Nutrient Requirement for the Degradation of Cellulose to Methane by a Mixed Population of Anaerobes

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A synthetic medium containing inorganic sources of N, P, Fe, S, Ca²⁺ and Mg²⁺, trace minerals and essential vitamins was developed for anaerobic degradation of cellulose to CH₄. A mixed culture obtained from sewage sludge and grown in batch culture in this medium degraded about 4 g cellulose l⁻¹ week⁻¹ and produced about 710 ml total gas per g cellulose degraded. The gas produced contained between 51 and 56% CH₄, the remainder being CO₂. For maximum degradation of cellulose to CH₄ and CO₂, the requirement for essential salts other than HCO₃⁻, Fe and S²⁻ was comparable to that of other anaerobes. NH₄⁺ or Na₂CO₃ at 20 to 24 mM and total Fe at 0.4 to 0.6 mM gave optimum conversion of cellulose to CH₄, while the inclusion of additional S²⁻ in the presence of 1.75 mM-SO₄²⁻ was not required.

INTRODUCTION

As part of a broader study of the conversion of cellulose to CH₄ and the micro-organisms involved in the process, a microbial ecosystem obtained from sewage sludge was maintained in the laboratory and tested for its ability to degrade various kinds of cellulose found in waste and to form methane (Khan, 1977). In these tests a synthetic medium generally used for the isolation and maintenance of anaerobes was used. However, cellulose degradation was slow and the yields of total gas and CH₄, respectively, were about 40 and 50% of the theoretical values based on stoichiometry, $n\text{C}_6\text{H}_{12}\text{O}_6 \rightarrow 3n\text{CH}_4 + 3n\text{CO}_2$. These comparatively poor yields of gas were not unexpected since, amongst the various factors controlling symbiotic-metabolic interaction between different species of micro-organisms present in an ecosystem, the composition of the medium is of prime importance in substrate utilization. For example, earlier work on washed cells obtained from this ecosystem has shown that the concentration as well as the nature of the sulphur source has a profound effect on the overall efficiency of the system (Khan & Trottier, 1978). Therefore, the work was extended to study the effect of various nutrients in the basal medium on the process and to determine their concentrations for optimum degradation of cellulose to methane.

METHODS

Culture maintenance. The culture was established from an inoculum obtained from a municipal digester by growth in a 30 l fermenter at 35 °C under anaerobic conditions. The culture was maintained under the 'repeated-fed batch culture' technique (Pirt, 1975) in a chemically defined medium (Table 1, medium II) containing trace minerals and vitamins but devoid of cysteine/Na₂S. The repeated-fed batch culture technique was used because of the difficulty involved in the continuous feeding of an insoluble substrate such as cellulose. This fermenter was fed at weekly intervals with cellulose at the rate of 1 g l⁻¹ week⁻¹ and medium was withdrawn to give a microbial residence time of 24 to 30 weeks. Under these conditions, the fermenter maintained a chemical oxygen demand (COD) of 3470 (± 412) mg l⁻¹, a volatile acid content of 0 to 10 mg l⁻¹

and a pH value of 6.9 ± 0.2 . The stability of this ecosystem with respect to these parameters and its ability to degrade cellulose and form methane over a period of 2 years indicated that the culture was well-established (Khan, 1977). Fermenter samples were filtered through six layers of cheesecloth to remove any undegraded cellulose and used as a subsequent inoculum. All procedures were carried out under an atmosphere of N_2/CO_2 (4:1, v/v); the gas mixture was passed over heated copper catalyst to remove traces of O_2 immediately before use.

Media and test conditions. The composition of the synthetic media tested are reported in Table 1. The effects of N, P, HCO_3^- , CO_3^{2-} , Cl^- , S^{2-} and Fe on the degradation of cellulose to CH_4 were studied by omitting the appropriate cation or anion from the medium and by adding it back in various concentrations. Since the volume of the inoculum formed 50 % of the total volume of the medium in these tests, the inoculum was analysed for the cations and anions under investigation, and the medium composition was adjusted to compensate for the carry-over ingredients. In all cases, the cellulose content and the concentration of trace minerals and vitamins were kept constant.

