MICROBIAL DYNAMICS DURING ANAEROBIC DIGESTION OF COW DUNG

P. Merlin Christy[*], L.R. Gopinath and D. Divya

Department of Biotechnology, Vivekanandha College of Arts and Sciences for Women, Tiruchengode- 637 205, Tamil Nadu, India.

Corresponding author: E.mail : pmerlinchristy@gmail.com Phone no: +91 7639493337.

ABSTRACT: The anaerobic digestion of cow dung was investigated for 30days in a laboratory scale batch reactor. Totally nine bacterial strains were isolated from cow dung slurry. The bacterial isolates were preliminarily examined by morphological characteristics and they were further confirmed by 16S rRNA sequencing and analyzed through BLAST. Microbial dynamics showed that hydrolytic bacterial population was initially increased and decreased towards the end of anaerobic digestion. But in methanogenic population, they were initially very low and increased gradually towards the end of anaerobic digestion. In Ruminococcus species the population was observed in fluctuation. The total solid (TS), volatile solid (VS) and chemical oxygen demand (COD) removals amounted to 46.12%, 43.14% and 45.23% respectively. Anaerobic digestion was feasible with pH about 7.17, temperature at 37.18^0C and maximum methane yield was about 64% at 30th day of digestion.
Keywords: Anaerobic digestion, Cow dung, Microbial dynamics, Population, Methane yield.

INTRODUCTION

Millions of tons of solid waste are generated each year from municipal, industrial and agricultural sources. Unmanaged organic waste fractions from farming, industry and municipalities decompose in the environment, resulting in large-scale contamination of land, water and air. These wastes not only represent a threat to environmental quality, but also possess a potential energy value that is not fully utilized despite the fact that they are cheap and abundant in most parts of the world. Anaerobic digestion is a suitable technology to treat the solid waste, waste water and it has been considered as a waste to energy technology. Anaerobic digestion consists of several interdependent, complex sequential and parallel biological reactions, during which the products from one group of microorganisms serve as the substrates for the next, resulting in transformation of organic matter mainly into a mixture of methane and carbon dioxide with minor quantities of nitrogen, hydrogen, ammonia and hydrogen sulfide [21,23,17]. In nature this process occurs in environments such as hot springs, swamps, paddy fields, lakes and oceans and the intestinal tract of animals [15]. The application of the anaerobic treatment process in waste management includes septic tanks, sludge digesters, industrial wastewater treatment, municipal wastewater treatment, hazardous waste management (aromatic and halogenated compounds), and agricultural waste management.

Methane fermentation is a complex process, which can be divided up into four phases: hydrolysis, acidogenesis, acetogenesis and methanogenesis. The degradation steps are carried out by different consortia of microorganisms, which partly stand in syntrophic interrelation and place different requirements on the environment [2]. The first group of microorganisms consist the hydrolytic bacteria. These organisms hydrolyses polymeric materials to monomers such as glucose and amino acids through extracellular hydrolytic enzymes (cellulase, xylanase, amylase, protease, lipase) they excrete.

A complex consortium of microorganisms participates in the hydrolysis and fermentation of organic material. Most of the bacteria are strict anaerobes such as Bacteriodes, 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*. 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 a 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 [27]. The environmental factors that are important in the process of anaerobic digestion include temperature, pH and buffering systems, retention time, process configuration, solubility of gases, availability of nutrients and the presence of toxic components in the process [7, 11, 24]. In the present study microbial dynamics, pH, temperature, biogas production and organic materials removal has been investigated for 30days in an anaerobic decomposing condition using cow dung as a substrate.

MATERIALS AND METHODS

Experimental set up and sample collection

The experiment was conducted in a pilot scale batch reactor. The capacity of the reactor was 20L. The reactor was equipped with inlet, outlet, thermometer, pressure gauge (Figure 1). Fresh cow dung samples were collected from the Feedlot slaughterhouse in Tiruchengode. The cow dung was blended with water at the ratio1:2 and loaded in to biogas unit. Anaerobic Digestion was carried out for 30 days. Samples from the reactor collected in the interval of 10 days (0, 10, 20, 30th days) for analysis.

