Role of formate in methanogenesis from xylan by *Cellulomonas* sp. associated with methanogens and *Desulfovibrio vulgaris*: inhibition of the aceticlastic reaction

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1. SUMMARY

Different methanogenic defined mixed cultures, including *Cellulomonas* sp. strain ATCC21399 as a hydrolytic and fermentative bacterium, were used to show that methane production could proceed from larchwood xylan as well as from cellulose. Via the different mixtures of bacteria used, the role of formate is described. It is shown that formate inhibits methanogenesis from acetate by pure cultures of aceticlastic methanogens.

2. INTRODUCTION

The anaerobic degradation of organic matter on a laboratory scale has been studied using glucose and cellulose degradation [1–6]. Cellulose (and therefore glucose) accounts for a high percentage of the composition of biomass [7], but relatively little reference has been made to the other important substrate, hemicellulose, and its main product of hydrolysis, xylose [8]. Hemicellulose is not only an important component of plant cellwalls, but may represent one of the main residues of the different industries processing wood or biomass [7,9].

This paper reports on the production of methane from xylan, using defined mixed cultures of bacteria involved in the anaerobic digestion of organic matter. An attempt is made to point out the importance of formate, as shown by the use of mixed and pure cultures.

3. MATERIALS AND METHODS

3.1. Organisms and growth conditions

The anaerobic techniques described by Hungate [10], Balch and Wolfe [11] and Balch et al. [12] were used throughout this study. Bacteria were grown anaerobically in aluminium-seal culture tubes (Bellco Glass, Vineland, NJ, U.S.A.). *Methanobacterium formicicum* MF was grown at 37°C under 5 psi of N_2-CO_2 (4:1) in the medium of Balch et al. [12]. *Methanosarcina barkeri* 227 was grown at 37°C under 30 psi of H_2-CO_2 (4:1) and *Methanosarcina thermophila* at 55°C, under 5 psi of N_2-CO_2 (4:1) in the medium described by Jones et al. [13], except that TES-buffer was replaced by bicarbonate buffer (4 g/l). *Desulfovibrio vulgaris* J.J. was grown at 37°C, as previously described [13].

Cellulomonas sp. strain ATCC21399 was grown

at 30°C under 5 psi of N_2-CO_2 (4:1) in the following medium: mineral solution No. 1 [12], 50 ml; mineral solution No. 2 [12], 50 ml; trace vitamin solution [12], 10 ml; trace mineral solution [12], 10 ml; $FeSO_4 \cdot 7H_2O$, 0.002 g; resazurin, 0.001 g; NH_4Cl, 1 g; xylan from larchwood (Sigma), 2 g; $NaHCO_3$, 7 g; yeast extract, 0.1 g; biotrypcase, 0.1 g; cysteine-HCl, 0.5 g. Anoxic medium was prepared as previously described [13].

Cellulomonas sp. strain ATCC21399 was used as a hydrolytic and fermentative bacterium able to produce acetate, ethanol and formate from xylan. Methanogenesis was expected from different mixtures of *Cellulomonas* and methanogenic bacteria such as: *Ms. barkeri* 227 as an aceticlastic methanogen, *Mb. formicicum* MF as a formate user.

In some experiments, *D. vulgaris* J.J. was added in methanogenic mixed cultures. This bacterium was able to degrade formate and to produce acetate from ethanol either in the presence or absence of sulfate on the basis of an interspecies hydrogen transfer [14].

3.2. Analytical procedures

Methane and acetate were assayed as previously described [15]. Ethanol was assayed using the same conditions as for CH_4. Formic acid was determined colorimetrically according to the method of Sleat and Mah [16]. Each liquid sample was prepared as previously described [15].

3.3. Experimental conditions

The basal medium used for all experiments was the same as described for *Cellulomonas* cultivation. In experiments containing both *D. vulgaris* and *Ms. barkeri* in the presence of sulfate, an excess of divalent iron was added to the medium to prevent inhibition of the aceticlastic reaction by high levels of soluble sulfides [15]. Xylan was added at the concentrations indicated. Experiments were performed in 60-ml serum bottles each containing 20 ml of medium. All experiments were made in triplicate and two controls without substrates were run for each experiment. Vials were incubated at 30°C except when specified.

For the sake of simplicity, associations of bacteria are designated as follows: C, *Cellulomonas* sp.; D, *D. vulgaris*; F, *Mb. formicicum*; B, *Ms. barkeri*; e.g., association CDB contained *Cellulomonas* sp., *D. vulgaris* and *Ms. barkeri*.

