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ORIGINAL ARTICLE

Stimulation of biomethanation by *Clostridium* sp. PXYL1 in coculture with a *Methanosarcina* strain PMET1 at psychrophilic temperatures

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Keywords
acetoclastic, biogas, *Clostridium*, coculture, low-temperature, methanogen, stimulation, volatile fatty acids.

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Abstract

Aim: Bioaugumentation of low temperature biogas production was attempted by addition of cold-adapted *Clostridium* and a methanogen.
Methods and Results: A psychrotrophic xylanolytic acetogenic strain *Clostridium* sp. PXYL1 growing optimally at 20°C and pH 5·3 and a *Methanosarcina* strain, PMET1, growing optimally on acetate and producing methane at 15°C were isolated from a cattle manure digester. Anaerobic conversion of xylose at 15°C with the coculture of the two strains was performed, and batch culture methane production characteristics indicated that methanogenesis occurred via acetate through 'acetoclastic' pathway. Stimulation studies were also undertaken to evaluate the effect of exogenous addition of the coculture on biogas yields at 15°C. Addition of 3 ml of PXYL1 at the rate of 12×10^2 CFU ml^{-1} increased the biogas 1·7-fold (33 l per kg cowdung) when compared to control (19·3 l per kg cowdung) as well as increased the volatile fatty acid (VFA) levels to 3210 mg l^{-1} when compared to 1140 mg l^{-1} in controls. Exogenous of addition of 10 ml PMET1 inoculum at the rate of $6{\cdot}8 \pm 10^2$ CFU ml^{-1} in addition to PXYL1 served to further improve the biogas yields to 46 l kg^{-1} as well as significantly brought down the VFA levels to 1350 mg l^{-1}.
Conclusions: Our results suggest that the rate-limiting methanogenic step at low temperatures could be overcome and that biogas yields improved by manipulating the population of the acetoclastic methanogens.
Significance and Impact of the Study: Stimulation of biomethanation at low temperature by coculture.

Introduction

Anaerobic bioconversion of organic matter to methane and carbon dioxide is accomplished by complex microbial systems in anoxic environments. The microbial communities are a mixture of species that ferment natural organic substrates to acetate, hydrogen and carbon dioxide and methanogens that form methane from these products (Miller *et al.* 2000). Temperature is regarded as one of the main factors influencing methanogenic degradation. Temperature not only has an effect on the methane production itself but also on the decomposition of organic materials from which methanogenic substrates are produced (Fey and Conrad 2000).

Breakdown of anaerobic wastewater treatment process as a result of decreasing temperature is often characterized by an increasing concentration of acetic acid or propionic acid (Kashyap *et al.* 2003). A high production rate of methane can be observed only after a decrease in volatile fatty acid (VFA) concentration. During our studies on psychrophilic anaerobic digestion, it was observed that high level of VFA accumulated, indicating that the methanogenic step could be inhibited at low temperatures (Akila and Chandra 2007). Much attention should, therefore, be paid to the acetate-utilizing methanogenic populations in psychrophilic processes to better understand the reactions and to improve their performance.

Anaerobic digestion of organic wastes with some additives to improve biogas yields has been given due importance for the last many years (Mandal and Mandal 1998; Singh and Singh 1996; Jarvis *et al.* 1997; Sanchez *et al.* 1994; Lalov *et al.* 2001; McDermott *et al.* 2001). The effects of seeding with bacteria to inhibit methane production by rumen micro-organisms have been studied (Lopez *et al.* 1999).

The effect of seeding with bacteria to improve low temperature biogas generation has been taken up in this study. The stable associations between carbohydrate fermenters and hydrogen utilizers have been well documented (Ruyet *et al.* 1984). In this paper, we report the direct conversion of xylose to methane and carbon dioxide by a coculture of *Clostridium* PXYL1 and *Methanosarcina* strain PMET1. This study also reports the results obtained from the enhancement of psychrophilic anaerobic digestion of cattle manure by exogenous addition of the cocultures.

Material and methods

Source of organisms

The following organisms were used for the study. Strain *Clostridium* PXYL1 (DSM 15427) and *Methanosarcina* strain PMET1 (>99% similarity to *Methanosarcina barkeri*, available on request) were enriched and isolated in our laboratory from an anaerobic batch digester running at 20°C.

Media and cultivation

The culture medium used for the enrichment and isolation of psychrotrophic xylanolytic organisms was prepared as described previously (Akila and Chandra 2003) with 0·5% birchwood xylan and 0·1% yeast extract as growth supplement. Methanogens were enriched on the medium containing acetate 3 g l^{-1} (Cairo *et al.* 1992). All cultivations were carried out at 20°C in 125-ml serum vials containing 50 ml of medium under an atmosphere of N_2/CO_2 (80/20, v/v) without shaking. Single colonies growing on rolltubes were separated, and purity of the cultures was routinely checked by microscopy.

