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Research Paper

STUDY OF DIVERSE METHANOGENIC AND NON-METHANOGENIC BACTERIA USED FOR THE ENHANCEMENT OF BIOGAS PRODUCTION

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To achieve a functioning and stable process with high methane production, it is important to create and maintain a beneficial environment for the activity of bacterial consortia of suitable species. Therefore, an experiment has been carried out to isolate methanogenic and nonmethanogenic bacteria from anaerobic biogas system. With this view, a microbial consortium and their relative performance with respect to individual species in consortia were studies with respect to methane generation. Eight bacterial species have been isolated from the biogas slurry prepared from cow dung. The morphological and microscopic studies have been carried out to study its gram staining properties. The biochemical tests of sugar fermentation, glucose, sucrose, lactose and dextrose shows that the isolate No. 1, 2 and 6 does not respond for this test but the remaining isolates showed positive response. Based on the fluorescence test, isolate 2, 6, 7, 8 are methanogenic and 1, 3, 4, 5 are nonmethanogenic in nature. The methane production of methanogenic and nonmethanogenic bacteria in consortia shows that methanogenic bacteria contribute more rather than nonmethanogenic bacteria. But when the studies were carried out by taking individual species of bacteria, it has been observed that the isolate No. 2 and 4 are most efficient to produce more methane, where isolate 2 is methanogenic and isolate 4 is nonmethanogenic. It shows that the individual species works in better way rather than in consortium in the biogas system. From the above study it was observed that the consortia "C" containing four different methanogenic bacteria are involved in the transformation of complex high molecular weight organic compounds to methane generation (76 %) in comparison to the Consortia "A" (23 %) and Consortia "B" (1%).

Keywords: Anaerobic digestion, Biogas plant, Methane, Methanogenic bacteria

INTRODUCTION

Biogas is a methane rich fuel gas produced by anaerobic breakdown or digestion with the help of methanogenic bacteria. Biogas produced by anaerobic digestion or fermentation of biodegradable materials such as biomass, manure or sewage municipal waste and energy crop. As per the Polprasert (1989), the overall

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reactions of organic matter conversion given below:

Organic matter $\rightarrow CH_4 + CO_2 + H_2 + NH_3 + H_2S$

Biogas technology may have the potential to short-circuit the 'energy transition' Leach (1987) describes from biomass to 'modern' fuels. Ranade *et al*., (1987) have successfully maintained a biogas plant of 25 litres. capacity, fed with market waste, in Pune, western India, suggested such a system to be a viable option for solid waste disposal in areas of rapid urbanization. In some areas, the plant may not be technically feasible all year round due to low winter temperatures that inhibit methanogenesis (Singh, 1985; Sudhakar and Gusain, 1991).

India has a long way to go to realize the benefits of biogas technology. China, through the creation of effective institutions and by placing an emphasis on training and education, has achieved widespread dissemination of biogas technology (Ruchen, 1981; Daxiong et al., 1990), though the social organization may particularly facilitate the spread of new, community-focused technologies. Chand and Murthy (1988) identified 50% of 1670 plants in the study as incapable of ever being made functional. Therefore, the detailed studies have been carried out by isolating the methanogenic/nonmethanogenic bacteria responsible for methane production.

MATERIALS AND METHODS

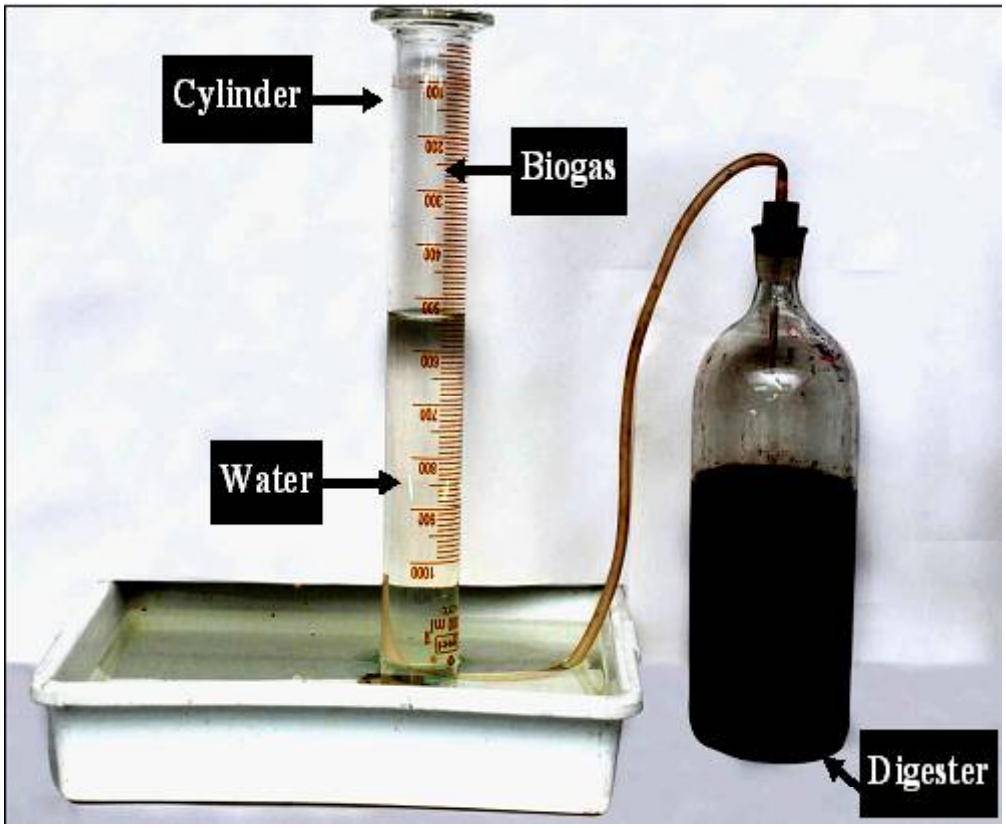
A bench scale study was conducted using cattle dung slurry to determine the volume of biogas generation based on the characteristics of organic material (cattle dung) an anaerobic conventional digester has been considered for generation of biogas. The test was carried out by digesting 500 ml of cattle dung and 1000 ml of water (ratio 1:2) mixed with 200 ml of well-digested sludge. Biogas generated from this mixture consists of methane due to digestion of sludge and cattle dung.

The cattle dung was placed in a 2.5 litre digester, together with a well-stabilized anaerobic sludge. Two glass tubes, one reaching into the cattle dung slurry, the other tube into the headspace, perforated the cork of the digester. The headspace of the digester is then connected with the measuring cylinder, with upside down position containing basic or acidic solution of 20% sodium sulphate. The biogas formed in the sample collected in the measuring cylinder, when an alkaline solution is used as the displacement liquid, CO_2 will be scrubbed from the biogas and only methane will be collected. Therefore, the displaced volume of the solution is then equal to the volume of methane generated. When using an acidic solution, CO_2 will not be absorbed and the displaced gas volume will be indicative of the total biogas generated. In parallel with the assay bottle a blank test was also conducted containing using the same amount of anaerobic sludge and tap water instead of cattle dung slurry. The difference in gas production between the two digesters gives total biogas produced due to cattle dung alone. The experimental set up of an anaerobic biodegradability test in the laboratory is shown in the Figure 1.

