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The Bacteriological Index of Bioslurry and the Fate of Pathogenic Bacterial Organisms during Anaerobic Digestion of Domestic Waste in a Biogas Plant

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Authors' contributions

This work was carried out in collaboration between the two authors. Author FCA designed the study, wrote the protocol, managed the literature searches and wrote the first draft of the manuscript. Author SAA performed the statistical analysis and managed the analyses of the study. Both authors read and approved the final manuscript.

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ABSTRACT

The bacteriological index of bioslurry and the fate of pathogenic bacteria during anaerobic digestion of domestic waste were determined. The wastes (food waste, vegetable waste, plantain peels, yam peels, and cow dung) were collected from households and markets within the Cross River University of Technology, Clalabar, Nigeria, and the analysis conducted at the Microbiology Department of the University. Bacteriological index was examined by enumerating the total heterotrophic bacterial (THB) count and bacterial diversity during the digestion period using viable count method on nutrient agar plate. The fate of pathogenic bacteria was analysed at 2 week interval for a period of 28 days using *Salmonella* and *Shigella* species as a case study. Results showed that the THB count decreased ($1.8x10^{10}$CFU/ml – $6.3x10^{8}$CFU/ml) over the 28 day period of anaerobic digestion. The isolation and identification of different bacterial species associated with anaerobic digestion of waste

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revealed the presence of aerobic, facultative anaerobic and anaerobic bacteria in Days 1, 14 and 28 respectively. *Pseudomonas* spp, *Bacillus* spp, *Lactobacillus* spp, *Klebsiella* spp, *Proteus* spp, *Escherichia coli and Staphylococcus* spp were among the organisms isolated on Day 1, which indicates that the initial microbial hydrolytic activities on the waste materials are mediated by aerobic and facultative anaerobic bacteria. The presence of *Staphylococcus* spp, *Enterococcus* spp, *Peptostreptococcus* spp, *Micrococcus* spp and *Fusobacterium* spp were present in the sample analysed on Day 14 showing that the digester was becoming anaerobic. Isolation of *Propionibacterium* spp, *Listeria* spp, *Erysipelothrix* spp and *Clostridium* spp on Day 28 showed that the digester has turned anaerobic, the stage at which biogas is produced. The result of the fate of pathogenic bacteria revealed that *Salmonella* and *Shigella* species decreased with time during the digestion process, with complete die off at Day 21. These indicate that anaerobic digestion enhances pathogen die off and could be applied as a waste treatment option in an integrated waste treatment management. A study on the metagenomics of the bioslurry will further reveal the uncultured and genomic diversity of associated microorganisms during anaerobic digestion.

Keywords: Bacteriological index; bioslurry; pathogenic bacteria; anaerobic digestion; domestic waste.

1. INTRODUCTION

Anaerobic digestion (AD) has given impetus to the search for renewable energy resources that could replace fossil fuels. It allows various organic waste materials and dedicated energy crops to be degraded and converted to a renewable energy carrier (biogas), and produces a nutrient-rich residue that can be used as fertiliser (biofertiliser) in agriculture [1,2,3]. Among various possible substrates for an economically feasible biogas production in Nigeria includes; domestic wastes, agricultural residues and sewage, water hyacinth, dung, urban refuse [4,2,5]. Pre-treatment of a substrate before anaerobic digestion increases biogas production and volatile solid reduction due to increased solubilisation [6,7].

Biogas refers to a gas produced by anaerobic digestion of biodegradable materials. It is mainly composed of Methane (CH_4), Carbon dioxide (CO_2) and other trace gases [8,9]. The efficacy of biogas can be better appreciated when it is cleaned and upgraded. The purification mainly consists of separation of water and hydrogen sulphide, and the upgrade consists of separation of carbon dioxide to raise the gas caloric value [8,9].