Growth and substrate utilization

Growth and substrate utilization were determined by monitoring increments of optical density at 540 nm and cell protein measurements (Lowry *et al.* 1951) as well as substrate utilization by determining total sugars (Dubois *et al.* 1956). In the case of methanogens, growth and substrate utilization were monitored by measuring the OD_{540} and methane production.

Microscopy and analytical methods

Morphological studies were undertaken by Gram staining and presence of endospores by Schaffer–Fulton's method (Deotsch 1981). Scanning electron microscopy was conducted as previously described (Albrecht *et al.* 1976).

Chemical analysis

Chemical oxygen demand (COD), cellulose and hemicellulose, total VFA, biogas and methane measurements were made as previously described (Akila and Chandra 2007).

Volatile fatty acid components by gas chromatography
The general procedure of Levett (1991) was used for the analysis of volatile products using gas chromatograph fitted with flame ionization detector. To 1 ml of the cell-free culture filtrate obtained by centrifuging broth at 12 000 ***g*** at 4°C for 1 h, 0·2 ml of aqueous H_2SO_4, 1 ml of diethyl ether was added and sealed in an Eppendorf tube, mixed on a vortex mixer for 10–15 s, centrifuged to break the ether/water emulsion, and aqueous and ether layers are allowed to separate. The upper ether layer (1 μl) was injected into the Nucon AIMIL Gas Chromatograph (5200 series; New Delhi, India) and compared with the retention times of peaks obtained with standard VFA mixture. Chromosorb 101 (60–80 mesh, length 6′, I.D. 2 mm) column was used with N_2 as carrier gas at a rate of 45 ml min^{-1} and H_2 at 35 ml min^{-1}. Injector and detector temperature were set at 210°C and column temperature was set at 190°C.

Gas analysis
The composition of the biogas as well as hydrogen in the head space was determined by a gas chromatograph (GC-AIMIL Nucon 5200 series) with a TCD (thermal conductivity detector) equipped with a steel column of Porapak Q. N_2 gas was used as the carrier gas at 30 ml min^{-1} and the oven temperature was kept at 190°C. The injector and detector temperature was maintained at 210°C each. Chromatograph peaks were compared with that of standard H_2 gas and standard biogas mixture obtained from Central Leather Research Institute, Madras.

Cocultivation experiments

Cocultivation experiments were performed with the isolates *Clostridium* PXYL1 and *Methanosarcina* strain PMET1 by using mineral base medium with yeast extract and xylose (0·5%) as carbon source and also contained cysteine-sulphide reducing solution, resazurin and bicarbonate. The pH of the medium was adjusted to 7–7·5, and

the cultures were incubated at 15°C without shaking. Strain PXYL1 and PMET1 were added in the ratio of 1 : 5 wherein 1 ml of PXYL1 contained 12×10^2 CFU and PMET1 $6{\cdot}8 \times 10^2$ CFU ml^{-1}. The gas phase was maintained with H_2/CO_2 (80 : 20). To rule out the formation of methane from H_2/CO_2, the coculture was grown on Formate – H_2/CO_2. The experiments were conducted in triplicates and subsequent transfers of the coculture to fresh medium were made to check the stability of coculture.

Stimulation studies

Batch experiments were conducted to investigate the effects of exogenous addition of the pure isolated cultures on biogas production at low temperatures. To fresh cattle manure, 10% inoculum from an adapted digester was added and served as control. Two rounds of experiments were conducted (i) to study the effects of exogenous addition of PXYL1 alone and (ii) to study the effects of the addition of both strains PXYL1 and PMET1. In the first batch of experiments, various inoculum levels of PXYL1 were added (1–10 ml) containing $1{\cdot}2 \times 10^2$ CFU ml^{-1}, in addition to the starter inoculum. In the second batch of experiments, besides the start-up culture and an optimum inocula of PXYL1, PMET1 was added at various concentrations ranging from 1 to 13 ml containing $6{\cdot}8 \times 10^2$ CFU ml^{-1}. At the end of 35 days, biogas, COD, total VFA, cellulose, hemicellulose as well as individual organic acids such as acetate, propionate and butyrate measurements were measured. All experiments were conducted in triplicates and the mean and ± standard deviation computed.

Results

Characteristics of strain *Clostridium* sp. PXYL1

A psychrotrophic xylanolytic acetogenic strain *Clostridium* sp. PXYL1 growing optimally at 20°C and pH 5·3 was isolated from a cattle manure digester (Akila and Chandra 2003). Enumeration of PXYL1 on solid medium with xylan as carbon and energy source was carried out. After 30 days of incubation at 20°C, an average of 12×10^2 CFU ml^{-1} were found.

