

Methanol Biosynthesis by Covalently Immobilized Cells of *Methylosinus trichosporium*: Batch and Continuous Studies

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The DEAE-cellulose linked cells of *Methylosinus trichosporium* displaying high specific methane mono-oxygenase activity ($66 \mu\text{mol}$ methane oxidized/h mg cells) were used for methanol biosynthesis from biogas derived methane in a batch and a continuous cell reactor. The optimum cell-to-carrier ratio was determined to be 0.5 g cells/g dry weight cellulose. Batch experiments indicated that 100 mM phosphate ion concentration was necessary to inhibit further oxidation of methanol; excess oxygen supply favored methanol accumulation with an increase in methane conversion efficiency to 27%. A pulse of 40 mM sodium formate at the end of 6 h resulted in restoration of methanol accumulation by regenerating NADH₂ required for the sustained activity of methane mono-oxygenase. Maximum methanol level of 50 $\mu\text{mol}/\text{mg}$ cells was obtained in the batch reactor. In a standard 50-mL ultrafiltration continuous reactor, the covalently linked cells produced methanol at a continuous rate of 100 $\mu\text{mol}/\text{h}$ for the first 10 h, after which the methanol accumulation rate fell low due to the depletion of NADH₂. The methanol accumulation could be stimulated by supplying sodium formate (40 mM) in either 20 or 100 mM phosphate buffer. Maximum methanol accumulation rate of 267 $\mu\text{mol}/\text{h}$ was obtained when 20 mM formate was supplied in the feed stream containing 100 mM phosphate ions, and this level of biosynthesis was maintained for over 72 h. The stoichiometric balance made at various points of formate addition indicated that the molar amount of methanol generated at steady state is dependent on the equimolar addition of sodium formate to the feed. The half-life $t_{1/2}$ and thermal denaturation rate constant K_d were computed to be 108 h and $6.42 \times 10^{-3} \text{ h}^{-1}$, respectively.

Key words: *Methylosinus trichosporium* • methanol biosynthesis • immobilization • batch and continuous studies

INTRODUCTION

The single-step oxidation of methane to methanol is catalyzed by the NADH-dependent methane mono-oxygenase (MMO) in the obligate methanotroph *Methylosinus trichosporium*. Although some oxidation of methane to carbon dioxide through methanol has been reported with cells of *Nitrosomonas*,^{1,2} the methanotrophs catalyze this reaction at a faster rate. Due to the ability of MMO to oxygenate a wide range of

n-alkanes, substituted alkanes, ethers, and aromatic, alicyclic, and heterocyclic compounds,^{3–5} yielding corresponding alcohols, the enzyme has recently been studied for such biocatalytic conversions (for review, see ref. 6).

The microbial route of methanol biosynthesis is much more complicated than ethanol fermentation as methanol gets further oxidized in methylotrophs to formaldehyde by NADH-independent methanol oxidase/dehydrogenase activity. It has previously been reported that high concentration of phosphate ions (>80 mM) selectively inhibited methanol oxidation activity in whole cells of *M. trichosporium* NCIB 11131, leading to extracellular methanol accumulation.^{7,8} The inorganic phosphate-dependent inhibition of enzyme activity was shown to be reversible and followed uncompetitive inhibition kinetics.⁷ A study on the effect of various inhibitors, metal ions, and adenine nucleotides on methanol oxidation activity indicated the enzyme activity to be stimulated by Mg²⁺ ions and inhibited by ADP and ATP.⁹ The purpose of this investigation was to use *M. trichosporium* cells (free and immobilized) for the biosynthesis of methanol from biogas derived methane, based on our studies on the regulation of methanol oxidation activity. Methanol biosynthesis was carried out in a small batch and a continuous reactor using the covalently linked cells. The basic studies on the operating conditions of the reactor and substrate requirement for coenzyme regeneration are described. Batch experiments were performed to optimize methane and oxygen input and substrate supplied for coenzyme regeneration. In continuous experiments effects of formate and phosphate ion supply and residence time on methanol accumulation rate were studied.