Biogas generation is mediated by microorganisms. The first step in the anaerobic digestion of complex organic substrates involves the breakdown of large molecules by hydrolysis [10,11]. Most of the bacteria belong in the classes of the *Clostridia* and *Bacilli*. The abundant species in biogas fermenter were members of the *Clostridia* (36%) and *Bacilli* (11%) classes, together with members of the *Bacteroidia* (3%), *Mollicutes* (3%), *Gammaproteo bacteria* (3%) and *Actino bacteria* (3%) classes [12]. Among the *Clostridia*, *Clostridium thermocellum* occurred most frequently. This species can hydrolyse cellulose efficiently by means of its extracellular cellulases, which are organised into cellulosomes [13]. An outstanding member of this class is *C. kluyveri*, which is unique among the *Clostridia*, because it uses ethanol and acetate as sole energy sources and converts these substrates to butyrate and H_2 [14]. A prominent and well-characterised species is *C. acetobutylicum*, which exerts cellulolytic, saccharolytic and H_2-producing activities. The fermentation pathways may yield organic acids such as acetate and butyrate (acetogenesis), or acetone, butanol and ethanol (solventogenesis) [15]. *Clostridium perfingens* generates lactate, acetate and butyrate from sugars, and through its [FeFe]-hydrogenase, it can also produce H_2 [16]. Similarly to *C. thermocellum, C. cellulolyticum* is a well-known strain that degrades cellulose to acetate and evolves CO_2 and H_2 [17]. *C. saccharolyticum* additionally possesses cellulolytic activity. The fermentation products include acetate, ethanol, H_2 and CO_2. *Thermoanaerobacterium thermosaccharolyticum* is a H_2-producing bacterium that has been reported to live in co-culture with *C. thermocellum*, the mixed culture producing more H_2 than the pure cultures [18,19]. *Ruminococcus albus* has been noted for its efficient cellulose degrading activity by cellulosomes; the major fermentation product is ethanol [20].

The volatile organic acids, CO_2 and H_2 generated by the acetogens are the substrates of methanogenesis carried out by special group of organisms, Archaea [21,22]. Aceticlastic and

hydrogenotrophic methanogens are distinguished in biogas fermenters [23]. The hydrogenotrophic archaea are capable of reducing CO_2 to CH_4, H_2 being used as an electron donor. Around 10% of the identified microbes in the biogas producing community are archaea [12]. In the domain of the archaea, the *Methanomicrobiales* order predominates in the community. Within this order, the most abundant species is *Methanoculleus marisnigri*. From the class of Methanococci, Methanococcus maripalidus is also a hydrogenotrophic methanogen [24]. Among the aceticlastic methanogens, Methanosarcina acetivorans was present in a relative majority. All of the identified Methanomicrobiales possess H_2 activating membrane-associated hydrogenases [25] and the relative wealth of hydrogenase-specific DNA reads corroborates the importance of these enzymes in the anaerobic degradation of organic material.

There is a need to examine the bacteriological index of anaerobic digestion for identification of the bacteria associated with the different stages of the biogas production. Biogas been a product of waste degradation and waste is known to habour some pathogenic microorganisms [26]. The fate of the pathogenic bacteria during biogas production is an area of concern to public health.

2. MATERIALS AND METHODS

2.1 Sample collection and preparation

Domestic wastes (food waste, vegetable waste, plantain peels, yam peels, and cow dung) were collected with the aid of hand gloves into containers, from households and markets within Cross River University of Technology, Calabar, Nigeria (www.crutech.edu.ng). The anaerobic digester was fabricated using a steel cylinder, following the design of Akubuenyi and Odokuma [9]. The waste samples were prepared by shredding the substrates separately using knife and mutter in order to improve system performance and enhance anaerobic digestion. Two hundred grams (200 g) each of the prepared samples were shuffled, mixed with water to the ratio of 1:3 and introduced into the anaerobic digester [9]. The set-up was allowed to ferment for bio-slurry production over a period of 28 days. The bioslurry sample was collected using a conical flask through the outlet channel of the digester, and was taken to the Microbiology laboratory for analysis.

2.2 Determination of Bacteriological Index

2.2.1 Enumeration of the total heterotrophic bacterial (THB) count of the bioslurry

The enumeration of the THB count was carried out on Day 1, by conducting a tenfold serial dilution of the bioslurry. One milliliter (1 ml) aliquot of the 10^{-6} and 10^{-7} dilution was pour plated on a nutrient agar in triplicate and incubated for 24 hours at 37°C. A subculture was carried out on a fresh nutrient agar in order to obtain pure colonies. The colonies were counted after 24 hours incubation, and recorded as colony forming units per milliliter (CFU/ml) [27]. These procedures was repeated on Day 14 and Day 28, but were incubated in an anaerobic jar to encourage the growth of facultative and anaerobic bacteria. They were also recorded accordingly.

