Research Paper

Methanol production from CO_2 by resting cells of the methanotrophic bacterium *Methylosinus trichosporium* IMV 3011

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Methanol production from carbon dioxide was successfully achieved using resting cells of *Methylosinus trichosporium* IMV 3011 as biocatalysts. Carbon dioxide was reduced to methanol and extracellular methanol accumulation has been found in the carbon dioxide incubations. However, resting cells of methanotrophs have a finite or intrinsic methanol production capacity due to a limiting supply of intracellular reducing equivalent. It has been found that the catabolism of stored Poly-β-Hydroxybutyrate (PHB) can provide intracellular reducing equivalents to improve the intrinsic methanol production capacity. The initial nitrogen and copper concentration in the culture medium were studied for the accumulation of PHB by *M. trichosporium* IMV 3011, to expand its potential uses in methanol production from carbon dioxide reduction. It appeared that the total methanol production capacity was increased with increasing PHB content in cells. Resting cells containing 38.6% PHB exhibited the highest total methanol production capacity. But higher PHB accumulation adversely affected the total methanol production capacity. The effects of methanol production process on the survival and recovery of *M. trichosporium* IMV 3011 were examined. The results showed that the methanol production from carbon dioxide reduction was not detrimental to the viability of methanotrophs.

Keywords: Carbon dioxide reduction / Methanol production / Methanotroph / Poly-β-hydroxybutyrate / Reducing equivalent

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Introduction

Methanol is used in a wide range of applications. Methanol can be produced chemically via methane, carbon dioxide, biomass, coal, heavy fuel oils etc. (Arakawa 1998, Cybulski 1994, Marchionna *et al.* 1998, Rozovskii and Lin 1999, Lange 2001). Strategies for conversion of carbon dioxide to methanol offer promising new technologies not only for recycling of the greenhouse gas but also for an efficient production of fuel alternatives. Enzymatically coupled sequential reduction of carbon dioxide to methanol, using a series of reaction catalyzed by three different dehydrogenases (formate dehydrogenase, formaldehyde dehydrogenases and methanol dehydrogenase), is particularly appealing (Obert and Dave 1999. In the process, the ability of the dehydrogenases to catalyze the reverse reactions in the presence of an excess of reducing equivalent has been exploited to facilitate reductions of carbon dioxide that are difficult to achieve using traditional chemical methods. However, the process presents some technical problems; for example, in order to keep the conversion process going, costly reduced nicotinamide adenine dinucleotide (NADH) must be used as reducing equivalent for each dehydrogenase-catalyzed reaction. NADH is depleted in the reaction and subsequently more NADH must be added. Also, it is very difficult to control

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the multi-step reaction in a given reaction sequence and to proportion the suitable enzyme dosage. In addition, intermediates of the pathway may be used in other reactions, diluted and exposed to bulk solvent where they may be degraded.

We have investigated whether microorganisms can be used as biocatalysts for the reduction of carbon dioxide to methanol, to overcome the above-mentioned drawbacks, since the enzymes are likely to be more stable in the cell than in the purified form and will ensure a continuing supply of NADH. Unfortunately, up to now, there are no known organisms whose normal biological role is reduction of carbon dioxide to methanol.

There are microorganisms called methane-oxidizing bacteria or methanotrophs that can utilize methane as their sole carbon source and energy source for growing. In these organisms, methane is oxidized via methanol, formaldehyde and formate to carbon dioxide with some formaldehyde being incorporated into cell biomass (Hanson and Hanson 1996). The first reaction in the methane oxidation pathway is catalyzed by methane monooxygenase (MMO). MMO utilize 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 dehydrogenases 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 (Hanson and Hanson 1996).

Reducing carbon dioxide to methanol is the reverse of the oxidation of methanol. In the previous works (Xin *et al.* 2004a , 2004b), we have explored the feasibility of using the methanotrophic cell for the methanol production from carbon dioxide. The results showed that carbon dioxide can be reduced into methanol by methanotrophs. It is possible and feasible to reduce carbon dioxide to methanol by methanotrophic whole cells containing formate dehydrogenase, formaldehyde dehydrogenase and methanol dehydrogenase, despite the fact that these enzymes normally oxidize their substrates *in vivo* or *in vitro*. Since MMO cannot effectively catalyze the reverse reaction of methane monooxygenation, extracellular methanol accumulation has been found in the carbon dioxide incubations.