MATERIALS AND METHODS

Organism

Methylosinus trichosporium 11131 used in the present study was obtained from NCIB (Aberdeen, U.K.). The culture was maintained on nitrate mineral salts (NMS)

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medium¹⁰ in an anaerobic steel container under an atmosphere of methane and air (1:1, v/v) at 30°C.

Growth

Large-scale cultivation of cells was carried out in a 2-L Biolaffite fermentor as described previously⁷ on NMS medium. Early stationary phase cells were harvested at 10,000g for 15 min at 4°C and washed twice with cold 20 mM phosphate buffer (pH 7.0). Cells were resuspended in the same buffer containing 5 mM MgCl₂ (at a cell concentration of 70–80 mg/mL) and used for immobilization.

Immobilization

The cells were immobilized on DEAE-cellulose (Sigma Chemical Co.) according to Griffith and Muir¹¹ with some modifications. Dry DEAE-cellulose was added to 15 vol of 0.5M HCl, allowed to stir for 30 min at 100 rpm, and the supernatant removed by suction filtration using a Büchner funnel. The DEAE-cellulose was then washed with distilled water until the pH of the washings was between 4.0 and 6.0. The washed preparation of DEAE-cellulose was suspended in 15 vol of 0.5M NaOH and stirred for 30 min at the same rpm; the alkali was removed by suction filtration and washed with distilled water until the washings were at neutral pH. The washed DEAE-cellulose was resuspended in 50 mM sodium phosphate buffer, pH 7.0, and glutaraldehyde (25%, w/v aqueous solution) was added to give a final concentration of 1% (w/v). After stirring for 2 h at room temperature, the DEAE-cellulose derivative was recovered by filtration and the excess glutaraldehyde removed by washing with the sodium phosphate buffer (50 mM, pH 7.0). The glutaraldehyde activated DEAE-cellulose was resuspended in buffer, and an equal volume of a suspension of whole cells containing 40 mg cells/mL of *M. trichosporium* in the same buffer was added. The clumps thus obtained were dispersed by shaking for 2 h at 100 rpm and kept at 4°C overnight. The immobilized preparation was washed with sodium phosphate buffer containing 0.5M NaCl to remove noncovalently bound cells. The immobilized whole cells were stored at 4°C and used for 15 days without any loss of methane mono-oxygenase activity.

Analytical Methods

Formate dehydrogenase activity was measured as described previously⁷ using sodium formate as the substrate. The activity is expressed as μmole CO₂ produced per hour per milligram cells.

Methane, carbon dioxide, and oxygen concentrations were determined chromatographically (GLC) using a molecular sieve 13x or 5A column in the thermal conductivity mode. Hydrogen served as the carrier gas at a

flow rate of 40 mL/min. The temperatures of the oven, detector, and injector were 40°C, 50°C, and 50°C, respectively, and current flow was 150 mA.

The calibrations were made with standard methane (5.1% in N₂), CO₂ (2.2% in H₂), and oxygen supplied by EDT research. The retention times for methane and carbon dioxide were 20 and 70 s, respectively. Oxygen was analyzed in a molecular sieve 13x column and had a retention time of 25 s.

Methanol was estimated chromatographically using a Porapak Q column in the flame ionization mode. Nitrogen was used as the carrier gas at a flow rate of 40 mL/min. The temperatures of the oven, detector, and injector were controlled at 140°C, 160°C, and 160°C, respectively. The retention time for methanol was 50 s. Absolute methanol (99.9%) from Prolabo (Paris) was used as the standard in the range 2.0–12.0 μmol/mL (or 0.02%–0.1%, v/v).

Batch Experiments

The batch experiments were conducted in 30-mL Subasealed conical flasks (under atmospheric pressure) containing 2–4 mg cells (in experiments involving free cells) or 18 mg cells (in experiments involving immobilized cells), after correcting for cell leakage, in 5 mL reaction mixture. It also contained 80–100 μmol/mL phosphate ions and 5 μmol/mL MgCl₂. The gaseous phase of the flask was replaced by methane (24–25 μmol/mL) and varying concentrations of oxygen (as described in the text). The flasks were incubated shaken (200 rpm) for 3 h at 35°C on a controlled temperature rotary shaker (Adolf Kühner, Switzerland). Gas–liquid chromatographic determinations of the gas phase (methane and oxygen) and aqueous phase (methanol) were carried out as listed above. All batch experiments were conducted in triplicate. The reported values are an average of three runs with a variation of 5–7% from the mean.