2.2.2 The Bacterial diversity of bio-slurry during anaerobic digestion

The bacterial diversity of the digester was determined by isolating and identifying the isolates following standard bacteriological procedures (27). The analysis was carried out at 2 week interval for a period of 28 days.

2.3 Determination of the Fate of Pathogenic Bacteria during Anaerobic Digestion

Salmonella and *Shigella* species which are known pathogenic bacteria were used as a case study. One milliliter [1 ml] of the bio-slurry sample was collected during the anaerobic digestion process and enriched on peptone water medium for 6 hours, after which 1 ml of it was incubated on *Salmonella-Shigella* agar medium for 24 hours at 37°C. This analysis was carried out at 7 day interval to determine the fate of the organisms. Isolation of *Salmonella and Shigella* species were carried out following the procedures of Cheesbrough [27].

2.4 Statistical Analysis

Data obtained where subjected to one-way analysis of variance (ANOVA). Differences between the mean values of the treatments were determined by Duncan new multiple range test (DNMRT) and the significance was defined at 0.05.

3. RESULTS AND DISCUSSION

3.1 Determination of Bacteriological Index

3.1.1 Enumeration of the total heterotrophic bacterial (THB) count

The result of the enumeration of bacterial isolates at 2 week interval showed that the THB count has the highest count on Day 1 ($1.8x10^{10}$cfu/ml), followed by Day 14 ($1.47x10^{10}$) and Day 28 ($6.3x10^{8}$) (Table 1). The bacterial load of the digester decreased with time throughout the study period.

The reduction of the THB count could be attributed to changes in physicochemical and biochemical characteristics of the digester. At the on-set of fermentation, the hydrolytic reactions that take place in the digester leads to acidification of the medium due to production of acidic substances. Poudel et al. [28] had reported that the load of total and faecal coliforms declined gradually during digestion. The research reported that in mesophilic anaerobic digestion of sewage sludge mixture, the total heterotrophic bacteria, total coliform and faecal coliform were reduced by 2.78, 4.53 and $5.16\log_{10}$ cfu/ml respectively in 30 days. The result also corroborates the finding of Ponugoti et al. [29] that up to $4\log_{10}$ reduction of total coliforms was observed from sewage sludge subjected to mesophilic anaerobic digestion.

The reduction in total heterotrophic bacteria could also be linked to the availability of nutrients, since the fermentation process was carried out through a batch system. The gradual exhaustion of nutrients in the digester could lead the process into a decline stage of microbial growth phase. This agrees with the finding of Kearny et al. [30], which reported that the rate at which viable numbers of enteric bacteria decline during semi-continuous anaerobic digestion is dependent upon the bacterial species and the availability of nutrients within the system. This physiological state of bacteria due to an insufficient level of nutrients to supply energy for growth and reproduction is termed starvation-survival state and can lead to a transition between balanced growth and either unbalanced or complete cessation of growth. The reduction can also be traced to the gradual loss of available oxygen and the on-set of microaerophilic and eventual anaerobic environment. Most heterotrophic bacteria found on Day 1 when the system was aerobic may not tolerate the absence of oxygen, as the digester turns anaerobic. This may lead to their death and eventual reduction in counts as observed in the enumeration of the total heterotrophic bacterial count.

3.1.2 Bacteriological diversity of bioslurry during anaerobic digestion

The isolation and identification of the bacterial species associated with anaerobic digestion revealed the presence of aerobic, facultative anaerobic and anaerobic bacteria in Days 1, 14 and 28 respectively (Table 2). The aerobic bacteria were mainly isolated on Day 1 analysis, the facultative anaerobes were isolated on Day 14 and the anaerobic bacteria and some facultative bacteria, on Day 28. Results further showed that there are different stages within which the waste and its by-product of utilisation are converted to biogas by different microbial activities.

The identification of *Pseudomonas* spp, *Bacillus* spp, *Lactobacillus* spp, *Klebsiella* spp, *Proteus* spp, *Escherichia coli and Staphylococcus* spp indicates that the initial microbial hydrolytic activities on the waste materials are mediated by aerobic and facultative anaerobic bacteria. The presence of *Staphylococcus* spp, *Enterococcus* spp, *Peptostreptococcus* spp, *Micrococcus* spp and *Fusobacterium* spp in the sample analysed on Day 14 indicates that the digester was becoming anaerobic. These organisms are known facultative anaerobes. This revealed that they are involved in the bio-conversion of waste materials to methane, carbondioxide and other gases present in biogas. The bacteriological analysis on Day 28 showed the presence of these anaerobic bacteria; *Propionibacterium* spp, *Listeria* spp, *Erysipelothrix* spp and *Clostridium* spp which indicates that the digester has turned anaerobic.

