Applied and Environmental Microbiology, Jan. 1984, p. 1–6
0099-2240/84/010001-06$02.00/0
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Vol. 47, No. 1

Methanogenesis from Sucrose by Defined Immobilized Consortia

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Received 6 June 1983/Accepted 3 October 1983

A bacterial consortium capable of sucrose degradation primarily to CH_4 and 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, *Escherichia coli*, produced acetate, formate, H_2, and 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 *Acetobacterium woodii* and *Desulfovibrio vulgaris*. The methanogenic stage was accomplished by the addition of the acetophilic methanogen *Methanosarcina barkeri* and the hydrogenophilic methanogen *Methanobacterium formicicum*. Results of studies with low substrate concentrations (0.05 to 0.2% [wt/vol]), a growth-limiting medium, and the five-component consortium indicated efficient conversion (40%) of sucrose carbon to CH_4. Significant decreases in yields of CH_4 and rates of CH_4 production were observed if any component of the consortium was omitted. Approximately 70% of the CH_4 generated occurred via acetate. Agar-immobilized cells of the consortium exhibited yields of CH_4 and rates of CH_4 production from sucrose similar to those of nonimmobilized cells. The rate of 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.

The degradation of carbohydrates to methane by defined mixed cultures has been studied by several investigators (8, 21, 26–28). Winter and Wolfe (27) studied the complete conversion of fructose to CH_4 and CO_2 with cocultures of *Acetobacterium woodii* and *Methanosarcina barkeri*. These authors also studied methane formation from glucose and fructose with different mixtures of *A. woodii* and various methanogenic bacteria (28). Winter (26) studied glucose degradation to CH_4 and CO_2 with various associations of *Bifidobacterium bifidus* or *Escherichia coli* as primary fermentative bacteria in association with *Desulfovibrio desulfuricans* and *M. barkeri*. Additional studies involving cellulose degradation by methanogenic mixed cultures have been reported (16, 18, 19, 25).

The immobilization of bacteria provides an attractive means of product separation from cellular material with retention of biocatalysts. However, few studies are available dealing with immobilized defined mixed cultures. Karube et al. (15) studied methane production by using agar-immobilized bacterial enrichments from digested sewage sludge, and Scherer et al. (22) studied methanol utilization by alginate-immobilized *M. barkeri*.

As a model system, we have constructed a mixed bacterial culture capable of sucrose degradation primarily to CH_4 and CO_2, with acetate as the key direct methanogenic precursor. In addition, we have studied the effect of immobilization on the performance of the defined consortium. This type of methanogenic consortium resembles mixed culture processes found in anaerobic environments such as sediments or anaerobic digestors (14, 23). Our experiments were designed specifically to test the ability of a consortium to carry out the biogenesis of methane in a nutrient-limited medium.

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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 125-ml serum bottles (Wheaton Scientific Co.). *E. coli* ATCC 12435 was grown at 37°C under an atmosphere of 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; K_2HPO_4, 10.0 g; mineral solution no. 2 (30), 10 ml; resazurin, 0.001 g. A filter-sterilized, O_2-free solution of sucrose was added before inoculation at a final concentration of 0.2% (wt/vol). *Desulfovibrio vulgaris* JJ, isolated from estuarine sediments, was grown at 37°C under an atmosphere of N_2-CO_2 (4:1) in a medium that contained the following constituents (per liter of distilled water): mineral solution no. 1 (1), 50 ml; mineral solution no. 2 (1), 50 ml; trace vitamin solution (30), 10 ml; $FeSO_4 \cdot 7H_2O$, 0.1 g; Trypticase, 1.0 g; $NaHCO_3$, 4.0 g; Na_2SO_4, 2.8 g; cysteine · HCl · H_2O, 0.5 g; $Na_2S \cdot 9H_2O$, 0.2 g. Sodium lactate was added at a final concentration of 40 mM. *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). *Methanobacterium formicicum* MF was grown at 37°C in medium 1 of Balch et al. (1) under 203 kPa of H_2-CO_2 (4:1). *M. barkeri* 227 was grown at 37°C under an atmosphere of 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; $FeSO_4 \cdot 7H_2O$, 0.002 g; resazurin, 0.001 g; trace mineral solution (30), 10 ml; vitamin solution (30), 10 ml; $NiCl_2 \cdot 6H_2O$, 0.5 mg; Na_2SeO_4, 3.7 mg; yeast extract, 1.0 g; Trypticase, 1.0 g; sodium acetate, 8.0 g; cysteine · HCl · H_2O, 0.5 g; $Na_2S \cdot 9H_2O$, 0.5 g. The medium was buffered with TES (*N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid) at a final concentration of 50 mM; the

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pH of the medium was adjusted to 7.0 with NaOH.

