BACTERIOLOGICAL ANALYSIS OF DIGESTATE FROM DIFFERENT WASTE STREAMS FOR PLANT GROWTH PROMOTING RHIZOBACTERIA

BY

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PG/LSC1817726

DEPARTMENT OF MICROBIOLOGY
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UNIVERSITY OF BENIN
BENIN CITY

NOVEMBER, 2022.

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A PROJECT REPORT SUBMITTED TO THE DEPARTMENT OF MICROBIOLOGY, FACULTY OF LIFE SCIENCES IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE UNIVERSITY OF BENIN, BENIN CITY, AWARD OF POSTGRADUATE DIPLOMA (PGD HONS) DEGREE

NOVEMBER, 2022.

CERTIFICATION

This is to certify that this project work was carried out by Ikponwosa Kelvin AIBANGBEE of the Department of Microbiology, Faculty of Life Science, University of Benin, Benin City.

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DEDICATION

This project work is dedicated to Almighty God for his grace and mercies throughout my period of study.

ACKNOWLEDGEMENTS

My sincere appreciation goes to Almighty God for his grace and mercies throughout my period of study.

I wish to acknowledge whole heartedly my project supervisor Prof. A. O. Emoghene and co-supervisor Dr. A. G. Ogofure for their patience and understanding towards me and the success of this project. May God Almighty richly bless you sir for your efforts.

My sincere appreciation goes to my head of department Prof. (Mrs.) F. I. Akinnibosun for her motherly role in the administration of the Department.

I want to also thank my lecturers for their mentoring throughout my stay in this school especially, Dr. (Mrs.). I. S. Obuekwe, Dr. A. G. Ogofure, Dr. (Mrs.) .B.I. Idemudia, Prof. B. O. Omogbai and Prof. (Mrs.) F. I. Akinnibosun for their assistance during the course of this project.

With deep sense of honor, I will not fail to appreciate my wonderful mum,, Mrs. M. Ailemoh and my siblings for their financial support and words of wisdom. My amazing brothers who were caring and supportive in all way round. I love you all.

I will not fail to appreciate my course mates, friends and colleges in medical biochemistry who have contributed in one way or the other to the success of the work, God bless you all.

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ABSTRACT

The need for an alternative to inorganic fertilizer is on the increase owing to the drawbacks associated with inorganic fertilizers. This study was therefore carried out to evaluate the bacteriological profile of digestates from different waste streams and the ability of the isolates to promote the growth of plants. Standard bacteriological methods were used to analyze digestates from different waste streams. Total heterotrophic bacterial count was obtained using pour plate method. The cultural, morphological and biochemical characteristics of the bacterial isolates were evaluated using standard differential media along with appropriate reagents. Plant growth promoting capacity of the isolates were evaluated using standard protocols for nitrogen fixation, phosphate solubilization, indole acetic acid and ammonia production. The results obtained from this study revealed that the total heterotrophic bacterial count (Log_{10} cfu/g) of digestate from different waste streams had values, which ranged from 4.91±0.02 for cattle rumen digestate to 4.41±0.03 for a combination of cattle rumen fruit and food waste digestate. The cultural, morphological and biochemical characteristics of bacterial isolates revealed the presence of *Enterobacter cloacae, Escherichia coli, Pseudomonas aeruginosa, Salmonella enterica, Proteus mirabilis* and *Bacillus cereus*. The distribution of bacterial isolates in the different waste streams revealed that *E. coli* was present in all digestates obtained from different waste streams. All bacterial isolates were found to possess plant growth promoting properties with 100 % capacity for nitrogen fixation and phosphate solubilization. While 66.7 % of the isolates had the ability to produce ammonia and indole acetic acid. The phosphate solubilization index of the isolates revealed that *E. coli* (5.97) and *P. aeruginosa* (5.76) had the highest solarization index amongst all tested bacteria in the study.

CHAPTER ONE

INTRODUCTION

Due to the loss of nutrients in the soil following its frequent usage, beginning from the late 19th and early 20th centuries, inorganic compounds containing nitrogen, potassium and phosphorus (NPK) were synthesized and used as soil fertilizers (Gupta *et al.,* 2015). These chemical fertilizers were then successfully used to replenish nutrient loss from the soil and consequently increase crop production (Savci, 2012). As of today over 300 million pounds of various chemicals are now produced in the form of fertilizers and pesticides under different brand names (Kumari *et al.,* 2014). Nutrient loss was not the only issue militating against increased crop production, they were other biotic factors such as pests and abiotic factors such as soil pH, climate, salinity, pollution etc. To control these pests use of chemical pesticides was employed successfully. Low soil fertility could threaten the security of food production and supply. Soil fertility is a major overriding constraint that affects all aspects of crop production. In the past years, inorganic fertilizer was advocated for crop production to ameliorate inherent low fertility of soils in the tropics. In addition to being expensive and scarce, the use of inorganic fertilizer has not been helpful in intensive agriculture because it is often associated with reduced crop yield after a long-term use, soil acidity, nutrient imbalance and their use is responsible for a significant proportion of the greenhouse-gas (GHG) emissions and water-pollution incidences from agriculture. The need to use renewable forms of energy and reduce costs of fertilizing crops has revived the use of organic fertilizers worldwide. Large quantities of organic wastes such as poultry manure are available especially in urban centers and are an effective source of nutrients for vegetables such as tomato (Adeyika and Agbede. 2009). Anaerobic digestion produces biogas and a very-wet residue called digestate which is a mixture of partially- degraded organic matter (OM),

microbial biomass and inorganic compounds (Jośe *et al*., 2011), and have been employed as an encouraged method of agricultural management for plant nutrition and productivity. With productivity not only a function of mineral input, but strongly linked with soil microbial communities since they form a fundamental part in retaining healthy soils by nature of the strong relationship between soil microorganisms, nutrient cycling, plant growth and disease suppression (Garbeva *et al*., 2004; Van-der-Heijden *et al*., 2007), digestate serve as an all-round soil conditioner and a better option for increased plant yield than synthetic chemical fertilizers. It is thought that current use of inorganic fertilizers is a major deleterious influence on the intrinsic soil microbial community and has been shown to cause decline in soil organic matter (Kibblewhite *et al*., 2008). Specifically, the addition of nitrogen can affect carbon transformations (Kibblewhite *et al*., 2008) thus adversely affecting the functioning of the microbial community with potentially considerable effects on specific functions. An example is methane metabolism: the diversity of methanotrophs is known to be lower in agricultural souls (Levine *et al*., 2011).

Hence, to manage and maximize the effectiveness of digestate as an efficient mitigator of problems associated with synthetic fertilizers amongst other functions, drying is primarily applied in order to reduce the digestate mass and consequently to produce a transportable and storable fertilizer (Salamat *et al*., 2020). Several publications demonstrated the effectiveness of digestate as suitable nutrient source in agriculture (Chantigny *et al*., 2008, Panuccio *et al*., 2016, Muscolo *et al*., 2017). Nonetheless, the quality (mineral content) of digestate is dependent upon characteristics of feedstock, digester process and treatment options (Aso, 2020).

AIM AND OBJECTIVES

The aim of this study was to evaluate the bacteriological profile of digestate from different waste streams for plant growth promoting rhizobacteria.

