CHARACTERIZATION AND PARTIAL PURIFICATION OF PECTINASE PRODUCED BY *ASPERGILLUS NIGER* USING BANANA PEEL AS CARBON SOURCE.

BY

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(17/27/MMI011)

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CERTIFICATION

This is to certify that this research project work was carried out by **Ahmed Olaitan YUSUF** with matriculation number **17/27/MMI011** under our supervision and that it is a fair reflection of the student's input.

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DECLARATION

I, Ahmed Olaitan YUSUF hereby declare that this research work titled "Characterization and Partial Purification of Pectinase produced by *Aspergillus niger* using banana peel as carbon source" was carried out by me under supervision of Dr. (Mrs). M.R. Adedayo and Dr. A.O. Sulyman. All resources used were adequately referenced.

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Ahmed Olaitan YUSUF Date

17/27/MMI011

DEDICATION

This research project work is dedicated to my loving son Yusuf Ahmad Kikiola, who has been a source of inspiration and motivation to me.

ACKNOWLEDGEMENT

All praises and adoration goes to Almighty Allah, the Creator of the universe, for His guidance and protections on me and my family.

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ABSTRACT

Pectinases are group of enzymes which causes the degradation of pectin. Pectinases are of industrial importance due to their biotechnological and commercial potentials in brewing industries, textile industries, food industries and paper industries among others. The present study was aimed at characterization and partial purification of pectinase produced by *Aspergillus niger* using banana peel as carbon source under submerged fermentation (SmF) system. Pectinolytic fungi were isolated from fruit wastes dumping sites. The isolates were characterized morphologically and microscopically using standard methods. The isolates were further subjected to screening for pectinolytic ability. Molecular identification was also carried out on the isolate with the best pectinolytic ability. The isolate with the best pectinolytic ability was identified as *A. niger* strain AH1 with GenBank accession number MK811422. Fermentation parameters optimized to increase pectinase activity include incubation period, salt supplements, additional carbon source, inoculum sizes, pH, moisture contents and temperature using standard methods. The crude pectinase obtained was purified by ammonium sulphate precipitation, dialysis and gel-filtration chromatography. Maximum pectinase activity of 575.37±0.67 U/mg/min/ml was obtained at day 3 of incubation, with addition of salt supplements, in the presence of sucrose as additional carbon source, 2 ml of 1×10^6 spores ml^{-1} of *A. niger*, pH 6, 100 ml moisture contents, and temperature of 50 ^{0}C. The specific activity increased with 1.38 fold while recovery yield was 8.94 %. The study confirmed that the isolated *A. niger* strain AH1 can produced maximum pectinase at optimized fermentation parameters and could be explored for pectinase production.

CHAPTER ONE

1.0 Introduction

Enzymes are bio-active compounds or catalyst that regulate many chemical reactions in living tissues and cells (Prathyusha and Suneetha, 2011). They are highly selective catalysts which can greatly accelerate both the rate and specificity of metabolic reactions, which range from the digestion of food to the synthesis of DNA. The use of chemical catalysts has been pursued for a very long time. Chemical catalysis though widely used was very cumbersome. The disadvantages this method possesses include need for high temperature and pressure for catalysis and the moderate specificity (Rajendra *et al*., 2016). These limitations were overcome by the use of enzymes. Enzymes work at milder conditions when compared to that required by chemical catalysts for operation. Also, enzymes are highly specific and catalyze reactions faster than chemical catalysts (Peter, 2015). Enzymes are produced from plant and animal sources but the microbial sources are generally the most suitable for commercial applications as a result of its bulk production capacity and easy manipulation for better product yield (Sivakumar *et al*., 2012). Solid-state fermentation (SSF) and submerged fermentation (SmF) are important fermentation methods employed for the production of microbial enzymes (Martins *et al*., 2000). Microbial enzymes are cheaper to produce and their enzyme contents are more controllable and reliable. Of greater importance is the production of microbial enzymes from lignocelluloses biomass which are cheap and readily available (Taylor *et al*., 2015; Asgher *et al*., 2018).

