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# Enhanced rate of methanol and acetate uptake for production of methane in batch cultures using *Methanosarcina mazei*

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**Abstract**

Batch cultures were performed for uptake of methanol, and acetate for production of methane and $CO_2$ by *Methanosarcina mazei* cultures. The growth, substrate consumption, methane and $CO_2$ production characteristics were analysed and compared. The maximum values of specific growth rate, specific rate of substrate consumption from methanol were 0.047–0.084 $ha^{-1}$, and 0.37–0.67 g methanol $g^{-1}$ cells $h^{-1}$, while the values of specific rate of methane and $CO_2$ formation were 13.38–19.99 mM methane $g^{-1}$ cells $h^{-1}$, and 5.46 mM $CO_2$ $g^{-1}$ cells $h^{-1}$, respectively. Similarly, maximum values of specific growth rate, and specific rate of acetate consumption were 0.059–0.096 $h^{-1}$, 0.49–0.74 g acetate $g^{-1}$ cells $h^{-1}$, respectively, while the values of specific rate of methane and $CO_2$ formation were, 5.30–9.90 mM methane $g^{-1}$ cells $h^{-1}$, and 5.30–11.0 mM $CO_2$ $g^{-1}$ cells $h^{-1}$ from its consumption. The values of substrate consumption kinetic parameters are significantly higher than those reported in other studies for *Methanosarcina* spp. and other acetoclastic methane producers. © 1998 Published by Elsevier Science Ltd. All rights reserved.

*Keywords:* Anaerobic; Methane; Renewable biomass

## 1. Introduction

Anaerobic digestion is used to treat many different wastes to reduce pollution and to produce methane (Cowan, 1992; Boopathy, 1996). Methane is one of the more renewable energy sources. It also has a special significance in the context of depleting energy reserves. In Pakistan, even though the availability of renewable biomass is abundant enough to produce sufficient methane, the country still has to import natural gas worth millions of dollars. Biomethanation of agro-industrial wastes and crop residues is a technological simple and economically viable process (El-Shinnawi *et al*., 1989; Cowan, 1992). Studies have been conducted on production of methane with a coculture of microbes utilizing plant materials/organic wastes (Ranade *et al*., 1987; Kalia *et al*., 1992; Deivanai and Bai, 1995) but yield of methane is relatively low. Previously, we demonstrated the potential of *Methanosarcina mazei* and five other methanogens for biogas production from locally-produced plant material utilizing mixed fermentative *Clostridium* strains (Tabassum *et al*., 1990; Tabassum *et al*., 1992), acetogenic organisms, namely *Desulfovibrio* sp. and *Syntrophomonas* sp. (Rajoka *et al*., 1996) and methane yields were higher than those reported by many other workers (Cho *et al*., 1995).

Acetate is an important intermediate in methanogenic degradation of organic matter (Fatehpur, 1987; Ahring and Westermann, 1988; Schinck, 1992; Stams *et al*., 1993; Dong *et al*., 1994). It accounts for 70–75% of the $CH_4$ formation in anaerobic digesters. Threshold values of acetate metabolism are 1.18 mM for *M. barkeri* (Westermann *et al*., 1989) and 1.62 mM for *Methanosarcina* sp. (Pavlostathis and Giraldo-Gomez, 1991). Acetate accumulation in the digesters retards propionate and butyrate biodegradation in synthrophic consortia (McInerney and Bryant, 1981; Ahring and Westermann, 1988; Goris *et al*., 1989; Fukuzaki *et al*., 1990; Wu *et al*., 1993). Higher concentrations of acetate are also not tolerated by acetogenic organisms such as *Syntrophomonas* spp. and *Desulfovibrio* spp. and methane producing organisms (Schink, 1992; Jetten *et al*., 1992; Dong *et al*., 1994; Chen and Hashimoto, 1996). For successful anaerobic disposal of wastes and

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to generate energy, it is essential to isolate strains of methanogens which can consume high concentrations of acetate at high rates.

