A Study on the Carbon Specificity of Methane Utilizing Mixed Culture by Growth Curve and Poly-β-Hydroxybutyrate Biosynthesis

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***Abstract* — Methane-utilizing mixed culture HD6T were cultivated in an open system using different carbon and energy sources, in order to produce poly-β-hydroxybutyrate (PHB). Shake-flask experiments shows that HD6T has common characteristics of carbon source in cell growth and PHB biosynthesis. With proper carbon source other than methane or methanol, the cells can survive, but PHB content decreases obviously. There is a limitation in utilizing carbon source to produce PHB with methane-utilizing mixed culture HD6T, namely, only carbon source will not cause great change of environment that cells has adapted to. The results shows that when 0.15% (V/V)/d of methanol is initially added at the atmosphere of methane, the biomass concentration and PHB yield are 1.84g/l(dry weight) and 0.44g/l(dry weight) respectively. Methanol at the atmosphere of methane may be the best carbon source for cell growth and PHB biosynthesis with Methane-utilizing mixed culture HD6T.**

Keywords - Poly-β-Hydroxybutyrate; methane-utilizing mixed culture; carbon source; growth curve

I. INTRODUCTION

Poly-β-Hydroxybutyrate (PHB) with the characteristics of biodegradability, biocompatibility, thermos-processibility, and flexible strengths are considered as a kind of biodegradable polymers. In the past twenty years, there was much research in the field of biodegradable materials and the development of production technologies mainly focused on the use of monocultures and sterile processes.[1,2]. However, the cost of PHB biosynthesis is so high that it is hard to compete with the common plastic. Methanotrophs are a group of bacteria that accumulate PHB intracellular utilizing cheap carbon source such as methane and methyl alcohol[3, 4]. Unlike chemical catalytic processes, which require high temperature and pressure, methanotrophs convert methane to multicarbon compounds at ambient conditions. A process utilizing methanotrophs that have the lower capital and operations expense instead of high temperature and pressure processes, has the potential to become economically viable in converting low cost substrate into value added chemicals [5] Whereas, there are always some problems blocked the industrial application for the methane-oxidizing bacteria. For instance, the source of pure bacteria is limited, the pure bacteria is easily infected in an open system. It is suggestion that accompany bacteria in the mixed system can reduce the substrate inhibition and improve the growth and metabolism of methane oxidative bacteria. Taking advantage of methane-utilizing mixed culture to biosynthesize PHB, it will promote the applied research on the industrialization and commercialization of PHB production.

At present, the research on methane-utilizing mixed culture has been made some progresses in the production of single cell protein [6,7], the treatment of biodegradable pollutants in the groundwater and the use of MMO activity. Only recently the use of mixed cultures for PHB synthesis has been put forward as a viable alternative. In this paper, the carbon-specificity of methane-utilizing mixed culture and PHB synthesis have been studied. The curve-fitting software in conjunction with a logistic growth model has been used to analyze growth data exactly and reliably.

II. EXPERIMENT

A. Microorganisms and culture medium

The methanotrophic strain applied was methane-utilizing mixed culture HD6T, which was isolated from soil samples of daqing oil fields, Heilongjiang Province, China. HD6T was cultivated as described by Zhang et al. [8].

The optimal mineral salt medium of HD6T (g/l): NH_4Cl(0.35),$KH_2PO_4 \cdot 7H_2O$(0.30),K_2HPO_4(0.25),NaCl(0.3), $MgSO_4 \cdot 7H_2O$(0.25),$CaCl_2 \cdot 2H_2O$(0.02),KNO_3(1.30),$FeSO_4 \cdot 7H_2O$(3×10^{-3}),$CuSO_4 \cdot 5H_2O$(5×10^{-3}),$MnSO_4 \cdot H_2O$(3×10^{4}), $ZnSO_4 \cdot 7H_2O$(2.8×10^{-4}),$Na_2MoO_4 \cdot H_2O$(2.5×10^{-4}), $FeCl_3$(1.0×10^{-3}), H_3BO_3(5.8×10^{-5}),KI(7.3×10^{-5}) and $CoCl_2 \cdot 6H_2O$(4.50×10^{-5}).

