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Identification and Evaluation of Effective Bacterial Consortia for Efficient Biogas Production

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Abstract: *Microorganisms play a vital role in processing organic matter and returning the chemical elements into active cycle. In these decomposers are effective in dismantling the complex organic matter through a sequential break down and release of energy. Biogas is one such process which occurs in the absence of oxygen and several groups of microorganism involve in degrade the organic compounds and release methane gas. To attain biogas with high methane concentration it is important to create and maintain the suitable bacterial consortia within the digester. Therefore, the present research was carried out to isolate different bacterial species from cow dung and construct four different bacterial consortia to analyze their efficiency of biogas production. The biogas production was carried out in a pilot scale batch reactor for 30 days using poultry droppings as a substrate and four different bacterial consortia in four separate digesters. Various hydrolytic enzymes, volatile fatty acids and biogas production were measured in the interval of 10 days. From the above study it was concluded that consortia 4 containing high amount methanogenic bacteria gave the highest biogas production with methane 79.45%.*

Key words: *Biogas, Consortia, Hydrolytic enzymes, Poultry droppings, Volatile fatty acids.*

I. Introduction

The demand for renewable fuels is increasing with growing concern about climate change, air quality, energy import dependence and the depletion of fossil fuels. Biogas is one of the versatile renewable fuels which can be used for power and heat/cool production or it can be upgraded to biomethane to be used as vehicle fuel [30]. Biogas is produced by anaerobic digestion of biological wastes such as cattle dung, vegetable wastes, sheep and poultry droppings, municipal solid waste, industrial waste water and land fill to give mainly methane (50-70%), carbon dioxide (20-40%) and traces of other gases such as nitrogen, hydrogen, ammonia, hydrogen sulphide, water vapour etc. [15]. It is smokeless, hygienic and more convenient to use than other solid fuels [4].

Methane fermentation is a complex process, which can be divided up into four phases: hydrolysis, acidogenesis, acetogenesis and methanogenesis [10, 17, 3, 22, & 23]. The degradation steps are carried out by different consortia of microorganisms, which partly stand in syntrophic interrelation and place different requirements on the environment [1]. The first group of microorganisms consist the hydrolytic bacteria. These organisms hydrolyses the polymeric materials to monomers such as glucose and amino acids through extracellular hydrolytic enzymes (cellulase, xylanase, amylase, protease, lipase) they excrete. Most of the bacteria are strict anaerobes such as Bacteriocides, Clostridia and Bifidobacteria. Furthermore, some facultative anaerobes such as Streptococci and Enterobacteriaceae take part. A second group of microorganisms are acidogenic bacteria. They convert the sugars and amino acids into carbon dioxide, hydrogen, ammonia and other organic acids. Third group of microorganisms consist acetogenic bacteria. The higher volatile fatty acids are converted into acetate and hydrogen by obligate hydrogen-producing acetogenic bacteria. Typical homoacetogenic bacteria are Acetobacterium woodii and Clostridium acetium. The accumulation of hydrogen can inhibit the metabolism of acetogenic bacteria. The maintenance of an extremely low pressure of hydrogen is essential for the acetogenic and hydrogen producing bacteria. Although many microbial details of metabolic networks in a methanogenic consortium are not clear, present knowledge suggests that hydrogen may be a limiting substrate for methanogens [2]. This assumption is based on the fact that addition of hydrogen producing bacteria to the natural biogas-producing consortium increases the daily biogas production. At the end of degradation process two groups of methanogenic bacteria produce methane from acetate or hydrogen and carbon dioxide. These bacteria are strict anaerobes and require a lower redox potential for growth than most other anaerobic bacteria. Only few species are able to degrade acetate into methane and carbon dioxide, e.g. Methanosarcina barkeri, Methanococcus mazei and Methanotrix soehngenii, where all methanogenic bacteria are able to utilize hydrogen to form methane [33].

