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Coupled effects of methane monooxygenase and nitrogen source on growth and poly-β-hydroxybutyrate (PHB) production of *Methylosinus trichosporium* OB3b

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ABSTRACT

The coupled effects of nitrogen source and methane monooxygenase (MMO) on the growth and poly-β-hydroxybutyrate (PHB) accumulation capacity of methanotrophs were explored. The ammonia-supplied methanotrophs expressing soluble MMO (sMMO) grew at the highest rate, while N_2-fixing bacteria expressing particulate MMO (pMMO) grew at the lowest rate. Further study showed that more hydroxylamine and nitrite was formed by ammonia-supplied bacteria containing pMMO, which might cause their slightly lower growth rate. The highest PHB content (51.0%) was obtained under nitrogen-limiting conditions with the inoculation of nitrate-supplied bacteria containing pMMO. Ammonia-supplied bacteria also accumulated a higher content of PHB (45.2%) with the expression of pMMO, while N_2-fixing bacteria containing pMMO only showed low PHB production capacity (32.1%). The maximal PHB contents of bacteria expressing sMMO were low, with no significant change under different nitrogen source conditions. The low MMO activity, low cell growth rate and low PHB production capacity of methanotrophs continuously cultivated with N_2 with the expression of pMMO were greatly improved in the cyclic $NO_3^-N_2$ cultivation regime, indicating that long-term deficiency of nitrogen sources was detrimental to the activity of methanotrophs expressing pMMO.

Introduction

Methane (CH_4) is an important greenhouse gas with approximately 20 times the impact of carbon dioxide (Rostkowski et al., 2012). It accounts for up to 23% of the total worldwide greenhouse emissions, and the percentage of CH_4 in the atmosphere has been growing rapidly in recent years (Wahlen, 1993). The amount of anthropogenic CH_4 emissions accounts for 63% of the global methane emissions (Kirschke et al., 2013). CH_4 is abundantly discharged during petroleum and natural gas extraction, oil refining and the biological degradation of organic matter in landfills and wastewater treatment facilities (Rostkowski et al., 2012; Zúñiga et al., 2011). Although CH_4 is usually used as a fuel, waste CH_4 from these plants is often flared or simply released to the atmosphere due to the elevated storage and transport costs of CH_4 (Khosravi-Darani et al., 2013). The environmental concerns aroused by the increasing CH_4 emissions have drawn more and more attention from scientists. Biological methods possess a great competitive advantage in the treatment of CH_4 emissions.

CH_4 can be used as sole carbon source and energy source by a subset of the methylotrophs known as methanotrophs.

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Methanotrophs are composed of two groups: type I (γ-proteobacteria) and type II (α-proteobacteria). Under nutrient-limiting conditions, such as nitrogen deficiency, CH_4 could be assimilated and stored within the cell in the form of poly-β-hydroxybutyrate (PHB) by type II bacteria via the serine pathway (Pieja et al., 2011a). PHB is a potential substitute for traditional plastics, because it has a series of virtues such as biodegradability, biocompatibility and mechanical properties similar to polypropylene (Rostkowski et al., 2012). It has been estimated that PHB production from biogas discharged by existing landfills and anaerobic digesters could theoretically replace 20%–30% of the total annual plastics market (DiGregorio, 2009).

The production of PHB is generally conducted in two stages: a continuous growth phase and a PHB accumulation phase under growth-limited conditions. The conditions under which bacteria are cultivated are likely to affect bacterial performance in the subsequent PHB production phase. It has been reported that different sources of nitrogen (NH_4^+–N, NO_3^-N and N_2–N) can be utilized by type II bacteria for cellular growth (Chu and Alvarez-Cohen, 1998). Generally, nitrate or ammonia that is added in media as an N source to culture bacteria. Ammonia can perform both as a nutrient for the growth of methanotrophs and as a competitive inhibitor of methane monooxygenase (MMO), due to the homology of MMO, via which methane is oxidized to methanol, and ammonia monooxygenase (AMO), which initiates the oxidation of ammonia in the ammonia-oxidizing bacteria. In terms of N_2-fixation, it is well known that nitrogenase activity is sensitive to oxygen. The oxygen partial pressure at which nitrogenase can be completely inactivated is varied for each species (Rostkowski et al., 2013). It was reported by some researchers that the nitrogenase of *Methylosinus trichosporium* OB3b (a typical II methanotroph) was not completely inactivated until the oxygen partial pressure increased to more than 0.25 bar (Murrell and Dalton, 1983).

There are two distinct forms of MMO enzymes: particulate MMO (pMMO), located in the cytoplasmic membrane, and soluble MMO (sMMO), within the cytoplasm. pMMO is found in almost all known methanotrophs, while sMMO is suggested to occur only in most type II methanotrophs, such as all known *Methylosinus* strains, and several type I methanotrophs (Pieja et al., 2011a). In methanotrophs capable of expressing both forms of MMO, sMMO is only expressed under copper starvation conditions, while pMMO is activated only when copper concentrations exceed 0.85 to 1 μmol/g (dry weight) of cells (Hanson and Hanson, 1996).

