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Relevance of Potential Microorganisms for Enhancing Biogas Production

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Abstract: *Bacteria were isolated from the sewage water for the purpose of enhancing biogas production. The sample was serially diluted up to 10^{-7} dilution, for the isolation of hydrolytic, acidogenic, acetogenic and methanogenic bacteria. However, nutrient agar was used for the isolation of hydrolytic bacteria, while basal media supplemented with specific substrate has been used for the isolation of acidogenic, acetogenic and methanogenic bacteria. After the isolation, the hydrolytic bacteria from the nutrient agar plates were selected on the basis of prominence for the preliminary screening of bacteria. After preliminary screening the efficient strains showed highest zone of clearance were selected for enzyme assay along with acidogenic, acetogenic and methanogenic bacteria. Based on the highest enzyme activity of hydrolytic (cellulase - 21.3U/ml, protease - 205.6U/ml, lipase-23.2U/ml), acidogenic (acetate-520U/ml), acetogenic (acetate dehydrogenase-325.2U/ml), methanogenic (methyl transferase-26.7U/ml) bacteria, the potential bacteria were selected and those were identified as Bacillus subtilis, Pseudomonas stutzeri, Brachybacterium sp. and Clostridium perfringens. Biogas production from poultry waste and cow dung along with four potential bacteria showed a highest methane concentration of 72%, which was relatively higher than that of control (58%). The analysis of biochemical compounds showed that there was a decrease in various compounds, which was also supported by the FTIR spectra obtained however the present study showed an increased biogas production efficiency of 18% was achieved through the addition of microbes, hence such kind approach with microbial addition would enhance biogas production in large extent in the meantime.*

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I. Introduction

Renewable energy helps to reduce global greenhouse gas emissions. This step leads to the production of lots of energy resources like solar energy, wind energy, tidal energy, hydro electric energy, biomass, and biogas. The major advantage of biogas over fossil fuel includes electricity generation, potentially help to reduce global climate change. The distinctive component of biogas are CH_4 (50-70%), CO_2 (20-25%), N_2 (0-5%), H_2 (0-1%) and H_2S (0-3%). The anaerobic digestion begins with conversion of insoluble carbohydrates, proteins and lipids in to simple sugars, fatty acids and amino acids by the hydrolytic bacteria. In the next stage acid forming bacteria (acidogens) convert the hydrolytic product in to simple organic acids, alcohols, carbon dioxide and hydrogen gas. Volatile acids are converted to acetate and hydrogen gas by acetogenic bacteria. **T**he final stage **by** methane forming bacteria produce**s** biogas from acetic acid or hydrogen and carbon dioxide**.**

Anaerobic digestion has four biological and chemical stages - hydrolysis, acidogenesis, acetogenesis and methanogenesis. Hydrolysis is the process of breakdown of complex organic molecules into simple sugars, amino acids, and fatty acids with the action of hydrolytic bacteria. The acidogenesis is the breakdown of the remaining components by acidogenic bacteria and again digested by acetogens and produce acetic acid. Methanogenesis converts into methane. Based on the solid content used for the process, systems can be categorized as high solid systems (>15%) and low solid systems (<15%). Hence the present study focused on the identification of potential bacteria for different stages of anaerobic digestion and evaluation of its efficiency on substrate for biogas production enhancement.

II. Materials And Methods

SAMPLE COLLECTION

Sewage water has been selected as the microbial source in this study since its simpler organic nature would allow the growth of all kinds of microorganisms. Therefore sewage sludge was aseptically collected in sterile bottle and was directly brought to the laboratory of Vivekanandha College, Tiruchengode, Tamilnadu where the further study being proceed.

BACTERIAL ISOLATION

The sample was serially diluted up to 10^{-7} dilution, for the isolation of hydrolytic, acidogenic, acotogenic and methanogenic bacteria. However, nutrient agar was used for the isolation of hydrolytic bacteria, while basal media supplemented with specific substrate has been used for the isolation of acidogenic, acetogenic and methanogenic bacteria. All the dilutions were spread plated on respective plates. All the nutrient agar plates were incubated at 37°C for 24 hours. After incubation, the obtained bacterial colonies were differentiated on the basis of colony characterization, followed by pure cultures of dominant colonies were obtained.