Pectinases are one of the important enzymes at the moment which constitutes 25 % of the global food enzyme market (Jayani *et al.,* 2005). They are a group of enzymes, which cause degradation of pectin that, are chain molecules with a rhamnogalacturonan backbone; associated with other polymers and carbohydrates in different mode of actions including trans-elimination and

hydrolysis (Jayani *et al.,* 2005; Favela-Torres *et al.,* 2006; Heidar *et al.,* 2011). In nature, pectinases are mainly found in plant tissue to facilitate the cell wall extension and softening some tissues during the maturation of part of the plant such as fruits. In addition, pectinase also catalyze the decomposition of plant residues and contribute to the carbon cycle and ecosystem stabilization. During the phytopathogenic process, pectinases are secreted copiously by microorganisms to infect the host plant (Whitaker, 2000; Obafemi *et al*., 2019). Pectinases have great biotechnological potential and can be used in many industrial processes (Tewari and Tewari, 2005; Zhong and Cen, 2005). Hence, applications of pectinases in various fields are widening, it is necessary to understand the properties and nature of these enzymes for efficient and effective usage (Sathyanarayana and Panda, 2003; Deng *et al*., 2019). In the food sector, pectins are primarily used as a gelling agent in the production of jam; and as stabilizers in the production of yoghurt (Koubala *et al*., 2006). In the pharmaceutical industry, these polysaccharides are used as drug delivery systems, which can also reduce the toxicity of the drugs (Morris *et al*., 2010). In industrial sector, pectinases are vastly applied in fruit juice extraction and clarification, textile and fiber processing and manufacturing, coffee and tea fermentations, industrial pectic wastewater treatment, paper making, purification of plant virus, poultry feed production and oil extraction (Saito *et al.,* 2004). Furthermore, these novel enzymes are also utilized in the extraction of specific plant bio-compounds from raspberry residues, tomatoes, grape skin and also apple skin (Neaga *et al.,* 2012). Pectinases can be produced by both submerged and solid state fermentation (SSF). Submerged fermentation is cultivation of microorganisms in liquid broth. It requires high volumes of water, continuous agitation and generates lot of effluents. SSF incorporates microbial growth and product formation on or within

particles of a solid substrate under aerobic conditions, in the absence or near absence of free water, and does not generally require strict aseptic conditions (Pandey *et al*., 2002).

Pectin was first isolated and described in 1825 by Henri Braconnot (Jayani *et al*., 2005). Pectin is one of the major components of the primary cellular walls in the middle lamella of plant tissues. The pectic matrix provides an environment for deposition, slippage and extension of the cellulosic-glycan network, and is the major adhesive materials between cells (Willats *et al.*, 2001). Pectin degradation leads to disassembly of the cellulose and hemicellulose network and plays an important role in fruit ripening (Lohani*et al*., 2004; Sañudo-Barajas *et al.*, 2009). Wall degrading enzymes are the major factors to initiate disassembly of cellular walls of harvested fruits (Miller and Fry, 2001). Involvement of pectic enzymes, such as polygalacturonase (PGase), pectin methyl esterase (PME) and β-galactosidase (β-Gal), in enzymatic disassembly of cellular walls has been widely reported (Nikolić and Mojovic, 2007; Prasanna *et al*., 2007; Rugkong *et al*., 2010; Wei *et al*., 2010; Almeida and Huber, 2011).

Globalization and quest for development in developing countries has led to more research on sustainable economy which has been focused on the utilization of renewable agricultural and industrial wastes (Martin *et al*., 2004). Agricultural and food wastes constitute themajor sources of pollution in many countries according to earlier report (Koubala *et al*., 2006). This is being controlled by biological degradation using microorganismsfor the biosynthesis of valuable compounds suchas proteins, polysaccharides, oligosaccharides, vitamins,hormones, enzymes and others as raw materials for medicaland industrial exploitations (El-Sheikh *et al*., 2009). Several microbes are capable of using these substances as carbon and energy sources for the biosynthesis of a vast array ofpectinolytic enzymes in different environmental niches (Fatima *et al*., 2019). Among the fungal species that have been exploited for the biosynthesis of pectinase are *A.*

foetidus (Gummadi and Panda, 2003), *A. niger* (Mongensen *et al*., 2009), *A. awamori* (Blandino*et al*., 2002),*Rhizopus, Trichoderma, Penicillium* and *Fusarium*spp. (Zohdi and Mehrnoush, 2013). But, the best-acknowledged microbial producers of pectinase are various species of *Aspergillus* and *Aspergillus niger* is the most productive (Singh and Rao, 2002).

