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Thermophilic degradation of cellulose by a triculture of *Clostridium thermocellum, Methanobacterium* sp. and *Methanosarcina* MP

(Methanogenesis; defined mixed culture)

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1. SUMMARY

The fermentation of cellulose at 55°C by different associations of the 3 bacteria *Clostridium thermocellum, Methanobacterium* sp. and *Methanosarcina* MP, was studied. *C. thermocellum* alone produced acetate, lactate, ethanol, H_2 and CO_2. The co-culture *C. thermocellum-Methanobacterium* sp. produced more acetate and less ethanol than the monoculture of *Clostridium.*

Methanosarcina MP used acetate only in the triculture including *Methanobacterium* sp. When methanol was added (5 mM) to the triculture, *Methanosarcina* MP had a shorter lag phase on acetate and degraded much more acetate. maximum methane production was 8.5 mmol CH_4/g cellulose degraded.

2. INTRODUCTION

Chemical and fuel production from anaerobic fermentations of cellulose deserve increased attention, because this organic compound is the most abundant natural polymer and comprises the majority of solid waste material. The anaerobic degradation of cellulose to methane and carbon dioxide is of current interest in terms of renewable energy for the future. To improve the overall performance of anaerobic digestion from cellulose, several authors have studied the degradation of cellulose by a mixed population of anaerobes [1–3]. A better understanding of the process was performed by studying cellulose degradation by defined methanogenic mixed cultures [4–8].

Interactions between chemoorganotrophs and methanogenic bacteria have been mostly studied in mesophilic systems. Weimer and Zeikus [4] reported the interaction between two thermophiles on cellulose, *C. thermocellum* and *Methanobacterium thermoautotrophicum.* Here we report on the conversion of cellulose to methane by *C. thermocellum, M. thermoautotrophicum* and *Methanosarcina* MP. The objective of this work was to determine the part played by each methanogenic partner and particularly the aceticlastic methanogen in the mixed triculture. This study was simplified, since the thermophilic *Methanosarcina* cannot convert methane from H_2-CO_2 as can the mesophilic *Methanosarcina.*

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3. MATERIALS AND METHODS

3.1. Chemicals

Gases were purchased from Airgaz (Marseille). All chemicals were of reagent quality unless otherwise stated. cellulose MN 300 (Machery and Nagel 300, thin layer chromatography grade) was used during this work.

3.2. Organisms

The organisms used were *C. thermocellum* NCIB10682 and *Methanosarcina* MP [9]. *Methanobacterium* sp. was isolated in our laboratory.

3.3. Culture media

The anaerobic techniques described by Hungate [10] and Balch et al. [11] were used throughout this study. A sodium bicarbonate-buffered medium was used instead of the phosphate-buffered medium of Weimer and Zeikus [4] since the total cellulose degradation (6 g/l) to CH_4 by the mixed defined culture *C. thermocellum-Methanobacterium* sp. *-Methanosarcina* MP was only successful in the bicarbonate medium.

The culture media contained the following compounds (g/l): cellulose, 6; $(NH_4)_2SO_4$, 1.3; K_2HPO_4, 0.3; $MgCl_2 \cdot 6H_2O$, 1.0; $CaCl_2$, 0.15; L-cysteine-HCl, 0.5; yeast extract (Difco, Baltimore, MD, U.S.A.), 2.0; resazurin, 0.001 and 0.03 ml of 5% $FeSO_4$ solution. The medium was prepared anaerobically, as described previously [12]. Media were dispensed in 20-ml aliquots into 60-ml serum bottles. N_2-CO_2 (80–20%) was the gas phase. After sterilisation (110°C, 30 min) and just before inoculation, 0.2 ml of 2% $Na_2S \cdot 9H_2O$ and 0.9 ml of 10% $NaHCO_3$ were dispensed into each vial. The final pH was 7.0.

