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Research Article

Poly-β-hydroxybutyrate Production by *Methylosinus trichosporium* OB3b at Different Gas-phase Conditions

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Background: The utilization of methane for production of Poly-β-hydroxybutyrate (PHB) not only cuts the emissions of greenhouse gases but also greatly reduces PHB production cost.
Objectives: The aim of this study was to determine the effects of gas-phase conditions on PHB production by *Methylosinus trichosporium* OB_3b.
Materials and Methods: Bacterial cultivation and PHB production were conducted in a series of sealed serum bottles. Nitrogen-free mineral salts medium was used to induce PHB production in the presence or absence of N_2 in the headspace.
Results: In the absence of N_2, the highest PHB content (i.e., 52.9% of the dry cell weight with a PHB concentration of 814.3 $mg.L^{-1}$) was obtained at a ratio of CH_4:O_2=2:1. Further study at different O_2 concentrations with a fixed CH_4 partial pressure in absence of N_2 showed that PHB accumulation by methanotroph could be tolerated high oxygen partial pressure and its respond to the variation of the oxygen concentration depends on the methane partial pressure. In presence of N_2, with headspace gas replenished only when oxygen was almost depleted, the degradation of intracellular PHB has appeared. In the regimen of updating headspace gas at the point when the PHB content began to decrease, the highest PHB content (i.e., 55.5% of the dry cell weight with 901.8 $mg.L^{-1}$ PHB concentration and 12.5 $mg.L^{-1}.h^{-1}$PHB productivity) was obtained at 0.2 atm O_2 and PHB accumulation was depressed with an oxygen concentration greater than 0.3 atm.
Conclusions: The methanotroph responses differentially to the increase in the oxygen partial pressure with regard to PHB accumulation either in the presence or in the absence of N2.

Keywords: Methylosinus trichosporium, Nitrogen Fixation, poly-beta-hydroxybutyrate

1. Background

Polyhydroxyalkanoates (PHAs) have attracted an increasing attention as an alternative to the traditional plastics, among which Poly-β-hydroxybutyrate (PHB) is the most widely studied and best-characterized homopolymer (1). However, the expansion of PHB utilization has been restrained due to their high production cost. Consequently, many research groups have devoted themselves to the development of the inexpensive feedstock and low-cost extraction methods. Techno-economic studies have shown that approximately 30-50% of the PHB production cost is mainly attributed to the expensive carbon sources (2). So, it could be greatly reduced if the waste organic carbon is used as an inexpensive and renewable feedstock, such as H2, methanol and cane molasses (1, 3-7). Methane, which is abundantly available during fossil fuels extraction and organic waste of the anaerobic degradation process, accounts for 20% of the worldwide greenhouse gas (GHG) emissions (8)and the global atmospheric methane ratio is increasing at an annual average of 1%(9) . Although some measures have to be taken in order to increase methane solubility and eliminate the possibility of the explosion during PHB production from methane, it has been estimated that using waste methane as feedstock might reduce the cost of PHB by approximately 30-35%(10). As well, PHB production from biogas discharged by the existing landfills and anaerobic digesters could theoretically replace 20-30% of the total plastics annual market (11). Methanotrophs, which utilize methane as the sole carbon source, mainly consist of two groups, type I and II, with different pathways, the ribulose monophosphate

(RuMP) pathway and the serine pathway, to complete carbon assimilation (12). PHB production has been reported to be restricted to type II genera (13), among which *Methylocystis* and *Methylosinus* are the most documented. Both methane and oxygen are important for methanotrophs (2)and variations of their partial pressure are likely to affect the activities and metabolisms of the methanotrophs. PHB are generally synthesized by microorganism under nutrient-limiting conditions and are consumed as a source of reducing equivalent under nutrient-sufficient conditions (12). Nitrogen deficiency is one of the most common ways to trigger the accumulation of PHB (14). The ability to fix molecular nitrogen has been reported to present in all type II genera (13). Methanotrophs can utilize N_2 as a sole nitrogen source for growth (15). It has been reported that *Methylosinus trichosporium* OB_3b grew slowly and accumulated about 6% PHB under N_2-fixing conditions (16). Shah *et al.* suggested that only 10% PHB was produced by *M. trichosporium* OB_3b when the mixtures of methane and air were supplied as substrate. However, after air was switched to pure oxygen with the same oxygen flux, the content of PHB was increased to 45% (17). It is obvious that the presence of N_2 could affect the PHB accumulation of the methanotrophs. Nevertheless, the accumulation of PHB by methanotrophs is always stimulated by removing the liquid medium nitrogen source in the previous studies (9, 13-15, 17). There is a little detailed-information on the impact of N2 on PHB production by methanotrophs.