Isolation of bacterial colonies from cow dung slurry

The cow dung samples from the biogas unit collected and homogenized. One gram of sample was dissolved in double distilled water and serially diluted up to 10^{-8}. Hydrolytic bacteria were isolated on nutrient agar, an acetogenic bacterium was isolated on basal medium [6] and methanogenic archaea were isolated on enrichment medium [14] by pour plate method. For the isolation of anaerobic bacteria the plates were kept in anaerobic chamber. After 48 hours of incubation the colonies seen on the medium were selected for microbial identification.

Identification of bacterial isolates

Morphological identification

Morphological characteristics examined include gram's staining, motility and shape for the preliminary identification of bacterial strains.

Molecular identification of bacterial isolates

16s rRNA sequencing was done for the identification bacterial isolates. The genomic DNA from the bacterial strains was isolated as per Sambrook *et al*. method [26]. The V3 and V4 regions of 16s rRNA were amplified with universal bacterial primers (341F, 5'-CCTACGGGAGGCAGCAG-3' with a GC clamp and 907R, 5'-CCGTCAATTCMTTTGAGTTT-3') and universal archaeal primers (A357F, 5'-CCCTACGGGGCGCAGCAG-3' with a GC clamp and A693R, 5'– GGATTACARGATTTC -3'). The amplified DNA fragments were separated on 1.5% agoarose gel. The amplicons were eluted from the gel using a Qiaquick gel extraction kit (Qiagen, Germany). The purified PCR products were sequenced using both the forward and reverse primers. The rRNA sequences were determined by the dideoxy Chanin-termination method using Big-Dye terminator kit using ABI 310 Genetic Analyzer (Applied Biosystems, USA). 16s rRNA sequences of the bacterial strains were deposited in the GenBank and analyzed using the BLAST program in GenBank at National Center for Biotechnology Information http://www.ncbi.nlm.gov/BLAST.

Analytical methods

The physico-chemical parameters analyzed were temperature, pH, Total solids (TS), Volatile solids (VS) and Chemical Oxygen Demand (COD) according to the standard methods [3]. The pH was measured by SUNTEX pH meter (SP-701). The temperature was measured by a thermometer.

Enumeration of bacterial population

The populations of hydrolytic bacteria, hydrogen producing acetogens (based on butyrate catabolism) and acetate, H_2 – CO_2 utilizing methanogens were enumerated by most probable number (MPN) technique.

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.

RESULTS AND DISCUSSION
Isolation and identification of bacterial isolates
Morphological identification

Totally nine bacterial species were isolated from cow dung slurry. Morphological studies showed that four isolates were gram positive and motile, five gram negative and non motile. The habitat diversity showed that one obligate aerobic, one facultative anaerobic and seven obligate anaerobic (Table 1). The cell of gram positive bacteria has a thick peptidoglycan and it cross linked with bacterial enzyme transpeptidase. It acts upon the proteins and peptides into amino acids, which is the part of hydrolysis phase generally hydrolysis occur in the presence of oxygen if the organism being strictly anaerobe would use hydrogen as an electron donor for supporting the hydrolysis phase. Gram negative bacteria contain thin peptidoglycan layer lacking enzyme transpeptidase therefore they depend upon the other bacteria in the anaerobic system. Methanogens lack peptidoglycan, a polymer that is found in the cell walls of the Bacteria but not in those of Archaea. Some methanogens have a cell wall that is composed of pseudopeptidoglycan. Other methanogens do not, but have at least one paracrystalline array made up of proteins that fit together like a jigsaw puzzle.

Molecular identification

16s rRNA gene sequencing analysis revealed that bacterial isolates were *Acetobacter syzygii, Bacteroides nordii, Clostridium perfringens, Methanobacterium formicicum, Lactobacillus acidophilus, Methanosarcina siciliae, Prevotella bivia, Porphyromonas asaccharolytica, Ruminococcus gnavus*. From the phylogenetic analysis of the representative bacterial clones, the micro-organisms in the phyla Firmicutes, Bacteroides, Proteobacteria and Euryarchaeota were observed (Table 2). Among them, the phylum Firmicutes and Bacteroidetes were dominant group and within the phylum Firmicutes and Bacteroidetes, class Clostridia and Bacteroidia were the dominant bacterial community. Microorganisms within the class Clostridia and Bacteroidetes have been frequently reported to be important throughout various anaerobic habitats and have the ability to degrade a wide variety of complex organic molecules, including proteins and carbohydrates. Clostridum and Bacteroides species isolated from rumen, digesters and natural habitats hydrolyze cellulose, hemi-cellulose and protein to produce VFAs, alcohol, CO_2 and H_2 [1].