Stock cultures of *C. thermocellum* were cultivated as described above. Stock cultures of *Methanobacterium* sp. were grown in the same medium without cellulose, with H_2-CO_2 (80-20%, 2 atm) as carbon and energy sources. Stock cultures of *Methanosarcina* MP were cultivated with sodium acetate $3H_2O$ (5 g/l) as substrate.

Experimental cultures were prepared by inoculating 0.5 ml of a 1-day-old culture of *Methanobacterium* sp. (60°C), 0.5 ml of a 4-day-old culture of *C. thermocellum* (60°C) and 2 ml of a 5-day-old culture of *Methanosarcina* MP (55°C). Average values of triplicate vessels are reported. All experiments were repeated at least twice. Results are expressed per vial (20 ml medium).

3.4. Analytical techniques

H_2 and CH_4 were measured by gas chromatography [13]. Volatile fatty acids were measured using a gas chromatograph equipped with a flame ionisation detector [13]. Cellulose concentrations were determined by measuring total carbohydrates by anthrone reaction [14].

4. RESULTS

4.1. Cellulose fermentation

Fig. 1 shows the time-course of cellulose utilisation in monoculture, co-culture and triculture. Cellulose degradation in the *C. thermocellum* culture or in the *C. thermocellum-Methanobacterium* sp. co-culture was essentially linear up to 4 days.

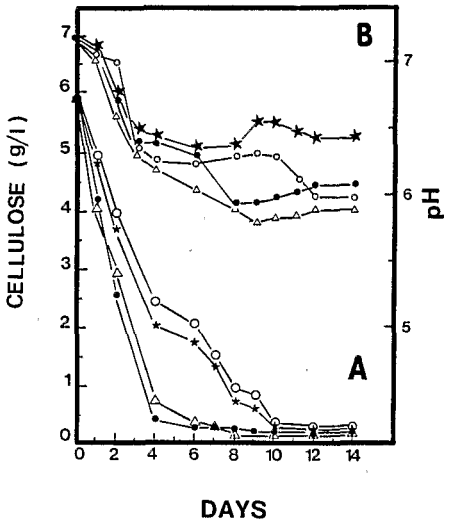

Fig. 1. Fermentation of cellulose by *C. thermocellum*, ●; *C. thermocellum* and *methanobacterium* sp., △; *C. thermocellum, Methanobacterium* sp. and *methanosarcina* MP, ○; *C. thermocellum, Methanobacterium* sp. and *Methanosarcina* MP+0.05 ml 2 M methanol, ★. (A), Cellulose degraded; (B), culture pH. Culture vessels were incubated at 55°C.

Addit on of *Methanobacterium* sp. to *C. thermocellum* h ad no effect on cellulose utilisation. In the tricul ure including *Methanosarcina* MP, cellulose was d egraded slower than in the co-culture. In all associ ations, more than 90% of the cellulose was degra ded.

With all 4 culture systems, cellulose fermentation was accompanied by a decrease in pH (Fig. 1). The pH drop was more pronounced when *C. thermocellum* was associated with *Methanobacterium* sp. In the triculture, the pH was maintained at 6.3 and decreased after 10 days of culture. When methanol was added to the triculture, the pH began to rise after 8 days and levelled off at pH 6.6.

