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Isolation and Characterization of Microorganisms with Hydrolytic Profile during Anaerobic Digestion and Biogas Production of Cow Dung and Rice Husk

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Abstract
Hydrolysis is important to biogas production as it involves breakdown of polysaccharides present in substrates. The Isolation and characterization of bacterial strains with hydrolytic profile during anaerobic digestion of CD (cow dung), RH (rice husk) and their mixture (CD:RH) was the subject of this research .The experiment lasted for 30 days using a 10 litre scale bio-digester. All physico-chemical parameters reduced significantly after digestion for CD (cow dung), RH (rice husk), and CD:RH (cow dung and rice husk) except moisture content that increased in all the substrates. Also, ash content (1.08-1.67 mg) and crude fibre (1.27-1.96 mg) increased in CD only. The pH ranges for the substrates were CD (7.0-7.5), RH (6.1-7.6), and CD:RH (6.1-7.8). Temperature ranges were CD (27.4^0C-33.5^0C), RH (27.2^0C-33.3^0C) and CD:RH (27.3^0C-33.4^0C). The total biogas production of the substrates were, CD (4327.65cm^3), RH (100cm^3), and CD:RH (4730.55cm^3). Percentage distribution of hydrolytic group of bacteria present in the digester include cellulolytic (37.50%), amylolytic (18.75%) , proteolytic (18.75%) and lipolytic bacteria (25%). Hydrolytic bacteria and fungi isolated were *Penicillium notatum, Micrococcus luteus, Pseudomonas aeruginosa, Klebsiella oxytoca, Aspergillus spp, Mucor spp, Bacillus subtilis, Proteus vulgaris, Clostridium spp, Peptostreptococcus spp* and *Enterobacter aerogene*. For hydrolytic groups of bacteria, the following had the highest activities in terms of diameter of cleared zones of inhibition: Amylolytic (*Enterobacter spp* - 15mm),lipolytic (*Pseudomonas spp* - 16mm),Cellulolytic (*Klebsiella spp* - 17mm) and Proteolytic (*Pseudomonas spp* - 14mm). Rice husk produced the least amount of biogas due to its high cellulose and lignin content. In conclusion, further investigation of hydrolytic groups of bacteria beyond 30 days of anaerobic retention period should be investigated.
Keywords: Hydrolysis, biogas, amylolytic bacteria, lipolytic bacteria, cellulolytic bacteria, proteolytic bacteria

1.0 Introduction

Biogas production is achieved via anaerobic digestion. Biogas production technology through anaerobic digestion is an alternative form of fuel and a good fertilizer source. The digestion process encompasses four stages namely: hydrolysis, acidogenesis, acetogenesis and methanogenesis. Organisms involved in each stage are referred to as hydrolyser, acidogen, acetogen and methanogen respectively(Zieminski *et al*.,2012).

The hydrolytic stage involves depolymerization of carbohydrates, proteins and lipids present in the substrate. Cellulase, protease, lipase and amylase were recognized as the major hydrolytic enzymes produced by hydrolysers. The hydrolytic stage is vital because large organic molecules are simply too large to be directly absorbed and used by microorganisms as a substrate or food source. Thus, needs to be broken down (Schnürer and Javis, 2010). “Cellulolytic” and “Amylolytic” are two common terms referring to anything capable of breaking down cellulose and amylose respectively. Enzymes responsible for the breakdown of cellulose and amylose are called “cellulase” and “amylase” respectively. Common cellulolytic and amylolytic bacteria in the hydrolytic stage of biogas production include the genera *Bacteriodes, Clostridium* and *Acetivibrio* (Cirne *et al*., 2007; Doi, 2008).

Amino acids (monomeric unit of protein) are obtained upon hydrolysis of proteins and peptides. “Proteolytic” is a term generally used for anything capable of breaking down protein. Enzymes responsible for the breakdown of proteins are called “proteases”. Common genera of proteolytic bacteria include *Clostridium, Peptostreptococcus, and Bifidobacterium* (Ramsay and Pullammanappallil, 2001). Lipolysis refers to the breakdown of fats. Enzymes responsible for the breakdown of fats are called “lipases”. Most of the known lipases are produced by aerobes or facultative anaerobes microorganisms. Strict anaerobes that secrete lipases include, among others, the genus *Clostridium* (Gupta *et al*., 2004; Petersen and Daniel, 2006). The aim of this study is to isolate and characterize hydrolytic bacterial strains during biogas production from cow dung (CD), rice husk (RH) and the mixture of cow dung and rice husk (CD:RH).

2.0 Materials and Methods

2.1 Sample collection

Fresh cow dung was collected from COLANIM farm, Federal University of Agriculture, Abeokuta (FUNAAB), Ogun State, Nigeria. The sample was collected in a sterile polythene bag and transported within 24 hours to the laboratory for sample analysis. Milled rice husk was obtained from the Ofada rice mill in Lafenwa market, Abeokuta, Ogun Sate, Nigeria.

2.2 Bio-digester Design and loading

A 10 litre laboratory scale anaerobic bio-digester was constructed. The bio-digester was constructed using Karki's Biogas model as a guide. It was designed to have three openings: one for slurry inlet, the second serving as gas outlet while the third was the slurry outlet. The gas produced in the bio-digesters were collected into sterile tyre tubes.

Nine (9) bio-digesters were constructed for the research. The slurry to water ratio in the bio-digesters were the same. An approximate 3 litres of slurry was fed into the bio-digester along with 3 litres of water while the remaining part of the bio-digester accounts for the gas space. A summary of the content of each bio-digester is given below:

- Bio-digester 1: 3 kg cow dung +3 litres of water
- Bio-digester 2: 1.5 kg rice husk + 1.5 kg cow dung + 3 litres of water
- Bio-digester 3: 3 kg of rice husk+ 3 litres of water

The bio-digesters were constructed in triplicates giving a total of nine (9) bio-digesters.