BIOGAS GENERATION USING BACTERIAL ISOLATES

The experiment was carried out in two different experimental sets: Set-I and Set-II. In Set-I, the consortia of 16 ml of eight isolates (2 ml of each), while in the Set-II contains four isolates methanogenic and nonmethanogenic separately in two digesters. The differentiation was on the basis of fluorescence test. In each digester 8 ml

Figure 1: Experimental Set up of Anaerobic Digester for Biogas Generation



culture (2 ml each) added in 1500 ml volume of cattle dung slurry and mixed without the addition of well-digested anaerobic sludge to check the potential activity of our isolates on biogas generation. Cattle dung slurry was used as control. This experimental set up uses three digesters which were given below.

Consortia "A" - Cattle dung slurry and 16 ml (2 ml of each) of eight isolates

Consortia "B"- Cattle dung slurry and 8 ml (2 ml of each) of four isolates

Consortia "C" - Cattle dung slurry and 8 ml (2 ml of each) of four isolates

PHYSICOCHEMICAL ANALYSIS

The influent and effluent samples from biogas plant were collected and analyzed for physicochemical parameters such as temperature, pH, alkalinity, suspended solids, volatile suspended solids, sodium, potassium, sulphate, Biochemical Oxygen Demand (BOD) and chemical oxygen demand (COD) were analyzed. The physicochemical analysis procedures were followed using Standard Methods (APHA, 1998, 19th Ed.).

BACTERIOLOGICAL ANALYSIS

Eight bacterial species were isolated from the biogas slurry and identified by standard bacteriological identification procedure. Microscopic examinations were carried out by gram staining and motility test. Biochemical tests were performed by inoculating broth culture of the isolates into the series of media. This includes following tests:

a) Sugar Fermentation Test

b) IMViC Test

- Indole Test
- Methyl Red Test
- Voges Proskauer Test
- Citrate Utilization Test

c) Enzyme Test

- Catalase Test
- Oxidase Test

d) Fluorescence Test

The colonies smaller than 0.5 mm was detected by long-wave ultraviolet light, Fluorescence is presumptive evidence for methanogenic bacteria, but definitive proof requires further characterization. To observe the presence of fluorescence in the isolated plates which were directly placed in Trans-illuminator and observations were noted.

Positive - Development of blue-green fluorescence

Negative - No fluorescence

SEM and Dot Blot

The scanning electron microscopy (SEM) provides with a magnified image of the specimen showing details not visible with a light microscope.

Total DNA of methanogens was isolated. It was then used as a template DNA for Polymerase Chain reaction (PCR) using archeal primer and furthers these PCR products were used as positive controls for Dot- blot technique.

RESULTS AND DISCUSSION

Characteristics of Wastewater

The samples of cattle dung slurry were collected from conventional anaerobic digester of bench scale system and analysis was carried out for physico-chemical characteristics as per Standard Methods (APHA, 1998). The Influent COD concentrations ranged between 27200 mg/l to 29400 mg/l with a mean value of 28300 mg/l while BOD values varied from 7616 mg/l to 9408 mg/l with mean values of 8512 mg/l. The concentrations of Suspended Solids (SS) ranged from 1248 mg/l to 1560 mg/l with an average of 1404 mg/l while Volatile Suspended Solids (VSS) ranged from 1200 mg/l to 1364 mg/l with an average of 1282 mg/l. The Effluent COD concentrations were ranged between 9450 mg/l to 10450 mg/l with a mean value of 9950 mg/l while BOD values varied from 2552 mg/l to 3344 mg/l with mean values of 2948 mg/l. The concentrations of Suspended Solids (SS) ranged from 489 mg/l to 582 mg/l with an average of 536 mg/l while Volatile Suspended Solids (VSS) ranged from 290 mg/l to 340 mg/l with an average of 315 mg/l.

It is computed that the average reduction of COD and BOD worked out as 64.86% and 65.47%, SS and VSS reduction 61.75% and 72.40% respectively. Anaerobic degradation is said to have occurred successfully when the COD reduction in proportion to the value of methane generated. It is an indication that the biogas generations are about 64.45% to 65.26%. The performance characteristics of some of the important physico-chemical parameters with respect to COD reduction and biogas generation are given in Table 1.

Bench scale study on anaerobic digestion was carried out in the laboratory using cattle dung to obtain appropriate concentration. The study was conducted with 1:3, 1:2 and 3:1 dilutions at various temperature ranges. The optimal functioning of

Table 1: Performance Characteristics of Influent and Effluent

Parameters	Influent		Effluent		% Reduction	
	Range	Mean	Range	Mean	Range	Mean
Temp. ^{0}C.	23-31	-	30-38	-	-	-
pH	7.2-7.4	-	7.3-7.6	-	-	-
Alkalinity	1983-2160	2072	3852-4083	3968	-	-
Sodium	197-225	211	187-162	180	-	-
COD	27200-29400	28300	9450-10450	9950	64.45-65.26	64.86
BOD	7616-9408	8512	3344-2552	2948	64.45-66.49	65.34
Suspended Solids (SS)	1248-1560	1404	489-582	535.50	60.81-62.69	61.75
VSS	1200-1364	1282	344-362	353	71.34-73.46	74.44
Phosphate	5.6-5.8	5.7	3.5-4.7	4.1	18.96-37.5	28.23
Potassium	554-579	566.5	480-486	483	13.35-16.06	14.7
Biogas generation	Range	Mean				
Gas yield m^3/kg COD removal	0.5-0.56	0.53				
CH_4 (%)	64-65	64.86				
CO_2 (%)	32-34	32.5				
Other gases (H_2, N_2, CO, O_2, H_2S)	1-3	2				

Note: All values are expressed in mg/l except Temperature and pH

biogas generation is obtained at 1:2 dilution rather than 1:3 and 3:1 dilutions. It shows that dilution ratio 1:2 is appropriate. The study was carried out in the winter season (February - March, 2011) and the total biogas generation was computed to be 8.140 L with a temperature ranged between 23- 31 °C (Figure 2). Similarly this study was extended up to summer season (April - May, 2011) with a temperature ranged between 30-38°C and the biogas generation worked out as 12.260 L presented in Table 2 and shown in Figure 3. It indicates that if temperature is increases or decreases then biogas generation follows its reactions. Methanogenic bacteria are very sensitive to small changes in temperature. As to utilization of volatile acids by methanogenic bacteria, a decrease in temperature leads to decrease of maximum specific growth of bacteria and ultimately reduction of biogas generation, while increase in temperature the biogas generation is observed more. Thus, mesophillic digester must be designed to operate at temperatures between 30°C and 35°C for their optimal functioning.