*Methanosarcina* spp. are some of the more versatile methanogens and can use $H_2/CO_2$, acetate, methanol, and methylated amines (Whitman *et al.*, 1992; Boopathy, 1996). *Methanosarcina maizei* is an important acetoclastic methanogen in anaerobic digesters. In combination with *Methanosaeta* sp., *Methanobacterium formicicum*, and two syntrophic fatty acid degraders, *M. mazei* has a role in granules formation in upflow anaerobic sludge blanket (Veiga *et al.*, 1997). *M. maizei* S-6 and *M. mazei* LYC are well-studied organisms but they yield low cell densities in media during cultivation (Boone and Mah, 1987; Yang and Okos, 1987; Vavilin and Lokshina, 1996). This work reports the use of *M. mazei* which grows to high cell densities and has a high consumption rate of methanol and acetate in batch culture.

## 2. Methods

### *2.1. Organism and growth conditions*

The mesophilic acetate utilizing bacterium, *M. mazei* (Fig. 1), was isolated from a biogas plant as described by Schink (1992). For this purpose, the serum vial enrichment procedure was used. The inorganic salts solution contained the following ($gl^{-1}$): $KH_2PO_4$, 0.5; $K_2HPO_4$, 0.5; NaCl, 1.0; $(NH_4)_2SO_4$, 0.5; $MgSO_4$, 0.1; $CaCl_2$, 1.0. Resazurin and acetate at a concentration of 0.001 g $l^{-1}$, 300 mM $l^{-1}$ and clarified rumen fluid were added. The medium was dispensed in aliquots of 40 ml into serum vials outgassed with a mixture of 80% $H_2$–20% $CO_2$. The vials were sealed with butyl rubber stoppers and autoclaved at 121°C for 15 min. Prior to inoculation, sterile, anaerobic stock solutions of $Na_2S.9H_2O$, cysteine–HCl, and $Na_2CO_3$ were injected through the stopper to give final concentrations of 0.03, 0.03 and 0.02%, respectively. The final pH of the medium was 7.0. A biogas fluid inoculum was added directly into the medium and cultures were incubated at 37°C. The organism enriched in this medium was stored for identification. It was identified by comparing the physiological and morphological properties of the organism with *M. mazei* reported by Boopathy (1996) and Boone and Mah (1987).

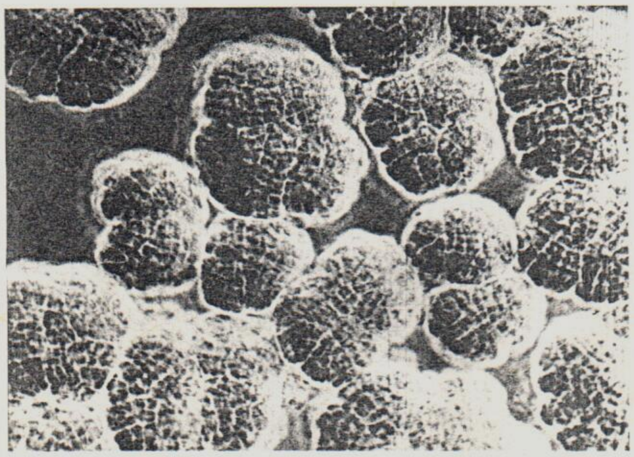

Fig. 1. Photomicrograph of *M. mazei* growing in acetate. Magnification 10 × 100.

The anaerobic cultures were maintained in basal medium containing minerals, vitamins, 0.5 g $l^{-1}$ $MgSO_4$ (as a substance to achieve uniform turbidity in the medium; Boone and Mah, 1987), sodium acetate (6.5 g $l^{-1}$) or methanol (5.0 g $l^{-1}$) and an $E_h$ indicator, resazurin (1 mg $ml^{-1}$, Khan, 1980; Sowers and Schreier, 1995). For this purpose, 45 ml amounts of medium were added in 125 ml serum vials and were closed with butyl rubber stoppers and aluminium caps using standard anaerobic culturing techniques (Sowers and Noll, 1995; Chen and Hashimoto, 1996; Boopathy, 1996) under the atmosphere of 20% $CO_2$–80% $N_2$ (v/v) gases. This stock culture (10%, v/v) was used as inoculum in all further experiments. All transfers were made with anaerobic culture technique using sterile syringes with 22-gauge hypodermic needles (Chen and Hashimoto, 1996).