The experiment was allowed to run for 30 days in continuous fermentation during and after which the following were recorded :

- The temperature of the bio-digester content and its pH recorded every three days
- Proximate analysis of the bio-digester content before and after the termination of the experiment.
- Collection of samples at 3 days interval for microbial analysis
- Volume of gas produced upon completion of the study.
- Separation of gas produced into its various components

Plate1: A 10 litre scale bio-digester

2.3 Isolation and Assessment of Bacterial Populations

Serial dilution was performed for the wastes by taking 1 g of each waste into a McCartney bottle containing 9 ml of sterile distilled water coupled with shaking to homogenise the suspension (10^{-1} dilution). Thereafter, 1 ml of aliquot from the 10^{-1} dilution was measured into another bottle containing 9 ml of sterile distilled water to obtain dilution 10^{-2} dilution. Further dilutions were carried out until a dilution level of 10^{-7} was reached. Samples were taken once every three (3) 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 24 - 48 hours at 35 ^{0}C. Colony forming units per gram (CFU g^{-1}) of bacterial growth between 30 - 300 colonies were enumerated. The colonies formed were sub cultured and identified using cultural, morphological, biochemical and molecular methods.

2.4 Isolation and Assessment of Fungal Population

For screening of fungi, dilutions of 10^{-3} to 10^{-4} were plated on Potato dextrose agar and Saboraud's dextrose agar supplemented with 100 $mgml^{-1}$ streptomycin and 15 $mgml^{-1}$ of penicillin (to inhibit bacterial growth). This was incubated for 72 to 96 hours. Total fungal counts were enumerated in CFU g^{-1} The colonies formed were sub cultured and identified using microscopic, colonial and molecular methods.

2.5 Characterization and identification of the isolates

Bacterial isolates were identified using standard biochemical tests with reference to Bergey's manual. The fungal isolates were identified based on cultural and morphological characterization with reference to de Hoog *et al.,* 2000 and Ellis *et al.,* 2007. Molecular characterisation by ribosomal DNA genes analysis (i.e 16ssRNA for Bacteria and 18ssRNA for Fungi) was also done using the method of Fowora (2013).

2.6 Physico-Chemical analyses of the cow dung (CD), rice husk (RH) and their combination(CD:RH)

Physico-chemical parameters analyzed were organic carbon, moisture content, total solids, total nitrogen, ash content, carbon/nitrogen ratio, crude fibre, volatile solid, crude protein, crude ash, biochemical oxygen demand (BOD) and chemical oxygen demand (COD) using standard method as described by the 20th edition of AOAC (2016)

2.7 Gas production analysis

A portable hand-held biogas analyser obtained from Beijing Shi'an Tech Instrument Co., Ltd having the ability to determine volume of gas produced and the percentage of constituents present in biogas was used.

2.8 Statistical analysis

The data obtained from the research were subjected to analysis using Microsoft Excel and Statistical Package for Social Sciences (SPSS) version 16.

3.0 Results

The total aerobic bacterial count showed that CD: RH had the highest count of 5.52 x 10^8 CFU g^{-1} while CD had the least count of 1.05 x 10^8 CFU g^{-1} .The total anaerobic bacterial count ranged from 1.10 x 10^8 CFU g^{-1} - 3.76 x 10^8 CFU g^{-1}. CD: RH had the highest anaerobic count while RH had the least anaerobic count. The total fungal count showed that CD: RH had the highest count of 4.73 x 10^5 CFU g^{-1} while CD had the least count. The variations of the above microbial counts with time is seen in **figures 1, 2 and 3**.

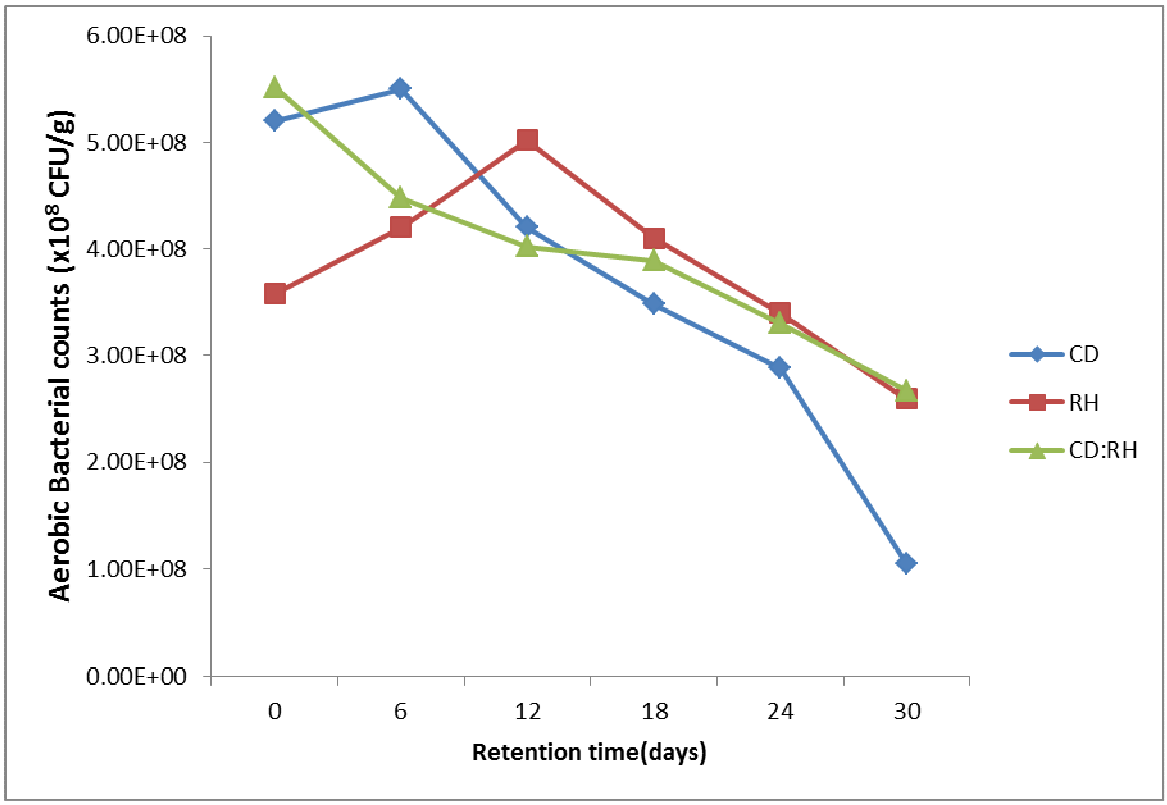
Figures 4 and **5** shows temperature and pH changes in digester content during anaerobic digestion of CD, RH and CD:RH. The overall temperature range of all the digesters was from 27.2 ^{0}C to 33.5 ^{0}C. The highest overall temperature (33.5 ^{0}C) was recorded in CD at the 21st and 30th day of digestion while the lowest temperature (27.2 ^{0}C) was recorded in RH at the 18th day of digestion. The overall pH range recorded in all the digesters was from 6.1 to 7.8. The lowest pH measurement (6.1) was recorded after the 27th day of digestion in CD: RH and 30th day of digestion in RH while the highest pH measurement (7.8) was recorded at the 1st day of digestion in CD:RH.

