BIOFERTILIZING POTENTIAL OF FEATHER HYDROLYSATE PRODUCED BY KERATINOLYTIC BACTERIA ON CULTIVATION OF *Zea mays* AND *Corchorus olitorius*

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ABSTRACT

Keratin-rich wastes in the form of feathers, hair, nails, and horn are highly available as by-products of agro industrial processing. Keratinases, which are produced by several bacteria that have been isolated from soils and poultry wastes, show potential use in biotechnological processes involving keratin hydrolysis. The aim of this study was evaluate biofertilizing potential of feather hydrolysate generated by keratinolytic bacteria on *Zea mays* and *Corchorus olitorius*. Chicken feathers and feather wastes were collected from feather dumpsite in Kure Market Minna, Niger State while soil sample was collected from abattoir waste dump site of Bosso, Minna. One gram (1 g) of soil sample was serially diluted and inoculated in 0.2 mL of the 10-fold dilution aliquot on the minimal medium containing: 2g/L of KH_2PO_4; 0.05 g/L of $MgSO_4$; 0.1g/L of $FeSO_4.7H_2O$; 0.1g/L of $CaCO_3$; 20 g/L of Keratin substrate and 20g/L of agar-agar for the selective growth of isolates, using the pour plate method. The plates were incubated at 37°C for 72 h. Distinct colonies were identified by cultural, morphological, biochemical and molecular characterizations. Three treatments [feather hydrolysate (FH), FH+ bacteria suspension, and FH+ NPK was applied along with positive control (NPK only) and negative control (water only). The treatments were tested on the growth indices for *Corchorus olitorius* and *Zea mays*. The isolates were identified as *Lysinibacillus fusiformis* strain NBRC15717 16S and *Bacillus pacificus* strain MCCC 1A06182. The highest yield of *Zea mays* was observed in NPK (61.2), followed by FH and suspension (32), The highest yield weight of *Corchorus olitorius* was observed in FH only **(**92.52) and the least in water **(**47.04). Proximate analysis of *Corchorus olitorius* and *Zea mays* revealed the highest moisture, fiber, ash, lipids, carbohydrate contents were highest in FH and NPK except for protein that was highest in FH only. NPK only had the highest height, followed by FH and NPK for *Corchorus olitorius* and the least in water residues while for *Zea mays*, NPK only had the highest height followed by FH + suspension and FH only. The result of the study showed that feather hydrolysates, obtained through microbial conversion of chicken feathers, increased growth and biomass yield of cultivated crops compared to a reference fertilizer (urea) and other by-products of the poultry agro-industry.

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CHAPTER ONE

1.1 INTRODUCTION

1.2 Background to the Study

Fertilizer has become essential to modern agriculture to feed the growing population and use of fertilizers, especially the chemical fertilizers has brought in blessings on humanity, which helped contain hunger and death in different areas of the world. Though chemical fertilizers increase crop production, their overuse has hardened the soil, decreased fertility, strengthened pesticides, polluted air and water, thereby bringing hazards to human health and environment as well (Laba *et al.,* 2018). It has already been proved how chemical fertilizers pose serious challenges to the balanced and sustainable growth of plants. Chemical fertilizer works faster and they can absorb the nutrients immediately which is One of the benefits because it works quickly and it is custom-made for requirement. Chemical fertilizers give the option of using phosphorus rich fertilizers (Colla *et al.,* 2017). However, the best effect of chemical fertilizer is for the first few years, after which the land becomes more or less unproductive. It is like steroid, boosting the patient for few years and then does not work. The three main nutrients that plants needs are nitrogen (N), phosphorus (P) and potassium (K) (Colla *et al.,* 2017). All nitrogen rich fertilizers contain either one or more of these nutrients. Potassium is essential for the bud placement and fruit plant quality. Potassium also has much to say on the plants ability to endure the winter. All these are now sourced from biotic communities like mangroves- still untapped by researchers and industry houses. The application of chemical fertilizers is an expensive method of supplying mineral nutrients to the crops. However, fertilizers are often washed from the field either by runoff (Grazziotin *et al.*, 2006). They can become unavailable to the crops through physical, chemical, or biological transformation. To compensate this, farmers apply more chemical

fertilizers than the plant needs, and the remainder is often released into the environment, polluting air and water (Łaba *et al.,* 2018). Organic fertilizers like sludge, compost, or manure have the advantage of being utilized by providing the nutrients that already available in the agro-ecosystem more effectively, and they require little input of energy to be processed. Furthermore, the mineral nutrients that are bound in organic materials may be more stable and not washed away quickly. Nutrients can be made more available by promoting certain types of organisms within the soil microbial community (Lange *et al.*, 2016). Chemical fertilizers which are now being used extensively since the green revolution have depleted soil health by making the soil ecology non inhabitable for soil micro flora and micro fauna which are largely responsible for maintaining soil fertility and providing some essential and indispensable nutrients to plants. They are natural fertilizers which are living microbial inoculants of bacteria, algae, fungi alone or in combination and they augment the availability of nutrients to the plants (Łaba *et al.,* 2018). The role of biofertilizers in agriculture assumes special significance, particularly in the present context of increased cost of chemical fertilizer and their hazardous effects on soil health (Kirkr *et al.*, 2002).

Biofertilizers are the products containing one or more species of microorganisms which have the ability to mobilize nutritionally important elements from non usable to usable form through biological processes such as nitrogen fixation, phosphate solubilisation, excretion of plant growth promoting substances or cellulose and biodegradation in soil, compost and other environments(Łaba *et al.,* 2018). In other words, biofertilizers are natural fertilizers which are living microbial inoculants of bacteria, algae, fungi alone or in combination and they augment the availability of nutrients to the plants (Holkar *et al.,* 2018).Considering these facts in this study, an attempt was made to use certain organic biostimulants, which are known to boost the growth and development of crop plants by

providing a sufficient amount of organic nutrients in the form of polypeptides, amino acids, and organic acids. The term “biostimulant” is relatively new, one broad definition was introduced by DuJardin (2015) as “Plant bio-stimulants are substances or materials, with the exception of nutrients and pesticides, which, when applied to plants, seeds or growing substrates in specific formulations, have the capacity to modify physiological processes in plants in a way that provides potential benefits to growth, development, or stress response.”

In a bibliographic analysis, Dujardin (2015) divided biostimulants into eight categories, such as humic substances, complex organic materials, beneficial chemical elements, inorganic salts including phosphate, seaweed extracts, chitin and chitosan derivatives, anti-transpirants, hormone-containing products, amino acids, peptides, and other N-containing substances.

These bio-stimulants can be made available to plants through foliar sprays or soil application. Biostimulants which are applied to foliar were shown to reach mesophyll cells by absorption through the cuticle and epidermal cells (Holkar *et al.,* 2018).

When applied to the soil, the absorption occurs through root epidermal cells and gets redistributed through xylem (Chen *et al.*, 2011). These biostimulants decrease the need for chemical fertilizers and have the capacity to satisfy the nutritional requirements of plants and also further result in higher yield (Tejada *et al.*, 2004).

Four major groups of biostimulants have been shown to affect root growth and nutrient uptake, namely, (1) Humic substances (2) protein hydrolysate and amino acid formulations (3) seaweed extract and (4) plant growth-promoting microorganisms. This study concentrated on the humic acid and protein hydrolysate.

1.2 Statement of Research Problem

Heavy chemical applications have led to adverse environmental, agricultural and health consequences. Although, chemical fertilizers increase crop production, their overuse has hardened the soil, decreased fertility, strengthened pesticides, polluted air and water, thereby bringing hazards to human health and environment as well (Laba *et al.,* 2018) Many efforts are being exercised to combat the adverse consequences of chemical farming. The need for chicken, wool, fish, birds, and reptiles in the food and textile industry is recognizable, but the proper disposal of waste products is also essential. The continuous use of these sources for human needs and the generation of loads of waste leads to the accumulation of waste in the ecosystem. The use of higher concentrations of poultry extended to environmental problems at regional to global scales (Gerber *et al.* 2007). Much of the applied fertilizer is released into the environment, causing environmental degradation. Aside from the fact that these chemical fertilizers uses in modern agriculture are not efficient they also pose serious challenges to the balanced and sustainable growth. Landscape degradation and local disturbance like odor, flies, and rodents are the main problems near the poultry farms. This is also causing water and soil pollution and adversely affect the lives of people living in nearby localities (Gerber *et al.* 2007). One way in which fertilizer use can be reduced without damaging plant nutrition is to enhance crop growth through the use of biostimulants.

1.3 Justification of the Study

The use of keratin biomass for the production of biofertilizers is one of the areas of research that still needs to be explored. The biofertilizers can be used as a replacement for the chemical fertilizers, responsible for changing microflora and fertility of cropland fields.

The management of keratin-based waste biomass by reconversion into commercially used products will not only save the ecosystem from a large amount of sludge but will also economically boost up the growth and development of crop plants. The green and eco-friendly methods used in this study for the extraction of keratin biomass with the minimal usage of harmful acids and chemicals is a promising approach.

1.4 Aim and Objectives

This study aimed to evaluate biofertilizing potential of feather hydrolysate generated by keratinolytic bacteria on *Zea mays* and *Corchorus olitorius.*

The objectives of this study were to:

I. isolate keratinase producing bacteria from feather dumpsite.

II. identify the isolates using biochemical and molecular methods.

III. produce feather hydrolysate using the isolates.

IV. test the biofertilizing ability of the produced hydrolysates on *Zea mays* and *Corchorus olitorius*

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Fertilizer

Fertilizers are responsible for approximately half of the world's crop production, supplying food, feed, fiber, and fuel for a global population that is expected to reach 9 billion before the middle of the 21st century. Most fertilizer materials come from concentrated supplies of naturally-occurring minerals that are mined or extracted from various ore deposits (Huang *et al.*, 2010). One exception is nitrogen (N) which is produced by combining N_2 from the air with natural gas (most common), coal, or naphtha to form anhydrous ammonia, which can be used directly as a fertilizer or converted to different other N fertilizers. Maintaining sufficient crop production depends upon a viable and efficient fertilizer industry throughout the world, to help provide the right nutrients, at the right rate, at the right time and in the right place (Huang *et al.*, 2010). This challenge must be met in a way that is economical for all parties from mine or fertilizer plant to field, is respectful of the environment, and considers social concerns for maintaining various ecosystem services for the general public (Nayaka *et al.*, 2013).

