Methanogenesis from Sucrose by Defined Immobilized Consortia

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A bacterial consortium capable of sucrose degradation primarily to \(\text{CH}_4\) and \(\text{CO}_2\) was constructed, with acetate as the key methanogenic precursor. In addition, the effect of agar immobilization on the activity of the consortium was determined. The primary fermentative organism, \(\text{Escherichia coli}\), produced acetate, formate, \(\text{H}_2\), and \(\text{CO}_2\) (known substrates for methanogens), as well as ethanol and lactate, compounds that are not substrates for methanogens. Oxidation of the nonmethanogenic substrates, lactate and ethanol, to acetate was mediated by the addition of \(\text{Acetobacterium woodii}\) and \(\text{Desulfovibrio vulgaris}\). The methanogenic stage was accomplished by the addition of the acetophilic methanogen \(\text{Methanothermobacterium barkeri}\) and the hydrogenophilic methanogen \(\text{Methanobacterium formicicum}\). Results of studies with low substrate concentrations (0.65 to 0.2% [wt/vol]), a growth-limiting medium, and the five-component consortium indicated efficient conversion (40%) of sucrose carbon to \(\text{CH}_4\). Significant decreases in yields of \(\text{CH}_4\) and rates of \(\text{CH}_4\) production were observed if any component of the consortium was omitted. Approximately 70% of the \(\text{CH}_4\) generated occurred via acetate. Agar-immobilized cells of the consortium exhibited yields of \(\text{CH}_4\) and rates of \(\text{CH}_4\) production from sucrose similar to those of nonimmobilized cells. The rate of \(\text{CH}_4\) production decreased by 25% when cysteine was omitted from reaction conditions and by 40% when the immobilized consortium was stored for 1 week at 4°C.

MATERIALS AND METHODS

Organisms and growth conditions. The anaerobic techniques described by Hungate (12) and Balch et al. (1, 3) were used throughout this study. Bacteria were grown anaerobically in aluminum-seal culture tubes (18 by 150 mm) (Bellco Glass, Inc., Vineland, N.J.) or in 250-ml serum bottles (Wheaton Scientific Co., E. coli ATCC 12435 was grown at 37°C under an atmosphere of \(\text{N}_2\) in medium containing the following constituents (per liter of distilled water): yeast extract, 2.0 g; Trypticase (BBL Microbiology Systems, Cockeysville, Md.), 2.0 g; \(\text{KH}_2\text{PO}_4\), 10.0 g; mineral solution no. 2 (30), 10 ml; resazurin, 0.01 g. A filter-sterilized, \(\text{O}_2\)-free solution of sucrose was added before inoculation at a final concentration of 0.2% (wt/vol). \(\text{Desulfovibrio vulgaris}\) IVJ, isolated from estuarine sediments, was grown at 37°C under an atmosphere of \(\text{N}_2\)-\(\text{CO}_2\) (4:1) in a medium that contained the following constituents (per liter of distilled water): mineral solution no. 1 (2), 50 ml; mineral solution no. 2 (2), 50 ml; trace vitamin solution (30), 10 ml; \(\text{FeSO}_4 \cdot \text{7H}_2\text{O}\), 0.1 g; Trypticase, 1.0 g; \(\text{NaHCO}_3\), 4.0 g; \(\text{Na}_2\text{SO}_4\), 2.8 g; cysteine • \(\text{HCl} \cdot \text{H}_2\text{O}\), 0.5 g; \(\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}\), 0.2 g. Sodium lactate was added at a final concentration of 40 mM. \(\text{A. woodii}\) ATCC 29683 was grown in M1 medium as described previously by Balch et al. (2). Fructose was added as substrate at a final concentration of 0.5% (wt/vol). \(\text{Methano­bacterium formicicum}\) MF was grown at 37°C in medium 1 of Balch et al. (1) under 203 kPa of \(\text{H}_2\)-\(\text{CO}_2\) (4:1). \(\text{M. barkeri}\) 227 was grown at 37°C under an atmosphere of \(\text{N}_2\) in medium containing the following constituents (per liter of distilled water): mineral solution no. 1 (1), 40 ml; mineral solution no. 2 (1), 40 ml; \(\text{FeSO}_4 \cdot \text{7H}_2\text{O}\), 0.002 g; resazurin, 0.001 g; trace mineral solution (30), 10 ml; vitamin solution (30), 10 ml; \(\text{NiCl}_2 \cdot 6\text{H}_2\text{O}\), 0.5 mg; \(\text{Na}_2\text{SeO}_3\), 3.7 mg; yeast extract, 1.0 g; Trypticase, 1.0 g; sodium acetate, 8.0 g; cysteine • \(\text{HCl} \cdot \text{H}_2\text{O}\), 0.5 g; \(\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}\), 0.5 g. The medium was buffered with TES (N-tris(hydroxymethyl)methyl-2-amino­ethanesulfonic acid) at a final concentration of 50 mM; the
pH of the medium was adjusted to 7.0 with NaOH.