For nutritional studies, various concentrations of methanol and acetate were used. After preparations, media were dispensed into bottles and made anaerobic as described in the previous section. The bottles were autoclaved keeping $N_2$ (100%, v/v) as the gas phase unless specified otherwise. After cooling, a 10% inoculum grown on the same carbon and energy source was employed to start the experiments. The temperature of the growth supporting medium was 30–37°C + 1°C. At different time intervals, gas and liquid samples were collected as described by Boopathy (1996) and analysed for quantification of end-products.

### *2.2. Quantification of end-products*

The end products of fermentation in the gaseous phase were determined as reported previously by other workers (Kalia *et al.*, 1992; Boopathy, 1996; Cho and Hashimoto, 1996) using Perkin Elmer, (Foster City, California, USA) and Gasukura, (Tokyo, Japan) gas chromatographs. The volume of total gases was determined by displacement of 20% saline acidified with 0.5% HCl. Methane volume was corrected for standard temperature and pressure conditions. Acetate and methanol were determined using a gas chromatograph as described by Nishio *et al.* (1993) after adjusting the pH of the supernatant from centrifugation of fermented medium (15000 rpm for 15 min) to pH 3.

Working conditions for gas chromatography were as follows: detector, flame ionization; column, stainless steel (1 m in length and 3 mm in diameter), packed with porapak QS (80/100 mesh), temperature of column, 190°C for acetic acid, and 150°C for methanol and carrier gas was nitrogen at flow rate of 30 ml $min^{-1}$. The values were calculated by comparison with the values for standards.

### 2.3. Determination of kinetic parameters

Overall, anaerobic digestion process conformed to first-order batch kinetics. For determining kinetic parameters for this fermentation process, the procedures followed by Lawford and Rouseau (1993), those given by Pirt (1975) or Yang and Okos (1987) were adopted. Dry cell mass (g $l^{-1}$) of *M. mazei* strain after growth on carbon sources, during the time course study (Figs 2 and 3), was determined on triplicate samples as described earlier. The growth yield coefficient ($Y_{x/s}$) was calculated as the dry cell mass per mass of substrate utilized from the test substrates. The volumetric rate of substrate utilization ($Q_s$), $CH_4$, $CO_2$, and cell mass productivities ($Q_p$) were determined from the maximum slope in plot of substrate (g $l^{-1}$), methane and $CO_2$ produced (mM $l^{-1}$), and cell mass (g $l^{-1}$) vs time of fermentation. The specific substrate uptake rate ($q_s$) was determined after Pirt (1975) by $Y_{s/x} \times \mu$. The product yield coefficients ($Y_{p/x}$ and $Y_{p/s}$) were calculated as the mM product produced (final value) per mass of cells formed and quantity of product per mass of substrate used from the carbon source. The specific rate of product formation ($q_p$) was calculated after Pirt (1975) by $Y_{p/x} \times \mu$.

### 2.4. Statistical analysis

Treatment effects were compared by the protected least significant difference method (Snedecor and Cochran, 1980). Significance of difference has been presented as ANOVA-II in the form of probability (*P*) values.