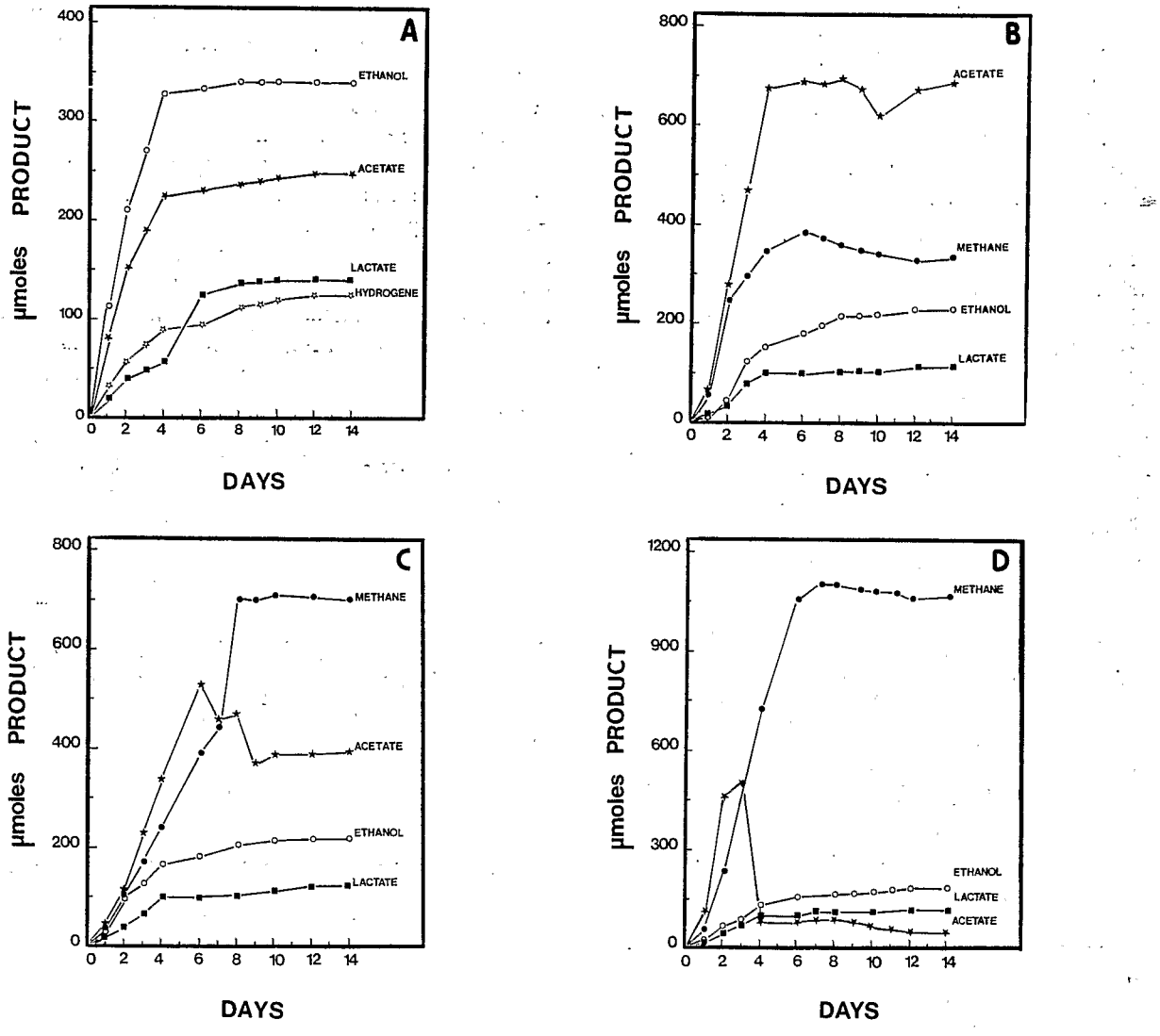

Fig. 2. Products of cellulose fermentation by *C. thermocellum* (A); *C. thermocellum* and *Methanobacterium* sp. (B); *C. thermocellum*, *Methanobacterium* sp. and *Methanosarcina* MP (C); *C. thermocellum*, *Methanobacterium* sp. and *Methanosarcina* MP+0.05 ml 2 M methanol (D). Culture vessels were incubated at 55°C. Results are expressed in µmol per 20 ml medium.