This result is in agreement with the findings of Poudel et al. [28], who isolated 22 bacteria belonging to eight genera: *Escherichia coli*, *Citrobacter foundii*, *Pseudomonas aeruginosa*, *Proteus vulgaricus*, *Salmonella* spp, *Shigella* spp, *Staphylococcus aureus* and *Enterobacter* spp from sewage sludge. The result also corroborates the findings of Lepeuple et al. [31] in his work on the levels of pathogen from treated biowaste. Benatti et al. [32] and Carrington [33] in a related, but separate studies reported similar genera of bacteria from sludge. *Pseudomonas*

spp, *Lactobacillus* spp, *Proteus* spp and *Escherichis coli* were not detected from Days 14 and 28 analyses. This could be attributed to their sensitivity to anaerobic conditions.

Some facultative anaerobes; *Staphylococcus* spp, *Enterococcus* spp, *Peptostrptococcus* spp, *Micrococcus* spp and *Fusobacterium* spp that were isolated on Day 14 were not detected on Day 28 when the digester system was anaerobic. In a related research, Côté et al. [34] reported that *Staphylococcus aureus* and *Pseudomonas aeruginosa* were detected only up to 10 days in both mesophilic and psychrophilic anaerobic digester. Apart from the influence of oxygen, increased pH levels as a result of production of acidic metabolites during the hydrolytic and acetogenic processes could account for the death of some of the organisms.

3.2 Fate of Pathogenic Bacteria during Anaerobic Digestion

The assessment of the fate of *Salmonella* and *Shigella* species over a period of 28 days showed that the pathogens decreased with time. *Salmonella* spp reduced from $4x10^1$cfu/ml on Day 1 to $1x10^1$cfu/ml on Day 14, and the organism was completely eliminated by Day 28. *Shigella spp* on the other hand reduced from $2x10^1$cfu/ml on Day 1 to $1x10^1$cfu/ml on Day 14, and its presence was completely eliminated by Day 28 (Table 3).

This reduction could be traced to changes in the physicochemical parameters of the digester during fermentation, as a result of microbial metabolism. It corroborates the finding of Maier et al. [35] that pathogen disinfection degree is influenced by a variety of interacting operational variables and conditions but it is highly dependent on time and temperature. It also agrees with the position of Fukushi et al. [36] that almost complete destruction of *Salmonella* spp was observed within 2 days of anaerobic digestion when pH was maintained below 5.5.

The result is in accordance with the finding of Salsali et al. [37] that the reduction of *Salmonella* spp. in digester effluents, when dosed with volatile organic acids, was found to depend on pH, temperature, the chain length of the acids, and the concentration and composition of the acids present. Increases in temperature appeared to increase the inhibitory effects of the volatile organic acids. At mesophilic temperatures, acidic pH resulted in a greater inhibition of *Salmonella* spp.; whereas at higher temperatures, neutral pH was found to be more inhibitory. They suggested that acid phase digesters that operate at elevated temperatures and low pH can achieve substantial reduction of *Salmonella* spp.

Kumar et al. [38] studied the survival of some pathogenic bacteria in anaerobic batch digesters at 18-25°C and 35°C under laboratory conditions. *E. coli* and *Salmonella typhi* survived at room temperature for up to 20 days, but the survival time was reduced to 10 days at 35°C. *Shigella dysenteriae* was a more temperature-sensitive organism, surviving for only 10 days at room temperature, and for 5 days at 35°C.