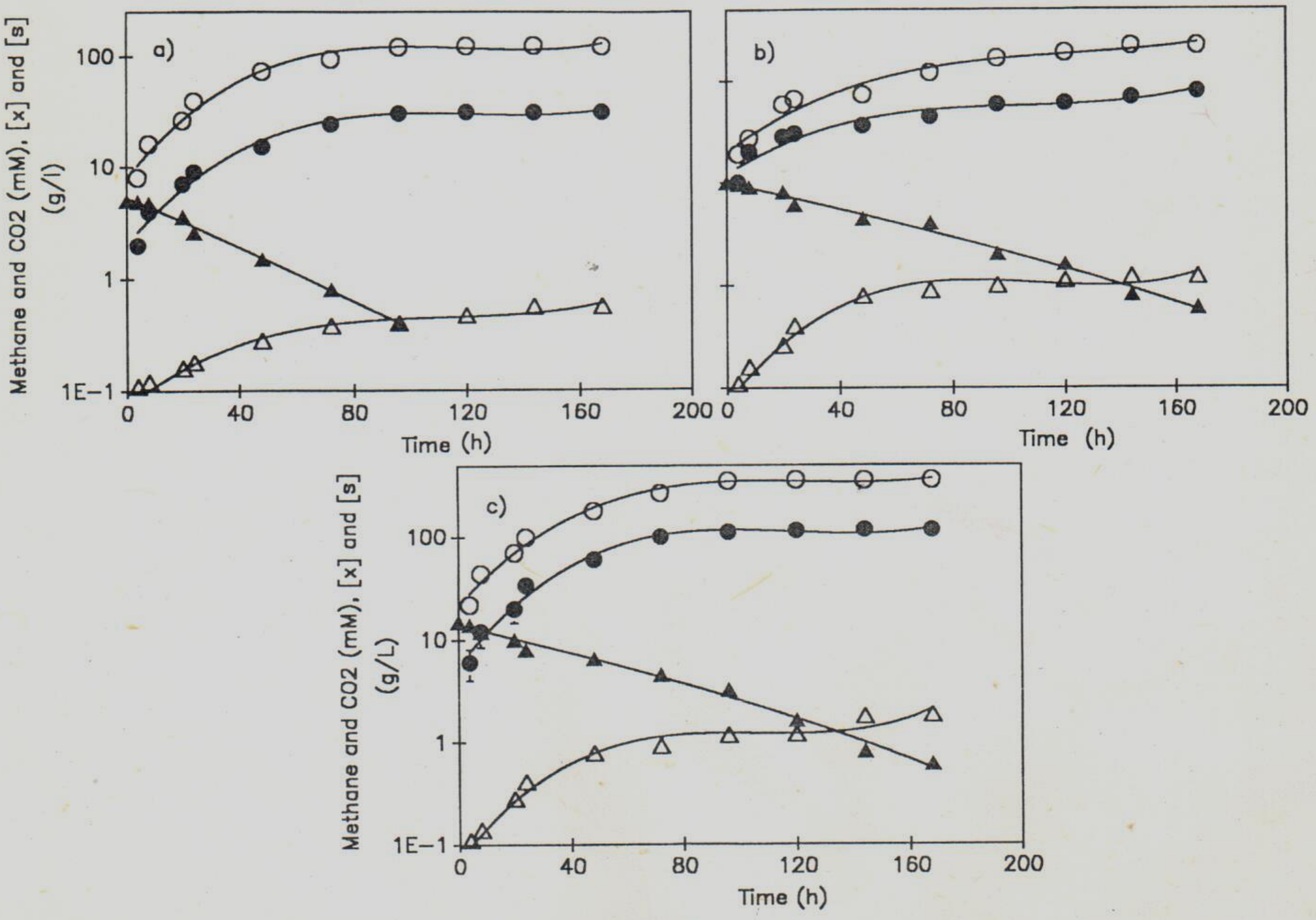


Fig. 2. Methane (○), cell mass (▲) and substrate utilization (▲) kinetics in fermentation of methanol used at (a) 5 g, (b) 10 g and (c) 15 g per litre. The results are means of three replicates with SD of 4.5–6.5% which are not visible due to log system used in plotting the data.

## 3. Results and discussion

### 3.1. Growth on methanol

*M. mazei* utilized methanol efficiently; methanol (1.5%, w/v) gave the highest cell yield [Fig. 2(a–c)]. The organism can grow on methanol [Fig. 2(a–c)] with a high substrate consumption, and product formation rates (Table 1). The increase in substrate conversion efficiency can be deduced from the responses of $\mu$, $t_d$, $Q_s$, $q_s$ and $Q_B$ and all values (Table 1) were comparable or better than those reported previously (Nishio *et al.*, 1984; Nishio *et al.*, 1993; Boopathy, 1996; Sanchez *et al.*, 1996; Vavilin and Lokshina, 1996). The values of $\mu$ and $q_s$ were comparable to those of mixed culture methanol uptake in continuous culture (0.041 $h^{-1}$ and 13.3 g methanol $g^{-1}$ cells $h^{-1}$; Nishio *et al.*, 1984) and *Methanosarcina* sp. (Boopathy, 1996).

