Temperature-Time Requirements For The Reduction Of Bacteria And Safety Of Digestate As A Biofertilizer.

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A PROJECT SUBMITTED TO THE DEPARTMENT OF MICROBIOLOGY, FACULTY OF LIFE SCIENCES, UNIVERSITY OF BENIN, IN PARTIAL FULFILMENT OF THE REQUIREMENT FOR THE AWARD OF BACHELOR IN SCIENCE (B.Sc.) DEGREE IN MICROBIOLOGY.

NOVEMBER, 2022.

Certification

This is to certify that this project work was carried out by **Grace Omotine GODFREY (Miss)** with the Matriculation Number **LSC1705525.**the Department of Microbiology, Faculty of Life Sciences, University of Benin, Benin City under my supervision.

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DEDICATION

This work is Dedicated to God almighty, for his love, and for the wisdom and strength to finish this research. All the praise be to his name.

ACKNOWLEDGEMENT

I am really appreciative of God's love, extraordinary favors, and bountiful supply throughout the course of this project work.

My profound gratitude goes to my exceptional project supervisor DR. C.E. OSHOMA whose assistance made this research a reality.

Saying thank you to the HOD Department of Microbiology, Prof. Mrs AKINOBOSUN. To my course adviser, DR. I.B. IDEMUDIA, thank you ma.

I also want to say a big thank you to my laboratory assistant MR SEAN OBASUYI for his assistance creating the drive to make sure I see to the completion of this project work. My appreciation also goes OMOEFE AFORKE, AJAYI JOAN, IGBONOMI EMMANUEL and other members of my group for their cooperation during the period of this project work.

I also want to say a big thank you to my friends who were of great support to me one way or another during this work. IROHA PROMISE, OKUNGBOWA MIRACLE, OKONYE CHUKWUEMEKA, BLESSING CHIKA, GLORY MOMOH.

I want to express my heartfelt gratitude to my great parents MR. and MRS. OGENE I. GODFREY for all their support, encouragement, financial assistance and their prayers which was of great help to me throughout the period of my B.sc program.thank you also to my wonderful siblings GOD'SLOVE GODFREY and PEACE GODFREY.

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Abstract

Digestate from cattle rumen is composed of microbial biomass, organic Matter and inorganic compounds derived from anaerobic digestion, can be used as an inorganic fertilizer or raw materials for biofertilizer production. This study was aimed at investigating the effect of temperature and time relationship towards reduction of bacteria in cattle rumen digestate and its resultant safety as biofertilizer. Cattle Rumen Digestate slurry (CRD) was obtained from National Centre for Energy and Environment in the University of Benin, Benin City, Edo State, Nigeria . The digestate was subjected to varying degrees of temp 28+2, 55, 60, 70

and 80 ℃ for a duration of 0,20,30, 40 and 60 min. The analysis carried out were for bacterial load using nutrient agar (NA) and Salmonella Shigella agar (SSA), physicochemical and heavy metal content. From the results, the lowest bacterial load of ($0.5\pm0.00\times10^5$cfu/ml) was recorded for exposure at 80 ^{0}C after 60min and *Salmonellae* load recorded with (NO GROWTH) were from digestate exposure at 70 and 80 ^{0}C after 40 and 60 mins respectively (for both temperatures) . Concentrations of nitrate, TK, TKN were highest at 60 ℃ and 80 ℃, TAN and TP were highest at 70 ℃ and 55℃ respectively, while 70 ^{o}C, heavy metals were lowest. This study study shows that digestate posses potential concerntration of nutrients and useful minerals which makes it suitable for agriculture land application.

CHAPTER ONE

1.0 INTRODUCTION

Digestate Exploitation has proven to be an economically feasible avenue for the cyclic use of large-scale and small-scale organic waste (Huang *et al*., 2016; Alkhalidi *et al*., 2019). It is a widely used system to convert organic material into energy-rich biogas and the residual sludge is known as digestate (Alburquerque *et al*., 2012). Digestate can be utilized as a biofertilizer for arable land, allowing for the cycling of plant nutrients and obviating the need for inorganic fertilizers that are dependent on fossil fuels. (Alkhalidi *et al*., 2019). The rapid development of biogas plants in European countries has increased the recycling of waste for energy generation, while producing large quantities of digestate (Franchino *et al*., 2016). There has been a tendency in the last decades of increased emphasis on improved sustainability in agriculture and preservation of natural resources, thus changing the focus of digestate processing from nutrient removal and disposal, to integrated nutrient recovery and recycling (Drosg *et al*., 2015). This contributes to promoting the circular economy in the agroindustrial sector (Suárez *et al.*, 2019).

These bio-fertilizers are used in agriculture as an alternative to chemical fertilizers, which are not environmentally friendly and can eventually reduce soil fertility.(Owamah *et al*., 2014). Bio-fertilizers are preparations containing latent cells of effective microorganisms that assist the absorption of nutrients by crop plants when applied via seed or soil by their interactions in the rhizosphere (Di Maria *et al*., 2017; Yasar *et al.*, 2017).

Digestate is produced by the anaerobic digestion (AD) of organic waste and is made up of microbial biomass, semi-degraded organic materials, and inorganic chemicals. Digestate can be utilized as a biofertilizer, an inorganic fertilizer, or a starting point for the development of biofertilizers.(Alburquerque *et al*., 2012; Roopnarain and Adeleke, 2017). AD is a regulated

degradation of organic waste in the absence of oxygen and in the presence of anaerobic microorganisms (Ojolo *et al*., 2007). AD is also a means of dealing with organic waste and at the same time meeting global energy needs, it minimizes bulk of organic matter to be disposed, generates digestate which is rich in nutrients and has agricultural value and produces biogas rich in methane which can be used directly as fuel or converted to compressed natural gas and liquefied natural gas. Heavy metal pollution, eutrophication, and a loss in soil quality are all results of an overreliance on inorganic chemical fertilizers.(Zhu *et al*., 2010; Owamah *et al*., 2014). Therefore, digestate is required as a bio-fertilizer in order to provide environmental benefits such as soil development, food quality and protection, as well as human and animal health. (Johansen *et al*., 2013). With several studies showing similar or higher yields in the use of digestate as an alternative to chemical fertilizer (Nkoa, 2014), this provides farmers with a new strategy that enables them to achieve the targeted objective of food security in Nigeria by growing food grain yields of high productivity (Food and Agricultural Organization, 2012). There are different varieties of digestate used as bio-fertilizers, and the main distinctions between them often lie in the raw materials used for digesting them, how they are employed, and where the microbes come from.(Garfi *et al*., 2011). In addition, the consistency of the digestate can be determined by both the organic and inorganic matter present in the substrates. During the AD procedure, the microbial communities consume much of the organic matter and transform some to inorganic compounds.For instance, in the digester, nitrogen that is accessible comes from the substrate, the atmosphere, or purging,is converted into ammonium and nitrates that remain in the digester until the completion of the AD process. The anaerobes in the digester do not use the inorganic matter during AD. Ammonium, an inorganic component which is essential for plant uptake is present in the digestate, thus makes digestate suitable for use as a fertilizer/soil conditioner (Bowen *et al*., 2014). Digestate usually contains microorganisms like

Pseudomonas, Klebsiella, Salmonella, Penicillium, Shigella, Bacteroides, Aspergillus, and Bacillus etc. some of which can be exploited in the production of bio-fertilizers because they hasten the microbial processes in the soil and increase the availability of nutrients that can be assimilated by plants (TNAU, 2008). *Klebsiella and Clostridium* species are free living nitrogen fixers while *Bacillus* and *Pseudomonas* species are phosphate solubilizers (Alfa *et al*., 2014).