Reducing carbon dioxide to methanol is energy intensive and requires a considerable amount of reducing equivalent to push the reaction along against energy laws. Resting cells of methanotrophs have a finite or intrinsic catalytic capacity for methanol production from carbon dioxide reduction due to a limiting supply of intracellular reducing power. The limitation due to reducing equivalent availability can be offset by adding NADH. 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 a variety of microorganisms under nitrogen, phosphate, or oxygen limiting condition. As shown in Figure 1, Methanotrophs may accumulate PHB by two possible pathway of carbon assimilation, the ribulose monophosphate pathway (RMP) and the serine pathway (Asenjo and Suk 1986). Methanotrophs are classified as type I or type II, depending on the differences in the membrane structure and in the assimilation pathways. Type II bacteria using the serine pathway are the most effective PHB producers (Wendlandt *et al.* 2001). The first step in the conversion of methane into PHB is carried out by nonspecific MMO enzyme systems (Asenjo and Suk 1986). In certain methanotrophs, such as

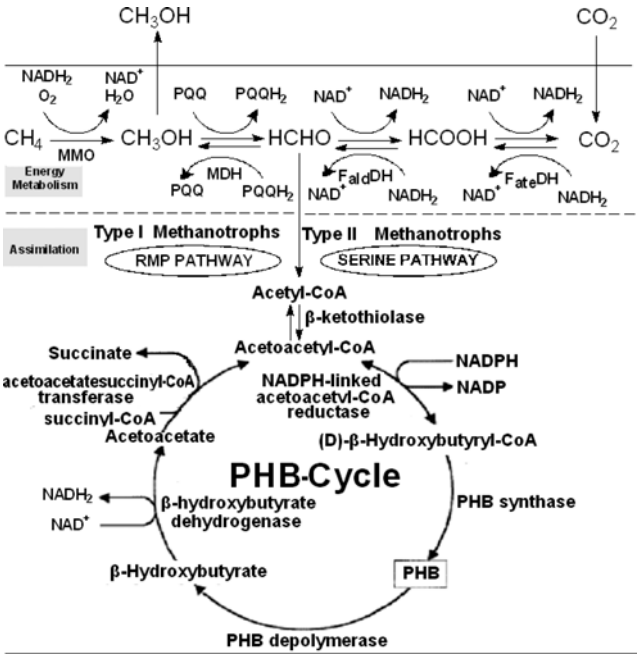

Figure 1. Proposed mechanism of methanol synthesis from carbon dioxide reduction by methanotrophic bacteria.
FateDH: formate dehydrogenase; F_{ald}DH: formaldehyde dehydrogenase ate ald; MDH: methanol dehydrogenase; MMO: Methane monooxygenase.

Methylosinus trichosporium OB3b and *Methylococcus cupsulatus* (bath), a soluble form (soluble methane monooxygenase, sMMO) is produced when Cu is inadequate, while a membrane bound particulate form (particulate methane monooxygenase, pMMO) of the enzyme is produced when sufficient Cu is present (Park *et al.* 1991, 1992, Stanley *et al.* 1983). Cells producing pMMO have a faster growth rate and higher catalytic activities with methane.

PHB is an internal reducing-energy storage polymer that can be used as an alternative reducing-energy source by a number of methanotrophs cultures under starvation conditions (Davis *et al.* 1964). In the PHB cycle pathways shown in Figure 1, the series of reaction involving the conversion of acetyl CoA to PHB and its depolymerization and oxidation back to acetoacetyl CoA, are confirmed by Korotkova (Korotkova and Lidstrom 2001). 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 equivalents via the action of the NAD^+-linked β-hydroxybutyrate dehydrogenase. A number of studies observed a correlation between Trichloroethylene (TCE) oxidation capacities and microbial PHB content (Henry and Grbić-Galić 1991, Henrysson and McCarty 1993, Chu and Alvarez-Cohen 1996), suggesting that PHB might be used as an alternative NADH source for TCE oxidation by methanotrophs.

The presence of endogenous reducing power reserves may have great significance in methanol production from carbon dioxide. Resting methanotrophic strains containing PHB as endogenous reducing power reserves may retain the ability to reduction carbon dioxide longer than strains without PHB.

In the present article, in order to assess better some practical applications of the resting cells in the production of methanol from CO_2 reduction, *M. trichosporium* IMV 3011 was studied to optimize the shake flask culture conditions for the PHB accumulation. The methanol production capabilities of methanotrophic bacteria with various PHB content were evaluated. The correlation between total methanol production capacities and microbial PHB content was investigated. Also, the effects of methanol biosynthesis process on survival and recovery of *M. trichosporium* IMV 3011 were examined.

Materials and methods

Microorganism and culture conditions

M. trichosporium IMV 3011 cells were obtained from the Russia Institute of Microbiology and Virology (Kiev. Ukraine). The following basal mineral salt medium was used for routine *M. trichosporium* IMV 3011 strain maintenance (g/l): NH_4Cl, 0.5; K_2HPO_4, 0.49; $KH_2PO_4 \cdot 7\,H_2O$, 0.40; $MgSO_4 \cdot 7\,H_2O$, 0.3; $CaCl_2 \cdot 2\,H_2O$, 0.02; KNO_3, 1.6; NaCl, 0.3; $FeSO_4 \cdot 7\,H_2O$, 0.004; $CuSO_4 \cdot 5\,H_2O$, 0.004; $MnSO_4 \cdot H_2O$, 0.0004; $ZnSO_4 \cdot 7\,H_2O$, 0.00034; $Na_2MoO_4 \cdot 2\,H_2O$, 0.00024; pH 7.0.