The data for product formation namely mM methane or mM $CO_2$ $g^{-1}$ cells, mM methane or $CO_2$ $g^{-1}$ substrate consumed, volumetric productivities or specific productivities of the products is shown in Table 1. *M. mazei* exhibited improved $Y_{p/s}$ and $Y_{p/x}$ of that reported by Nishio *et al.* (1984), Nishio *et al.* (1992), Nishio *et al.* (1993), Boone and Mah (1987) and Boopathy (1996) through volumetric uptake of methanol and $Q_p$ were significantly lower than that reported by Nishio *et al.* (1993) and higher than that reported by Boone and Mah (1987) and Boopathy (1996). Low $Q_s$ and $Q_p$ values were attributed to the low value of methanol used in these studies compared with that used by Nishio *et al.* (1993) and as observed by Chen and Hashimoto (1996) also, in product formation kinetics. The $q_p$ values of methane production from *M. mazei* on methanol medium were 13.4–14.7 mM $g^{-1}$ cells $h^{-1}$ and were improved 1.77-fold over that from mixed culture of sarcina growing on methanol in chemostat (8.33 mM $g^{-1}$ cells $h^{-1}$; Nishio *et al.*, 1984), and in batch culture (Boopathy, 1996).

The effect of methanol on cell wall components and ATP formation in the cell during reduction of methanol by hydrogen has been elucidated (Gottschalk, 1985; Weil *et al.*, 1989). Methyl reductase, the enzyme responsible for conversion of methanol to methane, requires ATP and proton-motive force. If less ATP or a lower proton-motive force is present, the

