Metagenomics of Bio Slurry Obtained From A Biogas Plant Using Next-Generation Dna Sequencing

Akubuenyi, F.C., [2]Odokuma, L.O., [3]Nwaokorie, F.O.
[1]*Cross River University of Technology, Calabar, Nigeria.* [2]*University of Port Harcourt, Nigeria,*[3]*University of Lagos, Nigeria.*

Abstract
Metagenomics of bioslurry obtained from biogas plant was carried out Next-Generation DNA sequencing. The DNA of the associated bacterial organisms was extracted using a ZYMO Research DNA extraction kit (Quick-gDNA™MiniPrep). They were sequenced by Next Generation Sequencing Technique to determine the nucleotide sequence of all microorganisms present in the sample using automated PCR cycle- Genome Sequencer™ FLX System from 454 Life Sciences™ and Roche Applied. Sequence analysis and alignment was performed using Vecton NTI suite 9 (InforMax, Inc.). The partial 16S rRNA gene sequences generated and subjected to BLAST analysis, and compared to GenBank database of the National Center for Biotechnology Information revealed the presence of Clostridium thermocellum, Clostridium tetani, Spirochaeta caldaria, Acinetobacter baumanni, Stenotrophomonas maltophilic, Prevotella ruminocola, Parabacterium distasonics, Clostridium cellulovorans, Mahella australiensis, Ethanoligenens harbinense, Odoribacter splanchnicus, Tanneralla forsythis, Clostridium stercorarium, Gramella forsetti, Geobacillus thermoleovorans Halibacterium modesticaldum and Veillonellaparvula, as the bacteria involved in biogas production. This implies that biogas production is mediated by these species of anaerobic bacteria.

Keywords: *Metagenomics, Bioslurry, Biogas plant, Next generation DNA sequencing*

INTRODUCTION

The disadvantages of the conventional Sanger sequencing technology, including its low throughput, high cost and operation difficulties, have limited its use in deeper and more complex genome analyses [1]. The recent introduction of Next Generation Sequencing (NGS) technology, with its high-throughput capacity and low cost, has largely overcome the problems, and the technology has been applied in various fields of life sciences, including forensics ([2], [3]), disease diagnosis [4], agrigenomics[5]and ancient DNA analysis [6]. Metagenomics is a guide from sampling to data analysis" and gives us a flow diagram of a typical metagenome project, containing the following steps: experimental design, sampling, sample fractionation, DNA extraction, DNA sequencing, assembly, binning, annotation, statistical analysis, data storage, metadata and data sharing [7].Using metagenomics, functional gene composition of microbial communities can be accessed [8]. Sequence comparison in metagenomics is done using BLAST, Megablast, BLAT and SSAHA. Then, analysis based on NCBI taxonomy is done.

Next Generation Sequencing and other new sequencing methods have led to three major improvements on the conventional technologies. First, they do not require bacterial cloning of DNA fragments; instead, they rely on the preparation of NGS libraries in a cell-free system. Second, instead of hundreds of sequencing reactions, they can parallelize the thousands-to-many millions of sequencing reaction. Third, the sequencing output is directly detected with no need for electrophoresis. The enormous number of reads generated by NGS enabled the sequencing of entire genomes at an unprecedented speed and thus it came to be widely used in various fields of life sciences.

The microbiology of biogas formation during anaerobic digestion of organicmatter is complex and involves interaction of different microorganisms. The first step of the digestion process involves the hydrolyses of the organic polymers of the substrate such as cellulose, other carbohydrates, proteins and lipids to low-molecular weight compounds, with Cellulolytic *Clostridia* and *Bacilli*bacteria playing important roles([9], [10], [11], [12]). Subsequently,

fermentative bacteria convertsthe low molecular weight metabolites into volatile fatty acids, alcohols, and other compounds which are then predominantly metabolized to acetate, carbon dioxide and hydrogen by syntrophic bacteria ([13], [14], [15], [16]). These compounds then serve as substrates for methanesynthesis which is mediated by methanogenic Archaea([17], [18]). Hydrogenotrophic Archaea reduces carbon dioxide tomethane using hydrogen as an electron donor, whereas aceticlasticArchaeaconverts acetate to methane ([19], 20]).