Physico-chemical parameters

pH and temperature dynamics during biogas production

Alkalinity is an important parameter in anaerobic digestion because it provides enough buffering capacity to neutralize any possible volatile fatty acids accumulation in the reactor and to maintain pH around 6.7 to 7.4 for stable operation [8]. The anaerobic degradation process is highly pH dependent because each of the microbial groups involved in the reactions has a specific pH range for optimal growth. The aspects influenced by pH include utilization of carbon and energy sources, efficiency of substrate dissimilation, synthesis of proteins and various types of storage material and the release of metabolic products from the cell [12]. The pH, temperature dynamics of the anaerobic digestion showed increasing or decreasing trends for 30 days. The initial pH during the study was around 7.11 which decreased up to 5.78 and increased after 20th day and reached 7.17 at 30th day (Figure 2). The initial decrease in pH is due to acid formation during hydrolysis and the later increase is owing to the production of volatile fatty acids and ammonia. The pH of acid-forming bacteria is around 6, while for the optimal pH for the methane-producing microbes is 6.8-7.2. The growth rate of methanogenic microbes decreases sharply below pH 6.6 [19].

The anaerobic degradation process is strongly influenced by temperature. Anaerobic digestion reactors are normally operated within the mesophilic (20-42^{0}C) and thermophilic ranges (42-75^{0}C) [29]. The hydrolysis and acidogenesis processes are not significantly affected by temperature, but he acetogenesis and methanogenesis stages are carried out by fewer specialized species of microorganisms are more sensitive to temperature. The temperature in the biogas unit ranges from 32-37^{0}C (Figure 2). The temperature fluctuations in the biogas unit showed always higher inside the unit than the surrounding atmospheric temperature, which shows the exothermic metabolism inside the unit. The temperature inside the digester has a major effect on the biogas production process.

Total Solid, Volatile Solid and Chemical Oxygen Demand dynamics during biogas production

Anaerobic digestion stabilizes the organic matter in wastewater solids and reduces pathogens, odors, total solids by converting part of the volatile solids fraction into biogas [18].

TS and VS destruction is a vital aspect in evaluating anaerobic digestion performance because the percentage of VS removal was increased as cumulative methane production was increased [1]. The TS degradation during anaerobic digestion of cow dung was about 46.12%, VS removal was 43.14% and COD removal efficiency was 45.23% (Figure 3).

Table 1. Morphological characteristics of bacterial isolates

Strain no.	Shape	Motility	Gram staining	Habitat
VBT23	Rod	Motile or non motile	Negative	Obligate aerobic
MC	Rod	Non motile	Negative	Obligate anaerobic
MG1	Rod	Non motile	Positive	Obligate anaerobic
MG2	Rod	Non motile	Positive	Obligate anaerobic
MG3	Rod	Non motile	Positive	Facultative anaerobic
MG4	Cocci	Non motile	Positive	Obligate anaerobic
MG5	Rod	Non motile	Negative	Obligate anaerobic
MG6	Rod	Non motile	Negative	Obligate anaerobic
MG7	Cocci	Non motile	Positive	Obligate anaerobic

Table 2. Bacterial species present in the biogas reactor compared by BLAST with NCBI

Strain no.	Accession no.	Identified organism	Closest sequence	Taxonomy	Ident %
VBT23	KJ000008	*Acetobacter syzygii*	Acetobacter syzygii gene strain NBRC 16604	Proteobacteria	99
MC	KF999872	*Bacteroides nordii*	Bacteroides nordii strain H6	Bacteroidetes	98
MG1	KF999873	*Clostridium perfringens*	Uncultured organism clone ELU0163-T374-SNIPCRAMgANa_000178	Firmicutes	98
MG2	KF999874	*Methanobacterium formicicum*	Methanobacterium formicicum strain TAF1	Euryarchaeota	98
MG3	KF999875	*Lactobacillus acidophilus*	Lactobacillus acidophilus strain NX2-6	Firmicutes	98
MG4	KF999876	*Methanosarcina siciliae*	Methanosarcina siciliae strain BEGN2	Euryarchaeota	99
MG5	KF999877	*Prevotella bivia*	Prevotella bivia strain JCM 6331	Bacteroidetes	99
MG6	KF999878	*Porphyromonas asaccharolytica*	Porphyromonas asaccharolytica strain JCM 6326	Bacteroidetes	98
MG7	KF999879	*Ruminococcus gnavus*	Ruminococcus gnavus strain A2	Firmicutes	99

