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Fermentation of Cellulose to Methane and Carbon Dioxide by a Rumen Anaerobic Fungus in a Triculture with *Methanobrevibacter* sp. Strain RA1 and *Methanosarcina barkeri*

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The fermentation of cellulose by a rumen anaerobic fungus in the presence of *Methanobrevibacter* sp. strain RA1 and *Methanosarcina barkeri* strain 227 resulted in the formation of 2 mol each of methane and carbon dioxide per mol of hexose fermented. Coculture of the fungus with either *Methanobrevibacter* sp. or *M. barkeri* produced 0.6 and 1.3 mol of methane per mol of hexose, respectively. Acetate, formate, ethanol, hydrogen, and lactate, which are major end products of cellulose fermentation by the fungus alone, were either absent or present in very low quantities at the end of the triculture fermentation ($\leq$0.08 mol per mol of hexose fermented). During the time course of cellulose fermentation by the triculture, hydrogen was not detected ($<1 \times 10^{-5}$ atm; <0.001 kPa) and only acetate exhibited transitory accumulation; the maximum was equivalent to 1.4 mol per mol of hexose at 6 days which was higher than the total acetate yield of 0.73 in the fungus monoculture. The effect of methanogens is interpreted as a shift in the flow of electrons away from the formation of electron sink products lactate and ethanol to methane via hydrogen, favoring an increase in acetate, which is in turn converted to methane and carbon dioxide by *M. barkeri*. The maximum rate of cellulose degradation in the triculture (3 mg/ml per day) was faster than previously reported for bacterial cocultures and within 16 days degradation was complete. The triculture was used successfully also in the production of methane from cellulose in the plant fibrous materials, sisal (fiber from leaves of *Agave sisalona* L.) and barley straw leaf.

In recent years, there have been several studies with cocultures of carbohydrate fermenters with methanogens which have claimed fermentation of carbohydrate solely to carbon dioxide and methane (11, 22). Using a combined culture of *Acetobacterium woodii* and *Methanosarcina barkeri*, Winter and Wolfe (22) were able to achieve complete degradation of fructose or glucose to carbon dioxide and methane. The success of their system depended on the ability of *A. woodii* to produce 3 mol of acetate per mol of hexose, so that in the combined culture the total yield of methane was 3 mol per mol of sugar. Recently Khan (11) claimed near theoretical yields of methane from cellulose (2.6 to 3.0 mol of CH_4 per mol of hexose) with *Acetovibrio cellulolyticus* in coculture with *M. barkeri*.

To our knowledge, no anaerobes have been documented that carry out a homoacetate fermentation of cellulose. However, there are many anaerobic bacteria which can ferment cellulose to produce electron sink products, such as lactate, ethanol, succinate, or propionate, apart from the methanogenic precursors acetate, formate, hydrogen, and carbon dioxide. Coculture of such organisms with hydrogen- but not acetate-utilizing methanogens produces a shift in the flow of electrons away from the formation of electron sink products to methane via hydrogen (it is postulated that hydrogen is produced via a pyridine nucleotide-linked hydrogenase), this is accompanied by an increase in acetate production (12, 19). However, there has been no study on how increased acetate production may be exploited by the addition of a second methanogen capable of cleaving the product to methane and carbon dioxide.

Recently, we showed that the interactions between a rumen anaerobic fungus and *Methanobrevibacter* sp. strain RA1 during cellulose fermentation were similar to those observed for interbacterial systems (5). The fermentations by the fungus alone and in coculture are summarized as follows: (A) fungus alone, 100 $C_6H_{12}O_6$

$\rightarrow$ 73 CH_3COOH + 38 CO_2 + 37 CH_3CH_2OH + 83 HCOOH + 35 H_2 + 67 $CH_3CHOHCOOH$; and (B) coculture, 100 $C_6H_{12}O_6 \rightarrow$ 139 CH_3COOH + 89 CO_2 + 10 CH_3CH_2OH + 3.5 $CH_3CHOHCOOH$ + 59 CH_4. (These measurements were made at the completion of fermentation as ascertained by no further increase in hydrogen [reaction A] or methane [reaction B]. The yield of carbon for reaction A was 90% and that for reaction B was 77%; therefore, these reactions do not represent balanced equations. The values for formate and hydrogen in reaction B were zero.) We also showed that the fungus readily attacked cellulose (5). In this study, we describe how increased acetate production by the coculture may be exploited by the addition of *M. barkeri* to cleave the product to methane and CO_2. Application of the three-culture system to the production of methane from cellulose in plant fibrous material is also described.

