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Biosynthesis of Methanol from CO_2 and CH_4 by Methanotrophic Bacteria

Jia-Ying Xin, Jun-Ru Cui, Jian-zhong Niu, Shao-feng Hua, Chun-Gu Xia, Shu-Ben Li and [1]Li-Min Zhu
State Key Laboratory for Oxo Synthesis and Selective Oxidation, Lanzhou Institute of Chemical Physics, Chinese Academy of Sciences, Lanzhou 730000, China
[1]College of Chemistry and Chemical Engineering, Donghua University, Shanghai 200051, China

Abstract: Methanotrophs can oxidize methane to carbon dioxide through sequential reactions catalyzed by a series of enzymes including methane monooxygenase, methanol dehydrogenase, formaldehyde dehydrogenase and formate dehydrogenase. Reducing carbon dioxide to methanol is an opposite reaction of methanol oxidation, which requires a considerable amount of energy. So far there are no known organisms whose normal biological role is reduction of carbon dioxide to methanol. In this paper, we report that bioconversion of carbon dioxide to methanol was successfully achieved using resting cells of methanotrophic bacteria of *Methylosinus trichosporium* IMV 3011 as biocatalysts. Extracellular methanol accumulation has been found in the carbon dioxide incubations. The conversion of carbon dioxide to methanol is energy-intensive and requires reducing equivalent to push the reaction along against energy laws. For long-term maintenance of methanol synthesis, methane was selected as a substrate for regeneration of reducing equivalent. By alternate reaction and regeneration, the results show that resting cell of *M. trichosporium* IMV 3011 can be used for many times to catalyze the reduction of carbon dioxide, in which the origin of the reducing equivalent is methane. It is possible to theoretically deduce that the overall reaction for methanol synthesis can be completed by biocatalysis using greenhouse gases (carbon dioxide and methane) as raw materials without adding hydrogen. This new route should be environmentally benign and a selective process operating at room temperature and normal pressure. Also, it is theoretically possible that the overall reaction can produce methanol without adding to the greenhouse effect.

Key words: Carbon dioxide reduction, methane oxidation, methanol production, methanotrophic bacteria, reducing equivalent regeneration

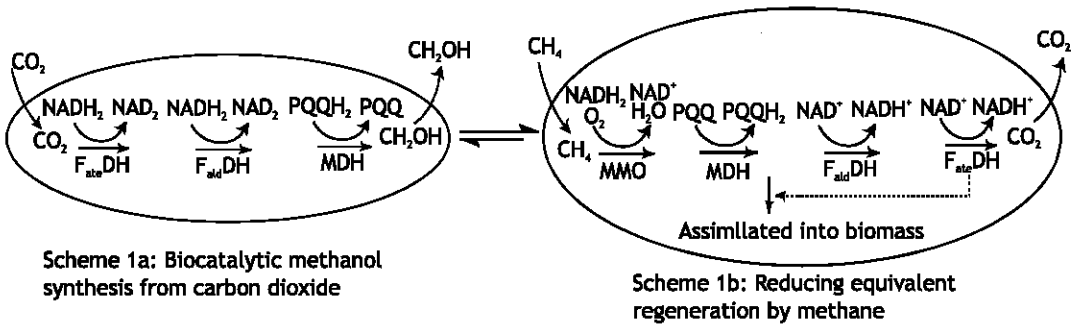
INTRODUCTION

Methanol is used in the chemical industry in a variety of ways. Methanol is a building block for other chemicals. It can be used directly as an alternative fuel source, as antifreeze and as a precursor to other compounds. Methanol can be produced chemically via methane, carbon dioxide, biomass, coal, heavy fuel oils etc[1-5]. Synthesis of methanol from carbon dioxide has several obvious advantages. For example, carbon dioxide is one of the greenhouse gases, which have been theorized to contribute to global warming. However, the conversion of carbon dioxide to methanol is fundamentally difficult to achieve under mild condition in chemical catalysis. Conventional methods for synthesizing methanol from CO_2 suffer from certain drawbacks such as inefficiencies, costs, high energy consumption and the need for special equipment adapted for high temperature or highly corrosive environments. Enzymatically coupled sequential reduction of CO_2 to methanol, using a series of reaction catalyzed by three different dehydrogenases, is particularly appealing[6]. 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 CO_2 that are difficult to achieve using traditional chemical methods. However, the process presents a number of 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 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.

Accordingly, a new technique for conversion of carbon dioxide to methanol that alleviates such drawbacks is desired.