There are 17 essential nutrients for crop growth. Three of them—carbon (C), hydrogen (H), and oxygen (O)—are supplied from air and water. The three macronutrients—N, phosphorus (P), and potassium (K) are mostly supplied from the soil, but soil deficiencies and crop removal must be replaced with supplemental sources—mostly fertilizers. A third group of secondary nutrients—sulphur (S), calcium (Ca), magnesium (Mg)— are no less essential, but are usually needed in smaller amounts as fertilizers. Finally, the micronutrients—boron (B), iron (Fe), manganese (Mn), zinc (Zn), copper (Cu), molybdenum (Mo), chlorine (Cl), nickel (Ni)—are needed in very small amounts,

but play essential roles as catalysts in metabolic processes of crop growth and development or play other key roles. Learning the way plants use each of the nutrients, and the source, rate, timing, and placement of each is important to nutrient management and optimizing crop production (De Vasconcelos *et al.*, 2019).

2.2 Biofertilizer

The term “Biofertilizer” or more appropriately a “Microbial inoculants” can generally be defined as preparation containing live or latent cells of efficient strains of Nitrogen fixing, Phosphate solublising or cellulolytic microorganisms used for application to seeds, soil or composting areas with the objective of increasing the number of such microorganisms and accelerate those microbial process which augment the availability of nutrients that can be easily assimilated by plants (Ertani *et al.*, 2014). Biofertilizer can provide an economically viable support to small and marginal farmers for realizing the ultimate goal of increasing productivity. Biofertilizer are low cost, effective and renewable source of plant nutrients to supplement chemical fertilizers (Gurav *et al.*, 2013). Microorganisms, which can be used as biofertilizer, include bacteria, fungi and blue green algae. These organisms are added to the rhizosphere of the plant to enhance their activity in the soil. Sustainable crop production depends much on good soil health. Soil health maintenance warrants optimum combination of organic and inorganic components of the soil. Repeated use of chemical fertilizers destroys soil biota. In nature, there are a number of useful soil microorganisms that can help plants to absorb nutrients (Ertani *et al.*, 2014). Their utility can be enhanced with human intervention by selecting efficient organisms, culturing them and adding them to soils directly or through seeds (Nayaka *et al.*, 2013). The cultured microorganisms packed in some carrier material for easy application in the field are called biofertilizers. Biofertilizers are living microorganisms of bacterial, fungal and algae origin. Their mode of action differs and can be applied alone or in combination.

2.3 Keratin

Keratin-rich wastes in the form of feathers, hair, nails, and horn are highly available as byproducts of agroindustrial processing. These keratin-rich wastes are difficult to degrade as the polypeptide is densely packed and strongly stabilized by several hydrogen bonds and hydrophobic interactions, in addition to several disulfide bonds (Holkar *et al.*, 2018). Keratin is the insoluble structural protein of feathers and wool which is known for its high stability, Keratin is insoluble fibrous protein found in hair, wool, feather, nail, horns and other epithelial covering. The keratinous wastes largely comprising of the insoluble structural protein "keratin" is increasingly accumulating in the environment mainly in the form of feathers, hair generated from various industries. They are also grouped into hard and soft keratins according to the sulfur content. Hard keratins found in appendages like feathers, hair, hoofs and nails have high disulfide bond content and are tough and inextensible. Whereas, soft keratins like skin and callus have low content of disulfide bonds and are more pliable (Holkar *et al.*, 2018) Despite the recalcitrance, keratin wastes can be efficiently degraded by a myriad of bacteria, actinomycetes and fungi due to the elaboration of keratinolytic proteases—keratinases. Keratinases are a class of proteases which have ability of cleaving different keratin substrate, These keratinous substrates are considered as one of the major factors which contribute to pollution, due to their recalcitrant nature. Besides their use in traditional industrial sectors like detergent, medicine, cosmetics, leather and feed (Farag *et al.*, 2004), they also find application in newer fields like prion degradation for treatment of the dreaded mad cow disease, biodegradable plastic manufacture and feather meal production and thus can be aptly called "modern proteases". Keratinolytic enzymes are widespread in nature and are elaborated by a compendium of microorganisms largely isolated from poultry wastes. A vast variety of bacteria, actinomycetes and fungi are known to be

keratin degraders. Among bacteria, degradation is mostly confined to gram-positives, including *Bacillus*, *Lysobacter*, *Nesternokia*, *Kocurica* and *Microbacterium*. However, a fewstrains of gram-negative bacteria, viz. *Vibrio*, *Xanthomonas*, *Stenotrophomonas* and *Chryseobacterium* have also been recently reported (Sangali *et al.*, 2002).In addition, a few thermophiles and extremophiles belonging to the genera *Fervidobacterium*, *Thermoanaerobacter, Bacillus* and *Nesternokia* have also been described (Adejumo *et al.*, 2018). Besides these, *actinomycetes* from the *Streptomyces* group, viz. *S. fradiae* using keratin as the sole source of carbon and nitrogen (Adejumo *et al.*, 2018).

Keratin utilization has been described in various organisms including water moulds, filamentous and non-filamentous bacteria and filamentous fungi. Along with bacteria and fungi, some insects incuding carpet beetles are well-known for keratin digestion (Zhan 2011). Bacterial strains that are capable of degrading cow horn have been investigated in several studies (Gupta and Ramnani, 2006). These bacterial strains produce keratinases which selectively degrade the keratin present in degrade the keratin present in cow horn and keratin producing microorganisms have the ability to degrade chicken feather, hair, nails, wool etc (Gradisar, 2005). This enzyme has been majorly produced by *Bacillus* species.

2.3.1 Keratinases

Keratinase is a certain class of proteolytic enzymes that has the capability to degrade insoluble, fibrous keratin substrate. Attention has been increasingly given to these enzymes due to their numerous potential applications which are connected to their ability to hydrolyse keratinous substrates (Brandelli, 2008). Keratinases are largely produced in a basal medium with a keratinous substrate. Keratinases are key protease enzymes; they hydrolyze both ‘soft’ (cytoskeletal materials in epithelial tissues, containing up to 1% sulphur) and ‘hard’ (protective tissues in hairs and nails, containing up to 5% sulphur)

keratins. Hence, in the past few decades, some research projects have focused on the activities of keratinases. The potential of keratinases in the biotechnological context has gained substantial and significant recognition since the beginning of the 21st Century: their substrate specificity and ability to attack highly cross-linked and recalcitrant structural proteins that resist common known proteolytic enzymes, such as trypsin and pepsin, make them valuable biocatalysts in industries that deal with keratinous materials (Karthikeyan *et al.*, 2007).

Keratinases are proteolytic enzymes that can hydrolyze keratins. Microbial keratinases are predominantly of the Metallo, serine, or serine-metallo type (Brandelli, 2008), with the exception of keratinase from yeast, which belongs to aspartic protease. Both Metallo and serine peptidases are endoproteases that cleave peptide bonds internally within a polypeptide. Metalloproteases are highly diverse, having more than 90 families. A characteristic feature of this type of enzyme is the involvement of a divalent ion (such as Zn^{2+}) for their catalytic activities, which are inhibited by metal chelating agents, transition, or heavy metals (Gupta and Ramnani 2006). Serine proteases fall into two broad categories based on their structure: chymotrypsin-like (trypsin-like) or subtilisin-like. The subtilisin subfamily is wholly inhibited by PMSF (phenylmethane sulfonylfluoride), antipain, and chymostatin (Tork *et al.*, 2013).

2.3.2 Sources of microbial keratinases

Keratin degraders can be found among diverse groups of microorganisms: from fungi to bacteria. These microorganisms are frequently isolated from keratin-rich environments such as soil and wastewater associated with the poultry industry and tannery (Monod *et al.*, 2002). However, due to the potential risk of infection, biotechnological applications of these fungi have not been widely explored. Many Gram-positive and Gram-negative bacteria are also found to be critical keratinase producers. From the Gram-positive

category, members of the *Bacillus* genus are the most prominent and prolific keratin degraders (Brandelli, 2008).

2.3.3 Keratinous substrates and their specificities

Keratinases from fungi, actinomycetes and bacteria have a wide range of substrates: from soft keratin such as stratum corneum to hard keratin such as feather keratin (Bálint *et al.*, 2005), sheep's wool, human and animal hairs (Chen *et al.*, 2011), nail, hoof and horn and azokeratin. Other substrates that are susceptible to keratinase degradation include collagen, elastin, gelatine (Tork *et al.*, 2013), albumin and hemoglobin. The chemical properties of their substrates strongly influence the substrate specificity of keratinases. As keratin is composed of 50-60% hydrophobic and aromatic amino acids (Coulombe *et al.*, 2002; Brandelli, 2008), keratinases appear to cleave preferentially hydrophobic and aromatic amino acid residues at the P1 position (Ramnani and Gupta, 2004).

2.4 Mechanism of Keratinolysis

Over the years, several hypotheses have been proposed to explain the mechanism of keratin degradation by microbial keratinases (Cai *et al.*, 2009). It is agreed that keratin degradation encompasses two main stages: deamination and keratinolysis. Deamination creates an alkaline environment for optimal enzymatic reactions by the alkaline proteases. The complex mechanism of keratinolysis that follows involves the cooperative action of sulphitolytic and proteolytic enzymes noted for their degradation activities on natural keratin substrates by purified keratinase from *Bacillus* sp (Fang *et al.*, 2013). Sulphitolysis changes the conformation of keratin and exposes more active sites, making them accessible for further digestion by alkaline protease and resulting in the release of soluble peptides and amino acids (Monod, 2008).

It is noted that purified keratinases are generally less effective in hydrolyzing native keratin, probably due to the removal of disulphide bond reduction components during the purification process. A suitable redox environment may be necessary for the active degradation of keratin. The presence of reducing agents stimulates keratin hydrolysis by purified keratinase. In a cell-bound redox system, the bacterial cells probably provide a continuous supply of reductant (e.g., sulphite) to break disulfide bridges (Fang *et al.*, 2013).