The objectives were to:

1. isolate and enumerate the bacteria present in digestates from different waste streams
2. identify bacterial isolates using biochemical means
3. evaluate bacteria isolates for plant growth promoting capacity

CHAPTER TWO

LITERATURE REVIEW

The secondary product of the anaerobic digestion is the slurry, termed as biogas digestate, or simply digestate. The digestate contains a huge number of organic compounds of both plant and microbial origin and numerous mineral elements. Its key characteristic is a low concentration of dry matter, ranging from a few percent to more than ten percent (Möller and Müller, 2012). The mineral composition of digestate depends, to a great extent, on the composition of substrate and the type of digestion process (Alburquerque *et al*., 2012). The concentration of mineral elements in digestate, in spite of the referenced announcement, is not high, but low (Möller and Müller, 2012, Ozores-Hampton and Peach, 2002). According to Möller and Müller, 2002, concentration of nitrogen in slurry ranges from 1.2 to 9 kg Mg^{-1} FW, and phosphorus from 0.4 to 2.6 kg Mg^{-1} FW. The recycled pathway of raw, liquid or solid form of digestate as fertilizers is the best way to close the cycle of nutrients, incorporated primarily into soil as mineral fertilizers (Arthurson, 2009).

Sources of Feedstock Used for Anaerobic Digestion

The feedstocks of anaerobic digestion processes include the following:

1. Sewage sludges: Liquid sludge, untreated sewage sludge, composted sludge, and lime treated sludge.
2. Animal wastes: Animal fats, animal blood, food remains, stomach contents, rumen contents, animal carcasses, and poultry, fish, and livestock manure.
3. Energy crops: Usually corn, maize, millet, and clover. This can be whole crops used in co-digestion or as waste (stems and stalks) from harvesting of these crops.

4. Municipal wastes: Food waste, coffee/tea filters, organic leftovers, bakery waste, and kitchen waste.
5. Agricultural wastes: Fruits, molasses, stems, plant straw, and bagasse (residue after crushing sugarcane or sorghum stalks).
6. Industrial wastes: Food/beverage processing waste, dairy wastes, starch/sugar industries wastes, slaughterhouse wastes, and brewery wastes (Chong *et al*., 2022).

These feedstocks require appropriate pre-treatments prior to the anaerobic digestion, owing to their possible presence of toxicity and contaminants. Instead of disposing the wastes to landfilling, the introduction of novel pre-treatment technologies can eventually recover the energy from these wastes and reutilize them. The ideal nutrient ratio, C/N ratio and carbon, nitrogen and phosphorus (C/N/P) ratio for anaerobic digestion is recommended to be from 20:1–30:1 and 100:5:1, respectively (Akyol *et al*., 2019). These are just some of the different sources that anaerobic digestate can come from. The chemical make-up of the digestate produced can vary depending on what feedstock is used.

Digestates from Anaerobic Digestion

Anaerobic digestion (AD) is a process whereby organic material is converted into energy-rich biogas and a plant nutrient-rich residue (digestate), which promote the growth of vegetables like tomatoes (Kajsa, *et al*., 2017). Anaerobic digestion (AD) generates two products: methane which can subsequently be used as a source of renewable energy, and digestate, which can be separated into a dry and liquid fraction suitable for land application (Walsh *et al.,* 2012).

The composition and characteristics of the anaerobic digestate depends on the characteristics of the feedstock or substrate, microbial community, operational conditions, configuration of

anaerobic digestion system, and digestate processing techniques. Anaerobic digestion can be adopted either as wet or dry, mesophilic or thermophilic, batch or continuous, single stage or multi-stage, co-digestion or mono-digestion process, which significantly influence the digestate characteristics, along with operational conditions (organic loading rate, trace element supply). Moisture content of the digestate is influenced by the choice between a wet and dry anaerobic digestion processes (Kim and Oh, 2011). Anaerobic digestion of animal manure before use as a fertilizer is generally considered positive, since the digestate obtained has higher proportions of mineralized plant-available nutrients than the untreated manure and digestion results in a significant odour reduction (Insam *et al*., 2015).

Use of Organic Amendments in Agriculture

The use of this organic amendments results in higher growth, quality and yield of crops. Organic amendments contain essential nutrients, vitamins, macronutrients, and factors of growth promotion such as indole acetic acid (IAA), gibberellic acid (GA) and beneficial microorganisms (Sreenivasa *et al.*, 2010). Organic amendments such as manures can improve soil-water-plant relations through modifying bulk density, soil water relation, total porosity, and consequently, increasing plant growth and water use efficiency (Sreenivasa *et al.*, 2010). Nileemas and Sreenivasa (2011) opined that the application of liquid organic manure as amendments promotes biological activity in soil and enhance nutrients availability to crop plants. Awad *et al.* (2012) stated that organic manure contains high levels of relatively available nutrients elements, which are essentially required for plant growth; moreover, it plays an important role for improving soil physical properties. Sustainability in agroecosystems involves environmentally friendly techniques based on biological and non-chemical methods (Bonato and Ridray, 2010).

Organic fertilizers such as poultry manure, cow dung, compost, cattle rumen, food waste, farmyard manure is being used as organic amendments and it's been reported to provide significant quantity of nutrients which are supply to the soil in slow rate over a long period which reduce the nitrate losses in drainage water (Sreenivasa *et al.,* 2010). It improves the activities of microorganism in the soil as well improves water movement and good aeration of the soil among many other advantages but the quantity, transportation, labor and cost still remain as the challenges of commercial use of organic fertilizers. Inorganic fertilizers are also been used by farmers in the production of crops and vegetables and many researchers have documented reports on the increased yield of various crops through its application (Akanbi, 2012). Among the commonly used inorganic fertilizers by vegetable farmers are the NPK and Urea but the scarcity and high price of these fertilizers remain a challenge facing crop production (Akanbi, 2012).

Effects of Digestate on Soil Properties and Biological Activity

There are only few studies about the effect of digestates on soil physical properties. Application of digestates improved soil properties by reducing the bulk density, increasing saturated hydraulic conductivity, moisture retention capacity of soils (Garg *et al.,* 2010), and aggregate stability (Beni *et al.,* 2012). Many reports indicate an enhanced soil microbial activity after field applications of digestates in comparison to inorganic fertilizers or untreated controls (Clements 2013).

Elste *et al.* (2010) reported that soil application of digestates enhanced the abundance and biomass of earthworms. Digestates with a high degradability of the organic matter such as clover-grass have a stronger effect on the short-term soil microbial activity than digestates with a low degradability such as silage maize. However, there are some indications that in a medium-term view of several months or even years, the differences in the effects of

application of digestates in comparison to the undigested feed stocks are minor or even negligible (Schauss 2011).

Poultry Manure as Organic Amendments

Poultry manure is used as soil amendment and contains fairly high nutrient composition particularly nitrogen than other sources of animal manure (Sunassee 2012). Poultry manure promotes and enhances the growth and yield of plants such as vegetables e.g tomatoes, because it is not all macro and micro nutrients that are readily available for the plant uptake, and this could bring about slow growth and poor yield (Ismail *et al.,* 2014).

