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Methanogenesis from lactate by a co-culture of *Clostridium formicoaceticum* and *Methanosarcina mazei*

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Summary. A co-culture of *Clostridium formicoaceticum* and *Methanosarcina mazei* converted lactate to methane and carbon dioxide at mesophilic temperatures and pH values near 7.0. Lactate was first converted to acetate by the homoacetogen, and then to CH_4 and CO_2 by the methanogen, with the second reaction as the rate-limiting step. The methane yield was about 1.45 mol/mol lactate. These two organisms formed a mutualistic association and may be useful together with the homolactic bacterium *Streptococcus lactis* to convert lactose to methane.

Introduction

Recently, two-phase anaerobic processes consisting of an acidogenic and a methanogenic phase in series have received increasing attention. Higher treatment efficiency and more reliable operation are attained for two-phase digestion than for the conventional, one-stage digestion, especially when a high-strength waste stream (such as whey) is to be treated. In the two-phase process, volatile fatty acids (mainly, acetic, propionic, and butyric acids) are usually formed in the acidogenic phase (Gough et al. 1987; Lo and Liao 1988). However, it has been reported that methanogenesis was enhanced when sugar hydrolysis was directed toward lactic acid rather than volatile fatty acids because more potential energy was available to the syntrophic bacteria (Pipyn and Verstraete 1981). Moreover, the formation of large amounts of propionic acid during acidogenesis should be prevented since propionic acid inhibits methanogenesis.

In general, no significant amounts of lactate can be detected in a normal anaerobic digestor at steady-state operation (Yang and Guo 1990). However, it is very likely that lactate may have been produced and immediately converted to acetate and other compounds by lactate-utilizing bacteria in some digestors (Yang et al. 1987). Recently, lactate was shown to be a major intermediary metabolite during biomethanation of whey lactose (Chartrain and Zeikus 1986a) and a major product from acidogenic fermentations of lactose (Kissalita et al. 1989) and glucose (Zoetemeyer et al. 1982).

There are several acetogenic anaerobes known to grow on lactate, including *Acetobacterium woodii* (Balch et al. 1977), *Clostridium formicoaceticum* (Andreesen et al. 1970) and sulfate-reducing bacteria *Desulfovibrio desulfuricans* and *vulgaris* (Bryant et al. 1977). The sulfate-reducing bacterium *D. vulgaris* was found to be responsible for the conversion of lactate and ethanol to acetate, the major methanogenic substrate, in a continuous whey digestor (Chartrain and Zeikus 1986b). This fermentation involved an interspecies H_2-transfer, usually with H_2-utilizing methanogens such as *Methanobacterium formicicum*. Lactate also may be converted to acetate by *A. woodii*. However, this organism cannot grow at pH values higher than 7.0 or temperatures higher than 32° C (Balch et al. 1977), and thus will not be important in most of the anaerobic digestors operated at near pH 7.0 and 37° C.

In contrast to other acetogens, *C. formicoaceticum* can neither use nor produce H_2 (Andreesen et al. 1970). It was previously shown that *C. formicoaceticum* homofermentatively converted 1 mol lactate to about 1.5 mol acetate at mesophilic temperatures (Yang et al. 1987). In the same paper, it was postulated that *C. formicoaceticum* might also be responsible for converting lactate to acetate in anaerobic digestion when the digestor pH was near neutral. The conversion of lactate to acetate by sulfate-reducing bacteria in a methanogenic environment has been well documented (MacInerney and Bryant 1981). However, to date no work has been reported on *C. formicoaceticum* grown on lactate in a methanogenic system. In this work, *C. formicoaceticum* was successfully co-cultured with acetoclastic methanogens to convert lactate to methane. The potential production of lactate as the intermediate product in two-phase anaerobic digestion of whey lactose is also discussed in this paper.

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Materials and methods

Microorganisms. *C. formicoaceticum* ATCC 27076 and two acetoclastic methanogens, *Methanosarcina mazei* strain S6 and *M. barkeri* strain 227, were used in this work.

Medium preparation and growth conditions. Anaerobic syringe techniques (Balch et al. 1979) were used in this study. *C. formicoaceticum* was cultivated in a previously described medium (Yang et al. 1987). The methanogens were cultivated in the same medium except that acetate or methanol (0.1 M) was used to replace lactate as the substrate and Na_2S was added to the medium as the sulfur source (Yang and Okos 1987). Cells were grown in 125-ml serum bottles containing 50 ml medium at 37° C. The initial pH of the medium was 7.0. Cultures were maintained in an active state by transferring 2% of the culture volume to a fresh medium every 2 weeks.

