APPLICATION OF ALUMINUM OXIDE NANOPARTICLE IN THE CULTIVATION OF SOME SELECTED ASPERGILLUS SPECIES FOR THE PRODUCTION OF PROTEASE ENZYME

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ABSTRACT

Chemicals are being utilized in a variety of businesses globally, which is having a severe impact on people's health. In today's society, environmentally acceptable replacements for these harmful compounds are used. This study applied aluminum oxide nanoparticles in the cultivation of some selected *Aspergillus* sp. in the production of protease enzyme. *Aspergillus niger*, *Aspergillus terreus* and *Aspergillus flavus* were isolated from the soil sample collected from the biological garden of the Federal University of Technology, Minna, Niger State. The pure isolates were cultivated using potato dextrose agar slant for 10 days. *Cymbopogan citratus* (lemon grass) was collected from the environment of Federal University of Technology, Minna, Niger State. The fresh lemon grass were diced and twenty-five (25g) was boiled with 200ml of distilled water and the filtrate was used to produce aluminum oxide nanoparticles. The spores of the isolated *Aspergillus* sp. were inoculated into sterile pre-culture medium that contains the aluminum oxide nanoparticles and was propagated in shake flasks for 24 hours. 7ml of the pre-culture medium was transferred into sterile production medium and propagated in shake flasks for 120 hours at 28°C. The spores of the enhanced *Aspergillus* sp. were harvested and introduced into the medium composed of (g/l): $CaCl_2.7H_2O$ (0.4), KH_2PO_4 (2.5), $MgSO_4.7H_2O$ (0.5), $ZnCl_2$ (0.1), NaCl (0.3), Casein (2.0) for protease enzyme production. The medium was incubated in a rotary shaker at 150rpm for 72 hours at 30°C. The cultures were centrifuged at 10,000rpm for 10 minutes. The fungal mycelia were removed and the supernatant was used as the enzyme extract. Different pH, ranging from 5.0, 6.0, 7.0, 8.0, 9.0 and 10.0 and different temperatures, ranging from 30º, 40º, 50º, 60º, 70º and 80º were used for the optimization of the protease enzyme. The *Aspergillus niger* had the highest yield of protease at 50ºC and *Aspergillus flavus* had the highest yield at pH 6.0.

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CHAPTER ONE

1.0 INTRODUCTION

1.1 Background to the Study

Enzymes are substances present in the cells of living organisms in minute amounts and are capable of speeding up chemical reactions (associated with life processes), without themselves being altered after the reaction. They accelerate the velocity of the reaction without necessarily initiating it (Oyeleke *et al.,* 2011; Engel, 2020). Enzymes are nature's sustainable catalysts. They are biocompatible, biodegradable and are derived from renewable resources (Sheldon and van Pelt, 2013). Enzymes constitute a large biological globular protein molecule responsible for thousands of metabolic processes that sustain life (Sheldon and van Pelt, 2013), and function as catalysts to facilitate specific chemical reactions within the cell. These reactions are essential for the life of the organism. There are three major sources of enzymes; the plant source, animal source and microbial source. Plant enzymes are derived from a variety of plants, for example; papain, animal enzymes are derived from animal glands, for example; trypsin, pepsin, and microbial enzymes are derived from microorganisms, for example; fungi and bacteria, through the process of fermentation, for example; amylase and protease. Microbial enzymes are preferred to those from both plant and animal sources because they are cheaper to produce, and their enzyme content are more predictable, controllable and reliable (Oyewole *et al.,* 2011).

Microbial enzymes produced from industries are selected from different groups of microorganisms and they include bacteria, fungi and yeasts. Many enzymes are produced in industries but most predominant enzymes that are produced on large scale in industries include protease, alpha-amylase, glucose isomerase and glucamylase (Nigam, 2015). Only few microoganisms including *Aspergillus* sp. have been reported

to possess the ability to produce raw starch degrading amylase (Oyewole *et al.,* 2011; Ayodeji *et al.,* 2017). *Aspergillus niger* and *Bacillus subtilis* have been reported to possess the ability to produce amylase and protease enzymes (Oyeleke *et al.,* 2011). *Bacillus subtilis* has been reported to have been used in the production of proteases, amylases and pencillinase enzymes (Bueno *et al.,* 2016). *Saccharomyces fragilis* has been reported to possess the ability to produce lactase and also *Saccharomyces cerevisiae* has been used in the production of invertase enzyme (Liu *et al.,* 2014). It has also been reported that amylase enzyme has been produced from *Aspergillus oryzae,* Pectinases and catalase enzymes has been produced from *Aspergillus niger,* Glucose oxidase enzyme from *Penicillium notatum* and Glucosidases enzyme from *Aspergillus flavus* (Vengadaramana, 2013).

Proteases are essential constituents of all form of life on earth including prokaryotes, fungi, plants and animals (Temam, 2017). They can be synthesized by plants, animals, and microorganisms constituting around 60% of the worldwide enzyme market (Nirmal *et al.,* 2011). However, proteolytic enzymes from microbial sources are preferred over the enzymes derived from plant and animals since they possess almost all characteristics desired for their biotechnological applications (Balakrishnan *et al.,* 2012). Hence microbial proteases represent one of the largest groups of industrial enzymes and account for approximately 60% of the total industrial enzyme sale in the world (Madhavi *et al.,* 2011; Temam, 2017).

Filamentous fungi have been utilized for the production of diverse industrial enzymes because these organisms exhibit the capacity to grow on solid substrates and secrete a wide range of hydrolyzing enzymes. Particularly, several species of *Aspergillus* have been exploited as important sources of extracellular enzymes including proteases (Li *et*

al., 2014). Products of *Aspergillus* sp. such as *Aspergillus niger*, *Aspergillus sojae*, and *Aspergillus oryzae* have acquired a Generally Recognized as Safe (GRAS) status from the US Food and Drug Administration, which has approved their use in the food industry (Heerd *et al.,* 2012). The production of proteases can be performed by Solid State Fermentation (SSF) and Submerged Fermentation (SmF) (Silva *et al.,* 2013). The application of SSF is of interest for fungi enzymes production due to its advantages in comparison to SmF, such as low fermentation technology, low cost, higher yields and concentration of the enzymes, and reduced waste output (Gastón *et al.,* 2016).

Some of the typical applications include enzyme use in the production of sweeteners, chocolate syrups, bakery products, alcoholic beverages, precooked cereals, infant foods, fish meal, cheese and dairy products, egg products, fruit juice, soft drinks, vegetable oil and puree, candy, spice and flavour extracts, and liquid coffee, as well as for dough conditioning, chill proofing of beer, flavour development, and meat tenderizing (Oyeleke *et al.*, 2011). The majority of currently used industrial enzymes are hydrolytic in action, being used for the degradation of various natural substances. Proteases remain the dominant enzyme type, because of their extensive use in the detergent and dairy industries (Neelam *et al.,* 2013). Various carbohydrases, primarily amylases and cellulases, used in industries such as the starch, textile, detergent, and baking industries, represent the second largest group of enzymes used in the industry (Neelam *et al.*, 2013). Enzymes also play a significant role in non-food applications. Industrial enzymes are used in laundry and dishwashing detergents, stonewashing jeans, pulp and paper manufacture, leather dehairing and tanning, de-sizing of textiles, deinking of paper, and degreasing of hides (Oyeleke *et al.*, 2011).

Metal oxide nanoparticles are being fundamentally used as a heterogeneous nanocatalyst in a variety of organic transformations as they contained high surface area than their bulk counterparts (Iravani, 2011; Kavitha *et al.,* 2013). Nowadays, metal oxide nanoparticles are attracting noteworthy interest as they can change the viable unconventional to conventional materials in various fields of solid state. Aluminium oxide is a compound of aluminium and oxygen with chemical formula of Al_2O_3. It is the most commonly occurring of several aluminium oxides, and specifically identified as aluminium (III) oxide. It is commonly called alumina. The oxides of aluminium materials are widely used in ceramics, refractories and abrasives due to their hardness, chemical inertness, high melting point, non-volatility and resistance to oxidation and corrosion (Tavakoli *et al.,* 2013; Malik *et al.,* 2014; Vadlapudi *et al.,* 2014). Predominantly, aluminum oxide nanoparticles (NPs) commonly known as alumina (Al_2O_3NPs) have trapped the awareness of many researchers due to its great catalytical activities. An alumina nanoparticle can be synthesized by using many techniques including ball milling, spray combustion, hydrothermal, sputtering, sol-gel, microwave and laser ablation (Rogojan *et al.,* 2011). Out of all the methods, hydrothermal method proved more helpful to obtain well shaped materials with designed texture and composition at low processing temperatures (Duraisamy, 2018). In general, alumina has many interesting properties such as catalyst, as high stability and hardness insulation, surface protective coatings, as composite materials with tunable mechanical properties (Duraisamy, 2018).

1.2 Statement of the Research Problem

The use of chemicals around the globe in different industries has increased tremendously, these chemicals has small but cumulative effect on the health of the people. This lead to the replacement of these toxic chemicals with environmental

friendly products and proteases are successfully considered as an alternative to chemicals and eco-friendly indicator, hence the necessity for this research.

1.3 Aim and Objectives of the Study

The aim of the study is to apply aluminum oxide nanoparticle in the cultivation of *Aspergillus* species for the production of protease enzyme.

i. To isolate the selected *Aspergillus* sp. (*Aspergillus niger*, *Aspergillus flavus* and *Aspergillus terreus*) from the soil.
ii. To synthesize aluminum oxide nanoparticle from *Cymbopogan citratus* (Lemongrass) leaf extract;
iii. To enhance the growth of the isolated aspergillus species using the aluminum oxide nanoparticle; and
iv. To produce protease enzyme using the enhanced *Aspergillus* species.

1.4 Justification of the Study

Proteases are being used as environmental friendly alternative in manufacturing industries and the selected *Aspergillus* sp. have been recorded to be excellent producers of proteases industrially. Microbial proteases serve as a better source of proteases than plants and animal sources because they can be cultured in large amounts in a short period, they are relatively cheap and there is continuous production of the desired products. This research focused on enhancing the morphology of the selected *Aspergillus* sp. with aluminum oxide nanoparticles to improve the protease enzyme yield of the organisms selected.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Enzyme

Enzymes are nature's sustainable catalysts. They are biocompatible, biodegradable and are derived from renewable resources (Sheldon and van Pelt, 2013). Enzymes constitute a large biological globular protein molecule responsible for thousands of metabolic processes that sustain life (Smith, 2000), and function as catalysts to facilitate specific chemical reactions within the cell. These reactions are essential for the life of the organism. The living cell is the site of tremendous biochemical activity called metabolism. This is the process of chemical and physical changes which go on continually in the living organism; enzymes facilitate life processes in essentially all life-forms from viruses to man.

Enzymes have been naturally tailored to perform under different physiological conditions. Build-up of new tissues (Lutolf *et al*., 2003), replacement of old tissues (Mizushima and Komatsu, 2011), conversion of food into energy (Van Beek *et al*., 2011), disposal of toxic materials (YalcinCakir *et al*., 2015), reproduction (Gerard *et al*., 2004) almost all the activities that can be characterized as "life." Enzymes act as life catalysts, substances that accelerate the rate of a chemical reaction. By reducing the activation energy (Ea) necessary to initiate the reaction, thus dramatically increasing the rate of reaction. Enzymes do not initiate reactions that would not naturally occur but they accelerate any reaction that is already underway. Enzymes enable the reaction to take place more rapidly at a safer, relatively low temperature that is consistent with living systems. During an enzyme-mediated reaction, the substrate physically attaches to the enzyme at its active site, allowing the substrate(s) to be converted to new product molecule(s).

Most enzyme reaction rates are millions of times faster than those of comparable uncatalyzed reactions. Enzymes are neither consumed by the reactions they catalyze, nor do they alter the equilibrium of these reactions. Enzymes are known to catalyze about 4,000 biochemical reactions (Schnell *et al*., 2006).

2.2 History of Evolution of Enzymes

The existence of enzymes has been known for well over a century. Biological catalysis was first recognized and described in the early 1800s, in studies of the digestion of meat by secretions of the stomach and the conversion of starch into sugar by saliva and various plant extracts (Binod *et al*., 2013). In 1835, Swedish chemist Jon Jakob Berzelius termed their chemical action as catalytic in nature. In 1860 Louis Pasteur recognized that enzymes were essential to fermentation but assumed that their catalytic action was inextricably linked with the structure and life of the yeast cell. Not until 1897 was it shown by German chemist Edward Buchner that cell-free extracts of yeast could ferment sugars to alcohol and carbon dioxide, Buchner denoted his preparation as zymase. The term enzyme comes from zymosis, the Greek word for fermentation, a process accomplished by yeast cells and long known to the brewing industry (Jones *et al.,* 1986). In 1876, William Kuhne proposed that the name 'enzyme' be used as the new term to denote phenomena previously known as 'unorganised ferments', that is, ferments isolated from the viable organisms in which they were formed. The word itself means 'in yeast' and is derived from the Greek 'en' meaning 'in', and 'zyme' meaning 'yeast' or 'leaven'. This important achievement was the first indication that enzymes could function independently of the cell. It was not until 1926, however, that the first enzyme was obtained in pure form, a feat accomplished by American biochemist James B. Sumner of Cornell University. Sumner was able to isolate and crystallize the enzyme urease from the jack bean. His work was to earn him the 1947 Nobel Prize. John H.