Morphological Characteristics of Bacterial Isolates

Total twelve isolates were obtained from the anaerobic digester in the present study using cattle dung. The isolates obtained during experimentation with their morphological characteristics are given in Table 3. Results obtained on the basis of morphological characteristics of twelve isolates, out of which only eight isolates (No. 1-8) were selected while the remaining four isolates (No. 9-12) were rejected due to the same morphological characteristics.

Figure 2: Biogas Generation During Winter Season (Feb.-March, 2011)

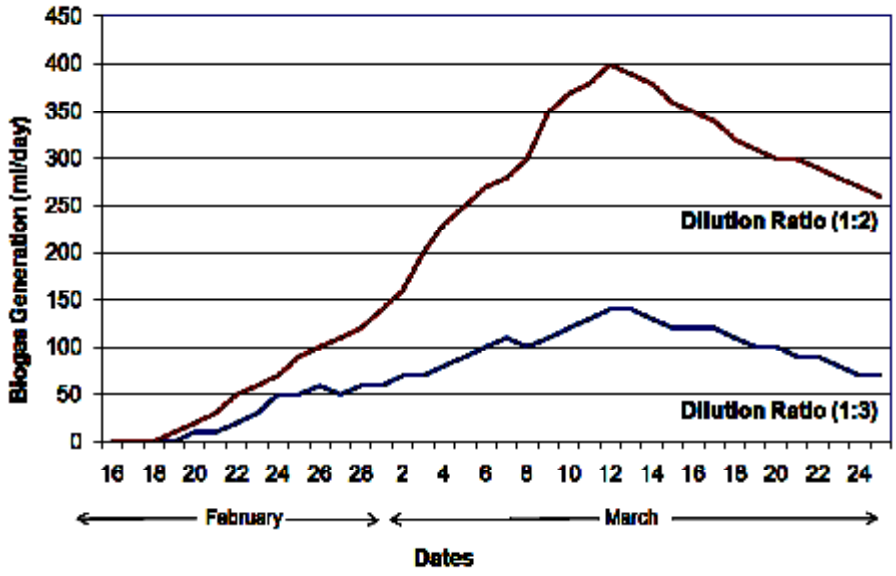


Table 2: Comparison of Biogas Generation During Winter and Summer Season

Sample Dilution Ratio	Winter Season (Temp. 23-31°C)		Summer Season (Temp. 32-38°C)	
	Total Vol. of Biogas generated in 38 days (L)	Vol. of Biogas (Avg.) generated (L/day)	Total Vol. of Biogas generated in 38 days (L)	Vol. of Biogas (Avg.) generated (L/day)
1:3	2.860	0.75	4.400	1.157
1:2	8.140	2.142	12.260	3.226

Figure 3: Biogas Generation during Summer Season (April- May, 2011)

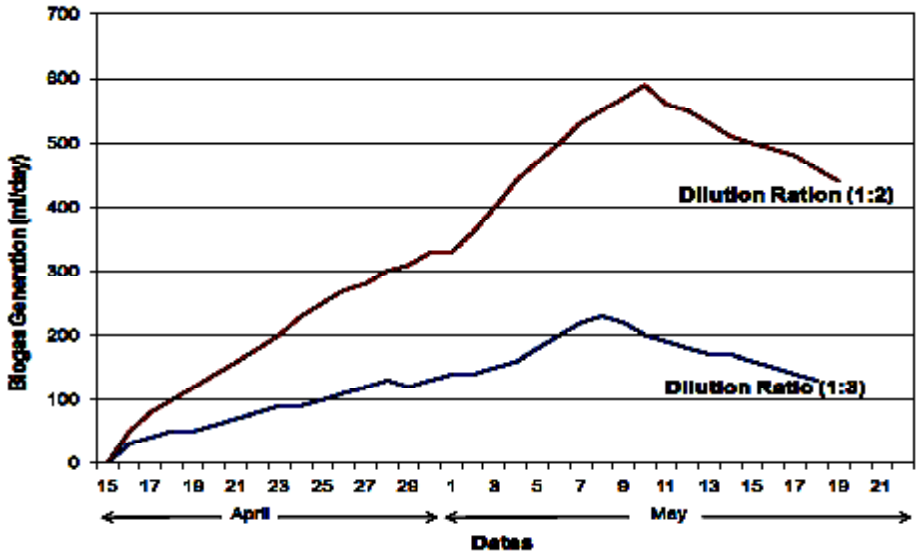


Table 3: Morphological Characteristics of the Bacterial Isolates

S. No.	Isolates	Morphological Characteristics	Gram Staining	Shape	Motility
1.	Isolate-1	Colourless, circular, granular, smooth, slow growing	+ve	Bacillus	Non-motile
2.	Isolate-2	Large, irregular, greenish fluorescent colonies	+ve	Coccobacillus	Non-motile
3.	Isolate-3	Yellowish, Moist, granular, smooth	-ve	Rods	Non-motile
4.	Isolate-4	Yellowish white, raised colonies, convex, translucent, Colourless	+ve	Cocci	Non-motile
5.	Isolate-5	Filamentous growth, yellowish white,	+ve	Bacillus	Motile
6.	Isolate-6	Slow growing, filamentous, blue-green fluorescent colonies	+ve	Rods	Non-motile
7.	Isolate-7	Greenish colonies, blue-green fluorescence, raised colonies	-ve	Coccoid	Motile
8.	Isolate-8	Yellowish white, raised colonies, filamentous, blue-green fluorescence	-ve	Rods	Non-motile

Identification of the Selected Isolates by Various Biochemical Tests

Biochemical tests (Sugar Fermentation, IMViC, Enzymes) were carried out and the result of the tests are shown in Table 4 and Table 5 respectively.

Fluorescence Test

Fluorescence test was carried out for the identification of methanogenic bacteria containing the F_{420} coenzyme which shows blue-green fluorescence by methanogenic bacteria and is readily distinguishable from the white-yellow fluorescence occasionally observed in non-methanogenic colonies. The isolates Nos. 2, 6, 7, 8 shows the blue-green florescence in ultraviolet light indicating the presence of methanogenic bacteria.

Scanning Electron Microscopy

The isolates showed methanogenic and non-methanogenic bacteria as shown in Table 6. On the basis of the results obtained from microscopic examination (Gram staining, Motility), optimum growth conditions, fluorescence test and dimension from scanning electron microscopy, it is concluded that the isolates obtained may be belonging to the following methanogenic bacteria as *Methanobrevibacter ruminantium* (Figure 4), *Methanobacterium formicicum* (Figure 5), *Methanosarcina frisia* (Figure 6); dimension 1.03-1.40 µm, *Methanothrix soehngenii* dimension 1.59-1.76 µm (Figure 7); and non-methanogenic bacteria as *Propionibacterium* dimension 1.02-1.56 µm (Figure 8), *Bacteroides*; 1.06-1.52 µm (Figure 9), *Peptostreptococcus* dimention *1.0 -1.02* µm (Figure 10), *Clostridium*; 6.13 µm (Figure 11).