B. Cultivation condition

All cultivations were performed in an open environment. Cultures were brought up in mineral salt medium at an agitation rate of 180 rpm, 30°C.

Culture with methane: HD6T were cultivated in 100 ml of the mineral salt medium using 250 ml shake flasks.Shake flasks were stoppered with rubber seal and gassed with methane and air(1:1,v/v)gas mixture .The gas phase was replenished every 12 h with the same gas mixture.

C. Biomass concentration

Biomass concentration evaluated using spectrophotometer (Shimadzu UV-2550, 600 nm) with mineral salt medium as the blank; (b)Dry weight cell:100 ml of the cell suspension was centrifuged at an agitation rate of 9000rpm for 10 min, then sediment was dried at 105℃ to a constant mass.

D. Poly-β-hydroxybutyrate analysis

Poly-β-hydroxybutyrate content: it was measured by gas chromatography. About 40 mg of dried cell powder was suspended in a mixture solvent including 4 ml of chloroform,4 ml of methanol containing vitriolic acid (15:85,v/v,vitriolic acid(conc.): methanol)and 20 mg of benzoic acid. After that suspension was water-bath heated at 100℃ for 4 h. After cooled to room temperature, 4 ml of distilled water was added and the samples were shaken for 30s. The heavier phase was directly analyzed on gas chromatograph(Agilent 6820 system with an FID and a capillary column of 0.23 mm×30 m; stationary phase,SE-54,USA).Pure PHB was used as standard sample.

E. MMO activity

The MMO activity were assayed by the epoxidation of propylene.The harvested cells from shake-flask cultures were washed twice with 20 mM phosphate buffer (PH7.0).The final pellet was resuspended in fresh buffer to obtain a density of 1-3 mg dwc ml^{-1}.The assay was carried out in 1ml of this washed cell suspension sealed in a 10ml reaction vial. The gasous phase of the vial was removed by vacuum and then replaced with a gas mixture of propene and air(1:4,V/V).The reaction was started by addtion of the gas mixture.Assay were performed at 32℃ on a water bath rotary shaker at 150rpm. Epoxypropane was measured by gas chromatography. Specific activities were expressed as nanomoles of epoxypropane formed per minute per milligram of dry weight cell(dwc).

III. RESULTS AND DISCUSSION

A. The effect of methanol Concentration on cell growth and PHB biosynthesis

As the low solubility of methane in liquid medium, there is a main obstacle for high cell density cultivation of methane-utilizing mixed culture. At high cell densities, the availability of methane is primarily limited by mass transfer from gaseous to aqueous phase [9]. In order to solve this problem, a potential method is to use a water soluble carbon source instead. Methanol although it has inhibitory effect on growth above certain concentrations, is a good alternative because of its infinite solubility in water and low cost. Methanol, despite being available substrate, is an inhibitory substrate and is toxic to cells at high concentrations. Hence, it is necessary to control the methanol concentration below toxic/inhibitory levels [10]. Under the action of alcohol dehydrogenase, methanol can be directly converted into formaldehyde, entering the carbon assimilation metabolism or deep oxidation.The addition of suitable amount of methanol can promote cell growth, but high concentration of methanol will restrain instead. In order to avoid inhibition of the high initial methanol addition on the cell growth, we adopted the intermittent feed way to provide carbon source, namely adding the methanol every 24 h to make sure the concentration of methanol was not exceed the tolerance range.

