# HYDROCARBONOCLASTIC AND P-SOLUBILIZING ISOLATES ASSOCIATED WITH THE EFFLUENT OF A BIOGAS DIGESTER


## ABSTRACT

The anaerobic digest of fresh gut contents of slaughtered beef cattle was screened for hydrocarbon-utilizing bacteria and fungi, and phosphate-solubilizing bacteria, using the spread plate method. Hydrocarbon-utilizing microbes were isolated on Bushnell Haas agar using the vapour-phase technique, while phosphate-solubilizing bacteria were isolated on Pikovkaya's agar. The hydrocarbon utilizing isolates were: *Ruminococcus* sp., *Clostridium* sp., *Pseudomonas diminuta*, and *Mucor hachijoensis*. The only phosphate-solubilizing isolate was the bacterium *Bacillus subtilis*. Physicochemical analysis of the effluent revealed: pH of 7.6, total solids of 9.83%, volatile solids of 7.24%, moisture content of 72.65%, ash of 2.59%, total organic carbon of 0.927%, total organic matter of 1.598%, total nitrogen of 0.238%, phosphorus of 0.0184%, potassium of 0.235%, and biological oxygen demand of 3.294 mg/L. The array of hydrocarbon utilizing and phosphate solubilizing microbes, and abundant nutrients present in the anaerobic digest of abattoir waste suggests its potential suitability as an agent of bioremediation of crude oil polluted sites and as a source of biofertilizer.

Keywords: biowaste, biostimulation, plant growth promoting bacteria, biofertilizer.

## INTRODUCTION

Recycling organic waste is essential to environment health and integrity. Anaerobic digestion (AD) is an established efficient means of decontaminating, stabilizing, and reducing the bulk of organic wastes. Anaerobic digestion yields biomethane (the combustible component of biogas) which is an added advantage to this process, hence anaerobic digestion is also called biomethanation. The relatively stable remnant after anaerobic digestion is called the digestate. Depending on the mode of anaerobic digestion employed and the nature of feedstock digested, the digestate could be either a solid or a liquid material (Makadi *et al*., 2012). Digestate contains a high proportion of macro and micro-nutrients necessary for plant growth. Though the microbial load in the digestate is drastically reduced (when compared to that of undigested feedstock) by anaerobic digestion, the digestate still contains a diverse microbial community that may be gainfully exploited for their inherent attributes. According to researchers, the high organic matter, improved available nutrients, and diverse microbial flora of the anaerobic digestate of organic wastes suggest its suitability for bioremediation of crude oil polluted sites and as a source of biofertilizers (Al Seadi *et al*., 2008).

The petroleum industry in Nigeria has created an economic boom, and at the same time led to various environmental dooms (Ibekwe *et al*., 2006). Pollution of the environment by petroleum products is an inevitable consequence of oil exploration, exploitation, distribution, and consumption. Large amounts of petroleum products handled and conveyed on land and across the world oceans create the possibilities of spillages and consequently land and sea contaminations (Ekhaise and Nkwelle, 2011). Biodegradation of hydrocarbons by either autochthonous or allochthonous populations of microorganisms represents one effective means of mitigating the impact of petroleum pollution in the environment (Leahy and Colwell, 1990). The ability to degrade or utilize hydrocarbon substrates is exhibited by a wide range of bacteria and fungi (Atlas, 1981). Though a lot of research has gone into bioremediation of petroleum contaminated sites using microorganisms, there is need for further studies with a view to developing more efficient options.

Phosphorus (P) is the second most important plant nutrient after nitrogen (Balakrishna *et al*., 2012). Phosphorus is essential for growth and productivity in plants: cell division, photosynthesis, development of good root system, and utilization of carbohydrates (Sharma *et al*., 2011). However, the most important function of phosphate in the plant system is

energy storage. Phosphates exist in nature as a variety of insoluble organic and inorganic forms (Balakrishna *et al*., 2012). Phosphate availability in soil is low because of its fixation as insoluble phosphates of calcium, aluminum, and iron. Since deficiency of phosphorus restricts the plant growth, to obtain optimum crop yields chemical phosphate fertilizers are widely used (Chabot *et al*., 1993). However, phosphorus supply through biological means is a viable alternative; phosphate solubilizing microbes (PSM) being potential candidates for dissolving the insoluble organic and inorganic phosphate compounds (Sundara Rao and Sinha, 1963). The objectives of this research are to screen the effluent of anaerobic digest of gut contents of slaughtered beef cattle for hydrocarbon utilizing bacteria and fungi, and for phosphate solubilizing bacteria.