Northrop and Wendell M. Stanley of the Rockefeller Institute for Medical Research shared the 1947 Nobel Prize with Sumner (Becker-Ritt *et al*., 2007). They discovered a complex procedure for isolating pepsin. This precipitation technique devised by Northrop and Stanley has been used to crystallize several enzymes (Gurung *et al*., 2013).

2.3 The Proteinaceous Nature of Enzymes

Enzymes are proteins and are nature's own biocatalyst and their function is determined by their complex structure. With the exception of a small group of catalytic RNA molecules, all enzymes are proteins which are made up of amino acids linked together by peptide bonds (Hawlader and Tareeq, 2014). By the early 1800s, the proteinaceous nature of enzymes had been recognized. Knowledge of the chemistry of proteins drew heavily on improving techniques and concepts of organic chemistry in the second half of the 1800s; it culminated in the peptide theory of protein structure, usually credited to Fischer and Hofmeister. However, methods that had permitted the separation and synthesis of small peptides were unequal to the task of purifying enzymes. Indeed, there was no consensus that enzymes were proteins. After isolation of a series of crystalline proteolytic enzymes beginning with pepsin by Northrop (1930), the proteinaceous nature of enzymes was established. They are high molecular weight compounds made up principally of chains of amino acids linked together by peptide bonds. Their catalytic activity depends on the integrity of their native protein conformation. If an enzyme is denatured or dissociated into its subunits, catalytic activity is usually lost. Thus the primary, secondary, tertiary, and quaternary structures of protein enzymes are essential to their catalytic activity (Seibert and Raushel, 2005).

2.4 Structure of Enzymes

Enzymes are proteins and, are agreeable to structural analysis by the methods of protein chemistry, molecular biology, and molecular biophysics. Like all proteins, enzymes are composed mainly of the 20 naturally occurring amino acids. The structures of enzymes can be elucidating by the physical methods such as Spectroscopic methods (Busenlehner and Armstrong, 2005), x-ray crystallography (Ogata *et al*., 2005), and more recently, multidimensional NMR methods (Chan *et al*., 2013). On the basis of arrangement of amino acids enzyme structure can be classified into following types,

2.4.1 Primary structure

The structure and reactivity of a protein are defined by the identity of the amino acids that make up its polypeptide chain, this amino acid sequence of the peptide chains is the primary structure of the enzyme (Alberts *et al.,* 2010).

2.4.2 Secondary structure

Secondary structure is due to the interaction of amino acids with each other in the same chain of protein. As a result, the protein chain can fold up on itself in two ways, namely α-helix or β-sheet resulting secondary structures (Fang *et al*., 2018).

2.4.3 Tertiary structure

The arrangement of secondary structure elements and amino acid side chain interactions that define the three-dimensional structure of the folded protein. So that specific contacts are made between amino acid side chains and between backbone groups. The resulting folded structure of the protein is referred to as its tertiary structure (Kuhlman and Bradley, 2019).

2.4.4 Quaternary structure and domains

Many enzymes consist of more than one polypeptide chain (or subunit) that aggregate to confer catalytic activity. In some enzymes the subunits are identical, in others they differ in sequence and structure. This description of subunit arrangement in such enzymes is called the quaternary structure. A typical enzyme is not an entity completely folded as a whole, but may consist of apparently autonomous or semiautonomous folding units called domains (Khandbahale *et al.,* 2019).

2.5 Sources of enzyme

Enzymes occur in all living organisms and catalyze biochemical reactions necessary to support life (Olempska-Beer *et al*., 2006). A wide array of enzymes is extracted from plant sources; they have many advantages including cost of production and stability of products (Hood, 2002). An ample range of sources are used for commercial enzyme production from a broad spectrum of plant species. Non-microbial sources provide a larger proportion of these, at the present time. Microbes are preferred to plants and animals as sources of enzymes because (Hasan *et al*., 2006):

i. They are generally cheaper to produce.
ii. Their enzyme contents are more predictable and controllable,
iii. Regular supply due to absence of seasonal fluctuations and rapid growth of microorganisms on inexpensive media.
iv. Plant and animal tissues contain more potentially harmful materials than microbes, including phenolic compounds (from plants), endogenous enzyme inhibitors and proteases.
v. Microbial enzymes are also more stable than their corresponding plant and animal enzymes and their production is more convenient and safer.

About fifty years ago, enzymes were being extracted strictly from animals like pig and cow from their pancreases (Vilček *et al*., 1994). Animal enzymes were multifold; they were not very stable at the low pH environment so that the enzyme product was destroyed before doing the job. To overcome this problem, plant enzymes were discovered, most important one is extraction of peroxidase from horseradish roots occurs on a relatively large scale because of the commercial uses of the enzyme (Veitch, 2004). Peroxidase can also be extracted from soybean, it is also having the common features with horseradish peroxidase (Ryan *et al*., 2006). Some plants like Cruciferous vegetables, including broccoli, cabbage, kale and collard and turnip greens and papaya are rich in catalase.

Wheat sprouts contain high levels of catalase (Marsili *et al*., 2004) and vegetarian sources of catalase include apricots, avocados, carrots (Gálvez *et al*., 2012). Catalase is also present in some microbes and bacteria (LeBlanc *et al*., 2011), *Aspergillus niger* culture also produces catalase enzyme (Sharma *et al*., 2012).

2.6 Proteolytic enzyme

Proteolytic enzymes catalyze the hydrolysis of the peptide bonds between amino acid residues of proteins. They are often referred to as proteases or peptidases. The International Union of Biochemistry and Molecular Biology, however, recommends the term peptidase (Theron and Divol, 2014). Proteases have been used for a long time for the benefit of humans. Hundreds of years ago humans learned to convert milk to curd by storing it in bags made of calf stomach. Rennet, whose main component is chymosin, a protease, was responsible for the conversion of milk to curd (Kumar *et al*., 2010). Cheese making is still considered a craft in non-industrial setups that relies on rennet for coagulating milk proteins.

With a growing concern over the reduction of the impact of industries on the environment, there is a visible shift toward less chemical-intensive processes. Enzymes have provided an eco-friendly alternative to harsh or toxic chemicals. The leather industry, for instance, has introduced alkaline proteases into the leather treatment process, which has successfully helped in reducing the pollution and water consumption in addition to improving leather quality (Rao *et al*., 1998). Enzymes as biological catalysts offer the advantages of high specificity and high catalytic activity over chemical catalysts. They also provide flexibility in terms of temperature of the reactions to be catalyzed, as enzymes of several kinds are available that are active at various temperatures. The proteolytic enzymes are used in the food, dairy, detergent, leather, and pharmaceutical industries and many others (Rao *et al*., 1998).

Most proteases are produced as a zymogen, the inactive form. Zymogens are activated by environmental cues or upon encountering their specific substrate, which may bring about a conformational change (Khan and James, 1998). Another strategy employed by nature is the use of sequences of up to 100 amino acid residues known as activation segments. The activation segments when cleaved allow the zymogen to function (Khan and James, 1998). Proteolytic enzymes are involved in crucial biological reactions occurring at the cellular to the organism level (Verdoes and Verhelst, 2016). At the cellular level proteolytic enzymes are involved in several pivotal biological reactions related but not limited to cell cycle, degradation of misfolded proteins, apoptosis, and mediation of immune responses. At the sub organism level their role has been highlighted in blood clotting and digestion of proteins in food. At the organism level proteases are recruited by viruses and pathogenic bacteria as well as insect pests to gain entry into their target host. Proteolytic enzymes also have an important ecological role in maintaining the nitrogen cycle. Proteases produced by microorganisms degrade the

dead organism and release the nitrogen assimilated in the form of proteins (Khan and James, 1998). This ability to play a wide array of roles may be attributed to the convergent evolution of myriad protein folds with active sites having similar structural platforms (Dhillon *et al.,* 2017). Proteolytic enzymes are ubiquitous because they are indispensable for any kind of living organism. They are produced by all organisms whether prokaryote or eukaryote (Rao *et al*., 1998).

Proteolytic enzymes are classified under the hydrolase category of enzymes. There are other criteria, also, by which the proteolytic enzymes are classified (Khan and James, 1998; Rao *et al*., 1998). Broadly, proteases may be grouped as exopeptidases or endopeptidases depending on the site of their action. Exopeptidases cleave peptide bonds near the termini of the polypeptide chains. Exopeptidases that cleave the bond from the N-terminus and produce a single amino acid residue or a di- or tripeptide are referred to as aminopeptidases. The exopeptidases that act on the C-terminus and produce either a single amino acid residue or dipeptides are referred to as carboxypeptidases. Endopeptidases cleave the bonds away from the termini. The proteolytic enzymes are classified according to the mechanism of action based on the catalytic amino acid residue involved, in four categories: (1) serine proteases, (2) aspartate proteases, (3) cysteine proteases, and (4) metalloproteases. A new class of threonine proteases was described in 2010 (Madala *et al*., 2011).

2.7 Sources of Proteolytic Enzymes

Proteolytic enzymes are produced by all life forms such as; plants, animals, and microorganisms. Proteolytic enzymes from all these sources have been used during some point or other in the course of human history. However, to meet the huge demand of industries, microorganisms have been the mainstay of the source of proteolytic enzymes. A high yield of proteolytic enzymes can be obtained by culturing

microorganisms, which grow in a short time and require less space compared to plant or animal sources.

2.7.1 Proteolytic enzymes from plants

Proteolytic enzymes in plants are involved in key physiological process like photoinhibition, photomorphogenesis in seeds, and senescence (Estelle, 2001). Cysteine proteases derived from plant sources, such as papain and bromelain from papaya and pineapple, respectively, have been used as meat tenderizers as well in baking and production of protein hydrolysates (Uhlig, 1998). The number of plant proteases used in industry is not very high, since 2005 proteolytic enzymes from plant sources have come into special focus for their therapeutic applications, as many of them are active over a wide range of temperature and pH (Dubey and Jagannadham, 2003).

2.7.2. Proteolytic enzymes from animals

Pancreatic trypsin, chymotrypsin, pepsin, and chymosin (rennet) are the most important industrial proteases of animal origin. Chymotrypsin from pancreatic juice is used for diagnostic purposes (Rao *et al*., 1998). These enzymes were being obtained from slaughtered cattle; however, to meet the demands from industry, recombinant versions are being produced and tested for efficacy. Trypsin is a serine protease and it is used in preparing medium for growing bacteria for research and industrial purpose (Rao *et al*., 1998). The performance of animal derived trypsin compared to recombinant trypsin for use in clinical applications was similar (Manira *et al*., 2014).

2.7.3 Proteolytic enzymes from microbial sources

Most microbial proteases are extracellular and thus are directly secreted into the fermentation broth, which makes the downstream processing easier to obtain the pure enzymes in bulk quantities compared with the proteases obtained from plants and

animal sources. The most widely used microbial proteolytic enzymes come from bacterial and fungal sources. Proteolytic enzyme-producing bacteria as well as fungi have been isolated from various sources like mangrove sediments, seawater, offshore oil fields, poultry compost, and industrial effluents (Savitha *et al*., 2011; Habbeche *et al*., 2014; Raval *et al*., 2014; Elhoul *et al*., 2015). The submerged fermentation technology was developed in the 1940s for bacteria and fungus (Vojcic *et al*., 2015). A decade later it provided an alternative for sourcing enzymes like rennet that were expensive and limited by the availability of slaughtered cattle. Bacterial proteolytic enzymes are generally active in neutral or alkaline pH. Members of the Bacillus and Streptomyces genera are the most commonly employed at the industrial level. Fungi, on the other hand, are versatile in enzyme production and produce acidic, neutral, and alkaline proteases. Bacterial neutral proteases are active in the pH range 5-8 and have low thermo stability compared to the bacterial alkaline proteases. Neutrase is a neutral proteolytic enzyme used in the food industry for protein hydrolysate production (Ou *et al*., 2010). Alkaline proteases from bacteria show optimal activity at alkaline pH and are quite thermostable, for example, proteases produced by *Bacillus licheniformis* and *Staphylothermus marinus* (Ellaiah *et al*., 2002). The bacterial proteases compared to fungal proteases possess higher thermostability and also higher reaction rate, except for enzyme produced by the thermophilic fungus *Malbranchea pulchella* (Vojcic, 2015).