Wilson *et al.* (2016) and Sharpley *et al.* (2010) described poultry manure as a mixture of bedding material (wood shaving, sawdust, grain husk, etc.) poultry dropping, feed, water, vaccine, and feather. It contains all 13 of the essential plant nutrients that are used by plants. These include nitrogen (N), phosphorous (P), potassium (K), calcium (Ca), magnesium (Mg), sulphur (S), manganese (Mn), copper (Cu), zinc (Zn), chlorine (Cl), boron (B), iron (Fe), and molybdenum (Mo). Plant nutrients originate from the feed, supplements, medications, and water consumed by the birds. Using poultry manure as a fertilizer for plants may provide a portion, or all, of the plant requirements (Griffiths, 2011). The amount of nutrients provided depends on the nutrient content of the manure (l kg of nutrient/t of manure) and the amount of manure applied (t of manure/hectare). The amount of manure applied per acre (called the application rate) is typically based on the nitrogen needs of the plants. However, phosphorous requirement can also be used to determine the application rate (Griffiths, 2011).

According to Tabler and Berry (2013), the composition of chicken manure varies according to age of the chicken, moisture content and age of the manure, kind and amount of litter, and storage and handling practices. The only sure way to know the composition is to analyze the

material. Conversion of the uric acid and urea to ammonia is rapid during the first 2 weeks after the addition of manure to a warm moist soil, but conversion of the organic forms of nitrogen to an available form is slow during the first 4 weeks after its addition (Griffiths, 2011). Phosphorus is primarily organic and becomes available as the manure decomposes, but all may not be available until the next cropping or season (Sharpley *et al.,* 2010). Other plant nutrients become available during decomposition of chicken manure and, like phosphorus, may not all be available until the next cropping season (Tabler and Berry, 2013).

Application of Poultry Manure (as Digestates) to Soils

Griffiths (2011) stated that chicken manure may be applied to the soil fresh or at any age. In general, commercially available manure is air dried, pulverized, and packed in plastic bags of varying sizes. The manure may be scattered on the surface of the soil and worked in with a rotary tiller, plow, spading fork, shovel, or similar tool. It should be mixed thoroughly and evenly so that no unmixed material remains in the soil. It should be applied in rows or hills as recommended for the type of crop grown. The manure should be mixed with or covered by soil to prevent offensive odors. Chicken manure, used wisely, brings excellent results as a top dressing for pasture and turf (Oyewole *et al.,* 2012).

Cattle Rumen Content as Digestates

Soil treated with cattle rumen digestate releases more nutrient into the soil which enhance growth of plants especially vegetables such as tomato. Rumen digestate supply nitrogen and has higher phosphorus content. Its limitation unlike other organic matter is the slow rate of decomposition (Ekpe *et al*., 2012). The rumen is an open chamber of fermentation, inhabited by microorganism that anaerobically digest complex compound of foodstuffs and generates

fermentation products (mainly acids) microbial cell mass for utilization by the host (Ekpe *et al*., 2011).

Rumen digestates are waste from abattoir obtained from cattle, sheep, goats that are presently a menace in most urban cities of developing countries. The digestate is made up of undigested fibrous materials example grasses that are still in their early stage of digestion. The digestate in the rumen is not uniform, but rather is stratified into gas, liquid and particles of different sizes, densities and other physical chrematistics. The rumen digestate is acted upon by a good number of microbes which include: Bacterial, Protozoa, Fungi, archaic and viruses and by mass account for 40-60% of total microbial matter in rumen (Awoden 2018). From analysis carried out by Ekpe *et al.* (2011); it was revealed that cattle rumen content contains percentage dry matter recovery of cattle rumen contents was 25.02%. Proximate analysis revealed that cattle rumen content contains: crude fiber (24.8%), nitrogen free extract (53.99%) and crude protein (15.41%). Gross energy was 0.72 Kcal/kg with an ether extract digestion coefficient of 82.48%.

Food Waste as Digestates

This category includes food not eaten, and food preparation leftovers from residences, commercial establishments such as restaurants, institutional sources like school cafeterias, and industrial sources like factory lunchrooms. Usually, most of the food wastes are disposed of in a landfill and are good source of nutrients to plants (Zhang *et al.*, 2011). Now it is a public concern for recycling food waste as the environmentally sound as well as cost-effective use of the biomass is difficult to manage (Takata *et al.*, 2012). Food waste also contains important nutrients and it would be more efficient to re-use them in agriculture. Composting is a well-known method to manage food waste for converting material like a

hygienic, humus-rich, and stable product that acts as soil conditioner as well as growth promoter for crop plants such as tomatoes (Lee *et al.*, 2011).

Digestate Management

Consequently, a huge volume of produced digestate leads to high cost of its storage to use as a fertilizer (Nkoa, 2014). An alternative solution is a separation of the raw slurry into solid and liquid phases (Makadi *et al*., 2012). The solid fraction of digestate has a much higher content of nutrients, but, at the same time, its C:N ratio increases. The main reason for this process is a considerable loss of its fine organic, as well as, mineral N fractions (Kolář *et al*., 2018). Based on the existing literature, solid digestate with high contribution of mineral N fraction (≥ ⅔ of total N content) should be treated as biofertilizer, while the one with low contribution (≤ ⅓), as a soil amendment (Nkoa, 2014, Teglia *et al*., 2011).

High moisture content along with limitations regarding digestate application make its management more essential. Mechanical dewatering technologies are usually applied in order to increase the dry matter content of digestate. However, the output still has considerable amounts of water, so that after mechanical separation, the percentage of the solid fraction is at most 35% (Turley *et al*., 2016). The drying of digestate either it is used as an organic fertilizer or as a solid fuel is regarded as a necessity, as it reduces the digestate volume and increases the dry matter content up to 90%. when a minimum final dry matter content of 80% is attained, regrowth and activation of the microorganisms can be avoided (Liu *et al*., 2017). Hence, the dried product can easily be stored for a long time and has a suitable particle flowability for applications using conventional farm fertilization machinery, as well (Turley *et al*., 2016). Also, drying increases the transportability of digestate and, thereby, its marketability. Furthermore, the NH_3 emissions from dried digestate with a higher dry matter content are lower compared with those from moist digestate. This is because NH_3 emissions

to the air correlate with the concentration of NH_4^+ ions in the liquid phase (Möller and Müller, 2012, Awiszus *et al*., 2018). Zeshan, (2014) studied the emission behavior of digestate with a high solid content (55%) under natural convection for 30 days in a temperature range of 30–33 C. They have shown that dried digestate exhibited 42% fewer emissions than raw digestate. However, the drying process is normally accompanied by a substantial nitrogen loss (Maurer and Müller, 2012).

Drying is also a necessary step for some post-treatment technologies such as composting (Liu *et al*., 2017) and pasteurization (Turley *et al*., 2016), as well. Additionally, it is possible to utilize dried digestate as a second-generation solid biofuel (Salamat *et al*., 2020). However, it should be noted that the calorific value of dried digestate is lower than that of wet digestate due to the volatilization of organic compounds. According to Kratzeisen *et al*. (2010), the calorific value of digestate pellets with a moisture content of 9.2–9.9% is comparable with that of wood.