In addition, whether methane is collected from natural gas or biogas, it is inevitable that some N_2 would be introduced and the purification is costly. So, it is meaningful to examine how N_2 affects the PHB synthesis ability of the methanotrophs. It has been suggested that nitrogenase activity of the methanotrophs is sensitive to the oxygen partial pressure (13, 15-18). Therefore, the effect of N_2 on the PHB synthesis ability of methanotrophs is probably O_2-dependent. If a high content of PHB could also be produced in the presence of N_2, the requirement for the purity of the methane and oxygen would be greatly reduced. As a result, the PHB production cost attributed to the substrate could be further reduced.

2. Objectives

In order to determine how PHB production of *M. trichosporium* OB_3b was affected by the gas-phase conditions in the absence and presence of N_2 and explore the possibility of accumulating high content PHB in presence of N_2, this study was performed in two steps. Firstly, it was investigated how PHB accumulation of methanotroph was affected by the variations of the oxygen and methane partial pressure in the absence of N_2, and secondly, the effect of the presence of N_2 on PHB synthesis was evaluated at different oxygen concentrations in two different headspace gas replenishment regimes.

3. Materials and Methods

3.1. Microorganisms and Culture Conditions

M. trichosporium OB_3b was kindly provided by *M. Kalyuzhnaya* (Lidstrom laboratory, University of Washington) and used throughout this study. *M. trichosporium* OB_3b was cultivated in the nitrate minimal salt (NMS) containing (per liter) KH_2PO_4 0.272 g, $Na_2HPO_4{\cdot}12H_2O$ 2.868 g, KNO_3 0.10 g, $MgSO_4{\cdot}7H_2O$ 0.10 g, $CaCl_2{\cdot}6H_2O$ 0.20 g and 2 mL of trace element solutions. The trace element solution was composed of (per 100mL): Na-EDTA 25 mg; $FeSO_4{\cdot}7H_2O$ 50 mg; Fe-EDTA 38 mg; $ZnSO_4{\cdot}7H_2O$ 40 mg; Cu-EDTA 10 mg; H_3BO_3 1.5 mg; $MnCl_2{\cdot}4H_2O$ 2 mg; $Na_2MoO_4{\cdot}2H_2O$ 26 mg; $CuCl_2{\cdot}2H_2O$ 30 mg; $NiCl_2{\cdot}6H_2O$ 1 mg; $CoCl_2{\cdot}6H_2O$ 5 mg. The initial pH of the medium was adjusted to 6.8 applying 1 M sodium hydroxide (19). An amount of 100 mL of the NMS medium and 5 mL of culture inoculums was introduced into a series of 300mL serum bottles which were capped with butyl rubber stoppers and screw top. Cultures were grown at 30 °C on the orbital shakers at 150 rpm under a CH4/O2 gas mixture (1:1, v/v). Headspace gas was replenished every 24 h by being subjected twice to the vacuum and replenished with the same gas mixture (CH4/O2 at a ratio of 1:1 v/v) to maintain a sufficient amount of the oxygen. The cell growth was monitored by measuring the gaseous composition in the headspace along with monitoring the optical density at 660 nm (V-560, Jasco International Co., Ltd., Japan) which was correlated with dry cell mass measured after lyophilizationfor 24 h.

3.2. PHB Production Studies

The nitrogen-free mineral salts (NFMS) medium, which was identical to NMS medium except for the addition of 0 mM KNO_3 to NFMS medium, was used to induce PHB production. Cell suspensions were harvested after about 5 d post-cultivation, washed twice with NFMS medium, and re-suspended in NFMS medium (OD_{660} of 1.5±0.05). Where after, the cell re-suspension solution was divided by transferring15 mL aliquots into a series of the 125 mL serum bottles. The serum bottles were capped with butyl rubber stoppers and screw top