Strain PXYL1 was a rod-shaped bacterium (Fig. 1a,b). Cells were 1–2 μm in width with sub-terminal spores (Akila and Chandra 2003). The bacteria were nonmotile with absence of flagella. The Gram reaction by conventional staining was positive at all stages of growth. The isolate was strictly anaerobic. Growth was observed even after pasteurization (20 min at 80°C). Strain PXYL1 grew well on xylose and xylan. The effect of temperature on growth was tested by determining the specific growth rates at various temperatures (Fig. 2). The optimum temperature for growth of PXYL1 was 20°C and the doubling time was 1·66 h on xylose and 2·52 h on xylan. There was no growth below 5°C and above 50°C. After 7 days, the growth and fermentation was complete (Table 1) and substrate utilization was 75% (glucose), 55% (cellulobiose), 80% xylan and 83% xylose. The end product of

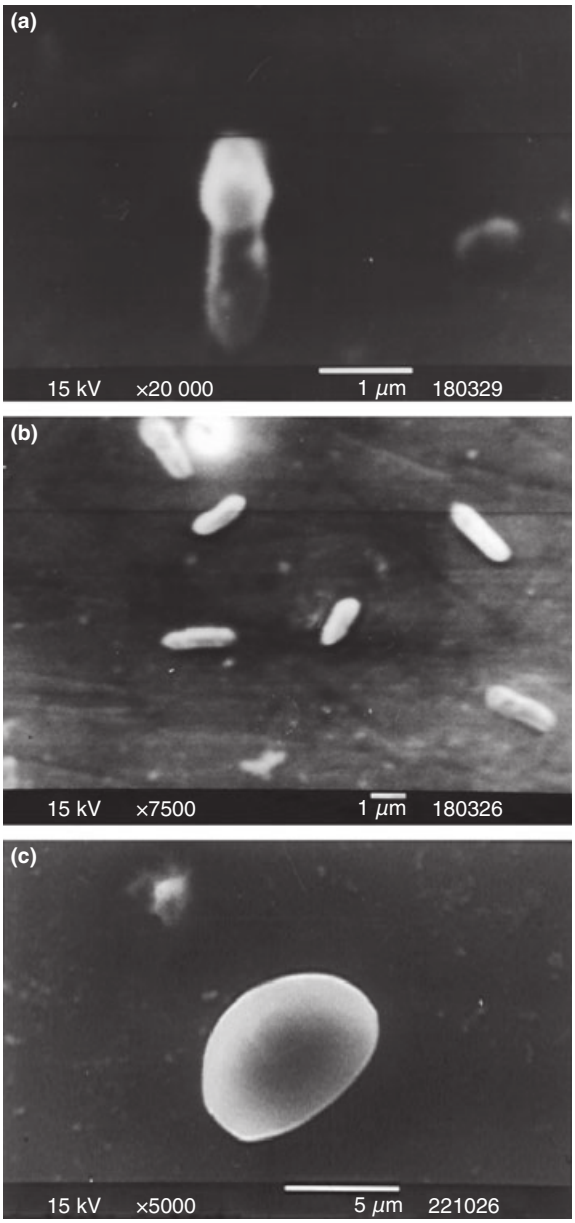

Figure 1 Scanning electron micrographs of (a) *Clostridium* strain PXYL1 with sub-terminal spore, magnification 20 000×, scale bar 1 μm (b) *Clostridium* strain PXYL1 showing rod-shaped cells, magnification 7500×, scale bar 1 μm (c) Methanosarcina strain PMET1, magnification 5000×, scale bar 5 μm.

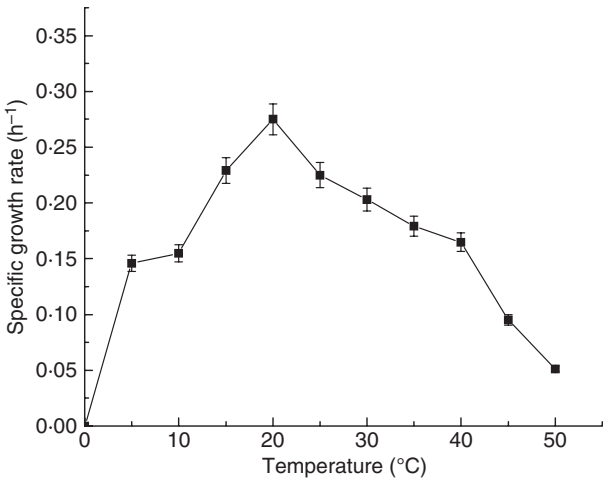

Figure 2 Specific growth rate of *Clostridium* PXYL1 at different temperatures (5–50°C) on xylan.

fermentation of PXYL1 on various substrates were analysed and the volume of the given substrate in 100 mmol. Acetate was the main product formed from all the substrates used by the strain PXYL1 and sometimes formate was formed, as is the case with cellulobiose. Carbon recovery was calculated from the products formed, the remaining substrate and excludes the cell carbon content. For birchwood xylan, the mole percentage of xylose was taken as 94·1 (Sigma, USA). The carbon recovery was 90% with glucose and xylose as substrate, 75% for cellobiose and close to 100 for xylan.