Under routine cultivation conditions, liquid cultures were grown in 50 ml medium in 500 ml Shake-flask. Shake-flask were stoppered with rubber seal and gassed with a methane: air (1:1, v/v) gas mixture. The gas-to-liquid ratio in the flasks was 9:1. The gas phase was replenished every 12 h with the same gas mixture. The cultivation of cells was carried out at 30 °C for about 96 h.

Under PHB accumulation cultivation conditions, various concentrations of $CuSO_4$, NH_4Cl and KNO_3 were added to mineral salt medium as described in the text for manipulation of PHB content in the cells and evaluation the effect of PHB on the bacterial capacity to synthesize methanol. Also, the cultivation time were prolonged as described in the text.

After vigorous shaking of the cultures to resuspend bacterial clumps, cells were harvested by centrifuge at 9,000 g for 10 min and washed twice with 20 mM phosphate buffer (pH 7.0) containing 5 mM $MgCl_2$ and resuspended in the same solution to give a cell density of 3.0 mg dry cell wt/ml.

PHB analysis

The PHB contents of cells were measured as described earlier [20]. Cell suspensions (three 200 µl replicates) were applied to Whatman glass fiber disks (GF/C, 2.1 cm). The fiber disks mounted on glass pins were dried at 105 °C for 10 min. The cells were digested in 150 µl of 5.25% sodium hypochlorite solution for 1 h and dried again. Warm chloroform was applied three times, and the disks were transferred to test tubes, sequentially washed twice with distilled water, ethanol, and acetone, and dried again in a 105 °C oven. Concentrated H_2SO_4 (2 ml) was added to the test tube, which was then sealed with Teflon-lined caps and heated in a water bath at 100 °C for 15 min. The absorbance of the reacted solutions was measured at 235 nm. An extinction coefficient of 15,500 $M^{-1}\,cm^{-1}$ (Ward and Dawes 1973) was used to calculate the PHB content of the cells. Cell-free blanks were treated with the same procedure. Pure PHB (Sigma) was used for standards and measured with the same method to check the validity of the extinction coefficient used.

Analytical methods

Methanol, formaldehyde and formate were determined chromatographically using a gas chromatograph equipped with a capillary GC column (0.23 mm × 30 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/h. The temperatures of the column, detector, and injector were 60 °C, 180 °C, and 180 °C, respectively. The retention time of methanol, formaldehyde and formate standards were 3.623, 4.242 and 4.506 min respectively. To estimate the concentration of methanol produced, 0.5 μl of the reaction solution was used for GC measurements. The concentration of methanol was calculated by using peak areas for the characteristic methanol band in the chromatogram. A calibration curve was established for aqueous methanolic solutions with known concentrations of methanol.

Formaldehyde was also determined by reaction with the acetylacetone reagent (Malashenko *et al*. 2000).

Methanol production by resting cell suspensions

Cells were harvested as described above. The methanol produced in the batch reaction was carried out as follows. Batch experiments were conducted in 100 ml sealed conical flasks (under atmospheric pressure) containing 10 ml washed cell suspension. The conical flask was tightly sealed with the Teflon-sealed septa. Reaction was initiated by replacing 50 ml of air in the head space of the conical flasks with 50 ml of CO_2 using a gas-tight syringe. The flasks were incubated at 30 °C in a rotary shaker (150 rpm). Reaction solution (0.5 μl) from the conical flasks was analyzed by GC for the formation of methanol at different time intervals.

Repetitive batch experiments and the measurement of total methanol synthesis capacities of cell suspension

The repetitive batch experiments were conducted in a 100 ml sealed conical flasks (under atmospheric pressure) containing 10 ml washed cell suspension. Reaction was initiated as described above. Methanol synthesis was stopped after 24 h. The contents of the conical flasks were centrifuged at 12,000 g for 2 min, the supernatants were removed, and the cells pellets were resuspended in fresh 20 mM phosphate buffer (pH 7.0) containing 5 mM $MgCl_2$. (at a same cell concentration of 3 mg dry weight cell/ml), 50 ml of air in the head space of the flask was replaced with 50 ml of CO_2 using a gas-tight syringe and the cycle was repeated. This cyclic procedure was continued until the subsequent methanol production ceased after seven or eight cycles. Total methanol production capacity is calculated by adding the cell-dependent methanol formation during each cycle.

Cell enumeration

The total number of cells was determined in 0.1 ml aliquots fixed with 0.1 ml of 4% formaldehyde. Direct counting was carried out with a Petrof Hausser bacteria counting chamber and a Nikon microscope. Samples for spread plate counts were taken from *M. trichosporium* IMV 3011 cell that catalyze 8 batches CO_2 reduction reaction. After serial dilutions, samples were plated onto basal mineral salt agar plates and incubated at 30 °C with an initial atmosphere of 50% methane in air. Colonies were counted after 10 days of incubation.