Firmicutes and *Methanomicrobiales* play a crucial role in hydrolysis, acetogenesis and methanogenesis representing key steps in anaerobic degradation of plant biomass [21]. However, additional taxa that were missed by previous studies, including members of the genera;*Streptococcus, Acetivibrio, Garciella, Tissierella,* and*Gelria* have been identified, which might also play a role in the fermentation process leading to the formation of methane [12]. Taking advantage of the CARMA feature to correlate taxonomic information of sequences with their assigned functions, it appeared that *Firmicutes*, followed by *Bacteroidetes* and *Proteobacteria*, dominate within the functional context of polysaccharide degradation whereas *Methanomicrobiales* represent the most abundant taxonomic group responsible for methane production [12].Leve´n*etal*. [22] reported the presence of *Firmicutes*(97.7%), *Bacteroidetes*(1.3%) and *Thermotogae*(1.0%) in the bioreactor fed with organic household waste.*Streptococcus* species were previously detected in different anaerobic habitats, especially in a mesophilic hydrogen-producing sludge and a glucose-fed methanogenic bioreactor ([23], [24]).

The contig sequences obtained from the metagenomics of biogas fermenter sample showed that *Methanoculleus* bacteria plays a dominant role in methanogenesis while *Clostridia* are important for hydrolysis of cellulosic plant biomass in a biogas fermenter [25]. Metagenome sequence data from a biogas-producing microbial community residing in a fermenter of a biogas plant provide the basis for a rational approach to improve the biotechnological process of biogas production [25]. Lee *et al*. [26] used 454 pyrosequencing of the V1, V2, and V3 regions of the 16S rRNA gene to assess the microbial community in seven full-scale reactors over time and observed that DNA sequences belonging to *Proteobacteria, Bacteroidetes, Firmicutes,* and *Chloroflexi*were the most abundant, and that the bacterial population was influenced by the digestion temperature.

The phenomenon of viable but non-culturable microorganisms, remain a great limitation in the enumeration and determination of the uncultured and genomicdiversity of microorganisms associated with the processes of biogas generation from domestic waste. This research seeks to add to that knowledge through the use of high throughput Next Generation Sequence technology.

MATERIALS AND METHODS

Sample Collection

The bioslurry was obtained from a spent biogas plant (Anaerobic digester) at the Microbiology Department of Cross River University of Technology, Calabar, Cross River State, Nigeria. The bioslurry sample was collected after 30 days of biogas production from the plant.

DNA Extraction

The DNA of the samples was extracted using a ZYMO Research DNA extraction kit (Quick-gDNA™MiniPrep). The procedure for extraction of the genomic DNA involved the addition of 4 volumes of Genomic Lysis Buffer to 1 volume of slurry sample (4:1) into a ZR Bashing Bead ™ Lysis Tube. The content of the tube was disrupted by mixing in a vortex mixer at maximum speed and allowed to stand at room temperature for 5 minutes.
The mixture was transferred to a Zymo-Spin ™ Column in a collection tube. It was centrifuged at 10,000 x g for 1 minute. The collection tube was discarded with the flow through.

The Zymo-Spin ™ column was transferred to a new collection tube. Two hundred microlitre (200µl) of DNA Pre-Wash Buffer was added to the spin column. The mixture was centrifuged at 10,000 x g for one minute. Five hundred microlitre (500 µl) of g-DNA Wash Buffer was added to the spin column. It was centrifuged at 10,000 x g for one minute.