The end product of fermentation of xylan by PXYL1 at different temperatures of 10, 20 and 30°C was determined (Table 2). It was observed that temperature influences end-product formation. While acetic acid was the predominant end product at lower temperatures of 10 and 20°C, butyric and propionic acids were also detected at the mesophilic range. The comparison of characteristics of some psychrophilic *Clostridia* with strain PXYL1 is given in Table 3.

Enrichment, isolation and characteristics of *Methanosarcina* strain PMET1

The morphological (Fig. 1) and biochemical characteristics of *Methanosarcina* strain PMET1 are given (Table 4). The effect of temperature on specific growth rate (Fig. 3) of PMET1 was typical of psychrotrophs, in that, between 5 and 50°C, specific growth rate increased with temperature up to a maximum value at 15°C (T_{opt}) with a doubling time of 15·89 h and thereafter decreased with temperature above T_{opt}. Cells grew well at pH between 6·5 and 7·5 with optimum growth at pH 7·0. Figure 4 shows the methane profiles at 15 and 37°C. Although

Table 1 End products of fermentation of PXYL1 on different substrates at 20°C

Products (mmol)*	Substrate (100 mmol)			
	Glucose	Cellobiose	Xylose	Xylan†
Acetate	43 ± 8	Traces	91 ± 3·2	90 ± 2·2
Lactate	24 ± 0·2	53 ± 2	52 ± 2·5	42 ± 4
Formate	147 ± 12	217 ± 3·6	75 ± 2	55 ± 2·9
CO_2	96 ± 7	108 ± 0·9	111 ± 3	150 ± 3
H_2	64 ± 3	19 ± 0·2	26 ± 0·8	45 ± 1
Substrate utilization (mmol)	75 ± 3	55 ± 2.	83 ± 5	80 ± 3·4
Cell protein (mg ml^{-1})	0·1 ± 0·001	0·08 ± 0·005	0·14 ± 0·007	0·18 ± 0
Percentage of carbon recovery‡	89 ± 1	73 ± 3	104 ± 6	108 ± 7

Experiment carried out in 100 ml serum vials with 50 ml medium and 50 ml head space.
*Analysis was performed at the end of 7 days.
†Mole percentage of xylose in Birchwood xylan taken as 94·1.
‡Excludes cell carbon content.

Table 2 End products of fermentation of xylan by PXYL1 at different temperatures

Temperature	Products (mmol)*								% Xylan utilization
	Formic acid	Lactic acid	Acetic acid	Propionic acid	Butyric acid	Ethanol	H_2	CO_2	
10°C	10 ± 0·9	84 ± 2	100 ± 2	ND	ND	ND	42 ± 3·1	90 ± 3	45
20°C	55 ± 1	46 ± 1	96 ± 2	ND	ND	ND	45 ± 2·7	150 ± 6	84
30°C	ND	45 ± 3	27 ± 1	96 ± 5	87·5 ± 5	ND	66 ± 4	165 ± 7	80

*Analysis was performed at the end of 7 days; products expressed the amount of the substrate in 100 mmol.
ND, Not detectable; H_2 and CO_2 was estimated in the total headspace.

Table 3 Comparison of characteristics of *Clostridium* strain PXYL1 with other psychrophilic clostridia reported