Methanogenic and non methanogenic microorganisms differ not only in terms of their nutritional and pH requirements, but also with respect to their physiology, growth and nutrient uptake kinetics and in their

particular ability to withstand environmental changes. The composition of microbial consortium depends on various factors such as substrate ingredients, temperature, pH, mixing or the geometry of the anaerobic digester. A clear understanding of the organization and behavior of this multifarious community is crucial for optimization of the performance and attainment of the stable operation of the process. [32]. The main aim of the study is to develop some successful bacterial consortium that can increase the methane concentration in biogas and to study the various hydrolytic enzymes, volatile fatty acids, and biogas production during 30 days of anaerobic digestion.

II. Materials and methods

2.1. Isolation of bacteria from cow dung

Fresh cow dung samples were collected in and around Namakkal and they were homogenized. One gram sample was dissolved in 100ml double distilled water for further analysis. From the homogenized samples, dilutions with double distilled water are made up to 10^{-8}. The poured nutrient agar plates are incubated at 37°C for 24 to 48 hours. After incubation, the number of colonies seen on the plates were selected for biochemical test and further analyzed through selective media. The selective media used for isolation of Enterobacterium cloacae-Eosin Methylene Blue agar, Pseudomonas aeruginosa - Cetrimide agar, Pseudomonas putida –Basal medium with hippurate [13], Bacillus subtilis-nutrient agar, Streptococcus bovis- membrane-bovis Agar [24], Streptococcus salivarius - Mitis Salivarius Agar with the addition of potassium tellurite, Clostridium tyrobutyricum- Bryant-Burkey Broth Base, Clostridium drakei- anoxic brain heart infusion (BHI) medium, [36], Methanospirillum hungatei- enriched medium [9], Methanosarcina thermophila- complex medium [37], Methanobrevibacter smithii [19], Methanococcus voltaei- defined medium [35].

2.2. Bacteriological analysis

The bacterial species isolated from fresh cow dung were detected by standard bacteriological identification procedure. Morphological characters examined include gram staining, shape and motility. Biochemical tests were performed by inoculating broth culture of the isolates into a series of media. These tests include IMIViC, Catalyze, Oxidase, Triple Sugar Iron, Sugar fermentation (glucose, sucrose, and lactose).

2.3. Preparation of bacterial consortia

The experiment was carried out by using four different bacterial consortiums. Consortia 1 were inoculated into the biogas unit 1 containing 1% of hydrogens, acidogens, and methanogens. Consortia 2 were inoculated into biogas unit 2, with 2% hydrogens were it is 1.5% higher than acidogens and methanogens. Consortia 3 were inoculated into biogas unit 3 restrain 2% acidogens which is 1.5% higher than hydrogens and methanogens. Consortia 4 were inoculated into biogas unit 4 have methanogens 1.5% higher than hydrogens and acidogens. The biogas unit without any bacterial consortium inoculum is named as consortia 5. The details of bacterial consortia preparation was given in Table 1.