This study intends to clarify how the cellular growth and PHB production ability of *M. trichosporium* OB3b are affected by the cultivation conditions with different nitrogen sources in the presence of sMMO or pMMO. In addition, it has been demonstrated that in the absence of copper, the use of nitrogen gas as the sole nitrogen source could enrich a methanotrophic community capable of producing 44% PHB (Pieja et al., 2011a). So, an understanding of the coupled effects of MMO and nitrogen source is also beneficial to the further improvement of the PHB accumulation capacity of mixed methanotrophic bacteria. PHB production with the simultaneous deficiency of nitrogen and phosphate was also studied, as the PHB production of methanotrophs is usually conducted under nitrogen-limiting conditions. In the end, an alternate nitrate and N_2 cultivation strategy was studied to explore whether the low activity of bacteria containing pMMO under continuous N_2-fixing conditions was induced by long-term nitrogen source deficiency.

1. Material and methods

1.1. Chemicals, bacterial strain and media

M. trichosporium OB3b was generously donated by M. Kalyuzhnaya (Lidstrom laboratory, University of Washington) and used for all experiments in this study. *M. trichosporium* OB3b were grown by using one of the following three growth media: NMS (nitrate mineral salt) (Xing et al., 2006), AMS (ammonia mineral salt), and NFMS (nitrogen-free mineral salts) media. 11.76 mmol/L $NaNO_3$ was added to NMS medium and 5.88 mmol/L $(NH_4)_2SO_4$ was added to AMS medium, while no nitrogen source was added to NFMS medium. 5 μmol/L or no Cu was added to the three media (NMS + 5 μmol/L Cu, NMS + 0 μmol/L Cu *etc.*) to determine the coupled effects of MMO and nitrogen source. The compositions of the three media were otherwise identical. The initial pH of the three media was adjusted to 6.8 with 1 mol/L sodium hydroxide. The growth of methanotrophs was conducted in 300 mL serum bottles. 100 mL of one growth medium was introduced into each bottle, and 5 mL of culture acclimated to the same medium was used as inoculum. For the serum bottles amended with NMS and AMS, cultures were grown under a 50% CH_4–50% O_2 atmosphere (*V*/*V*). The headspace gas of those bottles was updated every twenty-four hours by being subjected twice to vacuum and replenished with the same gas mixture (CH_4/O_2 1:1, *V*/*V*) to maintain sufficient oxygen. The headspace gas of serum bottles fed with NFMS medium consisted of 50% CH_4, 10% O_2 and 40% N_2 (*V*/*V*) and the headspace gas was replenished when the oxygen concentration was below 5% with the same method as described above. All of the serum bottles were capped with butyl rubber stoppers and screw tops. The serum bottles were then inoculated at 30°C on orbital shakers at 150 r/min. All cultivations were performed in a sterile manner.

Abiotic controls were adopted as negative control to confirm gas losses caused by leakage, which were less than 5%. Cell growth was monitored by measuring optical density at 660 nm (OD660, V-560, Jasco International Co., Ltd., Japan), which was correlated with dry cell weight (DCW) measured after drying at 105°C overnight. The variations of the headspace gas compositions were also determined to ensure sufficient oxygen.

1.2. PHB production

Experiments to compare the PHB production capacity of methanotrophs grown with three different N sources and two different MMO were performed in 125 mL sealed serum bottles. For cultures grown without Cu, NFMS medium, either lacking Cu (NFMS + 0 μmol/L Cu) or containing 5 μmol/L Cu (NFMS + 5 μmol/L Cu), was employed to induce PHB accumulation by the methanotrophs. PHB production of methanotrophs grown with 5 μmol/L Cu was conducted in NFMS + 5 μM Cu medium. In addition, NFMS medium without phosphate (NFMS + 0 μmol/L P) was also utilized to confirm whether the simultaneous deficiency of nitrogen and phosphorus sources could further promote the cellular PHB content of methanotrophs.

When the culture reached the late log-phase, cell suspensions were harvested by centrifugation (10,000 r/min, 5 min) and washed twice with NFMS medium. The resulting cell pellets were resuspended by the same NFMS medium with the same volume as the original cell suspension. Afterwards, the optical density was measured. Into each serum bottle 15 mL of cell resuspension solution was introduced, along with 50% CH_4 and 50% O_2 in the headspace. The headspace gases of all serum bottles were updated every twelve hours as described above. All serum bottles were incubated at 30°C on orbital shakers at 150 r/min.

Cell-free flasks were used as blank controls in each experiment. The gas compositions of each serum bottles headspace were monitored periodically. All of the PHB production experiments were conducted under sterile conditions. Duplicate serum bottles were sacrificed periodically every 72 hr. Cell suspensions were harvested by centrifugation (4700 r/min, 8 min) at 4°C and washed twice with deionized water. The resulting cell pellets were saved at −80°C for at least 8 hr and then lyophilized for 24 hr. Freeze-dried methanotrophic bacteria were conserved at −20°C in an airtight desiccator until the analysis of PHB.

1.3. Alternating nitrate and nitrogen gas as nitrogen source for the growth of methanotrophs

M. trichosporium OB3b was alternately cultivated with NMS medium and NFMS medium. Both NMS and NFMS medium were fed along with 5 μmol/L Cu. After being cultivated in NMS medium, 5 mL of the cell suspension was subjected to centrifugation and washed once with NFMS medium. Then, the resulting cell pellets was transferred to 300 mL serum bottles with 100 mL NFMS medium. When the cultures grown in NFMS medium reached late log-phase, 5 mL of cell suspension was inoculated in a 300 mL serum bottle containing 100 mL NMS medium. The culture was grown under the same conditions described above. After the culture was alternately cultivated with NFMS and NMS medium for five cycles, the characteristics of *M. trichosporium* OB3b were determined. The PHB production of the culture was evaluated in NFMS + 5 μmol/L Cu medium or NFMS + 0 μmol/L P medium under the same conditions mentioned above. Abiotic controls were adopted. The samples were taken and stored using the same method noted above.