Strain	Source	Substrate	(Opt) temp °C	Fermentation end products	Characteristics	Ref.
Clostridium fimentarium	Cattle manure digester	Glu, Fru, Mal, Ara, Xyl, Cellobiose, Gal and Man	20–25	Acetate, ethanol, lactate, formate,H_2 and CO_2	Gram-positive, oligosporous, motile rod, 0·5–0·6/2·1–5·0 m	Kotsiurbenko *et al.*, (1995)
Clostridium estertheticum	Vaccum-packed beef	Mono and di-sachharides and starch	15	Butyrate, acetate,formate, lactate, ethanol, butano, H_2 and CO_2	Gram-positive, motile with peritrichous flagella, ellipsoidal in a terminal-subterminal position	Collins *et al.*, (1992)
Clostridium vincentii	Pond sediment	Xylan,mono and disachharides; <0·5% NaCl	12	Acetate, formate, butyrate, H_2 and CO_2	Gram-positive, rod-shaped, motile,	Mountfort *et al.*, (1997)
Clostridium ultunense	Anaerobic digester sludge	Formate, glucose, cysteine, pyruvate, betaine, and ethylene glycol	37	Acetate, formate	Gram-negative, curved rods,. motile with polar flagellum	Schnurer *et al.* (1996)
Clostridium frigoris sp. Nov.	Antarctic microbial mat	Mono and di-saccharides	5–7	Butyrate, formate, lactate, acetate, ethanol, H_2 and CO_2	Gram-positive, rod shaped, motile with peritrichous flagella, terminal endospores	Spring *et al.*, (2002)
Clostridium lacusfryxelense Sp. Nov.	Antarctic microbial mat	Mono and di-saccharides	8–12	Butyrate, formate, acetate, lactate, ethanol, H_2 and CO_2	Gram-positive, rod-shaped, motile with peritrichous flagella, spherical to ellipsoidal spores in a terminal to sub-terminal position	Spring *et al.*, (2002)
Clostridium bowmanii Sp. Nov.	Antarctic microbial mat	Mono and di-saccharides	12–16	Butyrate, acetate, formate, ethanol, lactate, 1-butanol, H_2 and CO_2	Gram-positive, motile with peritrichous flagella, spherical endospores in a terminal to sub-terminal position	Spring *et al.*, (2002)
Clostridium psychrophillum	Antarctic microbial mat	Mono and di-saccharides	4	Lactate, ethanol, butanol, butyrate, H_2 and CO_2	Gram-positive, motile with peritrichous flagella, ellipsoidal in terminal to sub-terminal position	Spring *et al.*, (2002)
Clostridium algidixylanolyticum	Vaccum-packed lamb	Starch and xylan	25·5–30	Acetate, lactate, formate, ethanol, butyrate, H_2 and CO_2	Motile rods, elliptical sub-terminal spores	Broda *et al.*, (2000)
Clostridium PXYL1	Anaerobic digester sludge	Xylan, xylose, glucose etc.	20	Acetate, formate, lactate, H_2 and CO_2	Gram-positive, nonmotile,	Present study

Table 4 Phenotypic characteristics of methanogen strain PMET1 with other closely related psychrophilic methanogens

	PMET1	*Methanosarcina baltica* GS1-A^T	*Methanogenium frigidum*
Colony morphology	Brown, raised, irregular	NR	NR
Cell morphology	Irregular cocci (singly and in pairs) 4–6/2–4 μm	Cocci-singly and in pairs	Irregular nonmotile coccoids
Temperature optima	15°C	25°C	15°C
Growth at 4–10°C	+	+	+
pH optima	7·0	6·5	6·5–7·9
Substrate	++	+	–
Acetate	+/–	–	+
Formate	+/–	–	–
Methanol	+/–	–	++
H_2/CO_2			
Stimulatory factors	Yeast extract	2–4% NaCl	300–600 mmol l^{-1} Na^+

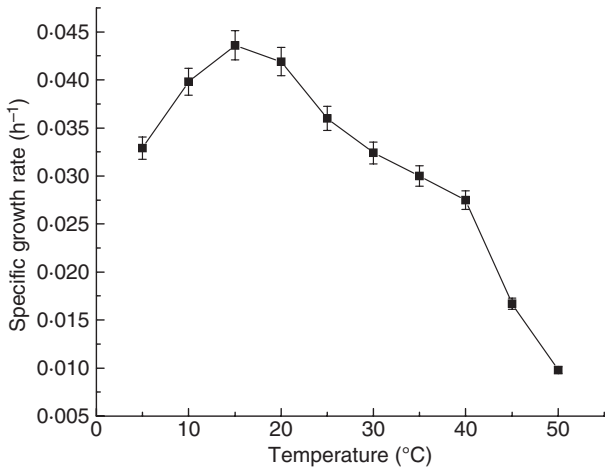

Figure 3 Specific growth rate of methanogen PMET1 at different temperatures (5–50°C) on acetate.