1.1 Aim and Objectives

This study was aimed at investigating the effect of temperature and time relationship towards reduction of bacteria in cattle rumen digestate and its resultant safety as biofertilizer.

Specific objectives were to;

1. determine the physicochemical parameters of cattle rumen digestate before and after treatment,

2. enumerate, characterise and identify bacteria from cattle rumen digestate,

3. determine the optimum temperature-time requirement of cattle rumen digestate for use as safe biofertilizer.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 **DIGESTATE**

Digestate contains more readily available nutrients than non-digested products which make it better for crop fertilization (Garfi *et al*., 2011).Different raw sources, such as residential,

municipal, and rural waste, are sufficient for the affordable manufacturing of digestate as biofertilizers,in contrast to chemical fertilizers, which require significant expenditures. (Curry and Pillay, 2012;Dai *et al*., 2013).

2.2 **EFFECT OF TEMPERATURE ON TREATMENT OF DIGESTATE**

Complex organic polymers like proteins and carbohydrates are first broken down by hydrolytic bacteria into more easily soluble monomers .(e.g. glucose and amin o acids). Acidogenic bacteria then consume the products of The biological decomposition and stabilization of organic molecules in anaerobic environments is known as anaerobic digestion (AD) (Chen et al., 2008). It is a waste treatment method that yields semi-solid or solid output (digestate), which is commonly used for agricultural fertilizer, and sustainable energy in the form of biogas (Angelonidi and Smith, 2015).

The methane (CH4) present in the biogas can then be used to fuel vehicles or burned to provide electricity and heat (Lymperatou et al., 2017; Passos et al., 2017). A group of anaerobic bacteria that collaborate effectively mediate the process (Li et al., 2011). Complex organic polymers like proteins and carbohydrates are first broken down by hydrolytic bacteria into more easily soluble monomers (e.g. glucose and amino acids). Afterward, acidogenic bacteria eat the byproducts of hydrolysis, converting them into simple fatty acid (e.g. lactic and butyric acids) and simple alcohols (e.g. methanol). Fermentation of the organic acids and alcohols occur through acetogenesis, forming acetic acid, hydrogen (H_2) and carbon dioxide (CO_2), which are the direct substrates for CH_4 production. Methanogenic bacteria then generate CH_4, with about 70% being produced from the acetic acid and the remainder from H_2 and CO_2 (Polprasert, 2007). By keeping organic waste out of landfills, anaerobic digestion lessens harmful effects like the emission of greenhouse gases, leachate, and stink (Environment Canada, 2013; Li *et al.,* 2011). The digestate can be utilized as a nutrient-rich fertilizer, depending on the substrate (Appels et al., 2011). Additionally, the digestate is rich in nitrogen, phosphorus, and potassium, making them available to plants for uptake (Koszel and Lorencowicz,

2015). Digestate is an organic soil additive that raises the soil's carbon stock (Westphal *et al*., 2016). This enhances the health and growth of plants. A substrate with a total solid content (TS) of more than 15% is required for solid-state digestion to take place. It is preferable to liquid-state digestion because it offers more ofpsubstrate variety, especially biomass composed mainly of lignin, cellulose, and hemicellulose. To Digesting waste in this form, reducing moving parts and decreased energy input for heating is required (Chaikitkaew *et al.,* 2015).

Due to its high lignocellulosic fiber content, animal manure's poor biodegradability and low CH4 yield make it difficult to use for the production of biogas (Andriamanohiarisoamanana *et al.,* 2017; Nasir and Ghazi, 2015; Raju *et al.,* 2013). Dairy cow excrement contains up to 40 to 50 percent fibrous lignocellulosic materials, primarily made up of partially digested plant stuff (Nasir and Ghazi, 2015; Rafique *et al*., 2010; Raju *et al.,* 2013). Dairy cow manure has a high lignocellulose content, although it may not be fully lignocellulosic because the rumen of cattle has digested a large percentage of the lignified material.It usually has a rather low TS concentration of 7–9% depending on the manure management system (Angelidaki and Ellegaard, 2003).Manure generally contains a lot of nitrogen in the form of ammonia, which is bad for AD. When employed as a single feedstock, manure's low carbon-to-nitrogen (C/N) ratio raises the risk of a process failure or inhibition (Hassan et al., 2016). The high water content of the manure also reduces the output of CH4. (Angelidaki and Ellegaard, 2003; Passos *et al*., 2017).In contrast, substrates high in lipids and/or quickly broken down carbohydrates. (Labatut et al., 2011). Pretreatment methods have been applied to lignocellulosic substrates to overcome these limitations and enable more decomposition of the organic matter. By removing the substrate's cell walls, removing the hemicellulose and lignin, and allowing for more efficient microbial hydrolytic activity, these procedures should increase the CH4 production (Nasir and Ghazi, 2015). One technique for raising the organic biomass's biogas output is thermal pretreatment. One of the earliest techniques recognized as

having the potential to improve AD, it has been effectively used in the industrial setting (Ariunbaatar et al., 2014; Carlsson et al., 2012). Thermal pretreatment is advantageous because it can recover heat from the pretreatment process and uses no extra chemicals (Carlsson et al., 2012; Zhang et al., 2014). There are gaps in our understanding of pretreatment, particularly as it relates to dairy cow dung. There are no optimal treatment combinations for particular substrates at their stated process parameters, such as TS and pH, despite the numerous investigations of pretreatment technologies at both the lab and industrial scales.(Appels *et al*., 2011). Additionally, for thermal pretreatment, little research has looked at temperature and time combinations that increase biogas yield, CH4 concentration, or shorten retention period (Ariunbaatar et al., 2014). Dairy cow manure thermal pretreatment has received relatively little research, with the majority of studies focusing on liquid manure (Passos *et al,* 2017). The majority of the research on the use of manure in AD has focused on its use as an inoculum, a source of nitrogen, or in conjunction with other biomass that contains less nitrogen (Appels *et al*., 2011). Manure is a desirable foundation substrate for codigestion because it has a high moisture content that works as a solvent for dry biomass (Andriamanohiarisoamanana *et al.*, 2017).

2.3 **Nutrient value and availability in digestate**

Digestate can successfully be used in place of mineral fertilizers because it is simple to use and apply (White et al., 2007). The nutrients in the feedstock have an impact on the digestate's fertiliser value. However, because digestate is the end product of a biological process, each digester tank-specific properties are present. Following storage, these features can change between batches from the same digester and even within a single batch of digestate (Frost and Gilkison, 2010).

2.4 **Microbiology in an anaerobic digester**

A multi-step process called anaerobic digestion of organic material is carried out by many groups of interdependent bacteria (Angelidaki et al., 2011). Hydrolysis, fermentation, acetogenesis, and methanogenesis are the four primary processes. Complex organic molecules like proteins, carbohydrates, and fats are hydrolyzed by extracellular enzymes released by hydrolytic bacteria during the hydrolysis stage to produce monomers like sugars, amino acids, and fatty acids. The second stage, fermentation, continues the degradation of the monomers into short-chain volatile fatty acids (VFA), alcohols, lactate, succinate, hydrogen gas, carbon dioxide, and ammonia by the fermentative bacteria. In the third phase, acetogenesis, syntrophic bacteria further break down the VFA, alcohols, and carbohydrates into hydrogen and acetate. Moreover, representatives from. Methanogens from the domain Archaea complete the process by producing methane, which is the final stage. Methanogens that produce methane primarily from acetate and carbon dioxide are known as acetotrophic methanogens, while hydrogenotrophic methanogens produce methane primarily from carbon dioxide and hydrogen gas. The initial inoculum, the feedstock, and the operating parameters all influence the activity and composition of the microbial population found in AD processes (Demirel & Scherer, 2008). Studies of bacterial communities have shown that Firmicutes, where the classes Clostridia and Bacilli predominate, and Bacteroidetes, which is often represented by the class Bacteroidia, are two of the most frequently seen phyla in digesters (Pierre & Wright, 2014; Sundberg *et al.*, 2013). Additionally, individuals from different phyla have also been found in lower abundances in various digesters operating on different substrates, e.g. phyla *Spirochaetes, Actinobacteria and Proteobacteria* (Li *et al*., 2014b; Sundberg *et al*., 2013).

