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The Methane Monooxygenase Intrinsic Activity of Kinds of Methanotrophs

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Abstract Methanotrophs have promising applications in the epoxidation of some alkenes and some chlorinated hydrocarbons and in the production of a biopolymer, poly-β-hydroxybutyrate (poly-3-hydroxybutyrate; PHB). In contrast with methane monooxygenase (MMO) activity and ability of PHB synthesis of four kinds of methanotrophic bacteria *Methylosinus trichosporium* OB3b, *M. trichosporium* IMV3011, *Methylococcus capsulatus* HD6T, *Methylomonas* sp. GYJ3, and the mixture of the four kinds of strains, *M. trichosporium* OB3b is the highest of the four in the activity of propene epoxidation (10.72 nmol/min mg dry weight of cell [dwc]), the activity of naphthalene oxidation (22.7 mmol/mg dwc), and ability in synthesis of PHB(11% PHB content in per gram dry weight of cell in 84 h). It could be feasible to improve the MMO activity by mixing four kinds of methanotrophs. The MMO activity dramatically decreased when the cellular PHB accumulated in the second stage. The reason for this may be the dilution of the MMO system in the cells with increasing PHB contents. It has been found that the PHB contents at the level of 1–5% are beneficial to the cells for maintenance of MMO epoxidation activity when enough PHB have been accumulated. Moreover, it was also found that high particulate methane monooxygenase activity may contribute to the synthesis of PHB in the cell, which could be used to improve the yield of PHB in methanotrophs.

Keywords Methanotroph · Methane monooxygenase · Poly-β-hydroxybutyrate · Reducing equivalent

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Introduction

There are microorganisms called methane-oxidizing bacteria or methanotrophs that can utilize methane as their sole carbon source and energy source for growing and that play important roles in the carbon cycle. Methanotrophic bacteria offer several advantages over other aerobic oxygenase-producing bacteria in degrading common groundwater contaminants, such as certain chlorinated aliphatic hydrocarbons, due to their high conversion rates and their utilization of methane as a nontoxic oxygenase inducer. They also have potential applications in the production of chemicals, such as epoxypropane, poly-β-hydroxybutyrate (poly-3-hydroxybutyrate; PHB) [1], methanol [2, 3], and select higher alcohols, due to their nonspecific methane monooxygenase (MMO) enzyme systems [4].

In these organisms, methane is oxidized via methanol, formaldehyde, and formate to carbon dioxide with some formaldehyde being incorporated into cell biomass [5]. The first reaction in the methane oxidation pathway is catalyzed by MMO. MMO utilizes two reducing equivalents to split the O–O bonds of dioxygen. One of the oxygen atoms is reduced to form H_2O, and the other is incorporated into methane to form methanol. Methanol from endogenous (methane oxidation via MMO) is oxidized via formaldehyde and formate to carbon dioxide by methanol dehydrogenase, formaldehyde dehydrogenase, and formate dehydrogenase. Most of the reducing power required for the metabolism of methane is produced by the oxidation of formaldehyde via formate to carbon dioxide. The carbon dioxide produced from methane oxidation is partly emitted and partly incorporated into cell biomass via the serine pathway [5]. In certain methanotrophs, such as *Methylosinus trichosporium* OB3b and *Methylococcus capsulurus* (Bath), a membrane-bound form of MMO (particulate methane monooxygenase [pMMO]) is produced when sufficient Cu is present, whereas a soluble form of MMO enzyme (soluble methane monooxygenase [sMMO]) is produced when Cu is inadequate [4, 6]. The sMMO oxidizes a wide range of hydrocarbons (e.g., *n*-alkanes, *n*-alkenes, aromatic and alicyclic compounds, and many chlorinated solvents). The pMMO, on the other hand, has narrower substrate specificity, but has higher activity with smaller hydrocarbons like methane, ethane, and propene [7–10]. Both sMMO and pMMO require O_2 and reducing equivalent NADH for their intracellular catalytic activity, as shown in Fig. 1; reducing equivalent NADH can be regenerated by the further oxidation of methanol to CO_2 [11].