Banana (*Musa sapientum*, family Musaceae) is a fruit crop of the tropical and subtropical regions of the world grown on about 8.8 million hectares (Mohapatra *et al*., 2010). It is possibly the world's oldest cultivated crop (Kumar *et al*., 2012). As a diet, banana is an affluent source of carbohydrate with calorific value of 67 calories per 100g fruit and is one of the most well-liked and widely traded fruits across the world (Emaga *et al*., 2008; Kumar *et al*., 2012).

1.1 Significance of the Study

During the processing of raw agricultural materials, a bulk quantity of agro-wastes is generated and thus provides a suitable platform for bio-production of enzymes. Pectinases are widely used in industries for liquefaction of pulpy products, clarification of juices, in paper making, treatment of industrial wastes etc. The production of pectinases using agro-wastes as substrate will reduce pollution of our environments by agro residues, facilitate low cost production of enzyme than the conventional method and have a great impact on the economy of Nigeria and reduce dependence on importation of enzymes.

1.2 Statement of Research Problem

Tons in millions of agro-waste residues are generated annually in many countries of the world. These agro-wastes including fruits peel are left to rot in the market area and on dumping sites, constituting a nuisance and eye sore to the community. Banana storage, cost and postharvest losses are critically high. Hence, considerable interest has been generated in recent years, for value addition to banana, such as the production of banana juice, wine, chips etc. During the

production of these products, banana peels accumulate in bulk posing serious environmental problems. To prevent this, it is therefore necessary to convert these wastes to useful end products like pectinases thereby adding value to banana while reducing the postharvest waste and environmental pollution.

1.3 Research Justification

Previous studies reported that fruit peel is rich in pectin and applicable in pectinases production through solid state and submerged fermentation. Due to the increasing demand for enzymes in industrial processes and high cost of importing the enzymes, this research is designed to study the potential of banana peel as a cheap raw material and carbon source in fermentation system to produce pectinases that meet industrial needs by locally employing fungal isolate obtained from fruit wastes dumping site.

1.4 Aim of the Study

To purify and characterized pectinase produced by *Aspergillus niger* using banana peel as carbon source.

1.5 Objectives of the Study

The objectives of the study are to:

- isolate *Aspergillus niger* from fruits waste dumping site;
- carry out morphological and molecular characterization of *Aspergillus niger* isolated;
- produce extracellular pectinase using isolated *A. niger* in submerged fermentation of banana peel;
- optimize culture parameters for pectinase production; and
- characterize and partially purify extracellular pectinase.

CHAPTER TWO

2.0 Literature Review

2.1 Pectinases

Pectinases are a group of enzymes involved in degradation of pectin that includes various enzymes classified into various classes and subclasses depending on the substrate specificity and mode of action, for example, methyl de-esterases, hydrolases, and lyases. Primarily, these enzymes are a heterogeneous group of related enzymes that hydrolyze the pectic substances, present mostly in plants (Murad and Azzaz, 2011). Pectinase production from bacteria including actinomycetes (Beg *et al*., 2000), yeast (Reid and Ricard, 2000) and fungi (Mohamed *et al*., 2006; Al-Najada *et al*., 2012) has been reported. The fungus produces these enzymes to break down the middle lamella in plants and extract nutrients from the plant tissue. Pectin esterase catalyzes the deesterification of methylester linkages of galacturonan backbone of pectic substances to release acidic pectins and methanol (Chellapandi, 2010). The resulting pectin is then acted upon by polygalacturonase (PGase) and pectin lyase (PL) (Idnurm and Howlett, 2001). Production, biochemical characterization, and applications of PL have been reviewed extensively (Yadav *et al*., 2009). Pectin esterases are found in plants, plant pathogenic bacteria, and fungi (Jayani *et al*., 2005), while PGases are widely distributed among fungi, bacteria, and many yeasts (Murad and Azzaz, 2011). They are also found in higher plants and some plant parasitic nematodes (Yadav *et al*., 2009). Polygalacturonases are important pathogenicity factors for other fungi, such as *Aspergillus flavus*, *Alternaria citri* and *Claviceps purpurea*, and for bacteria such as *Agrobacterium tumefaciens* and *Ralstonia solanacearum* (Di Matteo, 2006). The degradation of pectin chains by PGase loosens the primary cell wall allowing the cellulose-hemicellulose network to become more accessible for digestion and leads to the release of cell