The spin column was transferred to a clean 1.5ml microcentrifuge tube. Seventy microlitre (70 µl) DNA Elution Buffer was added to the spin column. This was incubated for 5 minutes at room temperature and then centrifuged at 10,000 x g for 30 seconds to elute the DNA. The elute DNA was transferred into a filter unit of Zymo-Spin ™ IV-HRC Spin Filter in a clean 1.5 ml microcentrifuge tube and centrifuge at exactly 8,000 x *g* for 1 minutes. The filtered DNA was the used for PCR and DNA sequencing.

DNA Amplification and Sequencing

DNA sequencing was performed by Next Generation Sequencing Technique to determine the nucleotide sequence of all microorganisms present in the sample using automated PCR cycle- Genome Sequencer™ FLX System from 454 Life Sciences™ and Roche Applied.Sequence analysis and alignment

was performed using Vecton NTI suite 9 (InforMax, Inc.). The partial 16S rRNA gene sequences generated with two primers set 27F: 5´-GAGTTTGATCCTGGCTCAG-3´ and 518R: 5´ -ATTACCGCGGCTGCTGG-3´ were subjected to BLAST analysis and sequences were compared to GenBank database of the National Center for Biotechnology Information (NCBI) ([27], [28]).

The partial 16S rRNA gene sequences generated with two primers set 27F: 5´-GAGTTTGATCCTGGCTCAG-3´ and 518R: 5´ -ATTACCGCGGCTGCTGG-3´ that were subjected to BLAST analysis and compared to GenBank database of the National Center for Biotechnology Information ([27], [28]) revealed the presence the following anaerobic organisms listed in Table 10.

RESULT

Table 1: BLAST analysis of 16SrRNA gene sequence obtained from the bioslurry and their taxonomic assignment

Sequence number	Sequence length	Ascension no.% of related microbe	identity	Identified E. value	Organism
1.	497	NC009012.1	88	1.00E-167	*Clostridiumthermocellum*
2.	527	NC004557.1	84	6.00E-96	*Clostridiumtetani*
3.	517	NC015732.1	76	2.00E-45	*Spirochaetacaldaria*
4.	524	NC009085.1	93	3.00E-174	*Acinetobacterbaumannii*
5.	531	NC010943.1	90	0.00E.00	*Stenotrophomonasmaltophilic*
6.	498	NC014033.1	87	2.00E-125	*Prevotellaruminocola*
7.	534	NC009615.1	80	5.00E-77	*Parabacteroidesdistasonics*
8.	493	NC014393.1	83	3.00E-119	*Clostridiumcellulovorans*
9.	512	NC009012.1	83	8.00E-130	*Clostridiumthermocellum*
10.	505	NC015520.1	83	2.00E-125	*Mahellaaustraliensis*
11.	490	NC014828.1	80	1.00E-93	*Ethanoligenensharbinense*
12.	518	NC015160.1	81	1.00E-107	*Odoribactersplanchnicus*
13.	529	NC015160.1	79	1.00E-97	*Odoribactersplanchnicus*
14.	519	NC015160.1	81	6.00E-111	*Odoribactersplanchnicus*
15.	526	NC016610.1	80	8.00E-100	*Tannerellaforsythis*
16.	531	NC015160.1	79	1.00E-107	*Odoribactersplanchnicus*
17.	507	NC020134.1	87	5.00E-156	*Clostridiumstercorarium*
18.	507	NC008571.1	78	8.00E-80	*Gramellaforsetti*
19.	522	NC016593.1	76	1.00E-47	*Geobacillusthermoleovorans*
20.	541	NC010337.2	77	2.00E-60	*Helibacteriummodesticaldum*
21.	507	NC013520.1	85	6.00E-116	*Veillonellaparvula*
22.	499	NC004557.1	98	0.00E+00	*Clostridiumtetani*
23.	525	NC004557.1	98	0.00E+00	*Clostridiumtetani*
24.	500	NC004557.1	98	0.00E+00	*Clostridiumtetani*
25.	499	NC004557.1	98	0.00E+00	*Clostridiumtetani*
26.	497	NC004557.1	98	0.00E+00	*Clostridiumtetani*
27.	487	NC016048.1	83	7.00E-115	*Oscilibactervalericigenes*
28.	505	NC013520.1	85	3.00E-119	*Veillonettaparvula*
29.	495	NC004557.1	97	0.00E+00	*Clostridiumtetani*
30.	498	NC004557.1	98	0.00E+00	*Clostridiumtetani*
31.	490	NC014828.1	86	2.00E-141	*Ethanoligenesharbinense*
32.	527	NC004557.1	97	0.00E+00	*Clostridiumtetani*
33.	502	NC004557.1	94	0.00E+00	*Clostridiumtetani*
34.	500	NC004557.1	96	0.00E+00	*Clostridiumtetani*
35.	508	NC009012.1	84	4.00E-132	*Clostridiumthermocellum*
36.	500	NC004557.1	98	0.00E+00	*Clostridiumtetani*
37.	503	NC004557.1	98	0.00E+00	*Clostridiumtetani*
38.	500	NC004557.1	98	0.00E+00	*Clostridiumtetani*
39.	508	NC016977.1	84	1.00-127	*Acidaminococcusintestine*
40.	493	NC009698.1	98	0.00E+00	*Clostridiumbotulinum*