4. RESULTS AND DISCUSSION

Two sets of experiments were done at 1 g/l and 5 g/l of xylan. Methane production at 1 g/l of xylan leveled off after 11 days of fermentation for the association CDB in the presence of sulfate (Fig. 1), and after one week for the others (Fig. 1). At 5 g/l, fermentation was completed after a longer period, ranging between 20 and 40 days (Fig. 2). At this concentration, there was a long lag phase with CF we could not explain in terms of the adaptation of *Mb. formicicum* to the use of formate since this bacterium was pregrown on formate.

Maximum methane production was obtained with the association CDB in presence of sulfate at 1 g/l and 5 g/l of xylan, respectively 8 μmol/ml and 50 μmol/ml. In these conditions, methane
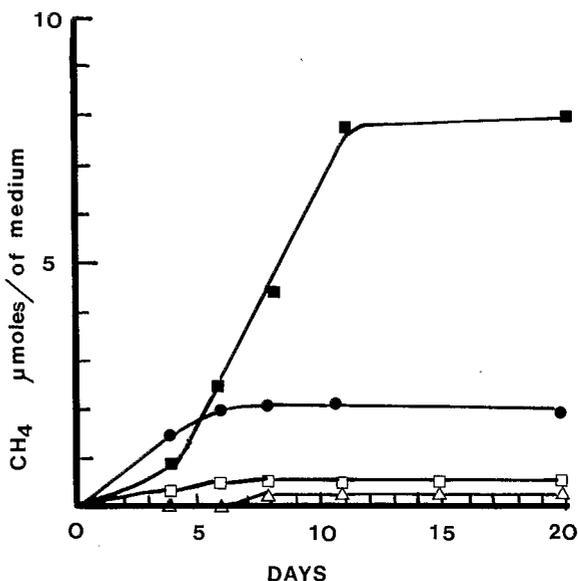
Fig. 1. Methane production from xylan (1 g/l) by different defined mixed cultures: △————△, CB; □————□, CF; ●————●, CDF in sulfate-free medium; ■————■, CDB in sodium sulfate and iron sulfate-containing medium (Na_2SO_4, 2 g/l; $FeSO_4 \cdot 7H_2O$, 4 g/l).
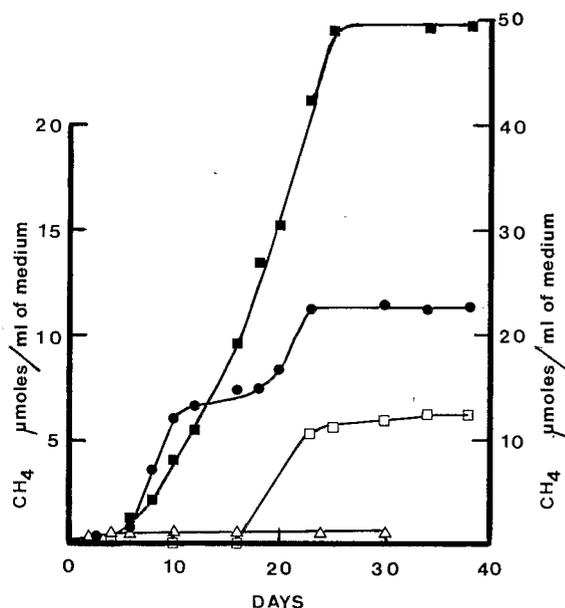
Fig. 2. Methane production from xylan (5 g/l). The left scale is for the following associations: □———□, CF; △———△, CB; ●———●, CDF in sulfate-free medium. The right scale is for: ■———■, CDB in sodium sulfate and iron sulfate-containing medium (Na_2SO_4, 2 g/l; $FeSO_4 \cdot 7H_2O$, 4 g/l).

production from xylan followed the commonly acknowledged pathways [17]: hydrolysis and acidogenesis by *Cellulomonas* sp., acetogenesis by *D. vulgaris* and methanogenesis by *Ms. barkeri* 227.

No 2-step kinetic reactions were observed for the association CDF in sulfate-free medium at 1 g/l of xylan (Fig. 1), contrary to the result obtained at 5 g/l with the same association (Fig. 2). Nevertheless, at 1 g/l, a 2-step kinetic reaction could be obtained in the earlier stage of fermentation. Such a pattern could be explained, either as a sequential use of formate and then ethanol by *D. vulgaris* in association with *Mb. formicicum* on the basis of an interspecies hydrogen transfer, or by the use of formate first by *Mb. formicicum* alone and then the oxidation of ethanol by the association of the sulfate reducer and the methanogen.