MATERIALS AND METHODS

Organisms. The rumen anaerobic fungus (strain PN1), obtained from the culture collection at the Department of Scientific and Industrial Research, Palmerston North, New Zealand, was described previously (5). The organism is closely related to the rumen phycomycete named *Neocallimastix frontalis* by Orpin (16). The phylogenetic position of these fungi remains to be determined, although the mode of zoosporogenesis from a monocentric rhizoid-bearing thallus is comparable to that of many chytrids. *M. barkeri* strain 227 was kindly supplied by R. A. Mah, Division of Environmental and Nutritional Sciences, School of Public Health, University of California. *Methanobrevibacter* sp. strain RA1 was obtained from our culture collection and has been described previously (5). This methanogen, which resembles *Methanobrevibacter ruminantium*, was isolated from a coculture with a rumen anaerobic fungus and was found to utilize H_2-CO_2 and, to a lesser extent, formate, but not acetate, for methane production (5).

Culture medium. The culture medium for growth of the fungus alone or in coculture with methanogens was as described previously (5). The gas phase was 70% N_2-30% CO_2. The medium was dispensed in 12-ml volumes in Hungate anaerobic culture tubes (140 by 16 mm), each containing 100 mg of Whatman no. 1 filter paper strips (15 mm long and 2 mm wide). In some media, filter paper was replaced by the same weight of sisal (fiber from the leaves of *Agave sisalona* L. commercially available as twine) or barley straw leaf. Pieces of the plant material were cut to approximately 15 mm in length and dried in a dessicator at 50°C before use. The medium used for the growth of *M. barkeri* on acetate was the 0.2% yeast extract medium of Mah et al. (14), except that sodium bicarbonate was omitted. Before coculture experiments, *M. barkeri* was cultured in the medium as described for the fungus, except that sodium acetate (50 mM) replaced cellulose.

Culture techniques. The techniques described by Hungate (10) were used for the maintenance and subculturing of the fungus-methanogen cultures. Experimental media were inoculated by transferring 0.05 of the volume of a 6-day-old culture of the fungus alone or stable coculture of the fungus and *Methanobrevibacter* sp. The stable coculture was obtained by recombining the pure culture of the methanogen with the fungus (5). The inoculum for the fungus alone was equivalent to approximately 3×10^3 colony-forming units (CFU) per ml of culture, and the inoculum for the coculture was equivalent to approximately 3×10^3 CFU of fungus and 1.4×10^7 CFU of *Methanobrevibacter* sp. per ml. For coculture studies with *M. barkeri*, the methanogen was added directly to experimental media in an amount equivalent to 0.1 mg (dry weight) of cells. CFU of the fungus in mono- or coculture were determined after 4 days of incubation in roll tubes inoculated with 5×10^{-2} to 5×10^{-4} ml of culture. CFU of *Methanobrevibacter* sp. were determined after 21 days of incubation in roll tubes inoculated with 5×10^{-5} to 5×10^{-7} ml of coculture. Glucose (0.1% wt/vol) was used as the growth substrate for the fungus and H_2-CO_2 (80/20 [vol/vol] in the gas phase) for *Methanobrevibacter* sp.

All incubations were at 38°C with the tubes maintained in a vertical position without shaking.

Analytical procedures. The determination of the fermentation products methane, hydrogen, CO_2, ethanol, acetate, lactate, and formate were as described previously (5). Cellulose in Whatman no. 1 filter paper and in plant materials was determined by the method of Updegraff (18). Lignin and hemicellulose in plant materials were determined by the methods of Goering and van Soest (9). Soluble carbohydrates were determined by a modification of the anthrone method, using glucose as the standard (1). For the dry-weight determination of *M. barkeri*, cells were harvested by centrifugation at 7,000 $\times$ *g* for 20 min at 0 to 2°C, washed twice with distilled water (no lysis occurred during washing), and added to preweighed planchettes. Planchettes and cells were then dried to a constant weight by incubation at 60°C in a vacuum oven and reweighed. The increase in weight was taken as the cell dry weight.

Chemicals. All chemicals were obtained from commercial sources and were of reagent grade.

