Isolation and presumptive identification of microbes in a typical fixed dome biodigester in Eastern Cape, South Africa

Mukumba Patrick1, Makaka Golden1 and Tazvinga Henerica2
1. University of Fort Hare, Physics Department, Private Bag X1314, Alice 5700, SOUTH AFRICA
2. Weather/Climate and Energy Research and Applications, Private Bag X197, Pretoria 0001, SOUTH AFRICA
*pmukumba@ufh.ac.za

Abstract
A research was undertaken to establish the typical microbial composition in a field based biodigester as a possible avenue to optimize the degradation process. A 4m³ digester was fed with cow dung. Sampling for microbial and biochemical analysis was done at three different stages of the study period; day 0, day 21 and day 42. It was critical to identify native microflora prior to digestion at the peak and end of digestion based on ideal retention period. Temperature, biogas yield and pH readings of cow dung were measured to comprehend and establish their influence on biogas formation. Based on the microorganism’s morphology and biochemical properties, the microbes were identified from Bergey’s Manual of Determinative Bacteriology and Bergey’s Manual of Systematic Bacteriology.

The research findings were dominated by firmicutes with the remainder being proteobacteria. Eight different pure colonies were isolated and the microorganisms suspected to be in the biodigester were: Bacillus cereus, Bacillus thuringiensis, Escherichia coli, Paenibacillus alvei, Enterobacter cloacae, Clostridium perfringens, Enterococcus faecalis and Klebsiella oxytoca. It is envisaged that the outcome of this research will help in the formulation of a starter culture to speed up the digestion startup process and troubleshooting efforts requiring microbial intervention.

Keywords: Biogas digester, cow dung, biogas, isolation, microbial composition.

Introduction
South Africa just like many developing countries is over dependent on convectional sources such as coal and firewood22. Increasing environmental concerns coupled with the high cost of electrical grid extension in far-fetched communities of South Africa imply that alternative sources of energy have to be explored17,18. The main biogas digesters available in South Africa are fixed dome, floating drum and balloon26. With a lifespan of at least twenty years, a fixed dome biodigester offers a solution that is not only affordable but sustainable and reliable25,31. These biodigesters are mostly applicable in cold climates such as Eastern Cape.

Anaerobic digestion is a process whereby microorganisms break down biomass wastes such as donkey dung in the complete absence of oxygen to produce biogas23. Biogas refers to the gas produced through microbial degradation of organic substances in the absence of oxygen.

Microorganisms are central for the formation of biogas; therefore, an appropriate environment must be established to ensure optimum yields30. The biogas process optimization is principally based on understanding the microbial community involved in the production chain. As noted by Mukumba et al19, use of microbial stimulants was one of the solutions to digester challenges in Eastern Cape, yet to date no work has been done to develop these starter cultures.

According to Manyi-Loh et al15 microbial species community tend to vary based on the physical and chemical factors despite the fact that they can perform degradation through the same four different stages. Put simply, the microbial community varies with substrate type, operational parameters and digester design16. Biogas is composed of methane and carbon dioxide as principal gases. There are also trace gases such as hydrogen, hydrogen sulphide, nitrogen, water vapor and siloxanes1,10.

Biogas provides clean energy for cooking, heating, lighting as well as fuel for vehicles and electrical generation when it is upgraded to biomethane21. Additionally, the effluent from the biodigester can also be used as nutrient-rich fertilizer29. Biogas production is a complex process due to the different reactions happening simultaneously with one reaction aiding or controlling the other. Bacteria-archaeal community working in syntrophy7,13 drives the reaction. The process is distinguished by four different stages namely hydrolysis, acidogenesis, acetogenesis and methanogenesis. Figure 1 shows biogas production process.

The ability to establish efficient and flexible processes in anaerobic digestion rests on understanding the diversity and function of various microorganisms that exist in a biodigester. This is essential for developing economies as it comes with lower implementation and running costs2. There are different types of biodigesters used in biogas production.
namely: industrial, balloon, floating drum and fixed dome biodigesters. Each type has its own pros and cons. However, the fixed dome type offers attractive advantages to project developers in developing countries such as long lifespan above 20 years, use of cheap available resources and stable temperature regulation.

Old fixed dome designs have double walls at the base whereas the latest version, a modified CARMATEC which is common in Africa is built using a single course throughout. They isolated and identified microorganisms by exposing them to variable temperature conditions and diverse feedstock in a laboratory scale biodigester which resulted in a shift and loss of the microbial community. For the archaeal group, results showed the dominance of Methanosarcina in all digester samples with Methanoculleus and Methanobacterium considered abundant at higher temperatures. The second group of microorganism: bacterial community had an antagonistic response to temperature increase. As the Bacteroidia decreased in relative abundance, there was an increase in Clostridia.

In another study, there was a change in the microbial community before and after digestion. Prior to digestion, the following bacteria were identified Proteus sp, Salmonella typhosa, Aerobacter cloacae, Escherichia coli, and B. subtilis. Clostridium perfringes and Salmonella typhimurium dominated the post fermentation substrate. It was also noted that the level of pathogenic microorganisms decreased, hence making the slurry an ideal and safe fertilizer replacement. The study also reported that biogas production is a function of time and microbial load.

