J. Biosci., Vol. 6, Number 3, September 1984, pp. 315–324.

Inhibition of methanogenesis and its reversal during biogas formation from cattle manure

G. P. KALLE and K. K. G. MENON
Hindustan Lever Research Centre, Andheri, Bombay 400 099, India

MS received 16 April 1984; revised 8 August 1984

Abstract. The composition of volatile fatty acids in the biogas digester based on cattle manure as substrate and stabilised at 25°C showed that it contained 87-88% branched chain fatty acids, comprising of isobutyric and isovaleric acids, in comparison to 38 % observed in the digester operating at 35°C.

Mixed cellulolytic cultures equilibrated at 25°C (C-25) and 35°C (C-35) showed similar properties, but rates of hydrolysis were three times higher than that observed in a standard biogas digester. The proportion of isobutyric and isovaleric were drastically reduced when C-25 was grown with glucose or filter paper as substrates. The volatile fatty acids recovered from C-25 (at 25°C) inhibited growth of methanogens on acetate, whereas that from C-35 was not inhibitory. The inhibitory effects were due to the branched chain fatty acids and were observed with isobutyric acid at concentrations as low as 50 ppm.

Addition of another micro-organism *Rhodotorula* selected for growth on isobutyric completely reversed this inhibition. Results indicate that the aceticlastic methanogens are very sensitive to inhibition by branched chain fatty acids and reduction in methane formation in biogas digester at lower temperature may be due to this effect.

Key words. Methanogenesis; methane inhibition; isobutyric; isovaleric; biogas; volatile fatty acids; *Rhodotorula.*

Introduction

Conversion of cellulosic manure to methane is a complex multistep process which operates under highly reducing conditions involving a large number of microorganisms. Basically it involves converting cellulose and other polymers in the biomass to fatty acids and then to methane (Chen *et al*., 1980; Zeikus, 1980; Mackie and Bryant, 1981). Under any given set of conditions, interaction between these microbial groups determine overall rate of methane formation and stability of the process. In rumen as well as in biogas digester, most of the methane is formed from acetate by aceticlastic methanogens whose metabolic activity is quite responsive to concentration of fatty acids. It is known, for instance, that concentration of volatile fatty acids (VFA) in excess of 2000 ppm inhibits methane formation in anaerobic digester (Chen *et al*., 1980; McCarty and McKinney, 1961; Kroeker *et al*., 1979). In addition, the entire process of biogas formation is sensitive to changes in temperatures, and it is a common experience

Abbreviations used: VFA, volatile fatty acids; IBA, isobutyric acid; IVA, isovaleric acid; TVM, total volatile matter, TS, total solids.

that the methane production is greatly reduced when the temperature falls below the ambient (25°C).

In this communication, we have presented evidence to show that, in addition to the concentration of VFA in the digester, its composition is equally important in determining inhibitory effects on methane formation. At lower temperatures (25°C), there is a significant accumulation in the digester of branched chain fatty acids such as isobutyric (IBA) and isovaleric (IVA) which inhibit methane formation. Experiments indicate that this inhibition is at the level of aceticlastic methanogens growing on acetate and can be reversed by incorporating another micro-organism which uses branched chain fatty acids for growth, suggesting an approach to improving the methane yields and making the system more tolerant to temperature changes.

Materials and methods

Enrichment of cellulolytic cultures

Mixed cellulolytic cultures were established in a 5L capacity anaerobic fed-batch digester (working vol 4 L) fitted with an inverted tube for collecting gas over 0·1 NHCl from which it was continuously tapped for GLC analysis of methane and carbon dioxide. Sources of organisms were buffalo dung (15-19 % solids) and rumen fluid. This was diluted twofold wt/wt and fed to the two digesters, one operated at 25°C and the other at 35°C for 10 weeks with an average retention time of 40 days. During this period, the culture was stabilised as determined by constant rate of methane and VFA formation. Mixed stabilised cultures were then transferred to the medium described by Hobson (1957) for cellulolytic anaerobes with only one variation in that filter paper (0·6 g) was used in place of cellulose powder. The cultures were then transferred once every fourth day in a batch digester (working vol 4 L) at 25°C and 35°C. They were labelled as C-25 and C-35 respectively and used as an inoculum for other digesters used in the study