Electron microscopy

After the samples had been washed (Na-phosphate buffer), pre-fixed (3% glutardialdehyde) and rinsed (Na-phosphate buffer), they were fixed using 1% osmium tetroxide. They were then washed a number of times, dewatered, subjected to block contrasting (phosphoric tungsten acid, uranyl acetate) and embedded in Durcupan. The sections produced using an ultramicrotome and an electron microscope (J EM2100cx, Japan) was used.

Results and discussion

Methanol production by resting cells pre-grown under routine cultivation conditions

M. trichosporium IMV 3011 is a strain of type II methanotroph (Xin *et al.* 2002). As shown in Fig. 1, in methanotrophs methane is oxidized to carbon dioxide in a linear pathway. Reducing carbon dioxide to methanol is the reverse of the oxidation of methanol. It has been found that resting cell suspensions of *M. trichosporium* IMV 3011 can reduce carbon dioxide to methanol, which accumulated in the reaction medium. No product peak other than methanol from carbon dioxide reduction was detected. For the whole cell-catalyzed pathway to synthesize methanol, three dehydrogenases catalyzing the sequential reduction of carbon dioxide to methanol may be in close proximity to one another within the cell. Thus, reducing equivalents, substrates and intermediates have shorter distances to travel to each enzyme. Product of one enzyme acts as a substrate of other, and is available for the active site of next enzyme without much diffusion. So no formaldehyde or formic acid was observed in the carbon dioxide incubations. In this study, methanol obtained from carbon

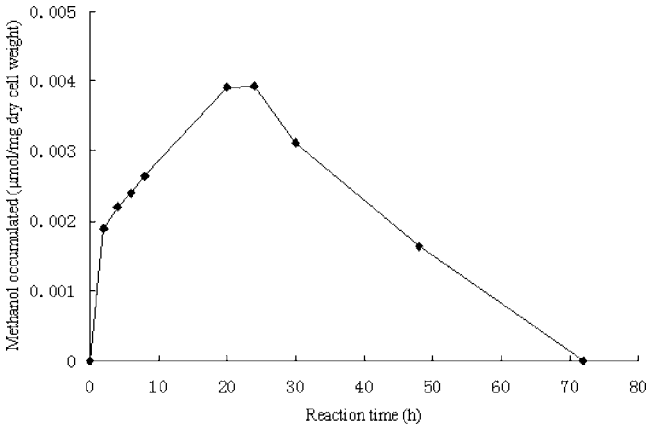

Figure 2. Time course of methanol production by cell suspensions of *M. trichosporium* IMV 3011.
M. trichosporium IMV 3011 cells were obtained from routine fermentation conditions for 96 h. Cell concentration: 3.0 mg dry cell wt/ml.

dioxide reduction was accumulated and excreted out of the cells. The production of methanol from carbon dioxide reached a maximum after 24 h of incubation. The amount of methanol slowly declined after further incubation (Fig. 2), perhaps through enzymatic or nonenzymatic degradation of methanol, depletion of intracellular reducing equivalents (eg. NAD(P)H or PQQH), the loss of CO_2 by leak and product inhibition etc.

The effect of product inhibition, enzymatic or nonenzymatic degradation of methanol and the loss of CO_2 by leak can be overcome or reduced by resuspending the cells in fresh medium and conducting a repetitive batch experiments as described in materials and methods section. However, in the repetitive batch methanol production experiments, cells also lost almost 100% of their initial methanol production ability after 4 repetitions of the process. This may be attributed to depletion of the endogenous intracellular reducing equivalents (eg. NAD(P)H or PQQH).

PHB as possible source of endogeneous reductants for CO_2 reduction

The ability of methanotrophs to transform CO_2 to methanol may be limited by reducing equivalent consumption. The transformation, however, is of no benefit to the cells as they typically consume reducing equivalent from this transformation. *M. trichosporium* IMV 3011 can accumulate PHB as carbon source and energy source reserve. Fig. 3 showed an electron micrograph of an ultra-thin section of cells of the strain *M. trichosporium* IMV 3011. PHB exists as discrete inclusions or granule in the bacterial cells.