Alkaline proteases have the largest market share as they are produced by the detergent and leather industries, which require these enzymes in huge amounts (Vojcic, 2015). Bacteria from the Bacillus genus have been a predominant source of alkaline proteases. Almost all proteases used in detergent formulations are subtilisins produced by *Bacillus* spp. (Vojcic, 2015). A new subtilisin-related recombinant proteolytic enzyme from a

fungus, *Malbranchea* sp., and produced by *Trichoderma reesei* has been introduced in detergent formulations (Vojcic, 2015).

Proteolytic enzymes from Bacillus and *Aspergillus* have replaced animal feces as a source of proteases in the leather-processing industry (Rao *et al*., 1998). Because of high demands for rennet in the dairy industry alternate sources for chymosin have been explored. Proteases from *Mucor michei, Bacillus subtilis*, and *Endothiaparasitica* are now used for cheese production by the dairy industry. A proteolytic enzyme from *Aspergillus oryzae* is used in the modification of wheat gluten (Rao *et al*., 1998). An extracellular solvent-stable alkaline metalloprotease from *Pseudomonas aeruginosa* has been characterized as having the potential for synthesis of enzymatic peptides (Jaouadi *et al*., 2013). Leucine aminopeptidases are being used in the production of protein hydrolysates. *Streptomyces aminopeptidases* have garnered interest for protein hydrolysate production in the food industry because of their stability, high enzyme activity, and broad substrate specificity (Rahulan *et al*., 2012).

2.8 Production of Proteolytic Enzymes

Enzyme-mediated processes are economically comparable to chemical process nowadays. Therefore, the reduction in the cost of enzyme production is a positive stimulus for the commercialization of enzyme-based processes. Proteolytic enzymes are one of the most important groups of industrial enzymes and account for nearly 60% of total enzyme sales (Turk, 2006). According to a global food enzymes market report, it is expected that the global food enzyme market will reach US$2.3 billion by 2018, and North America is expected to lead the market, followed by Europe and the Asiane Pacific countries (Vijayaraghavan *et al*., 2014).

Proteolytic enzymes find applications in industrial processes such as detergent, textile, leather tanning, dairy, pharmaceutical preparation, cosmetics, peptide synthesis, and

photography (Gupta *et al*., 2002). Proteolytic enzymes can be produced by plants, animals, and microorganisms. The inability of the plant and animal proteolytic enzymes to meet current world demands has led to an increased interest in microbial sources. The relative ease of genetic manipulation and biodiversity of microorganisms make them a highly used source of proteolytic enzymes. Microbial proteases account for 40% of the total worldwide enzyme sales (Turk, 2006). Microbial proteolytic enzyme production depends on the microorganism, medium composition, physicochemical properties, and the method of production. It is estimated that around 30-40% of the cost of production of industrial enzymes can be attributed to the cost of the growth medium (Joo *et al*., 2003).

Selection of the microorganism is important to obtain the desired product. The microorganism should be able to secrete large amounts of proteolytic enzymes and give adequate yields in a short time period. The production of proteolytic enzymes is also affected by the medium components such as carbon and nitrogen sources and supplementation of mineral salts. Easily metabolizable carbon sources such as maltose, starch, molasses, wheat bran, and coffee pulp and coffee husk enhance the production of proteolytic enzymes (Pandey *et al*., 2000). Corn steep liquor, soybean meal, fish meal, and yeast extract enhance the production of proteolytic enzymes, whereas free amino acids decrease the production (Blieva *et al*., 2003). Proteolytic enzyme production is also affected by the physicochemical properties such as pH, temperature, moisture content, incubation period, inoculation size, and aeration (Puri *et al*., 2002). Proteolytic enzyme production is enhanced by vitamins such as biotin and growth promoters such as 1-naphthylacetic acid (Tunga *et al*., 2001). Microbial proteolytic enzymes are produced by fermentation methods. The success of fermentation depends upon the

usage of low-cost raw materials, enzyme productivity, and ease of product recovery from the fermentation broth.

2.8.1 Solid-state fermentation

Solid-state fermentation (SSF) utilizes a solid substrate in the free form and the absence of liquid for the growth of microorganisms. Water is either adsorbed on a solid support or complexed with a solid matrix for the growth of the microorganisms. SSF is considered more natural than other types of fermentation, because it has conditions similar to those under which most microorganisms grow in nature. SSF has simple fermentation equipment and results in high volumetric productivity, relatively high concentration of product, less effluent generation, and relatively easy downstream processing (Renge *et al*., 2012).

Higher production of proteolytic enzyme can be achieved by using the best combination of medium components, such as carbon and nitrogen sources, metal ions, and surfactants, as well as by using optimized physicochemical properties such as pH, temperature, agitation, aeration, and inoculum size. Agro-industrial waste products such as wheat bran, rice bran, maize bran, gram bran, wheat straw, rice straw, rice husk, sawdust, corncobs, tea waste, aspen pulp, sugar beet pulp, peanut meal, groundnut oilcake, and mustard oil cake are generally used as the substrates for the SSF process for the production of proteolytic enzymes (Ramachandran *et al*., 2007).

2.8.2 Submerged fermentation

Submerged fermentation (SmF) uses a liquid fermentation medium with soluble nutrients. The substrate is dissolved or suspended in water, which is not a limiting factor. This fermentation process can be performed in shake flasks, a bench-scale fermenter, or an industrial-scale fermenter. In this type of fermentation, microorganisms

are able to utilize a variety of carbon sources such as maltose, corn starch. An industrial by product of the sugar industry, i.e., molasses, has been utilized extensively as a raw material for the carbon source. In addition, glucose is used as the main carbon source for the production of extracellular proteolytic enzymes in SmF with *Mucor mucedo* (Yegin *et al*., 2010).

Higher proteolytic enzyme production was achieved in medium supplemented with peptone, followed by beef extract, casein, yeast extract, tryptone, and $NaNO_3$, with soybean meal as the organic nitrogen source (Narayana and Vijayalakshmi, 2008). Scale-up studies of proteolytic enzyme production from *Serratia marcescens* grown on fresh whey were performed (Laca *et al*., 2008). Peanut meal was the best nitrogen source for proteolytic enzyme production compared to casein, peptone, and skim-milk powder (Sinha and Sinha, 2009).

2.8.3 Immobilized cell technology

Immobilization of microbial cells has become popular in the field of proteolytic enzyme production. Immobilized cell technology is often used to improve the bioprocess to get an enhanced yield of proteolytic enzymes as well as their long-term use and stability. Several natural and synthetic polymer matrices such as alginate, carrageenan, cellulose, agar, agarose, gelatin collagen, and polyacrylamide have been reported for immobilization of various microbe. Calcium alginate and polyacrylamide were found to be the best matrices for production of proteolytic enzymes (Kumar and Vats, 2010; Shivasharana *et al*., 2012). Gelatin and radiated gelatin also manifested better results compared with calcium alginate (El-Hadedy *et al*., 2014; Free, 2014; Chatterjee, 2015).

2.8.4 Industrial production scenario

The global market for technical enzymes, according to a report by Business Communications Company Research, which are usually used in bulk quantities, was estimated to be US$1 billion in 2010; for 2015 it has been estimated at US$1.5 billion. The leather industry, which uses large quantities of proteolytic enzymes, is the major consumer of industrial enzymes. Otto Ro¨hm is credited for using enzymes for the first time in industry.

He introduced enzymes from the pancreas of slaughtered animals to replace dog feces as a source of proteolytic enzymes in the leather industry. Some of the major companies that produce proteases are Novozymes, DSM, and DuPont Industrial Biosciences (Vojcic, 2015). The detergent industry is the largest consumer of proteases and the market for detergent enzymes is estimated at approximately V700 million (Vojcic, 2015).

Microbial production of the following enzymes by solid state fermentation and submerged fermentation is an ongoing research in Nigeria: Proteases, Xylanases, Amylase, Glucoamylases, Glucose Isomerase, Pectinases, Cellulases, Lipases, Lacases.

By the year 2025, the Enzyme Technology Division hopes to perfect the technology of microbial production of most Industrial enzyme needs of Industries in Nigeria in order to reduce importation, save foreign reserves and support the actualization of sustainable development goals (Ametefe *et al*., 2021)

2.9 Purification and Characterization of Proteolytic Enzymes

Proteolytic enzyme purification processes may vary from single-step purification to multistep procedures. The enzyme purification strategy is based on information about the level of purity required, source of enzyme, properties of the target enzymes, and accompanying impurities. It is also important to determine the scale of purification. The

first step toward obtaining pure enzyme is the isolation from the source, which is generally microbial cells, a plant, or an animal tissue. Most of the industrial proteases are now obtained from microbial sources. The extracellular proteases are easy to separate from the cells by filtration or centrifugation (Elhoul *et al*., 2015). The retentate obtained after filtration or the culture supernatant obtained after centrifugation is concentrated by ultrafiltration (Vidyasagar *et al*., 2007). Various properties of enzymes like solubility, charge, size, and binding affinity have been exploited at various stages of purification. Ammonium sulfate at high concentrations has been widely used to purify active forms of proteases by reducing the solubility, resulting in precipitation. Different proteins precipitate at different concentrations of salt; therefore, by making a concentration gradient of ammonium sulfate several fractions containing different proteins can be obtained (Barredo, 2005). A detergent-stable alkaline protease from *Streptomyces koyangensis* has been purified by using this method (Elhoul *et al*., 2015). Purification has also been done by ethanol-induced precipitation (Vidyasagar *et al.*, 2007). A thermostable alkaline protease produced by *Aspergillus niger* has been purified by this method (Coral *et al*., 2003). The precipitated protease is recovered by centrifugation followed by dialysis to remove the salt or solvent. Aqueous two-phase systems (ATPSs) have also been used for purification of proteases. ATPSs comprise two water soluble polymers or one polymer and salt (Amid *et al*., 2012). Sodium polyacrylate and polyethyleneglycol-based ATPS has been used for purification of proteases expressed by *Penicillium restrictum* (Barros *et al*., 2014). A modified organic solvent/salt-based ATPS using alcohol and salt combinations was used for purification of serine proteases from mango (Amid *et al*., 2012). This method has several advantages including low cost, low toxicity, and easy recovery of alcohol compared to polymer-based ATPS. Precipitated proteases are recovered by centrifugation followed by dialysis

to remove the salt. Intracellular proteases are isolated from the host cells by rupturing the cells by using a French press (Qoura *et al*., 2015). The cell extract is subjected to centrifugation to obtain a clarified cell-free extract.

The second step of purification, to remove most of the impurities from the isolated enzyme, is a chromatography technique applied based on charge, size, or binding affinity. Gel filtration efficiently separates the molecules based on size. The dialyzed enzyme obtained after precipitation is loaded onto a pre-equilibrated gel-filtration column. During elution small proteins are retarded to a greater extent compared to the larger proteins, which elute in the initial fractions. The fractions that contain the protease are pooled and concentrated. A fungal proteolytic enzyme present in the culture filtrate was precipitated by using ethanol and purified by gel filtration using Sephadex G-100 (Savitha *et al*., 2011)

The ion-exchange chromatography technique utilizes the charge on the protein to bind to the column matrix. The proteins are eluted by varying the pH, which may change the surface charge depending on the pI, or by increasing the ionic strength of the elution buffer to enhance the affinity of proteins toward the mobile phase (Elhoul *et al*., 2015; Qoura *et al*., 2015). The dialyzed filtrate obtained from filtration of the fermentation broth or the cell-free extract can be directly loaded onto an ion-exchange chromatography column. An alkaline protease was purified by a cation-exchange Mono-S Sepharose column by using a concentration gradient (0-500 mM) of NaCl (Elhoul *et al*., 2015). Characterization of proteolytic enzymes, or any enzyme for that matter, is necessary to determine the range of applications for which they could be utilized and to eliminate the conditions that could inhibit the enzyme reaction.

2.10 Properties of Proteolytic Enzymes

2.10.1 Optimum pH and temperature for proteolytic activity and enzyme stability

The optimum pH of a proteolytic enzyme may be determined by assaying the activity using various buffers over a range of pH, say, 4-12. The pH stability may be determined by incubating the enzyme in buffers of different pH and withdrawing aliquots at regular intervals to test the residual activity (Qoura *et al*., 2015). The activity of a serine protease from a haloalkalophilic bacterium tested in the pH range 8-12 showed not much variation in the pH range 8-11, though it rapidly dropped at a pH below 8 or above 11 (Raval *et al*., 2014). The enzyme retained its activity for 24 h in pH 8-11 without much loss. The optimum temperature for proteolytic activity is determined by assaying the enzyme at varying temperatures from 10 to 100°C. To determine the thermostability, the enzyme is incubated at various temperatures in a range 10-100°C for certain time periods and the aliquots are withdrawn to measure the residual activity. The activity of a serine protease from *Shewanella arctica* was tested in the temperature range 0 to 100°C (Qoura *et al*., 2015). Despite it being derived from a psychrophilic bacterium, the enzyme was active over a broad range of temperature from 0 to 80°C with a half life of 45 h at 40°C.