DISCUSSION

The metagenomics of the bioslurry which revealed the presence of various microorganisms (*Clostridium thermocellum, Clostridium tetani, Spirochaeta caldaria, Acinetobacter baumanni, Stenotrophomonas maltophilic, Prevotella ruminocola, Parabacteriumdistasonics, Clostridium cellulovorans, Mahella australiensis, Ethanoligenens harbinense, Odoribacter splanchnicus, Tanneralla forsythis, Clostridium stercorarium, Gramellaforsetti, Geobacillus thermoleovorans Halibacterium modesticaldum* and *Veillonellaparvula*) associated with biogas production showed that biogas production is mediated by anaerobic organisms.

This finding agrees with the position of Wirth *et al.*[29], who reported that predominant microbes contributing to the decomposition of organic matter include members of *Eubacteria*, class *Clostridia*, order *Clostridales*, family *Clostridaceae* and the *bacteriodes.* They showed that among the *Clostridia*, *Clostridium thermocellum* occurred most frequently. This species can hydrolyze cellulose efficiently by means of its extracellular cellulases, which are organized into cellulosomes ([30], [31]). *Clostridium perfingens* generates lactate, acetate and butyrate from sugars, and through its [FeFe]-hydrogenase, it can also produce H_2[32]. Similarly to *C. thermocellum, C. cellulolyticum* is a well-known strain that degrades cellulose to acetate and evolves CO_2 and H_2[33]. In a related study, Sundberg*et al.*[34] observed similar organisms (*Firmicutes,Actinobacteria, Proteobacteria, Chloroflexi, Spirochete,* and*Euryarchaeo)* from the study of the microbial community of 21 full-scale biogas reactors using 454 pyrosequencing of 16S rRNA gene sequences. Similar results were obtained by parallel construction of 16S rRNA and mcrAampliconlibraries and subsequent sequencing of cloned fragments [35].

*Clostridium stercorarium*was also implicated as one of the bacteria associated with biogas production. Itis a ubiquitous, thermophilic bacterial species. It degrades polysaccharides in plant biomass and produces acetate, ethanol, CO_2, and H_2, as well as minor amounts of lactate and L-alanine [36]. *C. stercorarium*has been detected in thermophilic biogas plants, in which it plays a major role in plant biomass degradation [37]. A great number of hemicellulases, glycosidases, and esterases are produced by *C. stercorarium*and have been investigated and cloned ([38], [39], [40]). The presence of Clostridium tetani could be traced to the inclusion of cow dung as substrate. *C. tetani* is found mostly in warm, damp areas, especially in manure treated soil, but can also be found in the intestines or feces of many animals, such as horse, sheep, and dogs. The bacterium is known mainly for its pathogenicity, causing the disease called tetanus. It relies on the breakdown of amino acids (driven into the cell with the help of sodium ion pumps) by various enzymes into pyruvate. The pyruvate can then be fermented into lactate as well as converted into acetyl-CoA. In a related study, Akubuenyi and Achor [41] have revealed the presence of *Clostridium, Propionibacteria, Listeria and Erysipelothrix*from bioslurry.