With the intention of enhancement of the capacity of carbon dioxide conversion to methanol, PHB was chosen as a source of reducing power in our experiment. To evaluate PHB as a possible source of reducing power, the effect of its monomer, 3-hydroxybutyrate, was examined as the test substrate. As shown in Fig. 4, addition of 3-hydroxybutyrate enhanced the methanol production capacity in the repetitive batch CO_2 conversion reaction. There are at least two possible explanations for the result. The first is that the PHB in resting cells can be used as a direct or indirect source of reducing power. The second is that PHB is a marker for a higher reducing power state in the cells; i.e., when internal reducing power is in excess of growth needs, the cells produce PHB. Such a higher reducing power

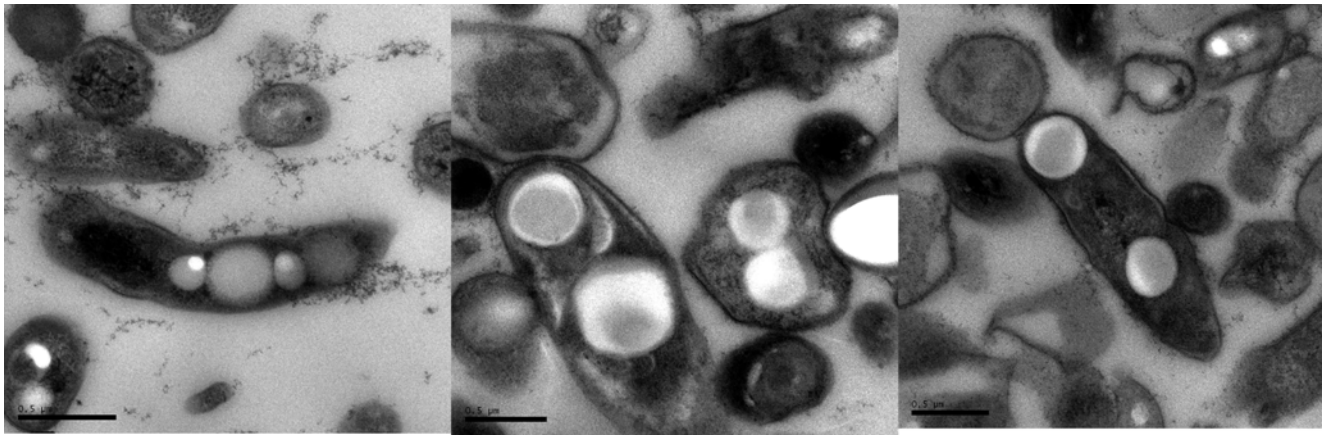

Figure 3. Transmission electron micrograph of *M. trichosporium* IMV 3011.
Lipid storage granules are the electron-dense (light-colored) inclusions inside the cells. *M. trichosporium* IMV 3011 contain PHB when grown under the routine conditions used in these experiments.

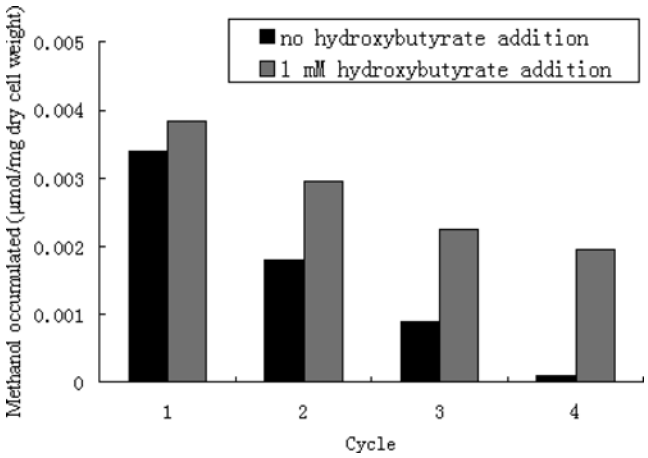

Figure 4. Repetitive batch synthesis of methanol from carbon dioxide with *M. trichosporium* IMV 3011
M. trichosporium IMV 3011 cells were obtained from routine fermentation conditions for 96 h. Cell concentration: 3.0 mg dry cell wt/ml.

state could then result in the higher methanol production capacities in the cells. The higher reducing power state could be represented by a high NADH-to-NAD^+ ratio or, indirectly, by other metabolites with capacity to produce NADH.

Effect of nitrate, ammonium and copper on PHB accumulation in growing cells

PHB formation can be affected by various conditions. It has also been reported that the synthesis of PHB is stimulated in cells grown under nutrient-limited conditions, including nitrogen, phosphate, or oxygen limiting condition (Shah *et al.* 1996). Also, the first step in the conversion of methane into PHB is catalyzed by methane monooxygenase (MMO). It has been found that the form and catalytic activity of MMO can be controlled by Cu (Park *et al.* 1991, 1992, Stanley *et al.* 1983). In an attempt to increase PHB accumulation in the earlier stages of fermentation, cell protein synthesis was limited by further reducing the level of nitrogen in the culture medium containing various concentration of Cu. The nitrogen limitation was imposed by decreasing the liquid medium KNO_3 and NH_4Cl concentration. As shown in Fig. 5, for cells cultured in medium containing 0 g/l, 0.002 g/l and 0.004 g/l $CuSO_4 \cdot 5\ H_2O$, a similar pattern associated with KNO_3 or NH_4Cl reduction was observed. Under these initial Cu concentration, the reduction of KNO_3 by factors of 2 (from 1.6 g/l to 0.8 g/l), 4 (from 1.6 g/l to 0.4 g/l) and 8 (from 1.6 g/l to 0.2 g/l) did not result in an obvious increase in PHB accumulation with 168 h fermentation. However, one-fifth NH_4Cl (0.1 g/l) resulted in the highest PHB accumulation with 168 h fermentation. Further reduction of NH_4Cl resulted in an obvious decrease in PHB accumulation with 168 h fermentation. This indicated that at NH_4Cl concentration lower than 0.1 g/l, the synthesis of the enzymes catalyzing the conversion of methane into PHB may be limited by further reducing the level of nitrogen in the culture medium. Based on the results, 1.6 g/l KNO_3 and 0.1 g/l NH_4Cl were chosen in the culture medium to use in subsequent studies.