1.4. Analytical methods

The gas compositions were analyzed by a gas chromatograph (GC7900, Techcomp Limited, China) equipped with a thermal conductivity detector, a Porapak Q and a Molecular Sieve column. Helium was used as the carrier gas and the standard curves for each gas were defined by injecting known amounts of pure gases into the equipment.

PHB content was determined by a gas chromatograph (GC7890II, Techcomp Limited, China) equipped with a flame ionization detector (FID) and a SE-54 column (length 30 m, internal diameter 0.32 mm, film thickness 0.25 mm). 5 to 10 mg of freeze-dried cell pellet was precisely weighed into a 10 mL screw-cap vial (HACH Products, USA). A method described by Chen et al. (2011) with slight modification was employed for the analysis of PHB. 1 μL of the heavier phase was injected into the gas chromatograph. Nitrogen was used as the carrier gas. The standard curve was set up by using a PHB standard (Sigma-Aldrich Chemical Co., USA).

The total MMO activity of whole cells was evaluated by a propylene-oxidation assay modified from the method described by Xing et al. (2006). 10 mL exponential cell suspension was harvested by centrifugation (10,000 r/min, 5 min) at 4°C and washed twice with 20 mmol/L cold phosphate buffer, followed by resuspension in the same buffer containing 5 mmol/L $MgCl_2$ and 20 mmol/L sodium formate. The assay was conducted with 10 mL cell suspension sealed in a 70 mL serum bottle and initiated by replacing 10 mL air with 10 mL propylene. The serum bottles were incubated at 30°C on a shaker at 150 r/min for 60 min. 1 mL cell suspension was centrifugated at 4°C to obtain the supernatant for future determination. Epoxypropane in the supernatant was measured by a gas chromatograph (GC7890II, Techcomp Limited, China) equipped with a FID detector and a SE-54 column (length 30 m, internal diameter 0.32 mm, film thickness 0.25 m). Nitrogen was used as the carrier gas. An external standard was employed for the quantification of epoxypropane. All samples were measured in duplicate. The enzyme activity was expressed as nmol of product formed per minute per milligram of dry cell mass. Moreover, to exclude the expression of sMMO in the presence of Cu with different nitrogen sources, a naphthalene assay was also employed, as naphthalene can only be oxidized by sMMO to 1- or 2-naphthol (Lontoh and Semrau, 1998).

The activity of nitrogenase was assayed by a modification of the acetylene reduction technique described by Murrell and Dalton (1983). 2 mL cell suspension was transferred to a 120 mL serum bottle, which was capped with a butyl rubber stopper and screw top. The headspaces of all bottles were subjected twice to vacuum and replenished with filtered argon gas before introduction of 2% O_2 and 1% acetylene gas. Then, 2% methanol was added as a readily available reducing substrate. The serum bottles were incubated at 30°C on a shaker at 150 r/min for 60 min. Duplicate serum bottles were prepared for each sample. Measurement of ethylene in the headspace was carried out by a gas chromatograph (GC7890II, Techcomp Limited, China) equipped with a FID detector and a Porapak N packed column (length 20 m, diameter 3 mm).

The nitrite was determined according to the N-(1-naphthalene)–diaminoethane photometry method. Hydroxylamine was analyzed by a spectrophotometric method as described previously (Frear and Burrell, 1955).

2. Results and discussion

2.1. Cell growth and other characteristics of *M. trichosporium* OB3b grown by different nitrogen source and MMO

As illustrated in Fig. 1, bacteria showed the lowest growth rate under N_2-fixing conditions and grew at the highest rate with the addition of NH_4^+, regardless of the presence or absence of Cu. The effects of MMO on the bacteria growth varied under different nitrogen source conditions. Under ammonia-supplied and nitrogen-fixing conditions, bacteria in media lacking Cu grew slightly faster than in media containing