However, if the methanol produced by MMO cannot be metabolized further to recycle reducing equivalent, the intracellular NADH becomes rate limiting. Therefore, an intracellular oxidation of exogenously supplied sodium formate to CO_2 is commonly used to regenerate NADH during in vitro assays of whole-cell MMO activity. Potential in situ bioremediation or in vitro bioreactor applications of methanotrophs in which large amounts of formate additions may not be environmentally acceptable, economically viable, or operationally practical require an alternate source of reducing equivalent. Hydrogen can be derived from coal or natural gas. It is oxidized rapidly by some hydrogenase enzyme systems to regenerate intracellular NADH and, therefore, is a potential inexpensive source of MMO reducing power [11–15]. It has been shown that the specific propene epoxidation rate of *M. capsulurus* (Bath) containing pMMO increased by an order of magnitude when H_2 was added as a reductant. However, a similar stimulation with H_2 could not be demonstrated reproducibly for sMMO-containing cells [16].

PHB, a naturally occurring biopolymer, is biodegradable and has tensile as well as thermal properties similar to those of synthetic plastic-like polypropylene [4]. PHB is accumulated as an intracellular carbon and energy storage material by a variety of microorganisms under nitrogen-, phosphate-, or oxygen-limiting condition. However,

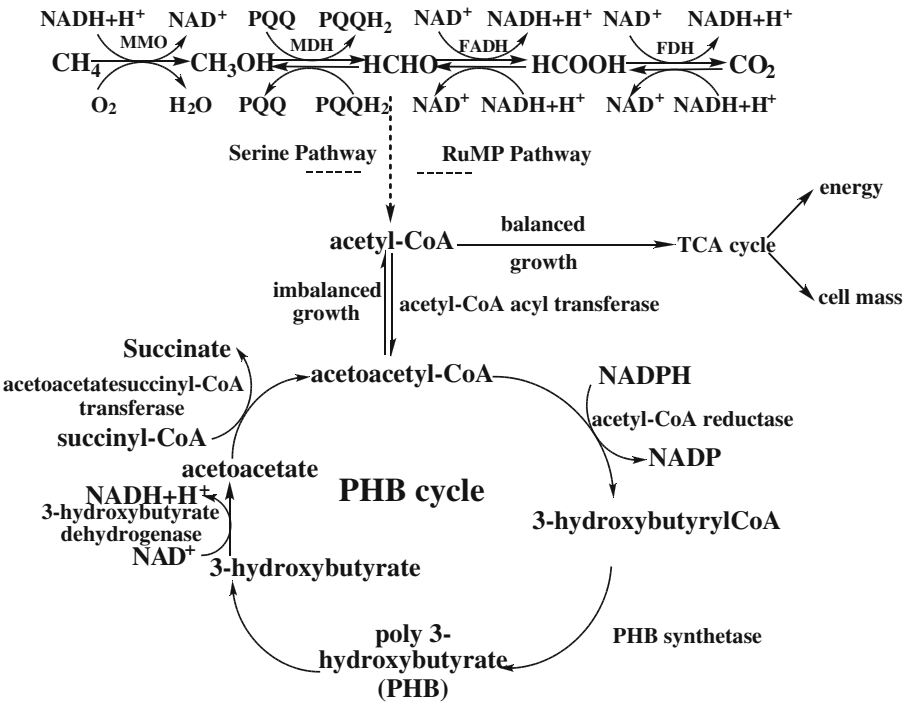

Fig. 1 Proposed metabolism pathway in methanotrophs. *FADH* formaldehyde dehydrogenase, *FDH* formate dehydrogenase, *MDH* methanol dehydrogenase, *MMO* methane monooxygenase, *balanced growth* cell cultivated in nutrients sufficient condition, *imbalanced growth* cell cultivated in nutrients deficiency condition