Anoxic medium was prepared by boiling the medium without adding reducing agents under a stream of N₂ or N₂-CO₂ (4:1) for 60 s. Reducing agents were added, the flask was stoppered, and the medium was dispensed inside an anaerobic glove box (Coy Manufacturing Co., Ann Arbor, Mich.). Media were sterilized by autoclaving at 105 kPa for 20 min.

Analytical procedures. Methane was analyzed in a Carle 9700 gas chromatograph equipped with a flame ionization detector and a 2-m stainless steel column packed with 8% SF-96 on Chromosorb WHP (80/100 mesh) and operated at 150°C. The carrier gas was N₂ at a flow rate of 25 ml min⁻¹. CH₄ was quantitated by comparing the peak height with known standards. H₂ and CO₂ were analyzed with a Packard 7800 gas chromatograph equipped with thermal conductivity detectors and a 2-m Porapak Q (80/100 mesh) column operated at 120°C. The carrier gas was N₂ at a flow rate of 30 ml min⁻¹. All gas sampling was performed with a pressure-lock syringe (Precision Sampling Corp., Baton Rouge, La.).

Acetate and ethanol were analyzed by gas chromatography as described for CH₄ determination, except that a 3-m column packed with SP-1000-1% H₃PO₄ on Chromosorb WAW (Supelco) was used for sample separation. Each liquid sample (1.0 ml) for organic acid and alcohol analysis was removed aseptically, centrifuged for 5 min at 10,000 x g to remove cells, and acidified with H₃PO₄ before injection; the recorded peak area was compared with known standards.

Glucose was determined colorimetrically with glucose oxidase (Sigma Chemical Co., St. Louis, Mo.) according to Sigma technical bulletin no. 510. Sucrose was assayed as described for CH₄ determination, except that a 3-m column packed with SP-1000-1% H₃PO₄ on Chromosorb WAW (Supelco) was used for sample separation. Each liquid sample (1.0 ml) for organic acid and alcohol analysis was removed aseptically, centrifuged for 5 min at 10,000 x g to remove cells, and acidified with H₃PO₄ before injection; the recorded peak area was compared with known standards.

Results

Sucrose fermentation by mixed cultures. Various combinations of E. coli, A. woodii, and D. vulgaris were cocultured to determine optimal conditions for the production of direct methanogenic precursors; the results are presented in Table 1A. The products of sucrose fermentation by the E. coli strain included acetate, ethanol, lactate, formate, CO₂, and H₂ (data not shown). Optimal acetate production occurred with the association of the three organisms (Table 1A); a shift in the final concentration of fermentation products from less ethanol and lactate formation to increased production of acetate was observed. These results are in agreement with previous observations (13, 18, 25, 31) and may be attributed to lactate oxidation to acetate by D. vulgaris or A. woodii, to ethanol oxidation by D. vulgaris, and to a shift in the fermentation pattern of E. coli toward increased production of oxidized compounds.