Continuous Experiments: Reactor Set-up

A standard 50-mL capacity ultrafiltration (UF) cell (Pharmacia, Sweden) was converted into a continuous stirred reactor (Fig. 1). The UF cell was filled with 50 mL of DEAE-cellulose immobilized microbial cell preparation suspended in 20 mM phosphate buffer (pH 6.4) at a density of 4 mg dry weight carrier/mL reactor volume. The top disc was sealed air tight, and two silicone rubber tubes (1 mm diameter) were inserted from the inlet hole of the UF cell. One of the silicone tubes was extended to the bottom of the cell and connected to a sparger made of a hard, plastic tube (1 mm diameter) ring with holes along the periphery. The pressure release vent of the UF cell was replaced with a glass capillary connected through a rubber stopper to be used as an outlet for the circulating gaseous

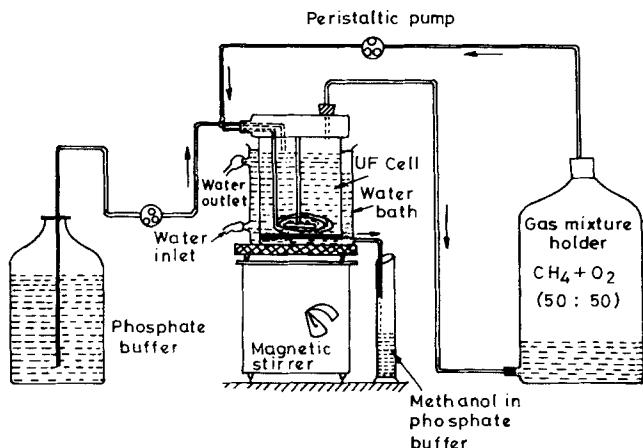


Figure 1. Continuous stirred tank reactor setup.

mixture. The temperature was maintained by circulating water at 35°C around the UF cell. A magnetic stirrer was used to stir the reactor contents.

The reaction was started by allowing mixture of gases (CH_4 and O_2 ; 1:1, v/v) to sparge through the UF cell. The gaseous mixture was continuously circulated by means of a peristaltic pump. The feed consisted of 100 mM phosphate buffer (pH 6.4) containing 5 mM MgCl_2 (and other ingredients, if required) and was fed continuously from the top of the UF cell by means of a variable-speed multichannel peristaltic pump. The second channel of the peristaltic pump was used to remove product continuously from the base of the UF cell. Samples were drawn at regular intervals to analyze for gas contents and methanol produced. The steady state was assumed to have been attained when the values of methane in the effluent, determined by successive sampling, levelled off to a constant value. The above procedure was repeated for different dilution rates.

RESULTS AND DISCUSSION

Batch Data

The immobilization of *M. trichosporium* whole cells by covalent linking to glutaraldehyde activated DEAE-cellulose was found most suitable for retaining MMO activity compared to the other immobilization methods tried in our laboratory such as physical adsorption or entrapment. No extracellular methanol accumulated in the latter two methods. The effect of changing the cell-to-carrier ratio on methanol accumulation and cell leakage is shown in Figure 2. Maximum methanol accumulation rate of 8.2 $\mu\text{mol}/\text{h}$ was obtained at 0.5 g cells/g dry weight cellulose, after which the rate fell sharply due to significant leakage of cells. The pH optimum was determined to be ranging from 6.2 to 6.6 in comparison to the reported value of 6.4 for free cells.⁷ Hence, immobilized cells appeared to be less sensitive to a pH change in this range for optimum methanol accumulation. The temperature optimum was 35°C, simi-