The result of the physico-chemical analysis of the substrate upon anaerobic digestion in **table 1** shows a reduction in Nitrogen content, Carbon content, Carbon/Nitrogen ratio, Ash content, Crude Fibre, Crude Protein, Fat Content, Total solids, Volatile solids, Biochemical oxygen demand (BOD) and Chemical oxygen demand (COD) except moisture content that increased in all the substrates. Ash content (1.08-1.67 mg) and crude fibre level (1.27-1.96 mg) increased in CD only.

Table 1: Physico-chemical analysis of CD, RH and CD: RH during 30 days of anaerobic digestion

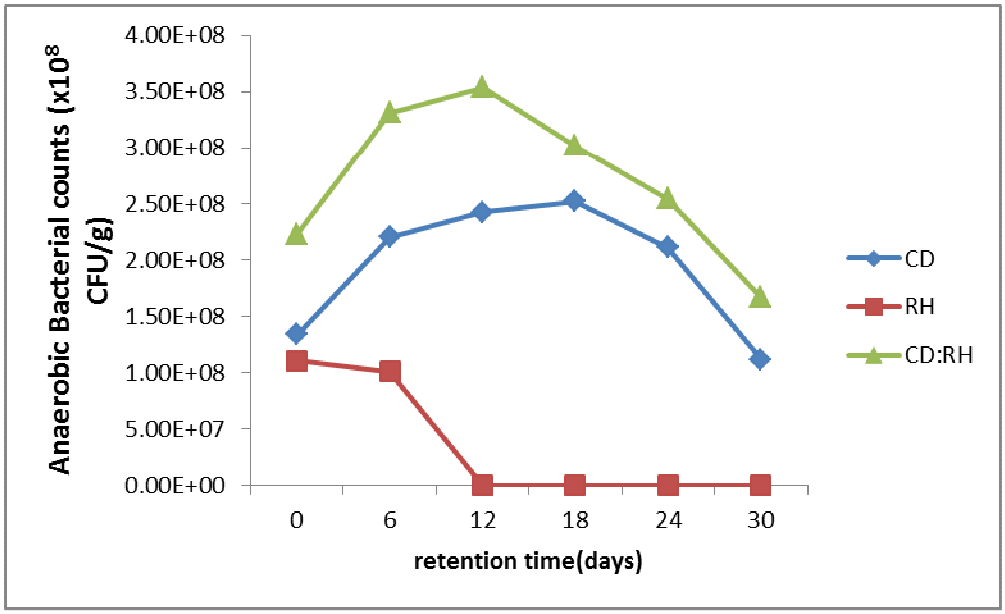
Parameter	CD		RH		CD:RH	
	Fresh slurry	Digested slurry	Fresh slurry	Digested slurry	Fresh slurry	Digested slurry
Nitrogen (%)	0.31	0.25	0.26	0.23	0.29	0.21
Carbon content (%)	7.79	5.81	6.29	5.16	8.22	5.55
Carbon/Nitrogen	25.12	23.22	24.21	22.42	28.33	26.44
Ash (g/100g)	1.08	1.67	2.86	0.12	1.45	0.51
Moisture (g/100g)	80.09	94.00	28.20	99.45	72.60	97.79
Crude fibre (g/100g)	1.27	1.96	4.16	0.21	1.88	0.89
Crude Protein (g/100g)	6.86	1.12	27.65	0.14	9.62	0.26
Volatile solid (%)	9.15	0.06	9.11	0.02	9.11	0.02
Total Solid (g/100g)	19.91	6.00	71.90	0.58	27.40	2.03
Fat Content (g/100g)	0.89	0.12	2.65	0.00	1.27	0.09
BOD (mg/L)	20.54	11.25	18.92	10.36	20.38	11.16
COD (mg/L)	7.30	4.01	7.10	3.89	6.79	3.72

Key : CD (cow dung), RH (Rice Husk), CD:RH (mixture of cow dung and rice husk).