utilization of methane as a carbon source by methanotrophs can also, perhaps, be exploited for the cost-effective commercial production of PHB [17]. PHB is an internal reducing-energy storage polymer that can also be used as an alternative reducing-energy source by a number of methanotroph cultures under starvation conditions. The first step in the conversion of methane into PHB is carried out by nonspecific MMO enzyme systems. Resting cells of methanotrophs containing sMMO and pMMO both have a finite or intrinsic catalytic capacity for propene epoxidation due to a limiting supply of intracellular NADH reducing power [4, 18–21]. The limitation due to reducing equivalent availability can be offset by adding an external source of metabolic electron donors such as sodium formate or hydrogen gas. Moreover, it has been suggested that the catabolism of stored PHB can also provide intracellular reducing equivalent to improve the trichloroethylene (TCE) transformation capacity assayed involving a methanotrophic mixed culture [20].

The work presented in this paper is part of an ongoing effort aimed at finding the differences of sMMO and pMMO activities of some kinds of methanotrophs and another way to regenerate reducing equivalent NADH to maintain the sMMO and pMMO activities. We studied the methanotrophs of *M. trichosporium* OB3b, *M. trichosporium* IMV3011, *Methylococcus capsulatus* HD6T, and *Methylomonas* sp. GYJ3 and compared their sMMO and pMMO activities. We also tried to find whether the stored PHB in the cell can provide NADH and contribute to keep sMMO and pMMO activities of the resting cell.

Materials and Methods

Microorganism and Cell Cultivation

(a) *M. trichosporium* OB3b was kindly donated by Prof. Xing Xinhui of the Institute of Biochemical Engineering, Tsinghua University of China; *M. trichosporium* IMV 3011 was obtained from the Russia Institute of Microbiology and Virology (Kiev, Ukraine); *M. capsulatus* HD6T was isolated from a soil sample around a natural gas well of Daqing, Heilongjiang Province; *Methylomonas* sp. GYJ3 was isolated from soil samples from the oil fields of Yumen, Gansu Province and cultivated as described by Shen et al. [22].

(b) *The mixture of the four kinds of strains*: In this work, four pure culture strains of *M. trichosporium* OB3b, *M. trichosporium* IMV3011, *M. capsulatus* HD6T, and *Methylomonas* sp. GYJ3 were co-used as inocula.

(c) *Nutrients sufficient (nutrients balanced) cultivation*: The basal medium employed for routine *M. trichosporium* OB3b strain maintenance was Higgins' nitrate minimal salts (NMS) [23]. *M. trichosporium* OB3b was used as inoculum for cultivation in a modified Higgins' salts medium. And *M. trichosporium* IMV3011 was cultivated in the medium for *M. trichosporium* IMV3011 itself (in grams per liter): 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), ethylenediamine tetraacetate (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}), $FeSO_4{\cdot}7H_2O$ (4×10^{-3}), $FeCl_3$ (1×10^{-3}), H_3BO_3 (6.2×10^{-5}), $CoCl_2{\cdot}6H_2O$ (4.8×10^{-5}), KI (8.3×10^{-5}). *Methylomonas* sp. GYJ3 and *M. capsulatus* HD6T were cultivated in the same culture as *M. trichosporium* IMV3011. Cells adapted to the respective medium, either lacking Cu (sMMO production) or containing 10 μM Cu (pMMO production), were used as inocula for cultivations in a modified salts medium. Shake flask studies were conducted at 30 °C in sealed 250-mL flasks equipped with a sidearm tube. The cultivation of the cells was carried out in 100 mL of liquid medium under a CH_4/air gas mixture (1:1, *v*/*v*) for 84 h.

(d) *Two-stage cultivation*: All of the strains were cultivated in the nutrients sufficient culture for 156 h (the first stage) and then the harvested cells were transferred into the nutrients deficiency (nutrients imbalanced) culture (in grams per liter): KH_2PO_4 (0.2), K_2HPO_4 (0.4), Na_2HPO_4 (0.74), $CaCl_2$ (0.024), $MgSO_4{\cdot}7H_2O$ (0.1), NH_4Cl (0.1), NaCl (0.3), KNO_3 (0.2), 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}), $FeSO_4{\cdot}7H_2O$ (1.6×10^{-4}), $FeCl_3$ (4×10^{-4}), H_3BO_3 (6.2×10^{-5}), $CoCl_2{\cdot}6H_2O$ (4.8×10^{-5}), KI (8.3×10^{-5}), and cultivated for 120 h (the second stage).