RESULTS

Cellulose utilization. The time courses of cellulose degradation in the different culture systems are shown in Fig. 1. With the anaerobic fungus alone, the rate of cellulose degradation was slowest, and only 53% of the initial cellulose was degraded at the end of the fermentation. Coculture of the fungus with *M. barkeri* gave an increased rate of cellulose degradation after an initial lag, and total cellulose degraded was increased (69%). However, the fastest rates of cellulose degradation were with the fungus-*Methanobrevibacter* sp. coculture and with a triculture system consisting of the fungus, *Methanobrevibacter* sp., and *M. barkeri* (3.0 mg/ml per day, maximum). Virtually all of the initial cellulose was degraded by the triculture ($\geq$98%), whereas with the fungus-*Methanobrevibacter*

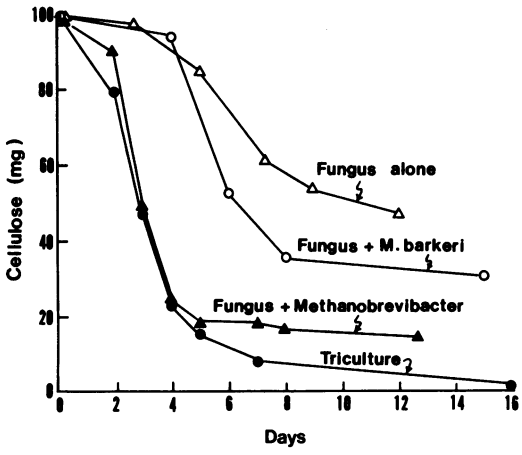

FIG. 1. Time course of cellulose degradation for various culture systems. Media (12 ml) containing 100 mg of cellulose were inoculated with 0.6 ml of fungus monoculture or fungus-methanogen culture. For all cultures, the amount of fungus inoculated was equivalent to approximately 1.8×10^3 CFU. *Methanobrevibacter* sp. (8.4×10^6 CFU) and the equivalent of 0.1 mg (dry weight) of *M. barkeri* were inoculated into the fungus-methanogen cultures. The incubation temperature was 38°C.

sp. coculture, 87% of the cellulose was degraded.

With all four culture systems, cellulose degradation was accompanied by a decrease in pH. The initial pH of the culture media was 6.9. At the end of fermentation the pH of the culture fluid from the fungal monoculture and coculture with *Methanobrevibacter* sp. was 6.0, whereas the values for the fungus-*M. barkeri* coculture and the triculture were 6.3 and 6.6, respectively.

Methane production. Methane production by the different fungus-methanogen culture systems is shown in Fig. 2. With the fungus-*M. barkeri* coculture, methanogenesis occurred after a lag of 4 to 5 days and the maximum rate of methanogenesis, 6.1 μmol/ml per day, occurred 10 to 12 days after inoculation. Total methane produced was about 550 μmol. With the fungus-*Methanobrevibacter* sp. coculture, rapid methanogenesis (12.0 μmol/ml per day, maximum) occurred after a short lag, and the total methane produced was 320 μmol. The time course of methanogenesis for the triculture showed two phases: an early methanogenic phase to 5 days and a second phase occurring from 5 to 16 days. An inflexion in the plot marked the shift from the first to the second phase. The maximum rate of methanogenesis for the first phase (12.5 μmol/ml per day) was similar to that for the fungus-*Methanobrevibacter* sp. coculture, whereas the rate for the second phase (7.2 μmol/ml per day, maximum) was similar to that for the fungus-*M. barkeri* coculture. The total methane for the triculture system (1.2×10^3 μmol) was greater than the methane obtained by summation of the values for the fungus-*Methanobrevibacter* sp. and fungus-*M. barkeri* cocultures (870 μmol).

Transitory accumulation of fungus fermentation products. In some cocultures, there was a transitory accumulation of hydrogen or acetate during cellulose fermentation. Coculture of the fungus with *M. barkeri* resulted in an increase in the partial pressure of H_2 to 0.04 atm (4 kPa) after 6 days of incubation, followed by a decrease to <0.01 atm (1 kPa) at 15 days. H_2 did not accumulate at any stage of incubation with the fungus-*Methanobrevibacter* sp. coculture or the triculture system (detection limit, 1×10^{-5} atm; 0.001 kPa). With the fungus-*M. barkeri* coculture, acetate accumulated to 230 μmol in 12 ml of culture medium by 8 days, followed by a decline to 50 μmol at 15 days. With the triculture system, acetate accumulated to 750 μmol (140 mol per 100 mol of hexose fermented) by 6 days but had disappeared by 16 days. Increasing the inoculum of *M. barkeri* (0.3 mg [dry weight] of cells added to 12 ml of medium) did not prevent the transitory accumulation of acetate in the triculture. In all of the fungus-methanogen cultures, there was no transitory accumulation of soluble carbohydrates during the course of cellulose fermentation.