The methanogenic and non-methanogenic bacteria are compared on the basis of shape, dimension, gram reaction, motility, optimal growth conditions and catalase test with the study carried out by Garcia, 1990; Holt, 1994; as shown in Table 7 and Table 8.

Various bacterial isolates were used in the present study to find out which bacterial isolate is useful for potential generation of biogas at 1:2 dilutions. The following 3 experimental set up with consortia A, consortia B and consortia C were used as given below:

Consortia "A"- Cattle dung slurry and 16 ml (2 ml of each) of 8 isolates (1 to 8)

Consortia "B"- Cattle dung slurry and 8 ml (2 ml of each) of 4 isolates (1, 3, 4, 5)

Table 4: Sugar Fermentation Test

S. No.	Isolates	Sugar Fermentation	Glucose	Sucrose	Lactose	Dextrose
1.	Isolate-1	Acid	-ve	-ve	-ve	-ve
		Gas	-ve	-ve	-ve	-ve
2.	Isolate-2	Acid	-ve	-ve	-ve	-ve
		Gas	-ve	-ve	-ve	-ve
3.	Isolate-3	Acid	+ve	+ve	+ve	+ve
		Gas	–ve	–ve	-ve	-ve
4.	Isolate-4	Acid	+ve	+ve	+ve	+ve
		Gas	+ve	+ve	-ve	+ve
5.	Isolate-5	Acid	+ve	+ve	-ve	-ve
		Gas	+ve	-ve	-ve	-ve
6.	Isolate-6	Acid	-ve	-ve	-ve	-ve
		Gas	-ve	-ve	-ve	-ve
7.	Isolate-7	Acid	+ve	+ve	+ve	+ve
		Gas	+ve	+ve	+ve	+ve
8.	Isolate-8	Acid	+ve	+ve	+ve	+ve
		Gas	+ve	-ve	+ve	+ve

Table 5: IMViC, Enzymes and Fluorescence Test

S. No.	Isolates	IMViC Test				Enzymes Test		Fluorescence Test
		Indole Test	MR Test	VPTest	CitrateTest	Catalase Test	Oxidase Test	
1.	Isolate-1	-ve	+ve	-ve	-ve	+ve	+ve	-ve
2.	Isolate-2	-ve	+ve	-ve	+ve	-ve	+ve	+ve
3.	Isolate-3	-ve	+ve	+ve	+ve	-ve	+ve	-ve
4.	Isolate-4	-ve	-ve	+ve	-ve	-ve	+ve	-ve
5.	Isolate-5	-ve	+ve	-ve	+ve	-ve	+ve	-ve
6.	Isolate-6	-ve	+ve	-ve	-ve	+ve	-ve	+ve
7.	Isolate-7	-ve	-ve	+ve	+ve	+ve	+ve	+ve
8.	Isolate-8	-ve	+ve	-ve	+ve	-ve	-ve	+ve

Table 6: Isolates Representing Genus of Bacteria

Isolates	Identified Bacteria in Biogas Digester		
	Methanogenic*	Isolates	Non-methanogenic**
Isolate-2	*Methanobrevibacterruminantium*	Isolate-1	*Propionibacterium*
Isolate-6	*Methanobacteriumformicicum*	Isolate-3	*Bacteroides*
Isolate-7	*Methanosarcinafrisia*	Isolate-4	*Peptostreptococcus*
Isolate-8	*Methanothrixsoehngenii*	Isolate-5	*Clostridium*

Note: *(Garcia, 1990); ** (Holt, 1994 and Breed, 1957)