4.2. Cellulose fermentation p oducts

When grown on cellulose *C. thermocellum* produced large amounts of ethanol and acetate. Lactate and H_2 were also detected (Fig. 2A). CO_2 was produced but not measured. Co-culture of the cellulolytic bacterium with *Methanobacterium* sp. resulted in an increase (from 240 to 650 μmol) in acetate concentration, whereas ethanol concentration decreased (Fig. 2B). H_2 did not accumulate at any stage of incubation. The fermentation products of the *C. thermocellum-Methanosarcina* MP co-culture were identical qualitatively and quantitatively to those of the *C. thermocellum* monoculture (data not shown). H_2 accumulated and acetate was not degraded by the aceticlastic methanogen.

Mixtures of *C. thermocellum* and *Methanobacterium* sp. were co-cultured in association with the aceticlastic methanogen *Methanosarcina* MP (Fig. 2C). On the 10th day of incubation, acetate degradation stopped. In contrast to this, the addition of methanol to the triculture system, in the early stages of growth, induced rapid utilisation of acetate after 2 days of incubation, and almost all acetate was used by *Methanosarcina* MP (Fig. 2D). In this triculture, 1100 μmol methane was produced. Small amounts of ethanol and lactate were measured. Methane production was about 3 times higher than in the *C. thermocellum-Methanobacterium* sp. co-culture. Maximum methane production was 8.5 mmol CH_4/g cellulose degraded.

5. DISCUSSION

The co-culture *C. thermocellum-Methanobacterium* sp. -*Methanosarcina* MP converted cellulose mainly to CH_4 and CO_2. In the triculture system, methanogenesis from cellulose occurred in two steps: acetogenesis and methanogenesis (from H_2-CO_2 or from acetate). The significant metabolic interaction during cellulose degradation between *C. thermocellum* and a hydrogenophilic methanogen has already been described by Weimer and Zeikus [4]. The use of H_2 by the methanogenic bacterium caused a shift of the electron flow, resulting in formation of less reduced fermentation products. Such interspecies H_2 transfers have been well documented with a variety of mixed cultures [15–18].

The focus of this work was to elucidate the role of *Methanosarcina* MP in the triculture system. Unlike mesophilic *Methanosarcina* species [19,20], *Methanosarcina* MP was unable to use H_2-CO_2 [9]. Thus, under thermophilic conditions, each methanogen had a well-defined role during cellulose degradation. In the triculture, the yield of methane was higher than for either co-culture. Furthermore, complete acetate degradation could be achieved in presence of methanol in the triculture. When both methanol and acetate were added to the grown medium, high ratios of assimilation versus catabolism of acetate were reported for *Methanosarcina* strains [21,22].

In our experiments, addition of methanol, a better metabolisable substrate for *Methanosarcina,* induced a rapid utilisation of acetate in the mixed defined culture. At the end of this experiment, nearly all acetate was converted to methane. In the absence of methanol, *Methanosarcina* MP started to degrade acetate 2 days later than in presence of methanol, so that the pH was less favourable for the aceticlastic reaction. Indeed, pH near 6.0 inhibited methanogenesis from acetate by *Methanosarcina* MP (9). On the 8th day of incubation, acetate was no more degraded by *Methanosarcina* MP and the pH dropped significantly.

In the triculture including *Methanosarcina* MP, the degradation of cellulose by *C. thermocellum* was slower than in the *C. thermocellum-Methanobacterium* co-culture. It seems more likely that the high dilution rate of the *Methanosarcina* inoculum (10%, v/v) rather than the activity of the aceticlastic methanogen, is responsible for this effect.

Methanosarcina MP did not use H_2-CO_2 or acetate when co-cultured with *C. thermocellum.* Acetate was used only when both methanogens were present. These results suggested inhibition of the aceticlastic reaction in *Methanosarcina* MP by H_2-CO_2 as reported for other *Methanosarcina* strains [23–25]. However, the conversion of H_2-CO_2 to methane by *Methanobacterium* sp. restored the aceticlastic activity by *Methanosarcina* MP.