The effect of the gas-phase conditions on the PHB production were first conducted without N_2, in which the headspace gas was renewed at every 24 h for 72 h to ensure the sufficient gas substrates. To study the effect of the applied ratio of the methane to that of oxygen at a constant pressure and in the absence of N_2, the headspace gas was refreshed by being subjected to the vacuum twice, replenished with a mixture of the methane and oxygen (CH_4/O_2; at the ratio of 3:1, 2:1, 1:1, 1:2, and 1:3 v/v, respectively) to restore an ambient

atmospheric pressure. To elucidate the effect of different oxygen concentrations at a pressure of 0.5 atm CH_4 without N_2, the headspace was first vacuumed, then methane was fed to a partial pressure of 0.5 atm followed by the addition of oxygen with the different partial pressures (oxygen partial pressure = 0.25, 0.33, 0.5, 0.67, and 0.75 atm, respectively). At last, helium was added to make sure that the same total pressure was reached in each bottle. The replenishment operation was repeated twice each time. In addition, the oxygen concentration effect (i.e., 0.2-0.6 atm, respectively) on PHB production was also conducted at 0.2 atm CH_4 to explore whether it was varied at the different methane concentrations.

For demonstrating the coupled effect of molecular nitrogen as well as different oxygen concentrations on PHB synthesis in two different headspace gas replenishment regimes, the headspace was first subjected to the vacuum, then methane was fed to the headspace at a partial pressure of 0.5 atm, oxygen at the partial pressure ranging from 0.1atmto 0.5 atm, and helium was added to restore an ambient atmospheric pressure. At last N_2 was fed to the headspace at a pressure of 0.3 atm applying a gas-tight syringe. In the first replenishment regimen, the headspaces gas was refreshed when the concentration of oxygen was below 5% (v/v). In the other regimen, the headspace gas was renovated every 12 h to inhibit the degradation of intracellular PHB.

All serum bottles were incubated at 30 °C on the orbital shakers at 150 rpm. The initial gaseous compositions were determined and the variations were monitored periodically. Duplicate serum bottles were sacrificed periodically for 72 h. The 10 mL cell suspensions were subjected to the centrifugation at 4 °C, washed twice with deionized water, lyophilized, and weighed before analysis of PHB.

3.3. Analytical Methods

The percent PHB was analyzed by a gas chromatography (GC7890 II, Techcomp limited, China) equipped with a flame ionization detector (FID) after digestion of the freeze-dried cell pellets (20). The headspace gas composition was determined by a gas chromatography (GC7900, Techcomp limited, China) equipped with a thermal conductivity detector. As well, statistical analyses were performed by PASW statistics release 18.0.0 (SPSS Inc., Chicago, Illinois). Spearman's rank correlation test was employed to determine the significance. ρ represents Spearman's correlation coefficient, n represents the number of points used, and Prepresents the significance.

4. Results

4.1. PHB Production with Different Ratios of Methane to Oxygen at Constant Pressure in the Absence of N_2

The changes of the percent PHB at different CH_4:O_2 ratios are illustrated in **Figure 1**. There was no obvious distinction in the PHB content among each ratio of methane to oxygen in the first 24 h. After that, a PHB content of CH_4:O_2 = 1:3 was the first to reach a plateau with a maximal PHB content of 35.2%, successively followed by CH_4:O_2 = 1:2 (40.7%), CH_4:O_2 = 3:1 (44.4%), CH_4:O_2 = 1:1 (49.5%), and CH_4:O_2 = 2:1 (52.9%). It was obvious that with an increase in CH_4:O_2 ratio from 1:3 to 2:1, gradually a higher maximal PHB content was obtained. Afterwards, the maximal content of PHB was decreased when the ratio was further increased to 3:1 (ρ = 0.689, n = 10, P = 0.027). The maximal PHB concentration and PHB productivity at CH_4:O_2 = 2:1 were 814.3 $mg.L^{-1}$ and 11.3 $mg.L^{-1}.h^{-1}$ respectively.