We have investigated whether intact microorganisms can be used as biocatalysts for the reduction of CO_2 to

Corresponding Author: Shu-Ben Li, State Key Laboratory for Oxo Synthesis and Selective Oxidation, Lanzhou Institute of Chemical Physics, Chinese Academy of Sciences, Lanzhou 730000, China



Scheme 1: The pathway of methanol synthesis from carbon dioxide (a) and reducing equivalent regeneration by methane (b)
$F_{ate}DH$: Formate Dehydrogenase $F_{ald}DH$: Formaldehyde Dehydrogenase
MDH: Methanol Dehydrogenase MMO: Methane monooxygenase

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 CO_2 to methanol.

There are microorganisms called 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. The reducing equivalents produced in these reactions are partly sent to the electron transport chain for ATP synthesis and partly used in the methane monooxygenase reaction. The CO_2 produced from methane oxidation is partly emitted and partly incorporated into cell biomass via the serine pathway[7].

Reducing CO_2 to methanol is the reverse of the oxidation of methanol. In this paper, we show that it is possible and feasible to reduce CO_2 to methanol by methanotrophic whole cells containing the enzymes formate dehydrogenase, formaldehyde dehydrogenase and methanol dehydrogenase, despite the fact that these enzymes normally oxidize their substrates *in vivo* or *in vitro* (Scheme 1a.). Since methane monooxygenase cannot effectively catalyze the reverse reaction of methane monooxygenation, extracellular methanol accumulation has been found in the CO_2 incubations. For maintenance of methanol synthesis for a long time, methane was selected as a substrate for reducing equivalent regeneration (Scheme 1b.).

MATERIALS AND METHODS

Culture of *Methylosinus trichosporium* IMV 3011: *Methylosinus trichosporium* IMV 3011 cells were obtained from the Institute of Microbiology and Virology (Kiev. USSR). The following nitrate mineral salt medium was used for the methanotroph large-scale cultivation, (g/l): NH_3Cl, 0.5; K_2HPO_4, 0.49; $KH_2PO_47H_2O$, 0.40; $MgSO_47H_2O$, 0.3; $CaCl_22H_2O$, 0.02; KNO_3, 1.6; NaCl, 0.3; $FeSO_47H_2O$, 0.004; $CuSO_45H_2O$, 0.004; $MnSO_4H_2O$, 0.0004; $ZnSO_47\ H_2O$, 0.00034; $Na_2MoO_4\ 2H_2O$, 0.00024; pH 7.0. Large-scale cultivation of cells was carried out in a 15 liter, continuous-gas feed fermentor (LH1075, UK) under an atmosphere of methane and air (1:10, v/v) at 32°C for about 96 h. Cells were harvested by centrifuge at 9,000 g for 10 min and washed twice with 20 mM phosphate buffer (pH 7.0). Cells were resuspended in the same buffer containing 5 mM $MgCl_2$ and used in batch experiments.

Analytical methods: Methanol formation from carbon dioxide was determined chromatographically using a gas chromatograph equipped with a capillary GC column (ϕ0.23 mm × 30 m; stationary phase, SE-54) and a flame ionisation detector (FID). Pure nitrogen served as the carrier gas at a flow rate of 75 ml min^{-1}. The temperatures of the column, detector and injector were 60, 180 and 180°C, respectively. To evaluate the concentration of methanol produced as a result of the cell suspension catalyzed reaction, 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.

Repetitive batch experiments: The batch experiments were conducted in a 100 ml sealed conical flasks (under atmospheric pressure) containing 10 ml washed cell suspension. The gaseous phase of the flask was replaced by carbon dioxide. The flasks were incubated and shaken at 32°C in a rotary shaker (150 rpm). Methanol synthesis was stopped after 24 h. The contents of the conical flasks

were centrifuged and the cells were resuspended in 20 mM phosphate buffer (pH7.0) containing 5 mM $MgCl_2$ (at a same cell concentration of 3 mg dry weight cell. ml^{-1}), then regenerated with methane and air (1:10, v/v) in a shaker operated at 32°C and 150 rpm. After 12 h regeneration, the cell suspension was reused for the next batch experiment. All values are average of three runs with a variation of 3-5% from the mean.