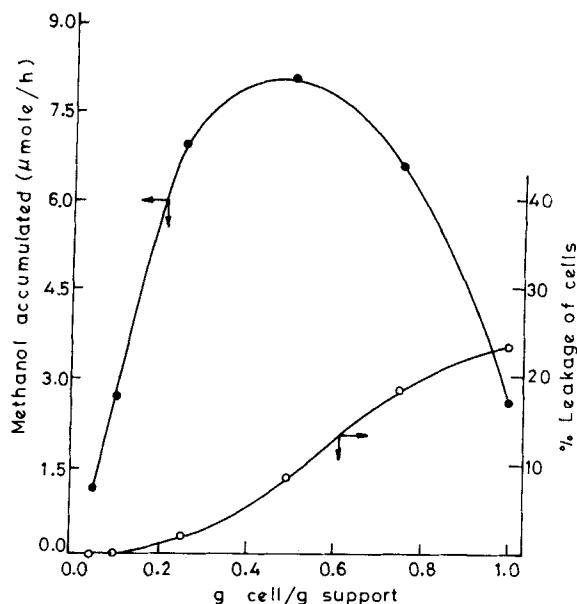
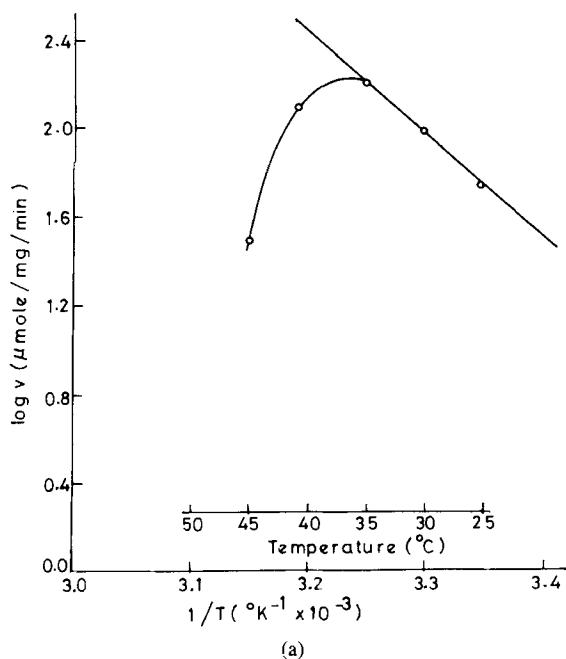


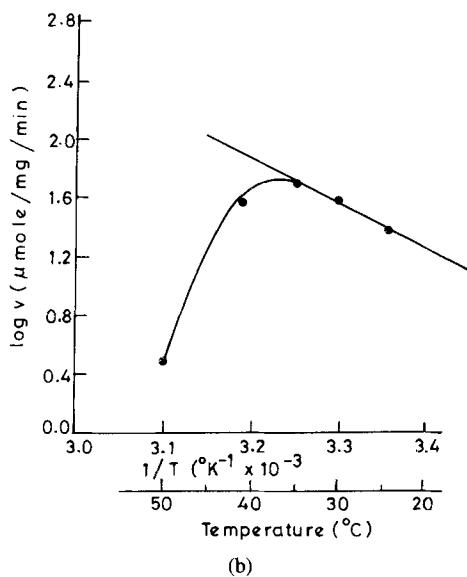
Figure 2. Effect of changing cell-to-carrier ratio on methanol accumulation.

lar to that observed for free cells. The temperature data were replotted in the form of an Arrhenius plot to calculate activation energy (E_a) for free and immobilized cells (Fig. 3). The values of E_a for free and immobilized cells were computed to be 19,537 and 14,800 cal/mol, respectively. The optimum concentration of phosphate ions required for inhibition of methanol oxidation activity was 100 mM, slightly higher than that required by the free cells (80 mM).⁷ Oxygen appeared to be the rate limiting substrate for methanol biosynthesis in immobilized cell preparations. The replacement of air with oxygen in batch cultures supported a total methanol accumulation rate of 8.92 $\mu\text{mol}/\text{h}$ mg cells, representing an increase in conversion efficiency over air supplied cells from 10% to 27% (Table I). This value is threefold higher than that reported by Hou¹² for propylene oxide synthesis using *M. trichosporium* OB3b. An increase in oxygen concentration was seen to result in decreased methane uptake rates. It has been reported that under oxygen excess (methane limiting) conditions, the enzyme MMO in *M. capsulatus* and *M. trichosporium* OB3b switches from soluble to particulate form^{6,13} and may exhibit altered reaction specificities toward different substrates. This may be responsible for the observed phenomenon.