BACTERIAL SCREENING

The Preliminary screening of hydrolytic enzymes such as cellulase, protease and lipase were performed by using the media such as CMC agar, protease specific agar and tributyrin agar for that, the pure cultured isolates were spot inoculated on each of three media ,and was then incubated at 37°C for 24-48 hours to accomplish substrate hydrolysis. After incubation, 0.1% Congo-red solution was added to CMC agar plates and was then undisturbed for 20 minutes. Followed by 1M sodium chloride solution was added to de stain the plates. The isolates produced zone of clearance after sodium chloride treatment was considered as cellulose procedures (Saraswati *et al.*, 2012). Conversely, mercuric chloride solution was added to explore the zone of clearance on protease specific medium after incubation. The zone of clearance obtained on tributyrin agar after incubation showed lipase producing isolates (Alnahdi, 2012).

ENZYME ASSAY

The efficient bacteria were inoculated in production media for the enzymes such as cellulase, protease and lipase. Basal media containing 0.1% carboxyl methyl cellulose as substrate was used as the production for cellulase. For cellulose assay, 1% CMC was used as substrate. The assay was carried out by DNS method and followed by optical density was measured at 575 nm (Saraswati *et al.*, 2012). One unit of the cellulase activity refers to the amount of enzyme that released 1µM of glucose. The efficient isolates were incubated in protease specific media broth for protease assay and assay was carried out by using the substrate casein. Finally, the optical density was observed at 660 nm against a reagent blank using tyrosine as standard (Lowery *et al*., 1951; Alnahdi, 2012). One unit of protease is defined as the amount of enzyme that released 1µg of tyrosine per minute under the standard conditions of supernatant solution for lipase assay 10% v/v olive oil emulsion in 2% v/v gum acacia was used and the reaction mixture was then titrated against 0.05N sodium hydroxide using phenolphalein indicator to find out librated fatty acids .one unit of lipase was determined as the amount of lipase required to liberate 1µM of fatty acids under assay conditions (Sirisha *et al.*, 2010).

BACTERIAL CHARACTERIZATION

Morphological and biochemical characterization were performed for the identification of potential bacteria gram's staining and spore staining were done for morphological analysis, while catalase, oxidase, sugar fermentation and IMViC tests were carried out for biochemical analysis, as per Bergy's manual .

BIOGAS PRODUCTION

A set of batch reactors were used for the study, for pilot scale production of biogas. The reactor has an inlet, outlet, sampling unit, thermometer, pressure gauge and gas collection unit. The cow dung and poultry waste has been used as substrates for present study, substrates were collected in cellophane bags from dairy farm and poultry farm locate near to Namkkal, Tamilnadu. One kilogram of cow dung and 1 kg of poultry waste was mixed with 15 litters of distilled water to achieve feasible degradation of substrate. The substrate mixture was then fed up to the pilot scale plant having 20 litres total volume. Appropriate media incubated with respective bacteria were used us inoculums. A final volume of 17 litres of substrate slurry was mixed with 250 ml of inoculums each of the four and that has been stirred well to achieve homogenization. The reactor without inoculums was run as control. The digesters were then air tighted to provide absolute degradation.

SUBSTRATE AND BIOGAS ANALYSIS

The substrate was collected at the interval of 20 days (0^{th}, 14^{th}, $21^{th}$and 30^{th} day) and analysed for the exploration of degradation rate of substrate at different time intervals. This substrate was analyzed for total pH, temperature, total dissolved solid(TDS), total suspended solid (TSS), total acidity, total alkalinity, polysaccharide, ammonical nitrogen, sugar test quantitative & qualitative, total volatile solid, cellulose & lignin, analysis. Biogas production has been carried out for 30 days and the digested samples during different time intervals (0^{th}, 14^{th}, 21^{th} and30^{th} day) has been collected and then analysed through gas chromatography (GC 3800). FTIR analysis was also done to know the functional group distribution of substrate before ($0^{th\ day}$) and after (30^{th} day) degradation.

III. Results

BACTERIAL ISOLATION

Plentiful bacterial colonies were observed on nutrient agar and other specific media. Based on the colony morphology of isolates, 7 bacterial colonies from nutrient agar (E1, E2, E3, E4, E5, E6 and E7) were selected for preliminary screening of hydrolytic bacteria. Conversely, 5 acidogeic bacteria (AD1, AD2, AD3, AD4 and AD5), 4 acetogenic bacteria (AC1, AC2, AC3 and AC4) and 4 methanogenic bacteria (M1, M2, M3 and M4) were selected from the whole bacteria obtained on the basis of prominence.

PRELIMINARY SCREENING

Preliminary screening for hydrolytic bacteria showed that E1 and E3 were efficient producers of cellulase, protease and lipase. The zone of clearance obtained after cellulase, protease and lipase screening (Table 1).