Enrichment of methanogens

Biogas digesters were set up as above at 25 °C and 35°C for 10 weeks using cattle manure as substrate. At the end of 10 weeks, 1 % sodium acetate and 0·5 % methanol were added once every third day along with fresh cattle manure (2·5 g solids per litre). Digesters were operated for 4 more weeks and the mixed cultures stabilised at temperatures (25°C or 35°C) and pH 6·8 were then transferred to the medium for methanogens. The mixed culture, so stabilised, converted about 80-85 % of acetate to methane.

Anaerobic culture techniques

The anaerobic culture techniques described by Hungate (1966) and modified by Miller and Wolin (1974) were strictly followed for both cellulolytic and methanogenic bacteria.

For studies with cellulolytic cultures, fed-batch laboratory digesters were set up at 25°C and 35°C with a capacity to handle 1·6 kg of 50 % slurry of the cattle manure (8 % total solids) and was replenished with 20 g of fresh manure (1·6 g total solids) every third

day. The cattle manure thus to be used as substrate and for subsequent additions was sterilised by autoclaving at 15 psi for 15 min. Inoculum C-25 and C-35 prepared as described above were added at 10 % level. Quantity of VFA and total reducing sugars were monitored in effluent every day. Effluents from digesters operating at 25°C and 35°C were collected, centrifuged at 78000*g* and sterilised by passing through Millipore membrane filters. Filtrates labelled VFA-25 and VFA-35 were respectively used in experiments as substrates for methanogenic bacteria.

In studies with methanogens, 450 ml of Smith and Hungate medium (pH 7·0) with 1 % sodium acetate, as a carbon source was taken in a 500 ml flask, flushed with nitrogen and inoculated with mixed cultures M-25 and M-35 as described above. The flask was fitted with a tube to collect the gas over a column of 0·1 N HCl. Flasks were incubated at 25°C and 35°C respectively and methane production was monitored. In some of the experiments, volatile fatty acids collected from digestion of cattle manure with C-25 and C-35 were used as substrates for methanogens. Methane production was also studied in the presence and absence of 1000 ppm IBA.

Experiments with Rhodotorula spp

The yeast belonging to *Rhodotorula* was isolated through standard soil enrichment techniques in Saboraud's medium containing 0·1 % ammonium isobutyrate. The isolate with ability to grow on isobutyrate under anaerobic conditions was selected for the study. It was routinely maintained in stab culture in the same medium in the presence of 1000 ppm of IBA. In studies with methanogens, *Rhodotorula* was grown in Saboraud's broth containing 1000 ppm IBA for 48 h and the cells were centrifuged, washed in distilled water and resuspended at a cell concentration of 10 g wet weight per litre of Smith and Hungate medium containing 1000 ppm of IBA. The cells were preincubated for 7 days at 35°C before transferring them to the digester containing methanogens (M-35) growing on 1 % sodium acetate.

Analytical methods

Cattle manure used as a substrate as well as the source of micro-organisms was analysed for protein, fat, crude fibre, total volatile matter (TVM), total solids (TS) according to the methods described in AOAC (1965). Volatile organic matter was measured by drying the sample in vacuum at 50°C and weighing before and after igniting it at 600°C. Loss in weight in grams was a measure of TVM.

Effluent from the digester was routinely monitored for reducing sugars (Bernfeld 1955), VFA (Rand *et al.* 1979) and total gas generated during digestion. Gas was monitored by GLC specially modified for the purpose. Gas samples were then injected in a chromosorb (80/100 mesh) packed column (6′ × 1/8″) to separate CH_4 and CO/CO_2. The separated components were then passed over a nickel catalyst where CO/CO_2 were reduced to methane by molecular hydrogen. All components were detected as methane. Thus, there were two peaks, one corresponding to methane and other corresponding to CO/CO_2 detected as methane. The details of the equipment and method are described by Subramaniam (1980). Values are expressed as per cent of the two peak areas. The VFA from the effluent was recovered by steam distillation. After acidification and extraction with ether, it was analysed by GLC.