The need for developing an indigenous, affordable, and environmentally friendlier means of ameliorating the hazardous effects of oil spill and soil infertility issues, coupled with the abundant availability of feedstock for prospective biogas plants justify this study. The effluent samples studied in this work were obtained from a laboratory-scale 30 days batch anaerobic digester operating at ambient mesophilic temperatures, used for biogas studies at a Research Laboratory Facility in Owerri. Microbiological investigations were limited to the isolation, characterization, and

enumeration of bacterial and fungal genera using microscopy and biochemical techniques.

## MATERIALS AND METHODS

### Sample collection

Effluent of anaerobic digest was collected from a laboratory-scale biogas facility located in a Research Facility in Owerri. The effluent was the by-product of a 30-days batch anaerobic digestion of gastro-intestinal (gut) contents of slaughtered beef cattle. Samples were collected using sterile opaque glass jars and stored at 4$^{o}$C until required for analyses.

### Physico-chemical analysis

Physico-chemical analysis was done using standard protocols as outlined in Handbook of Reference Methods for Plant analysis (Kalra, 1998).

### Microbiological analysis

*Enumeration and isolation of hydrocarbon utilizing microbes*

Aliquots (0.1 ml) of appropriate dilutions ($10^{-3}$ – $10^{-6}$) of effluent samples were plated in duplicate onto Bushnell – Haas mineral salt agar. This medium contained magnesium sulfate 0.2 g/L, calcium chloride 0.02 g/L, mono-potassium phosphate 1 g/L, dipotassium phosphate 1g/L, ammonium nitrate 1 g/L, ferric chloride 0.05 g/L, agar 15 g/L. The vapour-phase technique as described by Okpokwasili and Amanchukwu (1988) was used for the selective isolation of hydrocarbon utilizers. Sterile filter paper (Whatmann No. 1) saturated with filter sterilized Bonny light crude oil were aseptically placed unto the covers of the inoculated inverted plates and then incubated for 3 – 7 days at 30$^{o}$C. Hydrocarbons from the crude oil saturated filter paper were supplied in vapour-phase to the surface of the agar plate as sole source of carbon. Two set-ups were prepared, one was incubated under aerobic conditions, while the other was incubated under anaerobic conditions. Colonies were counted from duplicate plates and the average counts were recorded and used for the calculation of colony forming units per ml (CFU/ml) of effluent. Colonies of hydrocarbon utilizing species were selected based on their colonial morphology on the Bushnell – Haas agar plates. The isolates were purified by streaking on trypticase agar and potato dextrose agar plates for bacterial and fungal isolates respectively. Pure isolates were transferred onto trypticase agar and potato dextrose agar slants in Bijou bottles and stored at 4$^{o}$C in a refrigerator for further studies.

*Enumeration and isolation of phosphate solubilizing species*

Aliquots (0.1 ml) of appropriate dilutions ($10^{-3}$ – $10^{-6}$) of effluent sample were plated in duplicate onto Pikovskaya's agar amended with 0.05% natamycin (to prevent fungal growth). Pikovskaya's agar contained yeast extract 0.5 g/L, dextrose 10 g/L, calcium phosphate 5 g/L, ammonium sulfate 0.5 g/L, potassium chloride 0.2 g/L, magnesium sulfate 0.1 g/L, manganese sulfate 0.0001 g/L, ferrous sulfate 0.0001 g/L, agar 15 g/L. The set-up was incubated for 24 – 72 hours at 30$^{o}$C. Two set-ups were prepared, one was incubated under aerobic conditions, while the other was incubated under anaerobic conditions. Colonies were counted from duplicate plates and the average counts were recorded and used for the calculation of colony forming units per ml (CFU/ml) of effluent. Colonies of phosphate solubilizing bacteria were selected based on their colonial morphology on the Pikovskaya's agar plates. The isolates were purified by streaking on trypticase agar. Pure isolates were transferred onto trypticase agar slants in McCartney bottles and stored at 4$^{o}$C in a refrigerator for further studies.