RESULTS AND DISCUSSION

The feasibility of methanol bioconversion from carbon dioxide by methanotroph: The experiments were first conducted in a 100 ml sealed conical flasks (under atmospheric pressure) containing 10 ml cell suspension. The gaseous phase of the flask was replaced by gaseous CO_2. The flasks were incubated and shaken at 32°C in a rotary shaker (150 rpm). Chromatographic determinations of the liquid phase were carried out at different reaction times. It has been found that resting cell suspensions of *M. trichosporium* IMV 3011 can reduce CO_2 to methanol, which accumulated in the reaction medium. No product peak other than methanol from CO_2 reduction was detected. For the whole cell-catalyzed pathway to synthesize methanol, three dehydrogenases catalyzing the sequential reduction of CO_2 to methanol are in close proximity to one another within the cell. Thus, reducing equivalents, substrates and intermediates have shorter distances to travel to each enzyme. So no formaldehyde or formic acid was observed in the CO_2 incubations. The oxidation of methane is catalyzed by methane monooxygenases (MMO), which are classical monooxygenases that 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 CH_3OH[13]. In this study, methanol obtained from CO_2 reduction was accumulated and excreted out of the cells. The result led us to assume that the MMO is not able to catalyze the reverse reaction of the methane monooxygenation.

Control experiments with heat-killed cells indicated that the methanol was produced enzymatically. The production of methanol from CO_2 reached a maximum after 24 h of incubation in batch experiments. The amount of methanol slowly declined after further incubation, perhaps through enzymatic or nonenzymatic degradation of methanol, depletion of intracellular reducing equivalents (eg. NAD(P)H or PQQH) or due to product inhibition.

The effect of CO_2 concentrations: Various concentrations of CO_2 were used to examine the production of methanol by resting cell suspensions of *M. trichosporium* IMV 3011. Nitrogen was used to balance the rest of the gaseous phase. The amount of methanol produced was assayed after 6 h of incubation. A CO_2 concentration in the gaseous phase of 40% supported maximum methanol production. Higher CO_2 concentration did not stimulate nor inhibit the production of methanol. The reaction rate appears to be dependent upon the solubility of CO_2.

The effect of methane on the production of methanol: Of course, in the methanotrophic bacteria, methane can also be oxidized into methanol, but the product methanol is subsequently oxidized by dehydrogenases[8,9]. To obtained methanol from methane by methanotrophic bacteria, an inhibitor for methanol dehydrogenase must be used to prevent further oxidation. Also, equal molar reducer such as formate must be used to drive the reaction[8,9]. This in turn perhaps consumes more expensive material, defeating the purpose of the initial conversion.

In this paper, the effect of methane on the production of methanol was also studied. The production of methanol by cell suspension of *M. trichosporium* IMV 3011 was assayed in the presence of a given amount of methane. The initial concentration of CO_2 in the gaseous phase was kept constant (40%) in all of the experiments. As shown in Fig. 1. methane supply favors methanol accumulation. The result suggests that methane can be simultaneously oxidized into methanol during reduction of CO_2 to methanol and the further oxidation of methanol can be perfectly inhibited in the presence of an excess of CO_2. In contrast, no methanol was produced when methane and oxygen were used solely as substrates.

The effect of cell concentrations: The cell density in reaction solution also influences the rate of methanol production. The amount of methanol accumulated after 6 h incubation increased as the concentration of cell was increased up to about 3 mg ml^{-1} and no further increase in methanol production was observed at higher cell concentrations. This indicated that at cell concentrations higher than 3 mg ml^{-1}, the solubility of the gaseous substrates becomes the rate-limiting factor in the production of methanol.

Alternate reaction and regeneration of *M. trichosporium* IMV 3011: Converting CO_2 to methanol is essentially the reverse of the oxidation process. Whereas oxidation releases a large amount of reducing equivalent, logically the reverse would require a considerable amount. 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

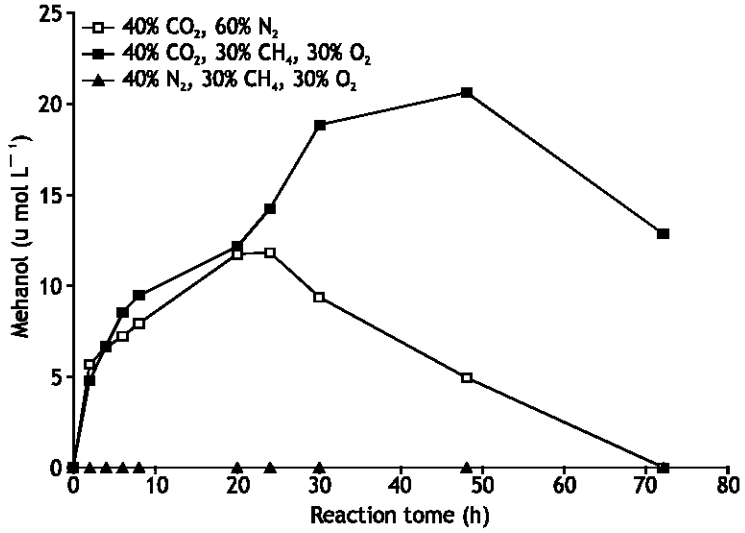


Fig. 1: Time course of methanol production by cell suspensions of *Methylosinus trichosporium* IMV 3011