Experiments were carried out in 160 ml serum vials (Miller & Wolin, 1974) containing 250 mg cellulose (acid washed, ashless), 20 ml of solution containing the desired amounts of salts and vitamins and 25 ml of inoculum. The medium was supplemented by anaerobically injecting 4 ml of water (control) or 4 ml of an aqueous solution containing the desired amount of cation or anion. Cysteine/ Na_2S solution or Na_2S solution alone (usually 1 ml per vial) was also introduced in a similar way whenever required. The volume of medium in each vial was always 50 ml. All solutions and water used in these tests were equilibrated and stored under N_2/CO_2 . Either N_2/CO_2 (4:1, v/v) or H_2/CO_2 (4:1, v/v) was used to maintain an anaerobic atmosphere inside the vials. A head-space atmosphere of H_2/CO_2 was used to determine the effects of test ingredients on the methanogenesis occurring as a result of CO_2 reduction. The volumes and composition of gases produced from cellulose degradation were corrected for the initial head-space gases. All vials were incubated at $35^\circ C$ with shaking. Results are averages for at least three tests carried out on different occasions. All tests were run in duplicate.

Since the inoculum derived from the stock culture contained a fairly high concentration of Fe in the form of insoluble FeS, which could not be removed readily, the inoculum was analysed for total Fe content and tested for its ability to degrade cellulose to CH_4 in an Fe-free medium. After incubation for 3 to 6 weeks, the fermenter liquid was analysed for Fe content and used as an inoculum for the next batch. This procedure was continued for up to six transfers using Fe-free medium each time. A control, containing Fe at the concentrations present in the initial inoculum, was concurrently run to rule out the possibility of any change in the inoculum.

Redox potential (E_h) was measured using a platinum/calomel electrode combination. Under the test conditions, E_h values of the medium were below -350 mV. The pH of the medium was generally between 6.7 and 7.0. Small quantities of propionic or butyric acids, occasionally detected in the fermenter liquids, were equated on a 1:1 basis with acetic acid for tabulation purposes.

Methods of analysis. Analyses were done to determine the volume and composition of gas in the head-space, the volatile acids and cellulose content of the fermenter liquids and the N, P, Fe, Cl^- , HCO_3^- and CO_3^{2-} contents of the inoculum. Gas volume was measured by a gas manometer. Gas composition was assayed according to van Huyssteen (1967) and volatile acids according to Ackman (1972) using gas chromatography. Details of these methods have been reported by Khan & Trottier (1978). For analyses, the total contents of each vial were centrifuged at $6000g$ for 20 min. The supernate was retained for the estimation of volatile acids, cations and anions. The pellet was washed three times by centrifugation and resuspension in water, dissolved in 72 % H_2SO_4 and the cellulose content was determined using the anthrone reagent (Herbert *et al.*, 1971). The supernate (described above) was used for the determination of N by the micro-Kjeldahl technique, P by 1-amino-2-naphthol-4-sulphonic acid, Fe by 1,10-phenanthroline, and Cl^- by $Hg(NO_3)_2$ as described by Orland (1965). Chemical oxygen demand (COD) determinations were made on well-mixed samples (Orland, 1965).

RESULTS AND DISCUSSION

The mineral composition of some of the defined media used for the isolation, characterization, cultivation and maintenance of anaerobes is given in Table 1. The nitrogen, phosphate, Ca^{2+} , Mg^{2+} , Cl^- and trace minerals and vitamin requirements of the mixed culture capable of degrading cellulose to CH_4 were basically the same as those of anaerobes of rumen (Bryant & Robinson, 1961; Caldwell & Bryant, 1966) and sewage (Kugelman & Chin, 1971) origin. Nitrogen from 3.5 to 38.0 mM and phosphate from 1.5 to 12.0 mM had little or no effect on the ability of the culture to degrade cellulose to CH_4 . Since nitrogen at

Table 1. Mineral and vitamin composition of media

All media were prepared according to Holdeman & Moore (1973) and contained 1 mg resazurin l⁻¹. Medium I was that described by Balch & Wolfe (1976), medium II by Bryant & Robinson (1961) and Caldwell & Bryant (1966), and medium III by Zeikus (1977).

Solution no.	Ingredients	Media			
		I	II (mg l ⁻¹ or ml l ⁻¹)	III	IV
1	K ₂ HPO ₄	450	296	1106	296
2	KH ₂ PO ₄	450	180	750	180
	NH ₄ Cl	—	—	900	680
	(NH ₄) ₂ SO ₄	450	450	—	150
	MgSO ₄ ·7H ₂ O	180	90	—	90
	MgCl ₂ ·H ₂ O	—	—	200	—
	CaCl ₂ ·2H ₂ O	12	60	—	60
	NaCl	900	45	—	—
3	Na ₂ CO ₃	—	4000	—	—
	NaHCO ₃	2500	—	—	2060
4	FeSO ₄ ·7H ₂ O	2	20	—	20
	FeCl ₂ ·4H ₂ O	—	—	—	—*
5	Trace mineral solution (v/v)	—	—	9†	10‡
6	Vitamin solution (v/v)	—	—	5§	10
7	Na ₂ S·9H ₂ O¶	500	250	500	—
	Cysteine·HCl·H ₂ O¶	500	250	—	—

* Added to bring the total Fe concentration of the inoculated medium to 0.4 mM when required.