Figure 4: Isolate No. 2- *Methanobrevibacter ruminantium*

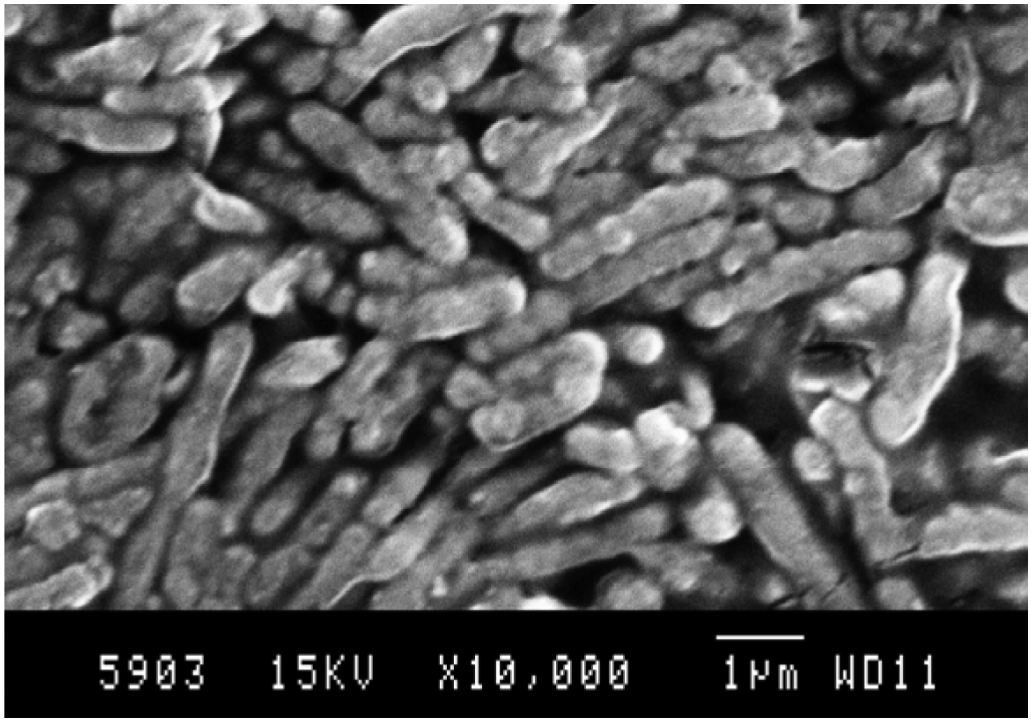


Figure 5: Isolate No. 6- *Methanobacterium formicium*

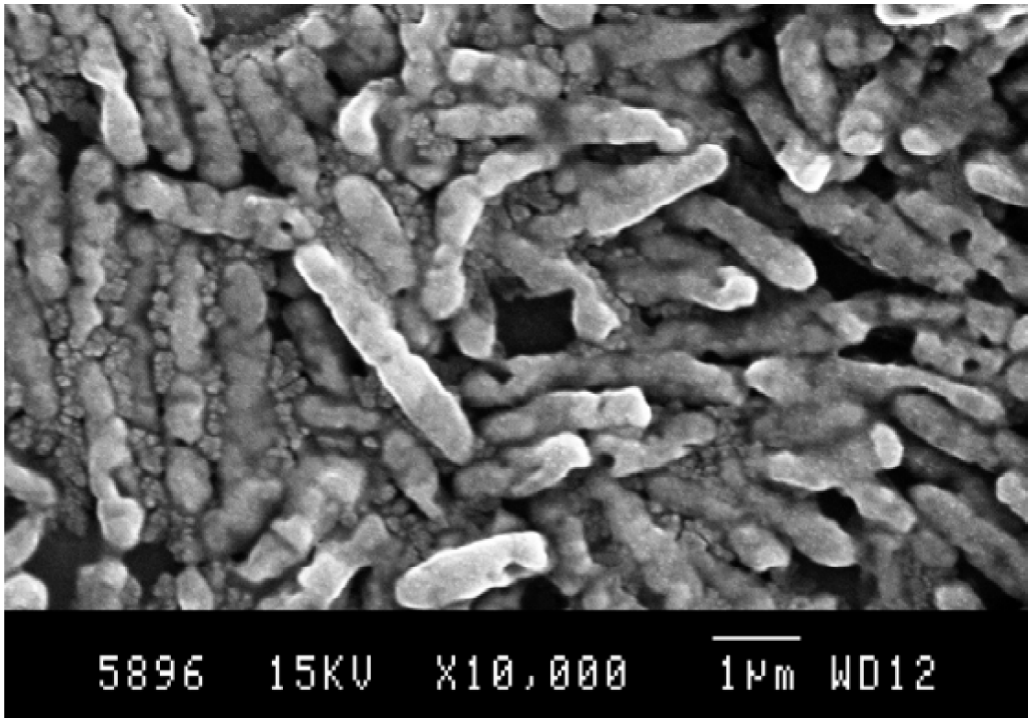


Figure 6: Isolate No. 7- *Methanosarcina frisia*

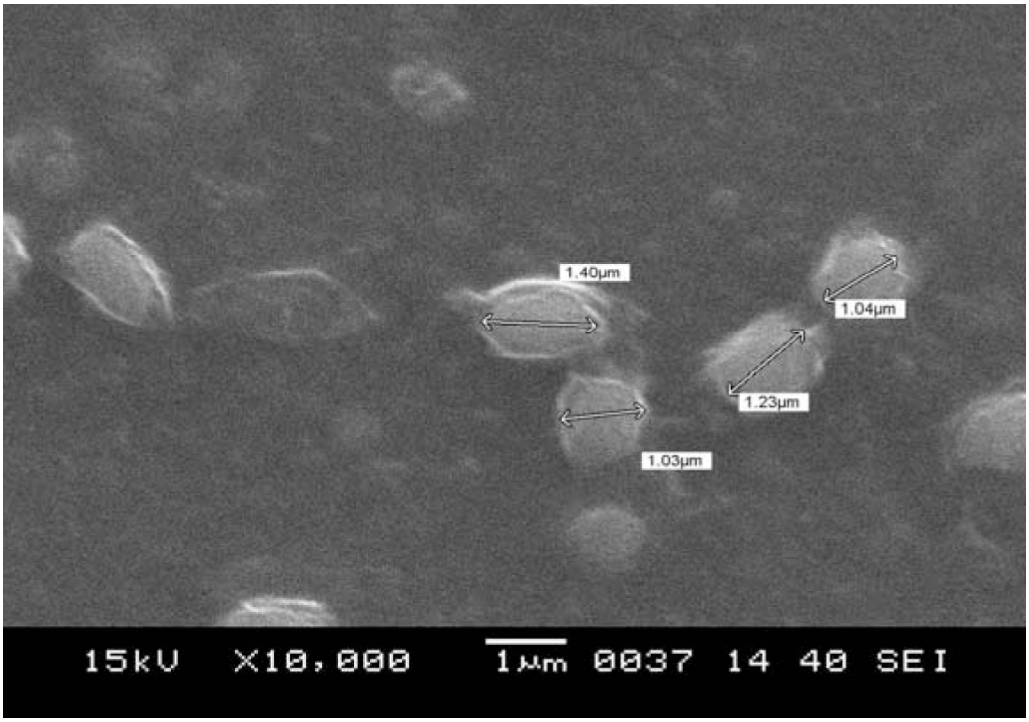


Figure 7: Isolate No. 8- *Methanothrix soehngenii*