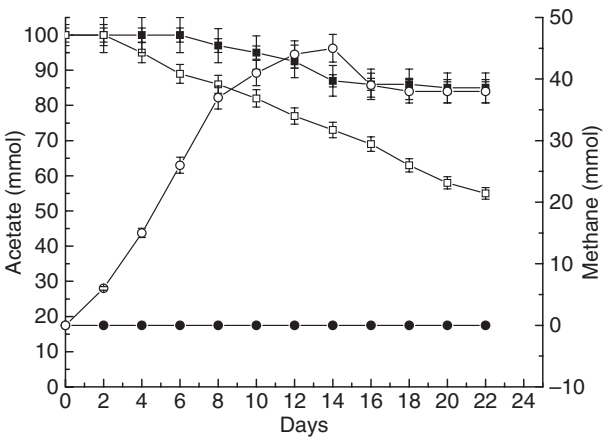

Figure 4 Methane profiles of PMET1 at 37 and 15°C when grown on acetate. Acetate: (■) 37°C and (□) 15°C; methane: (●) 37°C and (○) 15°C.

growth was initially observed at 37°C, no methane was detected in the headspace and the acetate utilization was only 15%, while at 15°C, the methane production was observed without a lag and up to 3 weeks and the acetate utilization was 65%.

Coculture studies

We integrated the fermentation of xylose to acetate and thereafter to methane with the *Clostridium* PXYL1–*Methanosarcina* PMET1 coculture. Coculture of the two species yielded efficient conversion of xylose to methane and carbondioxide. Figure 5b shows the acetate and methane production from xylose by the coculture for a period of 45 days. The cultures, despite the differences in the T_{opt}, were observed to stably co-exist. At 15°C, xylose was degraded to methane with a transient accumulation of acetate. The average ratio of methane to acetate for the total period was 0·621 ± 0·36. This is close to the expected ratio of 0·66 for the production of 3 mol of acetate and 2 mol of methane per 2 mol of xylose. Because acetate is degraded and stoichiometric amounts of methane are formed, the methanogenesis appears to be of the 'acetoclastic' type. To rule out the formation of methane from H_2/CO_2, the coculture was also grown on formate – H_2/CO_2 (data not shown). However, no methane was produced.

Stimulation studies

In order to stimulate biogas yields at low temperatures and also overcome the rate-limiting step of VFA degradation by methanogens, we studied the effects of exogenous addition of pure isolated cultures of xylanolytic strain PXYL1 and the methanogenic strain PMET1. It was observed that addition of the xylanolytic strain PXYL1 served to improve the biogas yields 1·7-folds (33 l per kg cowdung) with respect to control (19·3 l per kg cowdung) (Table 5). The

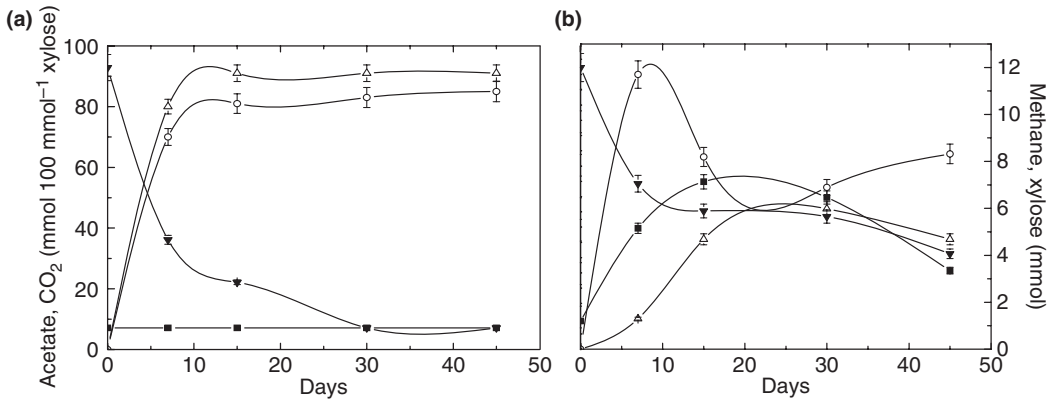

Figure 5 Time course of methane production from xylose by (a) PXYL1 alone and (b) coculture at 15°C, (■) methane; (○) CO_2; (△) acetate and (▼) xylose.

Table 5 Effect of addition of *Clostridium* PXYL1 on biogas generation on cattle manure slurry at 20°C at the end of 35 days

Batch*	Control	a	b	c	d	e
Biogas (l per kg cowdung)	19·3 ± 1	23·6 ± 2	33 ± 1	23 ± 2	23 ± 1	27 ± 2
Chemical oxygen demand (mg l^{-1})	2560 ± 50	2220 ± 150	1900 ± 34	1800 ± 47	1730 ± 45	1700 ± 40
Volatile fatty acid (mg l^{-1})	1140 ± 99	2780 ± 170	3210 ± 20	3230 ± 160	3890 ± 254	4110 ± 273
Cellulose (% w/w)	23 ± 0·9	23 ± 0·2	23 ± 2	23 ± 1	23 ± 0·9	23 ± 1
Hemicellulose (% w/w)	14 ± 0·6	14 ± 0·5	12 ± 0·5	10 ± 0·2	9 ± 0·3	9 ± 0·6