2.4.1 Optimization of keratinase production

Production of keratinase from a commercial perspective requires an integrated approach that combines optimal fermentation conditions, operational optimization, and effective downstream processing. Medium composition and culture conditions are the two crucial factors that affect the yield of an enzyme in a fermentation process (Farag and Hassan, 2004). The keratin source usually serves as the sole carbon and nitrogen source in a growth medium. The addition of separate carbon and nitrogen sources has been shown to increase enzyme production in some microorganisms (Brandelli, 2008) but suppress production in others. It is suggested that as each microorganism has its own optimal set of growth parameters, these conditions should be treated on a case-by-case basis (Cai *et al.*, 2009). The most significant parameters that affect keratinase production can be investigated using one factor-at-a-time method (Janaranjani *et al.*, 2010).

2.5 Sources of Keratin Biomass

Keratin biomass is derived from living organisms or their body parts after death. The major livestocks of keratin includes goatskins, sheepskins, cattle hides and buffalo hides as shown in Fig 1. Skin and its appendages such as nails, hair, feathers, wool, hooves, scales and stratum corneum are the richest sources of keratin (Kim *et al.*, 2001). It can be extracted from animal horns and hooves wool and human hairs (Figure 2.1). Several

million tonnes of feathers are produced annually. Hairs are the by-product of tanneries during the haircut process and 5% of dry hair weight generally recovered from raw material (Ebru *et al.*, 2019). The food industry produces millions of tons of keratin biomass. About 80% of human hair is formed of keratin only. It provides strength, flexibility, durability, and functionality to the hair in the form of different conformations (Bhaskara and Arthi, 2012).

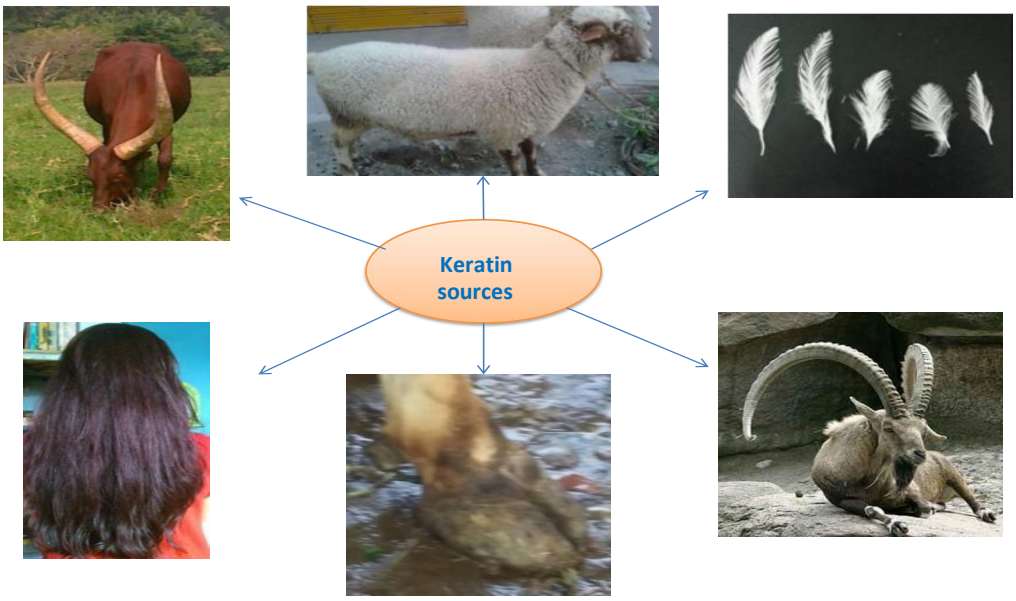

Figure 2.1- Major sources of Keratin biomass

2.5.1 Uses in the cosmetic industry

Keratin is hydrolyzed by acid, alkali, or enzyme, and keratin hydrolysate has considerable applications in cosmetic industries. Keratin based cosmetics have been reported as a treatment of skin and human hair. Keratin, with other natural polymers such as collagen, chitosan, silk fibroin, was used as a component of the blends for cosmetic applications (Karthikeyan *et al.*, 2007). The presence of keratin in the stratum corneum and hair cuticle helps in retaining moisture in the skin by interacting with cosmetics.

High molecular weight keratin proteins are the most attractive part of skincare applications due to their characteristics like hydrophilic and film-forming. A coating or

film on the skin is formed by a keratin molecule, which provides smooth and soft sensation (Karthikeyan *et al.*, 2007)

2.5.2 Uses as fertilizers

Plant metabolizing enhancer organic fertilizers was prepared by using sulfur bound amino acid solution. The composting of a chicken feather was also used to produce biofertilizers. Keratin is a good nitrogen source used to prepare the fertilizers. Bacteria and fungi produce keratinolytic enzymes, which help to degrade the waste biomass of Keratin. Both keratinolytic bacteria and fungi are proposed for use in composting. Among bacteria, the *Bacillus* genus produces plenty of keratinolytic enzymes, and actinomycetes also contribute to keratin degradation (Brandelli and Riffel, 2005). High amounts of nitrogen in feathers make them excellent to be used as fertilizers, but the presence of cystine linkages makes it difficult to degrade, so it made feather biomass less interesting to be used as a fertilizer (Gurav and Jadhav 2013). Some studies have been carried out to increase the utility of waste feathers as biofertilizers. In a previous study, the release of nitrogen was increased by the treatment of feathers with *Chryseobacterium* spp. These hydrolyzed feathers fertilizers were used for banana plants and can be applied as root dose and shoot dose for other cultivation crops (Gurav and Jadhav 2013). Feather waste treated with thermophilic actinomycetes strain was also used as a fertilizer for ryegrass cultivation (Vidyasagar *et al.*, 2007).

2.5.3 Keratins in biomedical applications

Cell adhesion sequences which normally present in fibronectin and cellular binding motifs present in keratin used as sites for cellular attachment this feature made keratin an ideal material for the synthesis of biomaterial in medical applications. Natural proteins considered healthy for skin and hair because of their hydrophilic nature. The monomeric units of natural keratin can be able to penetrate in the skin and hair cuticle and able to

nourish without any side effects. It is also used as a wound-healing agent because the oxidization of keratinous materials cleaves and oxidizes some of the disulfide linkages to form water-soluble peptides. The solid base for research in keratin directed to the growth of many biomaterials, which are keratin-based for use in biomedical applications (Riffel *et al.*, 2003). Keratin extracted from wool and human hair used to prepare the protein films, which was used to discover the structural and biological properties of self-assembled keratins from several years. Dissolved feather keratin can be used to develop protein fibers and 2D and 3D scaffolds, which can be used for tissue engineering (Saibabu and Niyongabo, 2013). The property of extracted keratin proteins to self-congregate and polymerize into complex 3D structures has led to their work as scaffolds for tissue engineering and fabricated keratin-based composite nanofiber by electrospinning used as an application in tissue engineering and regenerative medicines (Daniel *et al.*, 2014). Similarly, in another study, chicken feather extracted keratin for the formation of fabricated keratin films were used in controlled drug delivery systems (Daroit *et al.* 2009). Generally, protein fibers are preferred over cellulose and other synthetic fibers due to the unique property. Many attempts have been made to produce regenerated protein fibers from plants and animals. Feather keratin is one of the potential sources for regenerated fiber. Keratin extracted from feathers and developed regenerated fibers similar to sheep wool (Harison and Sundeep 2014). Because the presence of high crosslinking by cystine formulation of micro and nanoparticle from feather is difficult, but some researchers prepared micro and nanoparticles successfully from feather keratin. Keratin was converted into useful microparticle by treatment with ionic liquid, 1butyl-3-methylimidazoliumchloride. Treated feathers have low surface area but having higher ion sorption capacity than untreated feathers due to their hydrophilic nature. Harison and Sundeep (2014) developed nanoparticle (50-130 nm) from feather keratin, which showed

good biocompatibility and stability essential for controlled drug release. These particles can approach various organs of mice but maximally found in the kidney then liver and followed by the spleen. These keratin particles were water-stable, unlike other nanoparticles, and there is no need for any crosslinking or other chemical modification, thus suitable for medical application. These properties enhanced the utility of keratin-based nanoparticles in biomedical use. These also showed affinity towards other biomolecules, *e.g.,* Graphene oxide, and its derivative has potential applications in biomaterials. (Adejumo and Adetunji, 2018) used feather keratin to check the interaction of these molecules with that of graphitic layers. The result showed that the grafting by keratin enhances the attachment of *Escherichia coli* cells on graphitic films. Andriano *et al.* (2007) develop films by using feathers keratin, which was dissolved by using Na_2S. The yield of feathers after different times of hydrolysis was studied using different concentrations of Na_2S. Keratin has a molecular weight of 20 KDa extracted from a chicken feather, which was used to form films for controlled drug release applications. The film formed has good mechanical properties due to a large amount of cystine linkage. Recently, human hair keratin was used for keratin-based biomaterials, which have been utilized for tissue regeneration and human hair keratin to enrich human mesenchymal stem cells for clinical applications (Lee 2014).

2.5.4 Keratin in feedstock

Feather hydrolysate obtained by chemical, thermal hydrolysis of the chicken feather is rich in amino acids and polypeptides. Due to a similar composition with soybean protein and cottonseed protein, the hydrolysate used as a diet supplement for feeding ruminants. Enzymatic modification through enrichment with lysine leads to an increase in the feed nutritive value. Raw horns and hoofs are put on high pressure in a rendering plant to

make horns meal. Keratin is a useful protein that helps prepare the animal feed (Brandelli, 2008).

2.5.5 Keratin in environmental remediation

The hollow structure and alpha-helix are present in feathers for building uniform microporous material having a high surface area that was used as an electrode material, which is environment-friendly (Zhan and Wool 2011). Poole *et al.* in 2009 stated that the fibers obtained from keratin were environmentally friendly, renewable, and biodegradable. Development of keratin-based material has the potential for revolutionizing the bio-based green materials' world due to their biodegradability, biocompatibility, natural abundance, and mechanical durability (Poole *et al.* 2009; Balaji *et al.* 2012). The strength and modulus of the single feather are up to 300MPa and 6GPa, which is better than most of the wool fibers (Li and Wang 2013). Feathers can be used to made thermoplastic films for the packaging of food and other applications. However, the feathers are non-thermoplastic, so many chemical modifications are required to make them thermoplastic. (Reddy *et al.* 2020) reported the synthesis of thermoplastic by alkaline hydrolysis of the feather. To make a stable feather film, the synthetic monomer can be added to feathers. Grafting of a feather by acrylic monomers (methyl acrylate, methyl methacrylate and butyl acrylate) was also employed to improve the properties of feathers based thermoplastic (Jin *et al.* 2011). Other processes like acetylation and etherification were also used to develop other commercial products of feathers (Hu *et al.* 2011; Reddy *et al.* 2013; Shi *et al.* 2014). Feather degradation done by using microorganisms is a cost-effective and ecologically safe form of the continuously accumulated waste management (Vasileva-Tonkova *et al.* 2009).