† Trace mineral solution contained (mg l⁻¹): nitrilotriacetic acid, 4500; FeCl₂·4H₂O, 400; MnCl₂·4H₂O, 100; CoCl₂·6H₂O, 170; ZnCl₂, 100; CaCl₂, 20; H₃BO₃, 19; Na₂MoO₄·2H₂O, 10.

‡ Trace mineral solution was prepared according to Wolin *et al.* (1963) and contained (mg l⁻¹): nitrilotriacetic acid, 1500; MgSO₄·7H₂O, 3000; MnSO₄·H₂O, 500; NaCl, 1000; FeSO₄·7H₂O, 100; CoCl₂·6H₂O, 100; CaCl₂·2H₂O, 100; ZnSO₄·7H₂O, 100; CuSO₄·5H₂O, 10; AlK(SO₄)₂·12H₂O, 10; H₃BO₃, 10; Na₂MoO₄·2H₂O, 10.

§ Prepared according to Bryant *et al.* (1959).

|| Vitamin solution was prepared according to Wolin *et al.* (1963) and contained (mg l⁻¹): pyridoxine·HCl, 10; thiamin·HCl, 5; riboflavin, 5; nicotinic acid, 5; *p*-aminobenzoic acid, 5; biotin, 2; folic acid, 2; vitamin B₁₂, 0.5.

¶ Cysteine/sulphide solution prepared according to Holdeman & Moore (1973).

22 mM inhibits the growth of pure cultures of methanogens (Hobson & Shaw, 1976), we chose a nitrogen concentration of 15 mM which is comparable to that recommended for methanogens (Zeikus, 1977). Phosphate at 3 mM is recommended for growing rumen anaerobes (Bryant & Robinson, 1961; Caldwell & Bryant, 1966) and we used this concentration in further studies. These values gave a N/P ratio of 5:1 which is comparable to the proportions of these elements found in microbial cells (Luria, 1960). Since various pure and mixed cultures obtained from other ecosystems have been shown to tolerate fairly high levels of N, P, Mg²⁺, Ca²⁺, Na⁺, K⁺ and Cl⁻ (Hobson & Shaw, 1976; Patel & Roth, 1977; Kugelman & Chin, 1971), no attempts were made to study the toxic levels of these nutrients on this ecosystem. The toxic levels of Na⁺, K⁺, Ca²⁺ and Mg²⁺, respectively, were 300 mM, 150 to 200 mM, 125 to 150 mM and 125 mM for an anaerobic waste treatment process (Kugelman & Chin, 1971). For methanogens, Cl⁻ above 97 mM inhibits growth (Patel & Roth, 1977).

The optimum concentration of HCO₃⁻ for the degradation of cellulose to CH₄ was between 16 and 24 mM (Fig. 1). At higher concentrations, HCO₃⁻ depressed this process. Replacement of the optimum concentration of HCO₃⁻ by the same concentration of CO₃²⁻ inhibited both cellulose degradation and methane formation during the first week of incubation (Table 2). However, the deleterious effects of CO₃²⁻ on both of these parameters

Table 2. *Effect of sulphide, bicarbonate and carbonate on cellulose degradation and methane formation by mixed cultures*

The basic medium was medium IV without NaHCO_3 and Na_2S ; it was supplemented with NaHCO_3 (24 mm), Na_2CO_3 (24 mm) and Na_2S (0.5 mm) as indicated. The gas phase was H_2/CO_2 (4:1, v/v). The initial cellulose content was 5 g l^{-1} (27.8 mm as glucose equivalents).

Addition(s)	Incubation time (weeks)	Cellulose breakdown		Methane formation†		
		(mm)*	(% of initial value)	(mm)	(% of total gas)	[mol (mol glucose) $^{-1}$]
None (control)	1	18.3	66	43.8	51	2.4
NaHCO_3	1	23.6	85	43.9	45	1.9
Na_2CO_3	1	19.3	69	32.5	44	1.7
Na_2S	1	19.0	68	49.1	54	2.6
$\text{NaHCO}_3 + \text{Na}_2\text{S}$	1	19.0	68	40.4	51	2.1
$\text{Na}_2\text{CO}_3 + \text{Na}_2\text{S}$	1	8.7	31	17.5	49	2.0
None (control)	2	20.9	75	57.0	52	2.7
NaHCO_3	2	27.0	97	69.3	51	2.6
Na_2CO_3	2	26.5	95	65.8	51	2.5
Na_2S	2	23.2	84	67.5	54	2.9
$\text{NaHCO}_3 + \text{Na}_2\text{S}$	2	27.5	99	71.9	54	2.6
$\text{Na}_2\text{CO}_3 + \text{Na}_2\text{S}$	2	27.3	98	69.3	53	2.5

* Expressed as glucose equivalents.