Our data suggested that this triculture may be useful in a thermophilic bioconversion system.

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REFERENCES

[1] Khan, A.W. (1977) Can. J. Microbiol. 23, 1700–1705.
[2] Khan, A.W., Trottier, T.M., Patel, G.B. and Martin, S.M. (1979) J. Gen. Microbiol. 112, 365–372.
[3] Suchardova, O., Volfova, O., Krumphanzl, V. and Panos, J. (1981) Biotechnol. Lett. 3, 547–550.
[4] Weimer, P.J. and Zeikus, J.G. (1977) Appl. Environ. Microbiol. 33, 289–297.
[5] Khan, A.W. (1980) FEMS Microbiol. Lett. 9, 233–235.
[6] Laube, V.M. and Martin, S.M. (1981) Appl. Environ. Microbiol. 42, 413–420.
[7] Mountfort, D.O., Asher, R.A. and Bauchop, T. (1982) Appl. Environ. Microbiol. 44, 128–134.
[8] Laube, V.M. and Martin, S.M. (1983) Can. J. Microbiol. 29, 1475–1480.
[9] Ollivier, B., Lombardo, A. and Garcia, J.L. (1984) Ann. Microbiol. Inst. Pasteur 135B, 187–198.
[10] Hungate, R.E. (1969) in Methods in Microbiology (Norris, J.R. and Ribbons, D.W., Eds.) Vol. 3B, pp. 1767–1803. Academic Press, New York.
[11] Balch, W.E., Fox, G.E., Magrum, R.J. and Wolfe, R.S. (1979) Microbiol. Rev. 43, 260–296.
[12] Ollivier, B.M., Mah, R.A., Garcia, J.L. and Robinson, R. (1985) Int. J. Syst. Bacteriol. 35, 127–130.
[13] Garcia, J.L., Cayot, J.P., Ollivier, B., Trad, M. and Paycheng, C. (1982) Cah. ORSTOM. Sér. Biol. 45, 3–15.
[14] Hanson, R.S. and Phillips, J.A. (1981) in Manual of Methods for General Microbiology (Gerhardt, P., Murray, R.G.E., Costilow, R.N., Nester, E.W., Wood, W.A., Krieg, N.R. and Phillips, G.B., Eds.), pp. 328–364. ASM, Washington, DC.
[15] Bryant, M.P., Wolin, E.A., Wolin, M.J. and Wolfe, R.S. (1967) Arch. Microbiol. 59, 20–31.
[16] Iannotti, E.L., Kafkewitz, D., Wolin, M.J. and Bryant, M.P. (1973) J. Bacteriol. 114, 1231–1240.
[17] Latham, M.J. and Wolin, M.J. (1977) Appl. Environ. Microbiol. 34, 297–301.
[18] Wolin, M.J. (1974) Am. J. Clin. Nutr. 27, 1320–1328.
[19] Mah, R.A., Smith, M.R. and Baresi, L. (1978) Appl. Environ. Microbiol. 35, 1174–1184.
[20] Mah, R.A. and Smith, M.R. (1981) in The Prokaryotes (Starr, M.P., Stolp, H., Trüper, H.G., Balows, A. and Schlegel, H.G., Eds.), Vol. I, pp. 948–977. Springer Verlag, Berlin.
[21] Weimer, P.J. and Zeikus, J.G. (1978) Arch. Microbiol. 119, 175–182.
[22] Zinder, S.H. and Mah, R.A. (1979) Appl. Environ. Microbiol. 38, 996–1008.
[23] Smith, M.R., Mah, R.A. (1978) Appl. Environ. Microbiol. 36, 870–879.
[24] McInerney, M.J. and Bryant, M.P. (1981) Appl. Environ. Microbiol. 41, 346–354.
[25] Ferguson, T.J. and Mah, R.A. (1983) Appl. Environ. Microbiol. 46, 348–355.