Fig: 1 Biogas unit

Due to the abundance of anaerobic bacteria, they produce ammonia, H_2S, H_2 and ferment carbohydrates into acetate, butyrate, ethanol, CO_2 and H_2. Therefore, the higher TS and VS reduction was occurred. EL-Mashad and Zhang [13] reported that biogas production increase with an increase in COD removal and VS reduction, which can be explained by the fact that the methanogenic consortium acclimated very well and consequently leads to the digestion of organic matter (COD) and volatile solid (VS) under anaerobic condition.

Physical hindrance caused by accumulation of inorganic matter inside the bioreactor is determined by the VS/TS ratio. Chae *et al*. [9] reported that the VS/TS ratio of digester is an excellent indicator to ascertain the accumulation of unwanted materials and the adequacy of mixing system employed. VS/TS ratio during the anaerobic digestion was 93.53%. The highest VS/ TS ration indicates that the biogas reactor in under sufficient mixing.
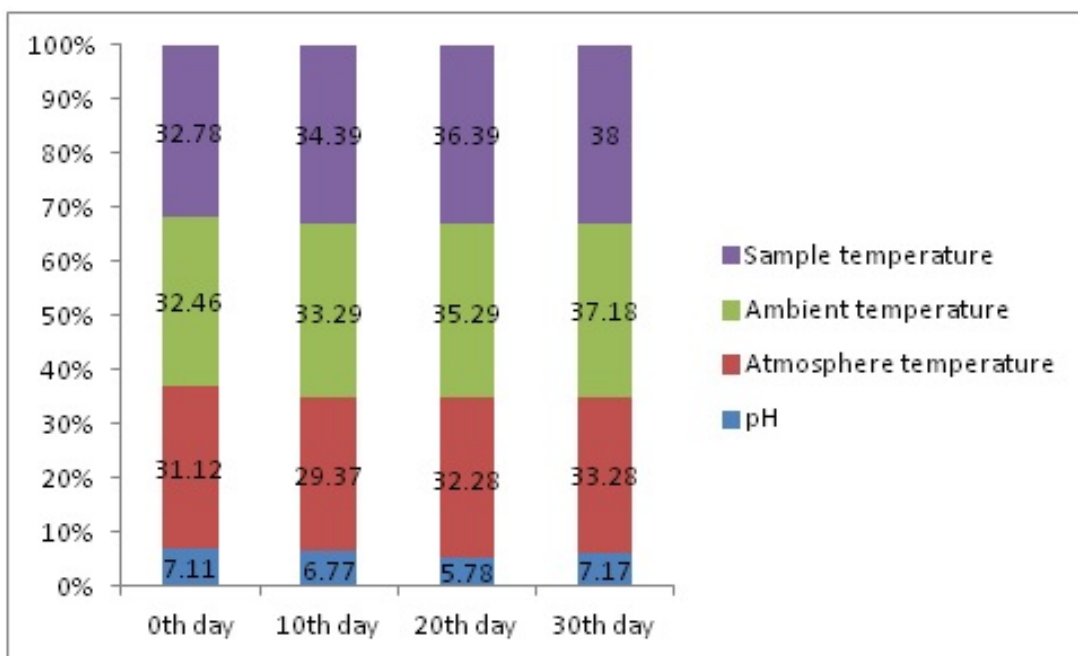