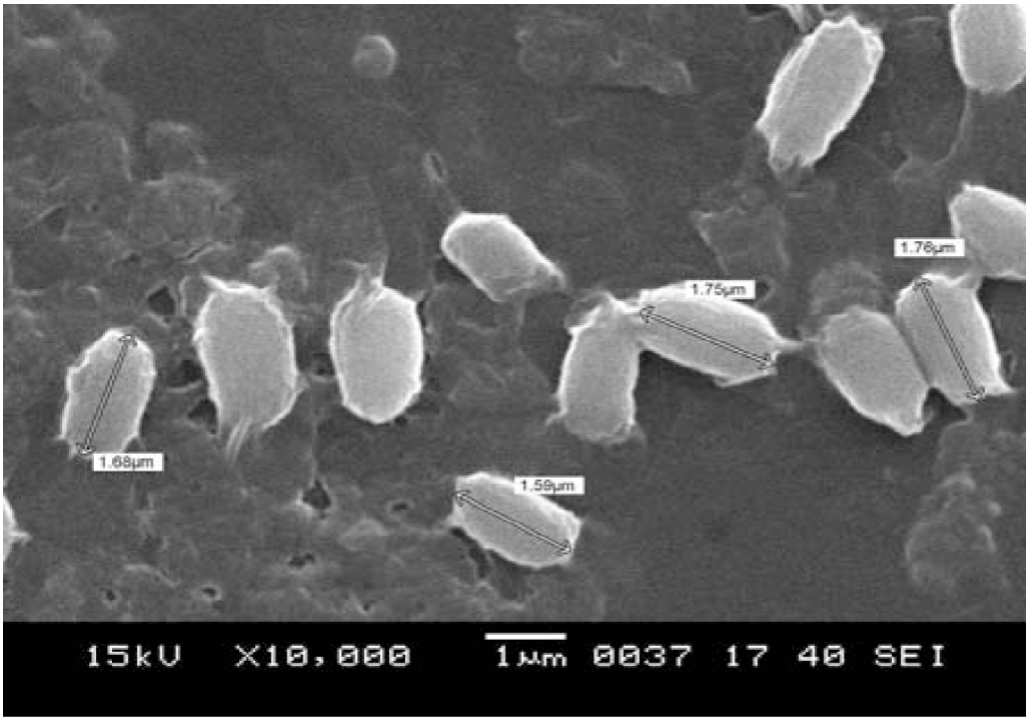


Figure 8: Isolate No. 1- *Propionibacterium* sp

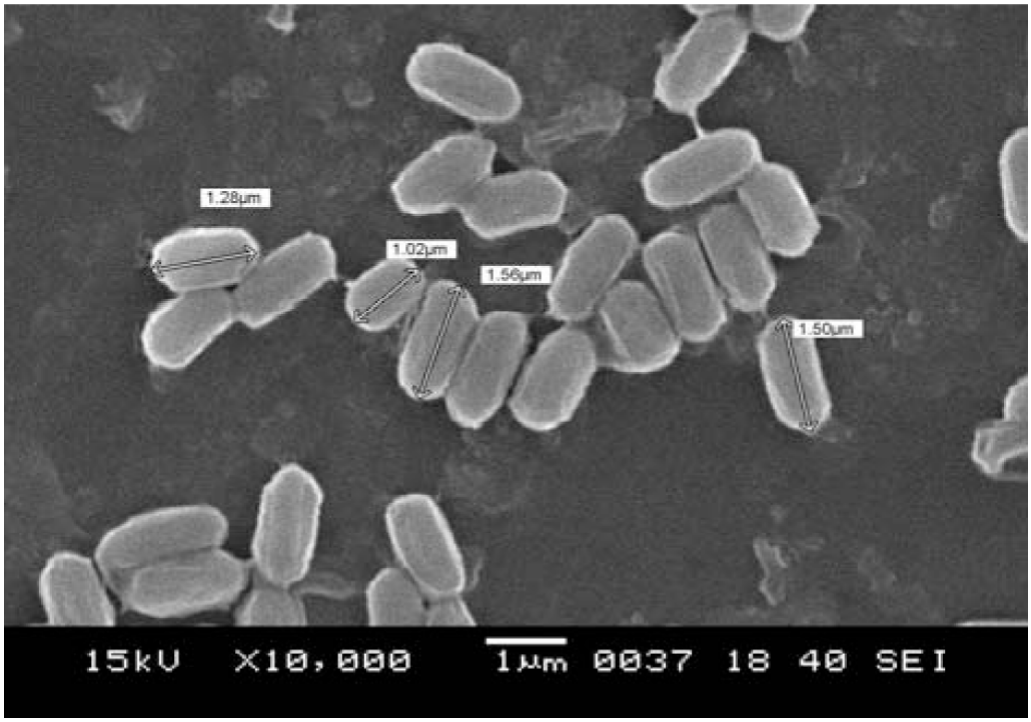


Figure 9: Isolate No. 3-*Bacteroides* sp.

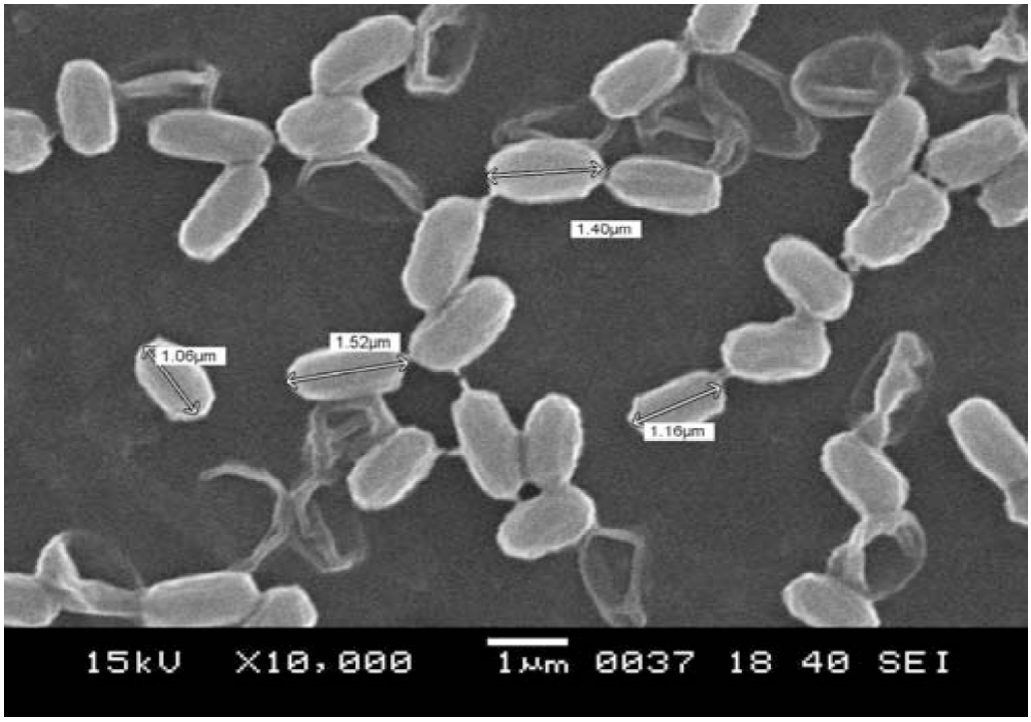


Figure 10: Isolate No. 4 - *Peptostreptococcus* sp.

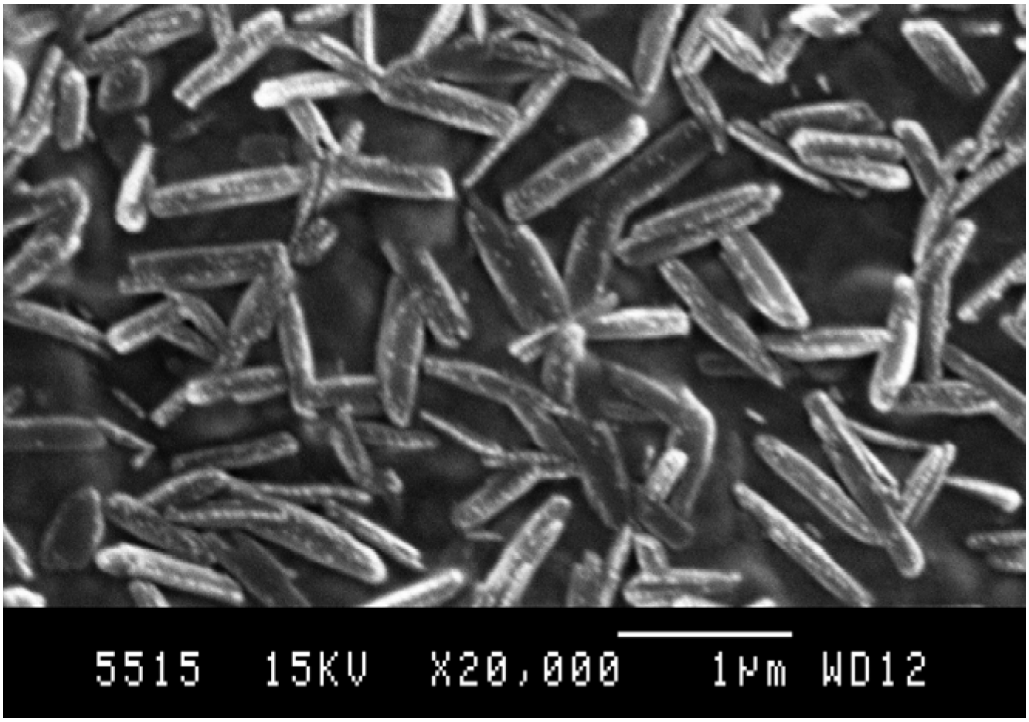


Figure 11: Isolate No. 5 - *Clostridium* sp.