Whole-Cell Enzyme Activity

MMO Activity

Bacteria were harvested by centrifugation at 9,000×*g* for 10 min at 4 °C. The cell pellets were washed twice with cold 20 mM phosphate buffer (pH 7.0). Cells were resuspended in the same buffer containing 5 mM $MgCl_2$ (at a cell concentration of 2–3 mg dry weight of cell [dwc]/mL) and used for the experiment [23].

The total MMO activity (sMMO and pMMO) of the intact cells was determined routinely by measuring the epoxidation rate of propene at pH 7.0 in sealed vials, without

providing any reducing equivalent. The assay was performed in 2 mL of cell suspension sealed in a 10-mL reaction vial. The gaseous phase of the vial was removed by vacuum and then replaced with a gas mixture propene–air (1:1, *v*/*v*). The reaction was started by addition of the gas mixture. Assays were performed at 35 °C for 6 h, epoxypropane formation from propene at different reaction times was measured by gas chromatography (GC; Agilent 6820 system, USA) equipped with a capillary GC column(Φ0.23 mm × 25 m; stationary phase, SE-54) and a flame ionization detector (FID). Pure nitrogen served as the carrier gas at a flow rate of 75 mL/min. The temperatures of the column, detector, and injector were 60, 180, and 180 °C, respectively. Quantification was performed using external standardization. Specific activity was expressed as nanomoles of epoxypropane formed per minute per milligram of dry weight of cell.

Chromatographic determinations of the liquid phase (epoxypropane) were carried out at different reaction times. Epoxypropane production was stopped after 6 h.

sMMO Activity (Naphthalene Oxidation Assay)

The activity of sMMO enzyme was evaluated by naphthalene assays modified from the method described by Brusseau et al. [24, 25]. The assay is based on the assumption that only sMMO can oxidize naphthalene to α- or β-naphthol in methane-oxidizing bacteria. The production of α- or β-naphthol is measured by reaction with tetrazotized *o*-dianisidine to form a purple naphthol diazo complex. The intensity of the naphthol diazo complex is measured as the absorption at 530 nm (*A*530). The assay was conducted by adding 3 mL of cell suspensions (20 mM phosphate buffer with cell concentration of 5–6 mg dwc/mL) along with 3 mL of naphthalene stock solution (0.0022 g/L at 25 °C).The mixture was incubated at 37 °C on a shaker at 150 rpm for 60 min before addition of 0.1 mL of freshly made 0.2% (*w*/*v*) tetrazotized *o*-dianisidine. The *A*530 of naphthol diazo dye was measured by a Hewlett Packard 8453 spectrophotometer within 2 min, since the absorbance starts to increase after 2 min. The concentrations of naphthol in aqueous solution are known to be proportional to the intensity of the naphthol diazo dye and were calculated by using the extinction coefficient of 38,000 M cm^{-1}. All samples were measured in duplicate. Reaction mixtures containing only cells (no naphthalene) were used as blank controls.

Poly-β-Hydroxybutyrate

Poly-β-Hydroxybutyrate Recovery After centrifugation or separation, 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 cell matter was removed by 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.

Poly-β-Hydroxybutyrate Analysis

(a) *PHB content*: PHB content was determined by gas chromatography [26]. 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 (conc.) and methanol), and 20 mg of benzoic acid and incubated at 100 °C for 4 h. After being 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 chromatography (Agilent 6820 system, USA, with a FID detector, a capillary column=0.23 mm×30 m, stationary phase, SE-54). Pure poly-3-hydroxybutyric acid was used.

(b) *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.