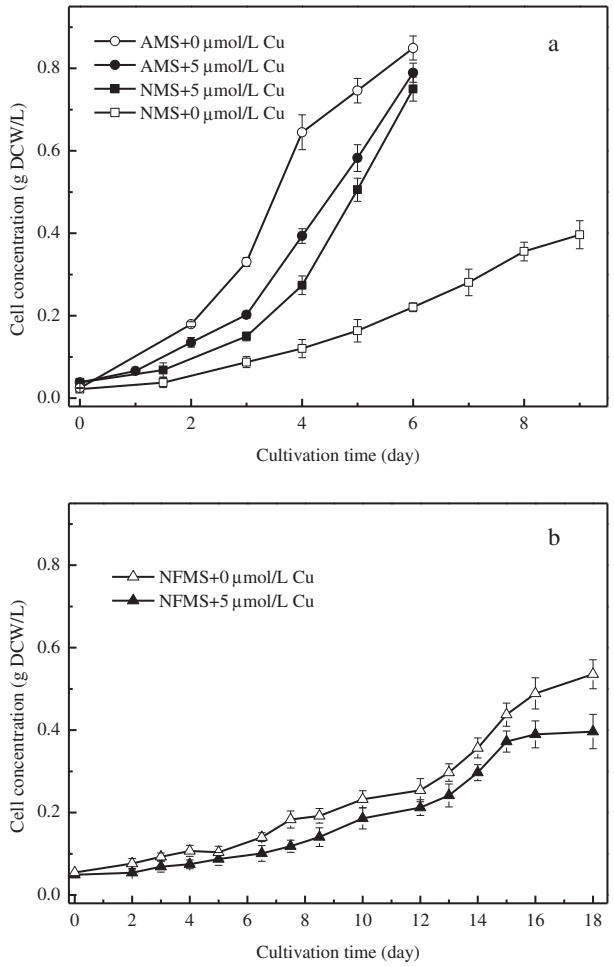

Fig. 1 – Growth curves of *M. trichosporium* OB3b grown with NH_4^+, NO_3^- (a) and N_2 (b) with 5 μmol/L Cu or 0 μmol/L Cu. AMS: ammonia mineral salt, NMS: nitrate mineral salt, NFMS: nitrogen-free mineral salts, DCW: dry cell weight.

5 μmol/L Cu. In contrast, with the supplement of nitrate, bacteria cultivated with 5 μmol/L Cu grew at an obviously higher growth rate. Other characteristics of each culture were also evaluated during the logarithmic phase (Table 1). Nitrogenase activity could be detected only under N_2-fixing conditions, which was consistent with the phenomenon observed by Murrell and Dalton (1983). Bacteria grown with different nitrogen sources exhibited distinct patterns with respect to net cell yield. Nitrate-supplied methanotrophs expressing pMMO showed 24.5% higher net cell yield than bacteria containing sMMO, while ammonia-supplied bacteria exhibited a contrary phenomenon. The net cell yields of both N_2-fixing bacteria were at low level (0.47–0.48 DCW/mg CH_4).

The consumption of methane is initiated by oxidizing methane to methanol via the catalysis of MMO. pMMO has been reported to have greater catalytic activity for methane than sMMO (Shah et al., 1996). In this test, the MMO activity of bacteria grown in NMS + 5 μmol/L Cu medium was 1.45 times that of bacteria in NMS + 0 μmol/L Cu medium, resulting in a higher bacterial growth rate in the medium containing Cu. A similar phenomenon was also observed in previous research (Park et al., 1992). In addition, a reducing equivalent is required in the methane oxidization process. It has been suggested that NAD(P)H can perform as an electron donor for both forms of MMO. However, pMMO can also obtain electrons directly from one or more electron transport proteins, a higher-potential electron donor (Bédard and Knowles, 1989; Hanson and Hanson, 1996; Strong et al., 2013), which might provide an explanation for why nitrate-supplied methanotrophs expressing pMMO showed a higher net cell yield than bacteria grown with sMMO.

The fact that the highest growth rate was sustained by ammonia demonstrated that ammonia played the role of nutrient instead of inhibitor for methanotrophic bacteria grown with sufficient carbon source. Moreover, both ammonia-supplied bacteria showed high MMO activity (74.03–78.84 nmol/mg DCW/min). Numerous similarities are shared by MMO and AMO. Nevertheless, it has been demonstrated that the gene encoding pMMO shows higher identity to the sequence of the AMO gene (Holmes et al., 1995). Therefore, in all likelihood, more ammonia would be fortuitously oxidized by pMMO with the consumption of reducing equivalent, followed by the generation of more hydroxylamine and nitrite. Indeed, the concentrations of hydroxylamine and nitrite detected in AMS medium containing Cu were about 2.3 and 1.5 times that in

Table 1 – Comparison of cell characteristics of *M. trichosporium* OB3b grown with different nitrogen sources and different methane monooxygenase (MMO).

Cultivation conditions	Net cell yield (mg DCW/mg CH_4)	Nitrogenase activity	CH_4/O_2 consumption ratio	MMO activity (nmol/mg DCW/min)	
				sMMO	pMMO
AMS					
5 μmol/L Cu	0.48 ± 0.02	–	1.47 ± 0.02	0	74.03 ± 2.17
0 μmol/L Cu	0.64 ± 0.01	–	1.31 ± 0.01	78.84 ± 1.56	0
NMS					
5 μmol/L Cu	0.61 ± 0.01	–	1.48 ± 0.02	0	81.57 ± 3.72
0 μmol/L Cu	0.49 ± 0.02	–	1.46 ± 0.03	33.32 ± 1.71	0
NFMS					
5 μmol/L Cu	0.48 ± 0.01	+	1.46 ± 0.01	0	30.87 ± 1.64
0 μmol/L Cu	0.47 ± 0.01	+	1.51 ± 0.02	34.72 ± 1.93	0

sMMO: soluble methane monooxygenase, pMMO: particulate methane monooxygenase.
+: the activity of nitrogenase was detected, –: the activity of nitrogenase was not detected.