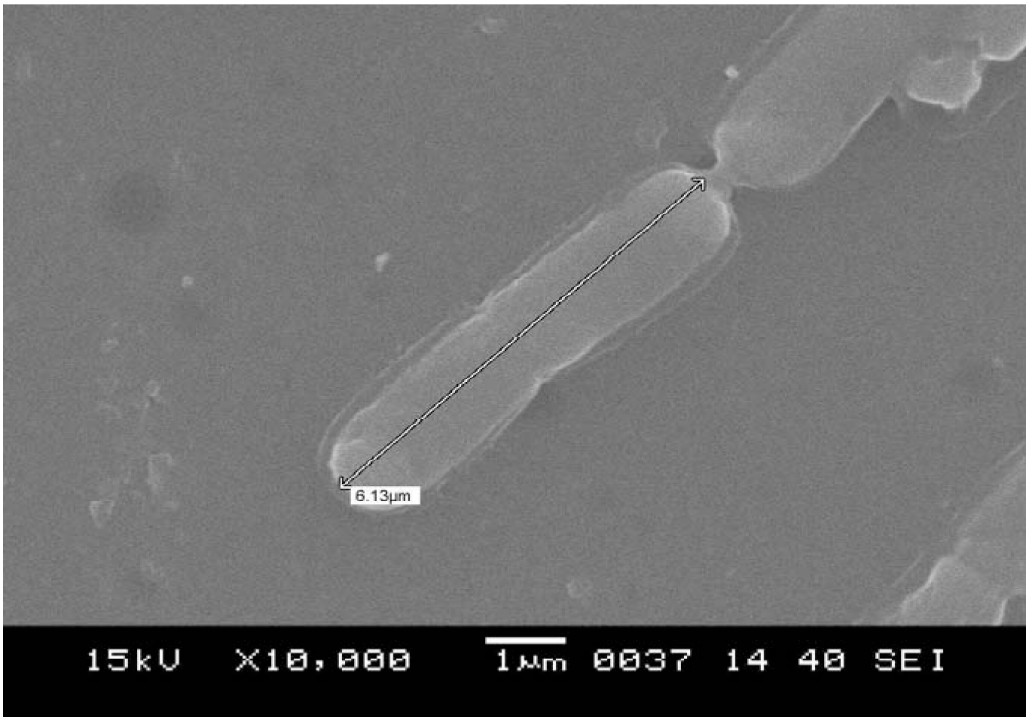


Table 7: Characteristics of Methanogenic Bacteria

Comparison with	Organism	Shape	Dimension (μm)	Gram reaction	Motility	pH	Temp. (°C)
Garcia, (1990)	*Methanobrevibacter ruminantium*	Coccobacillus	0.7×0.8-1.7	+ve	Non-motile	7.0	37-39
	Methanobacterium formicicum	Rods	0.4-0.8×2-15	+ve	Non-motile	6.6-7.8	37-45
	*Methanosarcina frisia*Pseudosarcina,	Coccoid	0.5-2.0	-ve	Motile	6.5-7.2	36
	Methanothrix soehngenii	Sheathed rods	0.8-1.2×2-3	-ve	Non-motile	7.4-7.8	35-40
Present Study (2011)	*Methanobrevibacter ruminantium*	Coccobacillus	-	+ve	Non-motile	7.2-7.4	37-38
	Methanobacterium formicicum	Long rods	-	+ve	Non-motile	7.0-7.5	37-38
	*Methanosarcina frisia*Coccoid	1.03-1.40	-ve	Motile	7.2-7.4	37-38	
	Methanothrix soehngenii	Rods	1.59-1.76	-ve	Non-motile	7.2-7.4	37-38

Table 8: Characteristics of Nonmethanogenic Bacteria

Comparison with	Organism	Shape	Dimension (μm)	Gram Reaction	Motility	Catalase Test
Holt (1994)	Propionibacterium	Pleomorphic rods, club shaped	0.5-0.8-1×5	+ve	Non-motile	+ve
	Bacteroides	Rod- shaped	–	-ve	Non-motile	–
	Peptostreptococcus	Spherical	0.5-1.2	+ve	Non-motile	-ve
	Clostridium	Rod- shaped	0.3-2.0×1.5-20	+ve	Motile	-ve
Present Study (2011)	Propionibacterium	Bacillus	1.02-1.56	+ve	Non-motile	+ve
	Bacteroides	Rod- shaped	1.06-1.52	-ve	Non-motile	-ve
	Peptostreptococcus	Cocci	–	+ve	Non-motile	-ve
	Clostridium	Bacillus	6.13	+ve	Motile	-ve

Consortia "C"- Cattle dung slurry and 8 ml (2 ml of each) of 4 isolates (2, 6, 7, 8)

In the experimental set up-I contain consortia "A", it indicates that the biogas generation (23.04%) may be because of the presence of some antibacterial substances produced by bacteria or group of bacteria, that inhibit the growth of biogas producers. Similarly in the experimental set up-II, consortia "B" also gives a very less biogas generation (1%) because of it was containing non-methanogenic bacteria while Consortia "C" gives maximum biogas generation (76 %) as it was containing methanogenic bacteria. The four isolates (2, 6, 7 and 8) of consortia "C" were giving comparatively very good results depicted in Figure 12.