2.11 Nanoparticles

Nanoparticles (NPs) and nanostructured materials (NSMs) represent an active area of research and a techno-economic sector with full expansion in many application domains. NPs and NSMs have gained prominence in technological advancements due to their tunable physicochemical characteristics such as melting point, wettability, electrical and thermal conductivity, catalytic activity, light absorption and scattering resulting in enhanced performance over their bulk counterparts. Ananometer (nm) is an International System of Units (Système international d'unités, SI) unit that represents

10−9 meter in length. In principle, nanomaterials (NMs) are described as materials with length of 1–1000 nm in at least one dimension; however, they are commonly defined to be of diameter in the range of 1 to 100nm. Today, there are several pieces of legislation in the European Union (EU) and USA with specific references to NMs. However, a single internationally accepted definition for NMs does not exist. Different organizations have a difference in opinion in defining NMs (Boverhof *et al*., 2015). According to the Environmental Protection Agency (EPA), NMs can exhibit unique properties dissimilarthan the equivalent chemical compound in a larger dimension (Abbass *et al.,* 2019). The US Food and Drug Administration (USFDA) also refers to NMs as "materials that have at least one dimension in the range of approximately 1 to 100 nm and exhibit dimension dependent phenomena" (FDA, 2011). Similarly, The International Organization for Standardization (ISO) has described NMs as a "material with any external nanoscale dimension or having internal nanoscale surface structure". Nanofibers, nanoplates, nanowires, quantum dots and other related terms have been defined based on this ISO definition (Bleeker *et al*., 2012). Likewise, the term nanomaterial is described as "a manufactured or natural material that possesses unbound, aggregated or agglomerated particles where external dimensions are between 1–100nm size range", according to the EU Commission.

2.11.1 Types of nanomaterials

Most current NPs and NSMs can be organized into four material-based categories (the references refer to recent reviews on these different categories of NMs).

i. **Carbon-based nanomaterials:** generally, these NMs contain carbon, and are found in morphologies such as hollow tubes, ellipsoids or spheres. Fullerenes (C60), carbon nanotubes (CNTs), carbon nanofibers, carbon black, graphene (Gr), and carbon onions are included under the carbon-based NMs category. Laser

ablation, arc discharge, and chemical vapor deposition (CVD) are the important production methods for these carbon-based materials fabrication (except carbon black) (Kumar and Kumbhat, 2016).

ii. **Inorganic-based nanomaterials:** these NMs include metal and metal oxide NPs and NSMs. These NMs can be synthesized into metals such as Au or Ag NPs, metal oxides such asTiO_2 and ZnO NPs, and semiconductors such as silicon and ceramics (Sannino, 2021).

iii. **Organic-based nanomaterials:** these include NMs made mostly from organic matter, excluding carbon-based or inorganic-based NMs. The utilization of noncovalent (weak) interactions for the self-assembly and design of molecules helps to transform the organic NMs into desired structures such as dendrimers, micelles, liposomes and polymer NPs (Awual *et al.*,2017).

iv. **Composite-based nanomaterials:** composite NMs are multiphase NPs and NSMs with one phase on the nanoscale dimension that can either combine NPs with other NPs or NPs combined with larger or with bulk-type materials (e.g., hybrid nanofibers) or more complicated structures, such as metal organic frameworks. The composites may be any combinations of carbon-based, metal-based, or organic-based NMs with any form of metal, ceramic, or polymer bulk materials (Ionov *et al*., 2021).

v. **Polysaccharide based nanomaterials**: The use of polysaccharide-based nanoparticles are additionally advantageous as they are found virtually in all living organisms and their application does not exhibit toxic effects to the organisms owing to their intrinsic biocompatibility and biodegradability that ensure safe therapies. These nanoparticles are commonly fabricated through ionotropic gelation and self-assembly of polyelectrolytes, a relatively simple procedure that does not

require the use of organic solvents and operated in mild temperature and pressure condition. They have good stability, safe, non-explosive and naturally available compared to metal nanoparticles (Salatin *et al.,* 2017; Saallah and Lenggoro, 2018).

2.11.2 Sources of nanomaterials

Sources of nanomaterials can be classified into three main categories based on their origin:

(i) Incidental nanomaterials, which are produced incidentally as a byproduct of industrial processes such as nanoparticles produced from vehicle engine exhaust, welding fumes, combustion processes and even some natural process such as forest fires; (Buzea *et al*., 2007)

(ii) Engineered nanomaterials, which have been manufactured by humans to have certain required properties for desired applications (Jeevanandam *et al.,* 2018) and

(iii) Naturally produced nanomaterials, which can be found in the bodies of organisms, insects, plants, animals and human bodies. However, the distinctions between naturally occurring, incidental, and manufactured NPs are often blurred. In some cases, for example, incidental NMs can be considered as a subcategory of natural NMs (Khan, 2020).

2.12 Methods of Synthesizing Nanomaterials

In generally synthesis of nanomaterial's can be classified two types, 1. Bottom-up approach 2.Top-down approach. In bottom-up approaches include the miniaturization of materials components (up to atomic level) with further self-assembly process leading to the formation of nanostructures (Aeila *et al.,* 2019). During self-assembly the physical forces operating at Nano scale are used to combine units into larger stable structures.

Ex: quantum dot formation during of nanoparticles from colloidal dispersion. In top-down approaches use larger initial structures, which can be externally controlled in the processing of nanostructures. Ex: ball milling and plasticized formation (Rajput, 2015). Various methods of synthesizing nanaomaterials is presented in figure 2.1

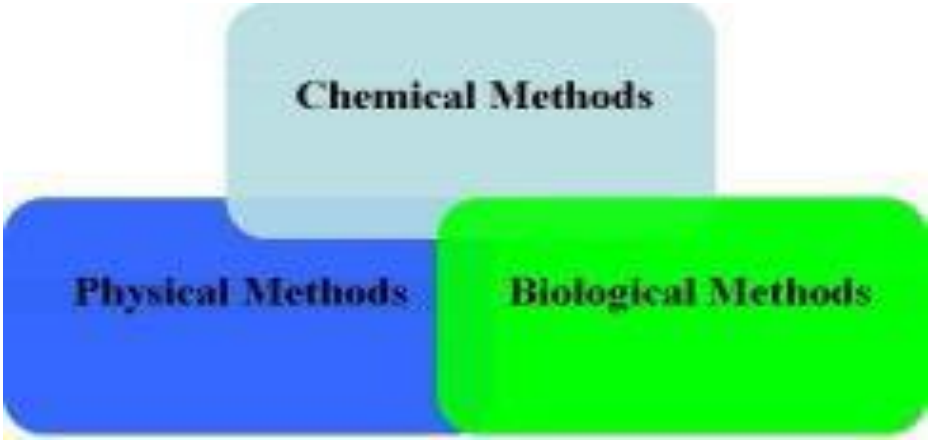

Figure 2.1: Methods of Synthesizing Nanomaterials **(**Patra *et al*., 2014**).**

2.12.1 Chemical methods

2.12.1.1 ***Chemical reduction method:*** In 1857, Michael Faraday, for the first time reported a systematic study of the synthesis and colors of colloidal gold using chemical reduction route. The chemical reduction of copper salts is the easiest, simplest and the most commonly used synthetic method for copper nanoparticles. In fact, the production of nano sized metal copper particles with good control of morphologies and sizes using chemical reduction of copper salts can be achieved (Ghorbani, 2014).

2.12.1.2 ***Micro emulsion/colloidal method:*** Hirai *et al.* (1992) observed that an appropriate amount of water, oil, surfactant and an alcohol- or amine-based co-surfactant produced clear and homogeneous solutions that Hirai called microemulsion. Microemulsion is a technique for the synthesis of nanoparticles in which two immiscible fluids such as water in oil (W/O) or oil in water (O/W) or water in supercritical carbon dioxide (W/Sc. CO_2) become a thermodynamically stable

dispersion with the aid of a surfactant. A typical emulsion is a single phase of three components, water, oil and a surfactant. Normally oil and water are immiscible but with the addition of a surfactant, the oil and water become miscible because the surfactant is able to bridge the interfacial tension between the two fluids. Microemulsion consists of surfactant aggregates that are in the ranges of 1nm to100nm. The location of water, oil and surfactant phases affects the geometry of aggregate. The micro-emulsion is said to be oil in water (O/W) if water is the bulk fluid and oil is in less quantity, with small amounts of surfactant. Similarly, the system is said to be water in oil (W/O), if oil is the bulk fluid and water is present in less quantity. The product of oil in water and surfactant (O/W) is called micelles, which is an aggregate formed to reduce free energy. Hydrophobic surfactants in nanoscale oil and micelles point towards the center of aggregate, whereas the hydrophobic head groups towards water, the bulk solvent. The water in oil microemulsion carries oil or organic solvent as bulk. The system is thermo dynamically stable and called reverse micelles (Ghorbani, 2014).

2.12.1.3 ***Sonochemical method:*** in the Sonochemical process, powerful ultrasound radiations (20kHz to 10MHz) were applied to molecules to enhance the chemical reaction. Acoustic cavitation is a physical phenomenon which is responsible for Sonochemical reaction. This method, initially proposed for the synthesis of iron nanoparticles, nowadays used to synthesize different metals and metal oxides. The main advantages of the Sonochemical method are its simplicity, operating conditions (ambient conditions) and easy control of the size of nanoparticles by using precursors with different concentrations in the solution. Ultrasound power affects the occurring chemical changes due to the cavitation phenomena involving the formation, growth and collapse of bubbles in liquid. The sonolysis technique involves passing sound waves of fixed frequency through a slurry or solution of carefully selected metal complex

precursors. In a solvent with vapor pressure of a certain threshold, the alternating waves of expansion and compression cause cavities to form, grow and implode.

Sonochemical reactions of volatile organometallics have been exploited as a general approach to the synthesis of various nano phase materials by changing the reaction medium. There are many theories presented by different researchers that have been developed to explain the mechanism of breakup of the chemical bond under 20KHz ultrasonic radiations. They have explained the sonochemistry process in these theories i.e., how bubble creation, growth and its collapse is formed in the liquid. One of these theories explains the mechanism of breaking of a chemical bond during a bubble collapse. According to one of these theories, bubble collapse occurs at very high temperatures (5000K-25000K) during the sonochemical process. Upon the collapse of the bubble, which occurs in less than a nanosecond, the system undergoes a very high cooling rate K/Sec. The organization and crystallization of nano -particles is hindered by this high cooling rate. The creation of amorphous particles is welled fined while the nanostructured particles are not clear. The reaction will occur in a 200nmring surrounding the collapsing bubbles if the precursor is a nonvolatile compound. The temperature of the bulk is lower compared to the ring, and temperature of collapsing bubble will be higher than the temperature of the ring. Sonoelectro chemical synthesis employs both electrolytes and ultra -sonic pulses for the production of nanoparticles (Satyanarayana and Reddy, 2018).

2.12.2 Physical methods

2.12.2.1 ***Pulse laser ablation:*** in this technique, a high-power pulsed laser beam is focused inside a vacuum chamber to strike a target in the material and plasma is created, which is then converted into a colloidal solution of nanoparticles. Mostly Second

Harmonic Generation (ND: YAG) type Laser is being used to prepare the nanoparticles. There are many factors that affect the final product such as the type of laser, number of pulses, pulsing time and type of solvent (Satyanarayana and Reddy, 2018).

2.12.2.2 ***Mechanical/High ball milling method:*** milling is a solid-state processing technique for the synthesis of nanoparticles. This technique was first used by Benjamin for the production of superalloys. In the milling process, raw material of micron size is fed to undergo several changes (Figure 2.2). Different types of mechanical mills are available which are commonly used for the synthesis of nanoparticles. These mills are categorized according to their capacities and applications. Due to mechanical limitations, it is very difficult to produce ultra-fine particles using these techniques or it takes very long time. However, simple operation, low cost of production of nanoparticles and the possibility to scale it to produce large quantities are the main advantages of mechanical milling. The important factors affecting the quality of the final product are the type of mill, milling speed, container, time, temperature, atmosphere, size and size distribution of the grinding medium, process control agent, weight ratio of ball to powder and extent of filling the vial (Satyanarayana and Reddy, 2018).

2.12.2.3 ***Mechanical chemical synthesis:*** In this process, chemical reaction is induced by mechanical energy. The chemical forerunners are mostly a mixture of chlorides, oxides and/or metals that react during milling or subsequent heat treatment to produce a composite powder in which ultrafine particles in a stable salt matrix are dispersed. These ultrafine particles are recovered by washing with suitable solvent from selective removal of the matrix (Aeila *et al.*, 2019).