Rhizobacteria with Plant Growth Promoting Activities

Bacillus subtilis

Bacillus subtilis is a common PGPR present in rhizo-sphere. The spores produced are long-lived and sustain several harsh environmental conditions; thus can be employed in diverse growth conditions (Nicholson *et al.*, 2000). It is one of the prominent biofilm- forming rhizobacteria, which help plants in nutrient uptake and root colonization (Sun *et al*., 2020). *Bacillus* spp. act as biostimulants through the production of phytohormones, auxin and cytokinin as well as expansin that contribute to plant growth and development (Zubair *et al*., 2019) by siderphore production, phosphate solubilization and nitrogen fixation Studies have been carried out for a better understanding of the plant growth potential

of B. *subtilis* based on genome sequencing, where strain EA-CB0575 increased the total dry weight (TDW) of tomato plants as compared to non-inoculated plants in a greenhouse study, which was associated with the presence of strain specific genes and metabolic pathways (Franco-Sierra *et al.*, 2020; Posada *et al.*, 2018). *Bacillus* spp. regulates nitrogen cycle and act as biocontrol via direct and indirect pathways (Radhakrishnan *et al*., (2017). The indirect method includes the formation of biofilm, plant growth promotion, competition for space and nutrients, and ISR. In the direct method of disease control, B. subtilis produce various lipopeptides, cell lytic enzymes, antioxidants and hormones which affect a wide range of fungi and bacteria (Wang *et al*., 2014). As metal purifiers, They also confer tolerance to abiotic and biotic stress on plants (Ramakrishna *et al*., 2020).

Pseudomonas aeruginosa

P. aeruginosa is a versatile and ubiquitous bacterium that has been recognized as an active antagonist of several bacterial and fungal plant pathogens, and has potential practical applications in agricultural systems (Chandra *et al*., 2020). This species can produce secondary metabolites such as indole acetic acid (IAA) and siderophores, and can also solubilize phosphate (Yasmin *et al.* 2017). *Research* on its interactions with plant-infecting fungi such as *Alternaria*, *Rhizoctonia*, and *Sclerotium* and oomycetes such as *Pythium* and *Phytophthora* have demonstrated that its production of phenazines plays an important role in controlling these pathogens (Gusain *et al*. 2015).

Enterobacter

Enterobacter contributes potentially to the development of a sustainable agricultural systems. Generally, *Enterobacter* function using three different mechanisms: synthesis of particular compounds for the plants, facilitation of certain nutrients uptake from the soil, and prevention

of plants diseases. The mechanisms of PGPR-mediated enhancement of plant growth and yield of many crops are not yet fully understood. However, possible explanations include (a) the ability to produce a vital enzyme, 1-aminocyclopropane-1-carboxylate (ACC) deaminase to reduce the level of ethylene in the root of developing plants thereby increasing the root length and growth; (b) the ability to produce hormones such as auxin, i.e., IAA; (c) a symbiotic nitrogen fixation; (d) antagonism against phytopathogenic bacteria by producing siderophores; and (e) solubilization and mineralization of nutrients, particularly mineral phosphates. (Maheshwari and Dinesh, 2011).

***Azospirillum* spp.**

Azospirillum species influence plant growth through versatile mechanisms; they include N2 fixation, phytohormone production (e.g., auxins, cytokinins, and gibberellins), increased nutrient uptake, enhanced stress resistance, vitamin production, siderophores and biocontrol (Steenhoudt and Vanderleyden 2000; Dobbelaere *et al*. 2003; Seshadri *et al*. 2000; Rodriguez *et al*. 2004). The phosphate solubilization feature is not entirely known yet as many attempts to determine its P-solubilizing capacities failed due to the experimental conditions (e.g., growth culture media). However, Seshadri *et al*. (2000) reported in vitro inorganic P-solubilization by *A. halopraeferans*. In addition, in vitro gluconic acid formation and P-solubilization from sparingly soluble phosphorus sources by two strains of *A. brasilense* (Cd and 8-I) and one strain of *A. lipoferum* JA4 were reported by Rodriguez *et al*. (2004).

Also, the PGP trait ACC (amino cyclopropene carboxylic acid) deaminase activity is absent in *A. brasilense* (Holguin and Glick 2003).

Azotobacter

Azotobacter is one of the most intensively investigated genera of PGPR, known to exploit atmospheric nitrogen for their cellular protein synthesis which is mineralized in the soil, imparting the crop plants a considerable part of nitrogen available from the soil source. *Azotobacter* spp. is sensitive to acidic pH, high salt concentration and temperature (Aquilanti *et al*., 2004). They pose advantageous impacts on the crop growth and yield through the biosynthesis of biologically active substances, instigation of rhizospheric microbes, production of phytopathogenic inhibitors, alteration of nutrient uptake and eventually magnifying the biological nitrogen fixation (Yan and Lenart, 2012). Research on *Azotobacter chroococcum* in crop production has shown its importance in improving plant nutrition and amelioration of soil fertility (Jnawali *et al*., 2015). Several strains of *Azotobacter* are found to be able to produce amino acids when grown in culture media supplemented with various carbon and nitrogen sources (González-López *et al*., 2005). Such substances produced by these rhizobacteria are implicated in several processes thus leading to plant-grown promotion (Jnawali *et al*., 2015).

CHAPTER THREE

MATERIALS AND METHODS

Study Area/sample collection

Study was conducted in rubber research institute of Nigeria, Iyanomo. Samples (untreated and treated effluents) were collected from rubber plantation after tapping and from the treatment plant to the laboratory for microbiological and physicochemical analysis. Duplicate samples were collected in sterile containers. Samples for chemical oxygen demand were collected in amber bottles.

Sterilization of Materials

Materials such as Petri-dishes, pipette, glass containers (conical flask, round bottom flask) and bottles were washed, drained and dried. They were wrapped with aluminum foil and sterilized in a hot-air oven at 160^{o}C for an hour. They were allowed to cool after sterilization before usage. An aseptic working environment was achieved with the use of Bunsen burner flame and disinfection of work surfaces with alcohol.

Preparation and Sterilization of media

All media used were obtained from Oxoid and were prepared according to manufacturers' instruction. The media used in this study include Plate count agar, Bacillus cereus agar (BCA), Eosin methylene blue agar (EMB), Mannitol salt agar (MSA), Salmonella Shigella agar (SSA), Pseudomonas cetrimide agar (PCA), Triple sugar iron agar (TSI), Simmons citrate agar (SCA) and Mueller Hinton agar (MHA).

Enumeration and Isolation of Bacterial and Fungal Isolates

The samples were serially diluted with a factor of 10 where 25g of sample was diluted in 225 ml of sterile distilled water (SDW) from whence 1 ml of aliquot was transferred to test tubes containing 9 ml of SDW even unto the fourth tube in the series. Thereafter, an inoculum volume of 1 ml from the fourth tube was transferred unto sterile petri dishes to which was added nutrient agar (supplemented with 1% fluconazole), while another set had potato dextrose agar (supplemented with 1% chloramphenicol). Replicates of samples (*n=3*) were prepared for both bacterial and fungal plates cultured using pour plate method. The formula employed for of dilution factor is given below in equation (1) (Ogofure and Igbinosa, 2021)

$$Dilution\ factor\ = \frac{final\ volume}{aliquot\ volume} \qquad (1)$$

$where: final\ volume = aliquot\ volume\ (sample\ volume)\ + diluent\ volume$

(Ogofure and Igbinosa, 2021)

Enumeration of the bacterial/fungal isolates was carried out using the formula delineated by Willey et al. (2008) and it is shown in the equation (2) below.

$$\frac{cfu}{g} = \frac{number\ of\ colonies\ x\ dilution\ factor}{volume\ of\ inoculum} \qquad (2)$$

Phenotypic Identification of Bacteria from Samples

Following pour plate culture of bacterial isolates from compost samples, single colonies were subcultured on tryptone soya agar and incubated for 24 h. at environmental temperature (28 ± 2^0C). The colonies were Gram stained and identified using standardized cultural and biochemical techniques as stipulated by Bridson (2006) in Oxoid manual. Differential Media (Oxoid) such as Chromogenic *Bacillus cereus* agar with chromogenic *Bacillus cereus* selective supplement, Sorbitol MacConkey agar with Cefixime-Tellurite Supplement, Eosine methylene blue agar, Pseudomonas cetrimide agar (supplemented with glycerol), *Salmonella*

Shigella agar, Mannitol salt agar, and triple sugar iron agar slants were used for successful isolation and culture of bacterial isolates from samples. Further confirmation of bacterial isolates were carried out using biochemical tests such as citrate (simon citrate agar {Micromaster}), indole, Oxidase, Urease, sugar fermentation, catalase, 3% KOH, Gas formation, and H_2S formation etcetera. Some of the bacterial isolates were further confirmed using molecular technique in a sequence of extraction of genomic bacteria DNA, agarose gel electrophoresis, PCR amplification and sequencing.