Cellulose fermentation to methane by the triculture. Compared with the two-culture systems the triculture showed an increase in carbon and electron flow to methane and carbon dioxide, and nearly equivalent amounts of the two gases

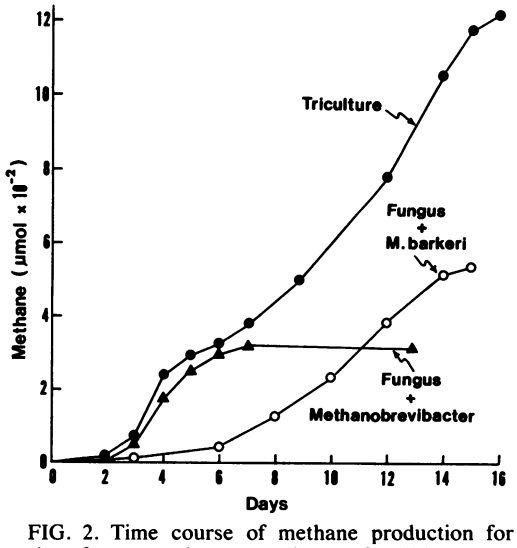

FIG. 2. Time course of methane production for various fungus-methanogen cultures. Conditions were as described for Fig. 1.

TABLE 1. Fermentation of cellulose by a rumen anaerobic fungus with *M. barkeri* in the absence and presence of *Methanobrevibacter* sp. strain RA1[a]

Product	Moles per 100 mol of hexose[b]	
	+*M. barkeri*	+*M. barkeri* and *Methanobrevibacter* sp. strain RA1
Acetate	16.7 ± 2.9	0
Lactate	28.6 ± 4.7	1.9 ± 0.2
Ethanol	25.2 ± 1.1	7.7 ± 0.5
Methane	127.9 ± 6.7	195.8 ± 7.2
Carbon dioxide	128.5 ± 4.9	206.4 ± 3.0
Hydrogen	<1	<0.05
Formate	76.0 ± 0.5	<2
Soluble sugars[c]	4.9 ± 0.3	6.3 ± 1.5
Carbon recovery (%)	88.6	77.1
Hydrogen recovery (%)[d]	86.5	76.2
Oxidation reduction index	1.08	1.01

[a] Determined at the completion of fermentation as ascertained by no further increase in methane production.

[b] Values are means of duplicate determinations ± 1 standard deviation.

[c] Determined as glucose equivalents.

[d] Determined by the procedure of Barker (2).

were formed (Table 1). Moles of CH_4 per 100 mol of hexose was greater than for the cocultures with *Methanobrevibacter* sp. or *M. barkeri* by 137 and 68 mol per 100 mol, respectively. Almost 2 mol of methane was produced per mol of hexose fermented in the triculture, and the fermentation products lactate, hydrogen, formate, and acetate were either absent or produced in very low quantities (<2 mol per 100 mol of hexose). Ethanol was produced in small amounts in the triculture (7.7 mol per 100 mol of hexose). Removal of both acetate and formate with concomitant decreases in electron sink products lactate and ethanol could only be achieved in the triculture and not with either two-culture system.

At the end of fermentation, some soluble sugars, equivalent to 6.3 and 4.9 mol of glucose per 100 mol of cellulose hexose, were present in the culture supernatants of the triculture and the fungus-*M. barkeri* coculture, respectively.

Production of methane from plant fiber cellulose by the triculture. Production of methane during the fermentation of different plant fiber substrates is summarized in Table 2. For comparison, results for the two-culture system (fungus plus *Methanobrevibacter* sp.) and the data for the fermentation of Whatman no. 1 filter paper are included. Most of the cellulose component of the plant fibers was degraded by 7 days, and by 19 days degradation was near complete. During this time there was little accumulation of soluble carbohydrates (<2 μmol per ml of culture medium), and hydrogen was not detected. When fermentation ceased, lactate, ethanol, acetate, and formate were either absent or present in very low quantities (0 to 25 μmol in 12 ml of culture medium). Total methane production from sisal was similar to that from filter paper as was the ratio of methane produced to cellulose fermented (2.14 mol of CH_4 per mol of hexose for sisal compared with 1.95 mol per mol for filter paper). However, for barley straw leaf, the ratio was substantially higher (3.11 mol per mol of hexose). indicating that another component was also fermented to methane, perhaps hemi-