TABLE 1: **Composition of microbes in different consortia of the experiment**

Species	CONSORTIA				
	1	2	3	4	5
Hydrogens					
Enterobacterium cloacae (%)	0.25 (50 ml)	0.50 (100 ml)	0.125 (25 ml)	0.125 (25 ml)	0.00
Pseudomonasaeru aeruginosa (%)	0.25 (50 ml)	0.50 (100 ml)	0.125 (25 ml)	0.125 (25 ml)	0.00
Pseudomonas putida (%)	0.25 (50 ml)	0.50 (100 ml)	0.125 (25 ml)	0.125 (25 ml)	0.00
Bacillus subtilis (%)	0.25 (50 ml)	0.50 (100 ml)	0.125 (25 ml)	0.125 (25 ml)	0.00
Total (%)	**1.00 (200 ml)**	**2.00 (400 ml)**	**0.50 (100 ml)**	**0.50 (100 ml)**	**0.00**
Acidogens					
Streptococcus bovis (%)	0.25 (50 ml)	0.125 (25 ml)	0.50 (100 ml)	0.125 (25 ml)	0.00
Streptococcus salivarius (%)	0.25 (50 ml)	0.125 (25 ml)	0.50 (100 ml)	0.125 (25 ml)	0.00
Clostridium tyrobutyricum (%)	0.25 (50 ml)	0.125 (25 ml)	0.50 (100 ml)	0.125 (25 ml)	0.00
Clostridium drakei (%)	0.25 (50 ml)	0.125 (25 ml)	0.50 (100 ml)	0.125 (25 ml)	0.00
Total (%)	**1.00 (200 ml)**	**0.50 (100 ml)**	**2.00 (400 ml)**	**0.50 (100 ml)**	**0.00**
Methanogens					
Methanospirillum hungatei (%)	0.25 (50 ml)	0.125 (25 ml)	0.125 (25 ml)	0.50 (100 ml)	0.00
Methanococcus voltaei (%)	0.25 (50 ml)	0.125 (25 ml)	0.125 (25 ml)	0.50 (100 ml)	0.00
Methanobrevibacter smithii (%)	0.25 (50 ml)	0.125 (25 ml)	0.125 (25 ml)	0.50 (100 ml)	0.00
Methanosarcina thermophila (%)	0.25 (50 ml)	0.125 (25 ml)	0.125 (25 ml)	0.50 (100 ml)	0.00
Total (%)	**1.00(200ml)**	**0.50 (100 ml)**	**0.50 (100 ml)**	**2.00 (400 ml)**	**0.00**
Grand Total (%)	**3.00 (600 ml)**	**3.00 (600 ml)**	**3.00 (600 ml)**	**3.00 (600 ml)**	**0.00**

2.4. Experimental design

A study was conducted using poultry droppings as substrate. The poultry droppings were diluted with tap water in the ratio of 1:5. The prepared slurry was loaded into 20L capacity biogas unit. The unit contains

thermometer to monitor the temperature, pressure gauge to check the pressure and gas collector for gas analysis. Samples were collected in the intervals of 10days for the analysis.

2.5. Different Enzymatic Assay of Consortia

For enzymatic assays the bacterial consortia were centrifuged at 10,000 rpm for 10 min. The supernatant was used for enzymatic assay. The experiments were carried out in duplicates and standard error was calculated.

Cellulase assay: Cellulase activity was assayed by incubating 1.0 ml of enzyme extract with 1 ml of 1% Carboxy methyl cellulose (CMC), 4 ml of sodium phosphate buffer (pH 9.0) at 50°C for 30 min. A control without enzyme was used in this assay. Released reducing sugar was measured by dinitrosalicylic acid (DNS) method. To this mixture 3 ml of DNS reagent was added and heated in a boiling water bath for 10 min. At the time of cooling 1 ml of freshly prepared 40% sodium potassium tartarate solution was added and the samples were read at 510 nm in U.V. Spectrophotometer. The enzyme activity was expressed as IU/ml [18].

Lipase assay: Lipase activity was assayed titrimetrically at pH 8.0 with a standard tributyrin as substrate. 1ml tributyrin was mixed with 3ml of Tris HCl (pH 8.0) to form emulsion. 1 ml of the enzyme was added to the emulsion. The mixture was incubated at 50°C for 30 min. The liberated fatty acids were titrated with 50mM NaOH. One unit of activity was defined as the amount of enzyme which liberated 1μM butyric acid per min under standard conditions.

Protease assay: The enzyme extract suitably diluted, was mixed with 50mM glycine - NaOH buffer (pH 9) to make 1 ml volume. 1ml of 1% casein (substrate) was added and incubated for 10 min at 60°C. The reaction was stopped by addition of 0.5 ml TCA (20%, w/v). The mixture was allowed to stand at room temperature for 30 min and filtered. 1 ml of the filtrate was mixed with 5 ml of 0.5M Na2CO3 solution. 0.5 ml of Folin & Ciocalteu's (phenol reagent) reagent was added and kept in dark to develop the blue color. It was estimated spectrophotometrically at 660nm against tyrosine as standard. One unit of protease activity was defined as the amount of enzyme required to liberate1 g tyrosine per milliliter in 1 min under the experimental conditions used.