As shown in Fig. 5, in addition to the Nitrogen depletion, a limitation of Cu may trigger further accumulation of PHB by this bacterium. These data are in agreement with previous reports that a Cu limitation triggers the accumulation of PHB in methane-grown bacteria (Shah *et al.* 1996). Because the stress caused by the NH_4Cl and Cu limitation resulted in a high cellular PHB content, Cu was further chosen as the effect factor to induce PHB accumulation in our experiment. *M. trichosporium* IMV 3011 was cultivated in mineral salt medium with various concentrations of $CuSO_4$. It was of interest to measure the PHB contents of batch-cultured cells in medium containing varying Cu concentration. As shown in Fig. 6, *M. trichosporium* IMV 3011 cells grown in mineral salt medium containing 0.002 g/l $CuSO_4 \cdot 5\ H_2O$ exhibit a highest PHB accumulation, a PHB accumulation of 41.0% was reached at 168 h. For cells cultured in mineral salt medium lacking Cu, a PHB accumulation of 7.5% (w/w) was reached at 168 h. The increased PHB contents in the presence of copper may be due to an improved efficiency with which *M. trichosporium* IMV 3011 produces energy (NADH and/or ATP) during methane oxidation to CO_2. However, further higher copper concentration adversely affect the PHB accumulation. $CuSO_4 \cdot 5\ H_2O$ concentration exceeded 0.002 g/l resulted in a lower PHB accumulation. When batch cultivation were carried out under 0.003 g/l and 0.004 g/l $CuSO_4 \cdot 5\ H_2O$ concentration, PHB accumulation was decreased to only 30.3% and 12.2% at 168 h, respectively. The reason for this may be an excess of copper suppress the large-stage rise in PHB. However, the reduction of $CuSO_4$ concentration from 0.004 g/l to 0.002 g/l resulted in slightly lower cell yields (data not shown). This observation demonstrates that copper has an important role in controlling the PHB accumulation of *M. trichosporium* IMV 3011. These data suggest that it is possible to increase the internal PHB content in cells by Cu concentration control. Hence, with regard to *M. trichosporium* IMV 3011, mineral salt medium containing 0.002 g/l $CuSO_4 \cdot 5\ H_2O$ was recommend for the PHB accumulation.

