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## Isolation and Characterization of Microorganisms Consortia Associated with Biogas Production from Spinach and Selected Fruits Waste as Substrates

By

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### ABSTRACT

*The research study was aimed at investigating microorganisms associated with biogas production using vegetable (Spinacia oleracea), banana peel, plantain peel, watermelon residue and pumpkin wastes as substrates. The samples were procured from different locations within Sokoto metropolis. The study was completed within a period of five weeks (35 days). Standard microbiological methods and fabricated anaerobic bio-digesters were used to screen the isolates and the wastes substrate for biogas production. Analysis revealed that the temperature within the digester ranged between 30°C and 34°C while the pH varied between 6.30 and 7.20 and 5.0 – 6.2 before and during/after the anaerobic digestion respectively. Anaerobic bacteria isolated were identified as Staphylococcus aureus, Micrococcus spp, Enterobacter spp, Escherichia coli, Citrobacter diversus, Bacillus firmus and Pseudomonas aeruginosa. The result showed that the percentage yield of biogas produced from the substrate is in the order: synergized mixture > plantain > banana > pumpkin > spinach > watermelon. The volume of biogas produced varied significantly ( $p < 0.05$ ) between the substrate treatments and the digestion internals (days). This study has confirmed the role of methanogens and other complementing bacteria in biogas production.*

Keywords: Agricultural Wastes, Biogas, Digester, Fermentative, Methanogens. .

### INTRODUCTION

The cost and scarcity of improved petroleum products used for industrial, agricultural and domestic fuels are drastically increasing, as this makes it difficult for most people to rise beyond subsistence level especially in developing countries like Nigeria, and these realities have led to a boost in the search for renewable and sustainable alternative to fossil fuels (Asikong *et al.*, 2016). Biogas is a flammable gas produced when organic materials are fermented under anaerobic condition (Baba *et al.*, 2012; Ezeohu *et al.*, 2005). It holds promise as an alternative energy source capable of being used for generating electricity, car fuelling, cooking among others (Madu and Sodeinde, 2001). The anaerobic digestion process involves four stages namely: hydrolysis, acidogenesis, acetogenesis and methanogenesis (Bitton, 2005; Gerardi, 2003; Nwuche, and Ugoji, 2008, Nwuche, and Ugoji, 2010). The process is carried out by consortia of microorganisms which partly stand in syntrophic interrelation (Angelidaki, 1993). Organisms involved in each stage are referred to as hydrolyser, acidogen, acetogen and methanogen respectively (Zieminski *et al.*, 2012). The first group of microorganisms are hydrolytic bacteria which hydrolyses the polymeric materials to monomers via extracellular hydrolytic enzymes namely cellulase, xylanase, amylase,

protease, lipase which they excrete (Gopinath *et al.*, 2014). The second group of microorganisms is acidogenic bacteria which convert sugars and amino acids into carbon (IV) oxide, hydrogen, ammonia and other organic acids. The higher volatile fatty acids are converted into acetate and hydrogen by obligate hydrogen-producing acetogenic bacteria. Hydrogen accumulation has the capability of inhibiting the metabolism of acetogenic bacteria hence, the maintenance of an extremely low pressure is essential for the acetogenic and hydrogen producing bacteria (Gopinath *et al.*, 2014). The 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 (Schink, 1997). The composition of these microbial consortiums depends mainly on factors like substrate ingredients, temperature, pH, mixing or the geometry of the anaerobic digester (Roland *et al.*, 2012). This study was initiated to isolate and characterize methanogenic microorganisms from the vegetable (*Spinacia oleracea*), banana, plantain, watermelon and pumpkin substrates as well as from the synergized substrates procured from different location in Sokoto metropolis.

## MATERIALS AND METHODS

### *Source of Samples*

The materials used for this research work were vegetable and fruit wastes collected from various locations within Sokoto metropolis. While pumpkin, spinach stalk, banana and plantain peel were collected from Ramin Kura market, fresh watermelon residues were collected from Miyetti Allah area both in Sokoto metropolis.

### *Media Used*

The media used in the study are starch agar, carboxymethyl cellulose agar, egg yolk agar and nutrient-gelatin agar procured from Biotech Lab Ltd, UK while triple sugar Iron agar was procured from Hardy Diagnostic, USA.