ENZYME ASSAY

The result of enzyme assay of hydrolytic enzymes for E1 and E3 shows that E1 was the potential producer of all the three hydrolytic enzymes. E1 showed the highest enzyme activity of 21.3 U/ml for cellulase, 205.6 U/ml for protease and 32.2 U/ml for lipase. Whilst E3 shows comparatively lower enzyme production (Figure. 1). Conversely, the acetate kinase assay of five acidogenic bacteria (AD1, AD2, AD3, AD4 & AD5) revealed that AD2 was the potential acidogenic bacteria. The AD2 showed highest enzyme activity of 520.4 U/ml when compared to the remaining acidogens, as described in Figure 2. Among the four acetogenic bacteria (AC1, AC2, AC3 & AC4) selected, AC3 showed highest activity of 325.2 U/ml for the enzyme carbonic anhydrase than the other three acetogens (Figure 3). Likewise, the methanogenic bacteria M1 show highest enzyme activity of 26.7 U/ml for methyl reductase in contrast to other methanogens such as M2, M3 & M4 (Figure 4).

MORPHOLOGICAL ANALYSIS

Morphological analysis of the potential isolates show that E1 was Gram positive rod shaped spore-forming bacteria. The acidogen AD2 was Gram negative rod shaped bacteria. The acetogenic AC3 was Gram positive rod shaped bacteria. While the methanogenic bacteria MI was Gram positive rod shaped bacteria as shown in Plate 2.

BIOCHEMICAL ANALYSIS

Biochemical analysis of E1 show that the organism was positive for methyl red, Voges-Proskauer, citrate, catalase, urease, starch hydrolysis tests, glucose and mannitol fermentation tests; while negative for indole and lactose fermentation tests. The acidogenic bacteria AD2 showed positive result for citrate, catalase, oxidase, glucose and lactose fermentation tests while negative results for indole, methyl red, Voges-Proskauer and mannitol utilization test. The acetogenic bacteria AC3 were positive for methyl red, catalase, and starch hydrolysis, glucose and lactose fermentation tests. AC3 was negative for indole, Voges-Proskauer, citrate, urease, oxidase, and sucrose utilization test. Similarly biochemical analysis of M1 shows that it was positive for Voges-Proskauer, hydrogen sulfide production, sucrose, lactose, and glucose utilization tests. While M1 was negative for mannitol, indole, methyl red and triple sugar ion test. However, based on the morphological and biochemical characteristics, E1, AD2, AC3 & M1 were identified as *Bacillus subtilis, Pseudomonas stutzeri, Brachybacterium sp.* and *Clostridium perfringens*, respectively (Table 2).

SUBSTRATE ANALYSIS

Substrate analysis was done in the interval of 0^{th} to 30^{th}. At the first day the pH will gradually decrease at the first days of analysis then increase at the last day. The temperature increases day by day at the time of substrate analysis. Total solid, volatile solid, lignin, cellulose, carbon content all is decrease gradually by increasing the day of substrate analysis. The nitrogen content is increased in this sequence of substrate analysis.

FTIR ANALYSIS

The important changes in the FTIR spectra are associated with the peak intensities at around 2910.6, 1714.7, 1541.1 cm^{-1}. At the 0^{th} day the peak range of cellulose and hemicellulose were 2910.6 cm^{-1} and1714.7 cm^{-1} respectively. The range of peak gradually decreases on the last day for cellulose and hemicellulose as 2883.6 cm^{-1} and 1687.9 cm^{-1} respectively. On the other side the peak of lignin gets increases from its 0^{th} day to last 30^{th} day as 1541.1 cm^{-1} to1556.6 cm^{-1} (Figure 5 & 6).

GAS ANALYSIS

The anaerobic digestion of organic material results in the production of biogas. The major component of biogas is methane gas, which gradually increases from 0^{th} day to the 30^{th} day. In case of natural digestion kept

as control shows slower growth rate then the digester having the organism as source. In 0^{th} day methane level in test sample is 2.32, which is increased by 72.5 on the 30^{th} day. In the control, the 0^{th} day production was 1.48 and its value on 30^{th} day was 54.7 which is less than that of test digester (Table 4).

IV. Discussion

The anaerobic digestion has been considered as a process for degrading a variety of polluting organic wastes for removal of contaminants. Not only used for removal of contaminants from the environment but also for saving energy. So, the anaerobic digestion is also called as the process in which biodegradable organic material is decomposed in the absence of oxygen to produce biogas (Pauss *et al.,* 1987). The organic matter can be degraded by the sequential action of hydrolytic, acetogenic and methanogenic bacteria to produce biogas.