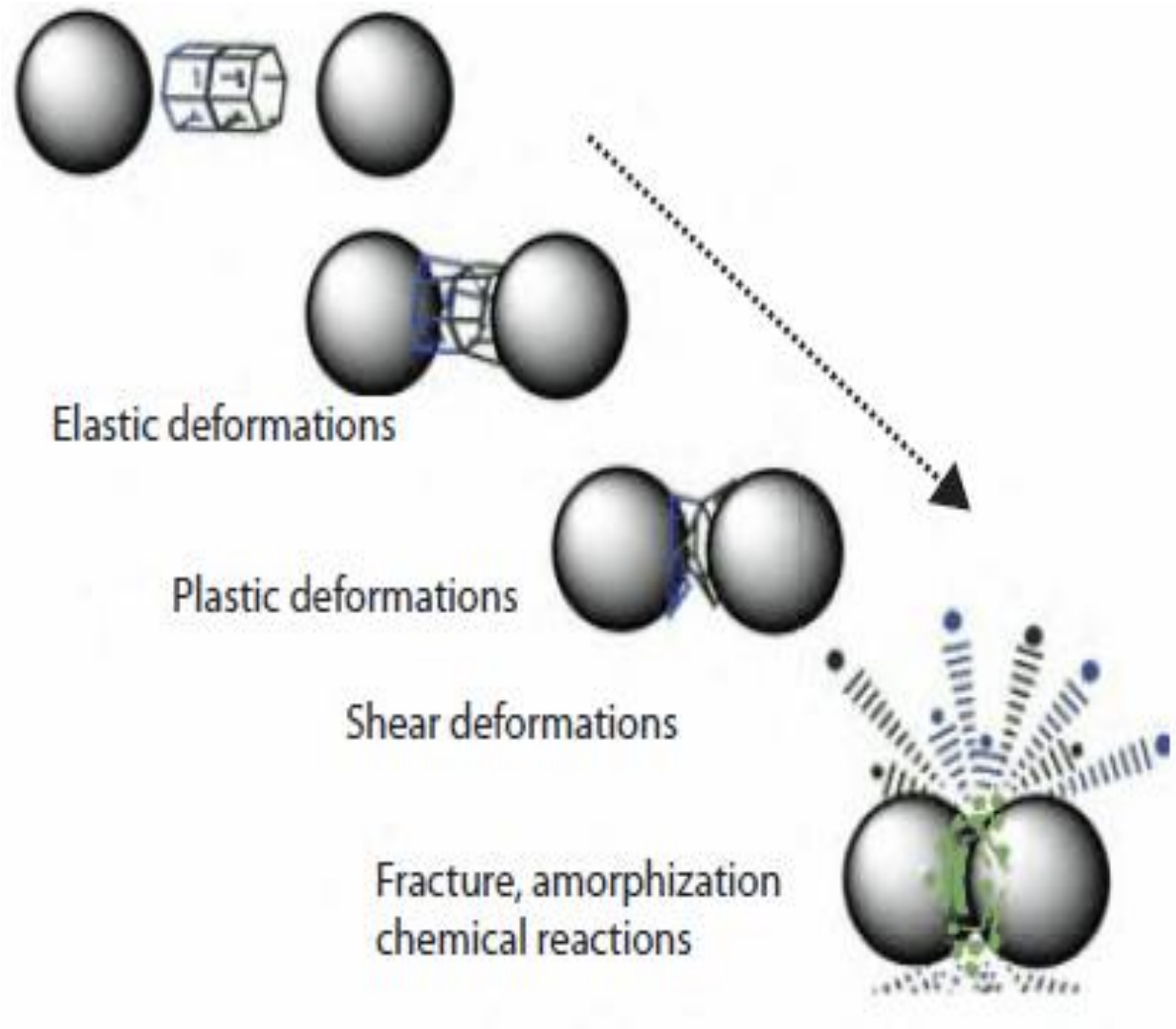

Figure 2.2: Synthesis of Nanoparticles using Ball milling method **(**Huot and Balema, 2012**).**

2.12.2.4 ***Pulsed wire discharge method*****:** Pulsed wire discharge (PWD) is a physical technique to prepare nanoparticles. Compared to all the other previously mentioned methods, metal nanoparticles synthesis by the PWD technique follows a completely different mechanism. In PWD, a metal wire is evaporated by a pulsed current to produce a vapor, which is then cooled by an ambient gas to form nanoparticles. Preparations of metal, oxide and nitride nanoparticles by PWD has been reported this method have potentially a high production rate and high energy efficiency. This process is not used

conventionally for common industrial purposes because it is not only very expensive but also impossible to use explicitly for different metals. It is mainly useful for those metals of high electrical conductivity that are easily available in the thin wire form (Satyanarayana and Reddy, 2018).

2.12.3 Biosynthesis of nanoparticles by microorganisms

It is recognized that microorganisms including bacteria, *Cyanobacteria*, *Actinomycetes*, yeast and fungi make inorganic nanoparticles such as gold, silver, calcium, silicon, iron, gypsum and lead. Because of their intrinsic potential, they produce nanoparticles, which are intra and/or extracellularly in nature (Asmathunisha and Kathiresan, 2013). However, due to extra processing phases like ultra-sonication and therapy with proper detergents, it is hard to extract the nanoparticles produced through intracellular biosynthesis (Sharma *et al*., 2007). As a result, screening of the microorganisms resulting in biosynthesis of nanoparticle extracellularly is necessary (Huang *et al*., 2009). Currently, microbial approaches in the production of nanomaterials of variable compounds are mostly restricted to metals, a few metal sulfide, and very little oxides. All of them are confined to the microorganisms of earthy source. Culture conditions determine the biological synthesis of nanoparticles through the utilization of microorganisms and consequently, it is necessary to standardize these circumstances for the production of nanoparticles in a large scale. While strict inspection over form, size, and combination of the particles is exercised, it is recognized that many microorganisms can produce metallic nanoparticles having characteristic features similar to nanomaterial's which are synthesized chemically (MubarakAli *et al*., 2012). It is hoped that by means of hydrolytic activity of the microorganisms, other metal oxides can also be formed. In conclusion, under moderate pressures and temperatures, nano-sized

materials can be produced by microorganisms. Moreover, it is inexpensive, undemanding, effective, energy-saving, and environment-friendly to make use of microbial procedure for the production of nanomaterials (Kathiresan *et al*., 2010).

2.12.4 Green synthesis

Green synthesis provides advancement over physical and chemical method as it is cost effective environment friendly, easily scaled up for large scale synthesis and in this method, there is no need to use high pressure, temperature, energy and toxic chemical (Malabadi *et al.,* 2012).

Various processes for the synthesis of nano and micro length scaled inorganic materials which have contributed to the development of relatively new and largely unexplored area of research based on the biosynthesis of nanoparticles (Salam *et al.,* 2012). Green synthesis of nanoparticles makes use of environmentally friendly non-toxic and safe reagent. Phytomining is the uses of hyper accumulating plants to extract a metal from the biomass to return an economic profit (Lamb *et al.,* 2001). Hyper accumulation species have a physiological mechanism that regulates the soil solution concentration of metals. Mechanism of biosynthesis of nanoparticles in plants may be associated with phyto-remediation concept in plants (Haverkamp *et al.,* 2007).

2.13 Aluminium Oxide Nanoparticles

The aluminum based nanomaterials comprise of metallic aluminum and aluminum oxide. The nanoparticles of metallic aluminum are highly reactive and are not adoptable in water remediation methods (Ghanta and Muralidharan, 2013; Ravindhranath and Ramamoorty, 2017). Aluminum oxide related nanomaterials are intensively used in diverse fields such as, in biomedicines, drug delivery systems, optics, electronic devices, and catalysts in various industrial synthesis (Ángela *et al*., 2014). As Albert

Cotton *et al*. (2007) rightly noted that though the aluminum oxide has simple stoichiometric formula, Al_2O_3, it has many manifestations such as various polymorphic forms and hydrated species and each has its own significance depending upon the nature and conditions of preparations (Albert *et al*., 2007) α-$Al_2O_3$and γ-Al_2O_3, are well-known forms. The α-Al_2O_3 form is obtained by heating hydrous Al_2O_3 above 1000°C and it has a crystalline structure with hexagonally packed oxide ion and octahedrally packed Aluminum ions such that each oxide is surrounded by four Aluminum ions (Albert *et al*., 2007). This form cannot be hydrolyzed. γ-Al_2O_3 is obtained by heating hydrous Al_2O_3 below 450°C and it possesses a defective structure with cation vacancies and hence, the surface has an affinity towards cations (Albert *et al*., 2007; Poursani *et al*., 2017). Another form, β- Al_2O_3 is considered to be a mixed oxide and the do pants being Na+ and Mg_2+ ions and this form exhibits clear ion exchanging properties and high conductivity; resulting in its wide use as a solid electrolyte in batteries (Albert *et al*., 2007). The hydroxides AlO(OH) and $Al(OH)_3$, are amphoteric and they exhibit both physical adsorption and ion-exchange depending upon the pH of the contacting solutions (Fentahun *et al*., 2014; Poursani *et al*., 2017)

Further, the spinning of $AlCl_4$ into fibers and then dehydrating it, result in oxide filaments that are stable up to 1400°C (Albert *et al*., 2007). Aluminum oxides when doped with other metallic ions, different colored gems are produced: ruby withCr3+and blue sapphire with Fe2+/Fe3+ and Ti4+. Al_2O_3on heating with alkali oxides at 1000°C, results in polymeric aluminum silicates such as $Na1_4[Al_4O1_3]$ and $Na1_7Al_5O1_6$ with the anions possessing tetrahedral AlO_4 with sharing edges (Albert *et al*., 2007). These compounds are known for their good ionic exchange abilities. Further, aluminum phosphate synthesized hydrothermally in presence of amines possesses cage-like

structures similar to that of zeolites. Contrary to zeolites, these structures are electro-neutral and have no ion exchange nature and hence they are used as molecular sieves with sizes as small as 12-13A° (Albert *et al*., 2007). Further, zeolites are aluminum silicates of the formula $M^{n+}_{x/n}(AlO_2)_x(SiO_2)_4].nH_2O$ and have cage-like structures with cavities occupied by large ions and water molecules which can be exchanged/adsorbed reversibly to the surface of zeolites and hence, zeolites acquire affinity towards cations (Albert *et al*., 2007).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Sample Collection

Fresh leaves of *Cymbopogan citratus* (Lemongrass) were collected from around the environment of Federal University of Technology Minna, Niger State, Bosso campus in sterile nylon bags. Five gram (5g) of soil samples were collected in sterile nylon bags from the biological garden of the Federal University of Technology Minna, Niger State, Bosso campus and taken to the Microbiology Laboratory for microbial analysis.

3.2 Media Preparation

The Potatoe Dextrose agar used for this work was prepared according to the manufacturer's instructions. The sporulation medium was prepared as follows: agar, 30 g l^{-1}; casein peptone, 5 g l^{-1}; malt extract, 20 g l^{-1}(Boruta and Bizukojc, 2016). The preculture medium contained the following: yeast extract, 8 g l^{-1}; lactose, 10 g l^{-1}; KH_2PO_4, 1.51 g l^{-1}; NaCl, 0.4 g l^{-1}; $MgSO_4 \cdot 7H_2O$, 0.51 g l^{-1}; biotin, 0.04 mg l^{-1}; $ZnSO_4 \cdot 7H_2O$, 1 mg l^{-1}; $Fe(NO_3)_3 \cdot 9H_2O$, 2 mg l^{-1}; 1 ml l^{-1} of trace elements solution containing $MnSO_4$, 50mg l^{-1}; $Na_2B_4O_7 \cdot 10H_2O$, 100 mg l^{-1}; $Na_2MoO_4 \cdot 2H_2O$, 50 mg l^{1}; $CuSO_4 \cdot 5H_2O$, 250 mg l^{-1} (Gonciarz and Bizukojc, 2014). The production medium was the same as the preculture medium but contained less yeast extract (4 g l^{-1}) and more lactose (20 g l^{-1}) (Gonciarz and Bizukojc, 2014). The pH value of all media was adjusted to 6.5 with the use of NaOH solution prior to sterilization. The media was sterilized for 20 min at 121°C.

Aluminum oxide (Al_2O_3) nanoparticles was sterilized separately and added to the preculture medium (6 or 12 g l^{-1}) together with fungal spores at the time of inoculation.

3.3 Isolation and Characterization of the Selected *Aspergillus* species.

One gram (1g) of soil obtained from the biological garden of the Federal University of Technology Minna, Niger State, Bosso campus was mixed in 9ml of sterile distilled water. Serial dilution was conducted, 1ml of dilutions 10^{-3} and 10^{-4} were poured into petri dishes and 10ml of potato dextrose agar (PDA) was poured into the same petri dishes and gently rocked to mix. The plates were incubated at 25°C for 48 hours. The fungal isolates were identified according to the taxonomic key of Heath, 2012, on the basis of their macroscopic and microscopic characteristics viewed through a microscope with a magnification of 40X objectives. The fungal isolates were maintained on PDA slants and stored at 4°C.