2.6 Protein hydrolysates

Plant growth stimulation and enhanced tolerance to biotic and abiotic stresses have been reported by the application of a variety of protein-based products. These plant stimulatory effects appear to be distinct from the nutritional effect of an additional nitrogen source (Ertani *et al.*, 2009).

Protein-based products can be divided into two major categories: protein hydrolysates and amino acids. protein hydrolysates are known to be a mixture of peptides and amino acids of animal or plant origin and individual amino acids such as glutamate, glutamine, proline and glycine betaine. Protein hydrolysates are prepared by enzymatic, chemical or thermal hydrolysis of a variety of animal and plant residues, including animal epithelial or connective tissues (Cavani *et al.*, 2006; Ertani *et al.*,2009), animal collagen and elastin (Cavani *et al.*, 2006), carob germ protein (Parrado *et al.*, 2008), alfalfa residue (Schiavon *et al.*, 2008; Ertani *et al.*, 2009), wheat-condensed distiller solubles, *Nicotiana* cell wall glycoproteins (Apone *et al.*, 2010), and algal protein (De Lucia and Vecchietti, 2012).

Protein/peptide and free amino acid contents of the hydrolysates vary in these preparations in the range of 1–85 % (w/w) and 2–18 % (w/w), respectively. The primary amino acids include alanine, arginine, glycine, proline, glutamate, glutamine, valine, and leucine. Siapton contains a high proportion of proline and glycine, while in carob germ hydrolysate, glutamine, and arginine predominate (Parrado *et al.*, 2008).

Non-protein components present in these hydrolysates may also contribute to the stimulatory effects on plants. For example, in addition to proteins, peptides and free amino acids, carob germ extract hydrolysate, is one of the types of protein hydrolysate, contained fats, carbohydrates, macro and micronutrient elements and at least six phytohormones, while an animal-based product, Siapton, another protein hydrolysate

commercially available form had a similar profile of proteins, amino acids, fats, and macro and micronutrients, but lacked carbohydrates and phytohormones (Parrado *et al.*, 2008).

An alfalfa hydrolysate was high in free amino acids (1.9 % w/w) and also contained macro and micronutrient elements and auxin and gibberellin-like activities based on a bioassay (Schiavon *et al.*, 2008). Ertani *et al.* (2013) reported the presence of triacontanol and IAA, both plant growth regulators, in the same alfalfa hydrolysate product and endogenous IAA in meat hydrolysate (Ertani *et al.*, 2013). Kauffman *et al* (2005), working with protein hydrolysate, derived from the enzymatic hydrolysis of animal membranes and containing 2 % (w/v) plant-available nitrogen, 21.3 % (w/v) free amino acids, peptides, nucleotides and fatty acids and 14.8 % (w/v) unknown organic matter, reported that the lipid-soluble fraction of the product produced an auxin-like response in vitro equivalent to 0.07 % (v/v).

2.6.1 Effect of protein hydrolysates on plant nutrient uptake and yield

Maini (2006) summarized the early studies with the first commercial protein hydrolysate from animal epithelial tissues, named as siapton. Maini (2006) presented data, enhanced N, P, K, and Mg contents in the grains of maize grown under Mgdeficient conditions and then treated with Siapton and Mg compared to that with Mg alone.

Tomato plants fertilized with Siapton or with a carob germ hydrolysate showed increased plant height and the number of flowers per plant compared to the control while only those fertilized with carob germ hydrolysate showed enhanced numbers of fruit per plant after 18 weeks growth in a greenhouse (Parrado *et al.*, 2008).

Koukounararas *et al.* (2013) reported recently that application of Amino16^{R}, a protein hydrolysate containing 11.3 % L-amino acids, to greenhouse tomatoes under varying

fertilization levels increased fruit yield and this was associated with increased fruit number or weight depending on the degree of fertilization. Papaya (*Carica papaya*) yields were increased by 22 % when plants were sprayed at monthly intervals with Siapton (Morales-Payan and Stall, 2003). Gajc-Wolska *et al.* (2012) found no effect of Aminoplant (Siapton) on yield of spinach (*Spinacia* oleracea) and endive (*Chicorium premium*), respectively, in field trials and Grabowska *et al.*(2012) showed an effect on carrot (*Daucus carota*) yield only for one variety in one year of three years of field trials and soluble sugars and carotenoids increased and nitrate content decreased in amino plant-treated carrot roots relative to the control in one year of two field trials.

An alfalfa hydrolysate increased leaf growth, foliar sugar content and decreased nitrate content of hydroponically-grown maize plants (Schiavon *et al.*, 2008) and in more recent studies, enhanced short-term growth of hydroponicallygrown maize in the absence and presence of salt stress (NaCl) was shown alfalfa protein hydrolysates increased K^+ content of leaves in the absence of NaCl and the presence of NaCl, enhanced Na^+ and decreased K^+ contents in roots and leaves (Ertani *et al.*, 2013).

Similarly, a meat hydrolysate derived from tanning residues increased short-term growth and micro-element content and decreased nitrate, phosphate, and sulphate content of hydroponically grown maize seedlings (Ertani *et al.*, 2013). Studies with individual amino acids suggest that they may play a significant role in regulating nitrogen acquisition by roots. Exogenously applied glutamine, in particular, decreased nitrate and ammonium influx and transporter transcript in barley roots (Fan *et al.*, 2006; Miller *et al.*, 2007).

In recent work, Ertani *et al.* (2014) reported that two biostimulants, one derived from alfalfa plants (AH) and the other obtained from red grapes (RG), were chemically characterized using enzyme-linked immunosorbent assays, Fourier transforms infrared

(FT-IR) and Raman spectroscopies. Two doses (50 and 100 ml l ^{1} for RG, and 25 and 50 ml l ^{1} for AH) of biostimulants were applied to *Capsicum Chinensis* L. Plants cultivated in pots inside a tunnel. Both biostimulants contained different amounts of indole acetic acid and isopentenyl adenosine; the AH spectra exhibited amino acid functional groups in the peptidic structure, while the RG spectra showed the presence of polyphenols, such as resveratrol (Miller *et al.*, 2007). These results revealed that at flowering, RG and AH increased the fresh weight of leaves and fruits and the number of green fruits, whereas, at maturity, the biostimulants affected mainly the fresh weight and number of red fruits.

At flowering, the leaves of the biostimulant-treated plants contained high amounts of epicatechin, ascorbic acid, quercetin, and dihydrocapsaicin, while at maturity, they exhibited high quantities of fructose, glucose, chlorogenic, and ferulic acids (Fan *et al.*, 2006).

Furthermore, green fruits exhibited high contents of chlorogenic acid, hydroxybenzoic acid, p-coumaric acid, and antioxidant activity, while both AH- and RG-treated red fruits were highly endowed in capsaicin. The 1H high-resolution magic-angle spinning (HRMAS)-nuclear magnetic resonance (NMR) spectra of red fruits revealed that both products induced the high amount of $NADP^{+}$, whereas RG also increased fumarate, ascorbate, glucose, thymidine, and high molecular weight species (Ertani *et al.*, 2014). These results suggested that AH and RG promoted plant growth and the production of secondary metabolites, such as phenols.

2.6.2 Hormone like activity of protein hydrolysate

Schiavon *et al.,* (2008) reported that alfalfa protein hydrolysate (EM) possessing gibberellin and auxin-like activity might promote plant nitrogen (N) nutrition in Zea

mays treatment with 0.01 or 0.1 mg l^{-1} protein hydrolysate for 48 h resulted in enhanced plant growth and leaf sugar accumulation.

The activity of a number of enzymes involved in carbon metabolism (malate dehydrogenase, MDH; isocitrate dehydrogenase, IDH; citrate synthase, CS) and N reduction and assimilation (nitrate reductase, NR; nitrite reductase, NiR; glutamine synthetase, GS; glutamate synthase, GOGAT; aspartate aminotransferase, AspAT) was significantly induced by protein hydrolysates supply to plants (Ertani *et al.*, 2014).

The auxin, like the effect of the protein hydrolysate, has been observed in the rooting experiment of tomato cuttings. The shoot, dry root weight, and root length were significantly higher by 21, 35, 24, and 26%, respectively, in tomato treated plants with protein hydrolysate at 6ml l ^{1} than untreated plants (Colla *et al.*, 2017).

Colla *et al.* (2017) reported that the application of protein hydrolysates at doses (0.375, 0.75, 1.5, and 3 ml l ^{1}) significantly increased the shoot length of the gibberellins deficient dwarf pea plants by an average value of 33% in comparison with control treatment.

2.6.3 Influence of protein hydrolysate on plant physiology and metabolism

Protein hydrolysates have been shown to stimulate carbon and nitrogen metabolism and to increase nitrogen assimilation. Maini (2006) reported that enhanced activity of NAD-dependent glutamate dehydrogenase, nitrate reductase, and malate dehydrogenase in maize following application of Siapton.

Schiavon *et al* (2008) showed that an alfalfa protein hydrolysate applied to hydroponically-grown maize increased the activity of three enzymes in the tricarboxylic acid cycle (malate dehydrogenase, isocitrate dehydrogenase, and citrate synthase) and

five enzymes involved in N reduction and assimilation (nitrate reductase, nitrite reductase, glutamine synthetase, glutamate synthase, and aspartate aminotransferase).

Ertani *et al.* (2009) compared the effects of hydrolysates from alfalfa (*Medicago sativa*) and meat flour on maize seedling growth and showed increased activities of glutamine synthase (GS) as well as nitrate reductase in leaves and roots compared to the control. Up-regulation of isoforms GS_1 and especially of GS_2, which is responsible for the assimilation of ammonia produced by nitrate reduction, was observed, consistent with a stimulatory effect of the hydrolysates on nitrogen assimilation.