There were different species of microorganisms have been identified in the sewage waste. The organisms identified are bacteria, the most abundant are protozoa, fungi and also viruses. Although there is a wide variety of a bacterium found in the sewage wastes. In the current study 7 bacterial colonies from nutrient agar (E1, E2, E3, E4, E5, E6 & E7) were selected for preliminary screening of hydrolytic bacteria. Conversely, 5 acidogenic bacteria (AD1, AD2, AD3, AD4 & AD5), 4 acetogenic bacteria (AC1, AC2, AC3 & AC4) and 4 methanogenic bacteria (M1, M2, M3 & M4) were selected from the whole bacteria obtained on the basis of prominence .But the predominant bacterial species were *E. aerogens, C. Farmer* (Adams *et al.,* 2005), *Pseudomonas fluorescens* (Bakere *et al.,* 2005), *Bacillus subtilis* (Saraswati *et al.,* 2012), *Bacillus licheniformis* (Tae *et al.,* 2000) anaerobic *Clostridium celerecrescens.* Biodegradation occur in nature regularly but the processes take place very slowly, hydrolysis is the process which takes place in biodegradation process. In earlier studies many cellulose hydrolyzing bacteria were isolated, which include *Enterobacter aerogen, Enterobacter cloaccae, S. marcescens, Citrobacter farmeri (*Adams and Boopathy*, 2005), Clavibacter agropyri (*Ramin *et al.,* 2008), *Cellulomonas species, Mycobacterium species, Kocuria varians, Bacillus cereus, Bacillus anthracis, Bacillus circulans, Bacillus vortex, Bacillus megaterium, Rhizobium, Brucellame litensis, Zhymomonasmobilis* and *Spirosomasps* (Wenzel *et al.,* 2002) could provide better result.

During hydrolysis cellulose, lipid and proteins have to be broken down in to its simpler form so cellulolytic, Lipolytic and proteolytic activity of the isolated colonies was mainly considered in this study. Lipid hydrolysis was effectively observed by the E1 and E3 strain than the other isolates which shows these isolates may also involve in hydrocarbon degradation. For proteolytic activity skim milk medium was used to see the zone of clearance.

The enzyme assay of hydrolytic enzymes for E1 and E3 shows that E1 was the potential producer of all the three hydrolytic enzymes. Whilst E3 shows comparatively lower enzyme production. The maximum protease activity (0.75 U/ml) was attained after 24 h (Kim *et al*., 1998). Protease production was found to be higher at 0.55U/ml and the isolates was identified as two different species of *Bacillus* (Brown *et al.,* 1991). By titration method the secondary screening of lipase is carried out using substrate as oleic acid. One unit of lipase was determined as amount of lipase required to liberate one µM of fatty acids per minute under assay conditions. Specific activity was determined as enzyme unit per mg of total protein concentration. Lipase from *B. Subtilis* that had a Km and Vmax value of 0.33 *mM* and 188 U/mg, respectively, when using oleic acid as substrate (Kambourova *et al*., 2003). Conversely, the acetate kinase assay of five acidogenic bacteria (AD1, AD2, AD3, AD4 & AD5) revealed that AD2 was the potential acidogenic bacteria. The AD2 showed highest enzyme activity of 520.4 U/ml when compared to the remaining acidogens. Among the four acetogenic bacteria (AC1, AC2, AC3 & AC4) selected, AC3 showed highest activity of 325.2 U/ml for the enzyme carbonic anhydrase than the other three acetogens. Likewise, the methanogenic bacteria M1 show highest enzyme activity of 26.7 U/ml for methyl reductase in contrast to other methanogens such as M2, M3 & M4.

Gram staining is the preliminary step for determining the acidogen, acetogen and methanogen followed by biochemical test (Kasten, 1981). The study followed the microscopic examination followed by biochemical testing. Specifically for the estimation of acidogens and acetogens carbohydrate fermentation test is done, by which acid and gas production is determined. For the methanogenic organisms IMViC, carbohydrate fermentation test, catalase and Oxidase test were done. However, based on the morphological and biochemical characteristics, E1, AD2, AC3 & M1 were identified as *Bacillus subtilis, Pseudomonas stutzeri, Brachybacterium sp.* and *Clostridium perfringens*, respectively.

As the biogas productions get started we know that the degradation of organic material takes place. In this process the cellulosic, hemi cellulosic material start breaks down to simpler form, in the same way the carbon content also get decrease and the major biogas methane content get increased gradually as its said in all earlier papers. In pH and temperature, for pH in the biogas units ranged from 7.4 to 8.2 and showed decreasing trend initially and increased after 30^{th} days. Ambient temperature varied from 32°C, 33°C, 35 °C, 39°C cow dung contained biogas unit showed initially decreased and increased day by day, compared to ambient temperature. Major parameters such as pH, temperature, VFA, TS, carbon ratio, nitrogen ratio these parameters

all depend on the anaerobic condition in biogas (Meherdad *et al.*, 2007) .The nitrogen content increase, but the carbon content will get decrease.