The synthesis of methanol by immobilized cells was at a constant rate of 8.75 $\mu\text{mol}/\text{h}$ mg cells for the first 2.5 h (Fig. 4), and the rate fell sharply after 6 h. In our earlier studies with free cells, the fall in the methanol accumulation rate was attributed to depletion of the endogenous NADH₂ pool, and methanol accumulation rate was maximally stimulated by supplying 40 mM formate to the reaction vessel.¹⁴ With immobilized cells, maximum stimulation in methanol accumulation rate to 8.6 $\mu\text{mol}/\text{h}$ mg cells occurred on addition of 40 mM



(a)



(b)

Figure 3. Temperature data of enzyme activity plotted as Arrhenius plot: (a) free cells; (b) immobilized cells.

formate at the sixth hour (Fig. 4). Total extracellular methanol levels of up to 50 $\mu\text{mol}/\text{mg}$ cells were observed at the end of 12 h. The in situ regeneration of NADH₂ has been demonstrated earlier by supplying methane metabolites to free cells¹⁵ and to immobilized cells.¹² In spite of an excess of methane and formate detected in the reaction vessel, the rate of methanol accumulation dropped sharply beyond 14 h. The reasons for this are not clear.

The MMO activity in immobilized cell preparations stored at 4°C was studied over a period of 15 days. The specific MMO activity of 54.2 μmol methane consumed/h mg cells (representing over 80% enzyme activity of freshly harvested cells, i.e., 66 μmol methane oxidized/h mg cells) after 2 weeks storage was obtained only on addition of sodium formate to the reaction mixture. Thus, NADH₂ supply appeared to be critical for MMO activity in the cells stored for a long period. The reported loss of MMO activity on storage³ at 4°C might be due to NADH₂ loss rather than to MMO enzyme instability.

Methanol Synthesis in a Continuous Reactor

The covalently linked immobilized cells produced methanol at a constant rate of 100 $\mu\text{mol}/\text{h}$ in a 50-mL reactor containing 100 mg dry cell weight after attaining steady state (Fig. 5). This rate of methanol biosynthesis continued for 11 h after which a fall in the rate was observed. Based on earlier batch studies, this was attributed to depletion of intracellular NADH₂ pool. The in vivo regeneration of NADH₂ in the continuous reactor was carried out by supplying different concentrations of sodium formate in 20 or 100 mM phosphate buffer, and the results are shown in Figure 5. A continuous feed of 20 mM sodium formate in 100 mM phosphate buffer resulted in sustained methanol accumulation of 267 $\mu\text{mol}/\text{h}$, giving a productivity of 5.34 mmol/h L. The reactor was maintained at this level of productivity for up to 70 h.

The interrelationship between formate supply, NADH₂ regeneration, and methanol accumulation was

Table I. Effect of O₂ concentration on methanol accumulation in immobilized *M. trichosporium* whole cells (batch).

| Particulars | Phosphate ions (mM) | Total oxygen ($\mu\text{mol} \times 10$) | Methane uptake ($\mu\text{mol}/\text{h mg}$) | Methanol accumulation ($\mu\text{mol}/\text{h mg}$) | Conversion (%) |
|----------------------|---------------------|--|--|---|----------------|
| 1. Free cells | 20 | 15.29 ^a | 53.9 | 2.72 | 4.22 |
| | 80 | 15.29 ^a | 49.0 | 4.73 | 9.80 |
| 2. Immobilized cells | 20 | 15.29 ^a | 66.2 | 0.40 | 0.58 |
| | 80 | 15.29 ^a | 62.1 | 2.10 | 3.30 |
| 3. Immobilized cells | 20 | 73.53 ^b | 9.8 | 0.00 | 0.00 |
| | 80 | 73.53 ^b | 33.4 | 8.92 | 26.70 |

^a Rest of volume balanced with nitrogen.

^b No nitrogen present.