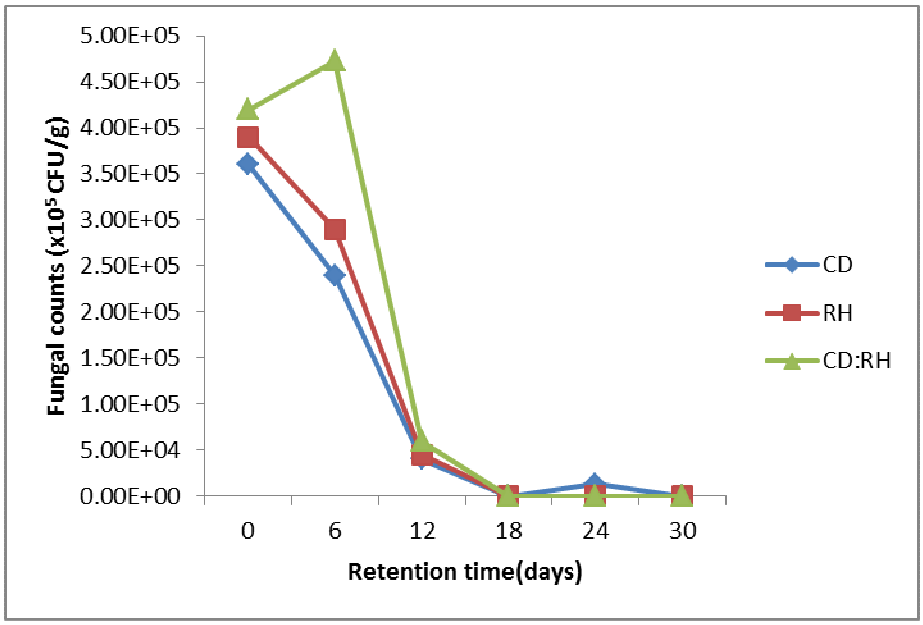
Key: CD = Cow dung; RH = Rice husk; CD:RH = Combination of cow dung and rice husk

Figure 1: Variation in the aerobic bacterial counts of the treatments with time



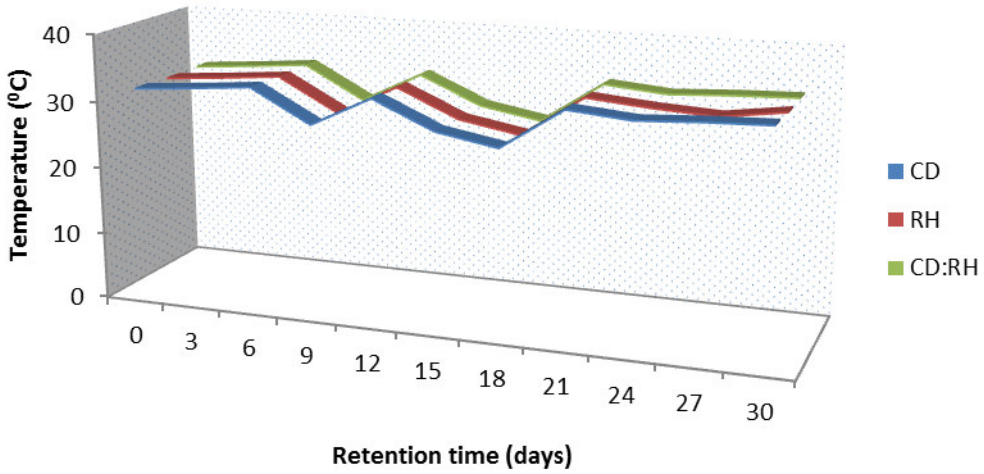
Key: CD = Cow dung; RH = Rice husk; CD:RH = Combination of cow dung and rice husk

Figure 2: Variation in the anaerobic bacterial counts of the treatments with time



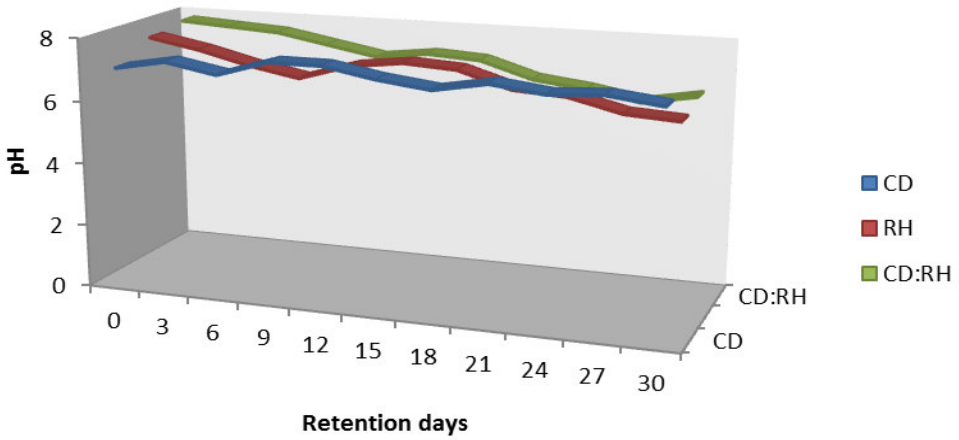
Key: CD = Cow dung; RH = Rice husk; CD:RH = Combination of cow dung and rice husk

Figure 3: Variation in the fungal counts of the treatments with time



Key: CD = Cow dung; RH = Rice husk; CD:RH = Combination of cow dung and rice husk