Amylase assay (DNSA 3, 5 dinitro salicylic acid methods): One ml of 1% starch was incubated with different dilutions of the enzyme extract and 1ml of citrate-phosphate buffer (pH 6.0) and was incubated at 50°C for 30 min. The reaction was stopped by adding 2 ml of DNS and kept in boiling water bath for 10 min and absorbance was recorded at 540nm against glucose as the standard. One unit of enzyme activity is defined as the amount of enzyme, which releases 1μmole of reducing sugar as glucose per minute, under the assay conditions (U/ml/min).

2.6. Measurement of Volatile fatty acids

All volatile fatty acid analyses were done through Gas Chromatograph (Hewlett Packard. HP 5890 series II) equipped with flame ionization detector (GC/FID), while the data analyses were done using the Chem Station (Hewlett Packard, software package). The column used was an HP-FFAP, 15m x 0.530 nm, I micron. The N2 flow rate was set at 1ml/min. the oven temperature programmed at 30^0C for 2 min, raised to 200^0 C at 25 C/min, and held at 240^0C for 1 min.

2.7. Gas analysis

The gas produced in the anaerobic digester was analyzed by using GC3800 gas chromatograph fitted with a flame ionization detector (FID); a glass column (1.6m long, 3mm i.d.) packed with 80/100 porpak Q; flow rate (N_2) 25ml/min; column temperature 32^0C; detector temperature 50^0C.

III. Results and Discussion

The anaerobic digestion is a sequential, complex biochemical process, in which organic compounds are mineralized to biogas which contains mainly CH_4 and CO_2 through a series of reactions mediated by several groups of microorganisms. The amount of gas produced varies with biochemical characteristics of organic wastes, consortia of microorganisms, pH, temperature, etc. However, anaerobic digestion progresses with several complex sequential and parallel biological reactions with many intermittent products through action of microorganisms producing substrates for other [21]. Cow dung was selected as a source for microbial isolation to increase the biogas production because it is a rich source of diverse group of microorganisms owing to diet of the ruminants which consist of high amounts of cellulosic matter [31].

3.1. Morphological Characteristics of bacterial Isolates

In the present research totally twelve bacteria were isolated from cow dung. By using these isolates, four different bacterial consortiums were developed and integrated into biogas unit to understand their roles in biogas production.The morphological characteristics of bacterial isolates were given in table 2.

TABLE 2: Morphological characters of microbes used for consortia

S. No.	Species	Morphology	Gram staining	Shape	Motility
1	Enterobacterium cloacae	White colonies Peritrichous flagellate	Negative	Rod	Motile
2	Pseudomonas aeruginosa	Spread pearl like	Positive		Motile
3	Pseudomonas putida,	Thin film oval Multiple non polar Flagelllate	Negative	Rod	Motile
4	Bacillus subtilis	Circular flagellate	Positive	Rod	Motile
5	Streptococcus bovis	Pairs or chain of cocci	Positive	Cocci	Non motile
6	Streptococcus salivarius	Circular Pairs or chain of peritrichous flagella	Positive	Cocci	Non motile
7	Clostridium tyrobutyricum	Irregular rod	Positive	Rod	Motile
8	Clostridium drakei	Chains of rod	Positive	Rod	Motile
9	M. hungatei	Yellow in color, circular in shape, and convex with lobate margins	Negative	Spiral	Motile
10	M. thermophila	Green and purple colony	Positive	Coccus	Non motile
11	M. voltaei	Single celled flgellates	Negative	Coccoid	Motile
12	M. Smithii	ovoid in pairs	Positive	Coccobacillus	Non motile