† Corrected for head-space gases using stoichiometry: $4\text{H}_2 + \text{CO}_2 \rightarrow \text{CH}_4 + 2\text{H}_2\text{O}$.

Fig. 1

Fig. 1. Effect of NaHCO_3 on cellulose degradation (○) and CH_4 formation (●) by mixed cultures in medium IV. The initial cellulose concentration was 5 g l^{-1} (27.8 mm as glucose equivalents); the head-space gas was H_2/CO_2 ; and cultures were incubated for 2 weeks.

Fig. 2

Fig. 2. Effect of Na_2S on cellulose degradation (○) and CH_4 formation (●) by mixed cultures in medium IV with (—) or without (---) NaHCO_3 . The initial cellulose concentration was 5 g l^{-1} (27.8 mm as glucose equivalents); the head-space gas was H_2/CO_2 ; and the cultures were incubated for 1 week.

disappeared during subsequent incubation. A higher than optimum concentration of HCO_3^- has also been shown to cause a decrease in the number of colonies of rumen anaerobes recovered from such media (Caldwell & Bryant, 1966). However, the inhibitory effect of CO_3^{2-} in the initial stages of fermentation was not due to differences in initial pH. The $\text{HCO}_3^-/\text{CO}_2$ buffer system appears to be more effective than the $\text{CO}_3^{2-}/\text{CO}_2$ buffer in the degradation of cellulose to CH_4 by mixed cultures. The importance of the $\text{HCO}_3^-/\text{CO}_2$

Table 3. *Effect of iron content of the medium on cellulose degradation, acid accumulation and gas formation by mixed cultures*

The basic medium was medium IV without Fe; FeSO_4 was added to give the required Fe concentrations. The gas phase was H_2/CO_2 (4:1, v/v). The initial cellulose content was 5 g l^{-1} (27.8 mm as glucose equivalents).

Fe content* (mm)	Incubation time (weeks)	Cellulose breakdown (mm)†	Acid accumu- lation (mm)‡	Total gas formation (mm)	Methane formation	
					(mm)	[mol (mol glucose) ⁻¹]
0.60	2	22.6	< 1	132.4	72.8	3.2
0.50	2	22.7	< 1	128.1	70.2	3.1
0.40	2	21.8	< 1	128.1	64.9	3.0
0.20§	3	21.2	9.0	126.2	60.1	2.8
0.10§	4	19.3	19.2	112.8	48.4	2.4
0.05§	5	18.0	21.9	109.6	43.7	2.4
0.02§	6	17.6	38.0	106.1	42.0	2.4

* Fe content of medium plus inoculum.

† Expressed as glucose equivalents.

‡ Total acids, expressed as acetic acid equivalents.

§ Addition of Fe to increase total content to 0.4 mm quantitatively restored the ability of the inoculum to degrade cellulose and form methane.

buffer in media used for the isolation, enumeration and growth of anaerobes is well known (Bryant, 1959).

Sulphide concentrations of up to 0.5 mM in medium containing $1.75 \text{ mM-SO}_4^{2-}$ had little or no beneficial effect on the degradation of cellulose to CH_4 (Fig. 2) but above 0.5 mM, S^{2-} inhibited CH_4 formation and above 0.8 mM it inhibited both cellulose degradation and CH_4 formation. The inhibitory effects of S^{2-} on these parameters were more pronounced in medium containing CO_3^{2-} than in medium containing HCO_3^- (Table 2). However, the deleterious effects of higher concentrations of S^{2-} on cellulose degradation to CH_4 disappeared during the second week of incubation, probably as a result of removal of S^{2-} as H_2S or FeS . Rumen micro-organisms (Emery *et al.*, 1957) and cellulose-degrading anaerobes present in sewage sludge (Khan & Trottier, 1978) utilize SO_4^{2-} as a sulphur source, but all methanogenic bacteria studied to date use only S^{2-} as a sulphur source (Zeikus, 1977). Since in medium containing SO_4^{2-} , S^{2-} formed by the reduction of SO_4^{2-} appears to serve as a sulphur source for the methanogens in mixed cultures (Khan & Trottier, 1978), and since the inoculum appears to contain enough S^{2-} for the growth of methanogens, no additional S^{2-} was included in this medium.