Results and Discussion

MMO activity of methanotrophs has been assayed under optimized the conditions of cultivated, such as a feed nitrate concentration, Higgin's "modified 1x medium" dilution rate of 0.064 h^{-1}, agitation speed, and gaseous substrate flow rates for methane and air/CO_2 (9:1) mixture [9]. From triplicate independent continuous culturing runs under these conditions, the cell density and whole-cell specific pMMO activity were 2.37±0.23 g/L and 206±15 nmol propene oxide formed/min mg dcw, respectively [9]. We studied the MMO activities of the four kinds of methanotrophs and the mixture of the four strains under the usual condition in batch cultivation.

Epoxidation Activity of MMO of Different Kinds of Methanotrophs

As shown in Fig. 2 and Table 1, the epoxidation activity of MMO is high in the initial 30 min of the reaction. The activity of MMO of the methanotrophs cannot keep for a long time without sufficient NADH supplied. As the result shows, the MMO activities of the strains are decreasing following the reaction time. Without enough reducing equivalents NADH or any exogenous substance supplied to regenerate NADH, the MMO activity of all strains drops to about 4 nmol/min·mg dwc and keeps at this value for some time, which occurs to all the four strains. Moreover, without an oxidizable agent to regenerate intracellular NADH levels, the MMO activities of all kinds of methanotrophs in the first 30 min of the reaction are at 10 nmol/min·mg dwc or so (Table 1). In contrast with the four pure strains, we found that the MMO activity of the mixture of the four strains (Mix) is higher than any of the pure strains. MMO activities vary in different pure strains, but when they are mixed, the MMO activity can be enhanced. It is speculated that symbiosis of these strains improves some properties and have more value in research.

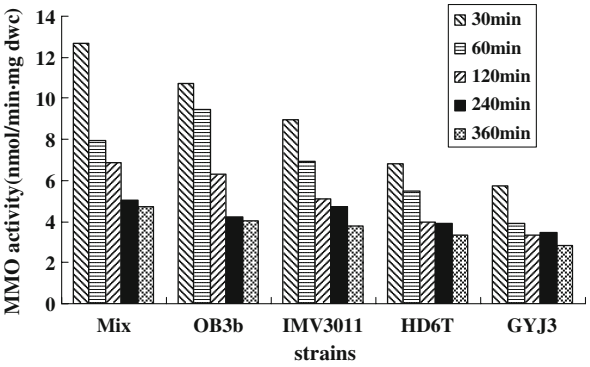

Fig. 2 Epoxidation activity of MMO at different reaction times of the four kinds of methanotrophs and the mixture of the four kinds of strains (*Mix*) cultivated for 84 h under nutrients sufficient condition

Table 1 Effect of Cu^{2+} on epoxidation activity of MMO at different reaction times of different kinds of methanotrophs cultivated for 84 h under nutrients sufficient condition.

MMO (nmol/min·mg dwc)	Mix[a] (Cu)[b]	Mix[c]	OB3b (Cu)[b]	OB3b[c]	IMV3011 (Cu)[b]	IMV 3011[c]	HD6T (Cu)[b]	HD6T[c]	GYJ3 (Cu)[b]	GYJ3[c]
30 min	12.7	6.49	10.7	9.09	8.98	8.11	6.84	7.71	5.73	6.09
60 min	7.96	6.29	9.45	9.86	6.91	6.54	5.48	5.75	3.93	3.61
120 min	6.89	5.80	6.32	6.01	5.11	4.34	3.99	5.04	3.34	2.76

[a] The mixture of the four kinds of strains

[b] Culture with Cu^{2+}

[c] Culture without Cu^{2+}

As mentioned earlier, there are two distinct types of MMO: a soluble, cytoplasmic enzyme complex (sMMO) and a membrane-bound, particulate enzyme system (pMMO). It has been found that, at low copper concentration/biomass ratios, the predominant form of the enzyme occurs in the soluble fraction of cell extracts (sMMO) and that, at high copper/biomass ratios, the predominant form of the enzyme occurs in the membrane-bound fraction (pMMO). Shah et al. have reported batch cultures of methane-grown *M. trichosporium* OB3b that biosynthesize exclusively pMMO have an approximately 1.5–2.2 higher whole-cell activity with propene as a substrate in comparison to cells that produce exclusively sMMO [9]. During the batch cultivation of *M. capsulatus* (Bath) in copper-containing medium, the MMO activity switches gradually from the particulate to the soluble form with increasing biomass. Furthermore, the batch culture cell density at which sMMO activity first appears is dependent qualitatively on the initial copper concentration.