*Identification of bacteria and fungi isolates*

Bacteria isolates were identified using the schemes of Bergey's Manual of Determinative Bacteriology (*Holt et al*., 1994), while fungi were identified by observing their macroscopic colonial appearance on agar plates and microscopic orientations and compared with the established identification keys of Pictorial Atlas of Soil and Seed Fungi (Watanabe, 2010).

## RESULTS AND DISCUSSIONS

**Table 1 Physicochemical analysis of effluent of anaerobic digest of gastro-intestinal contents of slaughtered beef cattle**

| Parameters | |
|---|---|
| pH(temperature) | 7.6(27$^{o}$C) |
| total solids (TS) (% of wet mass) | 9.83 |
| % volatile solids (VS) (% of wet mass) | 7.24 |
| moisture (% of wet mass) | 72.65 |
| ash (% of wet mass) | 2.59 |
| total organic carbon (TOC) (% of wet mass) | 0.927 |
| total organic matter (TOM) (% of wet mass) | 1.598 |
| biological oxygen demand (B.O.D) (mg/L) | 3.294 |
| nitrogen (N) (% of wet mass) | 0.238 |
| phosphorus (P) (% of wet mass) | 0.0184 |
| potassium (K) (% of wet mass) | 0.235 |

The results of eleven physicochemical parameters of effluent of anaerobic digest of slaughtered beef cattle rumen contents are shown in table 1.

**Table 2 Mean population densities of hydrocarbon-utilizing microbial isolates.**

| Isolate | Population density (CFU/ml) |
|---|---|
| *Ruminococcus* sp. | $15.2 \times 10^4$ |
| *Clostridium* sp. | $81.5 \times 10^3$ |
| *Pseudomonas diminutis* | $3.2 \times 10^6$ |
| *Mucor hachijoensis.* | $1.3 \times 10^3$ |

The mean population densities of the hydrocarbon utilizing microbial isolates from the effluent of anaerobic digest of the rumen contents of slaughtered beef cattle are shown in table 2. *Pseudomonas diminutis* and *Mucor hachijoensis* recorded the highest and least populations, respectively.

**Table 3 Mean population density of phosphate-solubilizing bacteria isolate.**

| Isolate | Population density (CFU/ml) |
|---|---|
| *Bacillus subtilis* | $33.5 \times 10^3$ |

Only one phosphate solubilizing bacteria was isolated from the anaerobic digest effluent shown in table 3.

The pH, nitrogen, phosphorus, and potassium values obtained in this work were similar to those reported by Gomez *et al*. (2007), Makadi *et al*. (2008),

and Stinner *et al*. (2008) in their separate works on cattle manure digestate. These values are greatly influenced by the anaerobic digestion (AD) operation parameters, such as organic load, temperature, and hydraulic retention time (HRT) (Al Seadi *et al*., 2008). The relatively high N content of the digestate is the consequence of the N concentration effect because of carbon degradation to $CO_2$ and $CH_4$, and N preservation during anaerobic digestion (Tambone *et al*., 2009). According to Makadi *et al.* (2012), available N ($NH_4$-N) is about 60 – 80% of the total N content of the digestate. Ammonium nitrogen ($NH_4$-N) is increased during anaerobic digestion of the protein-rich feedstock as reported by Menardo *et al*. (2011). The conversion of organic of N to $NH_4$-N allows its immediate utilization by crops (Hobson and Wheatley, 1992). On the other hand, the high phosphorus and potassium levels recorded in digestate effluent makes it a suitable supplement of these macronutrients in soils (Makadi *et al*., 2012). Borjesson and Berglund (2007) postulated all the phosphorus in the anaerobic digestate of cattle manure to be in available forms. The modified organic matter of the digestate makes digestate an excellent soil amendment over undigested manure. The pH of feedstock is increased during anaerobic digestion, and the alkaline pH of the resulting digestate is a useful property because of the worldwide problem of soil acidification (Madaki *et al*., 2012).