Mixtures of E. coli, A. woodii, and D. vulgaris, in association with a hydrogenophilic methanogen (M. formicicum), exhibited significant increases in acetate production from sucrose (Table 1B) compared with consortia without M. formicicum. Acetate production was optimal when the consortium contained all four organisms. Optimal CH₄ production occurred when M. formicicum was cocultured with E. coli and A. woodii; in the absence of an acetophilic methanogen, the amount of CH₄ produced represented a 15% recovery of carbon from sucrose. In all mixed cultures tested with M. formicicum, insignificant amounts of ethanol or lactate accumulated as final products of sucrose fermentation.

Mixtures of E. coli, D. vulgaris, and A. woodii were cocultured in association with the acetophilic methanogen M. barkeri, and the results are presented in Table 1C. The major products detected were CH₄ and CO₂. In mixed cultures without D. vulgaris, some ethanol was detected as a final product; ethanol was significantly reduced in mixed
### TABLE 1. Sucrose degradation by defined mixed cultures

<table>
<thead>
<tr>
<th>Organism*</th>
<th>Ethanol</th>
<th>Lactate</th>
<th>Acetate</th>
<th>H₂</th>
<th>CH₄</th>
<th>% C</th>
<th>as CH₄</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A.</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E. coli</td>
<td>8.3</td>
<td>4.0</td>
<td>11.2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.3</td>
</tr>
<tr>
<td>E. coli, A. woodii</td>
<td>7.0</td>
<td>0.6</td>
<td>12.0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.6</td>
</tr>
<tr>
<td>E. coli, A. woodii, D. vulgaris</td>
<td>2.0</td>
<td>ND</td>
<td>12.8</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2.0</td>
</tr>
<tr>
<td><strong>B.</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>E. coli, M. formicicum</td>
<td>1.1</td>
<td>14.6</td>
<td>0</td>
<td>7.3</td>
<td>11</td>
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<td></td>
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<tr>
<td>E. coli, M. formicicum, A. woodii</td>
<td>0.6</td>
<td>0.4</td>
<td>15.7</td>
<td>0</td>
<td>10.0</td>
<td>15</td>
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<tr>
<td>E. coli, M. formicicum, A. woodii, D. vulgaris</td>
<td>0.7</td>
<td>ND</td>
<td>17.1</td>
<td>0</td>
<td>7.4</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td><strong>C.</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E. coli, M. barkeri</td>
<td>2.2</td>
<td>0.4</td>
<td>0</td>
<td>15.5</td>
<td>23</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E. coli, M. barkeri, A. woodii</td>
<td>2.0</td>
<td>ND</td>
<td>0</td>
<td>17.3</td>
<td>26</td>
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<tr>
<td>E. coli, M. barkeri, A. woodii, D. vulgaris</td>
<td>0.7</td>
<td>ND</td>
<td>0</td>
<td>27.0</td>
<td>40</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>D.</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E. coli, A. woodii, D. vulgaris, M. formicicum, M. barkeri</td>
<td>1.0</td>
<td>ND</td>
<td>0</td>
<td>28.4</td>
<td>42</td>
<td></td>
<td></td>
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</tbody>
</table>

* Cultures were incubated at 37°C in basal medium for 10 days at an initial sucrose concentration of 0.15% (wt/vol).

Values do not represent a complete fermentation balance from sucrose; CO₂ and formate were not quantified.

### METHANOSIS BY DEFINED IMMOBILIZED CONSORTIA

The kinetics of sucrose degradation by specific mixed cultures are presented in Fig. 1A and B. At a sucrose concentration of 5 mM, rates of CH₄ production were 133 μmol day⁻¹ 100 ml⁻¹ when M. formicicum was cultured with E. coli, A. woodii, and D. vulgaris (Fig. 1A), compared with 330 μmol day⁻¹ 100 ml⁻¹ when M. barkeri was cultured with E. coli, A. woodii, D. vulgaris, and M. formicicum (Fig. 1B). Acetate accumulated in the consortium with M. barkeri and reached a final concentration of 17.1 mM. In the consortium with M. barkeri, acetate was detected in low concentrations (5 mM) as an intermediate product during the course of sucrose degradation, with final concentrations of less than 2 mM being detected.