Anoxic medium was prepared by boiling the medium without added reducing agents under a stream of N_2 or N_2-CO_2 (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 N_2 at a flow rate of 25 ml min^{-1}. CH_4 was quantitated by comparing the peak height with known standards. H_2 and CO_2 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 N_2 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 CH_4 determination, except that a 3-m column packed with SP-1000–1% H_3PO_4 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 × *g* to remove cells, and acidified with H_3PO_4 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 indirectly as glucose after treatment with invertase. The reaction was performed as described previously by Bergmeyer and Klotzsch (5), except that the amount of reactants was modified to adapt the method to small sample volumes (0.1 ml). Lactic acid was analyzed by the method of Hadzija (11).

Experimental conditions. The basal medium for mixed culture studies contained the following constituents (per liter of untreated ground water): vitamin solution (30), 10 ml; $NiCl_2 \cdot 6H_2O$, 0.5 mg; resazurin, 0.001 g; TES, 11.0 g; cysteine · HCl · H_2O, 0.15 g. The medium, adjusted to pH 7.2 with NaOH, was prepared anaerobically under an atmosphere of N_2 and sterilized by autoclaving at 105 kPa for 20 min. Ground water from a well that provides untreated water for biological purposes was used as a source of trace minerals. Analysis of untreated ground water revealed the following inorganic composition (milligrams per liter): SO_4^{2-}, 1.2; NO_3^-, 1.5; total PO_4^{2-}, 0.03; Fe^{3+}, 1.0; Ca^{2+}, 59.6; Mg^{2+}, 23.7; NH_4^+, 1.0; Na^+, 37.0; total CO_3^{2-}, 324. Mixed cultures were inoculated into medium contained in a 125-ml serum vial; the final reaction volume was 26 ml.

Bacterial cultures used for mixed culture experiments were harvested anaerobically at late-logarithmic phase by centrifugation at 15,000 × *g* for 15 min. Pelleted cells were suspended in an anaerobic wash solution that contained the following constituents (per liter of distilled water): NaCl, 7.0 g; TES, 11.0 g; cysteine · HCl · H_2O, 0.5 g; resazurin, 0.001 g. The pH of the solution was adjusted to 7.1 with NaOH before being made anoxic under N_2. After two washings, cells were resuspended in a small volume of wash solution, and a sample was removed for dry weight measurements. Each desired combination of washed cell suspensions was inoculated into a sealed serum vial that contained basal medium with 0.17 or 0.05% (wt/vol) sucrose. The final concentration of the bacterial components was as follows (in milligrams [dry weight] per liter of medium): *E. coli*, 90; *A. woodii*, 70; *D. vulgaris*, 65; *M. formicicum*, 90; *M. barkeri*, 350. The appropriate gas atmosphere (N_2) was added after removal of the vial from the anaerobic glove box. Vials were incubated at 37°C with shaking unless otherwise indicated.

Procedure for immobilization of cells. Inside an anaerobic glove box, various combinations of washed cell suspensions were made and added to sterile, cooled (45°C) anoxic basal medium, supplemented with 1.5% (wt/vol) Noble agar (Difco Laboratories, Detroit, Mich.). The solution was gently mixed, poured into a petri dish, and allowed to solidify and cool. Pellets (2.5 by 2.5 by 6 mm) of the solidified agar were prepared by extruding the gel through a stainless steel screen of specific mesh size. Each 125-ml serum vial with 20 ml of basal medium, supplemented with 0.05 or 0.17% (wt/vol) sucrose, received 6.0 g (wet weight) of the agar pellets. The serum vial was sealed with a butyl rubber stopper, flushed with N_2, and incubated at 37°C on a reciprocal shaker at 60 strokes min^{-1}.

Diffusion of sucrose. Sucrose diffusion through the agar matrix was measured according to the following procedure. Sucrose (0.2% [wt/vol] final concentration) was added to a sterile solution containing 1.5% (wt/vol) agar at 45°C, and the mixture was poured into a petri dish and allowed to solidify. Agar pellets, prepared by extruding the agar gel through a wire screen, were added to a flask containing glass-distilled water. Samples from the water phase were taken over a time period and assayed for sucrose diffusion from the agar pellets.

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, CO_2, and H_2 (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.