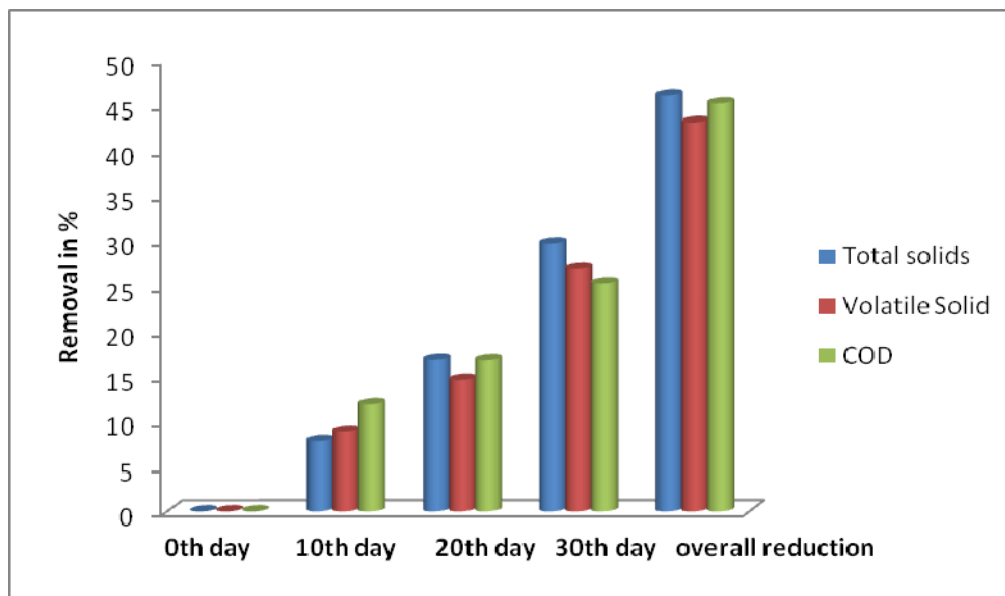**Fig: 2 Temperature and pH dynamics during biogas production**
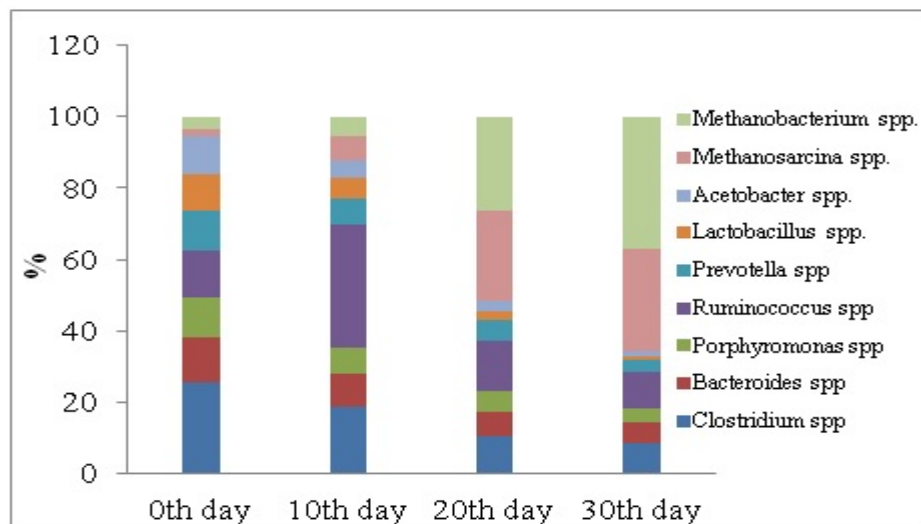

Fig: 3 TS, VS and COD dynamics during biogas production

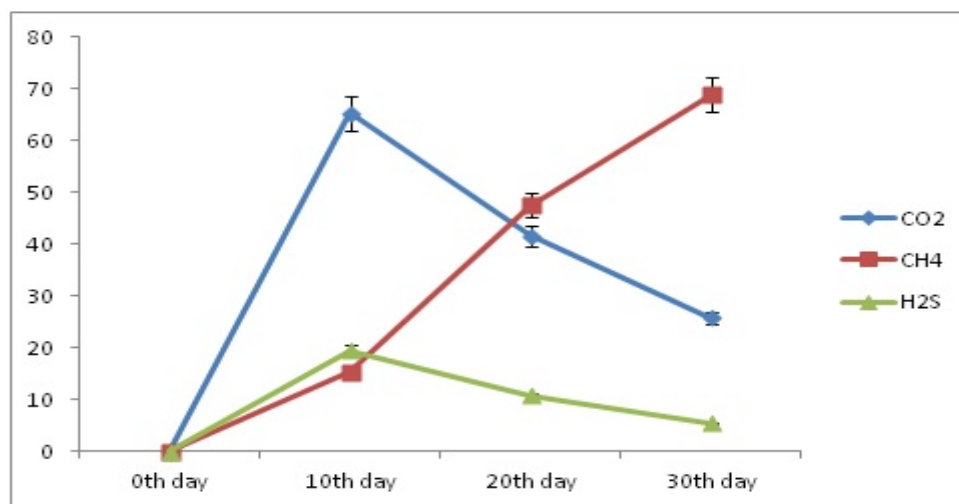**Fig: 4 Microbial dynamics during biogas production**