The identification of *Stenotrophomonasmaltophilic*from the metanogenomic analysis of the slurry is in line with Wang *et al.* [42] which reported the role of *Stenotrophomonasmaltophilic* in lignocelluloses degradation and its positive effect on biogas production. In a related study, Assih*et al.* [43] reported the isolation of *Stenotrophomonas*spp from an upflow anaerobic sludge blanket (UASB) reactor. This result agrees with the position of Yan *et al.* [44] which reported a mesophiliclignocellulolytic microbial consortium BYND-5, that are efficient in enhancement of biogas production. The result indicated that the bacterial groups represented in the clone library were the *firmicutes* (5.96%), the *bacteroides* (40.0%), *Deferribacteres* (8.94%), *Proteobacteria* (16.17%), *Lentisphaerae* (2.13%), *Fibrobacteraceae* (1.7%) and uncultured bacterium (25.1%).

*P*arabacteroides*distasionis* isolated from the slurry is a Gram-negative, non-spore-forming bacterium that produces volatile organic acids [45].

CONCLUSION

The presence of all bacteria associated with bioslurry, a by-product of anaerobic digestion of biodegradation waste can be revealed when the bioslurry sample is subjected to Next-Generation DNA Sequencing. The metagenomic analysis showed that the process is mediated by facultative anaerobes and anaerobes.

ACKNOWLEDGEMENT

I remain indebted to Prof Lucky Odokuma for all his support and guidance throughout this research work. I sincerely acknowledge the efforts of DrMrsFlourenceNwaokorie of Molecular Biology Division, Nigeria Institute of Medical Research, Yaba, Lagos, for her guidance during the molecular analysis aspect of this research. To my beloved wife, Chioma, I say thank you for everything.

REFERENCES

[1] M.J. Fullwood, C.L. Wei, E.T. Liu, and Y. Ruan, Next-generation DNA sequencing of paired-end tags (PET) for transcriptome and genome analyses. Genome Res, vol. 19, pp. 521–32, 2009.

[2] Y. Yang, B. Xie and Y. Jan, Application of Next-generation Sequencing Technology in Forensic Science. Genomic Proteomics Bioinformatics, vol. 12, pp. 190–197, 2014.

[3] J. Weber-Lehmann, E. Schilling, G. Gradl, D.C. Richter, J. Wiehler and B. Rolf, Finding the needle in the haystack: differentiating ‘‘identical’’ twins in paternity testing and forensics by ultra-deep next generation sequencing. Forensic SciInt Genet, vol. 9, no. 42–6, 2014.

[4] J.J. McCarthy, H.L. McLeod and G.S. Ginsburg, Genomic medicine: a decade of successes, challenges, and opportunities. SciTransl Med, vol. 5, no. 189sr4, 2013.

[5] M.E. Goddard, and B.J. Hayes, Mapping genes for complex traits in domestic animals and their use in breeding programmes. Nat Rev Genet, vol. 10, pp. 381–91, 2009.

[6] H.N. Poinar, C. Schwarz, J. Qi, B. Shapiro, R.D. Macphee and B. BuiguesMetagenomics to paleogenomics: large-scale sequencing of mammoth DNA. Science, vol. 311, pp. 392–4, 2006.