### *Preparation of Media and Reagents*

All the media and reagents used were prepared and preserved according to the manufacturer's specifications.

### *Preparation of the Raw Substrates for Microbial Screening*

- i. Pumpkin fruit, banana peel, plantain peel and watermelon residue: 10 g of the substrates were individually weighed and aseptically crushed into powder. It was transferred into 90 ml of sterile distilled water contained in a 100 ml volumetric flask. The mixture was agitated and allowed to settle.
- ii. Spinach (*Spinacia oleracea*) waste: The stalks of the vegetable were grounded with 10 milliliter aseptically transferred in a 90 ml of sterile distilled water contained in a 100 ml volumetric flask. The mixture was agitated and allowed to settle.
- iii. Substrate mixture: Equal quantity of all substrate was thoroughly grounded after which 10 milliliter was aseptically transferred into a 90 ml sterile distilled water in a 100 ml volumetric flask. The mixture was agitated and allowed to settle.

### **Isolation and Assessment of Bacterial Populations**

The method described by Aderonke *et al.*, 2017 was adopted with slight modification. The prepared substrates correspond to  $10^{-1}$  dilution. 10 ml of aliquot from the  $10^{-1}$  dilution was measured into another volumetric flask containing 90 ml of sterile distilled water to obtain  $10^{-2}$  dilution. Further dilutions were carried out until a dilution level of  $10^{-7}$  was reached. Samples were taken once every three days for total heterotrophic counts. For bacterial screening, dilutions  $10^{-5}$  to  $10^{-7}$  of the samples (upon serial dilution) were plated on starch agar, carboxymethyl cellulose agar, egg yolk agar and nutrient-gelatin agar (hydrolytic bacteria media). Plates were incubated for 48 hours at 35°C. Colony forming units per gram (CFU g<sup>-1</sup>) of bacterial growth between 30 - 300 colonies were enumerated. The colonies formed were sub cultured and identified using cultural, morphological and biochemical methods.

### **Characterization of the isolates**

Cultural and morphological characteristics of pure colonies were used to perform preliminary characterization (Holt *et al.*, 1994). The cell shape and arrangement characteristics were viewed under compound microscope after standard staining. 3% (w/v) KOH test (Gregersen, 1978) was used to determine the gram characteristics of the isolates. Among the biochemical tests conducted were catalase, coagulase, lactose, glucose, sucrose, citrate, indole, H<sub>2</sub>S production, urease, gas, methyl red, Voges-proskauer, spore formation, oxidase and motility tests

### **Digester Design**

Cylindrical cans were used as the digesters and plastic basins were used as water trough. To each can, a hole was bored on the lid of the can and a polyvinyl chloride tube was inserted into the hole and fixed with araldite adhesive to ensure that the digester was airtight. This served as the outlet for the generated biogas. The free end of the rubber tube was then inserted, through water contained in a partly filled water trough into an inverted measuring cylinder that has also been filled with water, to serve as the biogas collection system. The biogas was collected by downward displacement of water. The tins were labeled A, B, C, D, E and F corresponding to banana, plantain, watermelon, pumpkin, spinach and synergized mixture digesters respectively for the purpose of the study.

### **Preparation of Slurry and Loading of Digesters**

100 g of all substrate and mixture was fed into their respective digester and 400 cm<sup>3</sup> of water was added hence making up a ratio of 1:4. All digesters were stirred with their openings well sealed. The setup was monitored at least twice a day but the readings were taken every 24 hours at 12 noon every day for the 35 days retention period. The temperature and pH of the substrates were determined before and monitored throughout the retention period. Each procedure was performed in duplicate.

### **Measurement of Gas Production**

Biogas production was measured daily on volume basis as evidenced by the displacement of water in the inverted measuring cylinder that has been filled with water to serve as the biogas collection system

## STATISTICAL ANALYSIS

Statistical analysis of data obtained from the different treatments were carried out using a 2 way analysis of variance (ANOVA) and the means separated using the fishers least significant difference (LSD) at 5% significant level. All data were expressed as means  $\pm$  standard deviation of triplicate trials.

## RESULTS AND DISCUSSION

### *pH Variations of the Substrates*

Figure 1 shows the means variation in hydrogen ion concentration of the different substrates treatments before, during and after digestion.