4.2. PHB Synthesis with Different Oxygen Concentrations at the Fixed Methane Partial Pressures in Absence of N_2

In order to determine how oxygen partial pressure influences PHB production of the methanotrophs, the experiments were conducted at the two different methane partial pressures. **Figure 2** presents the variations of PHB content with different oxygen dosages at 0.5 atm of CH_4. It is noteworthy that the PHB synthesis ability of the Methylosinus trichosporium OB3b was limited at 0.25 atm O_2. When oxygen partial pressure was successively increased to 0.33, 0.5, 0.67 and 0.75 atm, the maximal contents were 41.5%, 48.9%, 51.5% and 52.3%, respectively. The accumulation of PHB was promoted by the higher oxygen concentration (ρ = 0.886, n = 10, P = 0.001). When the partial pressure of the oxygen was as high as 0.75 atm the conditions was still favorable for PHB synthesis, showing no inhibition. The PHB production at 0.2 atm CH_4 is illustrated in **Figure 3**. Similarly, the PHB accumulation of the methanotroph was also limited at low oxygen concentration (0.2 atmO_2). However, unlike the tests with 0.5 atm CH_4, the bacteria accumulated a higher content of PHB at 0.3 (38.2%) and 0.4 atm O_2 (40.9%) (ρ = 0.837, n = 6, P = 0.038) and the maximal PHB content was greatly decreased by 26.4% when oxygen partial pressure was further increased to 0.6 atm. It was obvious that PHB production of the methanotroph was strongly dependent on the oxygen concentration and the response to the variation of the oxygen concentration varied at different methane partial pressures, accordingly.

4.3. Coupled Effects of Molecular Nitrogen and Different Oxygen Concentration on the PHB Synthesis in two Different Headspace Gas Replenishment Regimens

In the first headspace gas replenishment regime, the headspace gas was refilled only when oxygen was almost depleted. The intracellular PHB contents in this regimen along with oxygen consumption curve are

plotted in Figur 4a and 4b. For an oxygen partial pressure of 0.1 atm, PHB content reached the plateau in 7h with a value of 5.5%. After the first oxygen supplement at 15.5h, the percent PHB value first increased gradually to 9.2% and then decreased slightly with the consumption of oxygen. Likewise, after the second oxygen supplement, the increase and decrease cycle of the PHB content was observed again. The similar behaviors were also observed at 0.2 and 0.3 atmO_2, as well. When the oxygen partial pressures were 0.4 and 0.5 atm (no headspace gas refreshment was performed), the PHB content was also increased at first and the maximal PHB content was obtained at 15.5h. But, afterward, the PHB level was decreased.

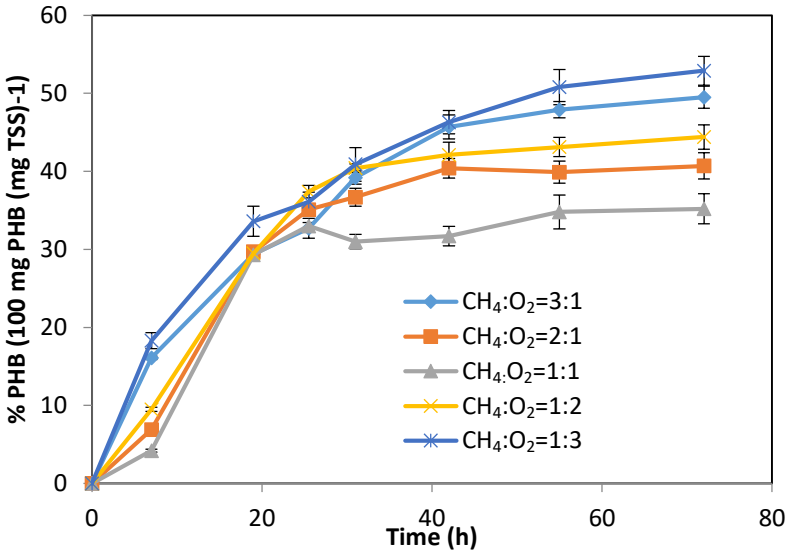

Figure 1. The time course of the percent PHB with the different ratios of the methane to the oxygen at constant pressure in the absence of N_2.

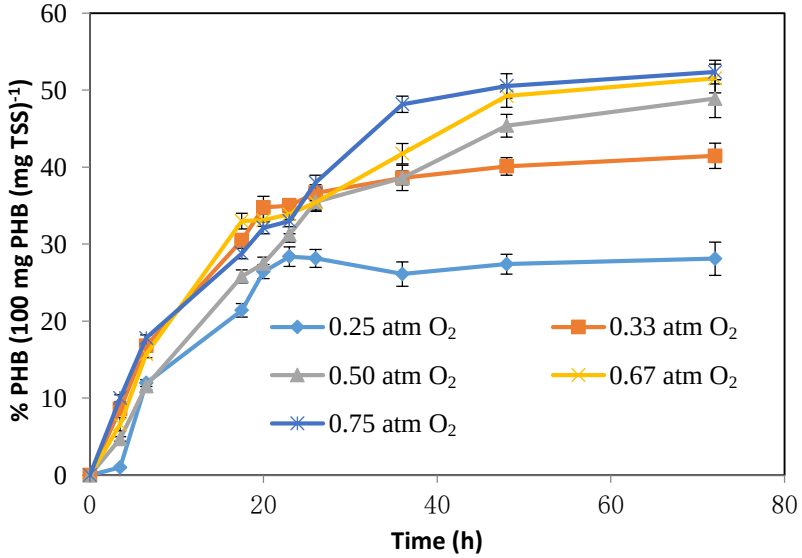

Figure 2. The profiles of the cellular PHB content at different oxygen concentrations with 0.5 atm CH_4 in the absence of N_2.