2.4. Biochemical characteristics of bacterial isolates

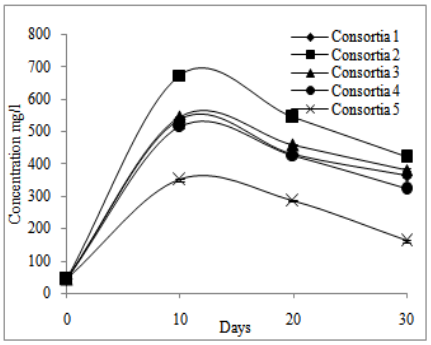
Biochemical characteristics of microorganism were studied to identify the genus and species of unknown bacteria. Microorganisms are extremely versatile and their range of metabolic capacities is very large [20]. These characteristics can be used to demonstrate the exceptional metabolic diversity of organisms. Biochemical characteristics of bacterial isolates were given in table 3. Iodole is a nitrogen metabolism test. Positive to indole test representing, bacteria that it can act upon amino acids and undergo deamination and hydrolysis leads to the formation of pyruvic acid and ammonia which leads to the production of methane and CO_2 which is main function of methanogens. The negative results showed that isolates were unable to produce indole as a result of aminoacid tryptophan breakdown due to lack of tryptophanase in the cell [12,11]. Carbohydrate metabolism of isolates was assessed by conducting tests involving Methyl red (MR), Vogues Proskauer (VP), Triple Sugar Iron (TRI). Methyl red test is used to identify bacteria that produce stable acid end products by means of mixed acid fermentation of glucose. Positive to MR test shows that they use mixed acid pathway to metabolize pyruvic acid to other acids such as lactic, acetic acids which indicates their presence and growth in acid phase during biogas production [7]. Negative to MR test indicates that it use butylene glycol pathway to metabolize pyruvic acid to neutral end-products. VP test is used to determine the ability of organism to produce a neutral end product, acetoin from glucose fermentation. Positive to VP test showed the ability of organism to produce acetoin during glucose fermentation instead of acids, which increase the pH above 5. Negative results showed its inability to produce non acidic or neutral end products from glucose metabolism. Positive to citrate is used to determine the capacity of the microorganism that utilize citrate as a sole carbon source which breaks down citrate to oxaloacetate and acetate and further into pyruvate and carbon dioxide and also produces ammonia with sodium citrate resulting alkaline condition. Alkalinity is an important parameter in anaerobic digestion because it provides enough buffering capacity to neutralize any possible Volatile fatty acids (VFA) accumulation in the reactor and to maintain pH around 6.7 to 7.4 for stable operation [5]. Negative to citrate test showed that it cannot act upon by the enzyme citrase. Triple sugar iron used to measure the ability of bacteria to utilize three sugars, glucose, lactose and sucrose and also the production of H_2S, which is the main function of anaerobic bacteria in biogas production. Catalase is an enzyme produce by many organisms which converts hydrogen peroxidases into water and oxygen and cause foaming due to release of oxygen. The bacteria that contain this enzyme are usually aerobic or facultative anaerobes. Oxidase is an enzyme involved in oxidation reaction of microorganisms due to the presence of cytochrome oxidase system in aerobic and facultative anaerobic bacteria [20, 14].

TABLE 3: Biochemical characters of microbes used for consortia

Species	Ind	MR	VP	Cit	Cat	Oxi	TSI	Glu	Suc	Lac
E. cloacae	- ve	- ve	+ ve	+ ve	+ve	-ve	+ ve	+ve	+ve	+ve
P. aeruginosa	- ve	+ve	- ve	+ve	-ve	+ ve	+ ve	-ve	-ve	-ve
P. putida	- ve	-ve	- ve	+ ve	+ve	+ ve	+ ve	+ve	+ve	+ve
B. subtilis	- ve	+ ve	+ ve	+ ve	+ ve	- ve	+ ve	-ve	+ ve	-ve
S. bovis	- ve	+ ve	+ ve	+ ve	- ve	- ve	- ve	+ve	+ve	+ve
S. salivarius	- ve	NA	+ ve	- ve	- ve	- ve	NA	+ve	+ve	+ve
C. tyrobutyricum	- ve	- ve	- ve	- ve	- ve	- ve	+ ve	+ve	+ve	+ve
C. drakei	- ve	- ve	- ve	- ve	- ve	- ve	+ ve	+ve	+ve	+ve
M. hungatei	+ ve	+ ve	- ve	+ ve	- ve	+ ve	+ ve	- ve	- ve	- ve
M. thermophila	- ve	+ ve	+ ve	+ ve	+ ve	+ ve	+ ve	+ ve	+ ve	+ ve
M. voltaei	- ve	+ ve	- ve	+ ve	+ ve	+ ve	+ ve	- ve	- ve	- ve
M. smithii	- ve	+ ve	- ve	+ ve	- ve	+ ve	+ ve	- ve	- ve	- ve