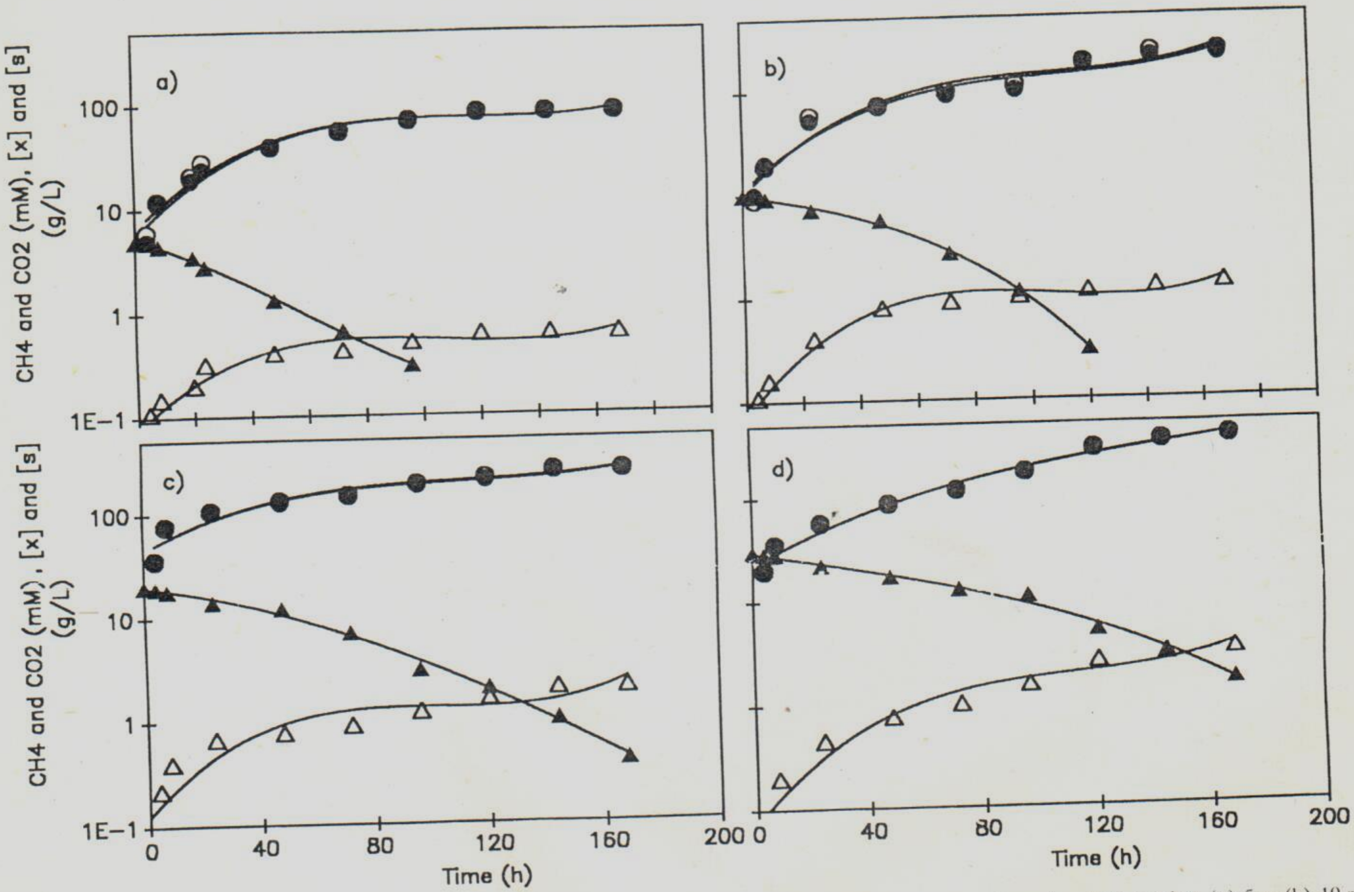


Fig. 3. Methane (○), carbon dioxide (○), cell mass (▲) and substrate utilization (▲) kinetics in fermentation of acetate used at (a) 5 g; (b) 10 g and (c) 15 g per litre. The results are means of three replicates with SD of 4.5–6.5% which are not visible due to log system used in plotting the data.

Table 1
Potential kinetic parameters for production of $CH_4$ and $CO_2$ during consumption of methanol in an anaerobic medium of pH 7.0 at 30°C in batch culture studies

| Kinetic parameter | Growth on different concentrations of methanol (g/l$^{-1}$) | | | Remarks |
|---|---|---|---|---|
| | 5 | 10 | 15 | |
| $\mu$ (h$^{-1}$) | $0.047 \pm 0.002^b$ | $0.082 \pm 0.002^a$ | $0.084 \pm 0.003^a$ | h.s. |
| $t_d$ (h) | $14.80 \pm 0.710^a$ | $8.600 \pm 0.420^b$ | $8.300 \pm 0.37^b$ | h.s. |
| $Q_s$ (g/l$^{-1}$ h$^{-1}$) | $0.110 \pm 0.005^c$ | $0.150 \pm 0.006^b$ | $0.254 \pm 0.006^a$ | h.s. |
| $Y_{x/s}$ (g g$^{-1}$) | $0.110 \pm 0.004^a$ | $0.120 \pm 0.005^a$ | $0.110 \pm 0.007^a$ | n.s. |
| $q_s$ (g s g$^{-1}$ cells h$^{-1}$) | $0.430 \pm 0.021^b$ | $0.670 \pm 0.032^a$ | $0.410 \pm 0.005^b$ | h.s. |
| $Q_B$ (g cells l$^{-1}$ h$^{-1}$) | $0.010 \pm 0.000^a$ | $0.020 \pm 0.001^a$ | $0.020 \pm 0.002^a$ | n.s. |
| $Q_{CH_4}$ (mM l$^{-1}$ h$^{-1}$) | $1.710 \pm 0.08^c$ | $3.510 \pm 0.08$ | $4.140 \pm 0.175^a$ | h.s. |
| $Y_{CH_4/x}$ (mM g$^{-1}$ cells) | $243.0 \pm 10.0^a$ | $241.0 \pm 9.5^a$ | $232.0 \pm 9.300^b$ | h.s. |
| $Y_{CH_4/s}$ (mM g$^{-1}$ s$^{-1}$) | $11.50 \pm 0.56^c$ | $13.80 \pm 0.58^b$ | $26.00 \pm 0.980^a$ | s. |
| $q_{CH_4}$ (mM g$^{-1}$ cells h$^{-1}$) | $13.38 \pm 0.55^c$ | $16.30 \pm 0.80^b$ | $16.79 \pm 0.820^a$ | h.s. |
| $Q_{CO_2}$ (mM l$^{-1}$ h$^{-1}$) | $0.360 \pm 0.001^c$ | $0.820 \pm 0.04^b$ | $1.620 \pm 0.076^a$ | h.s. |
| $Y_{CO_2/x}$ (mM g$^{-1}$ cells) | $100.0 \pm 4.50^a$ | $65.00 \pm 3.20^b$ | $65.00 \pm 3.100^b$ | h.s. |
| $Y_{CO_2/s}$ (mM g$^{-1}$ s$^{-1}$) | $1.120 \pm 0.04^b$ | $0.780 \pm 0.035^c$ | $7.80 \pm 0.340^a$ | h.s. |
| $q_{CO_2}$ (mM g$^{-1}$ cells h$^{-1}$) | $4.680 \pm 0.21^c$ | $5.200 \pm 0.24^b$ | $5.460 \pm 0.240^a$ | h.s. |