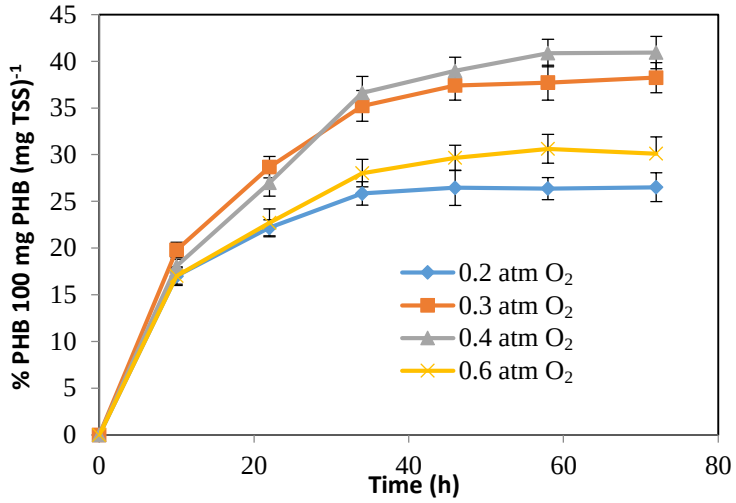

Figure 3. The time course of the percent PHB at different oxygen concentration with 0.2 atm CH_4 in the absence of N_2.

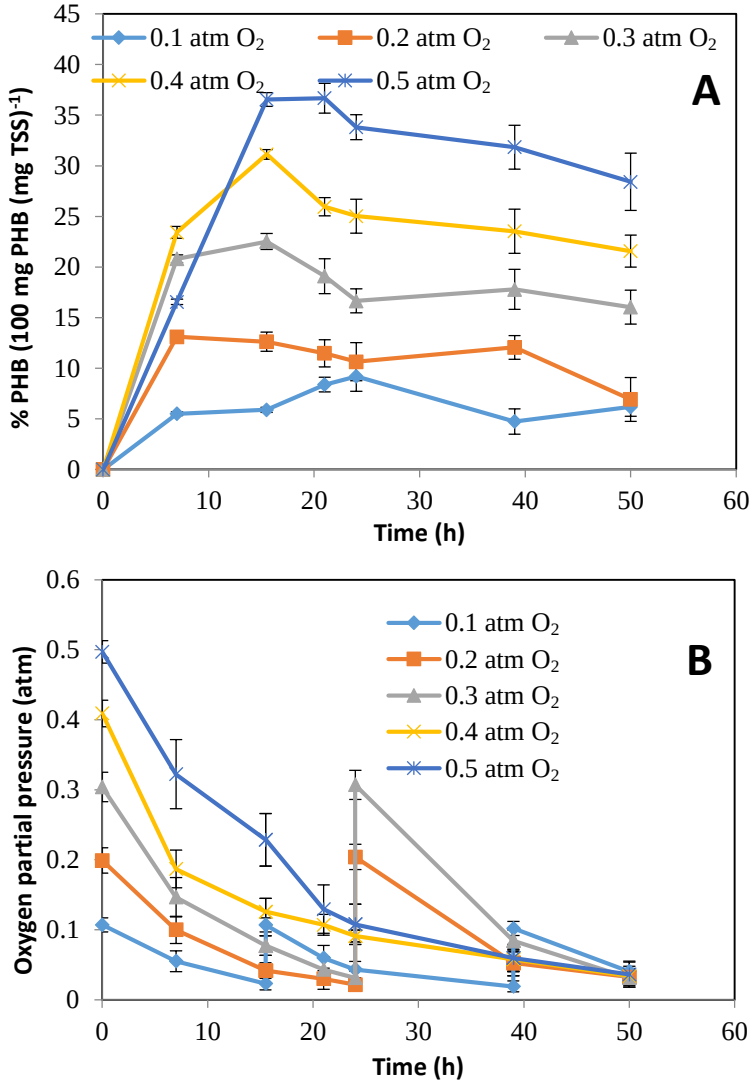

Figure 4. (A) The graph representing the variation in the percent PHB and (B) changes in the oxygen partial pressure in the presence of N_2. 0.5 atm CH_4, 0.3 atm N_2 and different concentration of the O_2 were introduced and the headspace was refreshed only when oxygen was almost depleted.