At the end of the experiment at 1 g/l of xylan, no formate or ethanol was detected, and almost stoichiometric amounts of CH_4 were produced (Table 1) assuming the following ratios:

Table 1

End-products formed at 1 g/l of xylan by different associations of bacteria (see text)

	Formate (mM)	Acetate (mM)	Ethanol (mM)	CH_4 (μmol/ml)
CF	0	6.1	2.2	0.6
CB	2.4	5.6	2.3	0.3
$CDF-SO_4$ [a]	0	8	0	2.0
$CDF+SO_4$ [b]	0	nd [c]	nd	0
$CDB+SO_4$ [b]	0	0	0	8.0

[a] In sulfate-free medium.
[b] In medium plus sulfate (Na_2SO_4, 2 g/l) and iron sulfate ($FeSO_4 \cdot 7H_2O$, 4 g/l).
[c] nd, Not determined.

$$4 \text{ formate} \rightarrow 1 \text{ } CH_4 \text{ (Table 2)} \qquad (1)$$

$$2 \text{ ethanol} \rightarrow 1 \text{ } CH_4 \text{ [18]} \qquad (2)$$
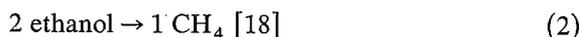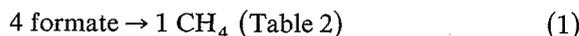
The use of formate by interspecies hydrogen transfer between *D. vulgaris* and *Mb. formicicum* could be a matter of controversy as the methanogen is a formate user (association CF, Table 1). Nevertheless, an alternative route could be suggested, i.e., carbon flow through formate in a sulfate-depleted environment, as described in Table 2.

In a medium containing an excess of sulfate, no CH_4 was produced by the association CDF, but all

Table 2

Alternative pathways for anaerobic formate uptake in sulfate-depleted environment

1. Direct use of formate by *Mb. formicicum*

$$4HCO_2^- + H_2O + H^+ \rightarrow CH_4 + 3HCO_3^-$$
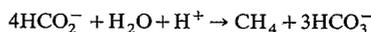
$$\Delta G'^0 = -130.4 \text{ kJ [18]}$$

2. Use of formate by interspecies hydrogen transfer:
   (i) half reaction completed by *D. vulgaris*

$$\left(HCO_2^- + H_2O \rightarrow HCO_3^- + H_2 \text{ } \Delta G'^0 = +1.3 \text{ kJ} \right) \times 4 \text{ [18]}$$
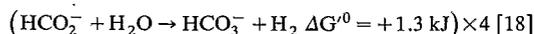
   (ii) half reaction completed by *Mb. formicicum*

$$4H_2 + HCO_3^- + H^+ \rightarrow CH_4 + 3 \text{ } H_2O \text{ } \Delta G'^0 = -135.6 \text{ kJ [18]}$$
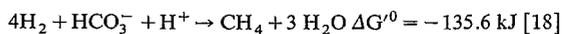
   (iii) sum of these reactions

$$4HCO_2^- + H_2O + H^+ \rightarrow CH_4 + 3HCO_3^-$$
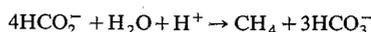
$$\Delta G'^0 = -130.4 \text{ kJ [21]}$$

the formate was used (Table 1). This would suggest that *D. vulgaris* displayed a higher affinity for formate than did *Mb. formicicum*. Therefore, in a way similar to the use of hydrogen in presence of sulfate [19], sulfate-reducing bacteria and formate-using methanogens seem to compete for formate. Thus, the presence of sulfate in anaerobic environments (e.g., anaerobic digestors, sediments) would divert the following compounds from direct methanogenesis to sulfatoreduction: hydrogen [19,20], acetate [21] and formate.

Another phenomenon was apparent the results (Table 1, Fig. 1): after several trials, methanogenesis from xylan never occurred when the association CB was used; only a very small amount of methane was produced and acetate was scarcely degraded (Table 1), even after a long period of incubation (one month). However, using the association CDB in the presence of sulfate (Table 1) or in its absence (Fig. 2), methanogenesis from acetate was not inhibited. The end-product formed by CB in the liquid phase were formate, acetate and ethanol (Table 1). Ethanol was not suspected to be an inhibitor at 2 mM, since the aceticlastic reaction had worked well in previous experiments, where ethanol appeared as an end-product in defined mixed cultures using *Ms. barkeri* 227 [13].