Combinations 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 CH_4 production occurred when *M. formicicum* was cocultured with *E. coli* and *A. woodii*; in the absence of an acetophilic methanogen, the amount of CH_4 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 CH_4 and CO_2. 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

Organisms[a]	Fermentation products[b] (μmol/ml)					% C as CH_4
	Ethanol	Lactate	Acetate	H_2	CH_4	
A.						
E. coli	8.3	4.0	11.2	0.3	0	
E. coli, A. woodii	7.0	0.6	12.0	0	0	
E. coli, A. woodii, D. vulgaris	2.0	ND[c]	12.8	0	0	
B.						
E. coli, M. formicicum	1.1		14.6	0	7.3	11
E. coli, M. formicicum, A. woodii	0.6	0.4	15.7	0	10.0	15
E. coli, M. formicicum, A. woodii, D. vulgaris	0.7	ND	17.1	0	7.4	11
C.						
E. coli, M. barkeri	2.2	0.4	0	0	15.5	23
E. coli, M. barkeri, A. woodii	2.0	ND	0	0	17.3	26
E. coli, M. barkeri, A. woodii, D. vulgaris	0.7	ND	0	0	27.0	40
D. *E. coli, A. woodii, D. vulgaris, M. formicicum, M. barkeri*	1.0	ND	0	0	28.4	42

[a] Cultures were incubated at 37°C in basal medium for 10 days at an initial sucrose concentration of 0.19% (wt/vol).
[b] Values do not represent a complete fermentation balance from sucrose; CO_2 and formate were not quantitated.
[c] ND, Not detected.

cultures with *D. vulgaris*, indicating ethanol oxidation to acetate. Methane production was optimal when *M. barkeri* was cultured with *E. coli, A. woodii*, and *D. vulgaris*. The final amount of CH_4 produced represented a 40% recovery of sucrose carbon as CH_4 compared with a recovery of 11% when *M. formicicum* was cultured with *E. coli, A. woodii*, and *D. vulgaris*. Acetate was detected only in trace amounts as a final fermentation product in mixed cultures with *M. barkeri*.

The final mixed culture tested was a combination of both methanogens (*M. barkeri* and *M. formicicum*) in coculture with *E. coli, A. woodii*, and *D. vulgaris*. The final yield of CH_4 (28.4 mmol/liter of medium) was slightly higher compared with the same consortium without *M. formicicum* (27 mmol/liter) and represented a 42% recovery of sucrose carbon as CH_4. The final yield of CH_4 from sucrose degradation by the complete consortium (28.4 mmol/liter) represented an increase of 14% compared with the predicted yield (24.5 mmol/liter) calculated from data of the complete consortium without *M. barkeri* (Table 1B and D).

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 CH_4 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 without *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 CH_4. 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 CH_4. The results presented in Fig. 1D indicate that the rate of CH_4 production for the immobilized consortium (320 μmol day^{-1} 100 ml^{-1}) and the total CH_4 produced (2,300 μmol 100 ml^{-1}) were almost identical to the data obtained for the cosortium 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 CH_4 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 CH_4 production for the consortium without *A. woodii* was

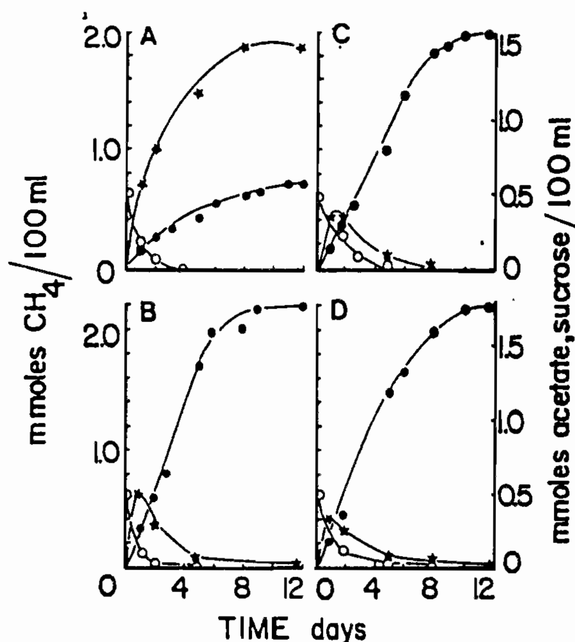

FIG. 1. Kinetics of sucrose degradation by defined mixed cultures. (A) Mixed culture of *E. coli, A. woodii, D. vulgaris*, and *M. formicicum*. (B) Mixed culture as in (A) with *M. barkeri*. (C) Agar-immobilized mixed culture of *E. coli, D. vulgaris, M. formicicum*, and *M. barkeri*. (D) Agar-immobilized mixed culture as in (C) with *A. woodii*. Symbols: O, sucrose; ●, CH_4; ★, acetate. Cultures were incubated at 37°C with shaking in basal medium (0.17% [wt/vol] sucrose).