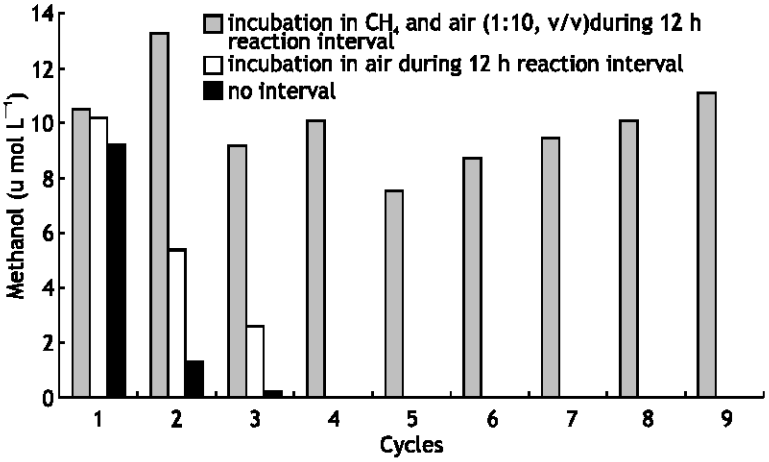


Fig. 2: Repetitive batch synthesis of methanol from CO_2 with *M. trichosporium* IMV 3011

they typically consume reducing equivalent from this transformation. As shown in Fig. 2, in the repetitive batch CO_2 conversion experiments, cells lost almost 100% of their initial methanol synthesis ability after 2 repetitions of the process.

For maintenance of methanol biosynthesis for a long time, methane was selected as a substrate for reducing equivalent regeneration. The inviable cell suspension of *M. trichosporium* IMV 3011 was *in vivo* regenerated with methane and air (1:10, v/v) in the flask for 12 h at 32°C. After the regeneration process, the cell suspension was continuously (or immediately) used to produce methanol. As shown in Fig. 3, the rapid resumption of reduction activity of the inviable cells suggests that a rapid process (reducing equivalent regeneration) was responsible, rather than slower processes (generation of additional cells or of dehydrogenase enzyme). As shown in Fig. 2, the inviable cell still partly retained the ability to synthesize methanol from CO_2 after 12 h incubation without methane. It is suspected that the methanotrophic bacteria may rely on their endogenous reserves (eg., lipid inclusions) for a source of electrons and energy during starvation.

As shown in Fig. 2, by alternate and intermittent regeneration of inviable cell, the repetitive batch methanol synthesis from CO_2 can be repeated 9 times with no obvious disappearance of methanol synthesis ability.

In the process of reducing equivalent regeneration, part of methane has been oxidized completely to CO_2. Methane is a trace gas in the atmosphere, contributing significantly to the greenhouse effect. It is 26 times more efficient in absorbing and re-emitting infrared radiation than CO_2[11,12]. The conversion of carbon dioxide to

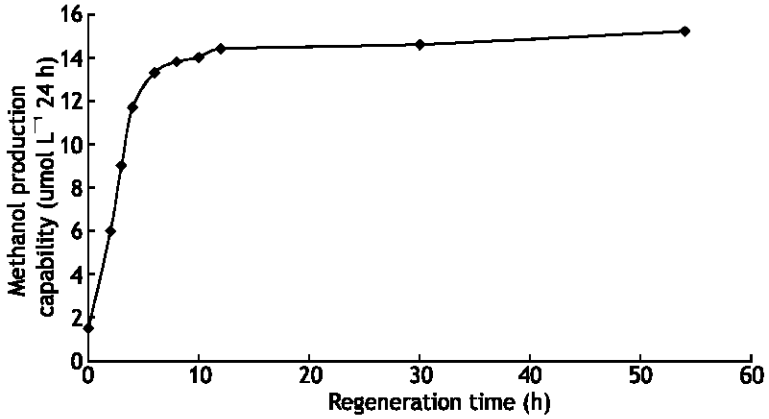


Fig. 3: Time course of methanol synthesis ability regeneration of cell of *M. trichosporium* IMV 3011 by methane and air (1:10, v/v)

methanol is energy intensive and requires reducing equivalent to push the reaction along against energy laws. Herein, the origin of the reducing equivalent is methane. Therefore, it is theoretically possible that the overall reaction can produce methanol in an efficient, environmentally friendly, renewable process without adding to the greenhouse effect.

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