medium lacking Cu (Table 2). It is well known that hydroxylamine and nitrite are highly toxic intermediates for the growth and activity of bacteria. As a result, a faint inhibition was observed in the growth of ammonia-supplied bacteria containing pMMO when compared with bacteria expressing sMMO. To resist the toxic intermediates of ammonia nitrogen metabolism, two metabolic pathways have been discovered in methanotrophs: in one pathway, as in ammonia-oxidizers, the formed hydroxylamine is first oxidized to nitrite by methanotrophs, and then the generated nitrite is reduced to nitric oxide, followed by the reduction of nitric oxide to nitrous oxide. However, unlike ammonia-oxidizers, electrons from hydroxylamine oxidation cannot support the growth of methanotrophs. In the other pathway, the formed hydroxylamine is directly reduced back to ammonia, a totally distinct detoxification mechanism (Stein and Klotz, 2011). A gene cluster encoding the enzyme participating in the second detoxification pathway was identified in *M. trichosporium* OB3b, while genes encoding proteins related to the first detoxification strategy have not yet been conclusively identified (Stein et al., 2010). However, the generation of N_2O was actually observed in this test (Table 2) and also has been detected in previous research (Lee et al., 2009). No matter which detoxification mechanism was employed to relieve the toxicity of hydroxylamine and nitrite, the capacity of *M. trichosporium* OB3b to assimilate ammonia would be facilitated, but at the cost of growing reducing equivalent demand. So the slightly lower net cell yield in AMS + 5 μmol/L Cu medium might be attributed to reducing equivalents consumed by the detoxification of hydroxylamine and nitrite.

The low growth rate of N_2-fixing bacteria indicated that only a limited nitrogen source could be provided by the N_2 fixation process, which was confirmed by the phenomenon observed previously that the depletion of nitrate led to a brief increase in PHB accumulation regardless of the presence of N_2-fixation (Shah et al., 1996). On the other hand, when compared with the assimilation of nitrate and ammonia, significantly more energy and reducing equivalents would be required in the process of N_2 fixation (Bodelier and Laanbroek, 2004; Orme-Johnson, 1985). Hence, it was not surprising that the net cell yields of N_2-fixing bacteria were low. Under N_2-fixing conditions, both methanotrophic bacteria showed weak MMO activity; but the MMO activity of N_2-fixing methanotrophs containing sMMO was 12.5% higher than that of bacteria expressing pMMO, which might then cause slightly faster growth in medium lacking Cu. The addition of nitrate or ammonia as a nitrogen source has been suggested to be essential for the expression of pMMO (Burrows et al., 1984). It has been observed that the depletion of nitrate was followed by an abrupt decrease in pMMO activity (Park et al., 1992). Therefore, the decrease of pMMO activity in NFMS medium might be attributed to the long-term deficiency of nitrogen source. Calculations have shown that in theory, the molar ratio of methane and oxygen consumption is equal to 1:1.5 (Asenjo and Suk, 1986), which was also confirmed in this study, as the ratio of O_2/CH_4 metabolized was around 1.5.

Table 2 – The concentrations of NH_2OH, NO_2^- and N_2O formed in the process of ammonia assimilation by methanotrophs.

Cultivation conditions	NH_2OH (mmol/L)	NO_2^- (mmol/L)	N_2O
AMS + 5 μmol/L Cu	0.076 ± 0.002	0.093 ± 0.003	+
AMS + 0 μmol/L Cu	0.033 ± 0.001	0.062 ± 0.002	+

+, N_2O was detected in the headspace gas.

2.2. PHB accumulation of *M. trichosporium* OB3b grown with different nitrogen sources and MMO

M. trichosporium OB3b grown with different nitrogen sources and MMO exhibited distinct PHB accumulation patterns (Fig. 2). For ammonia-supplied cultures (Fig. 2a), the highest maximal PHB content (45.2%) was obtained in NFMS + 5 μmol/L Cu medium with the inoculation of bacteria containing pMMO, which was slightly decreased to 42.2% in NFMS + 0 μmol/L P medium with the same inoculation and further reduced with the inoculation of bacteria expressing sMMO (26.6%–35.2%). In terms of bacteria cultivated with nitrate (Fig. 2b), the highest maximal PHB content (51%) was also obtained in NFMS + 5 μmol/L Cu medium with methanotrophs grown in media containing Cu. When translated to NFMS + 0 μmol/L P medium, the maximal PHB content of nitrated-supplied bacteria expressing pMMO was greatly decreased by 21% and comparable to that obtained in NFMS + 5 μmol/L Cu and NFMS + 0 μmol/L Cu medium containing cultures grown with NMS + 0 μmol/L Cu medium. Bacteria grown with NMS+ 0 μmol/L Cu accumulated PHB at a lower rate in NFMS + 0 μmol/L P medium, although the maximal PHB content (35.37%) only showed a slight decrease. The PHB production of N_2-fixing methanotrophs is illustrated in Fig. 2c. In contrast to nitrate- and ammonia-supplied bacteria, N_2-fixing cultures obtained the highest maximal PHB content (39.8%) in NFMS + 5 μmol/L Cu medium after being cultivated without Cu. The maximal PHB content of N_2-fixing bacteria with the expression of pMMO was only 32.1% in NFMS + 5 μmol/L Cu medium, which has a PHB accumulation trend similar to that of N_2-fixing bacteria containing sMMO in NFMS + 0 μmol/L Cu medium. The PHB production of N_2-fixing bacteria was seriously depressed under the conditions of simultaneous deficiencies of nitrogen source and phosphorus source. No PHB production was observed in NFMS + 0 μmol/L P medium containing bacteria grown by NFMS + 5 μmol/L Cu medium, and only 11% PHB was accumulated by N_2-fixing bacteria grown without Cu.