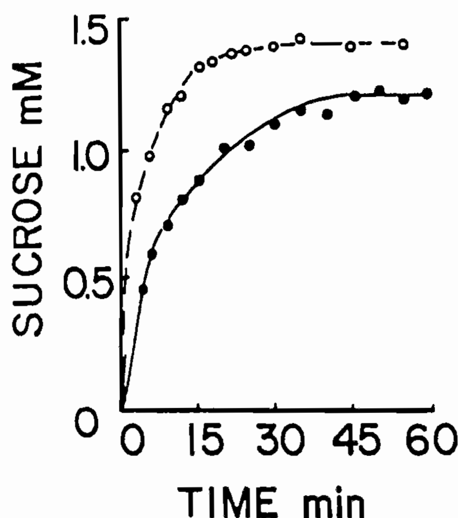

FIG. 2. Diffusion of sucrose through agar matrix. Pellets of solidified agar containing sucrose (0.5% [wt/vol]) were added to a solution of distilled water, and sucrose release was followed with time. Symbols: ●, agar pellets (6 by 6 by 6 mm); O, agar pellets (2.5 by 2.5 by 6 mm).

2,000 μmol 100 ml^{-1} (Fig. 1C); the maximal rate of CH_4 production was 220 μmol day^{-1} 100 ml^{-1}. These values are 13 and 30% less, respectively, compared with the results of the complete immobilized consortium (Fig. 1D). These results suggest that *A. woodii* significantly contributes to the pool of methanogenic precursors, either by the homoacetic 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 CH_4 production by the defined immobilized consortium. The rates of CH_4 production at 30 and 37°C at a sucrose concentration of 1.5 mM were similar (Fig. 3). At 25°C, however, a lag was noted before the onset of CH_4 production.

Pellets of the immobilized consortium were added to basal medium without sucrose and stored at 4°C under an atmosphere of N_2 for various lengths of time to determine the effect of cold storage on the activity of the consortium. Cold storage under N_2 in the absence of substrate significantly affected the rate of CH_4 production (Fig. 4). After 1 and 5 weeks of storage at 4°C, the rates of CH_4 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 CH_4 production by the immobilized consortium were tested, using basal medium prepared in various ways (Table 2). In aerobically prepared basal medium without cysteine, no CH_4 production was detected after 10 days of incubation at 37°C. Aerobically prepared basal medium without cysteine, prereduced by axenic *E. coli* fermentation, was inoculated with the immobilized consortium, and the rate of CH_4 production was reduced by 40% compared with controls. When the aerobically prepared basal medium without cysteine was aseptically bubbled with sterile N_2 at a rate of 100 ml min^{-1} for 15 min, the immobilized consortium exhibited a 25% reduction in the rate of CH_4 production. When basal medium with cysteine was used in a similar experiment, no difference in the rate of CH_4 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 CO_2 to acetate. *E. coli* was chosen as the primary organism responsible for the first step in the bioconversion of sucrose to CH_4 because the mixed acid fermentation of sucrose by *E. coli* primarily yields direct methanogenic substrates (acetate, formate, H_2, and CO_2) 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 homoacetic 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 CH_4 production from several methanogenic substrates. As indicated by the results, optimal CH_4 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% (wt/vol), ca. 42% of the sucrose carbon was recovered as CH_4. An estimate of the percentage of CH_4 arising from acetate was made from the difference in CH_4 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 CH_4 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

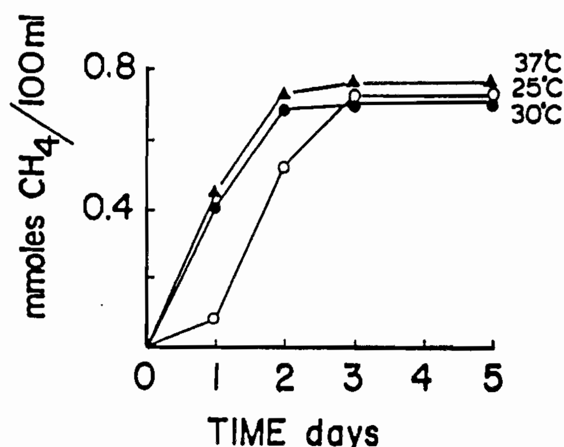

FIG. 3. Effect of incubation temperature on methanogenesis from sucrose (0.05% [wt/vol]) by the complete defined immobilized consortium (*E. coli, A. woodii, D. vulgaris, M. formicicum*, and *M. barkeri*). Symbols: O, 25°C; ●, 30°C; ▲, 37°C.