The reduction might also be due to the low initial load of the pathogens in the biowaste introduced into the digester. Bendixen [39] analysed large-scale digesters in Denmark and reported that the numbers of pathogens in the waste stream were reduced by 1-2 and by 4 $\log_{10}$ units during mesophilic and thermophilic digestion respectively. Côté et al. [34] reported that anaerobic digestion of swine manure slurry at 20°C for 20 days in an intermittently fed Sequencing Batch Reactor (SBR) reduced indigenous populations of total coliforms by 97.94–100%; reduced indigenous populations of *Escherichia coli* by 99.67–100%; resulted in undetectable levels of indigenous strains of *Salmonella*, *Cryptosporidium*, and *Giardia*. The research confirmed the reduction of indicator and pathogenic microorganisms by psychrophilic anaerobic digestion. *Salmonella typhimurium*, *Yersinia enterocolitica* and *Listeria monocytogenes* also declined more rapidly at 17°C than at 4°C during anaerobic digestion of cattle slurry (30). This showed that temperature is a major factor in pathogen reduction during anaerobic digestion. Thermophilic (50-60°C) or mesophilic (30-36°C) anaerobic digestion encourages more pathogen die-off than psychrophilic (<20°C) anaerobic digestion, though mesophilic anaerobic digestion is more common because of the stability of the process [40]. These findings indicate that anaerobic digestion could be an appropriate technique for the treatment of bio-slurry and sludge before final disposal. Sahlström et al. [41] shared this view in a study on bacterial pathogen incidence in sludge.

Table 1. Total heterotrophic bacterial count of the bio-slurry (CFU/ml)

Retention time (Days)	Mean THB (CFU/ml)	Methods
1	$1.8x10^{10}$	Viable count (27)
14	$1.47x10^{10}$	Viable count (27)
28	$6.3x10^{8}$	Viable count (27)

Table 2. Bacteriological index of the bio-slurry during the process of biogas production

Bacterial Isolates	Day 1	Day 14	Day 28
Pseudomonas spp	+++	-	-
Lactobacillus spp	+++	-	-
Klebsiella spp	+++	++	-
Proteus spp	+++	++	+
Bacillus spp	+++	++	+
Escherichia coli	+++	-	-
Citrobacter spp	++	-	-
Staphylococcus aureus	++	++	-
Salmonella spp	+	+	-
Shigella spp	+	-	-
Enterococcus spp	-	+++	-
Peptostreptococcus spp	-	+++	-
Micrococcus spp	-	+++	-
Fusobacterium spp	-	++	-
Corynebacterium spp	-	++	-
Bacteriodes spp	-	+	++
Sporolactobacillus spp	-	+	++
Stretobacillus spp	-	++	++
Propionibacterium spp	-	-	+++
Listeria spp	-	-	+++
Erysipelothrix spp	-	-	+++
Clostridum spp	-	-	+++

Key: *+++= Heavy growth; ++=Moderate growth*
+=Scanty growth; -=No growth

Table 3. Fate of *Salmonella* and *Shigella* species during a 28 day anaerobic digestion of domestic waste

Days	*Salmonella* spp (10^1CFU/ml)	*Shigella* spp (10^1CFU/ml)
1	4.17 ± 0.38^{d}	2.40 ± 0.32^{c}
7	2.47 ± 0.32^{c}	1.27 ± 0.27^{b}
14	1.13 ± 0.19^{b}	0.00 ± 0.00^{a}
21	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
28	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}

Means followed by the same letter(s) within a column are not significantly different (P< 0.05 from each other using Duncan's multiple range test (DMRT).

Table 4. Analysis of variance (ANOVA)

		Sum of squares	df	Mean square	F	Sig.
Salmonella spp	Between Groups	37.997	4	9.499	54.594	.000
	Within Groups	1.740	10	.174		
	Total	39.737	14			
Shigella spp	Between Groups	14.027	4	3.507	33.503	.000
	Within Groups	1.047	10	.105		
	Total	15.073	14			

Significant at P <0.05

The Table of Analysis of Variance (Table 4) showed that they is no significant difference between the number of *Shigella spp* and *Salmonella spp* across the 28 day anaerobic digestion of domestic waste.

4. CONCLUSIONS

The bacteriological index and fate of pathogenic bacteria during anaerobic digestion of domestic waste were determined. This was conducted at the Microbiology Laboratory of the Cross River State University of Technology, Calabar, Cross River State, Nigeria. The bacteriological index of the digestion process is characterised with reduction of total heterotrophic bacterial load over a period of time and the presence of different classes of bacteria; aerobes, facultative anaerobes and anaerobes at different fermentation stages. The fate of pathogenic bacteria during anaerobic digestion is die-off over a period of time. Anaerobic digestion technology could be applied as a waste treatment option, to reduce the incidence of waste related diseases. Further studies on the metagenomics of bioslurry will provide deeper knowledge on the uncultured and genomic diversity of microorganisms associated with anaerobic digestion of domestic waste.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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