From the results, it is found that the propene epoxidation abilities of pMMO (with Cu) of most strains are better than that of sMMO (without Cu) except *M. capsulatus* HD6T. To the strains except *M. capsulatus* HD6T, the expression of pMMO enzyme is more appropriate for epoxypropane synthesis. And in the second stage of the cultivation, the propene epoxidation activity of MMO drops down probably because the cells cannot maintain the normal metabolism under nutrients deficiency condition.

Without sufficient substance supplied, the cell cannot grow in a normal way but survive in another way. Hence, the activity of enzymes in normal metabolism has been decreased including MMO. Whereas NADH may serve directly as reducing power, storage polymers such as poly-β-hydroxybutyrate (PHB) may serve as an endogenous source of reductant in microorganisms. PHB can be accumulated as an intracellular carbon and energy storage material by methanotrophs under nitrogen-, phosphate-, and other nutrients-limiting conditions. As shown in Table 2, the epoxidation activities of MMO of these strains under nutrients deficiency condition do not decrease so dramatically as those in nutrients sufficient condition after reacting for 120 min. This may be attributed to the PHB accumulated in the second stage under nutrients deficiency conditions that maintain the activity of MMO.

Naphthalene Oxidation Activity of sMMO (without Cu) of Different Kinds of Methanotrophs

sMMO can oxidize naphthalene while pMMO cannot. Then sMMO activity in methanotrophs can be detected by naphthalene oxidation. From Fig. 3, it can be found that the naphthalene oxidation activity of sMMO (without Cu) of *M. trichosporium* OB3b in the nutrients balanced culture is the highest. But when the strains were cultivated in

Table 2 Epoxidation activity of MMO at different reaction times of different kinds of methanotrophs in two-stage cultivation.

MMO (nmol/min·mg dwc)	Mix[a] (Cu)[b]	Mix[c]	OB3b (Cu)[b]	OB3b[c]	IMV3011 (Cu)[b]	IMV 3011[c]	HD6T (Cu)[b]	HD6T[c]	GYJ3 (Cu)[b]	GYJ3[c]
30 min	6.43	5.17	5.71	3.98	6.88	4.99	6.63	4.94	5.10	4.09
60 min	6.22	5.25	5.90	3.12	7.09	4.96	5.77	4.64	4.86	4.18
120 min	6.15	4.72	5.94	3.94	7.03	5.23	6.73	4.97	4.32	3.82

Two-stage cultivation: 156 h under nutrients sufficient condition and 120 h under nutrients sufficient condition

[a] The mixture of the four kinds of strains

[b] Culture with Cu^{2+}

[c] Culture without Cu^{2+}

nutrients deficiency culture, the activities of their sMMO decrease except *M. capsulatus* HD6T. It is suggested that the sMMO activity is also much influenced by the nutrients condition. When the nutrients for the growth of cell are sufficient, the cell grows well with normal metabolism and good activities of different kinds of intracellular enzymes; while in nutrients deficiency condition, the cell cannot sustain the normal metabolism, and then the intracellular enzymes' activities are influenced and keep at low level.

Epoxidation Activity of MMO (with Cu and without Cu) and Intracellular PHB Contents of Different Kinds of Methanotrophs

In the PHB cycle pathways shown in Fig. 1, the series of reactions involving the conversion of acetyl CoA to PHB and its depolymerization and oxidation back to acetoacetyl CoA are confirmed by Korotkova [27]. The degradation of PHB in most bacteria is catalyzed by PHB depolymerase, β-hydroxybutyrate dehydrogenase, acetoacetate-succinate-CoA transferase, and β-ketothiolase. Degradation of PHB to acetoacetic acid would provide reducing equivalent via the action of the NAD^+-linked β-hydroxybutyrate dehydrogenase [28].