Figure 4: Temperature changes in the digester content during anaerobic digestion



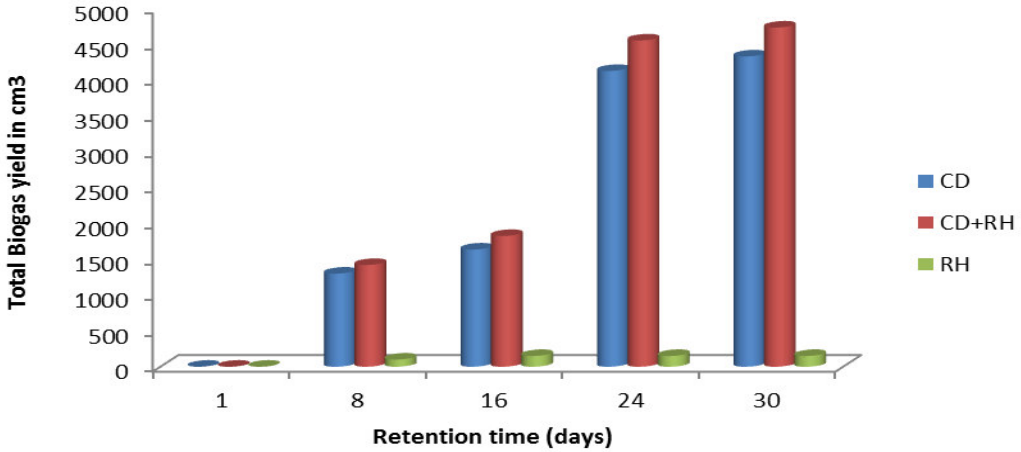
Key: CD = Cow dung; RH = Rice husk; CD:RH = Combination of cow dung and rice husk

Figure 5: pH changes in the digester content during anaerobic digestion

Table 2 shows the different species of bacteria and fungi present in the digester during the digestion process. The total biogas production produced by substrates during anaerobic digestion increased with days during fermentation. CD:RH had the highest gas production as shown in **figure 6** producing 4730.55cm^3 after 30 days of anaerobic digestion followed by CD with a volume of 4327.65cm^3 while RH produced the least amount of biogas after 30 days of anaerobic digestion with a volume of 100cm^3. The constituents of each gas produced as shown in **figure 7** were CD (62.4% CH_4, 37.4% CO_2, 0.2% H_2S), RH (100% CO_2), and CD:RH (73.8% CH_4, 25.8% CO_2, 0.4% H_2S).

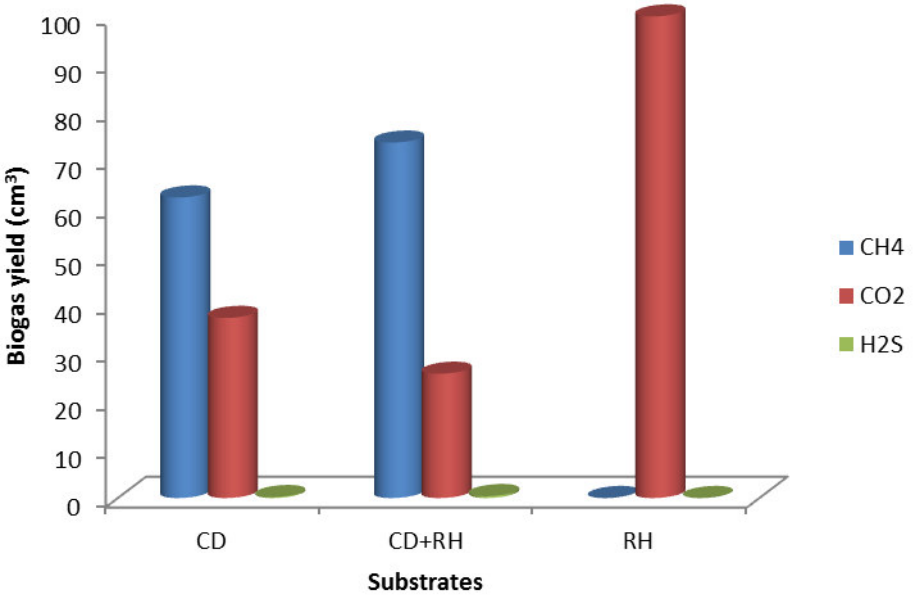
Table 2 Isolated group of microorganisms during 30 days of anaerobic digestion

S/N	Hydrolytic microorganisms			
1	**Cellulolytic**	**Lipolytic**	**Proteolytic**	**Amylolytic**
2	*Micrococcus luteus*	*Bacillus subtilis*	*Clostridium spp*	*Bacillus subtilis*
3	*Pseudomonas aeruginosa*	*Pseudomonas aeruginosa*	*Pseudomonas aeruginosa*	*Enterobacter aerogene*
4	*Klebsiella oxytoca*	*Micrococcus luteus*	*Peptostreptococcus spp*	*Pseudomonas aeruginosa*
5	*Aspergillus spp*	*Proteus vulgaris*		
6	*Penicillium notatum*			
7	*Mucor spp*			



Key: CD = Cow dung; RH = Rice husk; CD:RH = Combination of cow dung and rice husk

Figure 6: Total biogas produced by each waste at different days of anaerobic digestion



Key: CD = Cow dung; RH = Rice husk; CD:RH = Combination of cow dung and rice husk

Figure 7: Percentage of biogas constituents produced by wastes after anaerobic digestion

Figures 8,9,10 and **11** clearly describes hydrolytic profiles of digester's microflora. Summarily, the following had the highest activities in terms of diameter of cleared zones of inhibition: Amylolytic (*Enterobacter aerogene* - 15mm), lipolytic (*Pseudomonas aeruginosa* - 16mm), Cellulolytic (*Klebsiella oxytoca* - 17mm) and Proteolytic (*Pseudomonas aeruginosa* - 14mm).

Figure 12 gives the percentage distribution of hydrolytic microorganism of the digester feedstock during the period of digestion. Cellulolytic microorganisms were top in the digester with 37.50% followed by lipolytic bacteria, 25%, proteolytic and amylolytic bacteria both have 18.75%. **Table 3** shows the similarity of screened isolates sequences with those obtained from NCBI database Gene-bank.