Results

Composition of the cattle (buffalo) manure

Buffalo manure is a major source of the substrate in a biogas plant. It also provides the necessary inputs for cellulolysis as well as methanogenesis for the digester. Principal carbon source in this substrate is cellulose (and some hemicellulose) which comprises about 37-38 % of the dry matter. Other possible substrates for anaerobic digestion such as lipids, proteins etc. are in minor amounts (table 1). Together they constitute about 81-84 % of volatile organic matter which is digestible. Fresh manure contains about 10^4 bacteria per g, most of which have their origin in rumen fluid and can survive biogas system only under strict anaerobic conditions. Among the substrates, cellulose is relatively more resistant to enzyme attack, as compared with other substrates such as proteins or lipids and consequently, under anaerobic conditions its degradation becomes a rate limiting step in biogas production.

Table 1. Composition of cattle (buffalo) manure.

Analysis	1	2
	Per cent total	
Moisture	81	85
Total solids	19	15
Components	*Per cent dry weight*	
Crude fibre	37	38
Protein	5·6	6·0
Fat	1·4	2.0
Ash	16·3	19·0
volatile organic matter	84	81
	Per g of solids	
Total anaerobic bacteria	$1{\cdot}2 \times 10^4$	$1{\cdot}3 \times 10^4$

Profile of VFA produced at 25°C and 35°C in biogas digester

Anaerobic fed batch digesters set up for screening cellulolytic cultures were used for analysis of VFA in their effluent streams after they were equilibrated for about 10 weeks at 25°C and 35°C. The data presented in table 2 shows that the VFA accumulated at 25°C contained about 87-88% of its acids as IBA and IVA. The VFA at 35°C contained only 38% as branched chain fatty acids. Butyric acid was the major component of VFA accounting for 45·7% of the total. The digesters which were operated at temperatures less than 20°C showed very poor fermentation rates and practically no VFA accumulation (data not shown).

Production of VFA by purified C-25 and C-35 and its profile on different substrates

The conversion of cellulose and other organic constituents from the substrate to methane in a biogas digester involves a multitude of microbial systems and a study of

Table 2. Analysis of VFA from effluents and methane production in biogas digesters equilibrated at 25°C and 35°C.

	Per cent of total fatty acids*	
VFA	25°C	35°C
Acetate	Trace	Trace
Isopropionate	Trace	0·3
Propionate	2·5	0·6
Isobutyrate	32·6	16·7
Butyrate	3·6	45·7
Isovalerate	55·0	21·4
Valerate	1·1	3·9
Caproate	0·8	4·8
Unidentified	Trace	0·7
C_7(?)	4·4	2·3
Caprylic	Trace	3·6
Total gas (L/Kg dung added/day)	0·13	0·38
Methane (L/Kg dung added/day)	0·03	0·20

* Substrate for the biogas digester was cattle dung prepared as described in the text.

any one of them in isolation will not give a true picture of biochemical transformations taking place in the reactor. We have therefore chosen to study a mixed culture system which carry out important overall reactions leading to the formation of methane and CO_2. Figure 1 shows the rates of formation of sugar and VFA from cattle manure by C-25 at 25°C and C-35 at 35°C. In this experiment, sterilised cattle manure was used as substrate. For comparison, the effluent from a standard operating KVIC digester with a capacity to produce gas at a rate of 0·1 m^3/kg TVM/day was used as an inoculum. This digester has been operating at ambient temperatures and no effort was made to control temperatures. Fluctuations in temperature ranged from 26°C at night to 35°C during the day. Purified cultures showed almost three to four-fold improvement in fermentation rates when compared with the effluent culture from a standard KVIC biogas digester as measured by sugar and VFA content both at 25°C and 35°C.

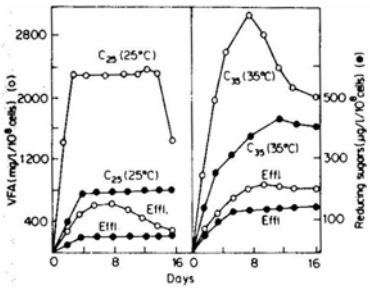

Figure 1. Hydrolysis of cellulose and other carbohydrates in cattle manure waste (dung) to reducing sugar and VFA by mixed cultures C25 at 25°C and C_{35} at 35°C. For comparison, the effluent from a standard KVIC digester was used as inoculum. Reducing sugar (●); VFA (○).