To test the possibility of more PHB accumulation in the presence of the N_2, the headspace gas was refilled every 12 hours, as the PHB content began to decrease after that. As shown in **Figure 5**, the different effects of the O_2

on PHB synthesis were observed. The highest maximal percent PHB value (55.5%) was obtained at 0.2 atm O_2. The decrease in the maximal PHB content occurred when oxygen concentration was successively increased to 0.5 atm as observed in the following pattern: 0.3 atmO_2 (47.0%) > 0.4 atm O_2 (45.9%) > 0.5 atm (37.3%). Then, the maximal PHB content slightly increased to 40.7% when oxygen concentration was further increased to 0.7 atm (ρ = -0.086, n = 10, P = 0.001). The inhibition of the higher concentration oxygen on PHB synthesis of the methanotroph appeared at 0.5 atmCH_4in the presence of N_2. **Table 1** provides details of the maximal PHB concentration and PHB productivity obtained in the regimen of refreshed headspace gas every 12 hours. It is noteworthy that the highest PHB concentration of the 901.8 mg.L^{-1} was obtained at 0.2 atm O_2 with a PHB productivity of 12.5 mg.L^{-1}.h^{-1}.

Table 1. The maximal PHB concentration and PHB productivity obtained in the regimen of the renovating headspace every 12 h in presence of N_2.

Oxygen concentration (atm)	Maximal PHB concentration (mg.L^{-1})	PHB productivity (mg.L^{-1}.h^{-1})
0.20	901.8 ± 21.3	12.5 ± 0.3
0.30	652.1 ± 31.4	9.1 ± 0.4
0.40	623.1 ± 24.1	8.7 ± 0.3
0.50	443.5 ± 19.1	6.2 ± 0.3
0.70	507.7 ± 23.8	7.1 ± 0.3

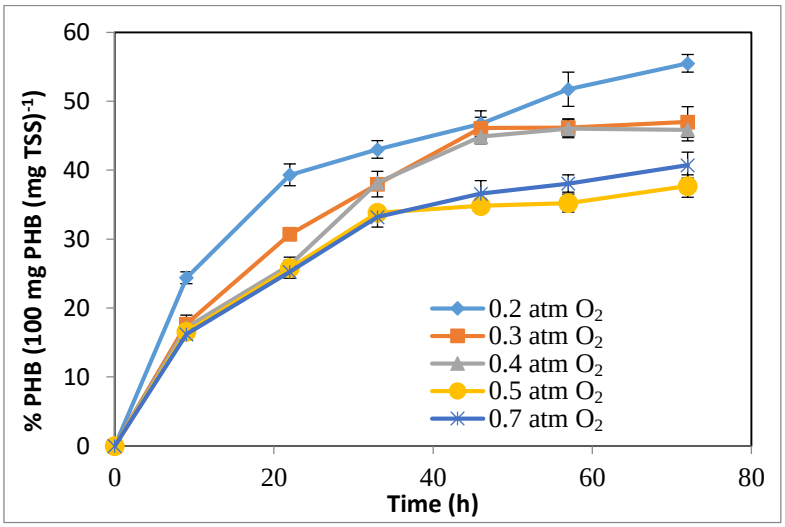

Figure 5. The effect of the oxygen concentration on PHB accumulation with headspace replenished every 12 h in the presence of N_2. The initial headspace was consisted of the 0.5 atm CH_4, 0.3 atm N_2 and different concentration of O_2.

5. Discussion

The PHB accumulation was first conducted with different ratios of the methane to the oxygen in the absence of N_2. It has been reported that methanotrophs prefers to grow at the level where both oxygen and methane are completely consumed (21). It has been calculated that the molar ratio of the consumed methane and oxygen consumed is equal to 1:1.5 in theory (22). In PHB production phase, the higher maximal PHB contents were more likely to be obtained with excessive methane at the higher ratios of methane to oxygen. The result was consistent with the previous research about PHB production from the mixtures of the volatile fatty acids (VFAs) that, an excess exogenous carbon source is favorable for the intracellular PHA accumulation of the activated sludge (23).