[7] T. Thomas, J. Gilbert, and F. Meyer, "Metagenomics - A guide from sampling to data analysis". Microbial Informatics and Experimentation, vol. 2, no. 1, pp. 3, 2012.

[8] J. Qin, R. Li and J. Raes, et al., "A human gut microbial gene catalogue established by metagenomic sequencing". Nature, vol. 464, no. 7285, pp. 59-65, 2009.

[9] L. Lynd, P. Weimer, W. van Zyl, and I. Pretorius, Microbial cellulose utilization: fundamentals and biotechnology. Microbiology and molecular biology reviews, vol. 66 pp. 506–577, 2002

[10] E. Bayer, J. Belaich, Y. Shoham and R. Lamed, The cellulosomes: multienzyme machines for degradation of plant cell wall polysaccharides. Annual Review of Microbiology.vol. 58, pp. 521–54, 2004.

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

[12] S. Jaenicke, C. Ander, T. Bekel, R. Bisdorf, M. Droge, K. Gartemann, S. Junemann, O. Kaiser, L. Krause, F. Tille, M. Zakrzewski, A. Puhler, A. Schluter, A. Goesmann, Comparative and joint analysis of two metagenomic datasets from a biogas fermenter obtained by 454- Pyrosequencing. Plosone, vol. 6, no. 1, pp. 1-15, 2011.

[13] H. Drake, K. Kusel and C. Matthies, Ecological consequences of the phylogenetic and physiological diversities of acetogens.Antonie Van Leeuwenhoek, vol. 81 pp. 203–213. 2002

[14] H. Shin and J. Youn, (2005).Conversion of food waste into hydrogen by thermophilicacidogenesis.Biodegradation. 16: 33–44.

[15] M. Myint, N. Nirmalakhandan and R. Speece, Anaerobic fermentation of cattle manure: Modeling of hydrolysis and acidogenesis. WaterResearch, vol. 41, pp. 323–332, 2007.

[16] D. Sousa, H. Smidt, M. Alves, and A. Stams, Ecophysiology of syntrophic communities that degrade saturated and unsaturated long-chain fatty acids. FEMS Microbiology Ecology, vol. 68 pp. 257–272, 2009.

[17] U. Deppenmeier and V. Muller, Life close to the thermodynamic limit: how methanogenicarchaea conserve energy. Results and problems in cell differentiation, vol. 45, pp. 123–152, 2008.

[18] R. Thauer, A. Kaster, H. Seedorf, W. Buckel, and R. Hedderich, Methanogenicarchaea: ecologically relevant differences in energy conservation. NatureReviewsMicrobiology, vol. 6, pp. 579–591, 2008.

[19] U. Deppenmeier, The unique biochemistry of methanogenesis. Progress in nucleic acid research and molecular biology, vol. 71, pp. 223–283, 2002.

[20] Y. Liu, and W. Whitman, Metabolic, phylogenetic, and ecological diversity of the methanogenicarchaea. Annals of the New York Academy of Sciences, vol. 1125 pp. 171–189 2008.

[21] L. Krause, N. Diaz, R. Edwards, K. Gartemann, H. Kromeke, et al., Taxonomic composition and gene content of a methane-producing microbial community isolated from a biogas reactor. Journal of Biotechnology, vol. 136, pp. 91–101, 2008.

[22] L. Leve´n, A. R. B. Eriksson and A.Schnurer,Effect of process temperature on bacterial and archaeal communities in two methanogenic bioreactors treating organic household waste. FEMS Microbiol.Ecol., vol. 59, pp.683–693; 2007.

[23] S. Dollhopf, S. Hashsham, F. Dazzo, R. Hickey, C. Criddle et al., The impact of fermentative organisms on carbon flow in methanogenic systems under constant low-substrate conditions. Applied microbiology and biotechnology, Vol. 56, pp. 531–538, 2001.