wall pectin-derived oligosaccharides, the oligogalacturonides (OGs), which in turn act as elicitors of a variety of plant defenses (Murad and Azzaz, 2011). Majority of fungal PGases are directly involved in plant pathogenesis and they are termed as "virulent factors" of fungal pathogens. Pectinases are reported to be the first enzymes to be secreted by fungal pathogens before invasion and colonization of plant cell walls (Idnurm and Howlett, 2001; Tomassini *et al*., 2009).

2.2 Biotechnological Applications of Microbial Pectinases

There has been several reports on biotechnological and industrial application of pectinases. Industrial processes, such as textile, plant fiber processing, oil extraction, treatment of industrial wastewater, containing petinacious material, etc have been carried out using the enzyme. They have also been reported to work on purification of viruses (Ahlawat *et al*., 2008) and in making of paper (Reid and Richard, 2004). However they are yet to be fully commercialized in Nigeria.

2.2.1 Fruit Juice Extraction

Over the years, largest industrial application of pectinases is in fruit juice extraction and clarification. A mixture of pectinases and amylases is used to clarify fruit juices and thus decreasing the filtration time up to 50 % (Kaur *et al*., 2004). Treatment of fruit pulps with pectinases also showed an increase in fruit juice volume from banana, grapes and apples (Kaur *et al*., 2004). Pectinases in combination with other enzymes such as cellulases, arabinases and xylanases, have been used to increase the pressing efficiency of the fruits for juice extraction (Gailing *et al*., 2000). Vacuum infusion of pectinases has a commercial application to soften the peel of citrus fruits for removal. This technique may expand in future to replace hand cutting for the production of canned segments (Favela-Torres *et al*., 2006).

2.2.2 Coffee and Tea Fermentation

Pectinase treatment accelerates tea fermentation and also destroys the foam forming property of instant tea powders by destroying the pectins (Saito *et al*., 2004). Pectinolytic microorganisms are used in the fermentation of coffee to remove the mucilaginous coat from the coffee beans (Rangarajan *et al*., 2012).

2.2.3 Textile Processing and Bioscouring of Cotton Fibers

Pectinases have been used in conjunction with amylases, lipases, cellulases and hemicellulases to remove sizing agents from cotton in a safe and eco-friendly manner, replacing toxic caustic soda used for the purpose earlier (Rajendran *et al*., 2011). Bio-scouring is a novel process for removal of non-cellulosic impurities from the fiber with specific enzymes. According to Hoondal *et al*. (2002) pectinases have been used for this purpose without any negative side effect on cellulose degradation.

2.2.4 Degumming of Plant Bast Fibers

Bast fibers are the soft fibers formed in groups outside the xylem, phloem or pericycle. For example Ramie and sunn hemp. The fibers contain gum, which must be removed before its use for textile making (Hoondal *et al*., 2002). The chemical degumming treatment is polluting, toxic and non-biodegradable. Biotechnological degumming using pectinases in combination with xylanases presents an eco-friendly and economic alternative to the above problem (Kapoor *et al*., 2001).

2.2.5 Waste Water Treatment

Vegetable food processing industries release pectin, containing wastewaters as by-product. Pretreatment of these wastewaters with pectinolytic enzymes facilitates removal of pectinaceous

material and renders it suitable for decomposition by activated sludge treatment (Hoondal *et al*., 2002).