On the other hand, as with ethanol, formate did not accumulate when the association CDB was used in the presence of sulfate. Unless an unknown product responsible for this inhibition was used by the sulfate reducer, formate is suspected to be a direct inhibitor of the aceticlastic reaction.

Formate was shown to be an inhibitor of methanogenesis from acetate by Baresi et al. [22] but this work was done with an enrichment culture, so an indirect effect could be the explanation.

To demonstrate the direct inhibition of the aceticlastic reaction by formate, experiments with pure cultures of methanogens were performed. As shown in Fig. 3, methanogenesis from acetate by *Mb. barkeri* 227 is inhibited by formate.

It appears (Fig. 3) that the extent of inhibition depended on the formate concentration in relation to a fixed concentration of acetate (2 g/l). The rate and the amount of methane decreased with increasing formate concentrations (Fig. 3). In neither case was hydrogen detected. To make cer-
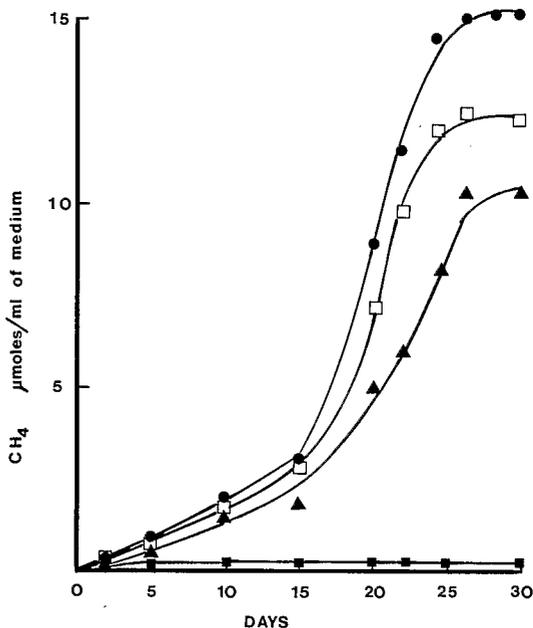
Fig. 3. Effect of different sodium formate concentrations upon methanogenesis of trihydrated sodium acetate (2 g/l), by *Ms. barkeri* 227 cultivated at 37°C: ●————●, 0 mM; □————□, 1.25 mM; ▲————▲, 2.5 mM; ■————■, 20 mM.

tain that this inhibition is not a feature peculiar to the mesophilic strain, experiments with an aceticlastic thermophilic methanogen, *Ms.thermophila,*
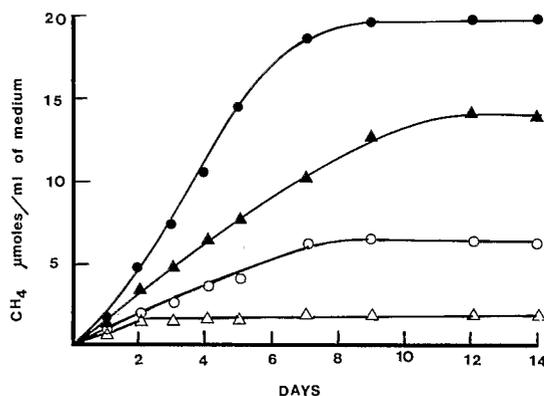
Fig. 4. Effect of 10 mM sodium formate on thermophilic methanogenesis of trihydrated sodium acetate by *Ms. thermophila* cultivated at 55°C. ●————●, Acetate 4 g/l, formate 0 mM; ○————○, acetate 4 g/l, formate 10 mM; ▲————▲, acetate 2 g/l, formate 0 mM; △————△, acetate 2 g/l, formate 10 mM.

were performed with 4 g/l and 2 g/l of trihydrated sodium acetate. As shown in Fig. 4, methanogenesis from acetate is inhibited by formate under thermophilic conditions. At a constant formate concentration, the extent of the inhibition depended on the acetate concentration (Fig. 4). Furthermore, the rate and total amount of evolved methane decreased to 0 (Fig. 4) and hydrogen was not evolved.

From these data, it is now clear that formate was responsible for the inhibition of methanogenesis in the mixed culture CB (Table 1); clearly, further studies need to be carried out on the inhibitory role of formate on anaerobic fermentations.

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