[24] A. Fernandez, S. Hashsham, S. Dollhopf, L. Raskin, O. Glagoleva, et al., Flexible community structure correlates with stable community function in methanogenic bioreactor communities perturbed by glucose. Applied and Environmental Microbiology.vol. 66 pp. 40-58, 2000.

[25] N. Diaz, M. Dondrup, R. Eichenlaub, K. Gartemann, I. Krahn, L. Krause, H. Kromeke, O. Kruse, J.H. Mussgnug H. Neuweger, K. Niehaus, A. Puhler, K.J. Runte, R. Szczepanowski, A. Tauch, A. Tilker and A. Goesmann, The metagenome of a biogas-producing microbial community of a production-scale biogas plant fermenter analysed by the 454-pyrosequencing technology. Journal of Biotechnology, vol. 136 no., 1–2, pp. 77-90, 2008.

[26] S.H. Lee, H.J. Kang, Y.H. Lee, T.J. Lee, K. Han, Y. Choi and H.D. Park, Monitoring bacterial community structure and variability in time scale in full-scale anaerobic digesters. J Environ Monit, vol. 14, pp. 1893–1905, 2012.

[27] Z. Zhang, S. Schwartz, L. Wagner and W. Miller, A greedy algorithm for aligning DNA sequences. J. ComputBiol, vol. 7, no. 1-2, pp. 203-214, 2000.

[28] S.Altschul, W. Gish, W. Miller, E. Myers and D.J. Lipman, Basic Local Alignment Search Tool.J.Mol.Biol.,vol. 215, no. 3, pp. 403-410, 1990.

[29] R. Wirth, E. Kovács, G. Moráti, Z. Bagi, G. Rákhely and K. Kovács, Classification of a biogas-producing microbial community by short-read next generation DNA sequencing. Biotechnol.Biofuels, vol 5, no.41, pp. 1-16, 2012.

[30] V.V Zverlov, J. Kellermann and W.H. Schwarz, Functional subgenomics of Clostridiumthermocellumcellulosomal genes: identification of the major catalytic components in the extracellular complex and detection of three new enzymes. Proteomics, vol. 5, pp. 3646–3653, 2005.

[31] D.N. Gold and J.J.V. Martin, Global view of the Clostridiumthermocellumcellulosome revealed by quantitative proteomic analysis.J.Bacteriol, vol. 189 pp. 6787–6795. (2007).

[32] M. Kaji, Y. Taniguchi, O. Matsushita, S. Katayama, S. Miyata, S. Morita and A. Okabe, The hydA gene encoding the H_2 evolving hydrogenase of Clostridiumperfingens: molecular characterization and expression of the gene. FEMS MicrobiolLett, vol. 181, pp. 329–336, 1999.

[33] E. Guedon, M. Desvaux, and H. Petitdemange, Improvement of cellulolytic properties of Clostridiumcellulolyticum by metabolic engineering. Appl Environ Microbiol, vol.68, pp. 53–58, 2002.

[34] C. Sundberg, W.A. Al-Soud, M. Larsson, E. Alm, S.S. Yekta, B.H. Svensson, S.J. Sørensen, and A. Karlsson, 454 pyrosequencing analyses of bacterial and archaeal richness in 21 full-scale biogas digesters. FEMS MicrobiolEcol, vol. 85, pp. 612-626, 2013.

[35] M. Krober, T. Bekel, N. Diaz, A. Goesmann, S. Jaenicke, et al., Phylogenetic characterization of a biogas plant microbial community integrating clone library 16S-rDNA sequences and metagenome sequence data obtained by 454- pyrosequencing. Journal of Biotechnology, vol. 142 pp. 38–49, 2009.

[36] M.L. Fardeau, B. Olivier, J.L. Garcia, and B.K.C. Patel, Transfer of Thermobacteroidesleptospartumand Clostridium thermolacticumas Clostridium stercorariumsubsp. leptospartumsubsp. thermolacticumsubsp. nov., comb.

nov.andC. stercorariumsubsp. thermolacticumsubsp. nov., comb. nov.Int. J. SystEvol. Microbiol, Vol. 51, pp. 1127–1131, 2001.