2.2.6 Paper and Pulp Industry

During papermaking, pectinase can deploymerizepectins and subsequently lower the cationic demand of pectin solutions and the filtrate from peroxide bleaching (Reid and Richard, 2004).

2.2.7 Animal Feed

Pectinases are used in the enzyme cocktail, used for the production of animal feeds (Hoondal *et al*., 2002). This reduces the feed viscosity, which increases absorption of nutrients, liberates nutrients, either by hydrolysis of non-biodegradable fibers or by liberating nutrients blocked by these fibers, and reduces the amount of faeces (Murad and Azzaz, 2011).

2.2.8 Purification of Plant Viruses

In cases where the virus particle is restricted to phloem, alkaline pectinases and cellulases can be used to liberate virus from the tissues to give very pure preparations of the virus (Ahlawat *et al*., 2008).

2.2.9 Improvement of Chromaticity and Stability of Red Wines

Pectinolytic enzymes added to macerated fruits before the addition of wine yeast in the process of producing red wine resulted in improved visual characteristics (color and turbidity) as compared to the untreated wines. Enzymatically treated red wines presented chromatic characteristics, which are considered better than the control wines. These wines also showed greater stability as compared to the control (Keller *et al*., 2006).

2.3 Pectic Substances

Pectic substances are complex, high molecular weight, acidic and negatively charged biomacromolecule (Jayani *et al*., 2005) which consist of 17 different monosaccharides and at

least seven different polysaccharides (Jacob, 2009). In plant cell wall, pectic substances and hemicelluloses act as the cementing agent for celluloses microfibrils in order to provide strength and stability to the structure of cell wall (Geetha *et al*., 2012). Generally, pectic substances are ubiquitous to plant kingdom and copiously found in the lamella between the primary cell walls of adjacent young plant cells in form of calcium pectate and magnesium pectate which are responsible for the cell cohesion (Jayani *et al*., 2005). In young and developing cell wall, pectic substances are synthesized in the Golgi apparatus from UDP-D-galacturonic acid during the early stage of plant growth (Hoondal *et al*., 2002).

2.3.1 Classification of Pectic Substances

According to Kashyap *et al*. (2001), pectic substances are classified based on the modification type of its backbone chain. Hence, different type of pectinolytic enzymes will react accordingly to this variety of pectic substances depending on its specificity and mode of action (Yadav *et al*., 2009).

2.3.1.1 Protopectin

Protopectin is the "parents" of the pectic substances. The hydrolysis of protopectin yield pectin or pectinic acid (Kashyap *et al*., 2001; Jayani *et al*., 2005). This kind of pectic substances is a water insoluble component and mainly found in the plant tissue predominantly in the middle lamella between the plant cells. The insolubility of this protopectin is due to its large molecular weight, salt bonding between the carboxyl group and also the ester bond structure between the carboxylic group of protopectinor the hydroxyl group of other constituents in the cell wall (Hoondal *et al*., 2002).

2.3.1.2 Pectinic Acid

Pectinic acid is composed of polygalacturonan chain that consists of more than 75 % of methylated galacturonate units. The salt of pectinic acid which is either neutral or acidic is known as pectinates (Jayani *et al*., 2005). Pectinic acid alone has a typical property in forming gel with acid or sugar under suitable condition. In addition, gel can also be formed with certain metallic ions or other compounds such as calcium salts if the pectinic acid is low in methoxyl content (Hoondal *et al*., 2002).

2.3.1.3 Pectin

Pectin is also known as polymethylgalacturonate. It is a soluble polymeric material which confers rigidity to the cell wall structure after binding together with cellulose (Jayani *et al*., 2005). Generally, 75% of the carboxyl groups of the galacturonate monomer are esterified with methanol (Hoondal *et al*., 2002).