3.4 Molecular Identification of Fungal Isolates

3.4.1 DNA extraction

A sterile mortal was used to collect 100mg of fungal mycelia, 1ml of DNA Extraction buffer (DEB) containing 0.05mg/ml proteinase K was added to the fungal mycelia and the mixture was macerated with a sterile pestle. The extract was transferred into 1.5ml eppendorf microcentrifuge tube, 50µl of 20% Sodium Dodecyl Sulphate (SDS) was added and allowed to incubate in a water-bath at 65°C for 30 minutes. The tubes were allowed to cool at room temperature, 100µl of 7.5M Potassium Acetate was added and the solution was mixed briefly. The solution was then centrifuged at 13000rpm for 10minutes and the supernatant was transferred into new fresh autoclaved tubes. Two-third (2/3) volumes of cold Isopropanol/ Isopropyl alcohol was added to the supernatant, the tubes were inverted 3-5 times gently and incubated at -20°C for 1 hour. The solution was centrifuged at 13000rpm for 10minutes and the supernatant was discarded. 500µl of 70% ethanol was added to the DNA pellet and centrifuged for 5minutes at 13000rpm, the supernatant was discarded carefully leaving the DNA pellet intact. Every

trace of ethanol was removed and the DNA pellet was dried at 37°C for 10-15minutes, the DNA pellets were then resuspended in 50µl of Tris-EDTA (TE) buffer and an aliquot of the DNA was taken and stored at 20°C for further laboratory analysis (Ahschul *et al.,* 1990).

3.4.2 PCR analysis

For the use of ITS gene in the characterization of fungi, ITS universal primer set which flank the ITS1, 5.8S and ITS2 region can be used for PCR. The PCR reaction cocktail consists of of 10 µl of 5x GoTaq colourless reaction, 3 µl of MgCl2, 1 µl of 10 mM of dNTPs mix, 1 µl of 10 pmol each ITS 1: 5' TCC GTA GGT GAA CCT GCG G 3'and - ITS 4: 5' TCC TCC GCT TAT TGA TAT GC 3''. primers and 0.3units of Taq DNA polymerase (Promega, USA) made up to 42 µl with sterile distilled water 8µl DNA template. The PCR was carried out in a GeneAmp 9700 PCR System Thermalcycler (Applied Biosystem Inc., USA), the PCR conditions includes a cycle of initial denaturation at 94°C for 5 min, followed by 35cycles of which each cycle comprises of 30secs denaturation at 94°C, annealing of primer at 55°C for 30secs, 1.5 min extension at 72°C and a final extension for 7min at 72°C (Lodish *et al.,* 2004).

3.4.3 Integrity test

The integrity of the amplified about 1.5Mb gene fragment was checked on a 1% Agarose gel which was 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 and 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 then poured into the tray, the gel was allowed

to solidify for 20minutes so as to form the wells. The 1XTAE buffer was poured into the gel tank to barely submerge the gel, two microliter (2µl) 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 (Lodish *et al.*, 2004).

3.4.4 Purification of amplified product

After gel integrity, the amplified fragments were purified using ethanol so as to remove the PCR reagents. Briefly, 40µl of PCR amplified products were placed in sterile 1.5µl eppendorf microcentrifuge tube and 7.6µl of 3M Na acetate and 240µl of 95% ethanol were added to it, the solution was mixed thoroughly by vortexing and kept at -20°C for at least 30minutes. The mixture was centrifuged at 13000rpm for 10minutes and 4°C, the supernatant was removed (invert tube on trash once) and the pellets were washed by adding 150µl of 70% ethanol and mix then centrifuge for 15 min at 7500rpm and 4°C. Again, all the supernatant was removed (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 and then re-suspend with 20 µl of sterile distilled water and kept in -20°C prior to sequencing. The purified fragment was checked on a 1.5% Agarose gel that ran on a voltage of 110V for about 1hr as previous to confirm the presence of the purified product and then quantified using a nanodrop of model 2000 from thermo scientific (Ahschul *et al.*, 1990).

3.4.5 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 was used for all genetic analysis (Ahschul *et al.*, 1990).

3.5 Preparation of Aluminium Oxide Nanoparticles Using *Cymbopogan citratus* (Lemongrass) Extract.

Fresh leaves of *Cymbopogan citratus* (Lemongrass) obtained from the school clinic environment of Federal University of Technology Minna, Niger State, Bosso campus were washed thoroughly with distilled water. Twenty-five gram (25g) of small pieces of finely cut leaves were dissolved in 200 ml distilled water and boiled for 30 mins. After cooling at room temperature, they were centrifuged at 12,000 rpm for 15 mins at 4°C and filtered through 0.45 lm PTFE filter. The filtrates were stored at 4–8°C and used as reducing and stabilizing agents.

Aluminium nitrate was used as precursor for the synthesis of aluminium oxide nanoparticles. To make sure of a complete reduction, aluminium nitrate was dissolved in aqueous extract of lemon grass in 1:4 ratio in conical flask under aseptic conditions with stirring at room temperature. The solution was subjected to microwave irradiation till colour change was observed. After 6–10 mins, yellowish brown precipitate was produced. The precipitates were centrifuged and washed with distilled water and methanol.

To obtain the dry powders of aluminium oxide nanoparticles the solution containing the nanoparticles were centrifuged at12,000 rpm for 15 mins, following which the pellet was re-dispersed in sterile double distilled water to get rid of any un-interacted biological molecules. This process of centrifugation and re-dispersion in sterile

deionized water was repeated 4–5 times to ensure better separation of the nanoparticles. The purified pellets were then dried in an oven at 80°C for 3–4 h (Ansari *et al.*, 2015).

3.6 Cultivation Conditions to Enhance the Growth of the *Aspergillus* sp. by Aluminum Oxide(Al_2O_3) Nanoparticles.

The *Aspergillus* sp. were inoculated onto agar slants and after 10 days of cultivation the spores were transferred into sterile liquid pre-culture medium together with Al_2O_3 nanoparticles. The pre-cultures were propagated in shake flasks (150 ml working volume, 500 ml total volume) for 24 hours and then 7 ml of the pre-culture was transferred into 150 ml of sterile liquid production medium. The subsequent shake flask cultivation was conducted for 120 h at 28°C (Tomasz and Marcin, 2019).

3.7 Screening for Proteolytic Activity

Proteolytic activity was detected by casein hydrolysis on agar plates containing YNB (DIFCO) medium supplemented with 0.5% of casein, 0.5% of glucose, and 2% of agar (w v-1), pH 7.0 (Rodarte *et al.*, 2011). The plates were incubated at 28ºC for 7-8 days. Enzyme activity was indicated by the formation of a clear zone around colonies after precipitation with 1 M HCl solution (Rodarte *et al.*, 2011).

3.8 Medium Preparation for Protease Enzyme Production

The medium used for protease enzyme production was composed of (g/l): $CaCl_2.7H_2O$ 0.4, KH_2PO_4 2.5, $MgSO_4.7H_2O$ 0.5, $ZnCl_2$ 0.1, NaCl 0.3, Casein 2.0; pH 6.0. The medium was autoclaved at 120°C for 20min (Oyewole *et al.*, 2011).

3.9 Culture Conditions for Protease Enzyme Production

Cultures were inoculated with 10^7spores mL^{-1} and incubated in a rotary shaker at 150rpm for 72h at 30°C in 250mL Erlenmeyer flasks with a working volume of 50mL.

The cultures were centrifuged at 10,000rpm for 10min to remove fungal mycelia and supernatants were used as the enzyme extract (Oyewole *et al.,* 2011).

3.10 Protease Enzyme Assay

Casein (1.0%) was dissolved in buffer (pH 8.0) by heating at 70°C. 2 ml of casein solution was mixed with 0.5 ml of culture supernatant and incubated at 45° C in water bath for 50 mins. The reaction was terminated by adding an equal volume of 10 % TCA followed by 10 mins holding time. Suspension was filtered through Watmann's filter paper. To 1ml of filtrate, 5ml of 0.5 Na_2CO_3 solutions and 0.5ml of 3-fold diluted folin reagent were added and mixed thoroughly. The colour development after 30 min of incubation at 30°C was measured as optical density (O.D) at 660 nm with blank as reference (Khan *et al.*, 2011).

3.11 Optimization of Kinetic Parameters for Protease Enzyme Production

3.11.1 Effect of pH on protease production

The effect of pH on the protease production was carried out using the following pH values of 5,6,7,8,9and 10 for all the *Aspergillus* sp. An assay was also be carried out based on Dinitrosalicyclic acid method (DNSA) (Oyeleke *et al*., 2011).

3.11.2 Effect of temperature on protease production

The effect of temperature on protease production was carried out using the following temperature values; 30℃, 40℃, 50℃, 60℃, 70℃, and 80℃ for all the *Aspergillus* sp. An assay was carried out based on Dinitrosalicyclic acid method (DNSA), (Oyeleke *et al*., 2011).

3.12 Statistical Analysis

Statistical analysis of data was carried out using Analysis of Variance (ANOVA) with SPSS version 17.

CHAPTER FOUR

4.0 RESULTS AND DISCUSSION

4.1 Isolation and Preliminary Screening of Isolates

The soil obtained from the biological garden of the Federal University of Technology Minna, Niger State showed various diversities of fungi present in the ecological niche with many isolates that can be utilized for the production of protease enzyme (Oyeleke *et al.,* 2011). The isolates were culturally and morphologically characterized (Table 4.1).

Table 4.1: Identification and Characterization of the Isolated Fungi.

Isolates Code No	Characteristics	Probable fungus
1	Colonies at 25°C attained a diameter of 5cm within 7 days, they consisted of initial white colour and then changed to black after a few days. The conidial heads appeared radial and they split into columns(biseriate). The conidiophore stipes were smooth-walled and hyaline.	*Aspergillus niger*
2	Colonies at 25°C attained a diameter of 5cm within 7 days, they consisted of a yellow-green colour. The conidia heads appeared radial, tending to split into loose columns with age. The conidiophores stipes were smooth-walled and hyaline.	*Aspergillus flavus*
3	Colonies at 25°C attained a diameter of 5cm within 7 days, they consisted of a dense brown-yellowish colour. The conidia heads appeared radiate tending to split into loose columns with age. The conidiophores stipes were smooth-walled and hyaline.	*Aspergillus terrus*

4.2 Molecular Characterization of the Selected Fungal Isolates.

The documented electrophoresis gel image is illustrated in Plate 1. The lane is labelled M (Molecular marker), and 1-2 representing DNA extracted from fungal isolate. The direction of the band migration from negative to positive electrodes is due to the naturally occurring negative charge carried by the sugar phosphate backbone (Lodish *et al.*, 2004). Lane 1 is more prominent compared to Lane 2. The fragment pattern from Plate 1 shows that the two bands were 600bp in size with reference to 1Kb DNA ladder.

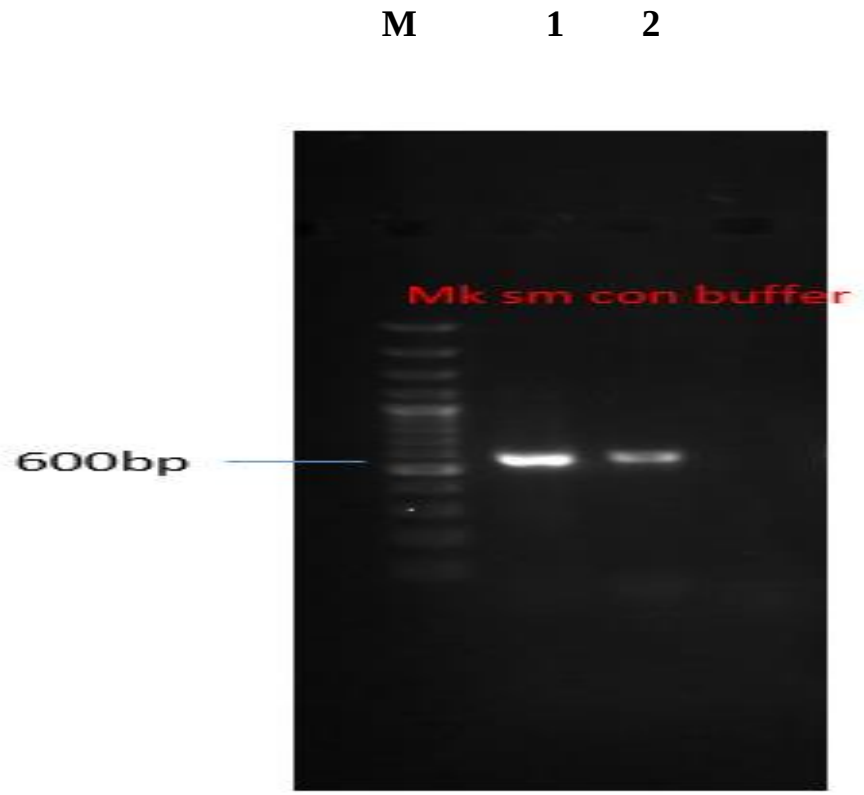

Plate 1: Gel electrophoresis micrograph of amplified product from *Aspergillus niger*

99% identical to *Aspergillus niger* strain MN945947

4.3 UV Spectroscopy of Aluminum Oxide Nanoparticle

UV spectroscopy reading of the synthesized aluminum oxide nanoparticles is illustrated in Figure 4.1. The peak 1 and 4 shows that the nanoparticles were detected at a wavelength of between 271 and 273nm. This depicts that the nanoparticles have been formed.