TABLE 2. Methane production from plant fibrous materials by a stable coculture of a rumen anaerobic fungus with *Methanobrevibacter* sp. in the absence and presence of *M. barkeri*

Substrate	% Composition			Culture system[a]					
				Fungus + *Methanobrevibacter* sp.			Fungus + *Methanobrevibacter* sp. + *M. barkeri*		
	Cellulose	Hemicellulose	Lignin	Incubation time (days)	Cellulose utilized (mg)	CH_4 (mmol)	Incubation time (days)	Cellulose utilized (mg)	CH_4 (mmol)
Whatman no. 1 filter paper	99.7	0	0	7	82.0	0.30	16	98.0	1.22
Sisal twine fiber	77.2	ND[b]	3.2	7	72.5	0.21	19	76.2	1.01
Barley straw leaf strips	37.0	51.0	7.9	7	33.8	0.17	19	36.0	0.70

[a] Media (12 ml) containing 100 mg of substrate were inoculated with 0.6 ml of fungus-*Methanobrevibacter* coculture (3×10^3 CFU of fungus per ml; 1.4×10^7 CFU of methanogen per ml). For the three-culture system the amount of *M. barkeri* added was equivalent to 0.1 mg (dry weight) of cells. Measurements were made until there was no further increase in methane production. Values are means of duplicate determinations. The error in determining the means was 2% or less.

[b] Not determined.

cellulose, since it was a major component of the substrate (51% of the dry matter). The contribution to methane by other components in the plant fiber substrates was not determined.

DISCUSSION

Coculture of an anaerobic fungus with methanogens offers new scope in the development of a fermentative system for the bioconversion of cellulose fiber to methane. The choice of the fungus resides in its ability to readily degrade either pure cellulose fiber or the cellulose component of plant fibrous material and to produce the appropriate precursors of methane when cocultured with methanogens.

This study describes how enhanced acetate production resulting from cellulose fermentation by coculture of an anaerobic fungus with *Methanobrevibacter* sp. may be exploited by addition of a second methanogen (*M. barkeri*) to cleave the acetate to methane and carbon dioxide. *Methanobrevibacter* sp. functions in the removal of hydrogen and formate and the resulting shift in electron flow away from the formation of electron sink products lactate and ethanol to methane is consistent with the presence of a hydrogenase in the fungus which catalyzes the production of H_2 from reduced pyridine nucleotide at low partial pressures of the gas. *M. barkeri* would also function in hydrogen removal but comparison of the data in Table 1, reaction B, and the data in Fig. 2 suggests that coupling was not as effective as with *Methanobrevibacter* sp.

With the triculture system, the yield of methane (almost 2 mol per mol of hexose) was higher than for either two-culture system, and complete cellulose degradation could be achieved. Furthermore, utilization of both acetate and formate for methane production could only be obtained with the triculture.

Cellulose degradation by the fungus in the triculture system was rapid (maximum rate equivalent to 3 g/liter per day) and greater than the rates obtained for bacterial cocultures. Using a thermophilic coculture of *Clostridium thermocellum* and *M. thermoautotrophicum*, Weimer and Zeikus (19) obtained a maximum rate for cellulose degradation equivalent to 0.7 g/liter per day. Recently Khan (11) obtained a maximum rate equivalent to 0.43 g/liter per day with *Acetovibrio cellulolyticus* in coculture with *M. barkeri*. Laube and Martin (13) were able to improve this rate to give 1 to 1.5 g/liter per day by the addition of *Desulfovibrio* to the coculture. The finding here that the cellulose component of sisal and barley straw leaf was readily degraded by the fungal coculture is consistent with previous studies (3) which showed rapid and extensive colonization of plant fibrous material by the anaerobic fungi in natural rumen digesta. This suggests that the anaerobic fungi may possess unique enzymatic properties, perhaps residing in the cellulase, to facilitate the rapid breakdown of plant fiber. The likely role of the fungi as important agents in lignocellulose digestion has already been suggested (3) and deserves investigation.