Table 3. Composition of VFA formed with different substrates by C-25 and C-35.

Substrate*	Concentration g/L	Culture	Temp. (°C)	pH	Per cent of VFA					Total branched chain fatty acids (%)
					Acetic	Propionic	Butyric	Isobutyric	Isovaleric	
Cattle manure	80	C-25	25	5·4	1·5	0·5	8·6	90·0	< 0·5	90·0
			35	5·2	20·5	—	17·12	52·3	10·46	62·8
		C-35	25	6·4	4·0	9·1	61·0	7·8	18·1	25·9
			35	6·6	1·9	7·7	70·3	6·5	13·5	20·0
Glucose	80	C-25	25	6·5	8·2	4·0	85·5	0·6	2·0	2·6
		C-35	35	6·5	< 5·0	< 5·0	95·0	< 5.0	< 5.0	< 5.0
Filter paper	6	C-25	25	5·8	< 5·0	—	69·0	24·8	0·7	25.5

* Experiments were carried out in batch culture (1 L) observing strict anaerobic conditions. The VFA was analysed after 10 days of incubation at respective temperatures.

Since the digesters operating at lower temperatures had shown accumulation of preferentially branched chain fatty acids with cattle manure as a substrate, a question may be asked whether the purified C-25 would also show a similar pattern, and if this property was true for other substrates as well. Results presented in table 3 clearly indicate that with cattle manure as substrate, mixed culture C-25 produced predominantly branched chain fatty acids at 25°C. The same culture grown at 35°C showed significant increase in acetic, butyric and isovaleric acids and reduction in IBA. A different VFA profile at a higher temperature by this culture is difficult to explain at present.

On the other hand, VFA produced by C-35 showed a pattern similar to that observed in digester operating at 35°C with butyric acid forming the predominant component, and its composition is not changed when the incubation temperature is 25°C. An interesting observation from the data presented in table 3 is the fact that the same culture C-25 switches over to produce straight chain fatty acids, predominantly butyric acid when glucose or filter paper is used as a substrate.

Effect of VFA-25 and VFA-35 on methane production by M-25 and M-35

Since the composition of VFA formed at different temperatures varies, how does it affect the methane formation? This was examined by collecting VFA from mixed cultures at C-25 at 25°C (VFA-25) and C-35 at 35°C (VFA-35), sterilising and using it as additive to methanogens growing on acetate at 25°C (M-25) and at 35°C (M-35). The data presented in table 4 shows a complete inhibition of methane formation from acetate by VFA-25 but not by VFA-35 suggesting a strong inhibitory effect of branched chain fatty acids, IBA and IVA present in VFA-25 on growth of aceticlastic methanogens.

Table 4. Effect of VFA-25 and VFA-35 on methane formation from acetate by M-25 and M-35.

Culture	Substrates (g/l)	Temp. (°C)	pH	Time (days)	Total gas (l)	Methane Volume (l)	Methane %
	Acetate						
M-25	10	25	6·8	14	4·8	3·45	71·8
				12	4·3	3·14	73·0
M-35	10	35	6·9	14	4·1	3·15	76·8
				15	4·2	3·63	73·0
	*VFA + Acetate**						
M-25	(VFA-25) 2·6	25	7·0	7	Nil	—	—
		35	6·8	7	Nil	—	—
	(VFA-35) 2·2	25	6·8	7	1·5	1·04	69·3
		35	6·8	7	2·2	1·50	67·8

* Concentration of acetate was 10 g/L. Concentration of VFA in effluent of the digester was 3·89 g/L for C-25 at 25°C and 2·2 g/L for C-35 at 35°C.