The cellulose content gets decrease during digestion, so the intensity of peak also gets decreased from 0^{th} to 30^{th} day. The peak around 2910.6 indicates the presence of sp^3 C-H stretching; this indicates the presence of cellulose (Jayaramudu *et al*., 2010). The peak 1714.7 shows the presence of C=C which indicates the presence of hemicellulose. However, the decrease in the range of peak in FTIR spectra point towards the highest degradation rate of hemicelluloses. In case of lignin, 1541.1peaks indicates symmetric C-H bonds. And the increase in the range of peak during digestion period has been supported by the release of lignin (Jayaramudu *et al*., 2013).

At high temperature of 39ºC microbes are not able to grow resulted into lower gas production compare to control. GC analysis shows presence of methane (72.5%), carbon dioxide (22.5%) and H_2S (4.12%) in the produced gas by methanogenesis (Gohel *et al*., 2013). Methane gas is vastly obtained gas in this process then other gases. This methane gas is only used as the biogas for many purposes.

V. Conclusion

The present study concluded that cellulolytic microorganism was successfully identified through cellulose Congo red agar medium; this revealed that the organism is *Bacillus subtilis*, acidogenic microorganism was identified as *Pseudomonas stutzeri*. Acetogenic bacteria were identified as *Brachybacterium* sp, methanogenic bacteria identified as *Clostridium perfringens*. The identified organism was used for biogas production using cow dung and poultry waste as substrate. And then the biogas that is methane is produced which is used for house hold works, also its eco-friendly.

Acknowledgement

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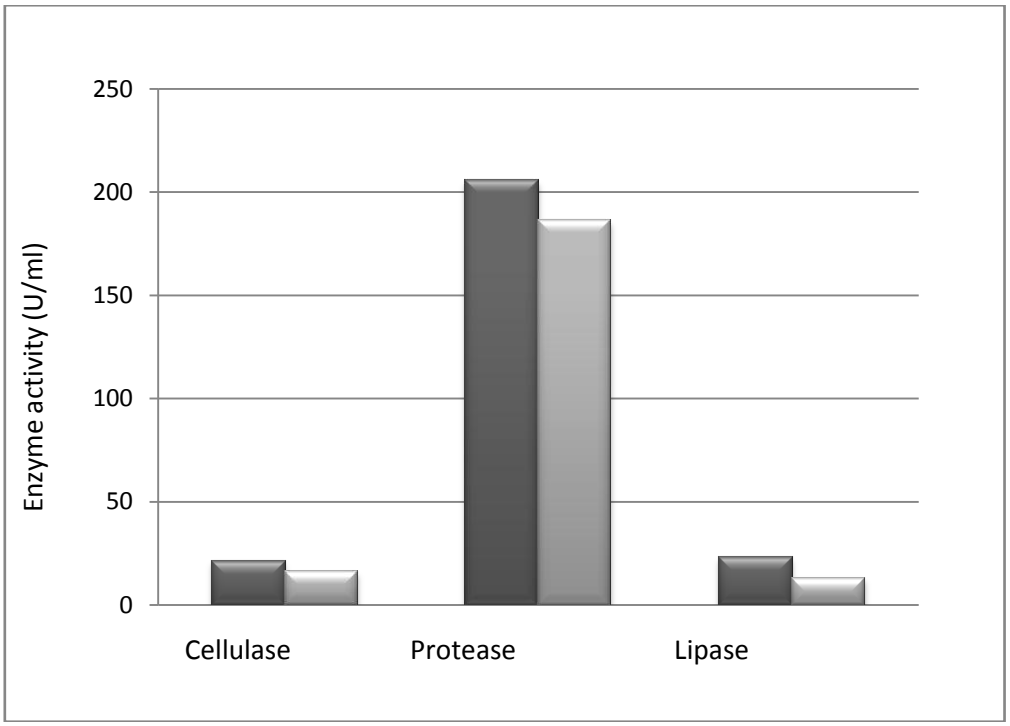