Using the lag phase duration and the maximum growth rate to characterize the bacterial growth curve is nowadays most widespread. The lag phase is an adaptive periods during which bacterial adjust themselves from a new environment into logarithmic growth phase. The more drastic the change happen in its ambient, the more time will be consume in that bacterial has to modify themselves to its new environment. Some main growth elements such as lag phase duration, maximal growth rate and maximal OD600 were analyzed to assess whether sudden elevated methanol added to inoculum increased the cell growth rate. In order to determine growth parameters reliably and exactly, the growth data were fitted by MATLAB 7.1 program using Logistic models. The Logistic model showed as follow:

$$y = \frac{A_2 - A_1}{1 + (\frac{x}{x_0})^p} + A_1 \quad (1)$$

Where A_1 is Concentration of inoculum(constant), A_2 is maximum concentration of cell growth(constant), p is Cell growth index(constant), x_0 is Proportional constant(constant), x is cultivation time(independent variable), y is Cell concentration (independent variable,determined by OD600).

The growth parameters calculated were lag phase duration and maximal growth rate. Lag phase duration was quantified

as the corresponding time obtained by intersection between the tangent at the logarithmic phase of the growth curve and the inoculum level. The comparison between fitted curve and experimental data showed as Figure1. The results of Fig.1 showed the fitted curve and experimental data were basically identical, the correlation index reached to 0.98(R^2=0.98), which suggested logistic model fit well to growth curve of methane-utilizing mixed culture.

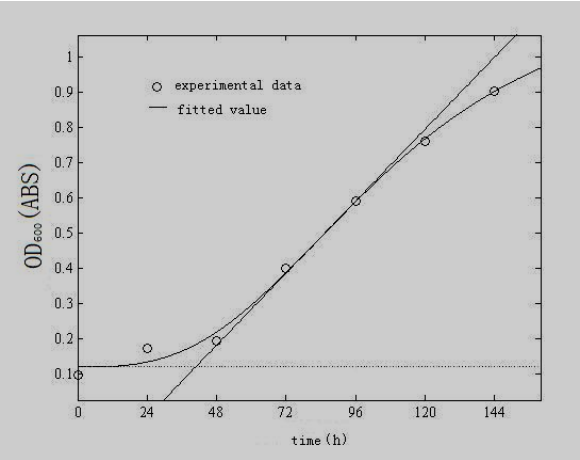

Figure 1. Comparison between fitted curve and experimental data

From Figure2, it can be seen that appropriate amount of methanol addition, cell density was improved obviously more than that of methane as carbon source. Adding 0.10%~0.20% (V/V) methanol every day was the most favorable for cell growth. Adding 0.050% (V/V) methanol every day can not supply enough carbon and energy source for cell growth, leading to cell density was relatively low. However, with other different high methanol concentrations addition, cell cannot metabolize methanol completely, which leaded to residual methanol accumulating too much, eventually cell growth was inhibited.

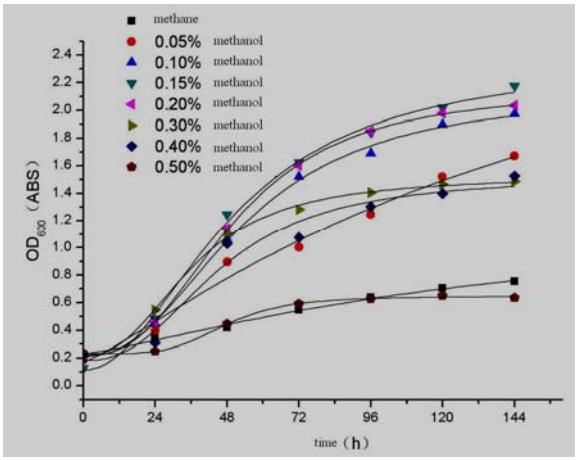

Figure 2. Absorbance fitting curves for cell growth at different concentration of methanol addition per 24h

In order to determine the impact of methanol concentration on cell growth further, we calculated the lag time, maximum growth rate, dry cell weight and PHB content, as shown in table 1.