For fermentor studies, the complete fermentor containing the unreduced medium (cysteine free) was autoclaved at 121° C for 25 min. After steam sterilization, the medium was boiled and flushed with filter-sterilized CO_2-N_2 gas (approx. 3 l/min) to remove oxygen. A separately sterilized cysteine/Na_2S solution was then added (final concentration 0.5 g/l) to reduce the medium. The medium pH was adjusted to 7.0 by varying the CO_2/N_2 ratio in the gas mixture after temperature cooled to 37° C. The anaerobiosis was then maintained by keeping a positive pressure (5 psig or 1.34 atm) inside the fermentor. The fermentors were gas-tight, and gassing was cut off at this point. A check valve was mounted on the exhaust gas condenser to allow gas outflow and to prevent pressure build-up during biomethanation. All fermentations were agitated at 150 rpm and maintained at 37° C.

Co-cultured fermentation. Methanogenesis from lactate using the co-culture of *C. formicoaceticum* and *M. mazei* (or *M. barkeri*) was studied with 5-l fermentors (New Brunswick Scientific, Edison, N.J., USA). The fermentor contained about 3 l medium, comprised, in part, of 0.1 M lactate and ~0.05 M methanol (or acetate). The fermentor was first inoculated with 100 ml cells of *M. mazei* (or *M. barkeri*), and the methanogens were allowed to grow for several days. At the end of the exponential phase of methanogenic growth, 50 ml cells of *C. formicoaceticum* were added into the fermentor to initiate the co-cultured growth on lactate. This procedure ensured that both organisms were in an active state and would grow simultaneously. Otherwise, the methanogens, which had an unpredictable, long lag phase, usually would start to grow much behind the acetogen, and co-cultured growth would not have been observed. The gas produced was collected and the gas volume measured in an inverted 4-l graduated cylinder immersed in a water trough. Following gas production, liquid and gas samples were removed aseptically for analyses.

Analytical techniques. Lactate and acetate were determined using HPLC as described by Yang et al. (1987). The growth of the acetogen was monitored by measuring the optical density at 660 nm (OD_{660}) on a spectrophotometer. In the co-cultured fermentation, cell clumps of the methanogens in the liquid samples were allowed to sediment before taking optical readings. The measured OD_{660} corresponded mainly to the cell density of *C. formicoaceticum*; thus growth was estimated by this method. The growth of methanogens was approximated by gas production (Yang and Okos 1987).

Both CH_4 and CO_2 were analyzed using a gas chromatograph (Varian 3300, Walnut Creek, CA, USA) equipped with a thermal conductivity detector. Two 1/8 in. stainless steel columns, 2-m Porapak N (80/100 mesh) and 4-m molecular sieve 5A (40/60 mesh), were used in series to separate the gas mixture. The detector temperature was 110° C, filament temperature 130° C, and column temperature 100° C. The carrier gas was He (40 psi) at a flow rate of 30 ml/min. The exit from the first column was connected to the sample side of the detector and then to the second column. The gas stream was then connected to the reference side of the detector. This flow scheme allowed the detection of all gas components, including CO_2, N_2, O_2 and CH_4, in the gas sample by only one injection. The corresponding retention times of these four gases were 1.7, 2.9, 3.6 and 5.2 min, respectively. No H_2, which was presumably unimportant in this work, was detectable by this method. The polarity of the detector signal was automatically switched at 2.6 min after CO_2 had been detected in order to obtain positive peaks on the recorder for all the gas components. The amount of methane produced was determined from the total gas volume under standard conditions (25° C, 1 atm) and the methane composition in the gas sample as measured by gas chromatography.

