Development of mixed inoculum for methane enriched biogas production

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Abstract Inocula were collected from four different sources such as Jajmau tannery waste treatment plant (ITW), Jajmau municipal waste treatment (IMW), Unnao distillery (IDW) and a batch reactor, in which the sludge of a field scale biogas reactor was added to cow dung slurry to develop inoculum (IBS). A combination of these mixed inocula were used for biogas production at 35°C in laboratory scale reactor (10 L capacity) and the average yield of biogas (0.547 Lg⁻¹ volatile solid (VS)) and methane (0.323 Lg⁻¹VS) in 41 d was higher in case of mixed inoculum IMW₁ (IMW+IBS), with maximum methane content in biogas (68% during 27–30 d), as compared to other mixed inocula as well as control i.e. ITW₁ (ITW+IBS), IDW₁ (IDW+IBS) and IBS. The corresponding yields of gas were biogas (0.505, 0.536 and 0.456 Lg⁻¹VS), methane (0.288, 0.305, and 0.245 Lg⁻¹VS) where as, the corresponding maximum methane content in biogas was 62% during 29–33d, 64% during 29–33 d and 62% during 27–29 d in ITW₁, IDW₁ and IBS.

Keywords Methanogens · Biogas · Mixed inoculum · Biogas reactor · Waste

Introduction

Methane fermentation is a versatile biotechnology capable of converting almost all types of carbonaceous organic materials to methane and carbon dioxide under anaerobic conditions. This is achieved as a result of the consecutive biochemical breakdown of organic materials to methane and carbon dioxide in an environment in which a variety of microorganisms, which include fermentative microbes (acidogens); hydrogen-producing, acetate-forming microbes (acetogens); and methane-producing microbes (methanogens), harmoniously grow and produce reduced end-products [8]. Anaerobes play important roles in establishing a stable environment at various stages of methane production, which offers an effective means of pollution reduction, superior to that achieved via conventional aerobic processes (Table 1). Although practiced for decades, interest in anaerobic fermentation has only recently focused on its use in the economic recovery of fuel gas from industrial and agricultural wastes. Presence of uncombustable gases (CO₂ and H₂S) reduces its calorific value. Therefore, it is necessary to remove or minimize the concentration of these gases before use of biogas.

Strevett et al. [33] have used a chemo-autotrophic methanogen (*Methanobacterium thermoautotrophicum*), uncoupled methanogenesis techniques and hollow fiber membranes, to convert carbon dioxide to methane, and effectively remove hydrogen sulfide from biological off-gases. Gas phase methane concentration was found to increase from 60 to 96%. O’Keefe et al. [25] have studied a methane enrichment process that involved air purging of recycled digester contents to strip CO₂ to increase methane content upto 90% in biogas. Reig et al. [27] studied chemically

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pretreated cellulose as feed and operated two anaerobic fluidized bed reactors at 35°C. Methane generation rate achieved was 1.09 Ld⁻¹ at a retention time of 8.3 h and at an influent substrate concentration of 1400 mg COD L⁻¹. Mashandete et al. [22] studied the effect of particle size on biogas yield from sisal fiber waste and confirmed that methane yield was inversely proportional to particle size. Methane yield increased by 23% when the fibers were cut to 2 mm size and was 0.22 m³ CH₄ kg⁻¹ volatile solids for untreated fibers. Lissens et al. [19] studied the enhancement of the anaerobic biodegradability and methane yields from different biowastes {food waste, yard waste, and digested biowaste already treated in a full scale biogas plant (DRANCO, Belgium)} by assessing thermal wet oxidation.

Biogas production depends on various factors like pH, temperature, hydraulic retention time (HRT), C: N ratio, etc [1, 4, 5, 17]. Lack of process stability, low loading rates, slow recovery after failure and specific requirement for waste compositions are some of other limitations associated with it. Anaerobic fermentation being a slow process, a large HRT of 30–50 d is used in conventional biogas plants. This leads to a large volume of digester and high cost of the system [34]. The decrease in gas generation during winter season has been reported, which poses a serious problem in the practical application of this technology [30]. All these have resulted in restricted application of biogas technology in rural areas. Thus, there is a need to improve the overall efficiency of anaerobic digestion process in the biogas plants. This could be done by several methods such as optimizing the various operational parameters, satisfying the nutritional requirements of microbes using different biological and chemical additives and by using inocula from

different biogas plants. This paper presents the results of lab scale experiment conducted in batch mode, to determine the biogas yield, and methane content in different reactors, using different mixed inocula from various sources.