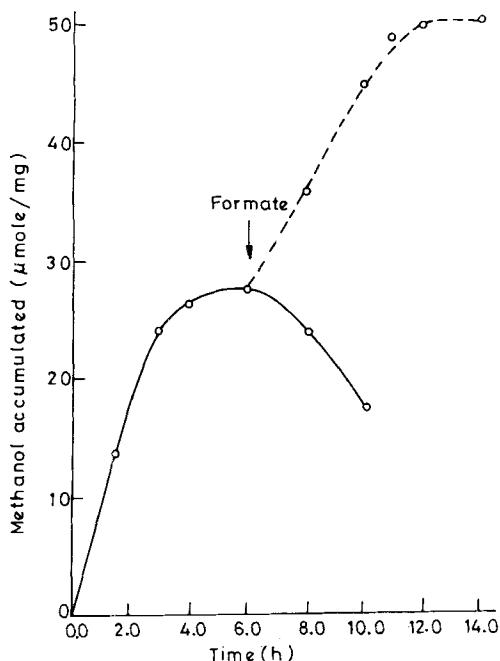


Figure 4. Effect of formate addition on methanol accumulation by immobilized cells. Formate, 40 mM, was added at the time indicated by the arrow.

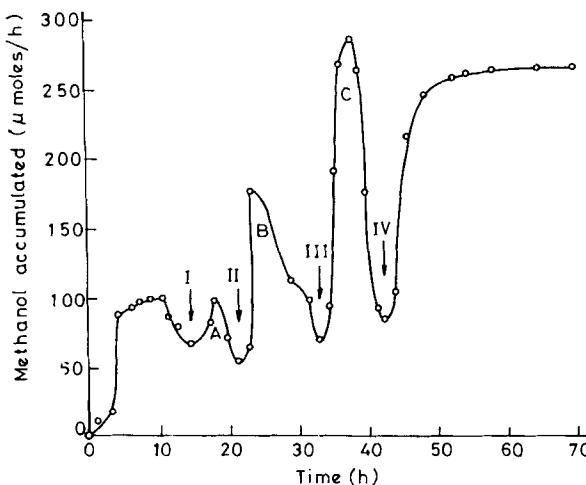


Figure 5. Formate addition and regeneration of NADH in a continuous reactor. The arrows indicate the time of addition of formate. Pulse I: 40 mM formate for 0.75 h in 20 mM phosphate buffer. Pulse II: 40 mM formate for 2.0 h in 20 mM phosphate buffer. Pulse III: 40 mM formate for 2.0 h in 100 mM phosphate buffer. Pulse IV: 20 mM formate continuously in 100 mM phosphate buffer.

studied by calculating the area under peaks *A*, *B*, and *C* in Figure 5 using the trapezoidal rule.¹⁶ The stoichiometric balance at each pulse of formate supply is given in Table II. A third pulse of 40 mM formate in 100 mM phosphate buffer resulted in 91.2% recovery of formate supplied in terms of methanol accumulation. Loss of nearly 90% formate was attributed to the further oxidation of methanol by partially inactivated methanol oxidation activity at 100 mM phosphate levels. An interruption in formate supply led to a fall in methanol

Table II. Stoichiometric balance on formate supply and methanol produced in continuous reactor.

| Pulse number | Formate supplied (mmol) | Methanol produced (mmol) | Amount of methanol produced per amount of formate supplied (%) |
|----------------|-------------------------|--------------------------|--|
| I ^a | 0.42 | 0.071 | 16.9 |
| II | 1.12 | 0.674 | 60.2 |
| III | 1.12 | 1.021 | 91.2 |

^a Defined in Fig. 5.

accumulation, indicating the interdependence between the two. A continuous feed of 20 mM formate in 100 mM phosphate buffer sustained continuous methanol biosynthesis up to 70 h. From the balance made in Table II, it can be concluded that molar amounts of methanol synthesized at steady state is driven by $\text{NAD}^+ + \text{H}_2/\text{NADH} + \text{H}^+$ cycling in resting cells and is dependent on equimolar addition of NADH regenerating substrate.