At a fixed methane partial pressure without N_2, the data indicated that limiting O_2 concentration negatively affected the PHB accumulation of the methanotrophs, which was also verified previously (2). So, it is important to ensure adequate oxygen pressure during the accumulation of the PHB. On the other hand, it seems that an overdose of oxygen might depress PHB synthesis of the methanotroph and the oxygen partial pressure that inhibited PHB production perhaps varied at different methane partial pressure, as well. It has been reported that with oxygen concentrations increasing from 20% to 60%, the methane oxidization rate was reduced by more than 23% for both types I and II methanotrophs (24). Henckel *et al.* reported that responses of the methane oxidation of the rice field soil to the increased oxygen concentration varies at high and low methane concentration, which is consistent with the phenomenon observed in this test (25). It has been reported that the molar ratio of the methane to that of oxygen should be maintained at a ratio ≥ 1:2 for an improved methane oxidation (26). In tests with 0.5 atm CH_4, the CH_4:O_2 ratio was maintained at a ratio ≥ 1:1.5 and the maximal PHB content was gradually increased with an increased oxygen con centrations. However, in the tests with 0.2 atm CH_4, when the oxygen partial pressure was increased to 0.6 atm (CH_4:O_2=1:3), the maximal PHB content was indeed significantly

decreased. Therefore, the lower PHB content of CH_4:O_2=1:3 was likely to be mainly caused by the inhibition of excess oxygen.

In the presence of N_2, with the headspace gas replenished only when oxygen was almost depleted, PHB was accumulated and degraded cyclically. It has been reported that with the different mixture of the methane and air as the gaseous substrate flow, once nitrate was exhausted, the percent value of PHB was improved apparently at first and then followed by a gradual reduction which was in agreement with the phenomenon presented in these investigations (17). It has been observed that only slow growth of the methanotrophs was performed with N_2 as a sole nitrogen source when compared with the nitrate- or ammonium-supplied bacteria (15), which indicated that N_2 could only provide a limited source of nitrogen. It seems that regardless of the oxygen partial pressure, the sudden removal of nitrate would result in a relative lack of nitrogen source and stimulates PHB accumulation at the beginning even though N_2 was added as a nitrogen source. It has been demonstrated that Type II methanotrophs have a complete tricarboxylic acid (TCA) cycle, which can utilize acetyl CoA produced from PHB degradation as the substrate to produce reducing equivalents (13). On the other hand, it is well known that a reducing equivalent is required in the process of energy-intensive N_2 fixation (27). Moreover, type II nitrogenase of the methanotrophs has been reported to tolerate an oxygen partial pressure up to 28% (28). Therefore, the degradation of PHB might be used as a source of reducing power to assimilate N_2.

When compared with PHB productions at 0.5 atm CH_4 without N_2, the maximal PHB content of 0.2 atm O_2 was significantly improved in the presence of N_2 with the headspace replenished every 12 h, which was likely attributed to the limited nitrogen source provided by the N2 fixation. A kinetic study of the PHB production by Protomonas extorque revealed that a nitrogen source was necessary, not only in the growth phase but also in the PHB production phase, as well (29). It was reported that the PHB accumulation of a recombinant Escherichia coli was improved significantly when a small quantity of the complex nitrogen source was added (30). With oxygen concentration progressively increasing to 0.7 atm, the maximal PHB content decreased obviously in the presence of N_2, which was so different from results observed at 0.5 atm CH_4 in the absence of N_2. It has been observed that the response of the methane oxidation to the increase of the oxygen concentration under N_2-fixing condition was opposite to that under nitrate-supplied conditions, which was supposed to be due to the effect of nitrogen metabolism on carbon metabolism (31). So, the reverse effects of oxygen partial pressure on PHB production between tests in the presence and in the absence of N_2 at 0.5 atm CH_4 might be attributed to the variations of the carbon metabolism.

In conclusion, both in the presence and in the absence of N_2, the maximal PHB content of *M. trichosporium* OB_3b could reach a high value. The responses of PHB accumulation of methanotroph to oxygen partial pressures in the absence of N_2 were opposite to that in the presence ofN_2. The production of high content PHB in the presence of N_2 would greatly reduce the requirement for the purity of the methane and pure oxygen could also be substituted by the air, leading to a further reduction in the PHB production cost.

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