TABLE 1. INFLUENCE OF DIFFERENT METHANOL CONCENTRATION ON THE GROWTH AND PHB CONTENT

Carbon source(V/V)	Maximum growth rate (h-1)	lag time(h)	Cell dry weight (g/L)	PHB content (%)
methane	0.014	4.0	0.34	9.11
methanol (0.05%)	0.029	2.4	0.68	11.32
methanol (0.10%)	0.033	8.9	1.08	16.05
methanol (0.15%)	0.043	10.2	1.22	21.53
methanol 0.20%)	0.038	12.8	1.14	15.42
methanol (0.30%)	0.034	15.2	0.62	14.27
methanol (0.40%)	0.027	18.2	0.57	11.93
methanol (0.50%)	0.017	27.1	0.49	8.84

The results showed that 0.05% (V/V) concentration of methanol , lag time was shortest, which was 2.4 h. Adding low concentration of methane helped methane-utilizing mixed culture to adapt to methanol culture quickly. But as cells grew into logarithmic phase, 0.05% (V/V)/d methanol addition cannot offer sufficient carbon source in cell proliferation, thus maximum specific growth rate and dry cell weight after stabilization was low. Whereas, due to methanotrophs in the mixed culture is difficult to adapt to the high initial methanol concentration, adding more than 0.3% (V/V) methanol will lengthen its lag time, which was longer than that of under the condition of methane(4.0h) . The results showed that at 0.15% (V/V)/d of methanol initial addition, cell growth and PHB content intracellular increased obviously, but lag time was a little longer(10.2h). Maximum OD600 was 2.176, cell density was higher significantly than that under the condition of methane cultured. Meanwhile dry cell weight was 1.22g/L, which was three times more than that under the condition of methane. Taking the above analysis into consideration, the strategy of methanol feeding has great influence on mixed bacteria growth and PHB

synthesis .So adding different concentrations of methanol at different phase can improve the efficiency of fermentation.

B. The effect of methane on cell growth and PHB biosynthesis in cultivation with methanol.

As the research suggested, in the process of methanotrophs cultivation , some methanol addition can promote the metabolism of cell , but some methanotrophs will lose the ability of methane metabolism[11,12]. Therefore, for HD6T, we used methane (V (methane: air) = 1:1) and methanol as carbon source to cultivate, and observed cell growth and PHB accumulation.

Table 2 showed that adding relatively small amount of methanol can improve cell growth at the atmosphere of methane. It is reported methanotrophs still has the ability to metabolize methane in the presence of methanol. To some extent, it can accelerate the oxidation of methane and promote the solubility of methane in water [13]. The addition of methanol can make up for the insufficient carbon source during cell growth, in which methane has low solubility in water .In this way, the bacteria can get more carbon source for growth and PHB accumulation.

It was proved that methane-oxidizing bacteria has positive effect on the accumulation of PHB, methane provided the necessary conditions for cell growth, but if only rely on methane as carbon source, the cells will spend a lot of energy to further oxidized methane, which is not conducive to the accumulation of PHB.

TABLE 2. THE GROWTH AND PHB CONTENT IN THE CELL AT DIFFERENT CONCENTRATION OF METHANOL WITH OR WITHOUT METHANE

	Cell dry weight（g/L）		PHB content (%)	
	0% CH_4	50% CH_4	0% CH_4	50% CH_4
methanol (0.05%)	0.72	0.85	16.22	17.58
methanol (0.10%)	1.17	1.27	16.01	19.14
methanol (0.15%)	1.26	1.38	21.69	24.05

The addition of methanol can reduce the consumption of NADH in cell growth, then make the reaction of methanol de-hydrogenation continuously, eventually provide sufficient carbon source for accumulation of PHB.