Concerning fungal isolates, cultural characteristics and microscopy were used for presumptive identification of the fungal isolates. Lactophenol cotton blue and wet mount staining was done for isolated fungi. Special fungal features such as the nature of spores, hyphae, conidia and other features were used during identification in line with recommendations by Barnet and Hunter (1996).

Morphology identification

The morphological identity of each bacteria isolate was obtained by Gram staining so as to know the gram reaction, cell morphology and arrangement by viewing under the microscope. The Gram stain procedure is as follows:

A smear of the bacteria isolate was made on grease free slide and heat fix by passing over flame. The smear was flooded with crystal violet which is the primary stain for 1min then washed with distilled water. Subsequently the slides were flooded with Lugol's iodine solution for 30sec and then washed off with distilled water. 95% alcohol was used for decolorization for 10sec and immediately washed off with distilled water. Finally, the smear was counter stained with saffranin for 1min and washed off. The slides were allowed to air dry before observing under the microscope using an oil immersion objective lens of ×100 magnifications.

Identification of Bacterial Isolates

Following pour plate culture of bacterial isolates from the samples, single colonies were subcultured on tryptone soya agar and incubated for 24 h. at environmental temperature (28 ± 2^0C). Differential Media (Oxoid) such as Chromogenic *Bacillus cereus* agar with chromogenic *Bacillus cereus* selective supplement, Sorbitol MacConkey agar with Cefixime-Tellurite Supplement, Eosine methylene blue agar, Pseudomonas cetrimide agar (supplemented with glycerol), *Salmonella Shigella* agar, Mannitol salt agar, and triple sugar iron agar slants were used for successful isolation and culture of bacterial isolates from samples. Further confirmation of bacteria identity were carried out using the following morphological characteristics and biochemical tests and identified using standardized cultural and biochemical techniques as stipulated by Bridson (2006) in Oxoid manual .

Gram Stain

Thin smears of the isolates were made on glass slides using a wire loop and were heat-fixed and allowed to cool. The smears were stained with crystal violet stain for a minute before washing off immediately with potable water. Then the smears were covered with Lugol's iodine for 30-60 sec and immediately washed off with water. The smears were rapidly decolorized with acetone or alcohol and washed rapidly with clean water after 5 seconds. Then the smears were stained with slaframine for 60 seconds and immediately washed off. The stained smears were allowed to air-dry after which a few drops of oil immersion were dropped on the smears after which they were viewed under the optical microscope using the 100x objective lens. The Gram-positive organisms were viewed as purple cells while the Gram-negative organisms were viewed as pink or red cells.

Biochemical Tests

These tests were conducted to determine the ability of the bacterial isolates to produce enzymes such as catalase, oxidase, and urease. Other biochemical tests were carried out to determine the ability of the bacteria to either utilize a sugar or substrate sources

Catalase (Hydrogen peroxide; H_2O_2) Test

The biochemical test was carried out to assess and detect if the enzyme catalase is present. Catalase is an enzyme that catalyzes the release of oxygen from hydrogen peroxide with a resultant effervescence. Catalase catalyzes the breakdown of toxic H_2O_2 (hydrogen peroxide) into water and oxygen, which are harmless. The enzyme is produced or expressed by all aerobic organisms and thus it is a useful test in differentiating members of the aerobic and anaerobic organisms.

Methodology: a drop of H_2O_2 (3 %) is placed on a grease free slide to which a loopful of the bacteria isolate is applied. Positive catalase activity was shown by effervescence, while no effervescence indicates absence of the enzyme.

Oxidase Test

The biochemical test is basically carried out to identify the presence of the artificial electron acceptor (cytochrome-c-oxidase), which is able to reduce oxygen. It is used to detect the presence of the enzyme in bacteria. That is, if certain oxidases which are required for the transportation of electrons between tetramethyl-p-phenylene-diamine (the redox dye) and electron donors in the bacteria are present or not.

Procedure: A whatman filter paper was soaked with a solution of 1% tetramethlyphenylene diamine hydrochloride. A 24 hours culture of the test isolate(s)

was smeared onto the impregnated filter paper. The presence of a purple colour, indicated a positive result.

Test for Urea Hydrolysis (Urease Test)

This was performed to show the capability of some bacteria to form an alkaline product (ammonia) via splitting urea under the influence or action of the enzyme urease.

Procedure: Urea was added to urease agar base before it was inoculated with the test organism in a slant. At optimum temperature incubation was done (37 ^{0}C) for 24- 48 hrs. The development of an intense pink/red color is indicative of a positive results while negative results show no colour."

Indole Formation Test

This biochemical test was performed to evaluate the capability of bacteria to produce indole via the hydrolysis of tryptophan. The spot indole test was used in this study to detect rapid indole producing organisms. This test is used to detect the presence of tryptophanase, an enzyme which catalyze the breakdown of tryptophan to release indole on reaction with cinnamaldehyde to produce a blue-green compound. When the enzyme is absent, there would be no colour production (indole negative).

Procedure: saturate the filter paper with 1% paradimethylaminocinnamaldehyde reagent. Use a loop to remove a colony of the culture to be tested from the agar surface and robbed on the surface of the filter paper already saturated with the reagent. Positive result is confirmed when a blue colour develop within 30 seconds. Most indole-producing organisms turn blue within 30 seconds to one minute. The development of a slightly pink coloration or none at all is indicative of a negative result.

Citrate Utilization Test (Simon Citrate Agar (SCA) Slant)

SCA slants were used for this biochemical testing procedure. It is usually performed to evaluate the capability of the bacterium to utilize citrate as its sole carbon source. The biochemical medium contains sodium citrate (sole carbon source), bromothymol blue (indicator) as well as ammonium dihydrogen phosphate (nitrogen source).

Procedure: prepare the medium as a slant using a test tube and culture the bacteria isolates to be tested and allowed to stand for 24 hours in an incubator. Development of a blue colour indicates a positive reaction to citrate while no colour change or if the green colour of the medium is retained, indicates a negative reaction.