Data available in the literature, suggest that protein hydrolyzates may promote nitrogen assimilation in plants through coordinated regulation of C and N metabolism. For instance, a protein hydrolyzate derived from alfalfa plants, enhanced shoot biomass production, soluble sugar accumulation, and nitrogen assimilation of hydroponically-grown maize plants (Schiavon *et al.*, 2008). Specifically, this biostimulant increased the activity of three enzymes (malate dehydrogenase, isocitrate dehydrogenase and citrate synthase) functioning in the tricarboxylic acid cycle (TCA) and five enzymes (nitrate reductase, nitrite reductase, glutamine synthetase, glutamate synthase, and aspartate aminotransferase) involved in N reduction and assimilation. RT-PCR confirmed the biostimulant-induced up-regulation of the genes coding for these enzymes (Ertani *et al.,*2014).

Further investigation revealed that the same biostimulant was found to improve the growth of maize plants cultivated under salinity stress by increasing the ratio of Na^+ and K^+ in leaves and the synthesis of flavonoids (Ertani *et al.*, 2013).

2.6.4 Protein hydrolysates and stress tolerance

There is considerable evidence that protein hydrolysates and specific amino acids including proline, betaine, their derivatives, and precursors can induce plant defense responses and increase plant tolerance to a variety of abiotic stresses, including salinity, drought, temperature and oxidative conditions (Ashraf and Foolad, 2007; Ertani *et al.*, 2013).

Kramer (1980) reported that the perennial ryegrass plants treated with a product based protein and exposed to prolonged high air temperature stress exhibited both improved photochemical efficiency and membrane thermostability than untreated plants. These results provided consistent and exciting results and showed that foliar applications of protein hydrolysates could positively influence plant tolerance to heat stress (Kauffman *et al.*, 2007).

Apone *et al.* (2010) reported that an amino acid/peptide/sugar mixture derived from plant cell walls induced the expression of three stress marker genes and two genes involved in the oxidative stress response in *Arabidopsis* plants and enhanced the tolerance of cucumber plants to oxidative stress.

Ertani *et al.* (2013) showed that an alfalfa hydrolysate applied to maize grown hydroponically under increasing salt stress increased plant biomass, reduced the activity of antioxidant enzymes and the synthesis of phenolics, but increased leaf proline and flavonoid content, phenylalanine ammonia-lyase (PAL) activity and gene expression relative to salt-stressed controls. Glycine betaine, the N-methyl-substituted derivative of glycine, and proline act as osmoprotectants or osmolytes stabilizing proteins, enzymes, and membranes from the denaturing effects of high salt concentrations and non-physiological temperatures (Ashraf and Foolad, 2007).

Accumulation of glycine betaine and proline is generally correlated with increased stress tolerance, and exogenous application of these compounds has been shown to enhance tolerance to abiotic stresses in a variety of higher plants, including maize, barley, soybean, alfalfa and rice (Chen and Murata, 2008).

In addition to their roles in stabilizing proteins and membranes, glycine, betaine, and proline have been shown to scavenge reactive oxygen species and induce expression of salt stress-responsive genes and genes involved in transcription factors, membrane trafficking and reactive oxygen species (Kinnersley and Turano, 2000).

Other amino acids affect tolerance to abiotic stresses. Exogenous application of glutamate and ornithine, precursors of proline, can also enhance tolerance to salt stress (Chang *et al.*, 2010). Arginine, which plays an essential role in nitrogen storage and transport in plants, has been shown to accumulate under abiotic and biotic stress (Lea *et al.*, 2006).

Amino acids and peptides play a role in the tolerance of plants to a range of heavy metals. Proline accumulation is induced in many plants subjected to heavy metal stress, and some metal-tolerant plants exhibit high constitutive proline content even in the absence of excess metal ions (Sharma and Dietz, 2006).

CHAPTER THREE

3.0 MATERIALS AND METHODS

The study was carried out in the Federal University of Technology, Minna, Niger State. The State is located in the North Central geopolitical zone of Nigeria and covers a landmass of 76,363 square kilometers. It lies between Latitude 8°.00-11° .30'N and Longitude 4º.00-8.00'E (Edogun *et al.,* 2017).

3.1 Source of Keratinous Waste

Chicken feathers were collected from the feather dumpsite in Kure Market Minna, Niger State, and transported to the grinding mill for processing into powder form; after that, it was stored at room temperature (30±2°C) and used for further studies.

3.2 Sample Collection

Soil sample was collected from the abattoir waste dump site of Bosso, Minna, and feather wastes were collected from the feather dump site of J.F Kure market, Minna.

3.3 Sources of the seeds

Jute mallow (*Corchorus olitorius*) and Maize(*Zea mays*) seeds were purchased from local dealers in Kure Market for the study.

3.4 Preparation of Media

The minimal medium was prepared as follows: 2g/L of KH_2PO_4; 0.05 g/L of $MgSO_4$; 0.1g/L of $FeSO_4.7H_2O$; 0.1g/L of $CaCO_3$; 20 g/L of Keratin substrate and 20g/L of agar-agar. The medium was sterilized at 121°C for 15 min, and then supplemented with 0.05g/L of sterile nystatin to inhibit the growth of fungi (Adetunji *et al.,* 2017).

3.5 Isolation of Keratin Degrading Micro-organisms

One gram (1 g) of soil sample was serially diluted using distilled water. Inoculation was done using 0.2 mL of the 10-fold dilution aliquot on the minimal medium for the selective growth of isolates, using the pour plate method.

The plates were incubated at 37°C for up to 72 h. Distinct colonies with characteristic morphological features were selected, isolated, and purified on yeast extract agar to obtain pure cultures. The pure cultures were stored on agar slants of yeast extract agar and minimal selective medium at 4 °C until needed.

3.6 Biochemical Characterization of the Isolates

The biochemical tests, viz. Gram staining, motility, indole production, methyl red, Voges Proskauer's, citrate utilization, sugar utilization, spore staining, catalase, oxidase, coagulase, urease, hydrogen sulfide, and hydrolysis were carried out according to the methods of Brenner *et al.* (Adetunji *et al.,* 2017).

3.7 Molecular Characterization of the Isolates

3.7. 1 DNA extraction

DNA was extracted using the protocol stated by (1). Briefly, Single colonies grown on medium were transferred to 1.5 ml of liquid medium and cultures were grown on a shaker for 48 h at 28 ºC. After this period, cultures were centrifuged at 4600g for 5 min. The resulting pellets were resuspended in 520 µl of TE buffer (10 mMTris-HCl, 1mM EDTA, pH 8.0). Fifteen microliters of 20% SDS and 3 µl of Proteinase K (20 mg/ml) were then added. The mixture was incubated for 1 hour at 37 ºC, then 100 µl of 5 M NaCl and 80 µL of a 10% CTAB solution in 0.7 M NaCl were added and votexed. The suspension was incubated for 10 min at 65 ºC and kept on ice for 15 min. An equal volume of chloroform: isoamyl alcohol (24:1) was added, followed by incubation on ice

for 5 min and centrifugation at 7200g for 20 min. The aqueous phase was then transferred to a new tube and isopropanol (1: 0.6) was added and DNA precipitated at –20 ºC for 16 h. DNA was collected by centrifugation at 13000g for 10 min, washed with 500 µl of 70% ethanol, air-dried at room temperature for approximately three hours and finally dissolved in 50 µl of TE buffer.

3.7.2 Polymerase chain reaction

PCR sequencing preparation cocktail consisted of 10 µl of 5x GoTaq colourless reaction, 3 µl of 25mM MgCl2, 1 µl of 10 mM of dNTPs mix, 1 µl of 10 pmol each 27F 5'- AGA GTT TGA TCM TGG CTC AG-3' and - 1525R, 5′-AAGGAGGTGATCCAGCC-3′ primers and 0.3units of Taq DNA polymerase (Promega, USA) made up to 42 µl with sterile distilled water 8µl DNA template. PCR was carried out in a GeneAmp 9700 PCR System Thermalcycler (Applied Biosystem Inc., USA) with a Pcr profile consisting of an initial denaturation at 94°C for 5 min; followed by a 30 cycles consisting of 94°C for 30 s, 50°C for 60s and 72°C for 1 minute 30 seconds ; and a final termination at 72°C for 10 mins. And chill at 4oC.GEL (2,3)

3.7.3 Integrity

The integrity of the amplified about 1.5Mb gene fragment was checked on a 1% Agarose gel ran to confirm amplification. The buffer (1XTAE buffer) was prepared and subsequently used to prepare 1.5% agarose gel. The suspension was boiled in a microwave for 5 minutes. The molten agarose was allowed to cool to 60°C and stained with 3µl of 0.5 g/ml ethidium bromide (which absorbs invisible UV light and transmits the energy as visible orange light). A comb was inserted into the slots of the casting tray and the molten agarose was poured into the tray. The gel was allowed to solidify for 20 minutes to form the wells. The 1XTAE buffer was poured into the gel tank to barely

submerge the gel. Two microliter (2 l) of 10X blue gel loading dye (which gives colour and density to the samples to make it easy to load into the wells and monitor the progress of the gel) was added to 4µl of each PCR product and loaded into the wells after the 100bp DNA ladder was loaded into well 1. The gel was electrophoresed at 120V for 45 minutes visualized by ultraviolet trans-illumination and photographed. The sizes of the PCR products were estimated by comparison with the mobility of a 100bp molecular weight ladder that was ran alongside experimental samples in the gel.

3.7.4 Purification of amplified product

After gel integrity, the amplified fragments were ethanol purified in order to remove the PCR reagents. Briefly, 7.6 µl of Na acetate 3M and 240 µl of 95% ethanol were added to each about 40µl PCR amplified product in a new sterile 1.5 µl tube Eppendorf, mix thoroughly by vortexing and keep at -20°C for at least 30 min. Centrifugation for 10 min at 13000 g and 4°C followed by removal of supernatant (invert tube on trash once) after which the pellet were washed by adding 150 µl of 70% ethanol and mix then centrifuge for 15 min at 7500 g and 4°C. Again remove all supernatant (invert tube on trash) and invert tube on paper tissue and let it dry in the fume hood at room temperature for 10-15 min. then resuspend with 20 µl of sterile distilled water and kept in -20^{o}C prior to sequencing. The purified fragment was checked on a 1.5% Agarose gel ran on a voltage of 110V for about 1hr as previous, to confirm the presence of the purified product and quantified using a nanodrop of model 2000 from thermo scientific.