Materials and methods

Reactors and operating conditions

A 10 L glass vessel batch reactor, whose mouth was sealed by a rubber cork having two holes, was taken (Fig. 1). One hole was having a glass tube dipped in reactor feed for extraction samples and another hole was connected to the top end of gas collector by silicon tubing. The bottom end of gas collector was connected to a measuring tube. Gas collector as well as measuring tube were filled with water. During gas formation, pressure increased inside the reactor, which created a pressure on water of gas collector and gas collection could be measured.

Microbial mixed cultures

Inocula were collected from Jajmau tannery waste treatment Plant (ITW), Jajmau municipal waste treatment plant (IMW), Unnao distillery (IDW) and 10 L laboratory batch reactor, in which the sludge of a field scale biogas reactor was added to cow dung slurry to develop inoculum (IBS).

Substrates (cow dung slurry)

A homogeneous mixture of cow dung slurry (CDS) having 92 gL⁻¹ total solids (TS) and 76 gL⁻¹ volatile solid (VS) was

Table 1 Literature data of biodegradable materials with high yield of methane

Biodegradable materials	CH ₄ Yield (m ³ kg ⁻¹ VS added)	References
Municipal solid waste		
Municipal solid waste	0.264	De Baere [6]
Municipal solid waste + Sewage sludge	0.187	DeBaere and Verstraete [7]
Fruit/ vegetable waste		
Spinach waste	0.316	Shiralipour and Smith [31]
<i>Beta vulgaris</i> leaves	0.297	Badger et al. [2]
<i>Sinapis alba</i> tops	0.300	Zubar [38]
<i>Helia heliantus</i> leaves	0.265	Gunaseelan [12]
<i>Calotropis procera</i>	0.280	Mahamat [20]
Grass feed		
Napier grass	0.310	Shiralipour and Smith [32]
<i>Cynadon dactylon</i>	0.112	Ghosh et al. [10]
<i>Triticum aestivum</i>	0.255	Richards et al. [28]
Millet straw	0.257	Mahamat [20]
Marine biomass feed		
<i>Ulva</i>	0.220	Habig et al. [13]
<i>Gracilaria tikvahiae</i>	0.300	Habig et al. [13]

made by mixing 20 kg of cow dung with 20 L of tap water and was sieved for removing large particles.

Composition of growth medium (DSMZ medium) of Savant et al. [31] Growth medium required for development of mixed methanogenic culture consisted of (g L^{-1}).glucose, 1.0; KH_2PO_4 , 0.25; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2; NaCl , 0.2; NH_4Cl , 0.2; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.025; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.001; trace element solution, 0.5; yeast extract, 0.5; sodium acetate, 0.5; sodium formate, 1; sludge fluid, 25; fatty acid mixture solution, 10; resazurine, 0.0005; cysteine $\text{HCl} \cdot \text{H}_2\text{O}$, 0.25; $\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$, 0.5. The pH of the growth medium was 6.7–7.0.

Sludge fluid (25 ml): 0.4% yeast extract was added to sludge from an anaerobic digester, and after gassing for a few minutes, it was incubated at 37°C for 24 h. Then the sludge was centrifuged at $10000 \times g$, the clear supernatant was put in screw-capped tubes under nitrogen gas. The sludge fluid was stored at ambient ($35\text{--}37^\circ\text{C}$) temperature in the dark.

Fatty acid mixture solution (10 mL) contained 0.5g L^{-1} each of valeric acid, isovaleric acid, 2- methylbutyric acid, isobutyric acid, distilled water 20 mL.

Trace elements solution (20 mL) in growth medium contained (g L^{-1}) Nitrilotriacetic acid, (neutralized with KOH), 1.5; $\text{MnSO}_4 \cdot 2\text{H}_2\text{O}$, 0.1; $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$, 0.2; $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 0.1; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.1; $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$, 0.025; $\text{Na}_2\text{SeO}_3 \cdot 0.2$; $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 0.1;

$\text{Na}_2\text{WO}_4 \cdot 2\text{H}_2\text{O}$, 0.1; pH of the trace elements solution was 7.5.

Development of inoculum in cow dung slurry and biogas production

Anaerobic techniques originally developed by Hungate [14] and modified by Miller and Wolin [23] were used. Methane producing four types of microbial cultures were obtained and using 25 mL roll vials containing 9 mL growth medium, were inoculated with 1 mL inoculum and incubated at 35°C for 7 d. Now, 250 mL of growth medium was inoculated with 10 mL inocula (prepared previously), under strict anaerobic condition using CO_2 . Microbial cultures obtained in the roll tube showed methane production. 250 mL of inoculum (IBS) was mixed separately with 250 mL of other three enriched methanogenic inocula (ITW, IDW and IMW), as shown in Fig. 2.