2.5 **Microbial composition**

Digestate is a live substance that is home to many different types of bacteria. For instance, the digestate contains microbes from the previous AD. By producing biogas after the digestate has been stored, these bacteria are still functioning. Additionally, the presence of organisms not specifically required for the biogas process, such as potentially harmful bacteria and fungus, is a risk (Sahlstrom *et al.,* 2008; Schnurer & Schnurer, 2006; Bagge *et al.*, 2005; Sahlstrom, 2003). Diverse species of these organisms may be dangerous not only to people handling digestate, but also to the soil and plant community, as well as to animals grazing on areas fertilized with digestate. Different substrates, such as farm and slaughterhouse wastes and waste from the food processing sectors, have been shown to include pathogenic bacteria such Listeria, Salmonella, Escherichia coli, Mycobacterium, Clostridium, Campylobacter, and Yersinia (Sahlstrom, 2003). In digestate, spore-forming Clostridia and fungi have also been found (Schnurer & Schnurer, 2006; Bagge *et al.,* 2005). The raw substrate can be pasteurized at 70 °C for one hour to minimize the burden of pathogens and their presence in the digestate. (Sahlstrom *et al*., 2008). The need for pasteurization depends on where the substrate is from. Animal byproducts, for instance, can be divided into three groups (European Parliament and Council, 2002). Bone marrow and other high-risk substrate from ill animals, among others, are under category 1, and these materials should be burned instead of being employed at all for biological treatment. Before further digestion, the category two substrate should be pasteurized at 70 °C for 1 hour. Slaughterhouse waste and manure are examples of category two substrates. However, according to the Commission of the European Communities, manure may be digested without heat treatment if it contains little to no salmonella, enterococaceae, or Escherichia coli (2006). Substrates like various organic home wastes, waste from the food processing sector, and slaughterhouse waste that are not covered by category two fall under category three. There is no requirement to heat-treat category three substrates before digestion (European Parliament and Council, 2002). After raw substrate was

pasteurized for one hour at 70 °C before being used in anaerobic digestion, Bagge et al. (2005) observed that the majority of bacterial pathogens examined had decreased. Temperature and HRT are important factors for bacterial pathogens, and the AD process itself results in a decrease in these pathogens. Higher process temperatures and longer HRT result in more effective inactivation (Sahlstrom *et al.*, 2008). It has also been demonstrated that the ammonia level is significant, with higher ammonia levels leading to better pathogen elimination (Ottoson *et al.,* 2008). However, it has been demonstrated that spore-forming bacteria may endure both anaerobic digestion and pasteurization. (Bagge *et al*., 2005). Regrowth of pathogens in digestate storage tanks at farm sites has also been reported (Bagge *et al*., 2005). Insufficient cleaning between runs of the digestate conveyance vehicle can result in pathogen contamination and regrowth (Bagge *et al.,* 2005). Additionally, Schnurer and Schnurer (2006) discovered that source-separated household waste intended for anaerobic digestion included fungal spores that were not inactivated by pasteurization at 70 °C for 1 hour. The amount of fungal spores decreased during the digestion of the heat-treated substrate, but some thermotolerant spores persisted and could wind up in the digestate, where they could pose a concern to people, animals, and the plant-soil system (Schnurer and Schnurer, 2006). In addition, it has been demonstrated that plant pathogenic fungus may endure anaerobic digestion (Bandte *et al*., 2013).

2.6 **Storage of digestate**

Digestate is created all year round, thus it must be preserved until the growing season, which is the only time it should be used as fertilizer (Normand *et al.*, 1996). Geographical location, soil type, winter rainfall, crop rotation, and national legislation controlling digestate/manure application will all affect the length of storage time needed (Svistoonoff *et al.,* 2003). For

instance, it is advised to have storage space for six to nine months' worth of digestate output in a temperate climate (Hocher et al., 2006). In some nations, the designated time for digestate storage is required (Svistoonoff *et al.,* 2003). Ammonia and methane gases are released when digestate is kept in open tanks, just like manure. If the liquid's surface is protected by a layer, these emissions can be decreased (Svistoonoff *et al.,* 2003). This layer may consist of a natural crust that is at least 10 to 20 cm thick, a layer of floating plastic debris, clay pebbles, or chopped straw, among other materials (Wang *et al.,* 2004). But keep in mind that as chopped straw breaks down, it might release methane. Digestate does not develop a surface crust during storage, in contrast to raw cattle slurry (Hocher et al., 2006). Utilizing flexible storage bags or covering the digestate storage tanks with airtight membranes are two more techniques that reduce both methane and ammonia losses (Photos 4 and 5). (Rippka *et al.,* 1979). In the biogas industry-developed nations of Europe (e.g. Germany, Denmark and Austria)To create covered digestate repositories, there are already financial incentives, with the primary goal of lowering emissions (Tien *et al.*, 1979)

2.7 **Methods of digestate application**

The same machinery that is used to apply separated liquid and raw slurry can also be used to apply digestate (Rai *et al.,* 2000). Similar to how farmyard manure is dispersed, separated solids can also be scattered using the same machinery (Omay *et al.,* 1993). Digestate must be used as fertilizer throughout the growth season to ensure its best results, and it must be applied with the right tools to achieve even coverage across the entire field and precise application rates (Xu *et al*., 2008). Additionally, this method will decrease ammonia volatilization. However, because digestate contains more ammonia than raw slurry, there is a greater chance that ammonia will volatilize during and after digestate distribution (Omay *et al.,* 1993). The best application techniques are consequently those that reduce the amount of

exposed surface area while yet ensuring topsoil contact (trailing hoses, trailing shoes, and injection) (Huang *et al.*, 2004). The advantages of decreased pollution, nutrient losses, and increased nutrient utilization outweigh the greater costs of these technologies when compared to splash plate spreading (Rai *et al*., 2000). German research demonstrates that the method that produced the lowest greenhouse gas emissions on arable land was digestate applied with a trailing hose, followed by quick shallow absorption (Wulf *et al.*, 2002). It was discovered that trailing-shoe applications had the lowest greenhouse gas emissions on grassland (Wulf *et al.,* 2002).