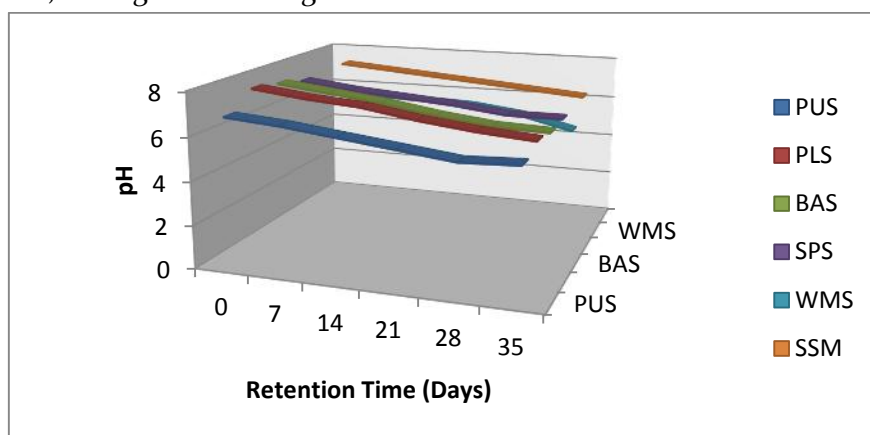


Figure 1: pH changes in the digester content before, during and after anaerobic digestion  
 Key: PUS = Pumpkin substrate, PLS = Plantain substrate, BAS = Banana substrate, SPS = Spinach substrate, WMS = Watermelon substrate, SSM = Synergized substrate mixture

The mean pH ranged between 6.3 – 7.2 before and 5.0 – 6.2 during and after the anaerobic digestion respectively. The result showed a marked decrease in the hydrogen ion concentration during the anaerobic digestion of the substrates. This is so because as digestion commences there is bound to be the formation of sulphide in the slurries due to the breakdown of biodegradable sulphur containing organic and inorganic compounds likewise the formation of fatty acids by acetogenic methanogens during the process of digestion (Bagudo, 2007). The observed pH is in agreement with the report of Yerima *et al.*, 2001 who stated that for biogas generation the methanogenus grow well when the pH is in the range of 6.8 – 7.8 and that the pH range for optimum biogas production has been reported as 6 –7 by Garba and Sambo, (1992). Similarly, the drop of pH observed in this study could have been as a result of the production of metabolites such as acetate, hydrogen gas, carbon dioxide and few other volatile fatty acids such as propionic acting on the substrates in the digesters (Tsunatu *et al.*, 2004). Research by (Anuputtikul and Rodtong, 2004) reported that there is a perfect link between the acidogenic and methanogenic phases when the pH remained at the range of 6.0-7.4 during the anaerobic digestion of substrates.

### *Temperature Variations of Substrates*

Figure 2 shows the mean temperature variation of the substrates before, during and during the digestion process.

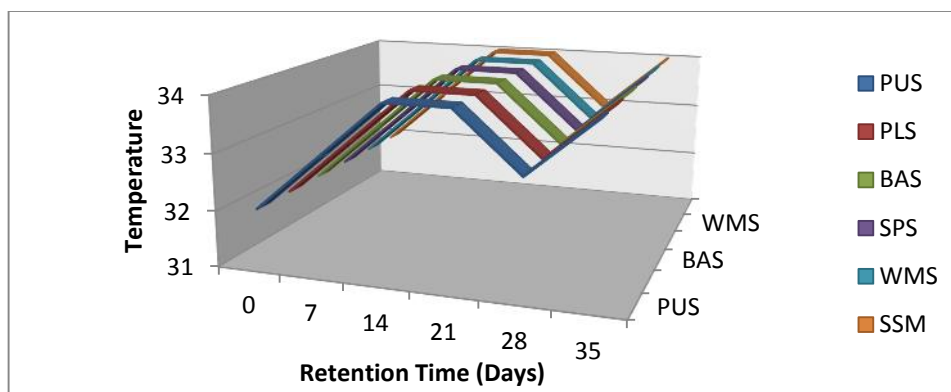


Figure 2: Temperature changes in the digester content before, during and after anaerobic digestion

Key: PUS = Pumpkin substrate: PLS = Plantain substrate: BAS = Banana substrate, SPS = Spinach substrate: WMS = Watermelon substrate: SSM = Synergized substrate mixture