Batch experiments were carried out in lab scale reactors with a working volume of 10 L. The digestion was carried out in four reactors, one was standard (IBS control) and another three were with different inocula along with cow dung slurry as substrate. All reactors were sealed with two-way cork and kept in a temperature-controlled chamber maintained at 35°C . The biogas production was recorded regularly (24 h interval), after 3 d of inoculation.

Fig. 1 Biogas reactor.

Fig. 2 Mixed inocula development and subsequent biogas production.

Analytical methods

The total gas production was measured by the water displacement technique and the biogas composition (e.g. CH₄, H₂S and CO₂) was periodically determined by a Netel Gas chromatograph with TCD detector, the stainless steel column was packed with Porapak Q; injector, oven and detector temperatures were 100°C, 50°C and 200°C, respectively. The nitrogen carrier gas flow was 30 mL min⁻¹.

Methane content in biogas was also analysed by the following procedure for comparison. The produced biogas was passed into 20% solution of potassium hydroxide, which absorbed carbon dioxide but not methane and hydrogen sulfide. This gas was then bubbled through a zinc acetate column, which absorbed hydrogen sulfide. The remaining volume of this gas was considered as methane. Cyber scan pH meter was used for pH determination. The total solids (TS) and volatile solids (VS) were determined by standard methods given by Greenberg et al. [9] and Williams [36].

Identification of microorganism

Genera of the methanogenic bacteria were identified by using the method prescribed by Rocheleau et al. [29].

Results and discussion

In anaerobic digestion of organic matter, various types of microorganisms take part in the processes of hydrolysis,

acid formation and methane production. Among these three steps, methanogenesis is considered as the most relevant one. In the first stage, namely hydrolysis, the particulate organic matter consisting of lipids, proteins and polysaccharides are converted to soluble form by microorganisms. The products of hydrolysis are converted to acetic acid, carbon dioxide and hydrogen by a group of microorganisms called acetogenic bacteria. Finally, methane is produced from these end products of acid formers by different types of microorganisms consisting of *Methanothrix soehngenii*, *Methanobrevibacter arboriphilus* and *Methanospirillum hungatei* [37]. The present investigation is based on batch process to study the effect of different inocula (collected from various sources) on biogas production. The methane content and biogas produced are shown in Table 2.

Daily biogas production in four different reactors

Figure 3 shows the daily biogas production in different reactors. When inoculum was transferred into fresh CDS, growth started after a period of time due to lag phase. The lag phase is a result of the physiological adaptation of the mixed inocula to CDS. Table 2 shows the cumulative biogas production in 41 d that is 208, 178, 193 and 169 L in reactors 1, 2, 3 and 4 (control), respectively. Thus it indicated that cumulative production of biogas obtained in reactor 1 was higher as compared to reactors 2, 3 and 4 (control).

Methane and carbon dioxide production in four different reactors

Figure 4 and Table 2, respectively indicate the daily and cumulative production of methane and carbon dioxide. The cumulative production of methane (123, 101 and 110 L) and carbon dioxide production (80, 73 and 78 L), respectively in reactors 1, 2 and 3 were higher than that of methane (91 L) and carbon dioxide (72 L) in reactor 4 (control). The highest methane and carbon dioxide production in reactor 1 could be a result of activation of acidogenesis and methanogenesis by mixed microbial cultures. Methane is produced from acetate via fermentation in which the acetate molecule is cleaved and the methyl group is reduced to methane with electrons derived from oxidation of the carbonyl group to

Fig. 3 Daily biogas production in different reactors.

Fig. 4 Methane and carbon dioxide production in different reactors using different inocula i.e. Reactor 1, IMW1; Reactor 2, IDW1; Reactor 3, ITW1; Reactor 4, IBS.

carbon dioxide [8]. The highest methane production in reactor 1 indicated the high methanogenic activity (conversion of acetate to methane) possibly due to the presence of various types of methanogenic population.

Total volatile solid reduction per day in different reactors

Table 2 and Figure 5 show the higher total volatile solid (TVS) reduction i.e. 380 g (50%) in reactor 1 than in reactor 4 (control) i.e. 370 g (49%) where as lower TVS reduction was obtained in case of reactor 2 i.e. 352 g (46%) and reactor 3 i.e. 360 (47%) in 41 d. The higher degradation efficiency of TVS in reactor 1 may be due to the presence of a consortium of highly active methanogenic organisms.