The three bacteria hydrocarbon utilizers isolated in this work were *Ruminococcus* sp., *Clostridium* sp., and *Pseudomonas diminutis*; while the only hydrocarbon utilizing fungal isolate was *Mucor hachijoensis*. This is similar to reports of other workers, in addition to the above hydrocarbon utilizers obtained in this work, Ogbonna *et al*. (2012) reported the presence of *Klebsiella sp*., *Penicillium sp*., *Saccharomyces sp*., and *Candida sp*. in the digestate of abattoir waste. Degree of diversity of the microbial flora of a given digestate is a function of type of feedstock, source of feedstock, operational temperature, and hydraulic retention time (Toerien and Hattingh, 1969). Among the hydrocarbon utilizers isolated in this work, *Ruminococcus sp*. and *Clostridium sp*. are anaerobic, *Mucor hachijoensis*. Facultative, while *Pseudomonas diminutis* is an aerobe. According to Gerardi (2003), some strains of *Pseudomonas* can persist in anaerobic digesters. This is possible because they have remarkably effective cytochrome systems which are able to scavenge oxygen. This enables *Pseudomonas diminutis* function optimally even at very low oxygen concentrations, imparting on them a competitive advantage even over micro-aerophiles (Varnam and Evans, 2000).

The hydrocarbon degrading potencies of the Bushnell Haas isolates of this work have been corroborated by other researchers as reported by Atlas

(1981). Similarly, the efficiency of cow dung in enhancing bioremediation of crude oil contaminated soils have also been reported by Njoku *et al*. (2008), Alfa *et al*. (2014), and Ofoegbu *et al*. (2015). In their work, Ogbonna *et al*. (2012) also reported the ability of abattoir waste to biodegrade polycyclic aromatic hydrocarbons (PAHs). The ability of anaerobic digestate to degrade hydrocarbons in contaminated sites lie mainly in its capacity of providing indigenous hydrocarbon degraders (in the hydrocarbons) with adequate nutrients for optimum performance (biostimulation), and its own consortium of allochthonous hydrocarbon degraders and facilitators that may supplement or/and complement the efforts of autochthonous hydrocarbon degraders (bioaugmentation).

The phosphate solubilizing potencies of species of *Bacillus*, *Aspergillus*, and *Mucor* have been reported by Alfa et al. (2014), and Karpagan and Nagalakshmi (2014). According to their reports *Bacillus* sp. showed the highest phosphate solubilizing capacity.

Alfa *et al*. (2014) reported that microorganisms belonging to the genera *Pseudomonas*, *Bacillus*, and *Aspergillus* were known to be free living nitrogen fixing organisms. Bacillus species also act as solubilizers for trace elements like silicates and zinc, as well as acting as plant growth promoters.

They also reported that *Pseudomonas* species are excellent plant growth promoting rhizobacteria.

Though the use of animal manure in enhancing the bioremediation of hydrocarbon contaminated sites and as a soil amendment has been extensively documented, however the use of its digestate after anaerobic digestion has not been so widely reported. Since anaerobic digestion extracts biogas from animal manure, sanitizes the manure by reducing its microbial load, stabilizes the manure and modifies and improves its physicochemical properties; hence the use of anaerobic digestate of animal manure may become the preferred choice in bioremediation and biofertilization.

## CONCLUSIONS AND RECOMENDATIONS

This study revealed the presence of different hydrocarbon utilizing microbes and a phosphate solubilizing bacterium in the anaerobic digestate of abattoir waste. It also revealed the rich nutrient contents of the anaerobic digestate of gut contents of slaughtered beef cattle. This discovery may suggest that effluents from biogas plants could be applied to the bioremediation of crude oil polluted environments and for soil amendment. Further studies should be able to measure their individual hydrocarbon degrading and phosphate

solubilizing capacities. Molecular studies may also be done to determine the location and proximity of genes responsible for desirable attributes.

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