[37] V.V. Zverlov, W. Hiegl, D.E. Köck, J. Kellermann, T. Köllmeier, and W.H. Schwarz, Hydrolytic bacteria in mesophilic and thermophilic degradation of plant biomass. Eng. Life Sci., vol. 10, pp. 528 –536,2010.

[38] J.A. Donaghy, K. Bronnenmeier, P.F. Soto-Kelly and A.M. McKay, Purification and characterization of an extracellular feruloyl esterase from the thermophilic anaerobe Clostridium stercorarium. J. Appl. Microbiol, vol. 88, pp. 458–466,2000.

[39] V.V. Zverlov, C. Hertel, K. Bronnenmeier, A. Hroch, J. Kellermann, and W.H. Schwarz, The thermostable alpha-L-rhamnosidaseRamA of Clostridium stercorarium: biochemical characterization and primary structure of a bacterial alfa-L-rhamnoside hydrolase, a new type of inverting glycosyl hydrolase. Mol. Microbiol, vol. 35, pp. 173–179,2000.

[40] V.V. Zverlov and W.H. Schwarz, Bacterial hydrolysis in anaerobic environmental subsystems—Clostridium thermocellumand Clostridium stercorarium, thermophilic plant-fiber degraders.Ann. N. Y. Acad. Sci., vol. 1125, pp.298–307,2008.

[41] F.C. Akubuenyi and S.A. Achor, The bacteriological index of bioslurry and the fate of pathogenic bacterial organisms during anaerobic digestion of domestic waste in a biogas plant.Microbiology Research Journal International.Vol. 25, pp. 1-9.

[42] W.D. Wang, Y.B. Song, Y.J. Wang, Y.M. Gao, R.Y. Jing, and Z.J. Cui, Biodiversity of mesophilic microbial community BYND 8 capability of lignocelluloses degradation and its effect on bigas production. Huan Jing KeXue, vol. 32, no. 1, pp. 253-8, 2011

[43] E.A. Assih, A.S. Ouattara, S. Thierry, J. Cayol, M. Labat and H. Macarie, Isolation of Stenotrophomonaspp from an upflow anaerobic sludge blanket (UASB) reactor. Intl. J. Sys. Evol.Microbiol, vol. 52, pp. 559-568, 2012.

[44] L. Yan, Y.Gao, Y. Wang, Q. Liu, Z. Sun, B. Fu, X. Wen, Z. Cui and W. Wang, Diversity of a mesophiliclignocellulolytic microbial consortium which is useful for enhancement of biogas production. Biores.Technol, vol. 111, pp. 49-54, 2012.

[45] M. Sakamoto and Y. Benno, Reclassification of Bacteroidesdistasionis,Bacteroidesgoldsteinii and Bacteroidesmerdae as Parabacteroidesdistasionis gen. nov.comb. nov.Parabacteroidesgoldsteinii comb.nov.andParabacteroidesmardae comb. nov.Int. J. Sys. Evolut.Microbiol.Vol. 56, pp. 1599–1605, 2006.

[46] F.C. Akubuenyi and S.A. Achor, The microbiological index of bioslurry and the fate of pathogenic bacterial organisms during anaerobic digestion of domestic waste in a biogas plant.Microbiology Research Journal International, vol. 25, pp. 1-9, 2018.

[47] S.G. Tringe, C. von Mering, and A. Kobayashi, et al., "Comparative metagenomics of microbial communities". Science, vol. 308, no. 5721, pp. 554–557, 2005.

[48] K.A. Weitz, S.A. Thorneloe, S.R Nishtala,S. Yarkosky and M. Zannes, The impact of municipal solid waste management on greenhouse gas emissions in the United States. J. Air Waste Manage. Assoc,vol.52 pp. 1000-1011, 2002