2.7.1 **Digestate separation**

Digestate can be manually extracted in a similar way to how animal manure is (Rai et al., 2000). Two outputs from separation—a liquid and a fibrous material—must be stored and managed separately (Tien *et al.*, 1979). To prevent any methane release, it is advised that the higher dry matter and fibrous portion be stored undisturbed or even composted (Wulf *et al.*, 2002). Only the dry materials that have been sorted are sent to the centralized biogas facilities, leaving the liquid component behind. This raises the feedstock's dry matter content. For feedstock with low volatile matter concentrations, such pig slurry and flushed dairy manure systems, this approach is especially suitable. According to research by Moller *et al.* (2007, fresh weight basis), combining whole pig manure with separated pig manure solids increased the generation of biogas per digester volume by more than double that of whole manure alone. Dilute feed, on the other hand, results in low biogas production, high transportation costs, and an additional energy demand for digestion heating. re- Decanter centrifuges give good differential partitioning of nutrients, particularly phosphorus, into the separated fibrous fraction . The use of chemicals to coagulate and/or flocculate the liquid prior to centrifuging can improve partitioning. However, decanter centrifuges have high capital and operating

costs; as a result their use tends to be limited to high volume systems such as large pig farms and centralised biogas plants (e.g. in Denmark). Complete conditioning of digestate is a stage beyond separation (Wulf *et al*., 2002). Three refined end products—pure water, concentrated mineral nutrients, and organic fibers—are the final results of thorough reconditioning. With the proper authorization, purified water may be utilized for irrigated agriculture, process water, or discharge into the surface water system. When nutrients need to be transported from agricultural areas with surplus manure to areas with nutrient deficiencies, complete conditioning is especially suitable (Omay *et al.,* 1993).. Membrane separation and evaporation are the two basic technologies employed in this process. (2008) (Xu *et al.*). Both are intricate and use a great deal of energy. For these reasons, only large-scale biogas plants, like those in the waste water treatment industry, are now seen as being economically viable (Omay *et al.,* 1993).

2.8 Plant Growth-Promoting Microbes in digestate

In addition to bacteria that fix nitrogen and dissolve phosphorus, there are others that can be employed as biofertilizers because they boost plant development by creating compounds that encourage growth (Bashan 1998). For instance, it was discovered that the rhizospheric strains of Bacillus pumilus and Bacillus licheniformis produce significant amounts of the physiologically active plant hormone gibberellin (Gutierez-Maero et al., 2001). Paenibacillus polymyxa, however, demonstrated a range of advantageous traits, including nitrogen fixation, phosphate solubilization, generation of antibiotics, cytokinins, chitinase, and other hydrolytic enzymes, as well as improvement of soil porosity (Timmusk et al., 1999). Additionally, it has been claimed that some Azospirillum species synthesize plant hormones. (Bashan *et al*., 1990; Bashan and Holguin 1997).These point to the potential of various bacteria as biofertilizers,

which may call for more research. The production of bioactive metabolites like siderophores and antibiotics, gaseous products like ammonia, and fungal cell wall-degrading enzymes that result in cytolysis, discharges of ions, membrane interruption, and suppression of mycelial growth and protein biosynthesis are just a few of the mechanisms used by rhizobacteria to inhibit the growth of phytopathogenic microorganisms (Idris et al., 2007; Lugtenberg and Kamilova 2009). For instance, certain strains of Pseudomonas can create antifungal metabolites such phenazines, pyrrolnitrin, pyoluteorin, and cyclic lipopeptides of viscosinamide that can shield sugar beet from Pythium ultimum infection. In order to better compete for iron, Pseudomonas fluorescens creates iron-chelating siderophores such pseudobactin and pyoverdin that bind and suck up ferric ions. This inhibits the development and spread of harmful microorganisms like *Pythium ultimum, Rhizoctonia batatticola, and Fusarium oxysporum* (Cox and Adams 1985; Leeman *et al*., 1996; Hultberg *et al*., 2000). *Pseudomonas aeruginosa* produces the siderophores pyoverdine, pyochelin, and salicylic acid and further induces resistance against Botrytis cinerea (on bean and tomato) and Colletotrichum lindemuthianum (on bean) (De Meyer and Höfte 1997; Audenaert *et al*., 2002). The extracellular chitinase and laminase produced by some Pseudomonas species, however, can lyse the mycelia of Fusarium solani. Additionally, biofertilizers offer defense against some insect pests, 303 plant diseases, and soilborne illnesses. For instance, Azotobacter infuses the soil with antibiotics that stop the spread of infections like Pythium and Phytophthora (Wani et al., 2013).

2.8.1 Phosphorus-Solubilizing Microbes in digestate

Phosphorus is the second-most restricting nutrient for plants after nitrogen in soil due to its high concentration yet availability of most of it (Schachtman et al., 1998). By relocating phosphorus from the inaccessible forms in the soil, phosphorus-solubilizing bacteria (PSB)

like Bacillus and Pseudomonas can boost the accessibility of phosphorus to plants (Richardson 2001). These microorganisms, along with specific soil fungi as Penicillium and Aspergillus, cause the soil's bound phosphates to dissolve by secreting organic acids that have a lower pH near them. Bacillus megaterium var. phosphaticum, a cheap rock phosphate, was applied to sugarcane and shown to boost sugar output and juice quality by 12.6% while lowering phosphorus needs by 25%, thereby further causing a 50% reduction of the costly superphosphate usage (Sundara *et al*., 2002)

2.8.2 Nitrogen-Fixing Microbes

Although nitrogen is most prevalent and abundant in the air, it can be difficult for plants to fix and absorb, making it a limiting nutrient. However, some microbes, some of which can interact in different ways with plants, can fix a significant amount of nitrogen (Claudine et al., 2009). This characteristics lowers losses due to denitrification, leaching, and volatilization and enables the effective plant uptake of the fixed nitrogen. These bacteria include:

(a) free to exist in the soil. Although measuring nitrogen fixation by free-living bacteria is challenging, it has been estimated to range from 3 kilogram N ha1 to 10 kg N ha1 in some plants, such as Medicago sativa (Roper et al. 1995). In culture media, Azotobacter chroococcum in arable soils may fix 2–15 mg N g1 of carbon source, and it also creates a lot of slime that aggregates the soil. Nevertheless, it has been discovered that free-living cultures of nodulating bacterial symbionts, such as Frankia, can fix atmospheric nitrogen in the rhizosphere of both their host and non-host plants. (Smolander and Sarsa 1990). For *Beijerinckia mobilis,* Method of innocullating leaf spray and seed soaking stimulated growth in barley plants by significant nitrogen fixation and other mechanisms of bacterial plant growth hormone synthesis (Polyanskaya *et al*. 2002). In India, free-living cyanobacteria (blue

green algae) have been used to produce up to 137 20–30 kg of nitrogen per hectare of rice (Kannaiyan 2002).

(b) Having symbiotic and other endophytic interactions with plants, such as those with cyanobacteria, Frankia, and rhizobia. Rhizobia bacteria are an important class of biofertilizers that include organisms like Rhizobium, Bradyrhizobium, Sinorhizobium, Azorhizobium, Mesorhizobium, and Allorhizobium. The ability of these bacteria to fix nitrogen varies by up to 450 kg N ha1 depending on the host legume species and strain (Stamford et al. 1997; Unkovich et al. 1997; Spaink et al. 1998; Vance 1998; Graham and Vance 2000). Rhizobial biofertilizers come in a variety of sterilizing carriers, including peat, perlite, mineral soil, and charcoal, and can be applied as powder, 145 liquid, or granular formulations (Stephens and Rask 2000). In a number of woody plants, the nitrogen-fixing actinomycete Frankia can also produce root nodules. (Torrey 1978;Benson and Silvester 1993).

When organic nitrogen is biologically converted to NH4 +, it is known as nitrogen mineralization (Nahm, 2005). Enzymatic breakdown of organic nitrogen, such as that found in animal manure and agricultural residues, is required for mineralization, which can occur under both aerobic and anaerobic conditions. Most organisms produce the enzymes necessary for mineralization. The mineralization of organic nitrogen in soil is crucial for soil fertility because it provides mineral N to the plants and soil microbes (Ros et al., 2011). Additionally, nitrogen mineralization indirectly affects the environment by raising the possibility of nitrate leaching and N2O losses. (Ros *et al*., 2011; Akkal-Corfini *et al*., 2010). The quantity of mineral N released in a soil during a specific period of time is referred to as the nitrogen mineralization capacity (NMC) (Ros *et al.,* 2011). NMC has been demonstrated to increase when organic fertilizer is introduced to soil in comparison to unamended control soil (I, II; Odlare *et al.,* 2008) and soil modified with mineral fertilizer (I, II). Given that many soil

microbes use fertilizer as a source of energy and nitrogen, this impact of the crop residues is probably a result of the input of organic material.