3.7.5 Sequencing

The amplified fragments were sequenced using a Genetic Analyzer 3130xl sequencer from Applied Biosystems using manufacturers' manual while the sequencing kit used was that of BigDye terminator v3.1 cycle sequencing kit. Bio- Edit software and MEGA

6 were used for all genetic analysis. These sequences were browsed in the database of the National Centre for biotechnological information (NCBI) (http://blat.ncbi.nlm.nih.gov/) via the blast option for possible matches

3.8 Inoculum development

A loopful of pure culture was inoculated into an inoculum medium consisting of 1% feather meal and 0.2% yeast extract (pH 7.5). The culture was incubated at 37°C and 100 r/min for 24h.

3.9 Preparation of feather hydrolysate

The hydrolysate was prepared by inoculating 1ml of inoculum into 19mL of fermentation medium (without agar and nystatin) in 100mL flasks. The flasks were incubated at 37°C at 100r/min for up to 120h. At 24 h interval, the whole flasks were taken out and the broth centrifuged at 500 r/ min at 10°C for 20 min, and the supernatants served as crude hydrolysates which were used without further purification, some of the broths were not centrifuged and they were used directly without further purification, these contain bacteria suspension. When not used immediately, the crude hydrolysates were stored at 4°C.

3.10 Experimental setup

Pots were filled with equal amounts of soil, the set up was arranged in a randomized complete block design. Three treatments [FH, FH+ bacteria suspension, and FH+ NPK was applied along with positive control (NPK only) and negative control (water only). There were replications for each treatment. After mixing FH into the soil, the soil was watered well and kept for two weeks. Ewedu seeds were sown in each pot. The pots were arranged in a randomized complete block design, and the positions were changed every

alternative day so that all the pots get equal sunlight. The plants were pruned one week after seed germination by keeping ten healthy plants in each pot.

After seven weeks from the emergence of plants, the plants were harvested by cutting the stems 1-cm above the ground. Visual symptoms were monitored carefully and noted throughout the growing period. After harvest, the plant height (cm), number of leaves, fresh weight (g) and dry weight (g) were measured and recorded.

3.11 Data Analysis

In all experiments, ANOVA test was conducted using the software package SPSS for Windows (SAS Inc., Cary, NC, USA). Duncan's multiple range test was performed at $P = 0.05$ on each of the significant variables measured

CHAPTER FOUR

4.0 RESULTS AND DISCUSSION

4.1 Results

4.1.1 Identification of keratinase producing bacteria

The results of the biochemical tests are summarized in Table 4.1. Morphological and biochemical characterization revealed the organisms to be *Bacillus cereus* and *Lysinibacillus fusinformis*.

Table 4.1 Biochemical Characterization of the Bacterial Isolates

	Bacterial Isolates			
Biochemical Test Reaction				
Gram's reaction	+		+	
Urease	+			
Citrate	+		-	
Hydrogen sulfide	-		-	
Motility	+		+	
Starch utilization	-		+	
Methyl red	-		-	
VP	+		+	
Nitrate reduction	+		-	
Spore formation	+		+	
Sugar utilization	A	G	A	G
Glucose	+	+	+	+
Fructose	+	_	+	-
Arabinose	+	_	-	-
Maltose	+	_	+	+
Lactose	+	_	-	-
Sucrose	+	_	+	+
Suspected Organism	*Lysinibacillus fusinformis*		*Bacillus cereus*	

(+) Indicates positive result and (-) Indicates negative result

4.1.2 Result of molecular identification

The sequence analysis revealed the isolate to be *Lysinibacillus fusiformis* strain NBRC15717 and *Bacillus pacificus* strain MCCC 1A06182, *Bacillus paranthracis* strain

MCCC 1A00395 *Bacillus cereus* strain ATCC 14579 *Bacillus cereus* strain JCM 2152 or

Bacillus cereus strain JCM 3010

Sequence for *Lysinibacillus fusiformis*

GGGGGGCTGAGTAACACGTGGGCAACCTACCTTATAGTTTGGGATAACTCCG
GGAAACCGGGGCTAATACCGAATAATCTGTTTCACCTCATGGTGAAACACTG
AAAGACGGTTTCGGCTGTCGCTATAGGATGGGCCCGCGGCGCATTAGCTAGT
TGGTGAGGTAACGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGG
TGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAG
CAGTAGGGAATCTTCCACAATGGGCGAAAGCCTGATGGAGCAACGCCGCGT
GAGTGAAGAAGGATTTCGGTTCGTAAAACTCTGTTGTAAGGGAAGAACAAG
TACAGTAGTAACTGGCTGTACCTTGACGGTACCTTATTAGAAAGCCACGGCT
AACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGA
ATTATTGGGCGTAAAGCGCGCGCAGGTGGTTTCTTAAGTCTGATGTGAAAGC
CCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGGAGACTTGAGTGCAGA
AGAGGATAGTGGAATTCCAAGTGTAGCGGTGAAATGCGTAGAGATTTGGAG
GAACACCAGTGGCGAAGGCGACTATCTGGTCTGTAACTGACACTGAGGCGC
GAAAGCGTGGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTA
AACGATGAGTGCTAAGTGTTAGGGGGTTTCCGCCCCTTAGTGCTGCAGCTAA
CGCATTAAGCACTCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAG
GAATTGACGGGGGCCCGACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCA
ACGCGAAGAACCTTACCAGGTCTTGACATCCCGTTGCCACTGTAGAGATATA
GTTTCCCCTTCGGGGGCAACGGTGACAGGTGGTGCATGGTTGTCGTCAGCTC
GTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGATCTTA
GTTGCCATCATTTAGTTGGGCACTCTAAGGTGACTGCCGGTGACAAACCGGA
GGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTACAGGCAT

Sequence for *Bacillus cereus*

GTCCTATACGACTTCACCCCAATCATCTGTCCCACCTTCGGCGGCTGGCTCCA
AAAGGTTACCTCACCGACTTCGGGTGTTACAAACTCTCGTGGTGTGACGGGC
GGTGTGTACAAGGCCCGGGAACGTATTCACCGCGGCATGCTGATCCGCGATT
ACTAGCGATTCCAGCTTCACGCAGTCGAGTTGCAGACTGCGATCCGAACTGA
GAACAGATTTGTGGGATTGGCTTAGCCTCGCGGCTTCGCTGCCCTTTGTTCTG
CCCATTGTAGCACGTGTGTAGCCCAGGTCATAAGGGGCATGATGATTTGACG
TCATCCCCACCTTCCTCCGGTTTGTCACCGGCAGTCACCTTAGAGTGCCCAAC
TGAATGCTGGCAACTAAGATCAAGGGTTGCGCTCGTTGCGGGACTTAACCCA
ACATCTCACGACACGAGCTGACGACAACCATGCACCACCTGTCACTCTGCCC
CCGAAGGGGAAGCCCTATCTCTAGGGTTGTCAGAGGATGTCAAGACCTGGTA
AGGTTCTTCGCGTTGCTTCGAATTAAACCACATGCTCCACCGCTTGTGCGGGC
CCCCGTCAATTCCTTTGAGTTTCAGTCTTGCGACCGTACTCCCCAAGGCGGAG
TGCTTAATGCGTTTGCTGCAGCACTAAAAGGGCGGAAAACCCTCTAACACTT
AGCACTCATCGTTTACGGCGTGACTACCAGGTATCTAATCTGTCGCTCCCCCA
CGCTTCGCGCTCAGCGTCAGTTACAGACAGAAGAGTCGCTCGCCACTGGTGT
CCTCAAATCTCTACGCATCACCGCTACCGTGAAATCCATCTCTCTTTCGCATC
AAGTCCCAGTTCCATGAC

Gel electrophoresis indicating a positive amplification of the 16S region of the bacteria isolates using 16S ribosomal universal primer, showed that the isolates have a band size of approximately 1500bp as seen in Plate 1.

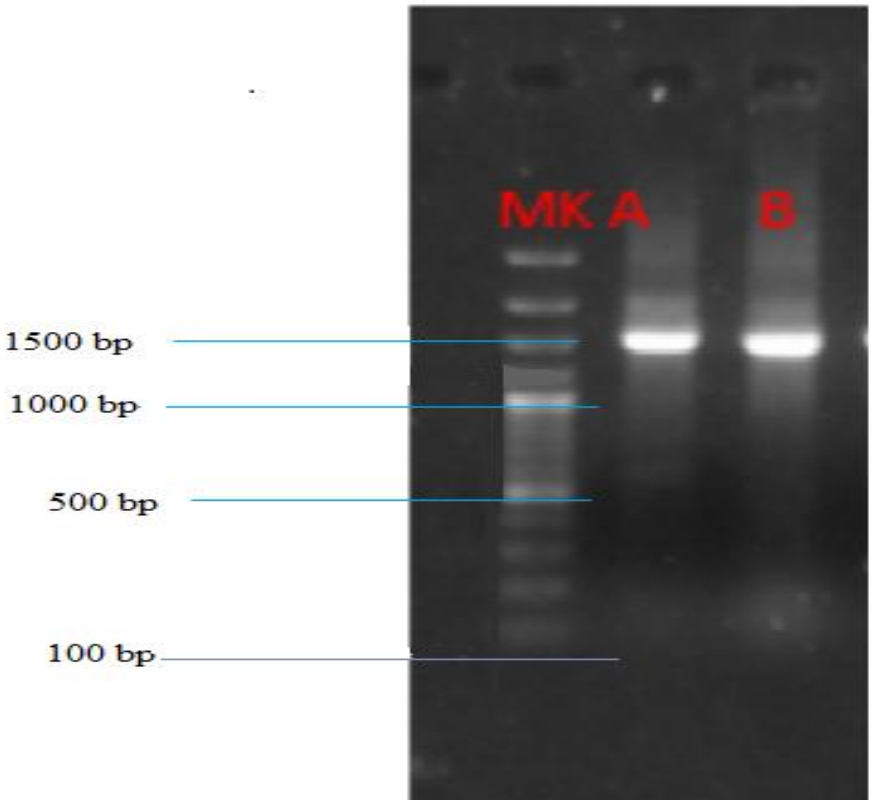

Plate 1: Agarose gel electrophoresis of the 16s rRNA of the bacterial isolates indicating approximately 1500bp

4.1.3 Yield and weight of crops

The results of the crop yield is presented in Table 4.2. The highest yield of *Zea mays* was recorded in those treated with NPK fertilizer (61.2a±0.00) while *Corchorus olitorius* treated with FH only evidenced the highest yield of crops of 92.52^{a} ±13.90 followed by those treated with NPK only with a yield of 90.75^{a} ± 15.01

Table 4.2: Yield weight of crops

Treatments	*Zea mays*	*Corchorus olitorius*
FH and NPK	13^{c}±0.00	70.5^{ab} ±23.0
NPK only	61.2^{a}±0.00	90.75^{a} ± 15.01
FH only	6.9^{d}±0.00	92.52^{a} ±13.90
FH and Sus	32^{b}±0.00	89.72^{a}±4.34
Water	4.9^{e}±0.00	47.04^{b}±2.01

The results of the total yield weight of *Corchorus olitorius* and *Zea mays* using the different treatments are displayed in Table 4. 2

Means that do not share a letter are significantly different
FH- Feather Hydrolysate
FH+susp- Feather Hydrolysate with Suspension
FH +NPK-Feather Hydrolysate with NPK
NPK- Positive Control
Water- Negative Control
Shoot height of the crops

4.1.4. Shoot height of crops

The results of the shoot height of each treatment are displayed in Figure 4.1. NPK only had the highest height (62 cm), followed by FH and NPK (61 cm) for *Corchorus olitorius* and the least in water residues (28 cm) while for *Zea mays* NPK only (64 cm) had the highest height followed by FH + suspension (62 cm) and FH only(60 cm).