2.5. Enzyme production by different bacterial consortia

During hydrolysis extra cellular enzymes (cellulase, amylase, protease, lipase) produced by microorganisms are utilized for degradation of the organic matter. The biogas production occurred only if efficient hydrolysis occurred [7]. The hydrolytic enzyme production was high at 10th day of digestion, in consortia 2 treated biogas unit. This is because of the dominance of cellulolytic population. Among various enzymes produced amylase was produced in maximum amount 650mg/l, followed by cellulase 445mg/l, protease 440mg/l and lipase 310mg/l (Fig. 1, 2, 3 & 4). Payel et al., 2011 [28] constructed eleven different microbial consortia and the compatibility of the bacterial strains within the consortia was checked by enzyme production (amylase, lipase, and protease). Among these consortium tested the consortia 3, 5, 12 were best producers of all these three enzymes.



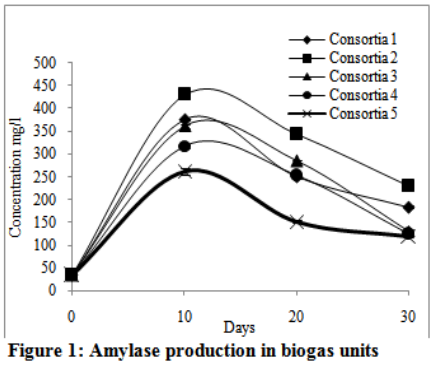
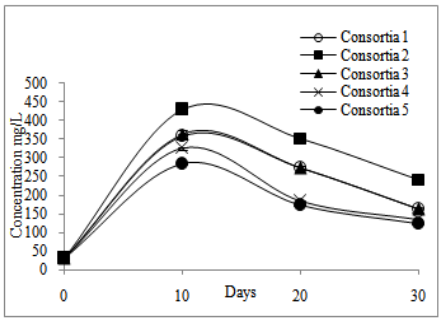


Figure 1: Amylase production in biogas units treated with different bacterial consortia

Figure 2: Cellulase production in biogas units treated with different bacterial consortia



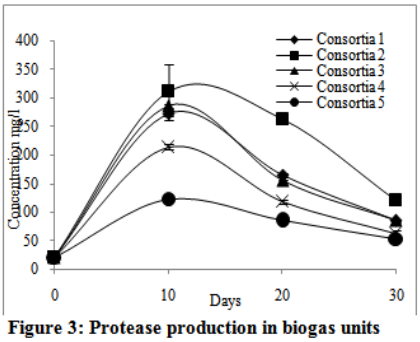


Figure 3: Protease production in biogas units treated with different bacterial consortia

Figure 4: Lipase production in biogas units treated with different bacterial consortia

2.6. Volatile fatty acids production by different bacterial consortia

Various factors like biogas potential of feedstock, design of digester, inoculum, nature of substrate, pH, temperature, loading rate, hydraulic retention time (HRT), carbon nitrogen ratio, volatile fatty acids (VFA) etc. influence the biogas production [16]. Depending on the anaerobic microbial species present and reactor conditions subsequent pyruvic acid fermentation can lead to the production of a number of C1 to C4 compounds such as volatile fatty acids (VFAs) e.g., acetic, propionic and butyric acids, other organic acids (formic and lactic), alcohols, ketones, and aldehydes [7,8, 27,6]. The volatile fatty acids production (acetic acid, propionic acid, butyric acid, valeric acid) was optimum at 20th day of digestion, in consortium 3 treated biogas unit. This is due to the high acidogenic population. Among the VFA production the products were chiefly acetic acid (1700mg/l) followed by butyric acid (1399mg/l), propionic acid (750mg/l) and valeric acid (125mg/l) (Fig. 5, 6, 7 & 8). Parawira et al., 2004 [26] investigated the production of volatile fatty acids by anaerobic digestion of potato waste. After 300h digestion of potato waste the fermentation products were chiefly (mg $^{g-1}$ total VFAs) acetic acid (420), butyric acid (450), propionic acid (140) and caproic acid (90), with insignificant amounts of iso-butyric acid, n-valeric acid and iso-valeric acids.