Figure 12: Biogas Generation using Bacterial Isolates in Consortia

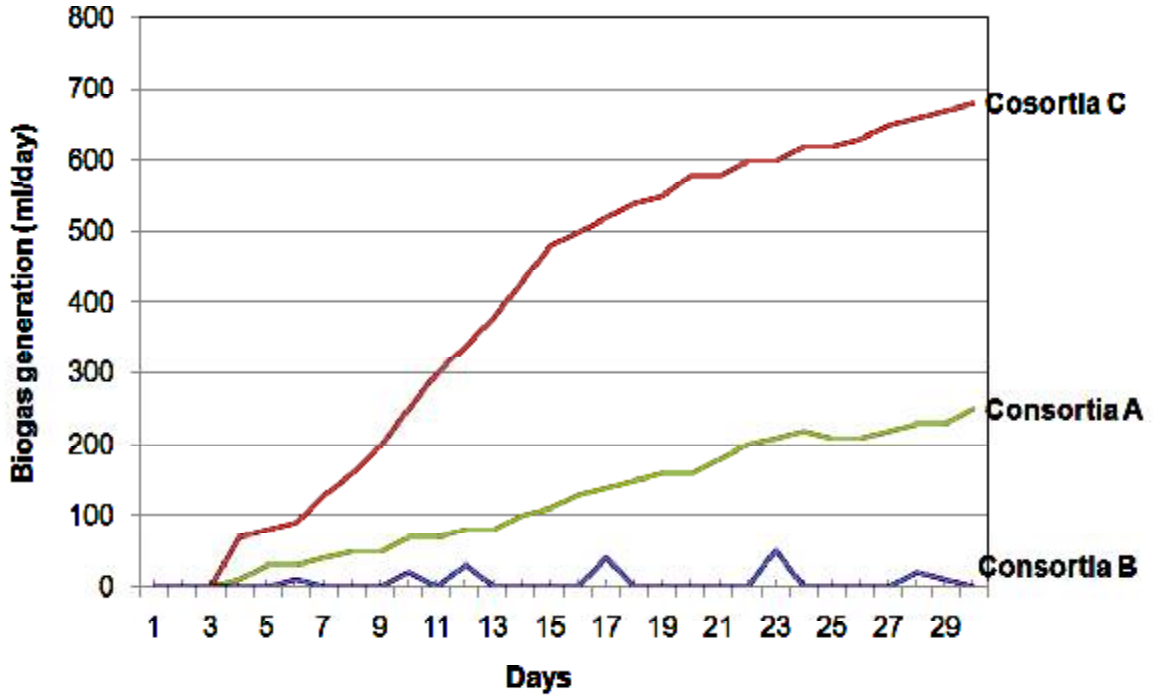
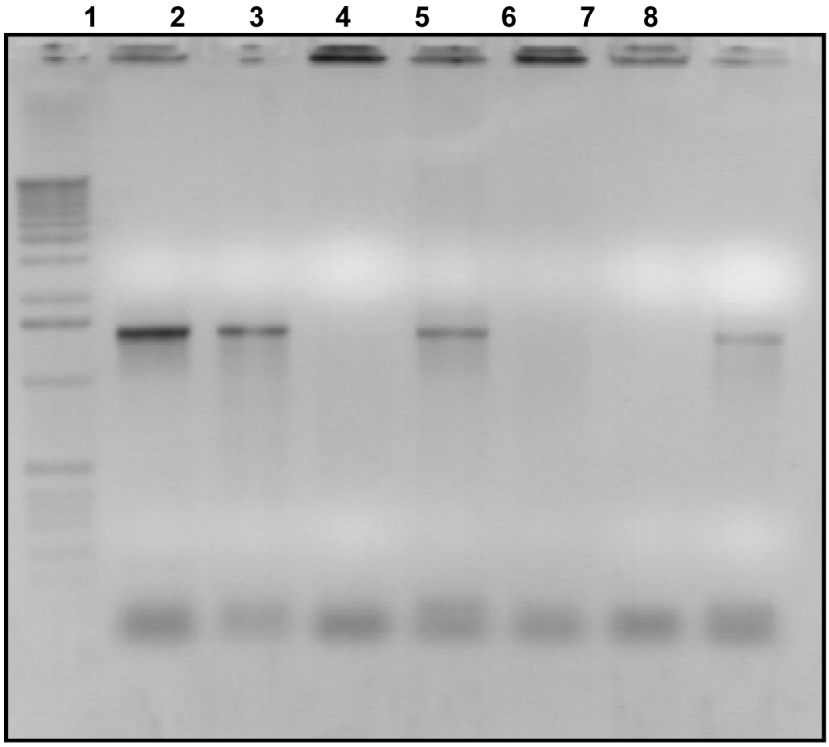


Figure 13. Gel Documentation Image of Isolated DNA after Electrophoresis



LANE 1- 1kb ladder
LANE 2- Isolate No. 2
LANE 3- Isolate No. 6
LANE 5- Isolate No. 7
LANE 8- Isolate No. 8

DNA Isolation

The laboratory scale studies were conducted for assessment of biogas generation potential using cattle dung it is concluded that cattle dung slurry of 1:2 dilution gives maximum biogas generation than 1:3 and 3:1 dilutions.

The DNA samples were visualized on a Vilber Lourmat UV trans-illuminator Kaiser RA-1, and photographed instantly by a solid-state camera attached to it. Figure 13 shows gel documentation image of isolated DNA after electrophoresis.

Gel Electrophoresis

Electrophoresis is a procedure which enables the sorting of molecules based on size and charge. In the case of nucleic acids, the direction of migration, from negative to positive electrodes, is due to the naturally-occurring negative charge carried by their sugar-phosphate backbone (Lodish, 2004).

Double-stranded DNA fragments naturally behave as long rods, so their migration through the gel is relative to their size or, for cyclic fragments, their radius of gyration. Single-stranded DNA or RNA tends to fold up into molecules with complex shapes and migrate through the gel in a complicated manner based on their tertiary structure. Therefore, agents that disrupt the hydrogen bonds, such as sodium hydroxide were used to denature the nucleic acids and cause them to behave as long rods again.

Gel electrophoresis of large DNA or RNA is done by agarose gel electrophoresis. Characterization through ligand interaction of nucleic acids or fragments may be performed by mobility shift affinity electrophoresis. Electrophoresis of RNA samples were used to check for genomic DNA contamination and also for RNA degradation. RNA from eukaryotic organisms shows distinct bands of 16s rRNA. Degraded RNA has less sharply defined bands, has a smeared appearance, and intensity ratio is less than 2:1. Therefore, the isolate No. 2, 6, 7 and 8 shows the presence of 16s rRNA densely.

CONCLUSION

- Various bacterial isolates were used for biogas generation using cattle dung in various dilutions.
- The experimental set up carried out by using consortia “A”, consortia “B” and consortia “C” to find out the suitable consortia to be used for maximum biogas generation.
- Consortia “A” contains all 8 isolates, Consortia “B” include the strict and facultative anaerobic bacteria *Bacteroides, peptostreptococcus, Clostridium,* and *propionibacterium* are involved in the hydrolysis and fermentation of organic compounds
- Consortia “C” include following Methanogenic bacteria *Methanobacterium formicicum, Methanobrevibacter ruminantium*, *Methanosarcina frisia*, and *Methanothrix soehngenii*.
- From the above study it can be concluded that the consortia “C” containing four different methanogenic bacteria are involved in the transformation of complex high molecular weight organic compounds to methane (76 %) in comparison to the Consortia “A” (23 %) and Consortia “B” (1%).

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