2.8.3 **Ammonia oxidation**

Ammonia oxidation, in which NH3 is converted to NO2 by ammonia-oxidizing bacteria (AOB), is the initial stage of nitrification (Ernst et al., 2008; Kowalchuk & Stephen, 2001). Due to their dual chemolithotrophic and autotrophic existence, the AOB are highly specialized and have complex cell machinery, making them a very sensitive collection of microorganisms that may react swiftly to changes in the soil environment (vanBeelen & Doelman, 1997). The AOB were believed to be the only ammonia-oxidizing organisms until quite recently. But over the past ten years, ammonia-oxidizing archaea (AOA) have also been identified and have been demonstrated to be quite abundant in soil (Leininger et al., 2006). It is hypothesized that they contribute more to ammonia oxidation overall than was first believed. (Kelly *et al*., 2011). The AOB, however, are more frequently seen in agricultural soils, or soils that have had significant disturbance, such as ploughing. Additionally, it has been demonstrated that nitrogen fertilization stimulates AOB more than AOA (Bissett et al., 2014). The increase in NO2 - concentration per unit of time under non-limited substrate concentration and ideal pH is known as potential ammonia oxidation (PAO) (Pell et al., 1998). It can be used to gauge the ammonia-oxidizing community's overall activity. Chlorate is added to the PAO assay at AOB-optimal conditions to prevent additional nitrite to nitrate conversion. This makes the approach simple and quick because nitrite is easily analyzed using an automated colorimetric method. By analyzing PAO, it is feasible to detect potential harmful substances, such as organic pollutants, pesticides, and heavy metals, in the environment before they become a problem (Odlare & Pell, 2009; Pell *et al*., 1998). Both favorable and unfavorable impacts on PAO have been documented when organic residues

(digestate, pig slurry, and cow dung) have been introduced to soil (I, II, IV; Odlare *et al.*, 2008; Leven *et al.*, 2006; Nyberg *et al.*, 2006). When compared to control soil (without fertilizer), mineral fertilizers, on the other hand, have only been observed to have stimulating effects on PAO (Odlare *et al.,* 2011). Nevertheless, the organic wastes in these tests had an even greater stimulating impact than the mineral fertilizer (I; Odlare *et al*., 2011).

CHAPTER THREE

3.0 SAMPLE COLLECTION

Cattle rumen digestate slury samples were obtained from National Center for Energy and Environment in the University of Benin, Edo state. Samples were collected in clean, sterilized lidded buckets and then quickly transported to the laboratory for analysis.

3.1 PROCESSING OF SAMPLE

At arriving the laboratory, the sample was introduced into a sterilized bucket with tightened lid and placed in the laboratory refrigerator before analysis. The sample (cattle rumen digestate) measured (100ml) was transferred into sterilized conical flasks aseptically and labelled with the different temperatures and Exposure duration (time). 28±2, 55, 60, 70 and 80 ^{0}C are the different temperatures assessed for,while the exposure durations(time) were 0, 20, 30, 40 and 60 mins respectively. For this experiment a water-bath (Biobase, SWB-110X, Shandong, China) was used for heat treatment of the samples at the different temperatures and time.Also, control samples which did not receive heat treatment was taken record of .

3.2 Mircobiological Analysis:

After processing, the samples were subjected to microbiological analysis and cultures were prepared using nutrient agar (NA) and Salmonella Shigella agar (SSA). The media were prepared according to instructions From the manufacturer Characterization and identification were achieved using the protocol outlined by Cheesbrough (2006). Nystatin was added to the agar preparations to inhibit fungal growth.

3.2.1 Enumeration and Isolation of Bacteria

A Measurement of 1ml of the heated digestate sample was collected and suspended in 9ml sterile peptone water. Then 10-folds of the serially diluted sample was pipetted aseptically into petri dishes and the molten medium specified for this experiment was poured into plates (pour plate method) and gently rocked. For incubation,the plates were transferred and stored in the incubator for 24hours after the media had solidified. After incubation, the plates were then observed for distinct colonies and enumeration was done based the number of visible counts (bacteria) on the poured plates. Enumeration was achieved by using the formula:

$$\frac{cfu}{ml} = \frac{number\ of\ colonies\ x\ dilution\ fold/series}{volume\ of\ inoculum}$$

(Willey *et al.,* 2012)

3.2.2 Nutrient agar (NA) and Salmonella Shigella agar (SSA):

Nutrient agar was used to culture for non fastidious organisms and also for heterotrophic bacteria plate counts. This medium was prepared from available dehydrated powder. In preparing the medium, 28g of nutrient agar powder was dissolved in 1 liter of distilled water in a cornical flask with lid secured with cotton wool and Aluminum foil paper it was evenly shaked and was autoclaved for sterilization at 121°C for 15mins.after autoclaving the

medium was cooled to temperatures 45-50 °C was despensed into petri dishes already containing inoculum and this was done aseptically using pour plate method.

While Salmonella Shigella agar was used for the isolation ,differentiation and cultivation of *Salmonella* spp. And some strains of *Shigella* spp. It was prepared from dehydrated powder. 60g of of the agar powder (salmonella shigella agar) was dissolved in 1litre of distilled water in a cornical flask (sterilized) and was covered with cotton wool and foil paper thoroughly mixed and boiled for 1min . The medium was left for cooling to (45-50^{0}C) and was aseptically depensed in into petri dishes already containing the inoculum using pour plate method and left to solidified.

3.3 Characterization and Identification of Isolated Bacteria

3.3.1 Phenotypic Identification of Bacteria from Samples

Following successful pour plate technique, isolation and culture was made from a single colony and characterized using cultural, morphological and biochemical methods using the Bergey's manual. Several tests such as Gram reaction, catalase, urease, indole, oxidase, sugar fermentation, citrate utilization, respective reaction on triple sugar iron agar tests were carried out to presumptively identify bacterial isolates.

3.3.2 Cultural Characteristics and Biochemical Tests

Colonies were examined for their cultural characteristics with reference to size, edge pattern, margin, surface texture, opacity, elevation, pigmentation, consistency and colour (pigmentation). Biochemical characterization tests were thereafter performed.

Gram staining was performed to differentiate the Gram positive from the Gram negative bacteria.

3.4 Biochemical Tests

Biochemical tests were carried out to aid the identification of the bacterial isolates as phenotypic (cultural) characteristics are not sufficient. The biochemical tests carried out are shown below;

3.4.1 Gram staining:

This test was carried out to verify the sort of cell the isolated bacterium had. The technique of Gram staining was used to distinguish between Gram-positive and Gram-negative bacteria. Gram-positive organisms are those that, after being decoloured, maintain the primary stain, whereas Gram-negative organisms do not. The cell wall composition is what prevents the stain from being retained.

Crystal violet is the (primary stain)used in gram stains, along with safranin, iodine, and alcohol (counter stain).

Procedure: Using an inoculating wire loop, a smear of the organism to be Gram stained was created on a clean slide. The slide was heated by passing it gently over the flame after being air dried. The smear was rinsed with distilled water after being dyed for one minute with 1% crystal violet. For one minute, gram iodine was added as a mordant, and then alcohol was added for another 30 seconds. After that, distilled water was used to rinse the slide. The slide was then air dried after being soaked with counter stain, safranin, and distilled water for one minute. The preparation's morphology (form), organization, and Gram result were noted while being examined under a microscope after oil immersion.