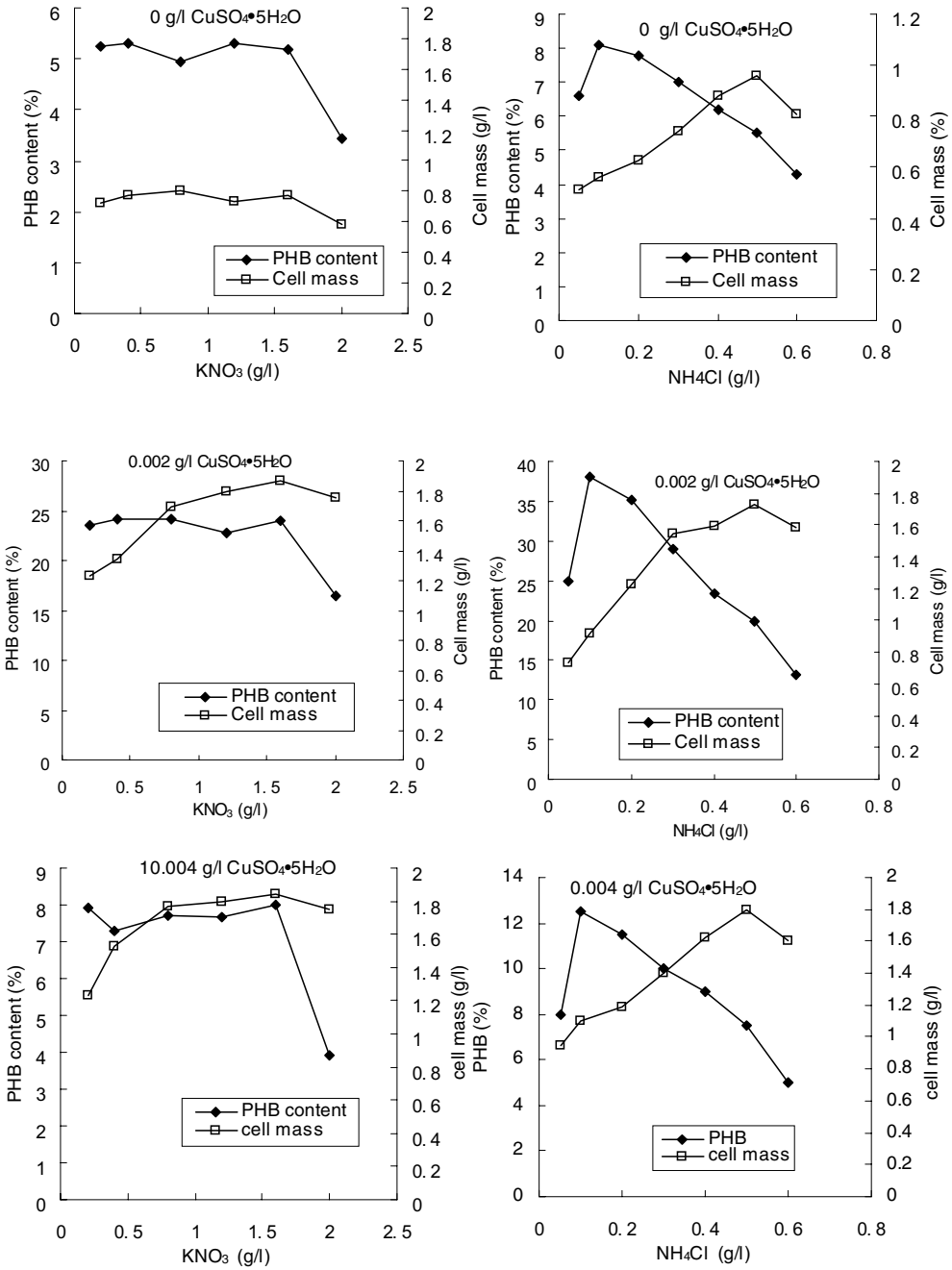

Figure 5. The effect of nitrogen concentration on growth and PHB accumulation. Cultivation time: 168 h.

PHB accumulation during growth in optimized culture medium and its effect on total methanol production capacity of the cells

PHB content in cells could be manipulated by incubation at different time. As shown in Fig. 7, for cells cultured in mineral salt medium containing 0.002 g/l $CuSO_4 \cdot 5\ H_2O$, 1.6 g/l KNO_3 and 0.1 g/l NH_4Cl, the PHB contents remained constant at 4.0% (wt/wt) during the first 48 h. This PHB level then rised markedly to 41% of the dry cell weight at 168 h.

PHB content and the methanol synthesis ability in a sample that was shaken in CO_2 were monitored as shown in Table 1, for cells obtained after 168 h cultivation in mineral salt medium lacking Cu, the cells contained a low level of PHB (7.5%). After 4 repetitions of the batch process (each cycle is 24 h in length), most of

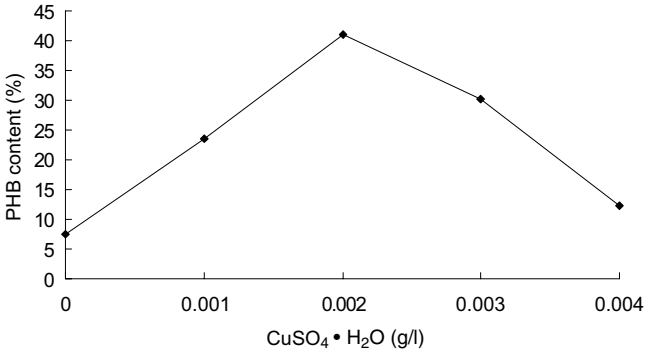

Figure 6. The effect of Cu concentration on PHB accumulation.

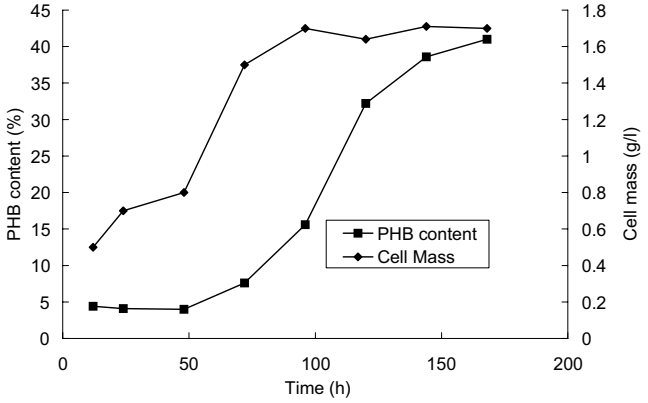

Figure 7. Growth and PHB accumulation of *M. trichosporium* IMV 3011.