Values are means of three sets of replicates. Within rows, values followed by different letters differ significantly ($P \leq 0.05$) according to Duncan multiple range test (DMRT).
n.s. = non-significant, s. = significant, h.s. = highly significant at $P \leq 0.05$.

production of methane is suppressed. Since 100% theoretical yields were obtained, ATP and proton-motive force may be produced at an optimal proportion.

### 3.2. *Growth on acetate*

This organism has the ability to utilize acetate at a high substrate conversion efficiency [Fig. 3(a–c)] but maximum growth occurred on acetate at 30°C and pH 7. These batch culture studies indicated that major components of gas phase were $CH_4$ and $CO_2$. The chemical analysis of the terminal medium indicated that only traces of acetate were present in the medium indicating that acetate conversion was taking place efficiently (100%). This organism can tolerate up to 4% sodium acetate in the medium but the maximum growth occurred at 3% level [Fig. 2(c)] and in this respect compares favourably with that reported by Fukuzaki *et al.* (1990). High tolerance to acetate is not consistent with that observed for *M. barkeri* which shows a lower growth on acetate than other substrates (Gottschalk, 1985; Yang and Okos, 1987; Jetten *et al.*, 1992). *M. mazei* LYC also used acetate inefficiently while *M. mazei* S-6 used acetate rapidly (Boone and Mah, 1987). Substrate consumption parameters namely $\mu$, $t_d$, $Q_s$, $q_s$ and $Q_B$ were improved over those reported by Nishio *et al.* (1984), Yang and Okos (1987), Fukuzaki *et al.* (1990) and Vavilin and Lokshina (1996)).

The organism produced methane from acetate on a quantitative basis (Nishio *et al.*, 1993) up to 3% concentration after which the production of methane on a theoretical yield basis declined although growth also declined after 3% and theoretical yields were not obtained (Fig. 3). *M. mazei* produced 122 mM methane g$^{-1}$ cells, with a maximum specific methane production rate of 9.9–10.9 mM g$^{-1}$ cells h$^{-1}$ on acetate. These product formation levels are significantly higher ($P \leq 0.05$) than those reported in *M. barkeri* (0.7 mM g$^{-1}$ cells h$^{-1}$; Fukuzaki *et al.*, 1990) and mixed culture of methanogens in continuous culture (Nishio *et al.*, 1984). The methane production coefficient ($Y_{p/s}$) was, however, comparable with that reported by Chen and Hashimoto (1996) and Sanchez *et al.* (1996).

The $q_p$ values of the organism on acetate medium (Table 2) were 9.9–10.9 mM $CH_4$ g$^{-1}$ cells h$^{-1}$ and were several-fold improved over *M. mazei* (1.1 mM g$^{-1}$ cells h$^{-1}$), *M. barkeri* (2.7 mM g$^{-1}$ cells h$^{-1}$) and mixed sarcina cultures (Nishio *et al.*, 1984; Fukuzaki *et al.*, 1990).

Methane is produced from acetate by *Methanosarcina* spp. by a decarboxylation reaction in which the methyl group is reduced to $CH_4$ and the carboxyl group is oxidized to $CO_2$. This reaction generates energy for ATP synthesis. The free energy yield from the aceto-clastic reaction ($-361$ kJ mol$^{-1}$) is insufficient for the formation of 1 M ATP per mole of acetate cleaved. Therefore, electron-transport phosphorylation is involved in the ATP formation and thus ATP requirement is met to produce methane from acetate (Gottschalk, 1985; Wei *et al.*, 1989) and theoretical yields have been obtained from acetate. A co-culture containing *M. mazei* (isolated in these studies) will be useful in those fermentation systems in which high