Fig: 5 Biogas dynamics during anaerobic digestion

Microbial dynamics during biogas production

Ramasamy *et al*. [25] stated that microbial diversity in biogas digesters is as great as that of rumen and he observed a clear differentiation existed in the type of cellulolytic bacterial distribution in the rumen and biogas digester. In rumen, Ruminococcus sp. alone accounted for 60% of the total population, whereas in biogas digester the predominant species belonged to the genera Bacteriodes and Clostridium rather than Ruminococcus. Later he reported that *Ruminococcus flavefaciens, Eubacterium cellulosolvens, Clostridium cellulosolvens, Clostridium cellulovorans, Clostridium thermocellum, Bacteroides cellulosolvens* and *Acetivibrio cellulolyticus* were some of the other predominant fermentative bacteria present in cattle dung-fed digesters. Sharda *et al*. [28] isolated four methanogenic and four non-methanogenic bacterial species (*Methanobrevibacter ruminantium, Propionibacterium, Methanobacterium formicicum, Bacteroides, Methanosarcina frisia, Peptostreptococcus, Methanothrix soehngenii, Clostridium)* from the biogas slurry prepared from cow dung.

The nature of the substrate determines the type and extent of the fermentative bacteria present in the digester. Totally nine bacterial species were isolated from cow dung in an anaerobic decomposing condition. Out of nine bacterial isolates six isolates were hydrolytic bacteria (*Bacteroides nordii, Clostridium perfringens, Prevotella bivia, Porphyromonas asaccharolytica*, *Ruminococcus gnavus*, *Lactobacillus acidophilus*) two isolates were methanogenic archaea (*Methanobacterium formicicum* and *Methanosarcina siciliae*) and one isolate was acetogenic bacteria (*Acetobacter syzygii*).

The microbial dynamics showed that Clostridium, Bacteriodes, Porphyromonas, Prevotella, Lactobacillus population were found to be high at 0th day and decreased progressively towards the end of the day (Figure 4). Because these are hydrolytic bacteria involve in the depolymerization of carbohydrates, proteins and lipids into monomers by their extracellular enzymes. So their population was seen high at hydrolytic stage. Cirne *et al*. [10] reported that during hydrolysis complex insoluble substrate such as polysaccharides are hydolysed into smaller units by a large number of hydrolytic microorganisms such as Clostridia, Micrococci, Bacteroides, Butyrivibrio, Fusobacterium, Selenomonas, and Streptococcus by secreting different hydrolyzing enzymes like cellulase, cellobiase, xylanase, amylase, protease and lipase. The population of acetogenic bacterium was gradually decreased towards the end of anaerobic digestion. The population of acetogenic bacteria should be high in its respective phase ie. acetogenic phase (20th day) but its population was negligible at 20th day (Figure 4). This may due to its slow-growing nature, sensitive to fluctuations in organic loads and environmental changes, and long lag periods are likely to be required for these bacteria to adjust to new environmental conditions [30]. The population of Methanosarcina and Methanobacterium species was very low up to 10th day and their population was stabilized at 20th day and rich at 30th day (Figure 4). This may due to initial adjustment in the microbial population from aerobic to anaerobic condition because methane is produced as a metabolic byproduct in anoxic conditions. *Methanobacterium formicicum* belongs to hydrogenotrophic methanogen and it use H_2-CO_2 or formate as a substrate for growth. *Methanosarcina siciliae* belongs to aceticlastic methanogen and it utilizes acetate as a source of energy. Methanogens are obligate anaerobes and very sensitive to environmental changes. Among the methanogenic population, the population of *Methanobacterium formicicum* was found to be high compared to the population of *Methanosarcina siciliae,* because hydrogen-utilizing methanogens have been found to be more resistant to environmental changes than aceticlastic methanogens therefore, methanogenesis from acetate has been shown to be rate limiting in several cases of anaerobic treatment of easily hydrolysable waste [4, 19]. The high diversity and dynamic activity of methanogens is favorable for maintaining the efficiency of the anaerobic digestion process. In Rumicoccus species the population was initially (0th day) low and got peak at 10th day and gradually decreased to the end of anaerobic digestion. Except Rumonococcus species, the population of other bacterial species were gradually increased or decreased from 0-30th day of anaerobic digestion. However, increase or decrease in bacterial population happened only after the 10th day of anaerobic digestion.