the PHB disappeared. Cells lost almost 100% of their initial methanol production ability. For cells obtained after 168h cultivation in mineral salt medium containing 0.002 g/l $CuSO_4 \cdot 5\ H_2O$, the cells contained a high level of PHB (41%). The cells containing 41% PHB lost one-third of their accumulated PHB after 4 repetitions of the batch process (each cycle is 24 h in length). Furthermore, they retained 53% of their methanol synthesis activity after 4 repetitions of the process (the total methanol production capacity was 0.026 (µmol/mg dry cell wt)). However, slight depletion of PHB has been found in the control batch with N_2 instead of CO_2. It is suspected that the methanotrophic bacteria may rely on PHB for a source of electrons and energy during starvation. Also, no methanol has been found in control batch with N_2 instead of CO_2.

Table 2 Summarizes the PHB contents and the corresponding total methanol production capacities for cell samples cultivated under optimum mineral salt medium at different time. The repetitive batch reaction was continued until the subsequent methanol production ceased after seven or eight cycles. Total methanol production capacity is calculated by adding the cell-dependent methanol formation during each cycle.

A correlation between the amount of PHB in the cells and the total methanol production capacity was found. The total methanol production capacity of resting cells containing 38.6% PHB was 1.9-fold greater than that of cells containing only 21.5% PHB. It is proposed that PHB serve as an endogenous source of electrons for CO_2 reduction. However, the total methanol production capacity slightly decreased when the cellular PHB contents exceeded 38.6%. The reason for this may be dilution of the dehydrogenase system in the cells with increasing PHB contents. Hence, with regard to *M. trichosporium* IMV 3011 the application of resting cells with a PHB content of 38.6% was recommend for the methanol synthesis from CO_2.

Survival and recovery of the cells after repeated batches of methanol production

In the present study, we examined the effects of methanol production process on survival and recovery of *M. trichosporium* IMV 3011. The control condition were obtained by replacing CO_2 with N_2. The total number of cells in a sample were monitored, samples for spread plate counts were taken from methanotrophic cell catalyzing 8 batches methanol production reaction. After serial dilutions, samples were plated onto agar plates and incubated at 30 °C with an initial atmosphere of 50% methane in air. Colonies were counted after 10 days of incubation. The total number of cells (direct counts) did not change obviously after 8 repetitions of the process. The recovery from CO_2

Table 1. The depletion of PHB level and the methanol synthesis ability of resting cells during repetitive batch synthesis of methanol.

Batch	Cells containing 7.5% of PHB		Cells containing 41.0% of PHB	
	Residual PHB in cell (%)	Methanol accumulated (µmol/mg dry cell weight)	Residual PHB in cell (%)	Methanol accumulated (µmol/mg dry cell weight)
1	4.1	0.0034	36.6	0.0036
2	2.3	0.0018	33.2	0.0030
3	<1.0	0.0009	29.8	0.0030
4	<1.0	0.0003	27.0	0.0027

Table 2. The PHB contents and the corresponding total methanol production capacities.

Culturing time at 30 °C (h)	PHB in cell (%)	Total methanol production capacity (μmol/mg dry cell wt)
96	21.5	0.016 ± 0.002
120	32.2	0.022 ± 0.001
144	38.6	0.031 ± 0.001
168	41.0	0.026 ± 0.002

Table 3. Direct counts and plate counts for *M. trichosporium* IMV 3011.

	Direct counts[a] (10^9 cells/mL)		Plate counts[a] (10^9 CFU/mL) after batch 8	Recovery[b] (%)
	batch 0	batch 8		
Control	5.32	5.21	4.25	80
CO_2 reduction reaction	5.01	4.86	3.80	76

[a] Results are the means ± standard errors for triplicate cultures. For direct counts, the standard errors of the means were no more than 5%.
[b] Recovery was calculated as (plate count after batch 8/direct count batch 0) × 100.

reduction reaction and control was 76% and 80% of the original number of cells, respectively. Recovery on agar plates incubated for 10 days with 76% indicated that a large fraction of the population remained culturable (Table 3). The results presented here show that CO_2 reduction can be not detrimental to the viability of methanotrophs. However, Cell growth on plates was slow, and some colonies were visible only after more than 9 days of incubation.

Conclusions

In this paper, we found that the catabolism of stored Poly-β-Hydroxybutyrate (PHB) can provide intracellular reducing equivalents to improve the intrinsic methanol production capacity. Although the PHB production capabilities of methanotrophs have been well documented, the concept of capitalizing upon this characteristic for the enhancement of methanol production from CO_2 reduction is novel.

The results shown that the cell of methanotrophic bacteria with appropriate PHB storage can long-term catalyze the reduction of CO_2, in which the origin of the reducing equivalent is hydrogen from methane. It is theoretically possible that the overall reaction can obtain methanol without adding to the greenhouse effect. Also, it's an efficient, environmentally friendly, renewable process.

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