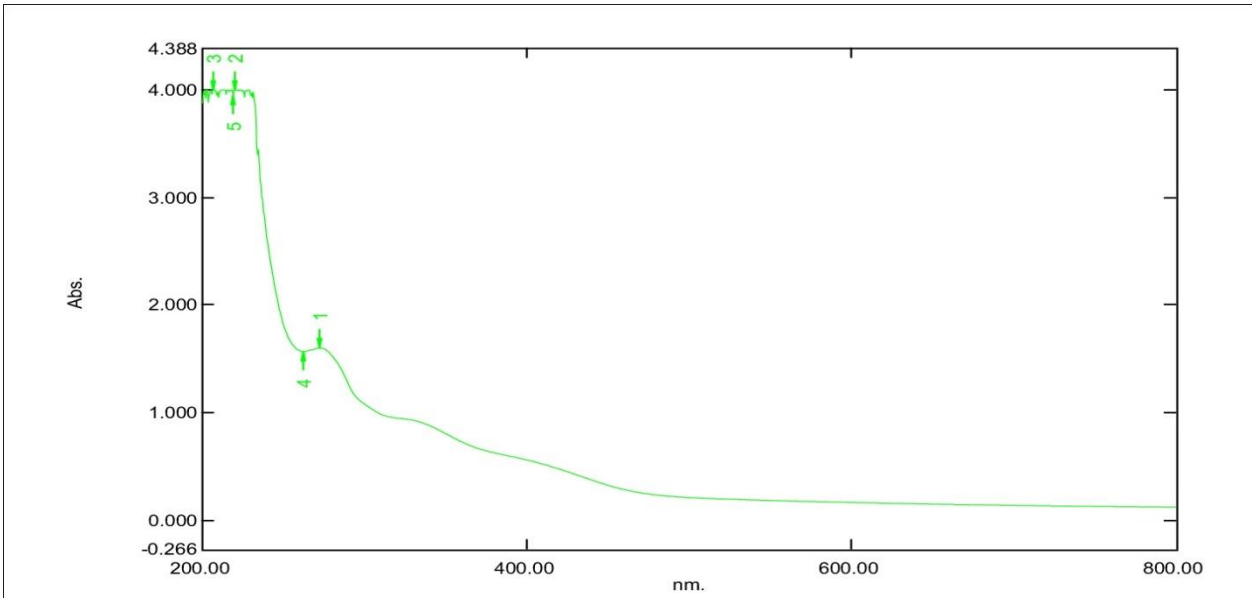

Figure 4.1 UV Spectroscopy Result of the Synthesized Aluminum Oxide Nanoparticles

4.4 Screening of the Fungi Isolates for Protease Activity by Plate Assay Method

Screening of fungi for their protease activity was carried out by the hydrolysis of substrate incorporating in the medium by plate assay method. After an incubation period, enzyme activities were detected by the appearance of zones around the fungal colonies. The protease enzyme assay of the nanoparticle cultivated *Aspergillus* sp. and *Aspergillus* sp. that were not cultivated by the nanoparticle was illustrated in Figure 4.2 below. Nanoparticle cultivated *Aspergillus* sp. had low clear zones when compared to the *Aspergillus* sp. that were not cultivated by the nanoparticle. The fungi species showed clear zones around the colony, *Aspergillus niger* had the highest clear zone at diameter 4.7 m for the species that was not cultivated by the nanoparticle and 4.0 m for the species that was cultivated by the nanoparticle. These isolated fungi are well-known producers of proteases (Oyeleke *et al*., 2011). This is in agreement with the report of Chandrasekaran *et al*., 2015 who reported similar clear zones around the *Aspergillus* species using plate assay method.

Table 4.2: Enzyme Activity of the Selected *Aspergillus* species.

Organisms	Enzyme Activity(mm)
Aspergillus niger	**4.5**
Aspergillus terreus	**3.9**
Aspergillus flavus	**3.5**
Cultivated *Aspergillus niger*	**4.0**
Cultivated *Aspergillus terreus*	**3.6**
Cultivated *Aspergillus flavus*	**3.2**

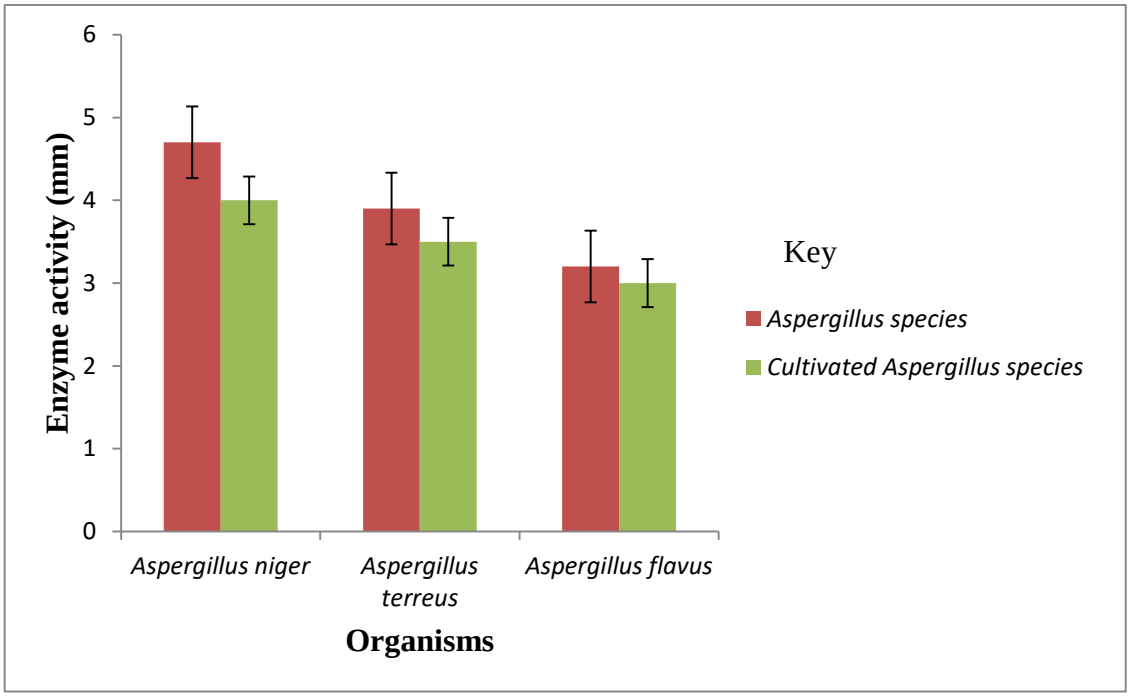

Figure 4.2: Proteolytic activity of the selected *Aspergillus* sp.

4.5 Optimization of Kinetic Parameters for Enzyme Production

4.5.1 Effect of Temperature on Protease Production

The effect of temperature on protease enzyme production by the nanoparticle enhanced *Aspergillus* sp. and the *Aspergillus* sp. that were not enhanced by the nanoparticle are shown in figure 4.3 and 4.4. Incubation temperature plays an important role in the metabolic activities of a microorganism. Even slight changes in temperature can affect enzyme production. The activity of protease increased as temperature of the medium was raised from 30˚C for all the *Aspergillus* sp., the activity increased until an optimum of 50˚C was obtained by *Aspergillus niger*. (Shumi *et al*. 2003) reported an optimum temperature of 40˚C and 50˚C respectively. A further increase in temperature resulted in decrease in enzymatic activity and thus the denaturation of the enzyme at higher temperature. Fungal proteases are usually thermo labile and show reduced activities at high temperatures (Shumi *et al*., 2003). Higher temperature is found to have adverse effects on metabolic activities of microorganisms.

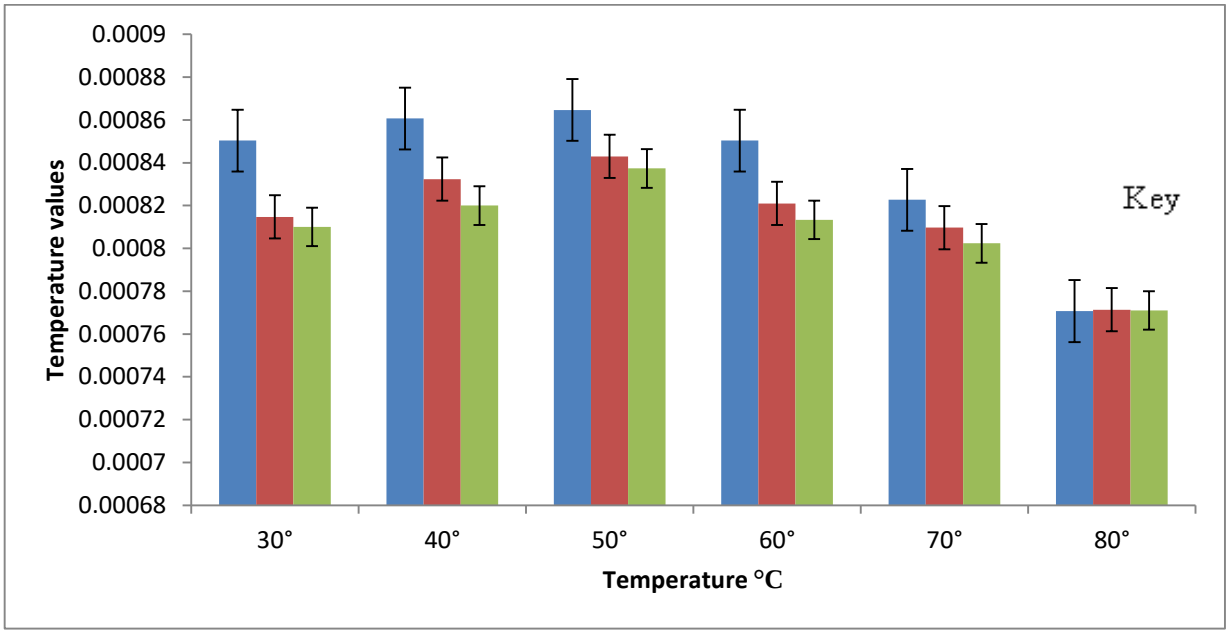

Figure 4.3: Effect of Temperature on Protease produced by the *Aspergillus species* not cultivated with Aluminum oxide nanoparticle

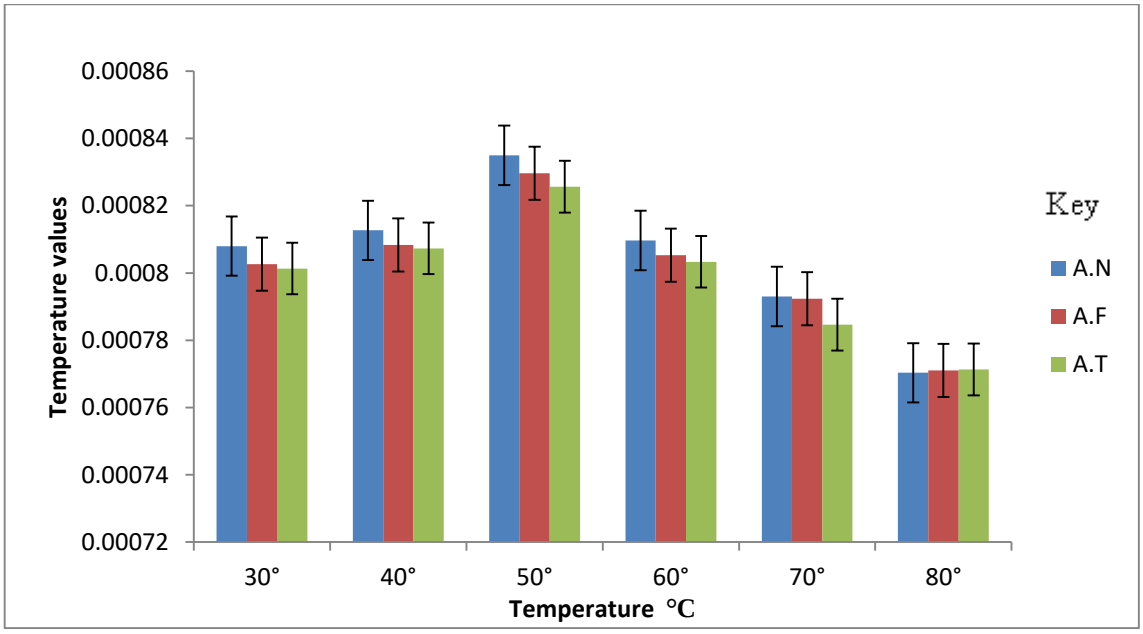

Figure 4.4: Effect of Temperature on Protease produced by the *Aspergillus* species cultivated by aluminum oxide nanoparticles.