An economic analysis has been performed and revealed that the production cost of PHB is affected more by intracellular PHB content rather than by other factors, despite the fact that substrate cost accounts for 30% of the production cost (Choi and Lee, 1997). Therefore, it was bacteria cultivated with NMS + 5 μmol/L Cu medium that had the best PHB production performance, although methanotrophs grown in AMS+ 0 μmol/L Cu medium showed the fastest growth rate. The highest PHB content was obtained by transferring bacteria cultivated by NMS + 5 μmol/L Cu medium to NFMS + 5 μmol/L Cu medium. Ammonia- and nitrate-supplied methanotrophs containing pMMO showed higher PHB production capacity than bacteria expressing sMMO, while N_2-fixing bacteria with the expression of sMMO achieved higher PHB content. It could be noted that the maximal PHB contents of bacteria expressing sMMO were low, with no significant change under different

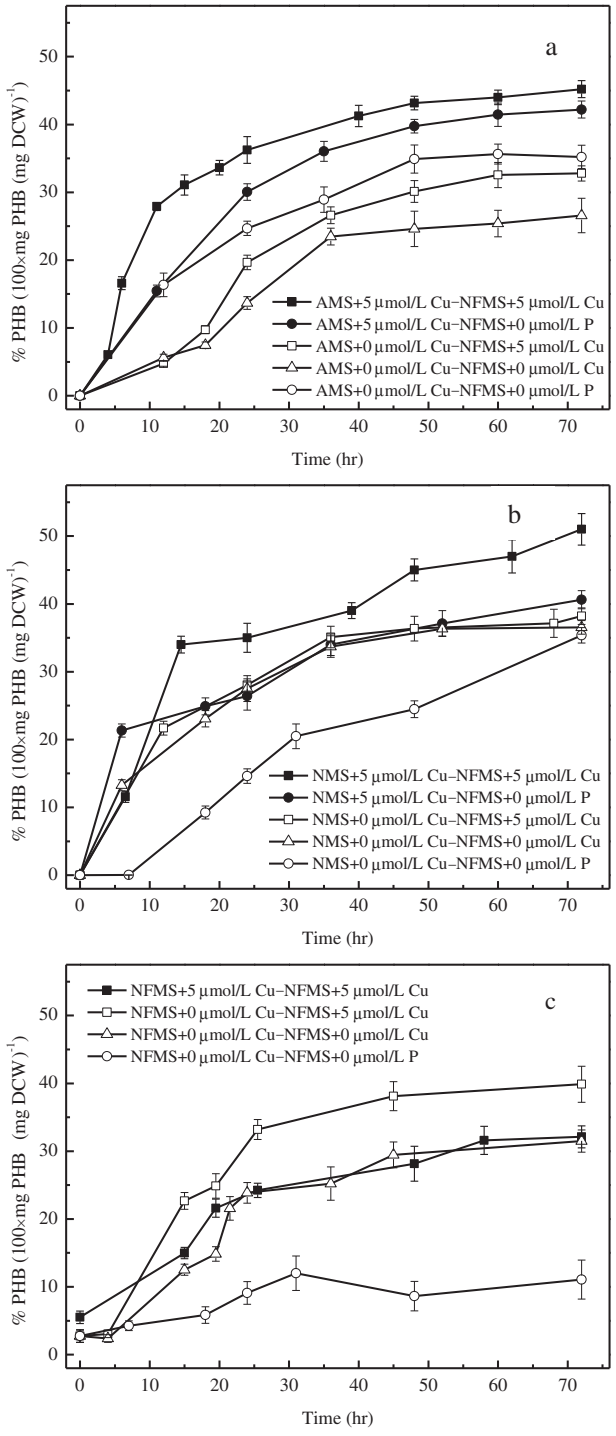

Fig. 2 – Profiles of the cellular poly-β-hydroxybutyrate (PHB) contents in different conditions for *M. trichosporium* OB3b grown with NH_4^+ (a), NO_3^- (b) and N_2 (c). AMS + 5 μmol/L Cu-NFMS + 5 μmol/L Cu represents that cultures were first grown in AMS + 5 μmol/L Cu medium and then PHB production was induced by NFMS + 5 μmol/L Cu medium, and so on.

nitrogen source conditions. Nevertheless, the PHB production capacity of methanotrophs expressing pMMO was greatly decreased after being cultivated with N_2, which might be also caused by depression of the activity of methanotrophs due to long-term nitrogen deficiency. As PHB production of bacteria grown with three different nitrogen sources was generally depressed under the conditions of simultaneous deficiency of nitrogen and phosphorus sources, it seemed that excessively harsh limitation of nutrients was detrimental to the PHB accumulation of methanotrophs. It is well known that reducing equivalents were required in the processes of methane oxidization and PHB accumulation (Pieja et al., 2011b). Severe environmental conditions might limit the activity of bacteria and result in inhibition of the formation of reducing equivalents. Subsequently, the deficiency of reducing equivalents depresses the oxidization of methane and the production of PHB. The decrease of PHB content in NFMS + 0 μmol/L P medium might also be partially caused by the reduction of pH, because the value of pH was indeed decreased to 5.6 at the end of the tests. From Fig. 2, it can be seen that N_2-fixing bacteria had a higher initial PHB content (2.5%–5.5%) than bacteria grown with nitrate and ammonia, which was consistent with a phenomenon reported previously (Chu and Alvarez-Cohen, 1998).

It has been reported that the growth of *M. trichosporium* OB3b could be accelerated with an appropriate increase in Cu concentration (Park et al., 1992). Therefore, tests on the effects of Cu concentration on the growth and PHB production capacity of nitrate-supplied methanotrophs were performed.