Biogas dynamics during anaerobic digestion

Anaerobic digestion consists of several interdependent, complex sequential and parallel biological reactions during which the products from one group of microorganisms serve as the substrates for the next, resulting in transformation of organic matter mainly into a mixture of methane and carbon dioxide [22]. Baba *et al*. [5] reported that cow dung is an effective feedstock for anaerobic digestion and could significantly enhance the cumulative biogas production. Considerable amount of anaerobic bacteria in the cow dung functions effectively to degrade the organic fraction from cattle manure even though pH was unregulated. Biogas dynamics during anaerobic digestion showed that the average percentage of CO_2 emission was high with 75% followed by methane 18%. This scenario changed after 20 days when the gas pressure stabilized with increasing emission of methane (65%) (Figure 5). The biogas production was initially low at 10th day due to the lag phase of methanogenic population and later it was stabilized at 20th day and gave peak value at 30th day because of the exponential growth of methanogens. The biogas production rate in batch condition is directly equal to specific growth of methanogenic bacteria [20]. Gopinath *et al.* [16] reported that treatment of different microbial consortia, the consortia 4 treated biogas unit gave utmost methane yield 79.45% at 30th day of anaerobic digestion since it contains high amount of methanogenic archaea.

CONCLUSION

The present work was carried out to exhibit the microbial dynamics during anaerobic digestion using cow dung as a substrate. Except *Acetobacter syzygii* the populations of other bacterial species were dominant at their respective phases. The pH decreases with acidogenic reactions and when the evolution of methane increases the pH increase, which is also reflected in the temperature. TS, VS and COD were reduced with increasing time. The emission of CO_2 was high at initial stage, which was reduced later with increasing release of CH_4.

ACKNOWLEDGEMENTS

REFERENCES

[1]   Ajay Kumar, J., Jianzheng, L., Qiaoying, B., Liguo, Z., Bowei, Z. 2012. Dry Anaerobic Digestion of Cow Dung for Methane Production: Effect of Mixing. Pakistan Journal of Biological Sciences 15, pp. 1111-1118.

[2]   Angelidaki, I., Ellegaard, L., Ahring, B.K. 1993. A mathematical model for dynamic simulation of anaerobic digestion of complex substrates: focusing on ammonia inhibition. Biotechnology and Bioengineering, 42, pp.159–166.

[3]   APHA., WEF., AWWA. 1995. Standard Methods for the Examination of Water and Wastewater, 19th ed. American Public Health Association, Washington, DC, USA.

[4]   Archer, D.B. 1983. The microbial basis of process control in methanogenic fermentation of soluble wastes. Enzyme and Microbial Technology, 5, pp.162-169.

[5]   Baba Shehu U. I. A., Nasir, I. 2012. Anaerobic digestion of cow dung for biogas production. ARPN Journal of Engineering and Applied Science, 7 (2), pp.169-172.

[6]   Balch, W.E., Schoberth, S., Tanner, R.S., Wolfe, R.S. 1977. *Acetobacterium*, a new genus of hydrogen-oxidizing, carbon Dioxide-Reducing, Anaerobic Bacteria. International Journal of Systemic Bacteriology, 27 (4), pp. 355-361.

[7]   Bjornsson, L., Murto, M., Mattiasson, B. 2000. Evaluation of parameters for monitoring an anaerobic co-digestion process. Applied Microbiology and Biotechnology, 54, pp. 844-849.

[8]   Callander, I.J., Barford, J.P. 1983. Recent Advances in Anaerobic Digestion Technology. Process Biochemistry, 18, pp. 24-30.

[9]   Chae, K.J., Yim, S.K., Choi, K.H., Park, W.K., Lim, K. 2002. Anaerobic digestion of swine manure: Sung-Hwan farm-scale biogas plant in Korea. 564-571.

[10]   Cirne, D.G., Lehtomaki, A., Bjornsson, L., Blackall, L.L. 2007. Hydrolysis and microbial community analyses in two-stage anaerobic digestion of energy crops. Journal of Applied Microbiology, 103, pp. 516-527.