Isolated one hundred and thirty-two bacteria were predominantly gram positive in a swine manure digester. They were classified under the following genera: Peptostreptococcus, Eubacterium, Bacteroides, Lactobacillus, Peptococcus, Clostridium and Streptococcus as well as two unidentified groups. Acetate was found as the sole end product of the several groups, hence acetoclastic methanogen was the major methane formation pathway.

Doloman et al. reported a varying microbial composition found in a UASB reactor fed with microalgal biomass. Samples were collected at different stages of anaerobic digestion. The DNA was extracted and subjected to next-generation sequencing using methanogen mcrA gene specific and universal bacterial primers. Bacteroidales, Pseudomonadales and Enterobacteriales were groups identified presumed responsible for substrate hydrolysis whereas the methanogenesis stage was dominated by Methanosarcina mazei.

Kirkegaard et al. studied the microbial community of large industrial scale digesters at 20 wastewater treatment plants over a six-year period. The sample from mesophilic digesters was found dominated by Euryachaeota phylum with Methanoseta as the most abundant genus followed by hydrogenoclastic methanogens genera such as Methanolinea, Methanospirillum, Methanobrevibacter and Candidatus Methanofastidiosa.

For the bacterial community, Chloroflexi was a dominant genera followed by Tetrasphaera and Candidatus Microthrix. Manyi-Loh et al. characterized microorganisms in a balloon-type digester fed with cattle manure and Firmicutes dominated findings with minor presence of Proteobacteria as well as Spirochaetes. Therefore, the microbial community in an underground fixed dome biodigester fed with fresh cow dung was investigated. This work will aid in the identification of microorganisms responsible for the production of biogas and can therefore be used to formulate commercial starter culture by industrialists or researchers. The use of commercial microbial stimulants will shorten start up time and also increase the biogas yields.

Material and Methods
An underground-modified Carmatec fixed dome biodigester was fed with cow dung. The Carmatec biodigester model was chosen for the study due to its low installation costs and better temperature regulations. The following materials were used: thermometer, Kern Mass balance, Platform Weighing scale (300kg), pH meter, 20L buckets, cooler box, McCartney bottle, test tubes, anaerobic jar and gas flow meter (JBD2.5-SA) used to measure biogas production.

Sample collection: A sample is a small portion which represents subjects under study. Slurry was aseptically collected from a biodigester using a sterile 200ml McCartney bottle. The bottle was first autoclaved before collection of the sample to ensure maximum sterility. The container was placed in a cooler box and immediately transported to the laboratory for analysis. Samples were collected at 3-day interval for 42 days.

Isolation and microbial population analysis: The media were prepared and inoculated following the Hungate technique of oxygen exclusion with some modifications. Nutrient broth and nutrient agar were used for the initial growth and screening of bacteria obtained from the biodigester. Upon arrival from sample collection, the workbench was prepared and disinfected using 70% alcohol. Serial dilutions were performed on the collected samples by taking 1g into a McCartney bottle containing 9ml of sterile distilled water (10⁻¹). The sample was shaken to homogenize the suspension. 1ml of aliquot was obtained from the 10⁻¹ using a sterile syringe and was added into another bottle containing 9ml distilled water to obtain 10⁻² dilution.

Similar dilutions were made until 10⁻⁸ was reached. For bacterial screening purposes, 10⁻⁴ to 10⁻² of the sample was inoculated onto already prepared nutrient broth. Anaerobic jar was used to incubate the bottles. Anaerobic Gas Pak was inserted in the anaerobic jar to eliminate residual oxygen. The jar was then incubated for a period of 24 – 48 hours at
35 °C. Streaking was done onto nutrient agar plates followed by incubation using the above conditions. In order to obtain pure bacterial isolates, the colonies were sub-cultured repeatedly on fresh plates of nutrient agar using streak plate method. Identification was done based on their morphological and biochemical characteristics.

**Results and Discussion**

**Morphological results:** Colony shapes and color were observed and recorded for all 8 samples using an electric light microscope. Most colonies showed cream colonies in nutrient agar. Table 1 shows morphological results of isolates.