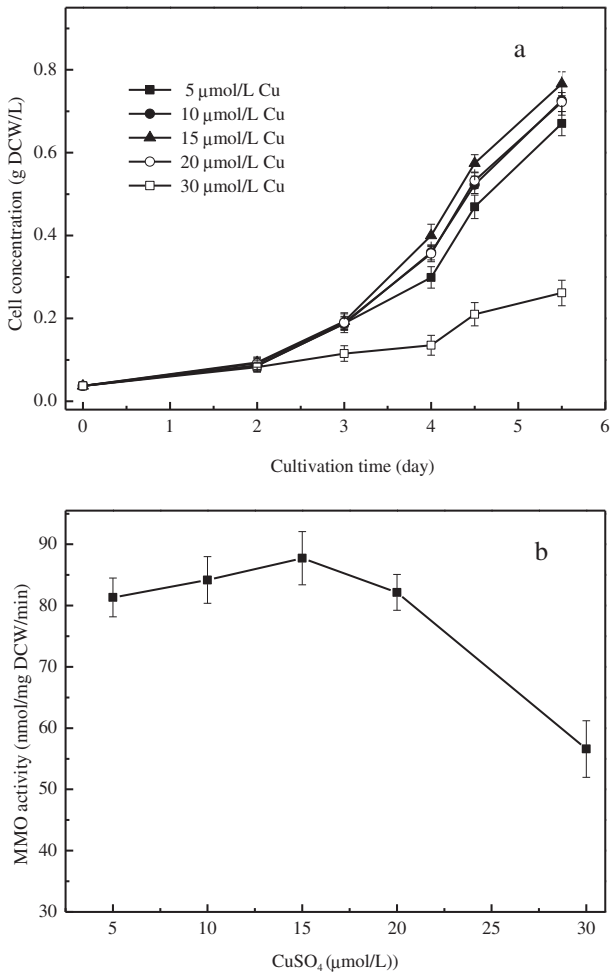

Fig. 3 – Effects of Cu concentrations on the growth curve (a) and methane monooxygenase (MMO) activity (b) of nitrate-supplied *M. trichosporium* OB3b.

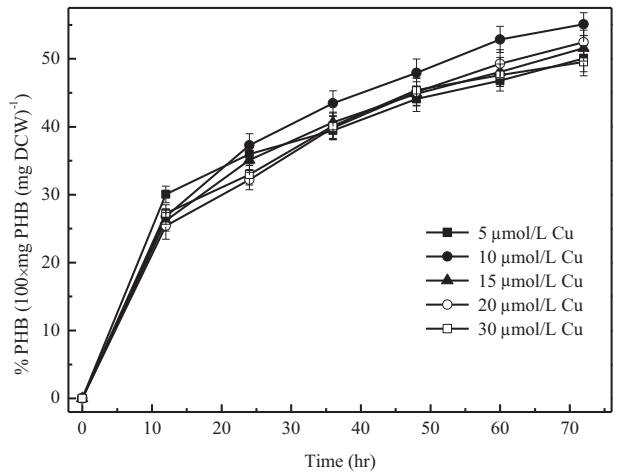

Fig. 4 – Profiles of PHB accumulation in different concentrations of Cu by *M. trichosporium* OB3b cultured with NO_3^- at 15 μmol/L Cu.

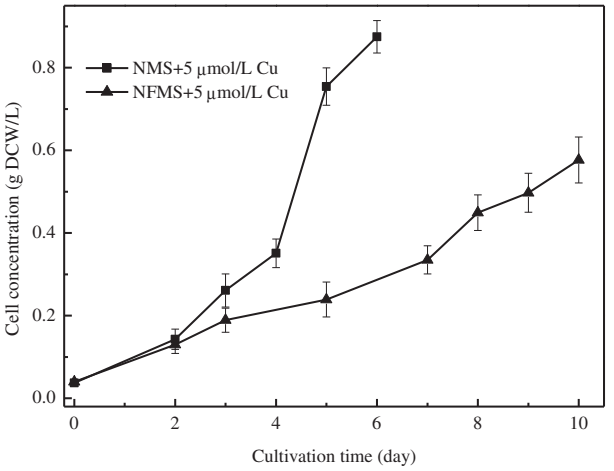

Fig. 5 – Growth curves of *M. trichosporium* OB3b alternately cultivated with NO_3^- and N_2 in the presence of 5 μmol/L Cu.

The growth rate of methanotrophs was slightly increased with Cu concentration increasing from 5 to 15 μmol/L, and greatly decreased in media containing 30 μmol/L Cu (Fig. 3a). Correspondingly, MMO activity was also monitored for varying Cu concentrations (Fig. 3b). A similar pattern was observed in the activity of MMO, as the MMO activity also peaked at 15 μmol/L Cu (87.73 nmol/mg DCW/min) and was dramatically lowered by 35.5% with the addition of 30 μmol/L Cu. In the PHB production phase, bacteria acclimated to 15 μmol/L Cu were used as test microorganisms. It seemed that the Cu concentration only had a slight effect on the PHB production capacity of methanotrophs (Fig. 4). The highest maximal PHB content (55.1%) was obtained with 10 μmol/L Cu, and there was no inhibition of PHB production with Cu concentrations up to 30 μmol/L. In addition, the naphthalene assay specific for sMMO did not detect any naphthol production in cells cultured with Cu, indicating that only pMMO could be expressed for concentrations of Cu more the 5 μmol/L regardless of the nitrogen source. It could be noted that the concentration of Cu not only regulated the expression of sMMO and pMMO but also affected the activity of bacteria expressing pMMO.