Triple Sugar Iron Agar Test

This test is used to evaluate the efficacy of bacterial isolates (particularly those of Gram-negative group) to fermentatively utilize glucose, lactose and/or sucrose as well as produce hydrogen sulfide (H_2S) gas. The composition of the medium include 1 part of glucose and peptone: 10 parts of sucrose: 10 parts of lactose. Phenol red and ferrous sulphate serves as an indicator for acidification of medium and H_2S production respectively. The medium was prepared according to manufacturer's instruction and it was prepared in tubes and placed in a slant position at an angle of about 60^0. The medium was allowed to solidify before it was inoculated with the test bacterium of interest. The bottom part of the tube (butt) was first inoculated by stabbing through the medium to the base and then the slant portion of the medium is inoculated next by streaking. The medium was incubated for 18-24 h. before the results were read using standard chart. Results for TSI is either by fermentation of glucose which could turn the entire medium to yellow (acidic) within 8 to 12 hours. The butt of the TSI agar will remains acidic even after 18 to 24 hours incubation period because of the presence of organic acids resulting from the fermentation of glucose under anaerobic

conditions in the butt of the tube. The slant reverts to alkaline state that is indicated by red color as the fermentation products gets oxidized to carbon dioxide (CO_2) and water (H_2O) and peptone in aerobic condition the slant undergoes oxidation releasing alkaline amines (Phenol red in alkaline pH turns red while in acidic pH turns yellow). The results and possible interpretation of the test is shown below

Table 3.1 Possible Scenarios for Triple sugar iron test

S.N.	Result (slant/butt)	Symbol	Interpretation
1	Red/Yellow	K/A	Glucose fermentation only, peptone catabolized.
2	Yellow/Yellow	A/A	Glucose and lactose and/or sucrose fermentation.
3	Red/Red	K/K	No fermentation, Peptone catabolized under aerobic and/or anaerobic conditions.
4	Yellow/Yellow with bubbles	A/A,G	Glucose and lactose and/or sucrose fermentation, Gas produced.
5	Red/Yellow with bubbles	K/A,G	Glucose fermentation only, Gas produced.
6	Red/Yellow with bubbles and black precipitate	K/A,G,H_2S	Glucose fermentation only, Gas produced, H_2S produced.
7	Yellow/Yellow with bubbles and black precipitate	A/A,G,H_2S	Glucose and lactose and/or sucrose fermentation, Gas produced, H_2S produced.
8	Red/Yellow with black precipitate	K/A,H_2S	Glucose fermentation only, H_2S produced.
9	Yellow/Yellow with black precipitate	A/A,H_2S	Glucose and lactose and/or sucrose fermentation, H_2S produced.
10	Yellow/Red	A/K	

Other Sugar Fermentation test :

Mannitol Fermentation was confirmed by growth on mannitol salt agar with ability to turn the pink medium into yellow.

Rhizobacterial Potential of Bacterial and Fungal Isolates

Screening for Indole Acetic Acid (IAA) production: This was determined by reaction of liquid culture of rhizobacterial isolates grown in 500 mg/L L-Tryptophan (the precursor for IAA biosynthesis) placed in tryptic soy broth (1 g/L MES hydrate, pH 6) and Salkowki's reagent. Inoculated broth was incubated at 30°C for 72h in a rotary shaker. After incubation broth was centrifuged at 3000rpm for 15min. Then 1.0 ml of the supernatant was mixed with 2.0 ml of Salkowski reagent (50 ml of 35% Perchloric acid + 1 ml of 0.5 M $FeCl_3$ solution), and the mixture was then incubated at room temperature for 25mins. Development of pink color after incubation at room temperature indicated IAA production (Patten and Glick, 2002; Kumar *et al*., 2012; Ngoma *et al*., 2013)

Screening for Ammonia production: Freshly grown bacterial cultures were inoculated in 10 ml nutrient broth and incubated at 30°C for 48h in a rotator shaker. After incubation, 0.5 ml of Nessler's reagent was added to each tube. The development of a yellow to brown colour indicated a positive reaction for ammonia production (Kumar *et al*., 2012)

Screening for Nitrogen fixation activity: A day old culture of bacterial isolates grown on nutrient agar was streaked on a Jensen's Nitrogen free medium otherwise knows as NFM (formulated via the addition of: 20g/L sucrose, 1g/L K_2HPO_4, 0.5 g/L $MgSO_4.7H_2O$, 0.5 g/L NaCl, 0.1 g/L $FeCl_3$, 0.005g/L $Na_2MoO_4.2H_2O$, 2g/L $CaCO_3$, 15g/L agar). Plates were incubated at 28°C for 1- 7 days. Growth on nitrogen deficient medium confirms the ability to fix nitrogen (Weselowski *et al*., 2016).

Screening for Phosphate Solubilization activity

Rhizobacterial cultures was spotted in triplicates of separately on the top of Pikovskya's agar (Micromaster) plates and incubated at 30°C for 3 days. A zone of clearing around the colonies after 1-3 days was scored as positive for phosphate solubilization. The diameter of

the halo zone and its bacterial colony from individual isolates was measured. The data obtained was used to calculate solubilization index (SI) using the formula below (Doilom *et al*., 2020).

$$\mathbf{SI} = \frac{\text{colony diameter} + \text{halozone diameter}}{\text{colony diameter}}$$

Statistical analysis

The data were analysed using the SPSS package version 21.0. All data are mean of three replicates. The mean, range and standard deviation of each parameter was determined. Unpaired student's *t* distribution test was used to determine significant differences on bacterial contamination on the hands of males and females

CHAPTER FOUR

RESULTS

The results obtained from this study revealed that the total heterotrophic bacterial count (Log_{10} cfu/g) of digestate from different waste streams had values, which ranged from 4.91±0.02 for cattle rumen digestate to 4.41±0.03 for a combination of cattle rumen fruit and food waste digestate (Figure 1). The cultural, morphological and biochemical characteristics of bacterial isolates revealed the presence of *Enterobacter cloacae, Escherichia coli, Pseudomonas aeruginosa, Salmonella enterica, Proteus mirabilis* and *Bacillus cereus* (Table 1). The distribution of bacterial isolates in the different waste streams revealed that *E. coli* was present in all digestates obtained from different waste streams (Table 2). All bacterial isolates were found to possess plant growth promoting properties with 100 % capacity for nitrogen fixation and phosphate solubilization. While 66.7 % of the isolates had the ability to produce ammonia and indole acetic acid. The phosphate solubilization index of the isolates revealed that *E. coli* (5.97) and *P. aeruginosa* (5.76) had the highest solarization index amongst all tested bacteria in the study.