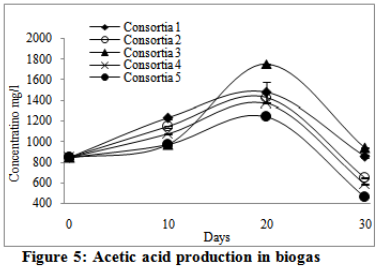


Figure 5: Acetic acid production in biogas treated with different bacterial consortia

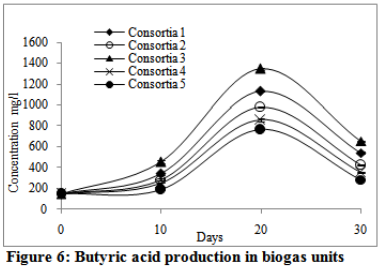


Figure 6: Butyric acid production in biogas units units treated with different bacterial consortia

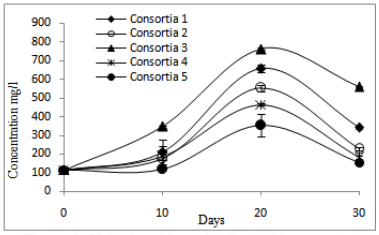


Figure 8: Valeric acid production in biogas units treated with different bacterial consortia units

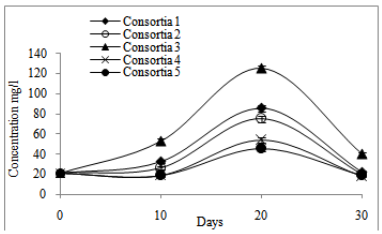


Figure 7: Propionic acid production in biogas

2.7. Biogas production by different bacterial consortia

The concentration and proportion of individual VFAs produced in the acidogenic stage is important in the overall performance of the anaerobic digestion system since, acetic and butyric acids are the preferred precursors for methane formation [11]. Bagi et al., 2007 [2] reported that microbial consortia added to the natural biogas producing microorganism brought about a significant increase in the biogas production at both mesophilic and thermophilic temperatures. Biogas production under the treatment of different bacterial consortia, the consortia 4 treated biogas unit gave utmost methane yield 79.45% (Fig.9) at 30th day of anaerobic digestion since it contains high amount of methanogenic bacteria. It showed that this microbial consortium aggressively involve in the transformation of complex high molecular weight organic compounds to methane. Sharda Dhadse et al., 2012 [34] examined methanogenic and non methanogenic bacterial consortia for maximum biogas production. They obtained maximum biogas production (methane 76%) in consortia "C" containing four different methanogenic bacteria when compare to other two bacterial consortia "A" & "B" containing facultative anaerobes.

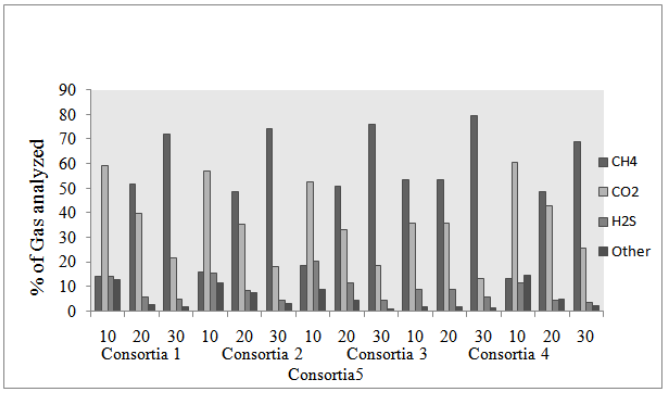


Figure 9: Biogas production under treatment of different bacterial consortia

IV. Conclusion

The experimental set up was carried out by using four bacterial consortia to find out the suitable consortia to be used for maximum biogas production. From the above study it was concluded that consortia 4 contains high concentration of methanogens gave maximum biogas production (79.45 % methane) when compared to other bacterial consortia.

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