The stored PHB in cells has been suggested to provide intracellular reducing equivalent to improve the TCE transformation capacity. The sMMO TCE degradation activity decreased to a negligible value following most of the PHB which was stored at a low level that disappeared without formate, while 30% to 40% of their TCE degradation activity was

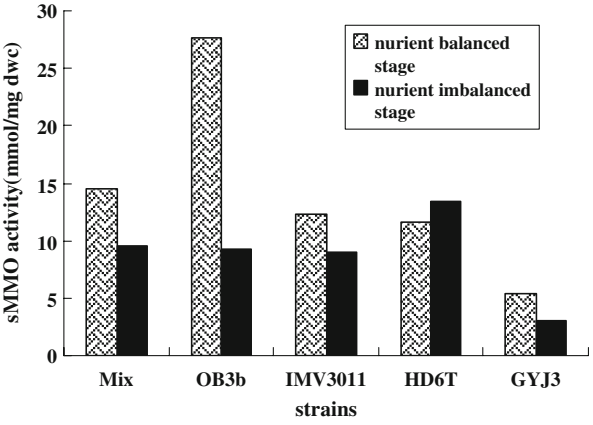

Fig. 3 sMMO activity of the four kinds of methanotrophs and the mixture of the four kinds of strains (*Mix*) cultivated for 84 h under nutrients sufficient condition and cultivated in two stages for 276 h

retained when the cells contained more PHB without formate after the same time [4]. In all cases, no free β-hydroxybutyrate was detected, indicating that the PHB lost upon storage was further metabolized by the cells. It has been suggested that intracellular reducing equivalent can also be provided by the catabolism of stored PHB to improve the TCE transformation capacity, but the data presented involved a methanotrophic mixed culture. A number of studies observed a correlation between TCE oxidation capacities and microbial PHB content [19, 20, 28, 29], suggesting that PHB might be used as an alternative NADH source for TCE oxidation by methanotrophs. Moreover, it can be suggested that another intrinsic catalytic capacity of MMO, including sMMO and pMMO, for propene epoxidation without adding any external source of metabolic electron donors is also influenced by catabolism of stored PHB.

Table 3 summarizes the PHB contents and the corresponding MMO activity of the cell samples. The MMO activities of different kinds of cells were assayed with the cell cultivated for 84 h when the activities reach the best level. From these results, it is found that the accumulating rules of the PHB are different from one strain to another. For example, the PHB content of *M. trichosporium* OB3b achieves a high yield when cultivated for 84 h. But when it comes to *M. trichosporium* IMV3011, it takes 156 h to get to the highest value. At this time, the PHB content of *M. trichosporium* OB3b has dropped down. So it is clear that the synthesis abilities of PHB are different with different methanotroph strains. To the Mix, *M. trichosporium* IMV3011 and *Methylomonas* sp. GYJ3, pMMO (with Cu) and sMMO (without Cu) have no obvious influence on PHB accumulation. To the other two, high MMO activity may be helpful to the PHB accumulation especially in normal growth condition. It can be concluded the PHB contents in cell between 1% and 5% are probably beneficial to the cells for maintaining the epoxidation ability of MMO of

Table 3 Epoxidation activity of MMO of different kinds of methanotrophs and PHB contents of different kinds of methanotrophs cultivated for different times in different conditions.