Immobilization of mixed cultures. Agar was chosen as a matrix to test the effect of immobilization of the defined consortia on sucrose degradation to CH₄. Two different-size pellets of solidified agar were tested to determine the characteristics of sucrose diffusion through the agar matrix, and the results are presented in Fig. 2. As expected, the smaller pellets (2.5 by 2.5 by 6 mm), having a greater surface/volume ratio, exhibited faster sucrose equilibration with the suspending medium than the larger pellets (6 by 6 by 6 mm). An equilibrium of sucrose between the agar matrix and the suspending fluid was reached after 20 and 35 min, respectively, for the small and large pellets. The small-size pellets were used throughout this study for immobilized consortia.

The complete consortium, consisting of E. coli, A. woodii, D. vulgaris, M. formicicum, and M. barkeri, was immobilized with Noble agar and tested to determine the effect on sucrose degradation to CH₄. The results presented in Fig. 1D indicate that the rate of CH₄ production for the immobilized consortium (320 μmol day⁻¹ 100 ml⁻¹) and the total CH₄ produced (2,300 μmol 100 ml⁻¹) were almost identical to the data obtained for the consortium of free cells (Fig. 1B). Thus, the agar-immobilization procedure did not affect the activity of the defined consortium. When Noble agar was substituted with Bacto-Agar (Difco) for immobilization, no differences in methanogenesis were noted.

**Influence of A. woodii in defined consortium.** To determine the importance of A. woodii on CH₄ production in the defined immobilized consortium, a comparison was made between the complete consortium and the consortium without A. woodii. At a sucrose concentration of 5 mM, the total CH₄ production for the consortium without A. woodii was...
production was 220 μmol day⁻¹ 100 ml⁻¹. These values are 13 and 30% less, respectively, compared with the results of the complete consortium (Fig. 2). Results suggest that A. woodii significantly contributes to the pool of methanogenic precursors, either by the homoeoacetate fermentation of glucose or fructose or by the oxidation of lactate to acetate.

Effects of temperature, storage, and reducing conditions on immobilized consortia. Several temperatures in the mesophilic range of growth were tested to determine the effect of temperature on CH₄ production by the defined immobilized consortium. The rates of CH₄ production at 30 and 37°C in a sucrose concentration of 1.5 mM were similar (Fig. 3). At 25°C, however, a lag was noted before the onset of CH₄ production.

Pellets of the immobilized consortium were added to basal medium without sucrose and stored at 4°C under an atmosphere of N₂ for various lengths of time to determine the effect of cold storage on the activity of the consortium. Cold storage under N₂ in the absence of substrate significantly affected the rate of CH₄ production (Fig. 4). After 1 and 5 weeks of storage at 4°C, the rates of CH₄ production were 40 and 60% lower, respectively, compared with the rate of a consortium tested immediately after preparation (control).

The effects of anaerobiosis and reducing conditions on CH₄ production by the immobilized consortium were tested, using basal medium prepared in various ways (Table 2). In aerobically prepared basal medium without cysteine, no CH₄ production was detected after 10 days of incubation at 37°C. Aerobically prepared basal medium without cysteine, produced by axenic E. coli fermentation, was inoculated with the immobilized consortium, and the rate of CH₄ production was reduced by 40% compared with controls. When the aerobically prepared basal medium without cysteine was aseptically bubbled with sterile N₂ at a rate of 100 ml min⁻¹ for 15 min, the immobilized consortium exhibited a 25% reduction in the rate of CH₄ production. When basal medium with cysteine was used in a similar experiment, no difference in the rate of CH₄ production was found compared with standard anaerobically prepared basal medium with cysteine.