Significant ( $p < 0.05$ ) variations in temperature was observed between the different substrate treatments before during the anaerobic digestion process. During the anaerobic digestion process, the mean temperatures within the digester ranged between 30°C and 34°C compared to the ambient (temperature before digestion) temperature. This observation is in agreement with that reported by Surnaso *et al.*, 2010, Aremu and Agarry, 2012, and El-Mashed *et al.*, 2003 who carried out research on biogas production using anaerobic biodigester from cassava starch effluent. Adelekan and Bamgboye, 2009 reported that the digestion temperatures within 28°C - 39°C range are favorable to the hemophilic bacteria populations and are well tolerated by anaerobic bacteria for maximum biogas production

**Morphological and Biochemical Characterization of the Isolated Microbial Isolates**

Tables 1 and 2 presents the morphological and biochemical characterization of the bacteria isolates.

Table 1. Morphological characteristics of bacteria isolates obtained from the substrates

Substrate	Isolate	Colony Morphology		Cell Morphology		
		Colour	Form	Gram Reaction	Arrangement	Motility
Banana Peels	BA <sup>1</sup>	White to Yellow	Oval	+	Grape-like (irregular) Tetrads	+
	BA <sup>2</sup>	Golden-brown	Rod	+		-
Pumpkin Fruit	PU <sup>1</sup>	Grey	Rod	-	Rod	+
	PU <sup>2</sup>	Off-white	Rod	+	Rod	-
Watermelon Residues	WM <sup>1</sup>	Rainbow	Rod	-	Rod	-
	WM <sup>2</sup>	Golden-brown	Rod	+	Tetrads	-
Plantain Peels	PL <sup>1</sup>	Off-white	Rod	+	Rod	-
	PL <sup>2</sup>	Semi-transparent	Rod	-	Rod	+
Spinach Stalks	SP <sup>1</sup>	Semi-transparent	Rod	-	Rod	+
	SP <sup>2</sup>	Grey	Rod	-	Rod	+
Synergized Mixture	SM <sup>1</sup>	Blue-green	Rod	+	Rod	+
	SM <sup>2</sup>	Golden-brown	Rod	+	Tetrads	-

Table 2. Biochemical characterization of the bacteria isolates obtained from the substrates

Substrate/Test	Banana Peels		Pumpkin Fruit		Watermelon Residues		Plantain Peels		Spinach Stalks		Synergized Mixture	
	BA <sup>1</sup>	BA <sup>2</sup>	PU <sup>1</sup>	PU <sup>2</sup>	WM <sup>1</sup>	WM <sup>2</sup>	PL <sup>1</sup>	PL <sup>2</sup>	SP <sup>1</sup>	SP <sup>2</sup>	SM <sup>1</sup>	SM <sup>2</sup>
Catalase	+	-	-	-	-	+	-	-	-	-	+	-
Coagulase	-	-	+	-	-	-	+	+	+	-	+	-
Lactose	-	-	+	-	-	-	+	+	+	-	-	-
Glucose	-	-	+	-	-	-	+	+	+	-	-	-
Sucrose	-	-	+	-	-	-	-	+	+	-	-	-
Citrate	-	-	+	-	+	-	+	-	-	+	-	-
Indole	-	-	+	-	+	-	-	-	-	+	-	-
H <sub>2</sub> S	-	-	+	-	+	-	-	-	-	+	-	-
Urease	-	-	+	-	-	-	+	-	-	-	-	-
Gas	-	-	-	-	-	-	+	+	+	-	-	-
MR	-	-	-	-	-	-	-	-	-	-	-	-
VP	-	-	-	-	-	-	+	-	-	-	-	-
Spore	-	-	-	-	+	-	-	-	-	+	+	-
Oxidase	-	-	+	+	-	-	-	-	-	-	-	-
Bacterial Isolated	SA	MS	ES	EC	CD	MS	EC	BF	BF	ES	PA	MS

Key: (+) = Positive, (-) = Negative, SA = *Staphylococcus aureus*, MS = *Micrococcus spp*, ES = *Enterobacter spp*, EC = *Escherichia coli*, CD = *Citrobacter diversus*, BF = *Bacillus firmus*, PA = *Pseudomonas aeruginosa*, BA = Banana, PU = Pumpkin, WM = Watermelon, PL = Plantain, SP = Spinach, SM = Synergized mixture

**Biogas Yield from Substrates**

Figure 3 shows the weekly volume of biogas yielded from the different substrates treatments.