Figure 1. Enzyme assay of hydrolytic enzymes

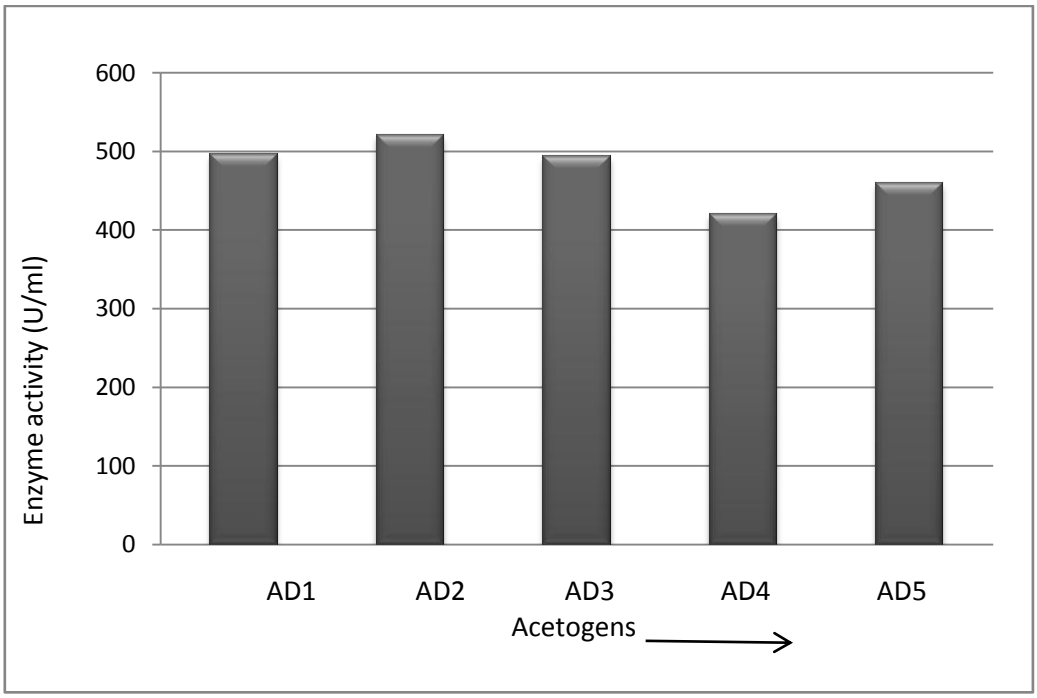

Figure 2. Assay of acetate kinase

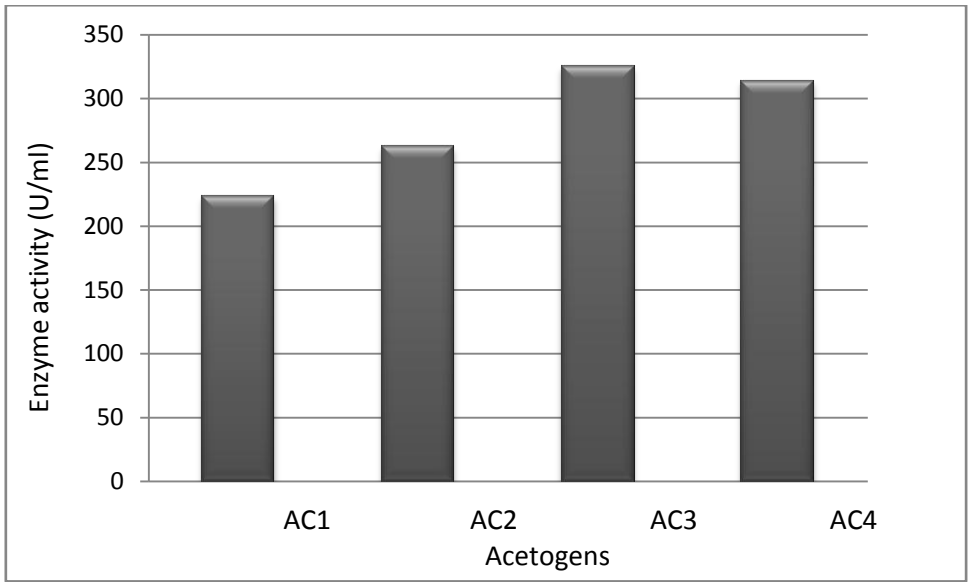

Figure 3. Assay of carbonic anhydrase

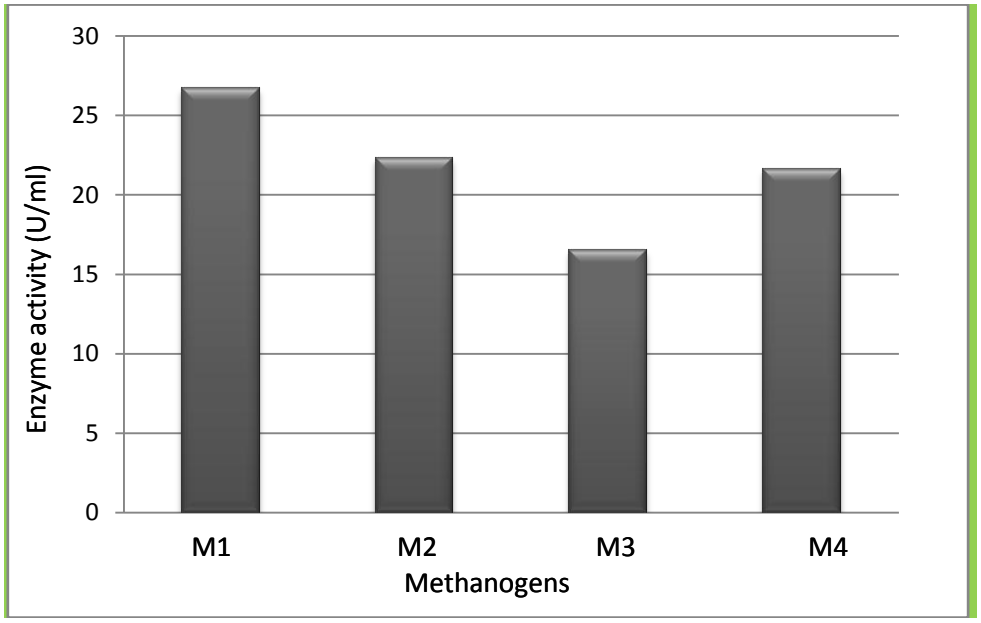

Figure 4. Assay of methyl reductase

Isolates	Cellulase (CMC)	Protease (PSM)	Lipase (TA)
E1	1.0	1.4	1.2
E2	0.6	1.1	0.6
E3	0.8	1.3	1.0
E4	-	0.9	0.8
E5	0.5	1.1	-
E6	0.2	-	0.7
E7	-	1.0	0.9

Table 1: Preliminary screening of hydrolytic bacteria

	Hydrolytic bacteria (E1)	Acidogenic bacteria (AD2)	Acetogenic bacteria (AC3)	Methanogenic bacteria (M1)
Indole	-	-	-	-
Methyl red	+	-	+	-
Voges-Proasker	+	-	-	+
Citrate	+	+	-	
Catalase	+	+	+	
Oxidase		+	-	
Urease	+		-	
Hydrogen sulphide production	-			+
Starch hydrolysis	+		+	
Triple sugar ion				-
Glucose fermentation	+	+	+	+
Lactose fermentation	-	+	+	+
Mannitol fermentation	+	-		-
Sucrose fermentation			-	+

Table 2: Biochemical analysis

	TEST				CONTROL			
Parameters	0^{th}	14^{th}	21^{th}	30^{st}	0^{th}	14^{th}	21^{th}	30^{st}
pH	7.4	6.9	4.8	8.2	7.5	7.0	5.8	7.9
Temperature	32.5	33.1	35.8	39.4	32.6	32.5	33.6	38.1
Ts (%)	94.6	85.3	35.2	22.4	94.6	90.2	52.5	40.6
Vs (%)	74.2	52.3	23.4	15.5	74.2	69.5	48.4	23.4
Carbon (%)	36.4	30.5	28.7	23.2	36.4	34.3	30.2	28.5
Nitrogen (%)	1.2	1.0	0.8	4.5	1.2	1.1	0.4	3.2
Cellulose	34.2	23.4	12.6	10.5	34.2	28.5	22.5	17.8
Lignin	12.3	14.6	15.0	15.2	12.3	11.6	12.2	12.8