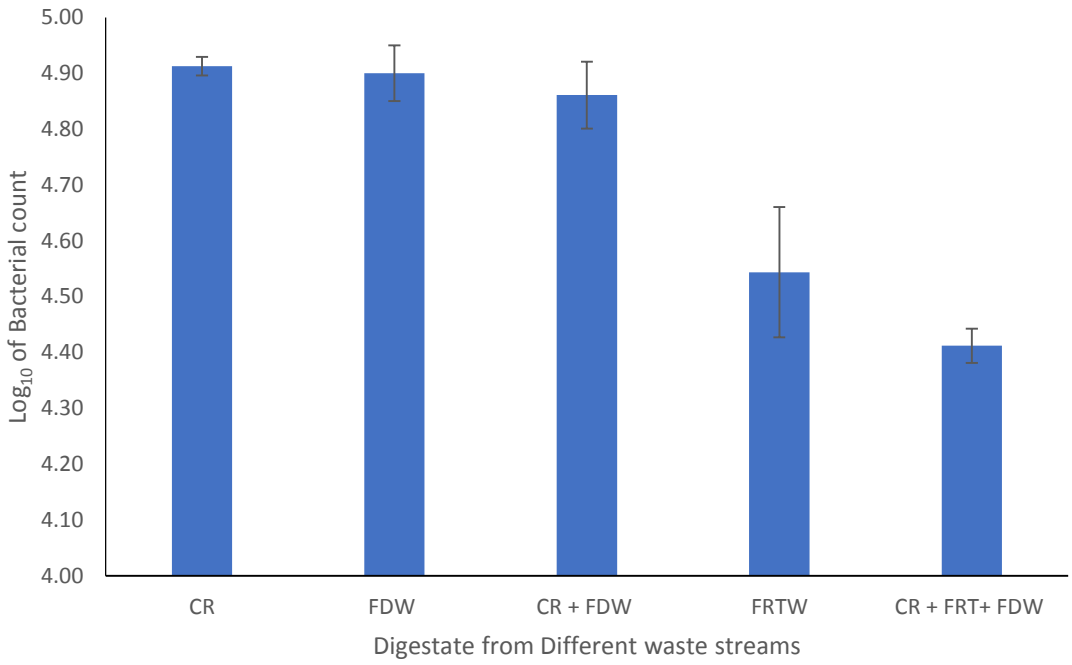

Figure 4.1. Total heterotrophic bacterial count (Log_{10} cfu/g) of digestate from different waste streams

Key: CR= Cattle rumen digestate

FDW = Food waste digestate

FRTW = Fruit waste digestate

CR + FDW = Cattle rumen + Food waste digestates

CR + FRTW + FDW = Cattle rumen + Fruit waste + Food waste digestates

Table 4.1. Cultural, morphological and biochemical characteristics of bacterial isolates

Parameters						
Elevation	Flat	Flat	Flat	Flat	Raised	raised
Margin	Undulate	Undulate	Undulate	Entire	Entire	Entire
Color	Cream	Cream	Cream	Cream	lemon	Cream
Shape	Irregular	Irregular	Irregular	Circular	Circular	Circular
Size	Large	Large	large	Medium	Medium	Medium
Gr. diff. agar	EMB	EMB	BCA	SSA	PCA	SSA
Colour	Purple	Green	Straw	Black	Green	Black
Morphological						
Gram stain	-	-	+	-	-	-
cell type	Rod	Rod	Rod	rod	rod	Rod
Arrangement	Disperse	disperse	disperse	disperse	disperse	pair/chains
Color	Pink	pink	purple	pink	pink	Pink
Biochemical						
KOH test	+	+	-	+	+	+
Indole	-	+	-	-	-	-
Citrate	+	-	+	+	-	-
Oxidase	-	-	-	-	+	-
Urease	-	-	-	+	+	-
Glucose	+	+	+	+	-	+
Sucrose	-	+	+	-	-	-
Lactose	-	+	+	-	-	-
Mannitol	-	-	-	-	-	-
Gas formation	+	+	-	+	-	+
H_2S formation	-	-	-	+	-	+
Identity	*Enterobacter cloacae*	*E.coli*	*Bacillus cereus*	*Proteus mirabilis*	*Pseudomonas aeruginosa*	*Salmonella enterica*

Key: Gr. diff. agar = growth on differential agar, EMB = Eosine methylene blue agar, BCA = Bacillus cereus agar, SSA = Salmonella Shigella agar, PCA = Pseudomonas cetrimide agar,

Table 4.2. Distribution of bacterial isolates from digestates from different waste streams

Parameters	*Enterobacter cloacae*	*E.coli*	*Bacillus cereus*	*Proteus mirabilis*	*Pseudomonas aeruginosa*	*Salmonella enterica*
CR	+	+	+	+	+	+
FDW	+	+	+	-	+	-
FRTW	-	+	-	+	+	+
CR+FDW	+	+	+	-	_	+
CR+FRTW+FDW	-	+	-	-	+	+

Key: + = Present; - = Absent

CR= Cattle rumen digestate

FDW = Food waste digestate

FRTW = Fruit waste digestate

CR + FDW = Cattle rumen + Food waste digestates

CR + FRTW + FDW = Cattle rumen + Fruit waste + Food waste digestates

Table 4.3. Plant growth promoting properties of bacterial isolates from digestates from different waste streams

Bacterial isolates	N_2 fixation	NH3 production	IAA production	Phosphate solubilization
Escherichia coli	+	+	+	+
Enterobacter cloacae	+	+	-	+
Bacillus cereus	+	-	+	+
Proteus mirabilis	+	-	+	+
Pseudomonas aeruginosa	+	+	+	+
Salmonella enterica	+	+	-	+
Total	100	66.67	66.67	100

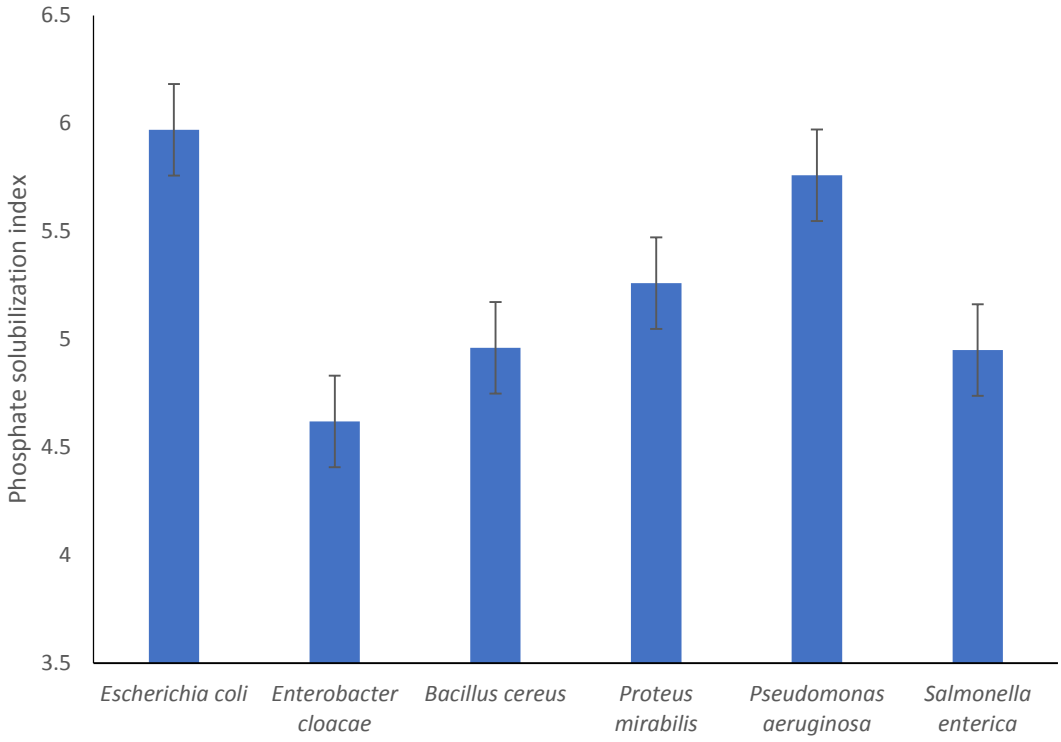

Figure 4.2. Phosphate solubilization index of bacterial isolates from digestates from different waste streams