A total Fe concentration in the medium of between 0.4 and 0.6 mM gave the best results for degradation of cellulose and production of CH_4 (Table 3). At lower Fe concentrations, the cellulose degrading ability of cultures was greatly diminished and acids accumulated in the medium. Since these lower concentrations of Fe did not affect the ability of this culture to reduce CO_2 to CH_4 , the accumulation of acids may indicate inhibition of the acetogenic phase at low Fe levels. These findings are in agreement with earlier work of Speece & McCarty (1964) showing that the addition of FeCl_3 at 0.6 mM lowered the concentrations of volatile acids accumulating in the digester. Iron is the least toxic of the common heavy metals and has no detrimental effects on anaerobic digestion up to 19.6 mM; above this concentration the toxic effects are minimal provided the pH of the medium is above 6.4 (Mosey & Hughes, 1975).

The total Fe contents given in Table 3 include both soluble and insoluble Fe, because it is difficult to remove precipitated Fe present in the inoculum and keep the Fe in a soluble state during incubation, particularly in mixed cultures. During the preparation of the medium, maintenance of strict anaerobic conditions is necessary to keep 0.3 mM or more Fe in solution. Under aerobic conditions, Fe present in the medium is precipitated as phos-

Table 4. *Effect of head-space gas and incubation time on cellulose degradation and gas formation in modified medium (IV) by mixed cultures*

Incubation time (weeks)	Head-space gas mixture (v/v)	Cellulose breakdown		Methane formation			Total gas formation		
		(mm)*	(% of initial value†)	From head-space gas‡ (mm)	From cellulose§ (mm)	[mol (mol glucose) ⁻¹]	From cellulose§ (mm)	[mol (mol glucose) ⁻¹]	[ml (g cellulose) ⁻¹]
1	H ₂ /CO ₂ (4:1)	15.7	57	18.0	47.4	3.0	89.5	5.7	716
1	N ₂ /CO ₂ (4:1)	21.8	78	—	58.8	2.7	121.1	5.6	708
2	H ₂ /CO ₂ (4:1)	25.6	92	18.0	78.9	3.1	142.1	5.6	704
2	N ₂ /CO ₂ (4:1)	26.7	96	—	76.3	2.9	149.5	5.6	710

* Expressed as glucose equivalents.

† Initial cellulose concentration was 5 g l⁻¹ (27.8 mm as glucose equivalents).‡ Head-space gases occupied 110 ml per vial at 37 °C. Calculations based on stoicheiometry: 4H₂ + CO₂ → CH₄ + 2H₂O, at 25 °C.§ Total gas, or total CH₄, minus CH₄ formed from head-space gases.

phate, carbonate or hydroxide. Even in medium prepared under strictly anaerobic conditions, and containing higher amounts of nitrilotriacetic acid as an Fe chelating agent, the H_2S formed as a result of reduction of SO_4^{2-} by mixed cultures reacts with Fe^{2+} to form insoluble sulphide. The equilibrium that exists between soluble and insoluble Fe depends on many factors, e.g. pH changes occurring as a result of the formation of acids from cellulose degradation, and the presence of chelating compounds produced by the micro-organisms. Formation of FeS may have additional beneficial effects on this fermentation in (i) serving as a reducing agent (Brock & O'Dea, 1977) and (ii) removing H_2S and S^{2-} which have toxic effects on both cellulose degradation (Khan & Trottier, 1978) and methanogenesis (Cappenberg, 1974).

Mixed cultures grown in the modified medium (Table 1, medium IV) degraded 92 to 96 % of the cellulose present and gave total gas yields of about 710 ml per g cellulose degraded (Table 4). The CH_4 content of the gas was 51 to 56 %, the remainder being CO_2 . These yields were about 95 % of the theoretical values based on stoichiometry: $n\text{C}_6\text{H}_{12}\text{O}_6 \rightarrow 6n(\text{CH}_4 + \text{CO}_2)$. The remaining 5 % carbon present in the cellulose, and probably some of the carbon supplied in the form of HCO_3^- , appear to be converted to biomass. Cellulose breakdown during the first week of incubation was faster in an N_2/CO_2 atmosphere than in an H_2/CO_2 atmosphere (Table 4). However, during subsequent incubation, these differences disappeared. In tests using H_2/CO_2 as the head-space gas, the formation of CH_4 from the head-space gases started in the first hour of incubation and was complete within 24 h. Although after the initial 24 h of incubation there was little or no H_2 left in the head-space, the possibility of initial contact of these organisms with H_2 resulting in slower growth or a longer lag period cannot be ruled out. The presence of H_2 during the initial 24 h did not affect the overall efficiency of the process.

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