	Mix[a] (Cu)[b]	Mix[c]	OB3b (Cu)[b]	OB3b[c]	IMV3011 (Cu)[b]	IMV 3011[c]	HD6T (Cu)[b]	HD6T[c]	GYJ3 (Cu)[b]	GYJ3[c]
MMO (nmol/min·mg dwc)[d]	12.7	6.49	10.7	9.09	8.98	8.11	6.84	7.71	5.73	6.09
PHB (%) of nutrients sufficient cultivation (84 h)[e]	1.92	4.88	11.0	5.82	3.52	2.86	5.19	3.9	1.33	1.08
PHB (%) of nutrients sufficient cultivation (156 h)[f]	0.61	0.65	3.74	5.97	4.35	8.65	4.22	2.55	0.87	0.94
PHB (%) of nutrients deficiency cultivation (120 h)[g]	8.07	4.22	1.50	1.89	10.7	1.89	5.33	0.95	1.21	1.62

[a] The mixture of the four kinds of strains

[b] Culture with Cu^{2+}

[c] Culture without Cu^{2+}

[d] The activities of MMO of different strains were assayed with the cell cultivated under nutrients sufficient condition for 84 h

The cell of different kinds of strains were cultivated in different cultures for different times:

[e] Cultivated under nutrients sufficient condition for 84 h

[f] Cultivated under nutrients sufficient condition for 156 h

[g] Cultivated under nutrients deficiency condition for 120 h

methanotrophs. But PHB content up to 10% has no measurable effect on the propene epoxidation capacities and may even decrease the MMO activity because of the dilution of the MMO system in the cells with increasing PHB contents. Tables 2 and 3 suggest that the more PHB are accumulated, the slower MMO activity decreases. Then, it can be concluded that the cellular PHB content has some influence on the maintenance of the MMO activity. Furthermore, the higher activity of pMMO (with Cu) of the cell probably promoted the accumulation of PHB in *M. trichosporium* OB3b, *M. trichosporium* IMV3011, and *M. capsulatus* HD6T. And it is indicated that copper probably influences the accumulation of PHB in the cell. Accordingly, PHB may be beneficial to the cells for maintaining MMO epoxidation activity when enough PHB have been accumulated, and high pMMO activity may promote the synthesis of PHB in the cell. This may be an effect factor to PHB accumulation which is helpful to the study on obtaining high yield of PHB in methanotrophs.

In addition, it also shows that the two-stage cultivation appropriate for the PHB accumulation of *M. trichosporium* IMV3011 may not be beneficial to the other strains (Table 3). After nutrients sufficient stage cultivation for 84 h, the PHB yield of 10% of *M. trichosporium* OB3b has been obtained. But when it comes to *M. trichosporium* IMV3011, it needs two-stage cultivation to reach this yield. It is indicated that the time for *M. trichosporium* OB3b to accumulate PHB is shorter than *M. trichosporium* IMV3011, and it is possible to find another appropriate condition for *M. trichosporium* OB3b to accumulate PHB more efficiently.

Conclusion

In this paper, we studied the MMO activity of four kinds of methanotrophs and their mixture without additional supply of reducing equivalent under the usual condition. Our major findings are summarized below:

1. The MMO activity and the sMMO activity assayed in this study varied with different kinds of methanotrophic bacteria. The activity of propene epoxidation of MMO and the activity of naphthalene oxidation of sMMO of *M. trichosporium* OB3b are the best of the four. Furthermore, the PHB accumulated capacities are different from each other. The PHB content of *M. trichosporium* OB3b could reach the highest level in the shortest time. Nutrients deficiency condition beneficial for *M. trichosporium* IMV3011 to synthesize PHB is not beneficial for the other strains.
2. The epoxidation activities of MMO and sMMO of the four kinds of methanotrophs in the first 30 min of the reaction are all at 10 nmol/min·mg dwc or so. And the activity of the Mix can reach the higher level. It could be feasible to improve the MMO epoxidation activity by mixing some kinds of methanotrophs. But their activities will drop dramatically with the reaction going on.
3. Although the epoxidation activities of MMO and sMMO of these strains cultivated with second stage under nutrients deficiency condition are lower than those cultivated under nutrients sufficient condition, the PHB accumulated in the second stage will probably help to prevent the activity from decreasing rapidly.
4. PHB is beneficial to the cells for the maintenance of MMO epoxidation activity when enough PHB have been accumulated, while high pMMO activity may contribute to the synthesis of PHB in the cell, which is probably beneficial for the production of PHB in methanotrophs.

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