Inhibition of methane formation by IBA and its reversal by Rhodotorula

Formation of methane from acetate was highly sensitive to inhibition by IBA even at concentrations as low as 50 ppm (figure 2). The inhibition was observed in the case of both the cultures M-25 and M-35 incubated at their respective temperatures of 25°C and 35°C. This inhibition was easily reversed by incorporating into the fermentation system *Rhodotorula* cells which were preincubated with 1000 ppm IBA presumably either by removing the inhibitor or converting it to a form easily metabolised by methanogens (figure 3). It is interesting to note that the methane production from acetate by methanogens was completely restored.

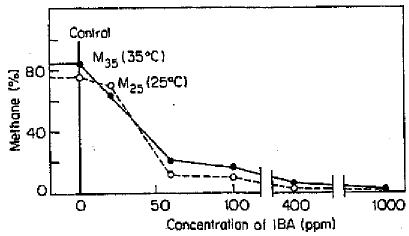

Figure 2. Inhibition of methane formation by IBA in M-25 and M-35 grown on acetate. Total incubation time - 2 days. Temperature of incubation was 25°C for M-25 and 35°C for M-35. Average concentration of IBA in a digester operating at ambient temperature varies between 40-60 ppm.

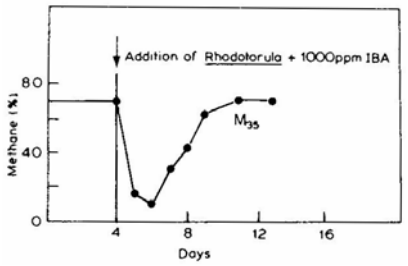

Figure 3. Reversal of inhibition of methane production by IBA using yeast *Rhodotorula*. The culture M-35 was stabilised at 35°C on acetate as a carbon source for 4 days. The IBA (1000 ppm) was added along with the preadapted cells of *Rhodotorula.* Details of the Experiment are presented in the text.

Discussion

The development of biogas generation system as an additional source of energy to meet the needs of rural areas of India has been given a high priority. Basic raw material for the biogas is cattle manure. The anaerobic digestion of organic constituents from the manure (table 1) to methane can be broadly considered to be due to the activity of three types of microbial systems *viz.* (i) fermentative microbes, which produce low molecular weight organic acids (VFA), (ii) acetogenic bacteria, and (iii) methanogens (Klass, 1984). We have presented evidence here to show that products of hydrolysis from the first two groups of micro-organisms have profound effects on methanogens which grow on acetate. About 72 % of methane comes from aceticlastic methanogens (Mackie and Bryant, 1981; Fathepure, 1983; Klass, 1984). It would therefore be expected that inhibition of metabolic activity of these organisms will affect methane production. Fermentative microbes selected for lower temperature (~ 25°C) produce VFA rich in

branched chain fatty acids, particularly IBA and IVA. Accumulation of these acids inhibits growth of aceticlastic methanogens on acetate and their ability to produce methane. The inhibitory effects can be reversed by incorporating into the system another organism *Rhodotorula* which utilises IBA under anaerobic conditions.

These observations are significant because they explain the extreme sensitivity of biogas generation system to slight variation in temperature and its stabilisation by incorporation of other micro-organisms with a capacity to use branched chain fatty acids *e.g. Bacteroides ruminogen, Ruminococcus albus, Ruminococcus flavifaciens, But yrivibrio fibrisolvens* etc. (Baldwin and Allison, 1983). These bacteria are normal constituents of rumen. Such an approach may also improve methane content of biogas since even in digester operating at 35°C, about 40 % of the total VFA comprise of branched chain fatty acids (table 2). The yeast *Rhodotorula*, though capable of growing on IBA, cannot survive at low O/R potentials (– 350 mv) which are essential for the optimal activity of methanogens (Taylor, 1975). Selection of anaerobes for growth on branched chain fatty acids should therefore be one of the important approaches to overcoming the problem of inconsistent production of methane in biogas digester due to temperature shifts.

Origin of branched chain fatty acids with sterilised cattle manure as a substrate needs to be explained, since it does not happen to any significant extent when glucose is used as a substrate even with the mixed culture C-25 which normally accumulates them upto 69-90% of total VFA (table 3). When pure cellulose in the form of filter paper is used as a substrate, the same culture accumulates about 25 % of the total VFA as branched chain fatty acids. This would suggest that at lower temperatures (25°C), polymers of glucose under anaerobic conditions have a tendency to be converted partially to branched chain fatty acids, a fact difficult to explain at present.

