB 的能力. 研究发现，通过添加适量的三羧酸（TCA）循环的抑制剂——苹果酸可以达到很高 PHB 产量的目的. 在细胞进行两阶段培养过程中，营养平衡和营养受限培养各进行 24 h，即加入不同浓度的苹果酸（前者控制在 5.7×10^{-4} g/L，后者控制在 0.03 g/L），可以使 PHB 的积累量达到 55%（未添加只能达到 40%）. 实验还通过对 PHB 合成中所需的酶的活性研究，从一定程度上证明了适当的抑制三羧酸循环对保持 PHB 合成的酶系的活性将更有利于 PHB 的合成.

关　键　词：聚-3-羟基丁酸酯；有机酸；甲烷利用菌；TCA 循环；酶活性