2.3. Cell growth, PHB accumulation and other characteristics of *M. trichosporium* OB3b alternately grown by nitrate and nitrogen gas

In order to explore the hypothesis that long-term nitrogen source deficiency was detrimental to the MMO activity, cell growth and PHB production capacity of methanotrophs expressing pMMO, methanotrophs were alternately cultivated with NO_3^- and N_2. As illustrated in Table 3, the MMO activity of N_2-fixing bacteria (75.51 nmol/mg DCW/min) was greatly improved in the cyclic $NO_3^-N_2$ cultivation strategy, accompanied by a significant increase in the net cell yield. Correspondingly, N_2-fixing bacteria grew at a significantly higher rate in the cyclic $NO_3^-N_2$ cultivation regime (Fig. 5), whose cell concentration at day 10 was 3.1 times that of bacteria in the continuous N_2 cultivation pattern. In addition, the growth rate of nitrate-supplied methanotrophs expressing pMMO was also slightly increased in the alternating nitrate and N_2 cultivation pattern, which might be attributed to some PHB being accumulated in the last N_2-fixing phase. It has been suggested that PHB can be used as a source of reducing equivalent for *Methylocystis parvus* OBBP to assimilate nitrogen in the presence or absence of methane (Pieja et al., 2011b).

The profiles of PHB accumulation of methanotrophs alternately grown with nitrate and N_2 are presented in Fig. 6. Not surprisingly, the maximal PHB content of N_2-fixing bacteria containing pMMO was increased to 40.5% in the cyclic $NO_3^-N_2$ cultivation regime. Concomitantly, nitrate-supplied bacteria accumulated PHB at a slightly higher accumulation rate, with a maximal PHB content equal to that obtained in the continuous nitrate cultivation pattern. As expected, the PHB accumulation of bacteria in cyclic $NO_3^-N_2$ cultivation regime was also depressed under the condition of simultaneous deficiency of nitrate and phosphate.

Table 3 – Comparison of cell characteristics of *M. trichosporium* OB3b alternately cultivated with NO_3^- and N_2 in the presence of 5 μmol/L Cu.

Cultivation conditions	Net cell yield (mg DCW/mg CH_4)	Nitrogenase activity	CH_4/O_2 consumption ratio	MMO activity (nmol/mg DCW/min)	
				sMMO	pMMO
NFMS	0.56 ± 0.02	+	1.48 ± 0.03	0	75.51 ± 2.87
NMS	0.63 ± 0.01	–	1.52 ± 0.01	0	76.91 ± 3.54

+: the activity of nitrogenase was detected, –: the activity of nitrogenase was not detected.

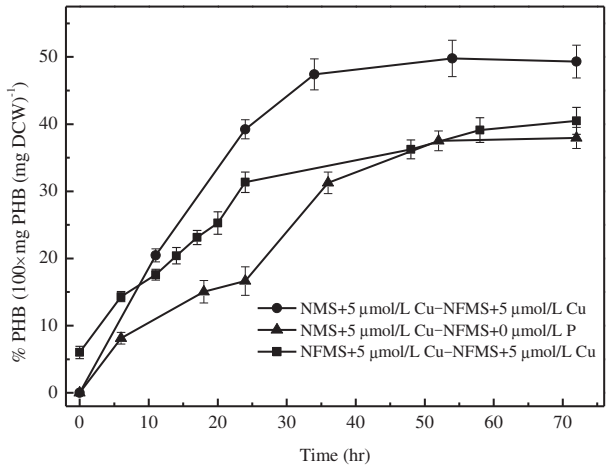

Fig. 6 – Profiles of the cellular PHB contents in different conditions for *M. trichosporium* OB3b alternately cultivated with NO_3^- and N_2 in the presence of 5 μmol/L Cu.

The results showed that the MMO activity, growth rate and PHB production capacity of N_2-fixing bacteria containing pMMO were greatly improved in the cyclic $NO_3^-N_2$ cultivation regime, which validated the supposition that long-term nitrogen source deficiency would depress the activity of methanotrophs expressing pMMO. The understanding of the reason causing the low MMO activity, low growth rate and low PHB percent of methanotrophs constantly grown with N_2 with the expression of pMMO was also meaningful for seeking appropriate processes to improve the PHB production performance of mixed N_2-fixing methanotrophs (Pieja et al., 2011a).

3. Conclusions

Ammonia-supplied *M. trichosporium* OB3b containing sMMO grew at the fastest rate, while the highest PHB content was obtained by transferring nitrate-supplied bacteria with the expression of pMMO to NFMS + 5 μmol/L Cu medium. The slightly lower growth rate and lower cell yield of ammonia-supplied bacteria expressing pMMO might be attributed to high similarity between the gene encoding pMMO and the sequence of the AMO gene. The MMO activity, growth rate and intracellular PHB content of bacteria expressing pMMO were greatly decreased after being continuously cultivated with N_2. However, in the cyclic $NO_3^-N_2$ cultivation regime, the activity of N_2-fixing bacteria expressing pMMO was significantly increased, which validated the supposition that long-term nitrogen source deficiency would depress the activity of methanotrophs expressing pMMO.

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