Results and discussion

Fermentation kinetics

Figure 1 shows a typical set of time-course data of the co-culture grown on lactate. In this fermentation, lactate was converted to acetate by *C. formicoaceticum*, and then to methane and carbon dioxide by *M. mazei*. The medium pH decreased from 7.0 to 6.7 initially, due to the production of acetic acid, and then increased to 7.3, due to acid consumption. The conversion of acetate to methane was the rate-limiting step in this mixed culture fermentation. The methane content in the gas produced was about 53%, with the balance as CO_2. No H_2, ethanol, or formate was found throughout the co-cultured fermentation, indicating that *C. formicoaceticum* performed the same homoacetic fermentation as that previously found under pure culture conditions (Yang et al. 1987). The co-culture of *C. formicoaceticum* and *M. barkeri* also had the same fermentation pattern. The co-cultured fermentation thus can be represented by the following two reactions in series:

$$\text{Lactate} \xrightarrow{C.\ formicoaceticum} 1.5\ \text{acetate} \xrightarrow{M.\ mazei} 1.5\ CH_4 + 1.5\ CO_2$$

It is known that *C. formicoaceticum* has a pH optimum at 7.6 and will stop growth at pH 6.3 (Tang et al. 1989). *M. mazei* also has an optimum pH around 7.0

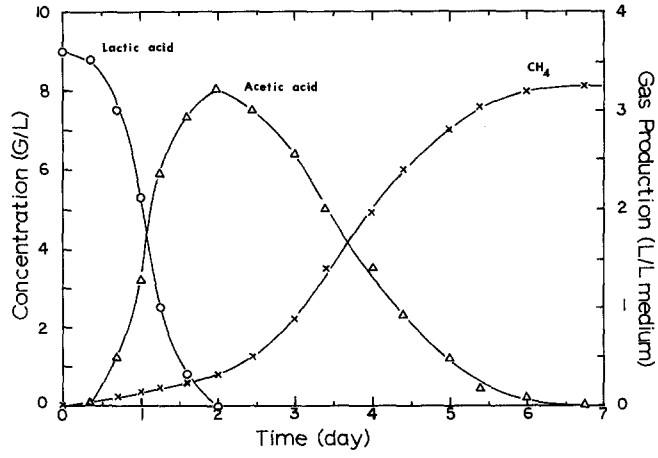

Fig. 1. Fermentation kinetics of a co-culture of *Clostridium formicoaceticum* and *Methanosarcina mazei* grown on lactate. The zero time reflects the time of inoculation of *C. formicoaceticum*, about 4 days after methanogen inoculation

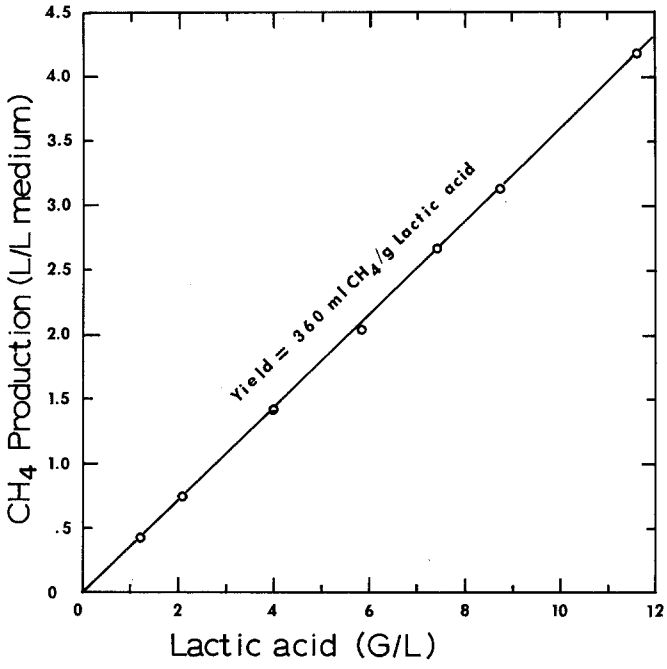

Fig. 2. Methane yield from co-cultured fermentation of lactic acid

(Yang and Okos 1987). The removal of acetic acid by the methanogen may keep the medium pH from decreasing, which is desirable for both acetogenic and methanogenic growth. As also shown in Fig. 1, the acetogenic rate was much higher than the methanogenic rate. It is thus necessary to have a methanogenic population higher than that of the acetogen in order to maintain balanced growth (i.e., no acetate accumulation) for the co-culture.