**DISCUSSION**

Methanogenesis from biopolymers in natural ecosystems occurs in three basic steps (29): fermentation, acetogenesis, and methanogenesis. The acetogenic step, for our purposes, is defined as intermediary reactions producing acetate, i.e., via oxidation of organic acids or alcohols to acetate, or oxidation of hydrogen and reduction of CO₂ to acetate. E. coli was chosen as the primary organism responsible for the first step in the bioconversion of sucrose to CH₄ because the mixed acid fermentation of sucrose by E. coli primarily yields direct methanogenic substrates (acetate, formate, H₂, and CO₂) and indirect (ethanol, lactate) methanogenic precursors. A. woodii and D. vulgaris were chosen to fulfill the acetogenic step of the metabolic scheme; both bacteria are capable of lactate oxidation to acetate, although by different mechanisms. In addition, A. woodii may play an additional role in the production of methanogenic precursors by a homoeoacetate fermentation of hexose. Further, D. vulgaris oxidizes ethanol to acetate and reduces sulfate to sulfide; it is known that sulfide is required by most methanogens for growth and that reducing conditions are required for activity (6, 29, 32). The methanogenic step was effected by M. barkeri and M. formicicum to ensure optimal CH₄ production from several methanogenic substrates. As indicated by the results, optimal CH₄ production from sucrose was obtained by the complete consortium (E. coli, A. woodii, D. vulgaris, M. barkeri, and M. formicicum). At a sucrose concentration of 0.2% (w/vol), ca. 42% of the sucrose carbon was recovered as CH₄. An estimate of the percentage of CH₄ arising from acetate was made from the difference in CH₄ produced by the consortium in the presence and absence of M. barkeri. Since M. barkeri was the sole acetate-degrading organism present. Calculations revealed that 70% of the CH₄ was produced from acetate, and this value is in good agreement with data from various ecological studies (14), indicating that our consortium may be representative of the type of mixed cultures found in natural fermentative-methanogenic habitats.

The performance of the constructed consortium was significant considering that conditions for bacterial growth were severely limited in our experiments. Only trace

![FIG. 2. Diffusion of sucrose through agar matrix. Pellets of solidified agar containing sucrose (0.5% [w/vol]) were added to a solution of distilled water, and sucrose release was followed with time. Symbols: O, agar pellets (6 by 6 by 6 mm); O, agar pellets (2.5 by 2.5 by 6 mm).](image)

![FIG. 3. Effect of incubation temperature on methanogenesis from sucrose (0.05% [w/vol]) by the complete defined immobilized consortium (E. coli, A. woodii, D. vulgaris, M. formicicum, and M. barkeri). Symbols: O, 25°C; O, 30°C; A, 37°C.](image)
METHANOGENESIS BY DEFINED IMMOLIZED CONSORTIA

FIG. 4. Effect of storage time of the complete immobilized consortium on methanogenesis from sucrose (0.05% [wt/vol]). Agar pellets of the consortium were added to basal medium without substrate and stored at 4°C for 1 week (B), 3 weeks (C), and 5 weeks (D) before the addition of substrate and incubation at 37°C. (A) Control to which substrate was added immediately after preparation of the consortium.

TABLE 2. Effects of anaerobiosis and reducing conditions on methanogenesis from sucrose*

<table>
<thead>
<tr>
<th>Condition</th>
<th>Total CH₄ production (mmol/100 ml)</th>
<th>Maximal rate of CH₄ mmol day⁻¹ (100 ml⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anaerobic</td>
<td>0.8</td>
<td>0.4</td>
</tr>
<tr>
<td>N₂ sparged*</td>
<td>0.8</td>
<td>0.4</td>
</tr>
<tr>
<td>N₂ sparged*</td>
<td>0.5</td>
<td>0.3</td>
</tr>
<tr>
<td>Aerobic, E. coli 1</td>
<td>0.5</td>
<td>0.23</td>
</tr>
<tr>
<td>Aerobic</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* Pellets of the defined immobilized consortium (E. coli, A. woodii, D. vulgaris, M. formicicum, and M. bacterii) were added to basal medium (0.05% [wt/vol] sucrose), prepared as indicated, and incubated at 37°C with shaking for 10 days.
* Medium gassed with N₂ at a flow rate of 100 ml min⁻¹ for 15 min.
* Medium without cysteine.

Acknowledgments

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Literature Cited