*The different batches are: (control), 10% inoculum of cold-adapted cattle slurry; the rest had additions of strain *Clostridium* PXYL1 besides the 10% adapted slurry – (a), +1 ml PXYL1; (b), +3 ml PXYL1; (c) +5 ml PXYL1; (d), +7 ml PXYL1; (e), +10 ml PXYL1; wherein, 1 ml PXYL1 corresponded to 12×10^2 CFU.

inoculum level of 3 ml was found to produce the highest yield and further increase in inoculum did not significantly improve the amount of biogas. The VFA levels were found to increase with increase in the inoculum levels and showed a 2·8-fold increase (3210 mg l^{-1}) on addition of 3 ml of the PXYL1 inoculum when compared to 1140 mg l^{-1} in controls. While cellulose appeared to be resistant to degradation, the hemicellulose content showed a further decrease, with the addition of the xylanolytic strain (Table 5). Same was the case with the percentage of COD reduction.

Addition of the coculture as 3 ml PXYL1 and 10 ml PMET1 was optimal (Table 6) and further improved the biogas yields by 2·3-fold (46 l per kg cowdung) over the control, and 1·4-fold over the addition of PXYL1 alone. There was a concomitant reduction (2·38-fold) in the total VFA content (1350 mg l^{-1}) (Table 6) with respect to the addition of PXYL1 alone (3214 mg l^{-1}). An analysis of

Table 6 Effect of addition of coculture (PXYL1 + PMET1) on biogas generation on cattle manure at 20°C at the end of 35 days

Batch*	Control	a	b	c	d	e	f	g
Biogas (l per kg cowdung)	20 ± 1	33 ± 2	32 ± 3	34 ± 2	36 ± 2	36 ± 2	46 ± 2	45 ± 1
Chemical oxygen demand (mg l^{-1})	2450 ± 150	1890 ± 80	1880 ± 100	1870 ± 230	1880 ± 200	1860 ± 170	1880 ± 170	1880 ± 100
Volatile fatty acid (mg l^{-1})	1145 ± 190	3214 ± 210	1865 ± 220	1752 ± 200	1750 ± 190	1750 ± 270	1350 ± 100	1400 ± 110
Acetate (mmol l^{-1})	120 ± 11	185 ± 20			90 ± 6 (Batch f)			
Propionate (mmol l^{-1})	42 ± 7	65 ± 3			11 ± 2 (Batch f)			
Butyrate (mmol l^{-1})	11 ± 0·9	34 ± 1			6 ± 0·5 (Batch f)			

The different batches are: (control), 10% inoculum of cold-adapted cattle slurry; the rest had additions of strain *Clostridium* PXYL1 (3 ml) plus different inoculum levels of PMET1, besides the 10% adapted slurry – (a), +3 ml PXYL1; (b), +3 ml PXYL1 + 1 ml PMET1; (c), 3 ml PXYL1 + 3 ml PMET1; (d), 3 ml PXYL1 + 5 ml PMET1; (e), 3 ml PXYL1 + 7 ml PMET1; (f), 3 ml PXYL1 + 10 ml PMET1; (g), 3 ml PXYL1 + 13 ml PMET1; wherein, 1 ml PXYL1 corresponded to 12×10^2 CFU and 1 ml PMET1 corresponded to $6{\cdot}8 \times 10^2$ CFU.

the individual organic acids (Table 6) showed that exogenous addition of xylanolytic strains significantly increased the content of acetic (185 μmol ml^{-1}), propionic (65 μmol ml^{-1}) and butyric acids (34 μmol ml^{-1}) when compared to the control values of 120, 42 and 11 μmol ml^{-1} respectively. Addition of the coculture brought down the content of the individual VFAs to 90, 11 and 6 μmol ml^{-1} respectively.

Discussion

Methane fermentation at low temperature has recently become a subject of considerable interest. Research on improved strategies for anaerobic digestion of wastes generally studies the complex microbial populations that result from anaerobic enrichments of micro-organisms from native habitats. During our previous studies on low temperature anaerobic digestion (Akila and Chandra 2007), we observed that at high organic loading rates, VFA accumulated and contributed to the VFA in the effluent with corresponding less than theoretical levels of methane. As a part of a study to improve biogas production at low temperatures, the xylanolytic and methanogenic bacteria were isolated to get a better insight into the microbiology of the different species and to understand the anaerobic digestion process at psychrophilic temperatures.