The dilution rate D of the continuous reactor was varied from 0.2 to 1.0 h^{-1} (corresponding to a flow rate of 10–50 mL/h) to study the most suitable residence time for methanol biosynthesis. The specific product formation rate v and methanol production as a function of dilution rate are shown in Figure 6. A maximum specific product formation rate of 25 $\mu\text{mol}/\text{h mg cells}$ and total methanol production of 175.0 μmol was obtained at a dilution rate of 0.5 h^{-1} . Further increase in dilution rate resulted in a sharp decline in methanol accumulation, indicating a specific contact period between cells and substrate for methanol biosynthesis.

The stability of immobilized microbial cells in continuous operation was studied for over 88 h (Fig. 7). The leakage of the cells and microbial contamination were microscopically examined throughout the operation. The immobilized preparation was stable and did not disintegrate or lose cells. The slope in Figure 7 was used to calculate the half-life $t_{1/2}$ and the thermal denaturation rate constant K_d . The computed values were 108 h and $6.42 \times 10^{-3} \text{ h}^{-1}$, respectively.

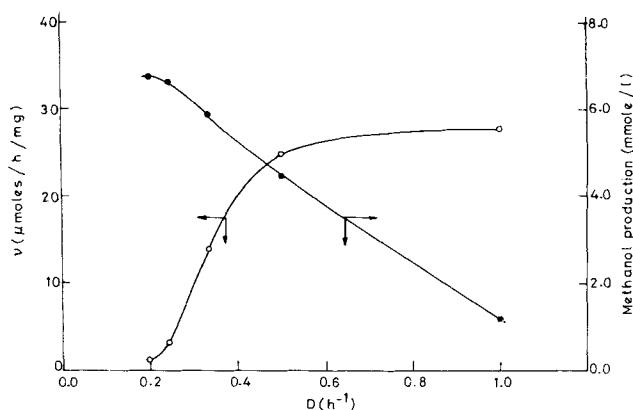


Figure 6. Effect of dilution rate on methanol biosynthesis by cells.

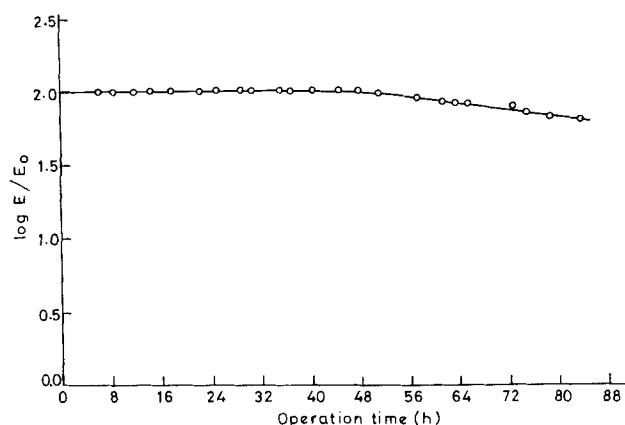


Figure 7. Operational stability of the immobilized cells in the continuous reactor: E/E_0 , MMO activity measured at the indicated time divided by enzyme activity of freshly immobilized cells.

CONCLUSION

The feasibility of using covalently linked cells of *M. trichosporium* for continuous biosynthesis of methanol from methane is described. While the pH and temperature optima remain the same between free and immobilized cells, higher phosphate ion concentration (100 mM) is required for maximum methanol biosynthesis in immobilized cells. Facilitated reaction rates are observed in immobilized cells as indicated by lowered activation energy requirements, i.e., 14,800 cal/mol as against 19,537 cal/mol for free cells. In batch and continuous studies, the rate of methanol accumulation falls low after a certain initial period due to depletion of the intracellular NADH₂ pool. The reducing power could be regenerated by supply of sodium formate in the feed stream to the reactor. Both phosphate ions and duration of supply of formate affect methanol biosynthesis in the continuous reactor. Although several reasons for the instability of immobilized methanotrophs have been proposed,⁶ our studies indicate that thermal deactivation may be responsible for slow loss of enzyme activity.

With the recent cloning of a structural gene for the *r*-subunit of protein A of MMO (identified as the site of hydrocarbon oxidation) from *M. capsulatus* into *Escherichia coli*,^{17,18} it may be possible to develop highly selective biochemical catalysts for controlled oxidation of alkanes to alcohols. The continuous supply of reducing power in such a system will remain a major area of interest.

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