Average production of biogas and methane per day in four different reactors

Table 2 indicates the average methane and biogas production per day as a function of digestion time in different reactors (inoculated by different inocula). The average biogas and methane production is defined as the cumulative biogas and methane production divided by time (41 d). The average biogas and methane production in reactors 1, 2 and 3 (5.07, 4.34, 4.7 and 3, 2.47, 2.68 Ld⁻¹, respectively), were higher than reactor 4 (control) i.e. 4.12, 2.2 Ld⁻¹.

Average yield of biogas and methane in four different reactors on the basis of volatile solid consumed

Table 2 depicts that the average yield of biogas and methane (cumulative amount of biogas and methane divided by

Fig. 5 Production of methane and reduction of volatile solid in different reactors using four different inocula i.e. Reactor 1; IMW1, Reactor 2; IDW1, Reactor 3; ITW1, Reactor 4; IBS (control).

Table 2 Production of various gases by using mixed inocula, obtained from four different batch reactors, each containing 10 L cow dung slurry

Reactor	Average daily production (Ld ⁻¹)		Total VS (g) in 10 L		Reduction (%)	Cumulative production (L) in 41 d				Average yield (Lg ⁻¹ VS)		Maximum methane content (%) in biogas (during)	
	Biogas	Methane	Initial	Final		Biogas	Methane	Carbon dioxide	Other gases H ₂ S & NH ₃	Biogas	Methane		
Reactor 1	5.07	3.00	760	380	50	208	123	80	5	0.547	0.323	59.17	68% (27–30 d)
Reactor 2	4.34	2.47	760	408	46	178	101	73	4	0.505	0.288	56.9	62% (29–33 d)
Reactor 3	4.70	2.68	760	400	47	193	110	78	5	0.536	0.305	57.02	64% (29–32 d)
Reactor 4	4.12	2.21	760	390	49	169	91	72	6	0.456	0.245	53.6	62% (27–29 d)

amount of total volatile solid consumed) is a function of digestion time in different reactors (containing different inocula). It is interesting to note that a higher average yield of biogas in the range of 0.456–0.547 Lg⁻¹ VS was obtained as compared to earlier workers. Rajasekaran et al. [26] achieved 252.5 L kg⁻¹ VS yield of biogas with cow dung and digested slurry. Marique et al. [21] reported a yield of 417 Lkg⁻¹ VS biogas under thermophilic conditions, Webb and Hawkes [35] reported 0.372 m³ biogas kg⁻¹ of VS using poultry litter with cow dung. In these experiments, the higher average yield of methane was obtained in the range of 0.245–0.323 L g⁻¹ VS in 41 d of digestion in different reactors, where as Moller et al. [24] achieved 148 L kg⁻¹ VS yield of methane using dairy cattle manure. A higher yield of biogas and methane was obtained from reactors 1, 2 and 3 i.e. 0.547, 0.505, 0.536 and 0.323, 0.288, 0.305 L g⁻¹ VS, than that of reactor 4 (control) i.e. 0.456 and 0.245 Lg⁻¹ VS, respectively. The IMW₁ reactor produced maximum methane yield (0.323 L g⁻¹VS) and maximum methane content (68%) in biogas during 27–30 d. Marique et al. [21] reported 60 % methane content in biogas, whereas Karim et al. [18] reported 67 % methane content in biogas using animal waste as substrate. The maximum yield of biogas and methane obtained using inoculum IMW₁ in reactor 1 was due to presence of *Methanosarcina* sp. and *Methanosaeta* sp. along with usual varieties of methanogenic bacteria. These two archaea are capable of metabolizing acetate, where as *Methanosarcina* sp. is also able to grow on other substrates such as hydrogen, carbon dioxide, formate, methanol and methylamines [3, 15]. Threshold concentration, the maximum concentration below which a species is no longer able to degrade a particular substrate (Großkof et al. [11]), can be used as an approximated indicator of the predominance of species in a system. According to Jetten et al. [16], *Methanosaeta* sp. shows a much lower minimum threshold for acetate utilization.

Conclusion

The experimental results indicated that the mixed inoculum IMW₁ (IMW + IBS) used in reactor 1, produced maximum methane content in biogas. It appears that the mixed microbial population (inocula obtained from municipal waste and biogas plant adapted to cow dung slurry) increased the rate of reaction of hydrolysis, acidogenesis and methanogenesis, as a result of efficient degradation of organic matter into methane due to the presence of diversified methanogenic organisms, especially *Methanosarcina* sp. and *Methanosaeta* sp. Further, with this mixed inoculum it may be possible to further improve the biogas yield with enriched methane

content, through optimization of process parameters and by changing biodegradable feed substrates.

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