Isolation of cold-active strains

Clostridium PXYL1 is a 'psychrotrophic' anaerobic spore-forming xylanolytic bacterium growing optimally at 20°C (Akila and Chandra 2003). This organism fermented xylan and many soluble sugars (glucose, cellobiose, xylose, arabinose, etc.) and was noncellulolytic. Fermentation products were acetate, formate, lactate, hydrogen and carbon dioxide. Strain PXYL1 has features that are typical of the homoacetogenic bacteria because acetate was the main fermentation product formed. Taxonomically, the homoacetogenic group constitutes an extremely heterogeneous group, which includes the genus *Clostridium* (Schnurer *et al.* 1996). Temperature was found to influence end-product formation, with acetate being the predominant end product at low temperatures, indicating that the fermentation was channelized to the formation of more acetate at low temperatures (Table 2).

A psychrotrophic Gram-positive irregular coccoid methanogen, which showed close similarity to a *Methanosarcina* sp., was also isolated from the cattle manure digester. Acetate served as the main substrate for methanogenesis by PMET1 in a mineral base medium, which converted it to methane and carbon dioxide most efficiently at 15°C than at 37°C.

Coculture studies

In a microbial scenario, these two anaerobic strains (*Clostridium* and methanogen) could co-exist and degrade xylose to methane and carbon dioxide. In the coculture, an initial increase in the acetate concentration coincided with the degradation of xylose (Fig. 5). And because acetate was degraded and stoichiometric amounts of methane were formed during cocultivation of the strains PXYL1 and PMET1, it seems clear that the pathway was of methanogenesis was of the 'acetoclastic' type. There is a delicate balance between acetate production and utilization, by the coculture. Unless the acetate is used as soon as it is formed from xylose, the medium pH will decrease and will be inhibitory to the acetate utilization by PMET1. Acetate production and its conversion to methane were found to occur effectively without much lag in the liquid suspension of the coculture. Table 7 gives the literature review of species so far reported that occur in coculture with methanogens in efficient methane production.

Stimulation studies

Exogenous pure culture additions to acid-forming stage can be used to change the product array (Miller *et al.* 2000). In most of the production processes, propionate and

Table 7 Studies on different species that exist in coculture with methanogens

Co-culture (source)	Type of association	Reference
Clostridium ultenense – methanogenic isolate MAB1 (swine manure digester)	Acetate oxidizing – hydrogenotrophic	Schnurer *et al.* (1996)
Clostridium bryantii – various H_2-utilizing methanogens	C4-C11 Fatty acid oxidation – hydrogenotrophic	Stieb and Schink (1985)
Desulfotomaculum – Methanobacterium thermoautotrophicum (thermophilic UASB reactor)	Propionate oxidizing: hydrogenotrophic	Imachi *et al.*, (2000)
Ruminococcus albus: *Methanobrevibacter smithii*, *Methanosarcina barkeri*	Cellulose: H_2-utilizing	Miller *et al.* (2000)
Clostridium PXYL1: PMET1	Xylose–acetate–methane	Present study

acetate accumulate to high concentrations, which are toxic for the methanogens (Vavilin and Lokshina 1996). Although addition of the xylanolytic strain *Clostridium* PXYL1 improved biogas generation, it resulted in a significant accumulation of acetate in the medium. For successful degradation of wastes to generate energy, it is, therefore, essential to isolate such strains of methanogens, which have high acetate consumption rates. *Methanosarcina* ssp. are the most versatile methanogens and can use H_2/CO_2, acetate, methanol and methylamines (Boopathy 1996) and along with other syntrophic strains can be exploited for improved product formation (Miller *et al.* 2000). Exogenous addition of optimal inocula of *Clostridium* PXYL1 (3 ml) and *Methanosarcina* PMET1 (10 ml) resulted in the efficient conversion of the hemicelluloses to acetate and concomitantly to methane, probably because of optimal numbers of the respective fermenters. The methane yield from the exogenous addition of the cocultures at the psychrophilic temperature was 46 l per kg cowdung when compared to the standard value of 36 l kg^{-1} (Singh and Singh 1996).

Our research is significant in many respects. First, cultures enriched and isolated from a low-temperature cattle manure digester in our laboratory had cold-active microbes for production of methane. Secondly, this is the first description of a psychrotrophic xylanolytic *Clostridium* sp. PXYL1 that could exist in coculture with an acetate-utilizing methanogen and may have multiple functions in methanogenic ecosystems in terms of syntrophic and nonsyntrophic fermentations. Thirdly, our study shows that the populations of the methanogenic communities can have a decisive effect on the rate of methanogenesis at low temperatures. In the present study, we speculate that low temperatures selected 'for' preferentially, the acetate utilizing methanogens. Development of economically viable process with such enrichment cultures can improve biomethanation at low temperatures and thereby improve the quality of life in remote rural areas in the developing countries.

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