Methanogenesis from acetate appeared to be limiting in the triculture, since acetate temporarily accumulated. *M. barkeri* is known to grow slowly on acetate; the specific growth rate is about 0.4 $days^{-1}$ in medium with yeast extract and Trypticase (17). Acetate dissimilation in methanogenic ecosystems has been suggested to be inhibited or repressed by H_2, and only after low partial pressures of the gas are achieved (<0.01 atm; 1 kPa) does degradation occur (15). However, this would not explain the acetate accumulation in our triculture, because H_2 never exceeded 1×10^{-5} atm (0.001 kPa). Transitory accumulation of acetate without concomitant increases in H_2 has also been observed in other methanogenic ecosystems, such as benzoate enrichments (7; D. O. Mountfort and M. P. Bryant, unpublished data).

Attempts to substantially improve the rate of methane production in the triculture by increasing the inoculum of *M. barkeri* did not produce any marked effect.

One feature of the fungus-*Methanobrevibacter* sp. coculture has been its stability, allowing it to be maintained by routine transfer. As yet, a stable triculture that can be routinely transferred has not been obtained. Presumably this was due to the slow growth of *M. barkeri* and to the loss of viability of the fungus after prolonged incubation (in the presence of *Methanobrevibacter* sp., the fungus tended to die out if incubated for longer than 7 days).

The greater cellulose degradation by the triculture compared with the two-culture systems and the monoculture of the fungus was most likely due to the methanogens acting together to remove products, which could have inhibited the growth of the fungus. The data in Fig. 3 suggest a possible role for the electron sink products lactate and ethanol, and perhaps formate, in the regulation of cellulose degradation. Hydrogen may have had an indirect effect by controlling electron flow in lactate and ethanol formation through the pyridine nucleotide-linked hydrogenase reaction. It appears unlikely that acetate alone would have had an inhibitory effect at elevated concentrations, but inhibition of cellulose degradation due to a pH decrease resulting from its accumulation together with other acids cannot be ruled out.

The carbon and hydrogen recoveries for the

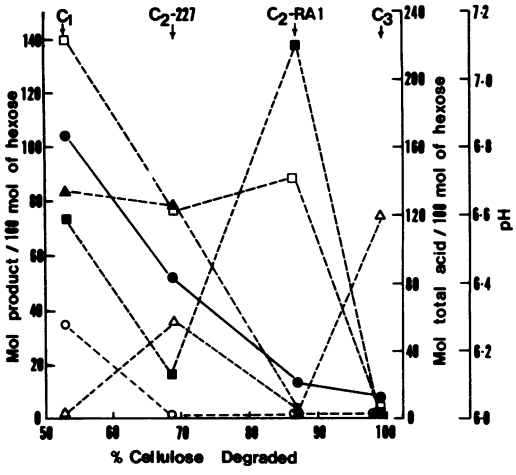

FIG. 3. Effect of product formation and pH on cellulose degradation. The values for cellulose were obtained from Fig. 1 and represent the percentage of substrate degraded at the end of fermentation by the various culture systems. Data for the corresponding product formation were taken from reactions A and B and Table 1. Abbreviations: C_1, fungal monoculture; C_2-227, coculture of fungus with *M. barkeri*; C_2-RA1, coculture of fungus with *Methanobrevibacter* strain RA1; and C_3, triculture. Symbols: ■, acetate; ▲, formate; ○, hydrogen; ●, sum of electron-sink products lactate and ethanol; □, total acid; and △, final pH (initial pH, 6.9). Data for CH_4 and CO_2 are omitted for clarity, since these products appeared to have no obvious effect on cellulose degradation.

triculture fermentation were low. In a previous study, the carbon recovery for cellulose fermentation by the fungus alone was shown to be greater than 90% (5). One possible explanation for the lower value reported here is that one or more products of the fungus were assimilated into cell biomass of the methanogens. Previous studies have shown that both CO_2 and acetate may contribute to cell carbon of methanogens (6, 8, 20, 21) and that as much as 60% of the cell carbon in *M. ruminantium* may be derived from acetate (6). The near theoretical value for the oxidation-reduction index of the triculture fermentation is consistent with acetate, which has a zero oxidation state, being an assimilated product.

In the gut fermentation of mammals, the ability of the anaerobic fungi to penetrate deeply into plant tissues not normally accessible to bacteria (4) has suggested that they have a special role in fiber digestion. In the present work, the ability of an anaerobic fungus to utilize either pure cellulose or the cellulose component of plant fibrous material has been demonstrated. Coculture with methanogens resulted in the conversion of cellulose to methane and carbon dioxide and at the same time there was an increase in the rate and extent of cellulose degradation. The combination of two methanogens to give an increased yield of methane, together with the invasive properties of anaerobic fungi, may offer distinct advantages in fermentative systems for the conversion of plant fiber to methane.

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