[11]   Demirel, B., Yenigun, O. 2002. Two-phase anaerobic digestion processes: A Review. Journal of Chemical Technology and Biotechnology, 77, pp.743-755.

[12]   Elefsiniotis, P., Oldham, W. K. 1994b. Influence of pH on the acid-phase anaerobic digestion of primary sludge. Journal of Chemical Technology and Biotechnology, 60, pp. 89-96.

[13]   El-Mashad, H.M., and Zhang, R. 2010. Biogas production from Co-digestion of dairy manure and food waste. Bioresource Technology, 101, pp. 4021-4028.

[14]   Ferry, J.G., Smith, P.H., Wolfe, R.S. 1974. *Methanospirillum*, a New Genus of Methanogenic Bacteria, and Characterization of *Methanospirillum hungatii* sp.nov. International Journal of Systematic Bacteriology, 24, pp.465-469.

[15]   Garcia, J. L., Patel, B.K.C., Ollivier, B. 2000. Taxonomic, phylogenetic, and ecological diversity of methanogenic Archaea. Anaerobe, 6, 205-226.

[16]   Gopinath, L.R., Merlin Christy, P., Mahesh, K., Buvaneswari, R., Divya, D. 2014. Identification and Evaluation of Effective Bacterial Consortia for Efficient Biogas Production. IOSR Journal of Environmental Science, Toxicology and Food Technology, 8 (3), pp.80-86.

[17]   Gujer, W., Zehnder, A.J. B. 1983. Conversion processes in anaerobic digestion. Water Science and Technology, 15, pp.127-167.

[18]   Merlin Christy, P., Gopinath, L.R., Divya, D. 2014. A review on anaerobic decomposition and enhancement of biogas production through enzymes and microorganisms. Renewable and Sustainable Energy Reviews, 34, 167-173.

[19]   Mosey, F.E., Fernandes, X.A. 1989. Patterns of hydrogen in biogas from the anaerobic digestion of milk sugars. Water Science and Technology, 21, pp.187-196.

[20]   Nopharatana, A., Pullammanappallil, P.C., Clarke, W.P. 2007. Kinetic and dynamic modelling of batch anaerobic digestion of municipal solid waste in a stirred reactor. Waste Management, 27, pp.595-603.

[21]   Noykova, N., Muller, T.G., Gyllenberg, M., Timmer, J. 2002. Quantitative analysis of anaerobic wastewater treatment processes: Identifiability and Parameter Estimation. Biotechnology and Bioengineering, 78, pp. 89-103.

[22]   Parawira, W. 2004. Anaerobic treatment of agricultural residues and waste water application of high-rate reactors. Doctoral Dissertation. Sweden, Department of Biotechnology, Lund University.

[23]   Pavlostathis, S.G., Giraldo-Gomez, E. 1991. Kinetics of anaerobic treatment. Water Science Technology, 24, pp.35-59.

[24]   Rajeshwari, K.V., Balakrishnan, M., Kansal, A., Kusum Lata., Kishore, V.V.N. 2000. Stateof-the-art of anaerobic digestion technology for industrial wastewater treatment. Renewable and Sustainable Energy Reviews, 4, pp.135-156.

[25]   Ramasamy, K., Nagamani, B., Sahul, H. 1990. Biogas production technology. Tech Bull; Fermentation Laboratory, Tamil Nadu Agricultural University, Coimbatore. 1, pp.91.

[26]   Sambrook, J., Fritsch, E. F., Maniatis, T. 1989. Molecular Cloning: A Laboratory Manual, Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

[27]   Schink, B. 1997. Energetics of syntrophic cooperation in methanogenic degradation. *Microbiology and Molecular Biology Reviews,* 61, 262–280.

[28]   Sharda, D., Kankal, N.C, Bharti K. 2012. Study of diverse methanogenic and non methanogenic bacteria used for the enhancement of biogas production. International Journal of Life Sciences Biotechnology and Pharma Research, 1 (2), pp.176-191.

[29]   Van Lier, J.B., Rebac, S., Lettinga, G. 1996. High rate anaerobic wastewater treatment under psychrophilic and thermophilic conditions. In: Proceedings of the IAWQ-NVA International Conference on advanced wastewater treatment, Amsterdam, 23-25 September.

[30]   Xing, J., Criddle, C., Hickey, R. 1997. Effects of a long-term periodic substrate perturbation on an anaerobic community, Water Research, 31, pp. 2195-2204.