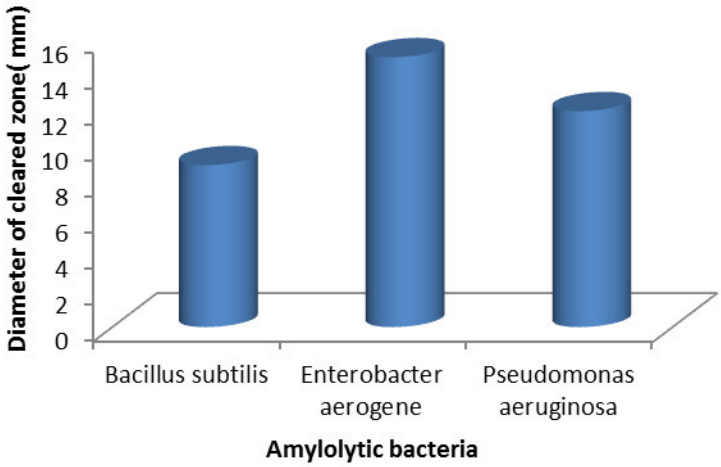


Fig. 8. Amylolytic bacteria isolated during anaerobic digestion of wastes

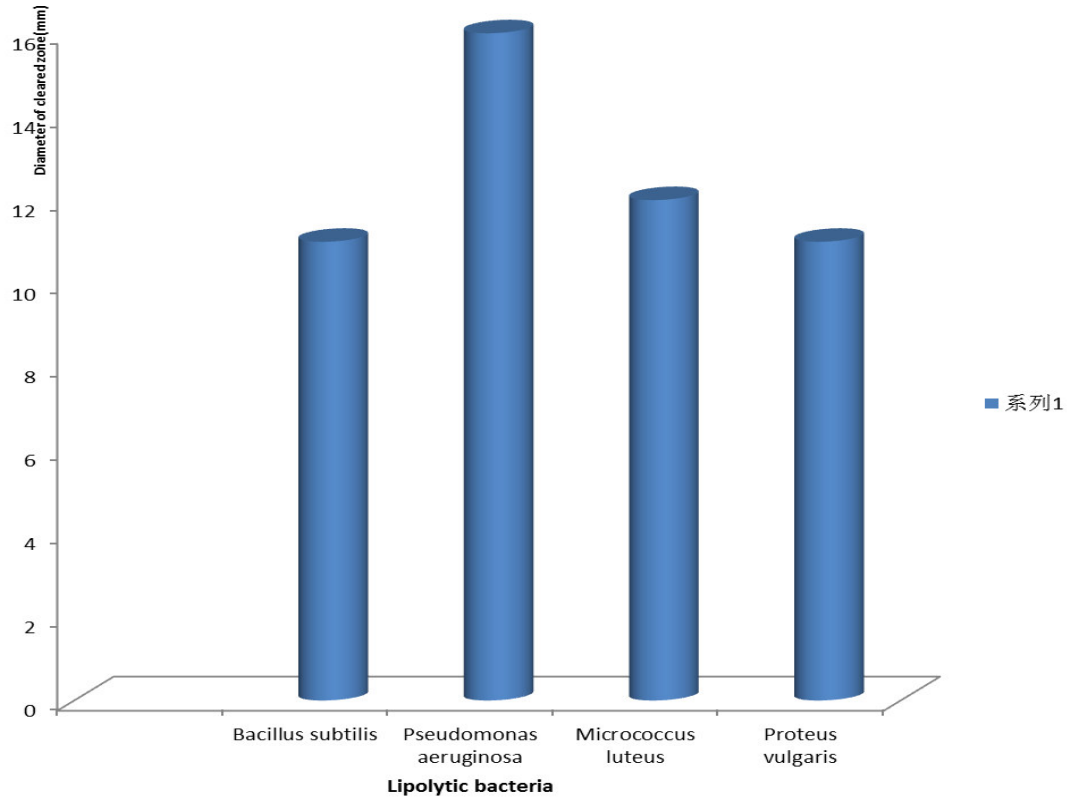


Fig. 9. Lipolytic bacteria isolated during anaerobic digestion of wastes

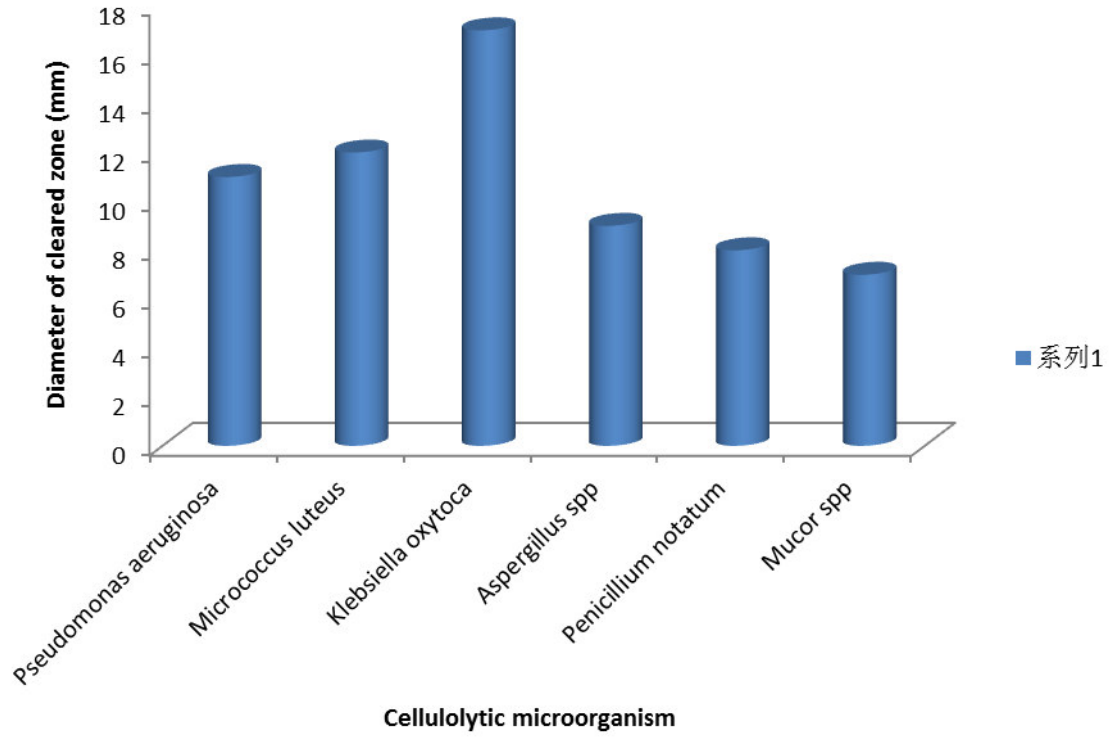


Fig.10. Cellulolytic microorganism isolated during anaerobic digestion of wastes

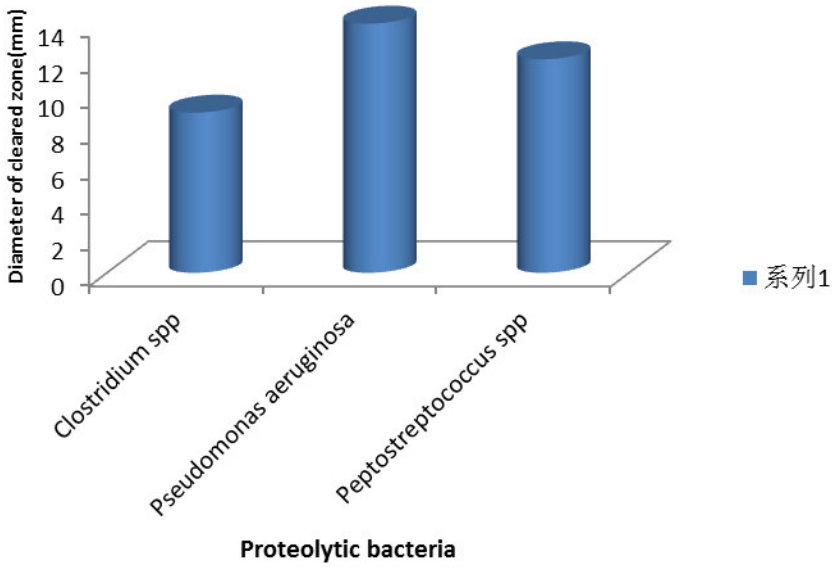


Fig.11. Proteolytic bacteria isolated during anaerobic digestion of wastes

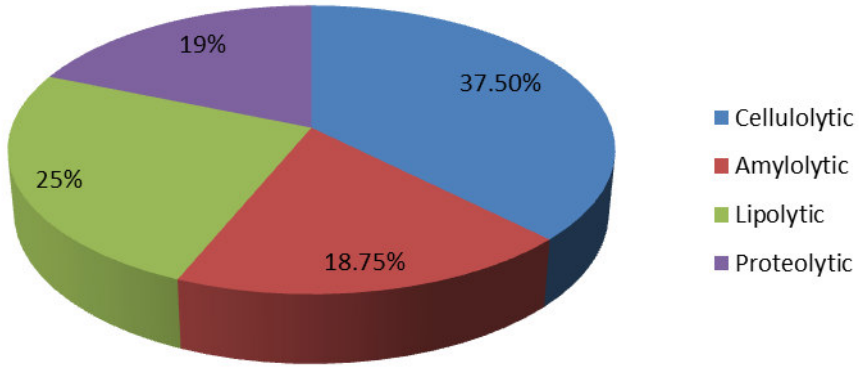


Figure 12. Percentage distribution of hydrolytic microorganisms in the digester during 30 days of anaerobic digestion.

Table 3 The similarity of DNA of sequences with sequences obtained from NCBI database Gene-bank

S/N	Identified organism	Identity (%)	Accession no
1	*Pseudomonas aeruginosa PA1*	98	NC_022808.2
2	*Pseudomonas aeruginosa PA7*	95	NC_009656.1
3	*Pseudomonas aeruginosa MTB-1*	95	NC_023019.1
4	*Aspergillus niger*	95	KF414527

4.0 Discussion

The overall temperature range of all the digesters was from 27.2^0 C to 33.5^{0}C. The highest overall temperature (33.5^{0}C) was recorded in CD at the 21st and 30th day of digestion while the lowest temperature (27.2^{0}C) was recorded in RH at the 18th day of digestion. The overall pH range recorded in all the digesters was from 6.1 to 7.8. The lowest pH measurement (6.1) was recorded after the 27th day of digestion in CD: RH and 30th day of digestion in RH while the highest pH measurement (7.8) was recorded at the 1st day of digestion in CD:RH. The temperature of the digester remained constant at mesophilic range. This was similar to that of Dahunsi and Oranusi (2013) who reported a temperature range of 22.0ºC - 30.5ºC. Frequent rainfall during the research period was responsible for the non-steady and lowered temperature readings. The result of the physico-chemical analysis of the substrate upon anaerobic digestion shows a reduction in Nitrogen content, Carbon content,