3.4.2 Oxidase test

This is mainly used to differentiate between *Pseudomonas* from other Gram-negative rod bacteria. Oxidase test was carried out to identify bacteria species that will produce cytochrome oxidase enzyme. *Staphylococcus aureus* and *Escherichia coli* which are Gram-positive and Gram-negative respectively were employed as control. A piece of filter paper using sterilized loop and 2-3 drops of freshly prepared oxidase reagent (1% aqueous tetramethyl-3-phenyl nediamine dichloride) was added. A smear of the isolates were dropped on the filter paper using a sterile wire loop. A positive oxidase test is indicated by purple colouration within 10 seconds.

3.4.3 Urease test.

This is used to test organisms that have the abilities to produce the enzyme urease which catalyzes the breakdown of urea to produce ammonia. The test is usually used to differentiate organisms like *Proteus mirabilis* from other non-urease positive organism. A sterilized medium was dispensed into test tubes aseptically and the test bacteria isolated were inoculated into the medium and incubated at 37 ^{0}C for 24 hours. A change in colour from yellow to red-pink confirmed the presence of urease.

3.4.4 Indole production test

Which isolate can separate indole from the tryptophan in peptone water was determined using this assay. The test is typically used to distinguish between different types of Gram-negative bacteria, particularly enterobacteriaceae. A liter of distilled water was used to dissolve five grams of peptone broth that was readily accessible commercially. The medium was then autoclaved for 15 minutes at 121 0C to ensure its sterility. Each bacterial isolate was injected into the peptone broth after the 4 ml of the medium had been poured into a sterile test tube. After 24 hours of incubation at 37°C, a few drops of KOVAC reagent were applied to the

inoculation media. The red coloration that appears right away at the top of the test tube indicates a positive result.

3.4.5 Citrate utilization test

This test is used to identify which of the isolate can utilize citrate as the sole source of carbon for metabolism. Simon's citrate agar was the medium used for this test. In the preparation, commercially available Simon's citrate agar 24.28g was dissolved in 1litre of distilled water and sterilized by autoclaving at 121^0C for 15 minutes. The medium was dispensed into test tubes and the test organism was inoculated by stabling the medium on the tubes using sterile straight inoculation wire containing culture. The tubes were incubated at 37^0C for about 24 hours. Positive result is indicated by a change in colour from green to bright blue.

3.4.6 Catalase test

This test looks for the catalase enzyme to see if it's present or not. Hydrogen peroxide is broken down by the catalase enzyme, releasing free oxygen gas and causing the creation of water. The bacterial isolates that were spread out on a slide received a few drops of newly prepared, 3% hydrogen peroxide. Gas bubble creation suggested catalase enzyme activity was positive.2H2O2'n 2H2O+O2

3.4.7 Sugar fermentation and production of gasses using Triple sugar iron agar (TSI)

The manufacturer's instructions were followed when making TSI, and the prepared media was put in a test tube and kept in an inclined position to solidify. Using a sterile loop, the test bacteria was injected onto the medium's slant and butt before being left to incubate for 18–24 hours. The results were interpreted based on the formation of acid or alkaline in the slant or butt area of the tube, and the existence of air bubbles in the slant or but region verified the generation of gas. Further, the medium's blackening indicated the presence of hydrogen

sulfide. In accordance with the usual microbiological methodology and other conducted biochemical tests, a prepared lab chit was utilized for result interpretation.

3.4.8 Statistical Analysis

The data were analysed using the SPSS package version 21.0. All data are mean of two replicates. The mean and standard deviation of each parameter was determined.

CHAPTER FOUR

4.0 RESULTS

Tables 1 shows the total heterotrophic bacterial counts from cattle rumen digestate on nutrient agar (NA) The highest count was recorded at room temperature (28 ± 2^0C). At this temperature, the total heterotrophic bacterial count at 0 min was (43.00±4.24×10^5cfu/ml); And after 60mins exposure duration, the plate recorded overgrowth. Across the temperature range of 55^0C-80^0C, the lowest Total heterotrophic count was recorded at 80^0c after 60mins (0.5±0.00×10^5cfu/ml) while the highest was recorded at 55^0C after 20min (11.75±1.06×10^5cfu/ml).

Table 2 shows the total heterotrophic *Salmonella Shigella* counts from cattle rumen digestate on Salmonella Shigella agar (SSA) the highest count was recorded at room temperature (28±2^0C) At this temperature, the total heterotrophic *Salmonella Shigella* count was (13.00±2.85×10^3cfu/ml) after 60min. At 0min, the count was (6.00±1.41×10^3cfu/ml). Across the temperature range of 55^0C-80^0C the highest count recorded was (3.25±0.35 ×10^3 cfu/ml) at 55^0C after 20min while the lowest counts were at 70^0C and 80^0C after 40 and 60min respectively for both temperatures, recorded with (NO GROWTH) this signifies microbial elimination at these temperatures and times.

Table 3 shows the PH of the sample and physicochemical parameters analyzed at different temperatures and Exposure duration. The pH ranged from 7.20 - 8.20. The lowest pH was recorded at 28±2^0C on exposure for 0 min (7.20 ± 0.00) While the highest pH was recorded

at 70^{o}C on exposure for 20, 30 and 60 min. Physicochemical parameters analyzed include: total ammonium nitrogen (TAN), Nitrate (Nit), Total Khedjahl nitrogen (TKN) total phosphorus (TP) total potassium (TK), electrical conductivity (EC). For total ammonium nitrogen, nitrate, total khedjahl nitrogen, phosphorus and potassium, electrical conductivity, the general trend was a subsequent decreases in recorded values as temperature increased.

Table 4 shows the heavy metal content of cow rumen digestate at the different temperatures and times. The Zinc (Zn), Nickel (Ni), Lead (pb), Chromium (Cr), Copper (Cu) and Cadmium (Cd) content was assessed. Zinc Nickel, Lead, Chromium and Cadmium showed a different trend where decrease was only recorded at 60mins exposure duration for all temperature. However, the recorded values remained same at 70^{o}C and 80^{o}C.

Table 1: Total heterotrophic bacteria count (x10^5cfu/ml) from Cattle Rumen Digestate at different Temperatures and Exposure Durations on Nutrient agar (mean ± S.D.)

Time (min)	Temperature (℃)				
	28±2	55	60	70	80
0	43.00±4.24	-	-	-	-
20	-	11.75±1.06	9.25±3.18	2.25±1.77	2.50±1.41
30	-	6.25±1.06	6.75±2.47	1.50±0.00	2.25±0.35
40	-	5.75±1.06	3.25±1.06	1.50±0.71	1.25±0.35
60	Overgrowth	2.75±1.77	1.75±1.06	1.25±1.06	0.30±0.00

Table 2: *Salmonella shigella* Count (x10³ cfu/ml) from Cattle Rumen Digestate at different Temperatures and Exposure Durations on salmonella shigella agar (mean ± S.D.)