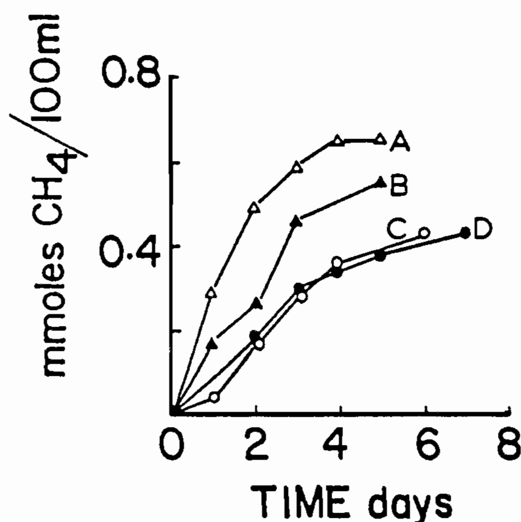

FIG. 4. Effect of storage time of the complete immobilized consortium on methanogenesis from sucrose (0.05% [wt/vol]). Agar pellets of the consortium were added to basal medium without substrate and stored at 4°C for 1 week (B), 3 weeks (C), and 5 weeks (D) before the addition of substrate and incubation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

amounts of essential growth nutrients, including sources of nitrogen and phosphorus, were provided through the addition of untreated ground water. In addition, dilute substrate concentrations were tested in most experiments. However, results of our studies indicated that the bacterial consortium was nevertheless efficient in the conversion of sucrose to CH_4 in growth-limiting medium.

There is much evidence to support the premise that interspecies H_2 transfer reactions, as first reported by Iannotti et al. (13), are important in the performance of our consortium. A dramatic shift in the levels of fermentation products of sucrose degradation by *E. coli* was noted when *E. coli* was cocultured with either methanogen. This phenomenon has been well documented with a variety of mixed cultures (7–9, 13, 18, 21, 31). The H_2-utilizing organisms *M. formicicum* and *D. vulgaris* also may play a role in the performance of acetate utilization by *M. barkeri*. It has been reported (4) that the presence of H_2 inhibited CH_4 production from acetate in enrichment cultures containing methanosarcina; if this is the case, then methanogenesis from acetate would be optimized if H_2 was removed by the H_2-consuming bacteria.

Several types of matrices for whole-cell immobilization have been investigated, including gels of agar (15, 20), carrageenan (24), alginate (17, 20, 22), gelatin (10), and polyacrylamide (15). Alginate and agar gels are experimentally desirable because of their ease of use and nontoxic characteristics. Agar immobilization of the defined consortium had no apparent effects on sucrose degradation, rate of CH_4 production, or final yield of CH_4 compared with nonimmobilized mixed cultures. With agar-immobilized consortia, the influence of *A. woodii* and *D. vulgaris* on CH_4 production from sucrose was evident, specifically at higher sucrose concentrations. Whereas both organisms significantly contributed to the pool of methanogenic precursors, *D. vulgaris* probably played an important role by production of sulfide. The use of immobilized bacterial consortia may prove to be an effective means of retaining specific biocatalysts within continuous-flow methanogenic systems.

TABLE 2. Effects of anaerobiosis and reducing conditions on methanogenesis from sucrose[a]

Condition	Total CH_4 production (mmol/100 ml)	Maximal rate of CH_4 mmol day^{-1} 100 ml^{-1}
Anaerobic	0.8	0.4
N_2 sparged[b]	0.8	0.4
N_2 sparged[c]	0.5	0.3
Aerobic, *E. coli* prereduced[c]	0.5	0.25
Aerobic[c]	0	0

[a] Pellets of the defined immobilized consortium (*E. coli, A. woodii, D. vulgaris, M. formicicum*, and *M. barkeri*) were added to basal medium (0.05% [wt/vol] sucrose), prepared as indicated, and incubated at 37°C with shaking for 10 days.

[b] Medium gassed with N_2 at a flow rate of 100 ml min^{-1} for 15 min.

[c] Medium without cysteine.

ACKNOWLEDGMENTS

We thank V. Gabriel for excellent technical assistance.

This work was supported by U.S. Department of Energy grant DE-AC02-80ER 10681.

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