Methane yield

The methane yield from lactate was determined from seven batch fermentations, each initially containing a different lactate concentration. At the end of gas production, the total amount of methane produced was determined. Figure 2 shows a methane yield of 360 ml/g lactic acid consumed (1.45 mol CH_4/mol lactate), as found in these experiments. The acetate yield from lactate by *C. formicoaceticum* has been determined to be 0.98 g/g (or 1.47 mol/mol) lactic acid consumed (Yang et al. 1987). The methane yield from acetate was previously found to be about 367 ml/g (or approx. 0.98 mol/mol) acetic acid used by the methanogens (Yang and Okos 1987). Thus, the yield values were not changed under the co-cultured conditions as compared to the pure culture fermentations (see Table 1). The same methane yield was obtained for *M. mazei* and *M. barkeri* (data not shown).

Effect of coculture on fermentation

As indicated by the unchanged fermentation kinetics and product yields, the interaction between these two organisms is seemingly like a simple commensalism; i.e., the second microbe uses the product of the first one as a growth substrate. No other interactions seemed to occur in this mixed-culture fermentation. H_2 can neither be produced by *C. formicoaceticum*, nor be used by *M. mazei*. There was no interspecies H_2-transfer involved in the biomethanation of lactate as that observed for the co-culture of sulfate-reducing bacteria and hydrogenophilic methanogens (Bryant et al. 1977).

However, *C. formicoaceticum* had a slightly higher growth rate under the co-cultured conditions as compared with the pure culture fermentation (Fig. 3A). It is known that both acidic pH and acetate inhibit the growth of *C. formicoaceticum* (Tang et al. 1989). The increased fermentation rate was attributed to the removal of acetate by the methanogens (Fig. 3B). Thus, both organisms benefited from the co-cultured growth, suggesting a mutualistic relationship for these two organisms grown together.

Table 1. Product yields from various mono- and co-cultured fermentations

Culture	Substrate	Products	Product Yield
1. *Streptococcus lactis*	Lactose	Lactate	~0.95 g/g lactose[a] ~3.8 mol/mol lactose
2. *Clostridium formicoaceticum*	Lactate	Acetate	0.98 g/g lactic acid[b] 1.47 mol/mol lactate
3. *Methanosarcina mazei*	Acetate	CH_4, CO_2	367 ml CH_4/g acetic acid[c] 0.983 mol CH_4/mol acetate
4. Co-culture of 2 and 3	Lactate	CH_4, CO_2	360 ml CH_4/g lactic acid[d] 1.45 mol CH_4/mol lactate
5. Mixed cultures of 1, 2, 3	Lactose	CH_4, CO_2	~5.5 mol CH_4/mol lactose[e]

[a] Experimental results from Tang et al. (1988)
[b] Experimental results from Yang et al. (1987)
[c] Experimental results from Yang and Okos (1987)
[d] Experimental results from this work
[e] Expected results based on the yields in 1, 2, and 3

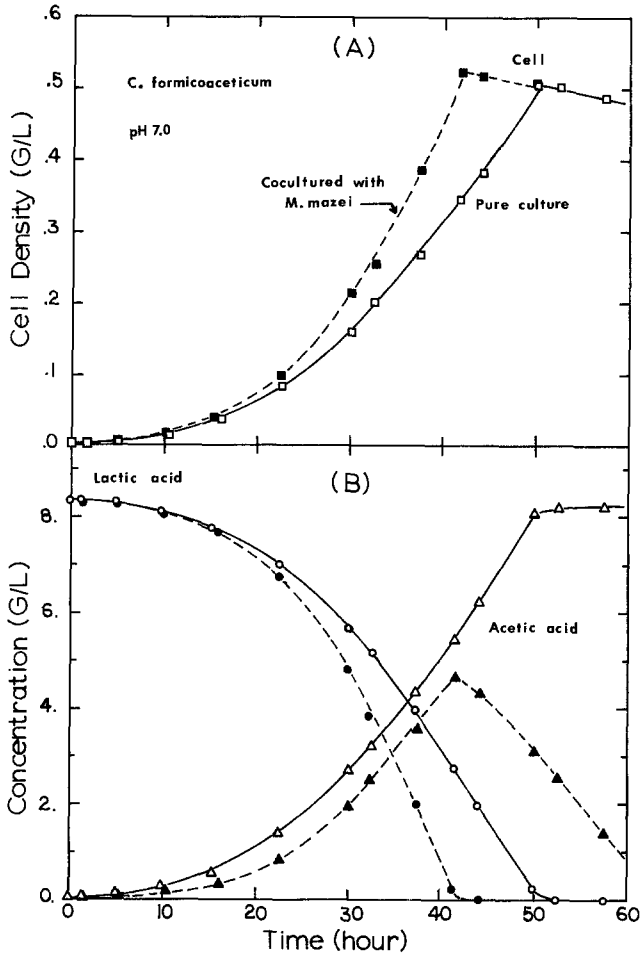

Fig. 3. Effect of co-cultured growth on homoacetic fermentation and growth of *C. formicoaceticum* under co-cultured conditions (----) compared to that under pure culture conditions (——)