Table 2
Potential kinetic parameters for production of $CH_4$ and $CO_2$ during consumption of acetate in an anaerobic medium of pH 7.0 at 30°C in batch culture studies

| Kinetic parameter | Growth on different concentrations of acetate (g/l): | | | | Remarks |
|---|---|---|---|---|---|
| | 5 | 10 | 20 | 30 | |
| $\mu$ ($h^{-1}$) | $00.059 \pm 0.003^b$ | $00.062 \pm 0.002^b$ | $00.08 \pm 0.005^{ab}$ | $00.10 \pm 0.004^a$ | h.s. |
| $t_d$ (h) | $14.800 \pm 0.720^a$ | $08.600 \pm 0.420^b$ | $08.30 \pm 0.410^c$ | $07.20 \pm 0.34^d$ | h.s. |
| $Q_s$ ($g\ l^{-1}\ h^{-1}$) | $00.050 \pm 0.002^d$ | $00.130 \pm 0.005^c$ | $00.21 \pm 0.001^b$ | $00.33 \pm 0.01^a$ | h.s. |
| $Y_{x/s}$ ($g\ g^{-1}$) | $00.120 \pm 0.006^a$ | $00.120 \pm 0.055^a$ | $00.11 \pm 0.004^a$ | $00.11 \pm 0.04^a$ | n.s. |
| $q_s$ ($g\ s\ g^{-1}$ cells $h^{-1}$) | $00.490 \pm 0.030^d$ | $00.520 \pm 0.030^c$ | $00.74 \pm 0.030^b$ | $00.87 \pm 0.04^a$ | h.s. |
| $Q_B$ (g cells $l^{-1}\ h^{-1}$) | $00.001 \pm 0.000^a$ | $00.020 \pm 0.000^a$ | $00.02 \pm 0.000^a$ | $00.02 \pm 0.00^a$ | n.s. |
| $Q_{CH_4}$ (mM $l^{-1}\ h^{-1}$) | $00.930 \pm 0.004^d$ | $01.21 \pm 0.061^c$ | $003.02 \pm 0.114^a$ | $02.000 \pm 0.10^b$ | h.s. |
| $Y_{CH_4/x}$ (mM $g^{-1}$ cells) | $90.000 \pm 4.100^c$ | $91.00 \pm 3.500^c$ | $122.00 \pm 5.500^a$ | $114.00 \pm 5.80^b$ | h.s. |
| $Y_{CH_4/s}$ (mM $g^{-1}\ s^{-1}$) | $10.800 \pm 0.450^b$ | $10.90 \pm 0.470^b$ | $12.800 \pm 0.610^a$ | $12.800 \pm 0.60^a$ | s. |
| $q_{CH_4}$ (mM $g^{-1}$ cells $g^{-1}$) | $05.300 \pm 0.230^d$ | $05.64 \pm 0.280^c$ | $09.900 \pm 0.450^b$ | $10.900 \pm 0.51^a$ | h.s. |
| $Q_{CO_2}$ (mM $l^{-1}\ g^{-1}$) | $00.350 \pm 0.012^d$ | $01.26 \pm 0.050^c$ | $03.020 \pm 0.120^a$ | $02.870 \pm 0.11^b$ | h.s. |
| $Y_{CO_2/x}$ (mM $g^{-1}$ cells) | $90.000 \pm 4.300^c$ | $91.00 \pm 4.600^c$ | $120.00 \pm 5.500^a$ | $115.000 \pm 4.1^b$ | h.s. |
| $Y_{CO_2/s}$ (mM $g^{-1}\ s^{-1}$) | $10.800 \pm 0.410^c$ | $11.00 \pm 0.350^c$ | $12.00 \pm 0.450^b$ | $13.000 \pm 0.55^a$ | h.s. |
| $q_{CO_2}$ (mM $g^{-1}$ cells $h^{-1}$) | $05.300 \pm 0.300^d$ | $05.64 \pm 0.250^c$ | $09.720 \pm 0.420^b$ | $11.000 \pm 0.42^a$ | h.s. |

Values of means of three sets of replicates. Within rows, values followed by different letters differ significantly ($P \leqslant 0.05$) according to DMRT. n.s. = non-significant, s. = significant, h.s. = highly significant at $P \leqslant 0.05$.

concentration of acetate inhibits the methane production process.

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