Table 3. Substrate analysis

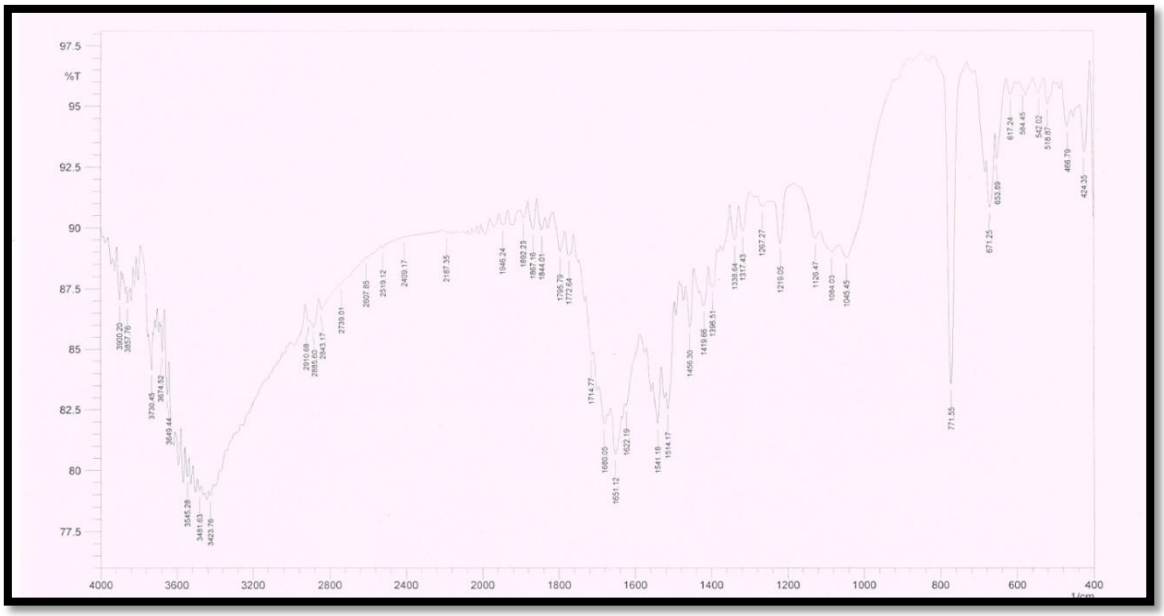

Figure 5. 0^{th} day FTIR analysis

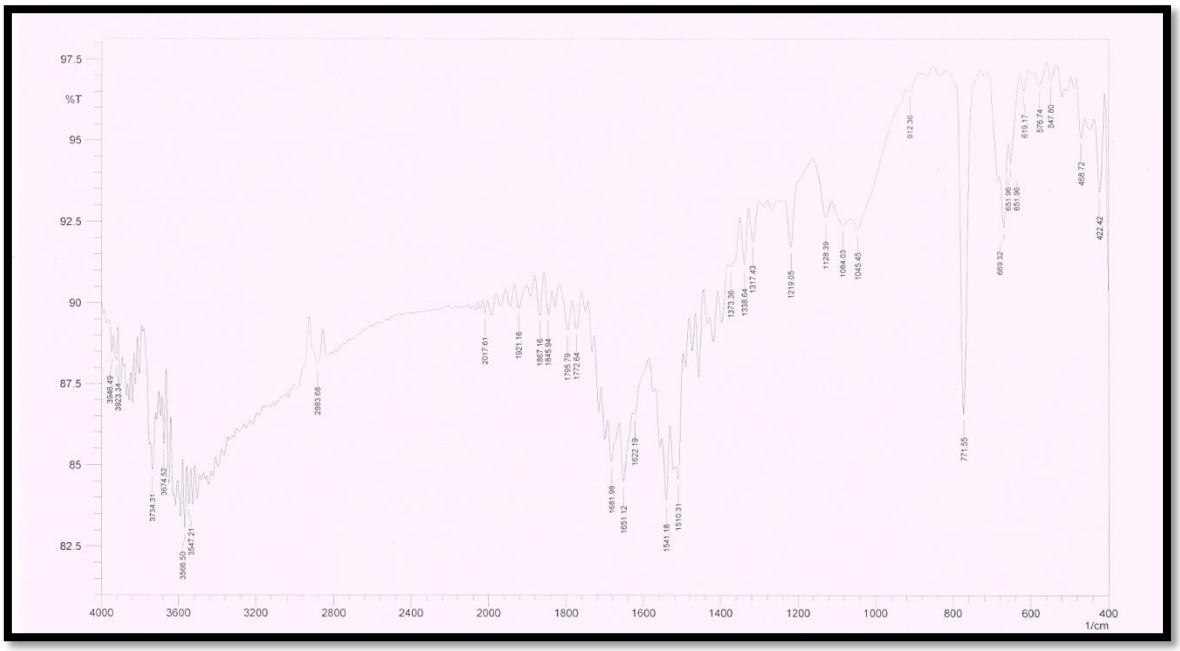

Figure 6. 30th day FTIR analysis

Gases/Day	0th		7th		14th		21st		30th	
	TEST	CONTROL	TEST	CONTROL	TEST	CONTROL	TEST	CONTROL	TEST	CONTROL
CO_2	70.45	75.68	58.34	64.38	43.25	52.39	28.78	38.79	22.5	36.4
CH_4	2.32	1.48	12.34	8.76	28.67	18.38	56.98	48.53	72.5	54.7
H_2S	18.29	15.18	16.57	12.78	15.98	15.28	8.34	6.59	4.12	5.9
OTHERS	8.94	7.34	12.75	14.08	12.1	13.95	5.90	6.09	3.01	4.1

Table 4. Biogas characterization

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