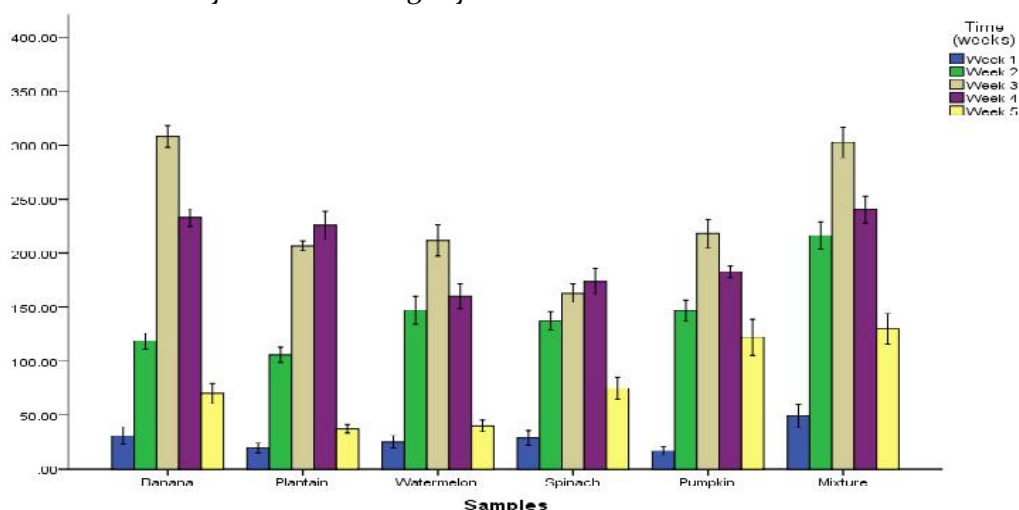


Figure 3: Mean weekly volume of biogas produced from each substrate

The biogas yield varied significantly ( $p < 0.05$ ) between the substrate treatments and the digestion intervals (days). Maximum biogas yield was obtained within 21 days (3<sup>rd</sup> week) of digestion for the banana, watermelon, pumpkin and the synergized substrate mixtures. That of

plantain and spinach substrates was recorded within 28 days (4<sup>th</sup> week) over the digestion period of 35 days. According to (Jaenicke *et al.*, 2011), members of methanogens frequently dominate methanogenic sub-communities in different anaerobic digester systems and this research has shown that different methanogenic sub-communities within biogas producing consortia are crucial in the anaerobic degradation process for synthesis of methane (Dhevagi *et al.*, 1992). These findings are in line with that of Demirel and Scherer, 2008 who reported that the anaerobic transformation of organic wastes is a process which involves many different groups of bacteria such as hydrolyzing, acidifying, acetogenic and methanogenic bacteria which in the final stage produce CO<sub>2</sub> and methane, which are the main products of the digestion process. The peak in the volume of biogas within 21 – 28 days is as a result of the acclimatization of the biogas producing microorganisms after the hydrolysis of the substrates by the hydrolyzing organisms. The decline in the volume of biogas produced subsequently is as a result of the decline in the activities mainly of methanogens as well as other factors like decrease in pH (Figure 1) and increase in temperature (Figure 2) of the medium, deposition of microbial metabolites, gradual exhaustion of available nutrient from the substrates and the replacement by organisms that tend to utilize some of the products of their actions (Asikong *et al.*, 2016). The highest percentage yield of biogas was observed in the co-digestion of all substrate and this may be due to the varying proximate composition of the substrate, positive synergetic effect of the co-digestion of the substrate providing more balanced nutrients, increased buffering capacity, and decreased effect of toxic compounds (Mata-Alvarez *et al.*, 2000; Li *et al.*, 2009; Jianzheng *et al.*, 2011).

## CONCLUSION

This research confirms that organic waste materials from banana peels, plantain peels, watermelon residues, pumpkin fruit and spinach stalks can be utilized by microorganisms for biogas production. The usage of these substrates for biogas production is possible due to the accessibility by cellulolytic, lipolytic, proteolytic and amylolytic microorganisms which play a vital role in biogas production from organic wastes.

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