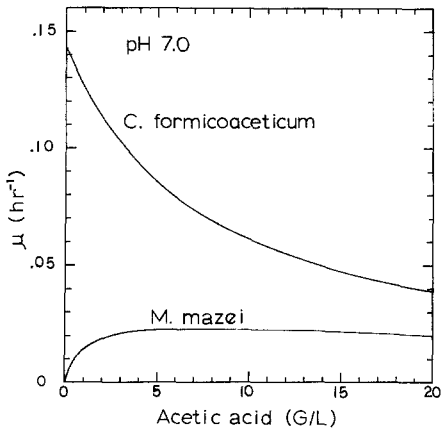

Fig. 4. Comparison of specific growth rates (μ) of *C. formicoaceticum* and *M. mazei* at various concentrations of acetic acid

Effects of acetic acid

The effects of acetic acid on the specific growth rate (μ) of *C. formicoaceticum* and *M. mazei* have been studied under pure culture conditions. Acetate was previously found to be a non-competitive inhibitor of *C. formicoaceticum*, and the inhibition kinetics can be represented by the following equation (Yang et al. 1988b):

$$\mu = \frac{\mu_m K_p}{K_p + A} \tag{1}$$

where μ_m is the maximum specific growth rate, K_p is the product inhibition constant, and A is the concentration of acetate. At pH 7.0, $\mu_m = 0.144\ h^{-1}$, and $K_p = 0.12$ M. The specific growth rate was found not to be affected by lactate when its concentration was higher than 0.01 M (Yang et al. 1988b).

For *M. mazei*, the growth substrate acetate became inhibitory to cell growth at high concentrations. The kinetics can be represented by the following equation (Yang and Okos 1987):

$$\mu = \frac{\mu_m A}{K_s + A + A^2/K_i} \tag{2}$$

where K_s is the substrate saturation constant and K_i is the substrate inhibition constant. At pH 7.0, $\mu_m = 0.029\ h^{-1}$, $K_s = 0.017$ M, and $K_i = 0.81$ M.

Figure 4 shows the predicted specific growth rates of these two organisms at pH 7.0 and various acetate concentrations, as calculated from the kinetic equations given above. Thus, at pH 7.0, the homoacetogen always has a specific growth rate higher than that of the aceto-clastic methanogen. The difference in the growth rate is larger at a lower acetate concentration. This implies that the methanogenic population must be much higher than that of acetogens in the co-culture in order to prevent the accumulation of acetic acid during the course of batch fermentation, or in order to convert most of the substrate acid to methane in a continuous culture. This also explains why the *C. formicoaceticum* metabolizes lactate faster, and probably grows faster, in a co-culture, compared to growth alone.

The results from this study indicate that *C. formicoaceticum* will be able to convert lactate efficiently to acetate in a methanogenic environment. Recently, the development of defined starter cultures for biomethanation of whey permeate has received increasing interest (Chartrain et al. 1987; Zellner et al. 1987; Yang et al. 1988a). No homoacetogen can utilize lactose. However, lactose can be readily converted to lactate by homolactic bacteria (e.g., *Streptococcus lactis)* with a product yield of 90% or higher (Tang et al. 1988). Therefore, methane can be produced from lactose by first converting lactose to lactate, and then using the co-culture of *C. formicoaceticum* and *M. mazei* to convert lactate to methane. Because of the homofermentative metabolism, the expected methane yield from this process would be about 5.5 mol CH_4/mol lactose degraded (see Table 1), which is higher than those found previously with heterofermentative metabolism, 4.65–5.10 mol CH_4/mol lactose (Chartrain et al. 1987). Also, as mentioned earlier, it is desirable to have lactate, instead of volatile fatty acids, as the intermediate for the two-phase anaerobic process. It will be feasible to do this by using the co-culture reported in this paper for the second (methanogenic) phase.

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