TABLE 3 CARBON-SPECIFICITY OF METHANE-UTILIZING MIXED CULTURE (INITIAL OD600 IS ABOUT 0.048)

Carbon source	OD_{600}		Cell dye weight(g/L)		PHB content (%)		MMO activity (nmol/(min mg dwc))	
	50% CH_4	0% CH_4	50% CH_4	0% CH_4	50% CH_4	0% CH_4	50% CH_4	0% CH_4
metha-nol	2.16	1.90	1.84	1.21	24.05	21.7	3.66	2.76
ethanol	0.74	0.65	0.33	0.28	8.19	5.57	0.22	0
Isopro-panol	0.74	0.64	0.30	0.22	7.42	8.56	0.16	0
butanol	0.67	0.46	0.29	0.20	6.38	5.25	0.13	0
formic acid	0.17	0.15	0.08	0.12	3.66	4.15	0	0
glycer-ol	1.47	0.93	0.65	0.41	5.49	7.21	0.33	0
glucose	2.27	2.16	1.42	1.32	4.87	6.99	0.56	0
metha-ne	0.76	/	0.36	/	9.11	/	3.58	/

C. Carbon-specificity of methane-utilizing mixed culture

To investigate the effect of carbon source to methane-utilizing mixed culture,0.15% (v/v) methanol and the same molar volume of other carbon source were added to the mineral salt medium respectively, such as ethanol, isopropanol, butanol, formic acid, glycerol, glucose, or methane (methane: air= 1:1) as the sole carbon source. After

120h cultivation, cell concentration, PHB content and MMO activity were determined to inspect the carbon-specificity of methane -utilizing mixed culture.

As shown in table3, carbon compounds can be used as carbon source for methane-utilizing mixed culture. When carbon source was methane plus methanol or methane plus glucose, cell grew best and concentration is at maximum, OD600 is 2.16 and 2.27 respectively. The above methane-utilizing mixed culture were inoculated to medium again, using methane (methane: air =1:1) as the sole carbon source instead, after 72h cultivation, OD600 were 0.75, 0.05, 0.07, 0.08, 0.05, 0.08 and 0.07 respectively. Results showed that the mixed bacteria after transferring carbon source (except methanol) cannot grow well and no MMO activities were tested in the cells, suggesting methanotrophs in the mixed system may be lost under the cultivation of other carbon source. Compare with methane as sole carbon source, cells cultivated after 120h cultivation in mineral salt medium using methanol as growth substrate, the MMO activity decreased greatly. Once cells harvested from the methanol cultivation were subcultured to methane cultivation, the MMO activity increased with time and recovered to 3.98nmol/(min mg dwc) within 72h. These results indicated that to maintain the growth stability of methanotrophs in mixed culture, it is essential to supply methane and methanol in the process of cultivation. Methane is important for methanotrophs to keep higher MMO activity. From table3, it showed that adding some carbon source properly, cells survived, but the content of PHB decreased significantly, which indicated that methanotrophs may be dominant bacteria who accumulated PHB in mixed system. It is possible that some other carbon source addition is beyond the tolerance of the cells growth or can not be utilized by the cells, leading to cell growth restriction and PHB content reduced greatly. Although formic acid is also a kind of C1 carbon source, it cannot be used by cell growth, the reason may be that addition of formic acid changed the pH value in medium significantly, cell cannot endure high PH surrounding.

IV. CONCLUSION

The C1 carbon compounds such as methane or methanol is necessary carbon source to maintain cell growth and PHB accumulation of Methane-utilizing mixed culture HD6T. It is essential to supply methane or methanol in the process of cultivation to make sure methanotrophs who may be dominant bacteria accumulated PHB occurred in the mixed system. The addition of methanol can promote cell growth and PHB biosynthesis. There was a limitation in the utilization of carbon source to produce PHB by methane-utilizing mixed culture HD6T, it may only use carbon source that will not cause environment change greatly, in which cells has adapted to. It has been found that methanol-grown cultures of methane-utilizing mixed culture HD6T represent a microorganism agent for the biosynthesis of PHB. Methanol concentration has great effect on Lag time and maximum growth rate of cell growth, which are two important parameters for improving fermentation efficiency .To make the application of methane-utilizing mixed culture HD6T more commercially feasible, it is important to increase biomass productivity. It is recommended that further work be mentioned on achieving optimal methanol feeding strategy by fermentation control.

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