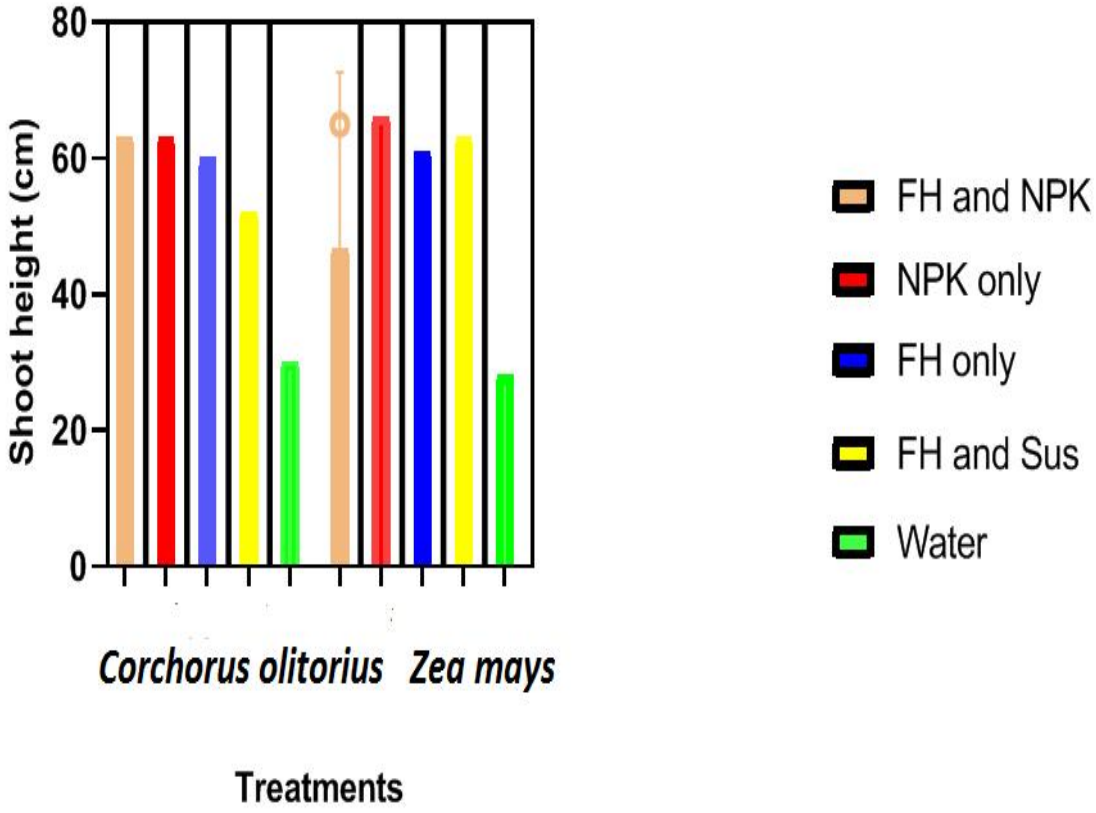

Figure 4.1. Heights of *Corchorus olitorius* and *Zea mays*

FH- Feather Hydrolysate
FH+susp- Feather Hydrolysate with Suspension
FH +NPK-Feather Hydrolysate with NPK
NPK- Positive Control
Water- Negative Control

4.1.5 Proximate contents of *Corchorus olitorius* and *Zea mays*

Proximate analysis of *Corchorus olitorius* and *Zea mays* (Tables 4.3 and 4.4) revealed the moisture (18.61%), fiber (19.12%), ash (6.10%), lipids (8.62%), carbohydrate (59.34%) contents were highest in FH and NPK except for protein that was highest in FH only (59.34%; This is similar to the results obtained with *Zea mays* having moisture, fiber, ash, lipids, and carbohydrate and protein contents of 19.61%, 19.12%, 6.10%, 8.62%, 59.34% and 58.28% respectively

Table 4.3: Proximate Contents (%) of *Corchorus olitorius*

	Moisture	Fiber	Ash	Lipid	Carbohydrate	Protein
FH only	9.21±6.92^{b}	2.16±0.01^{a}	3.53±0.01^{a}	3.29±0.01^{a}	56.31±0.01^{a}	59.34±0.01^{a}
FH and Susp	13.21±0.01^{ab}	2.16±0.01^{b}	3.53±0.01^{b}	3.29±0.01^{a}	56.31±0.01^{b}	56.31±0.01^{b}
FH and NPK	18.61±0.01^{a}	19.12±0.01^{c}	6.10±0.01^{c}	8.62±0.01^{b}	59.34±0.01^{b}	56.31±0.01^{b}
NPK	15.61±0.01^{ab}	18.31±0.01^{c}	5.10±0.01^{c}	8.62±0.01^{b}	52.32±0.01^{c}	52.32±0.01^{c}
Water	13.30±0.01^{ab}	2.18±0.01^{c}	2.18±0.01^{d}	2.18±0.01^{c}	10.33±0.01^{d}	10.33±0.01^{d}

FH- Feather Hydrolysate
FH+susp- Feather Hydrolysate with Suspension
FH +NPK-Feather Hydrolysate with NPK
NPK- Positive Control
Water- Negative Control

Table 4.4: Proximate Analysis of *Zea mays*

	Moisture	Fiber	Ash	Lipid	Carbohydrate	Protein
FH only	9.21±6.92^{b}	2.16±0.01^{a}	3.53±0.01^{a}	3.29±0.01^{a}	56.31±0.01^{a}	58.28±0.01^{a}
FH and Susp	13.21±0.01^{ab}	2.16±0.01^{b}	3.53±0.01^{b}	3.29±0.01^{a}	56.31±0.01^{b}	57.21±0.01^{b}
FH and NPK	19.61±0.01^{a}	19.12±0.01^{c}	6.10±0.01^{c}	8.62±0.01^{b}	59.34±0.01^{b}	56.31±0.01^{b}
NPK	16.61±0.01^{ab}	18.31±0.01^{c}	5.10±0.01^{c}	8.62±0.01^{b}	52.32±0.01^{c}	52.32±0.01^{c}
Water	13.30±0.01^{ab}	2.18±0.01^{c}	2.18±0.01^{d}	2.18±0.01^{c}	10.33±0.01^{d}	10.33±0.01^{d}

FH- Feather Hydrolysate
FH+susp- Feather Hydrolysate with Suspension
FH +NPK-Feather Hydrolysate with NPK
NPK- Positive Control
Water- Negative Control

4.1.6 Harvested crops

The result of the harvested crops (*Corchorus olitorius* and *Zea mays*) after different treatment are presented in plate 2 and 3

Plate 2 : *Corchorus olitorius* leaf after each treatment

NPK ONLY

WATER ONLY

FH ONLY

FH+ SUSP

FH AND NPK

FH- Feather Hydrolysate
FH+susp- Feather Hydrolysate with Suspension
FH +NPK-Feather Hydrolysate with NPK
NPK- Positive Control
Water- Negative Control

Plate 3: *Zea mays* of Each Treatment

4.2 DISCUSSION

Chicken feathers constitute about 8% of the total body weight of a particular chicken, which contain 90% of total crude protein. Large amount of chicken feathers (i.e., 20 million tons) are generated on a weekly basis by the poultry industries, most of which are discarded into the environment where they persist and constitute environmental problem owing to the fact that they are difficult to degrade naturally (Nurdiawati *et al.*, 2019). However, degradation of chicken feathers can be enhanced through enzymatic actions produced by microorganisms. Microorganisms are ubiquitous, their presence in a particular environment is a function of the role(s) they play to impact either positively or negatively to the ecosystem. Microorganisms often evolve devising means they can employ to utilize the available substrate in their immediate environment; a typical example is the production of enzymes to breakdown complex substrates into simple usable forms. As such, microorganisms with potentials of transforming chicken feathers into useful industrial and agricultural by-products have been explored.

Various studies including but not limited to Kowalczyk *et al.* (2017), Nagarajan *et al.* (2018), and Nurdiawati *et al.* (2019) have all reported potentials of keratinolytic bacterial in the production of chicken feather hydrolysate through the enzymatic action of keratinase. The major environmentally friendly agents derived from microorganisms widely used in chicken feather degradation are disulphide reductases, proteases and keratinases commonly produced by Gram positive bacteria (Kowalczyk *et al.*, 2017). In this study, genus of *Bacillus* and *Lysinibacillus* commonly found in soils were isolated and characterized. The bacterial strains from *Bacillus* sp. include *B. cereus* ATCC 14579, *B. cereus* JCM 2157, *B. cereus* JCM 3010, *B. pacificus* MCCC 1A06182, and *B. paranthracis* MCCC 1A00395. Whereas *Lysinibacillus* sp. such as *L. fusiformis* NBRC15717, *L. fusiformis* DSM9828, *L. mangiferihumi* M-GX18 and *L. sphaericus*

MBRC 15095 were all isolated and characterized at molecular levels. The variation of *Bacillus* sp., reported in this study is similar to that of Kowalczyk *et al.* (2017), which reported the presence of varying bacterial species such as *B. cereus* and *B. pseudofirmus* among other bacterial species that degrade feathers in poultry.