The practical importance of our observations is quite apparent because they provide an explanation as to why addition of reducing sugars like sugar cane molasses should improve methane production at lower temperatures in biogas digesters (Subramanian, 1977). On the basis of the results presented here, it is fair to assume that in molasses, the presence of invert sugar may favour the formation of straight chain fatty acids e.g. butyric acid rather than branched chain fatty acids, thereby improving the rates of methane formation.

It is interesting to note that in all cases, under conditions which reduce accumulation of branched chain fatty acids, there is a predominant shift to produce large amounts of butyric acid, a probable precursor to acetate formation during anaerobic digestion. There is a compelling evidence to suggest that acetogenic bacteria *e.g. Butyribacterium methylotrophicum* accumulates predominantly butyric acid and not acetate when grown on C_1 compounds such as methanol, CO_2 and formate (Kerby *et al*., 1983). This apparently is derived from condensation of two acetyl coenzyme A molecules to yield one butyrate. In methanogenesis, butyrate is a precursor for acetate, a natural substrate for aceticlastic methanogens.

Acknowledgements

Authors acknowledge assistance of Mr. C. Satischandra, (Miss) C. Desa, (Mrs) Shanta Achar and (Miss) K. K. Nayak.

References

Baldwin, R. L. and Allison, M. J. (1983) *J. Animal Sci.,* **57** *Suppl.,* 2, 461.
Bernfeld, P. (1955) in *Methods in Enzymology,* (eds S. P. Colowick and N. O. Kaplan) (New York: Academic Press) Vol. 1, p. 149.
Chen, Yud-len, Varel, V. H. and Hashimoto, A. G. (1980) *Ind. Eng. Chem. Prod. Res. Dev.,* **19,** 471.
Fathepure, B. Z. (1983) *FEMS Microbiol.,* **19,** 151.
Ghose, T. K. and Bhadra A. (1981) *Process Biochem.,* **16,** (Oct/Nov), 23.
Hobson, M. (1969) in *Methods in Microbiology,* (eds J. R. Norris and D. W. Ribbons) (New York: Academic Press) Vol. 3 B, p. 133.
Hungate, R. E. (1966) *The Rumen and its Microbes* (New York: Academic Press).
Kerby, R., Niemczura, W. and Zeikus, J. S. (1983) *J. Bacteriol.,* **155,** 1208.
Klass, D. L. (1984) *Science,* **223,** 1021.
Kroeker, E. J., Schutte, D. D., Sparling, A. B. and Lapp, H. M. (1979) J. *Water Pollut. Control Fed.,* **51,** 718.
Mackie, R. I. and Bryant, M. P. (1981) *Appl. Environ. Microbiol,* **41,** 1363.
McCarty P. L. and McKinney, R. E. (1961) *J. Water Pollut. Control Fed.,* **33,** 233.
Miller, T. L. and Wolin, M. J. (1974) *Appl. Microbiol.,* **27,** 985.
Official Methods of Analysis of Association of Agricultural Chemists (1975), (eds W. Horwitz, A. Senzel, Helen Reynolds and D. L. Park) (A.O.A.C., Wash. D. C.) pp. 129-138.
Rand, M. C, Arnold, E. and Taras, Michel, J. (1979) *Standard Methods for the Examination of Water and Waste Water,* (Pub. American Public Health Association, American Water Works Association and Water Pollution Control Federation) p. 320.
Smith, P. H. and Hungate, R. E. (1958) *J. Bacteriol,* **75,** 713.
Subramaniam, S. K. (1977) *Biogas Systems in India* (New Delhi: Management Development Institute) p. 13.
Subramaniam, T. S. (1980) *Proc. Indian Inst. Chem. Eng.,* (Dec) p. 73.
Taylor, G. T. (1975) *Process Biochem.,* **10,** 29.
Zeikus, J. C. (1980) *Ann. Rev. Microbiol,* **34,** 423.