Time (min)	Temperature (℃)				
	28±2	55	60	70	80
0	6.00±1.41	-	-	-	-
20	-	3.25±0.35	3.00±0.71	0.75±0.35	0.50±0.00
30	-	2.50±0.00	2.25±1.06	0.50±0.00	0.25±0.35
40	-	1.50±0.71	1.50±0.00	No growth	No growth
60	13.00±2.85	0.50±0.00	0.50±0.00	No growth	No growth

Table 3: Physicochemical Parameters of Cow Rumen Digestate at Different Temperatures

Temp (℃)	Time (min)	Properties						
		pH	EC (s/m)	TAN (mg/l)	NIT (mg/l)	TKN (%)	TP (mg/l)	TK (mg/l)
28±2	0	7.20±0.00	841±5.66	1.79±0.057	0.045±0.0029	0.046±0.0064	45.88±3.37	465±4.24
	60	7.40±0.14	844±3.536	2.46±0.679	0.039±0.0042	0.055±0.015	3.37±2.821	432±4.243
55	20	7.95±0.07	591±5.66	2.55±0.003	0.30±0.131	0.043±0.002	43.27±29.08	363.5±2.12
	30	8.05±0.07	733.5±70.00	1.91±0.021	0.021±0.050	38.305±0.004	411±0.91	46.01±1.41
	40	8.00±0.00	624±11.31	4.49±0.031	0.025±0.301	31.705±0.001	31.71±1.07	350.5±6.37
	60	7.90±0.14	604±5.66	2.74±0.001	0.032±0.001	36.00±0.002	36.00±0.47	371.5±2.12
60	20	8.10±0.00	486±29.08	3.87±0.005	0.028±0.04	31.00±0.011	31.00±2.04	490±4.24
	30	8.00±0.00	796.5±0.91	1.74±0.193	0.029±0.13	37.10±0.007	37.11±26.08	5141.5±339.92
	40	8.15±0.07	554±1.07	3.26±0.011	0.03±0.74	29.595±0.004	27.59±1.24	545.5±2.12
	60	8.10±0.00	594±0.47	2.81±0.004	0.225±0.16	27.185±0.003	29.19±0.94	459.5±1.02
70	20	8.20±0.00	586±7.07	5.02±0.22	0.048±0.040	0.028±0.005	16.98±0.96	325±2.83
	30	8.20±0.00	773±16.97	3.67±0.35	0.025±0.002	0.025±0.009	22.31±2.59	376±25.06
	40	8.15±0.07	778±5.66	3.67±0.26	0.022±0.001	0.028±0.019	22.76±1.32	378.5±10.68
	60	8.20±0.00	750.5±34.65	4.02±0.30	0.019±0.002	0.036±0.004	20.69±1.81	308.5±101.12
80	20	7.85±0.07	814±15.56	2.6±0.19	0.035±0.003	0.049±0.001	34.60±1.51	446±11.31
	30	7.75±0.07	810±5.66	2.95±0.141	0.034±0.002	0.029±0.005	20.982±0.79	412±19.80
	40	7.90±0.00	811.5±3.54	3.13±0.050	0.03±0.0001	0.014±0.430	30.57±18.92	420.5±3.54
	60	8.10±0.14	807±0.71	3.29±0.021	0.028±0.003	0.445±0.010	28.16±0.54	388.5±3.54

and Time

Parameters: EC=electrical conductivity, TAN=total ammonium nitrogen NIT=nitrate, TKN=total khedjahl nitrogen

TP=total phosphorus,TK=total potassium
Values= mean± S.D.

Table 4: Heavy metal Properties of Cattle Rumen Digestate at Different Exposure times

Temp (℃)	Time (min)	Heavy metal				
		Zn (mg/l)	Ni (mg/l)	Pb(mg/l)	Cr (mg/l)	C
28+2	0	13.29±0.93	2.46±0.113	0.34±0.021	0.73±0.021	6.
	60	441.73±612.74	1.29±0.169	0.26±0.127	0.625±0.007	4.
55	20	20.90±2.06	1.76±0.17	0.09±0.006	0.34±0.02	8
	30	2.39±1.87	2.39±0.50	0.14±0.042	0.39±0.03	1
	40	28.27±1.29	1.99±0.23	0.17±0.006	0.34±0.08	1
	60	26.12±2.03	1.65±0.12	0.161±0.006	0.24±0.03	1
60	20	14.52±0.36	1.9±0.06	0.14±0.04	0.41±0.02	5
	30	12.72±354.44	2.21±6.37	0.15±1.40	0.52±0.14	5
	40	15.32±0.60	2.28±0.24	0.17±0.02	0.53±0.08	6
	60	12.18±0.64	1.68±0.16	0.15±0.05	0.46±0.02	7
70	20	319±0.04	0.79±0.24	0.08±0.007	0.27±0.021	2
	30	4.17±0.79	0.81±0.05	0.014±0.026	0.34±0.007	2
	40	4.16±0.26	0.71±0.10	0.504±0.004	0.38±0.007	2
	60	3.98±0.33	0.61±0.02	0.112±0.566	0.26±0.064	2
80	20	8.38±1.20	1.41±0.04	0.26±0.04	0.58±0.01	4
	30	7.13±0.68	1.51±0.22	0.30±0.10	0.54±0.01	3
	40	6.12±0.03	1.39±0.22	0.21±0.00	0.65±0.02	3
	60	5.64±27.58	1.47±0.22	0.24±0.01	0.63±0.05	3

Parameters: Zn=zinc, Ni=nickel, Pb=lead, Cr=chromium, Cu=copper, Cd=Cadmium

Values=mean ± S.D.

Table 5: morphological and structural characteristics of bacteria isolated from cattle rumen digestate

	1	2	3	4	5	6	7	8	9	10	11	12	13
Gram stain	-	-	-	-	-	-	+	+	+	-	-	-	-
Shape	Bacilli	Bacilli	Bacilli	Bacilli	Bacilli	Bacilli	Bacilli	Cocci	Coccci	Bacilli	Bacilli	Bacili	Bacilli
Arrangment	Singly/pairs	Singly/pairs	Singly/pairs	Singly/pairs	Singly/pairs	Singly/pairs	Singly/Pairs	Singly/Pairs	Chains	Pairs	Pairs	Pairs	Pairs
Colour	Blue/green	Colourless	Greyish white	Greyish white	Grey	Greyish white	Greyish white	Creamy	Light yellow	Colourless	Black	Grey	Pink
Margin	Irregular	Smooth	Smooth	Smooth	Smooth	Smooth	Irregular	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth
Elevation	Flat	Flat	Slightly raised	Flat	Convex	Flat	Convex	Flat	Lowconvex	Low convex	Low convex	Convex	Convex
Catalase	+	-	+	+	+	+	+	-	-	+	+	+	+
Oxidase	+	-	-	-	-	-	-	-	-	-	-	-	-
Indole	-	+	+	-	-	-	-	-	-	-	-	VAR	-
Urase	-	+	-	-	VAR	-	-	-	-	-	-	-	-
Citrate	-	+	-	+	+	+	+	-	+	-	-	-	-
Lactose	-	-	+	-	+	+	-	+	+	-	-	-	+
Gas	+	+	+	+	+	+	-	-	-	-	+	+	+
Vp	-	+	-	+	-	+	+	+	-	-	-	-	+
Mr	-	+	+	-	+	-	-	-	+	+	+	+	-
Glucose	-	+	+	+	+	+	+	+	+	+	+	-	+
Mannitol	+	-	+	+	+	+	+	+	-	+	+	+	-

Xylose	-	+	+	+	+	+	-	+	-	+	+	-	-
Opacity	Opaque	Transparent	Opaque	Translucent	Translucent	Opaque	Opaque	Opaque	Transparent	Translucent	Transparent	Opaque	Transparent
Possible isolate	*P.aeruginosa.*	*Proteus. spp.*	*Escherichia coli.*	*Enterobacter spp.*	*Citrobacter spp.*	*Klebsiella pneumoniae*	*Bacillus spp.*	*Enterococcus spp.*	*Streptococcus spp.*	*Yersinnia spp.*	*Salmonella typhi*	*Shigella spp.*	*Salmonella parathyii*