Bacillus sp. are often associated with production of feather hydrolysate, their ability to survive and persist in nutrient scarce environment such as the soil is a function of their spores and cell structures as well as their ability to synthesize enzymes that can utilize substrate present in their environment (Lang *et al.*, 2016). Li *et al.* (2013) also reiterates the importance of *Bacillus* sp. in chicken feather degradation. *Bacillus* sp. have been reported to utilize chicken feathers as substrate to obtain carbon and nitrogen as their main source of energy during growth with a resultant production of feather hydrolysate (Chouyyok *et al.*, 2005). Akcan (2012) reported *Bacillus* sp. to be isolated from soil exposed to chicken feathers. Similarly, earlier study by Prakasham *et al.*, (2006) reported the presence of *Bacillus cereus* CC-1 in soil sample, which was used to efficiently degrade 90% of chicken feather within 4 days of treatment periods. Elsewhere, study by Okoroma *et al.*, (2012) also isolated strains of *Bacillus* sp. (i.e., DF3, DF2b and DF1a) from soil with great potentials to utilize keratinase and protease in the hydrolysis of chicken feathers. In spite of the isolation of other genuses of bacteria such as *Curtobacterium, Acinetobacter, Paenibacillus, Phyllobacterium,* and *Serratia* from soilby Paul *et al.* (2014) however, *Bacillus* sp. were the most abundant and most effective in degrading chicken feathers followed by *Lysinibacillus* sp., which were also isolated in this study. Similarly, study conducted by Nnolim *et al.* (2020) isolated strains of *Brevibacillus* sp. Nnolim-K2, *Chryseobacterium* sp. FPF-8, and *Bacillus* sp. FPF-1 from soil. However, *Bacillus* sp. FPF-1 was the most efficient in terms of enzyme (i.e., keratinase) production and activities in degrading chicken feather.

The bioconversion of large amount of chicken feathers by *Bacillus* sp. and *Lysinibacillus* sp. to yield feather hydrolysate does not only alleviate environmental pollution caused by this chicken feathers but also provide biofertilizer for crop production among other important industrial applications. Crops requires both macro- and micronutrients for good produce. Farmers in Nigeria often used Urea and NPK fertilizer to improve crop yield. However, these fertilizers can constitute to environmental problems especially when they are washed off into water bodies such as rivers and lakes causing algal bloom among others. To prevent this occurrence, biofertilizers such as chicken feather hydrolysate can be used as alternative to improve crop production. As mentioned earlier, keratinolytic and proteolytic bacteria such as Bacillus sp. and *Lysinibacillus* sp. have the potentials to provide crops with nitrogen, ammonium, peptides, and amino acids, made available in the form of feather hydrolysate (Lange *et al.*, 2016). Nitrogen is important in the development and growth of plants as such, their availability for plant use cannot be overemphasized.

Result from this study indicated a general increase in crop yield treated with NPK fertilizers, feather hydrolysate + NPK (FH + NPK) fertilizers, feather hydrolysate + microbial suspension (FH + SUSP) and feather hydrolysate (FH) than negative control (i.e., those treated with only water) for both *Zea mays* and *Corchorus olitorius* used in this study. This result concurs to findings obtained by Anitha and Eswari (2012). In their study, the groundnut plant treated with feather hydrolysate and chemical fertilizer all had a better crop yield compared to the negative control. The reason for this crop yield is quite simple, plants treated with feather hydrolysate and chemical fertilizers (urea or NPK) are exposed to abundant nitrogen and other macro nutrients necessary for their development and eventually yielding good produce. Likewise, studies by Daniel *et al.*

(2014) and Adriano (2007) all reported high yield from plants treated with feather hydrolysate and chemical fertilizers respectively.

The crops yield as depicted in Table 4.2 simply shows the nutrient demands of varying plants. *Corchorus olitorius* is a vegetable, which nutrient requirements were met by FH evidenced with a highest crop yield of 92.52 ±13.90% followed by 90.75 ± 15.01% produced by plants treated with NPK fertilizer. This was not the case for *Zea mays* as results showed a higher (61.2±0.00 %) yield for plants treated with NPK fertilizer against a low yield of 6.9±0.00 % recorded for FH. However, this percentage yield was found to be greater when *Zea mays* were treated with FH + SUSP. This increase could be related to microbial activities since *Bacillus* sp. have been reported in the past to improve soil fertility making nutrients available for plant use through synthesis and secretion of extracellular enzymes, which liberates nutrients such as nitrogen, carbon, phosphorus and potassium among other micronutrients (Adejumo and Adetunji, 2018). Studies by Adejumo and Adetunji, 2018) also demonstrated a higher crop yield when FH+SUSP was used to improve rice production. In their reports, it was stated that FH+SUSP also helps in encouraging the proliferation of plants beneficial microorganisms and soil quality. This is in agreement with results obtained by Nagarajan *et al* (2018). In their study, macronutrients such as K, N and P were found to be highest in soils with 1.5% compost of chicken feathers. The water holding capacity of the soil was also improved as the percentage compost chicken feathers increases creating a conducive environment for plant growths (Nagarajan *et al.*, 2018).

Aside just promoting the growth of plants, the use of FH solely in crop production also ensures the development of quality plants nourished with abundant proteins, which is typical of this study. Proximate results of *Corchorus olitorius* and *Zea Mays* depicted in Table 3 and 4 respectively, indicates high content (59.34±0.01%) of protein when FH

was used solely as the source of nitrogen and carbon for *Corchorus olitorius.* Likewise, the protein content of *Zea mays,* which was reported to be highest in the treatment that contains only FH. A lowest protein content was observed in negative control of both *Corchorus olitorius* (10.33±0.01%) and *Zea mays* (10.33±0.01%), which strongly indicates that the use of FH as a sole source of nitrogen and carbon in plant cultivation positively increase the protein content of the plant. No wonder, FH is used in animal feed as a cheap but reliable source of protein supplying animals with over 20 free amino acids (Gençkal 2004). Venkata *et al.* (2013) studied the nutritional constituents of FH, in their result, they documented that strains of *Bacillus aerius* NSMk2 were important in making available free amino acids numbered up to 17 out of which 8 belongs to group of amino acids regarded as essential. They include: tryptophan, valine, phenylalanine, threonine, methionine, isoleucine and lysine.

CHAPTER FIVE

5.0 CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

Feather hydrolysates, obtained through microbial conversion of chicken feathers, increased growth and biomass yield of cultivated crops compared to a reference fertilizer (urea) and other by-products of the poultry agro-industry. With the increase in population and a need for more urbanization and industrialization the loss of agricultural land is predicted to increase rapidly in the coming decades. As agricultural production intensified with increase in human populations over the past few decades, producers became more and more dependent on agrochemicals for crop protection which poses serious threat to the environment and human health. Bacterial degradation of feathers can be a good choice to derive usable byproducts from feather waste. The present study revealed excellent finding of thermoduric and alkaliphile *Bacillus cereus* CC-1 with good protease (keratinase) activity. Poultry industry use many amino acids for feed formulation for better growth of poultry animals and in this present finding *Bacillus cereus* was able to degrade feathers efficiently and release a significant amount of amino acids which could be positively considered for feather meal production. Apart from high amino acids content feather hydrolysate also shows antioxidant activity which gives an added advantage to be used as feed additive. Chemical fertilizers have several harmful effects which include water pollution, chemical burn to the crops, increased air pollution, acidification of soil and mineral depletion. Therefore, there is increasing demand for organic fertilizer. Organic fertilizers are pollution free and nontoxic. Feather hydrolysate contains nitrogen and phosphorus which helps it to be an excellent Biofertilizer. *Lysinibacillus fusiformis* and *Bacillus cereus* were identified as feather degrading

bacteria from this study. The isolated bacteria were able to produce feather hydrolysate which was subsequently used as a bio-fertilizer. The result of this study showed that the biofertilizer produced from the hydrolysis of feather had a positive effect on the growth of the selected vegetables. Furthermore, there was a synergistic effect between the feather hydrolysate and the chemical fertilizer (NPK).

5.2 Recommendations

1. The use of keratin biomass for the production of biofertilizers is one of the areas of research; it still, needs to be explored.
2. The greatest challenge for agricultural researchers in the current century is to meet the increasing world population and to reduce the use of synthetic chemicals which poses serious threat to the environment and human health.
3. Manipulating the isolate for better production of feather hydrolysate in large scale should be encouraged and also sourcing for other keratin-wastes for better production and to increased growth and biomass yield of cultivated crops.

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APPENDICES

Appendix I: Nucleotide Sequence blast of *Lysinbacillus fusiformis*

Description	Max score	Total score	Query cover	E value	Per.ident
***Lysinibacillus fusiformis* strain NBRC15717 16S ribosomal RNA.**	2037	2037	99%	0.0	99.64%
***Lysinibacillus fusiformis* strain NBRC15717 16S ribosomal RNA.**	2037	2037	99%	0.0	99.64%
***Lysinibacillus fusiformis* strain DSM 2898 16S ribosomal RNA.**	2037	2037	99%	0.0	99.64%
***Lysinibacillus mangiferihumi* strain M-GX18 16S ribosomal RNA.**	1982	1982	99%	0.0	98.75%
***Lysinibacillus sphaericus* strain NBRC 15095 16S ribosomal RNA**	1977	1977	99%	0.0	98.66%

Appendix II: Nucleotide Sequence blast of *Bacillus* sp

\Description	Max score	Total score	Query cover	E value	Per. ident	Accession
***Bacillus pacificus* strain MCCC 1A06182 16S ribosomal RNA**	2268	2268	99%	0.0	98.83%	NR_157733.1
***Bacillus paranthracis* strain MCCC 1A00395 16S ribosomal RNA**	2268	2268	99%	0.0	98.83%	NR_157728.1
***Bacillus cereus* strain ATCC 14579 16S ribosomal RNA (rrnA)**	2268	2268	99%	0.0	98.83%	NR_074540.1
***Bacillus cereus* strain JCM 2152 16S ribosomal RNA (rrnA)**	2268	2268	99%	0.0	98.83%	NR_113266.1
***Bacillus cereus* strain JCM 3010 16S ribosomal RNA**	2268	2268	99%	0.0	98.83%	NR_115714.1