CHAPTER FIVE

DISCUSSION

The need for an alternative to inorganic fertilizer is on the increase owing to the drawbacks associated with inorganic fertilizers. This study was therefore carried out to evaluate the bacteriological profile of digestates from different waste streams and the ability of the isolates to promote the growth of plants. The results obtained from this study revealed that the total heterotrophic bacterial count (Log_{10} cfu/g) of digestate from different waste streams had values, which ranged from 4.91±0.02 for cattle rumen digestate to 4.41±0.03 for a combination of cattle rumen fruit and food waste digestate. The cultural, morphological and biochemical characteristics of bacterial isolates revealed the presence of *Enterobacter cloacae, Escherichia coli, Pseudomonas aeruginosa, Salmonella enterica, Proteus mirabilis* and *Bacillus cereus*. The distribution of bacterial isolates in the different waste streams revealed that *E. coli* was present in all digestates obtained from different waste streams. All bacterial isolates were found to possess plant growth promoting properties with 100 % capacity for nitrogen fixation and phosphate solubilization. While 66.7 % of the isolates had the ability to produce ammonia and indole acetic acid. The phosphate solubilization index of the isolates revealed that *E. coli* (5.97) and *P. aeruginosa* (5.76) had the highest solarization index amongst all tested bacteria in the study. The results obtained in this study is in agreement with several reports in literatures where plant growth promoting properties of most of the aforementioned bacteria. Babalola *et al.* (2007) carried out experiments in pot cultures in to investigate the growth promotion activities of *Enterobacter, Klebsiella oxytoca* and *Pseudomonas* on maize plant suffering from *Striga hermonthica* infection. According to their findings some of the inoculants expressed statistically significant increase in growth over the control plus they also improved the agronomic characteristics of the maize. This can only be

possible when the bacteria can promote plant growth. More so, in a certain study, seeds of maize, beans, lettuce and cucumber were coated with a *Bacillus* sp. for a 12-month duration in South Africa by Ugoji *et al.,* (2006) who reported a decline in microbial densities of the treated seeds as the 7th month signifying that the treatments protected the seeds from pest infestation. According to Adesemoye and Ugoji (2006) in a research conducted in Nigeria, reported that inoculation methods; coating and soaking gave statistically similar outcomes when *Pseudomonas* sp. was used to inoculate three plants: okra, tomato and African spinach. They also reported that *Pseudomonas* is a very versatile and robust PGPR with great potentials in Nigeria. Adesemoye *et al.* (2008) using *B. subtilis* and *P. aeruginosa* as representatives of their genera respectively, compared their plant growth promoting potentials. They reported no significant differences between their overall perfomances but added that *B. subtilis* may be relatively more effective since it can produce endospores. This is comparable to the Bacillus species isolated in this study. Jida and Assefa, (2011) reported presence of efficient N-fixing and lentil-nodulating rhizobium in Ethiopian soils and selected 30 PGPR symbionts of such rhizobia. Under greenhouse environment, they reported that the isolates had the following characteristics: phosphate solubilization (16.7 %) and Indole Acetic Acid production (36.7 %), and they expressed tolerance to abiotic stress such unfavorable pH, heavy metal toxicity, and secretion of biocontrol agents was evident in majority of the isolates screened (Jida and Assefa, 2011). Galal *et al.* (2001) in a study conducted in Egypt, demonstrated that *Azospirillum lipoferum* and *Bacillus megaterium* in consortium can meet the nitrogen (N) and phosphorus (K) needs of wheat plants in balanced proportions. In a similar study in Egypt, El-Azouni (2008) recounted that the Nitrogen (N) and Phosphorous (P) uptake, dry weight and yield of soybean had increased significantly over the control after inoculation with P-solubilizing fungi *Aspergillus niger* and *Penicillium italicum. Rhizobium leguminosarum* has been shown to colonize the roots of rice plant

endophytically in a farm rotation agricultural system involving rice and berseem clover (*Trifolium alexandrinum*) (Yanni *et al.,* 2001). The authors posited that in addition to carrying out endophytic colonization of rice, *R. leguminosrum* can provide 25–33 % of the endorsed rate of Nitrogen supply expected from Nitrogen fertilizer for rice (Yanni *et al.,* 2001).

CONCLUSION

The use of digestates have shown to be better than those of inorganic fertilizers and since this has been proven in the sense that it contains plant growth promoting bacteria, it can be safe to consider it as a viable alternative to inorganic fertilizer. More research into this fact is needed in order to give it the popularity and general acceptance it deserves.

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APPENDIX 1

CULTURE MEDIA

SALMONELLA, SHIGELLA AGAR (SSA)

Lab-lemco powder	5.0g/L
Peptone	5.0g/L
Lactose	10.0g/L
Bile salts	8.5g/l
Sodium citreate	10.0g/L
Sodium thiosulphate	8.5g/l
Ferric citrate	1 .0g/L
Brillant gree	0.00033g/L
Neutral red	0.025g/L
Agar	15.0g/L

BILE AESCULIN AGAR

Peptone	14.0g/L
Bile salts	15.0g/L
Ferric citrate	0.5g/L
Aesculin	1.0g/L
Agar	15.0g/L

MUELLER-HINTON AGAR

Dehydrated infusion from beef	300.0g/L
Casein	17.5g/L
Starch	1.5g/L
Agar	17.0g/L

MAC-CONKEY AGAR

Formula gm/litre

Peptone 20.0
Lactose 10.0
Bile salts 5.0
Sodium chloride 5.0
Neutral red 0.075
Agar 12.0
pH 7.4 ± 0.2

GRAM STAINING AND BIOCHEMICAL REAGENTS

STAIN AND REAGENT

Gram stain

The Gram stain was prepared using two stains (crystal violet and safranin or carbol fuchsin), Gram's iodine, and a decolorizing agent (ethyl alcohol).

A. Gram crystal violet

Solution A

Crystal violet - 2.0 g

Dissolved in ethanol (95%) - 20.0 ml

Solution B

Ammonium oxalate - 0.8 g

Distilled water - 80.0 ml

Gram iodine

Iodine (crystalline) - 1.0 g

Potassium - 2.0 g

Distilled water - 300.0 ml

3.0g of medium was dissolved in 300.0 ml of distilled water.

It is very important to note that; crystalline iodine, potassium and distilled water were combined to produce iodine solution and that Gram's iodine solution was stored in a dark bottle and protected from light so that it does not degrade.

Decolorizer

95 % ethyl alcohol was used.

Gram safranin

Safranin-O (certified) - 0.25 g

Ethyl alcohol (95 %) - 100.0 ml

Working solution:

Safranin stock solution – 10.0ml

Distilled water – 90.0 ml

Biochemical reagents

Indole medium

Peptone – 20.0 g

Sodium chloride – 5.0 g

Distilled water – 1000 ml

pH – 7.4

25.0 g of indole medium was dissolved in 1000 ml of distilled water and autoclaved for 15 min at 121 ºC and dispensed aseptically into sterile test tubes.

Oxidase reagent (Kovac's oxidase)

Amul-alcohol – 15.0 ml

p-dimethyl-aminobenaldehyde – 0.5 ml

Concentrated HCl – 50ml

Small quantity of Kovac's reagent was prepared by dissolving the aldehyde into alcohol and adding the acid slowly and then kept inside the refrigerator.

Catalase test

3% Hydrogen peroxide