<table>
<thead>
<tr>
<th>Isolate #</th>
<th>Morphology of isolates</th>
<th>Shape</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Ovoid in pairs</td>
<td>Coccus</td>
</tr>
<tr>
<td>2</td>
<td>Creamy white colonies</td>
<td>Rod</td>
</tr>
<tr>
<td>3</td>
<td>Large creamy white intact colonies</td>
<td>Rod</td>
</tr>
<tr>
<td>4</td>
<td>White, non-transparent colonies</td>
<td>Coccus</td>
</tr>
<tr>
<td>5</td>
<td>White colonies</td>
<td>Rod</td>
</tr>
<tr>
<td>6</td>
<td>Light white colonies</td>
<td>Rod</td>
</tr>
<tr>
<td>7</td>
<td>White</td>
<td>Rod</td>
</tr>
<tr>
<td>8</td>
<td>Cream colored colonies</td>
<td>Rod</td>
</tr>
</tbody>
</table>

**Table 1**

Showing morphological results of isolates

<table>
<thead>
<tr>
<th>Isolate #</th>
<th>Gram stain</th>
<th>Motility</th>
<th>Indole</th>
<th>VP</th>
<th>Citrate</th>
<th>Oxidase</th>
<th>Catalase</th>
<th>Gelatin hydrolysis</th>
<th>MR</th>
<th>Tentative Isolate</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td></td>
<td>Bacillus cereus&lt;sup&gt;a,b&lt;/sup&gt;</td>
</tr>
<tr>
<td>2</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td></td>
<td>Bacillus thuringiensis&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>3</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td></td>
<td>Clostridium perfringens&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>4</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td></td>
<td>Enterococcus faecalis&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>5</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
<td>Klebsiella oxytoca&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>6</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td>Paenibacillus alvei&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>7</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td></td>
<td>E. coli&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>8</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td></td>
<td>Enterobacter cloacae&lt;sup&gt;a,b,c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

**Legend:** VP- Voges Proskauer, MR- Methyl Red; a- Day 0; b- Day 21; c- Day 42; + Positive; -Negative

**Fig. 1:** Biogas production process (own source)
Biochemical results: Table 2 shows the biochemical test results of the isolates. Table 2 shows that gram positive was dominant as five out of eight isolates tested positive. Based on these results, it was evident that most of the microorganisms required for biogas production are mostly motile. Figure 2 shows pH and biogas production yield (m³) over the 42 day period. Figure 3 shows biogas yield (m³) and temperature readings over a 42 day period.

Microbial community identified concurs with the findings of Monyi-Loh et al16 which was dominated by Firmicutes. Proteobacteria was the second group of bacteria present. The results slightly differed on the major bacteria phylum found by other authors in which both Firmicutes and Bacteroidetes were repeatedly identified as major phyla33. Bacillus cereus is a facultative bacterium that converts glucose into lactate, succinate, acetate and ethanol6. Its existence in samples collected at day 0 and day 21 can be linked to the intermediate products of biogas production as shown in figure 1. Bacillus thuringiensis, a soil borne bacterium converts glucose into lactate under anaerobic conditions. It can carry out metabolism in aerobic conditions as well27.

Clostridium perfringens produces acetate from sugars as well as hydrogen through its high turnover [FeFe] – hydrogenase. Its ability to produce mentioned intermediates can be associated to its existence at day 21 and day 2112. At day 0, Enterococcus faecalis, Bacillus cereus and Klebsiella oxytoca were identified in which the former has a specific role in the early stages of biogas production. Enterococcus faecalis is known to hydrolyze plant polysaccharides. Fructose and lactose are converted to acetate and ethanol8,35. Production of biogas was high with increasing temperature. This trend agrees with the findings of Sibiya et al32.
Increasing temperature had an overall positive performance within the mesophilic range, as the biogas yield was high. Chances of collision between reacting molecules are increased when temperature rises and thus enhancing reaction rates. The pH appeared to be decreasing between the 3rd and 12th day as shown in figure 2 due to anaerobic fermentation.

Presence of Enterobacter cloacae at day 0 could be a contributing factor for low pH along with other undetectable microorganisms which produce a variety of acids from carbohydrates, lipids and proteins. The pH started shifting towards the neutral and the rate of biogas production increased sharply between the 9th and 18th day. The pH later decreased probably because of aceticlastic methanogens.

Rabah et al. noted that a pH between 6.8 and 7.2 was optimum for biogas production although we observed higher yields between pH 6.9 and 7.4. An increase in temperature was accompanied by increased biogas yield between the 6th and 18th day as shown in figure 3. A rise in temperature increases the biological activity of microbes present in the biodigester as evidenced by an increase in biogas yield. The drop in biogas yields after the 24th day, despite a corresponding temperature increase could be attributed to volatile fatty acids leading to acidification of the biodigester which inhibits biogas production.

Conclusion
The results obtained indicate that abattoir waste contains critical microorganisms responsible for an immediate plant start up. It was also noted that microbial diversity is related to the type of waste and operational conditions. The different intermediates produced by these microorganisms’ show syntrophic relationships that exist amongst them.

Identification of microbes responsible for degradation of substrates at different stages will enable optimization of the process leading to higher biogas yields and higher substrate degradation rates.

Acknowledgement
The authors wish to acknowledge the financial support from Govan Mbeki Research and Development Centre of the University Hare, South Africa.

References
17. Monyai P. and Chivanga S., Overcoming the household energy gap in South Africa, IMIESA, 45(6), 45-47 (2020)


29. Rowse L.E., Design of small-scale anaerobic digesters for application in rural developing countries, University of South Florida Scholar Commons graduate theses and dissertations (2019)


(Received 13th July 2020, accepted 28th September 2020)