Carbon/Nitrogen ratio, Ash content, Crude Fibre, Crude Protein, Fat Content, Total solids, Volatile solids, Biochemical oxygen demand (BOD) and Chemical oxygen demand (COD) except moisture content that increased in all the substrates. Ash content (1.08-1.67 mg) and crude fibre level (1.27-1.96 mg) increased in CD only. The reduction in BOD and COD agrees with the reports by Dahunsi and Oranusi (2013) indicating that anaerobic digestion is a potent method of reducing these parameters and pathogens from sludge or wastewater. Carbon to nitrogen ratio was also determined. An optimum C:N ratio of between 20:1 and 30:1 has been suggested in previous studies to be adequate for optimum gas production. If the C:N ratio is very high, the nitrogen will be consumed rapidly by methanogens to meet their protein requirements and will no longer react on the leftover carbon content of the material leading to less gas production (Schnürer and Jarvis, 2010).The reduction in total solids and volatile solids may be due to the utilisation of the waste by the microorganisms. This agrees with the reports of Oyeleke *et al*., (2003) who stated that, the total solids and volatile solids reduce as methane yield increases. The study showed that co-digested CD:RH had the highest biogas production followed by CD and RH only. RH produced the least amount of biogas. This is corroborated by the works of Kalia *et al.*(2000) and Momoh (2004) who reported that the composition of biogas as well as biogas yields depend on the substrates owing to differences in material characterisation in each feed material. Hence, given the high cellulose and lignin content of RH, it is not surprising that it is resistant to enzymatic degradation and hence, biogas production as explained by Iyagba *et al*. (2009).

Four groups of hydrolytic microrganisms were isolated from the digester. These include; cellulolytic, amylolytic, proteolytic and lipolytic microrganisms. Of the four groups of bacteria isolated, fungal presence was only recorded in the cellulolytic category where *Aspergillus spp ,Mucor spp* and *Penicillium spp* were isolated. This agrees with the point of Agunwamba (2001) that bacteria are mainly responsible for anaerobic digestion of biogas process. The absence of fungi in later stages justified the points that methane-producing microorganisms are mainly bacteria. Isolated hydrolytic bacteria where divided into four (4): cellulolytic, amylolytic, proteolytic and lipolytic bacteria. Identified cellulolytic microorganism were *Klebsiella spp, Pseudomonas spp, Micrococcus spp* , *Mucor spp, Aspergillus spp and Penicillium spp*. Identified amylase producing bacteria were *Bacillus spp*, *Enterobacter spp* and *Pseudomonas spp*. This results tallies with the work of Mazzucoteli *et al.*(2013) who isolated *Bacillus*, *Serratia*, *Enterococcus*, *Klebsiella*, s*Stenotrophomonas*, *Lactococcus,* and *Escherichia* genera as cellulose and amylase producing bacteria.

Isolated proteolytic bacteria include *Clostridium spp, Pseudomonas spp and Peptostreptococcus spp*. This is supported by the work of Ramsay and Pullammanappallil (2001) who claimed that the genera *Clostridium, Peptostreptococcus, and Bifidobacterium* are proteolytic bacteria. *Bacillus spp, Pseudomonas spp, Micrococcus spp, and Proteus spp* were the identified lipolytic bacteria .

5.0 Conclusion

Penicillium notatum, Micrococcus luteus, Pseudomonas aeruginosa, Klebsiella oxytoca, Aspergillus spp, Mucor spp, Bacillus subtilis, Proteus vulgaris, Clostridium spp, Peptostreptococcus spp and *Enterobacter aerogene* are the hydrolytic microorganisms involved in biogas production. Also, the research showed that the combination of cow dung and rice husk (CD:RH)is best for biogas production. This research revealed that anaerobic digestion of wastes is a potential method of reducing the proximate parameters tested and pathogens from substrates. Also, anaerobic digestion is a viable way of converting agricultural wastes (either after harvesting in plants or during livestock breeding in animals) to useful ventures. The study also revealed that *Pseudomonas aeruginosa* is a versatile hydrolytic microorganism with its presence in the four groups of the isolated hydrolytic microorganisms. The high cellulose and lignin content of rice husk is responsible for its low biogas production. Hence, further investigation beyond 30 days retention period should be investigated.

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APPENDIX

1. Isolation of cellulolytic bacteria

Cellulolytic bacterial strains was isolated using CMC (Carboxy-methyl cellulose) medium supplemented with 1% CMC (Hi Media) at incubation temperature of 37 ^{0}C for 24 hours as described by Yucai *et al.*(2011). The composition of the CMC agar media include potassium chloride 0.2g , ammonium di hydrogen phosphate 1 g, yeast extract 1 g , carboxymethylcellulose 26 g, agar 3 g and magnesium sulphate hepta hydrate 1 g and 1000 ml distilled water.

2. Isolation of amylolytic bacteria

Amylase producing bacteria were screened for their ability to hydrolyse starch using soluble starch as substrate. 25 g of starch powder was suspended in 1 L of water and mixed thoroughly. The mixture was heated with frequent agitation and boiling for a minute until the powder was completely dissolved. The mixture was further autoclaved. Upon incubation, the plates was flooded with 1 % iodine. The clear zones around the well (8 mm or more) indicates a positive amylase activity (Cheesbrough, 2006).

3. Isolation of Lipolytic bacteria

Gelatin agar was prepared by adding gelatin to sterilized nutrient agar of temperature around 45-50 ^{0}C and was plated on sterile petri dishes. A pure colony of bacteria isolates was inoculated on gelatin agar and incubated at 37 ^{0}C for 48 hours. After incubation, lysis around the colonies was observed which indicate lipolytic action (Cheesbrough, 2006).

4. Isolation of Proteolytic bacteria

Egg yolk media was prepared by adding egg yolk to sterilised nutrient agar of temperature around 45 – 50 ^{0}C and was plated on sterile petri dishes. A pure colony of bacteria isolates was inoculated on egg yolk medium agar and incubated at 37 ^{0}C for 48 hours. After incubation, lysis around the colonies was observed which indicate proteolysis (Cheesbrough, 2006).