4.5.2 Effect of pH on Protease Production

The effect of pH on protease enzyme production by the nanoparticle cultivated *Aspergillus* sp. and the *Aspergillus* sp. not cultivated by the nanoparticle are shown in figure 4.5 and 4.6. The activity of protease increased as the pH of the buffer was increased. The protease produced was optimally active at pH 8.0 and pH 10.0 by the *Aspergillus nige*r that was not cultivated with the nanoparticle and the *Aspergillus flavus* that was cultivated with the nanoparticle respectively. This finding was in line with the works of Oyeleke *et al.,* 2011 on protease activity who reported optimum pH of 8.0. Thermo alkaline proteases are the most commonly used of the alkaline proteases because they function at a pH range of 7.0 – 12.0 and a temperature of 40˚C– 60˚C (Beg *et al.,* 2003).

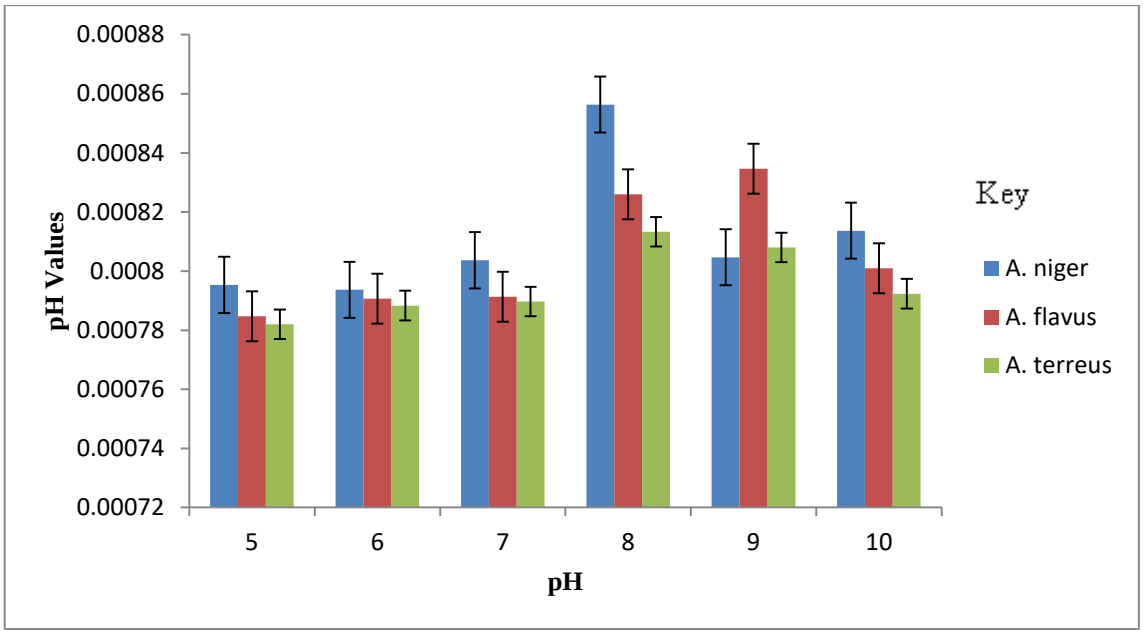

Figure 4.5: Effect of pH on Protease produced by the *Aspergillus species* not cultivated with Aluminum oxide nanoparticle

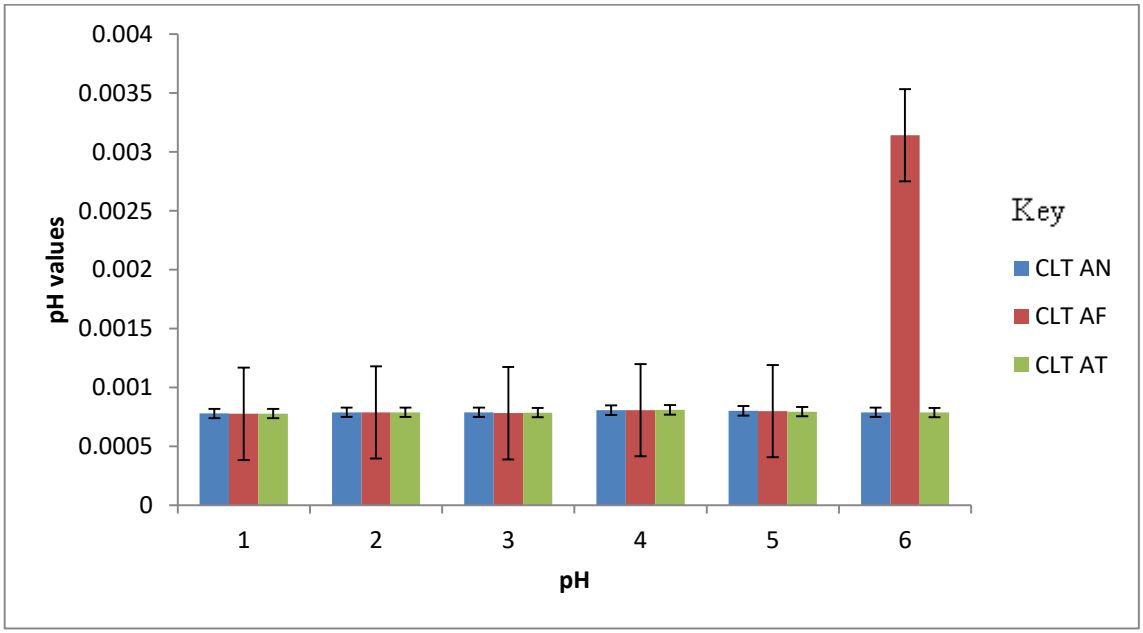

Figure 4.6: Effect of pH on Protease produced by the *Aspergillus species* cultivated by aluminum oxide nanoparticles.

4.6 Discussion

In this present study, fungal isolates were morphologically, culturally amd microscopically identified to be species of *Aspergillus*: *Aspergillus* niger, *Aspergillus flavus, Aspergillus terreus* with the blackish, greenish and brownish yellow colouration observed on plate while they had a septate hypae and biseriate conidiophores. This is a general characteristic of species of Aspergillus, and this result corroborates with the findings of Aziz and Zainol (2018) who isolated similar species of fungi from the soil. These species of *Aspergillus* have been known to be ubiquitous and commonly isolated from the soil. Due to their ability to degrade plant cell walls rapidly, this *Aspergillus* sp. have been used to produce a number of important industrial enzymes such as cellulases, hemicellulases, and proteases that are used commercially in the food industry.

Molecular characterization was carried out in order to identify which species the isolated *Aspergillus* genus belonged to, and the organisms were identified to be *Aspergillus flavus*, *Aspergillus terreus* and *Aspergillus niger*.

The isolated fungi were screened for their protease activity, the nanoparticle cultivated *Aspergillus species* had lower clear zones compared to the control, this shows that enhancing *Aspergillus species* with nanoparticles do not increase their protease activity. This could be as a result of the green synthesis of the aluminum oxide nanoparticle. *Aspergillus niger* had the highest protease activity, this conforms with the observation of Chandrasekan *et al.* (2015), who reported higher protease activity in *Aspergillus niger*.

In this study, formation of Aluminium oxide nanoparticles using *Cymbopogan citratus* (lemon grass) extraxct was viewed by a colour change from brown to yellowish brown. Similarly, Ansari *et al*. (2015) reported that Aluminium oxide nanoparticles exhibited marked colour change from brown to yellowish brown in aqueous solution which is due

to excitation or surface plasma resonance. By using UV-vis spectrum, the maximum absorbance peak was 273 nm. Duraisamy (2018) also reported absorption spectra of Aluminium oxide nanoparticles to range from 271 to 273 nm. This result agrees with the findings of Goutam *et al*. (2018) who synthesized Aluminium oxide nanoparticles using leaf extract of rosa.

In this present study, there was an increase in the protease enzyme produced by the organisms cultivated with Aluminium oxide nanoparticles and the organisms not cultivated with Aluminium oxide nanoparticles as the temperature increased. Both produced protease enzyme at optimum temperature of 50°C, at temperature above 50°C the activities of the enzyme decreased while at 80°C, the activities remained constant. According to Sabu *et al*. (2012), since enzyme is a secondary metabolite produced during exponential growth phase, the incubation at high temperature could lead to poor growth and thus a reduction in enzyme yield. This result is in agreement with the work of Shumi *et al*. (2003) who reported maximum enzyme activity at 50°C temperature.

In this study, the protease enzyme activity increased as pH of the buffer was increased, optimum temperature of the protease enzyme produced by the *Aspergillus* sp. not cultivated with Aluminium oxide nanoparticles was at pH 8.0 while the optimum temperature of the protease enzyme produced by the *Aspergillus* sp. cultivated with Aluminium oxide nanoparticles was at pH 10.0. It has been reported widely that protease production from microbial source can be acidic or alkaline protease as reported by many researchers depending on the organism and source of the isolation. Studies carried out by Sethi and Gupta (2015) maximum protease enzyme activity was observed in medium of pH 9.0 in case of *Penicillum chrysogenum* and *Aspergillus niger*. This result is similar to the discovery of Oyeleke *et al*. (2010) who recorded optimum pH 8.0 for protease enzyme produced by *Aspergillus niger*.

CHAPTER FIVE

5.0 CONCLUSION AND RECOMMENDATION

5.1 Conclusion

The *Aspergillus* species (*Aspergillus niger, Aspergillus flavus* and *Aspergillus terreus*) were successfully isolated from the soil and were identified using macroscopic and microscopic characteristics. The organisms were further identified molecularly.

Aluminum oxide nanoparticle was produced from *Cymbopogan citratus* (lemon grass) and the Ultraviolet (UV) spectroscopy detected the nanoparticle at wave length between 271nm and 273nm.

The nanoparticle was able to enhance the aspergillus species by showing changes in the morphology of the fungal species.

Aspergillus niger cultivated with the aluminum oxide nanoparticle was able to produce protease enzyme at an optimum temperature of 50°C. *Aspergillus flavus* cultivated with the aluminum oxide nanoparticle was able to produce protease enzyme at an optimum pH 10.

The protease enzyme produced by the nanoparticle enhanced *aspergillus species* recorded lower yield compared to the protease enzyme produced by the *aspergillus* species that were not enhanced by the nanoparticle.

5.2 Recommendation

The successful use of aluminum oxide nanoparticle for controlling fungal morphology, to enhance the production of protease still needs further research as only one paper was dedicated to it and lovastatine was the major enzyme produced from the research.

Research on controlling microbial morphology to enhance the production of enzymes should be encouraged to improve industrial enzyme production and application.

5.3 Contribution to Knowledge

Chemicals are being utilized in a variety of businesses globally, which is having a severe impact on people's health. This study applied aluminum oxide nanoparticles in the cultivation of some selected *Aspergillus* sp. in the production of protease enzyme which can lead to reduced waste production, lower energy consumption and overall reduced environment impact/ *Aspergillus niger* had the highest clear zone at diameter 4.5mm for the species that was not cultivated by the nanoparticle and 4.0mm for the species that was cultivated by the nanoparticle. The nanoparticle cultivated *Aspergillus niger* had the highest protease enzyme yield at 50℃ and the nanoparticle cultivated Aspergillus flavus had the highest yield at pH 6.0. This research study has shown that aluminium oxide nanoparticles can be used in the cultivation of the selected organisms: *Aspergillus niger*, *Aspergillus flavus* and *Aspergillus terreus* in the production of protease enzyme and also increase the yield of the enzyme produced by 25%.

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APPENDICES

APPENDIX A: Nucleotide Sequence of the Fungal Isolate.

TAAACCTGCGGAAGGATCATTACCGTGCGGGTCCTTTGGGCCCAACCTCCCA

TCCGTGTCTATTGTACCCTGTTGCTTCGGCGGGCCCGCCGCTTGTCGGCCGC

CGGGGGGGCGCCTCTGCCCCCCGGGCCCGTGCCCGCCGGAGACCCCAACAC

GAACACTGTCTGAAAGCGTGCAGTCTGAGTTGATTGAATGCAATCAGTTAA

AACTTTCAACAATGGATCTCTTGGTTCCGGCATCGATGAAGAACGCAGCGA

AATGCGATAACTAATGTGAATTGCAGAATTCAGTGAATCATCGAGTCTTTGA

ACGCACATTGCGCCCCCTGGTATTCCGGGGGGCATGCCTGTCCGAGCGTCAT

TGCTGCCCTCAAGCCCGGCTTGTGTGTTGGGTCGCCGTCCCCCTCTCCGGGG

GGACGGGCCCGAAAGGCAGCGGCGGCACCGCGTCCGATCCTCGAGCGTATG

GGGCTTTGTCACATGCTCTGTAGGATTGGCCGGCGCCTGCCGACGTTTTCCA

ACCATTCTTTCCAGGTTGACCTCGGATCAGGTAGGGATACCCGCTGAACAA

A