Legend:VAR=variable, +=Positive, -=negative

Table 6: Frequency of Occurance of Isolated Bacteria from Cow Rumen Diestate at different temperatures and exposure duration

Isolate	Frequency
Psuedomonas aeruginosa.	11.66%
Proteus spp.	10%
Escherichia coli.	5.83%
Enterobacter spp .	8.63%
Citrobacter spp.	6.86%
Klebsiella pneumonia	8.20%
Bacillus spp	12%
Enterococcus spp	8.03%
Streptococcus spp	7.5%
Yersinia spp	3.33%
Salmonella typhi	8.06%
Shigella flexneri	3.64%
Salmonella paratyphi	5.83%

CHAPTER 5

5.0 DISCUSSION

The digestate obtained from the anaerobic farmentation of bi products from animals have shown to be an important option to commercial And industrial fertilizers. This study was carried out to assess the potential of converting used up or spent cattle rumen digestate into biofertilizers enriched and safe for farmland application. The sample (cattle rumen) was tested for it Coliform and salmonellal load,important mineral compounds such as as nitrate, total potassium, total ammonium nitrogen and total phosphorus, tested for the presence of heavy metals (Zinc, Lead, Chromium, Cadmium, Copper and Nickel) and it contamination profile .

The highest total heterotrophic bacteria count was recorded at (28 ± 2^0C for 0min) and at this same temperature for 60min (overgrowth) was recorded. Across temperature ranges 55-80^0C,the highest count was recorded at 55^0C after 20mins.while the lowest count was at the highest temperature 80^0C after 60mins. For the total heterotrophic *Salmonella Shigella,* the highest count was at (28 ± 2^0C for 0min). While across the the Temperature ranges of 55-80^0C the highest count was at 55^0C after 20mins while the lowest were at the highest temperatures 70^0C and 80^0C for 40 and 60mins (for both temperatures). Non thermophilic bacteria are unable to proliferation at these temperatures, this could be one of the reasons why the higher temperatures recorded the least counts. (Russel,2013). Acceptable bacteria limit on digestate is 1000cfu/g as reported by (Nag *et al.,*2021). From this study, exposure for 60mins at 80^0C results to be the most suitable Time and temperature treatment .as the counts here we're below the acceptable limits of bacteria contamination in digestate used as a biofertilizer for application on farmland . similar to this study elimination of bacteria in digestate in digestate occured at 60_75^{0}C for 30mins as recorded by (muhamud *et al.,*2016). The Exposure duration and

temperature ranges did not seem to have affected the PH change. The lowest pH recorded for this study was (7.20) While the highest was (8.20).PH is important for microbial proliferation and stability of mineral compounds in solutions. This point to the probability that the PH of the sample was not responsible for the change recorded in the concerntration of the assessed physicochemical parameters as there was significant difference recorded across the temperature and exposure time ranges.(Yinazou *et al.,*2016).

The effect of the treatment protocol on these quantities were seen to vary across the different tested exposure duration and temperature. Some of the low concerntrations were after 60mins at 80^0C. The concerntration of Nitrate, Total potassium ,total khedjahl nitrogen were highest at 60^0C and 80^0C while Total ammonium nitrogen,Total potassium, Total phosphorus were highest at 55^0C and 70^0C respectively. The concerntration of Total khedjahl nitrogen, total potassium, total phosphorus and total ammonium nitrogen were above the minimum accepted limits as compared with the report by Ezemagu *et al.,*(2021). The presence of the nitrogenous compounds indicates that the biofertilizer produced contain chlorophyll, Essential components of Amino acids, nucleic acids and adenosine triphosphate (ATP)as the energy transfer molecule.(Ezemagu, 2020; Mitter *et al.,*2021).

The biofertilizer produced Will assist soil microbes in converting insoluble phosphorus into readily available forms for plant when it is applied to the soil through a number of mechanism of solubilization and mineralization (Alori *et al*.,2017). As for the potassium present in the biofertilizer produced it will contribute to the functioning constitution of protein synthesis which will be available for uptake by plant when applied to farmlands.For the concerntration of heavy metals that were analyzed,Cr,Cd,and Pb were the highest at 80^0C, Cu at

55^0C while Zn and Ni were highest 60^0C.this is similar to the report of Angar *et al.,*(2021) ; Zhao *et al.,(*2017). which postulates that temperature can have different effects on heavy metals content as also shown in the present study.at 70^0C the concerntration recorded for TK,Tp, heavy metals,and nitrogenous compounds were lowest. This corresponds or is similar to the work of (Batista *et al.,*2021).

The values for Cr,Cu,Pb,Ni,Zn and Cd were below the acceptable minimum as reported by Ezemagu *et al.,*(2021). This indicates that the biofertilizer produced can be acknowledged as safe for agricultural use .The presence of low heavy metal content in the produced biofertilizer are useful and acceptable because,they are involved in the biochemical and physiological reactions like oxidative stress protection, respiration, photosynthesis in plants and water oxidation.(Castro *et al*.,2018; Nagayoti *et al*.,2010). Alkaline condition is suitable for the supply of nutrients in digestate needed for agricultural purposes as reported by Ezemagu *et al*.,2020. The isolated bacteria from this study include *Psuedomonas aeruginosa, Proteus* spp, *Escherichia coli, Enterobacter* spp, *Citrobacter* spp*, Klebsiella pneumoniae, Bacillus* spp*, Enterococcus* spp, *Streptococcus* spp, *Yersinia* spp, *Salmonella typhi,Shigella flexneri*,and *Salmonella paratyphi*. *E.coli* and *Salmonellae* are an important indicator bacteria, thier presence records for bacterial contermination of Faecal origin.this shows that the sample used for this study can contribute to Environmental contamination if not processed properly. The microbial decomposition of this sample in anerobic condition is an effective way of processing it safely (Alfa *et al.,*2014).

Some of the other Isolated bacteria are of clinical importance as they have been recorded to cause environmental and food_borne diseases (Alfa *et al.,*2014; Russo and Johnson.,2018).

Sun *et al.,*(2022) Isolated *Bacillus* sp from anaerobic digestion substrates and the processing fertility.also the other Isolates were isolated from used up/spent digestate as investigated by (Rabah *et al.,*2010).

It is important to note that before used up digestate is applied as an option to commercial fertilizer,it should first be assessed for it load of microbial contamination and concentration of useful minerals and nutrients present in it and the type of microbial responsible for the contamination. treating the sample with heat may lead to reducing the level of contaminationand also affecting negatively the concerntration of important parameters . therefore, it is important to determine a balance where the level of contamination will not be consequential and the concerntration of important parameters will be enough when applied as a biofertilizer . From this study, the balance was recorded to have existed between the ranges of 80-70^{0}Cafter 30mins Exposure duration. And it corresponds with the results by Taylor *et al*.,(2010).

5.1 CONCLUSION

The sample (cattle rumen digestate)was exposed to different degrees of heat treatment, temperatures 55,60,70,80^{0}C and exposure durations 20,30,40,60mins simply to analyze for the best temperature and exposure time suitable for realizing biofertilizer as safe for application on agricultural lands. After 30mins of exposure to heat treatment at 80^{0}C. The bacteria load of the digestate recorded significant decrease below the acceptable limits;also the concerntration of

heavy metals recorded decrease at this temperature (80^0C) after 60mins of exposure duration. In this study cattle rumen digestate has been shown to posses the potential to become an alternative to commercial fertilizer, as it shows reasonable concentrations of nutrients and useful minerals.

More research is necessary and recommend to arrive at a better final product that will contain low heavy metal concentration and lower contamination rate by application of more innovative treatment technique.

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