Previous research had reported that pectin is copiously found in plants including fruits and vegetables (Chaudhry and Suneetha, 2012). Approximately, 0.5 – 4 % of total plant fresh weight was constituted of pectin (Jayani *et al*., 2005). According to Jacob (2009), pectic substances play an imperative role in the plant cell growth and cell differentiation during the development of the plant tissue. New materials will be laid down and the old materials will be removed or degraded by the pectinolytic enzymes. While in an immature fruit, the pectin binds to the cellulose microfibrils which contribute to the stability and rigidity of the cell wall. However, during the ripening stage, the main backbone chain or the side chain of pectin is changing due to the naturally occurring pectinolytic enzymes reaction (Martin *et al*., 2004). Consequently, the rigid cell wall is softened since the pectin becomes more soluble resulting from the natural chain modification when the fruit turns to ripe (Kashyap *et al*., 2001).

2.4 Structure of Pectin

2.4.1 Primary Structure of Pectin

According to Chaudhry and Suneetha (2012), chemically, pectic substances are a complex colloidal acid polysaccharides with a long unbranched galacturonic acid backbone chain and linked together by α, 1-4 linkages (Figure 1). This is the basic unit of pectic substances which is also known as homogalacturonan (HG). Galacturonic acid which is the monomer of this polysaccharide can be methyl esterified at C-6 and the hydroxyl group at C-2 or C-3 can be acetylated (Reddy and Sreeramulu, 2012).

2.4.2 Secondary Structure of Pectin

The secondary structure of pectin (Figure 2) is composed of two main regions which are "smooth region" (homogalacturonan) and "hairy regions" (rhamnogalacturonan I and II) (Pedrolli *et al*., 2009). Homogalacturonan constitutes the backbone chain of pectin and composed of α, 1-4 linked D-galacturonic acid residues. Rhamnogalacturonan I is a highly branched region which comprised of a huge number of side chain α, 1-2 linked L-rhamnopyranose residues. Meanwhile, the more complex Rhamnogalacturonan II is predominantly found in primary cell wall of plants and plays a significant role as a signal molecule in the development of plant cell wall. This biomacromolecule form a very flexible, extended and curve (worm like) structure in solution (Yadav *et al*., 2009)

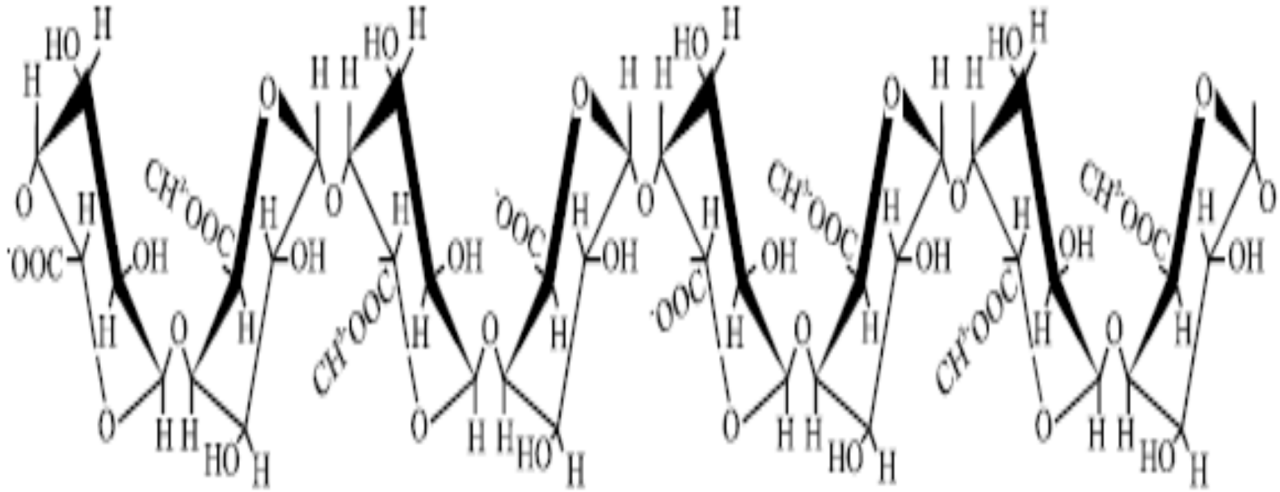

Figure 1: Primary structure of Pectin (Jacob, 2009)

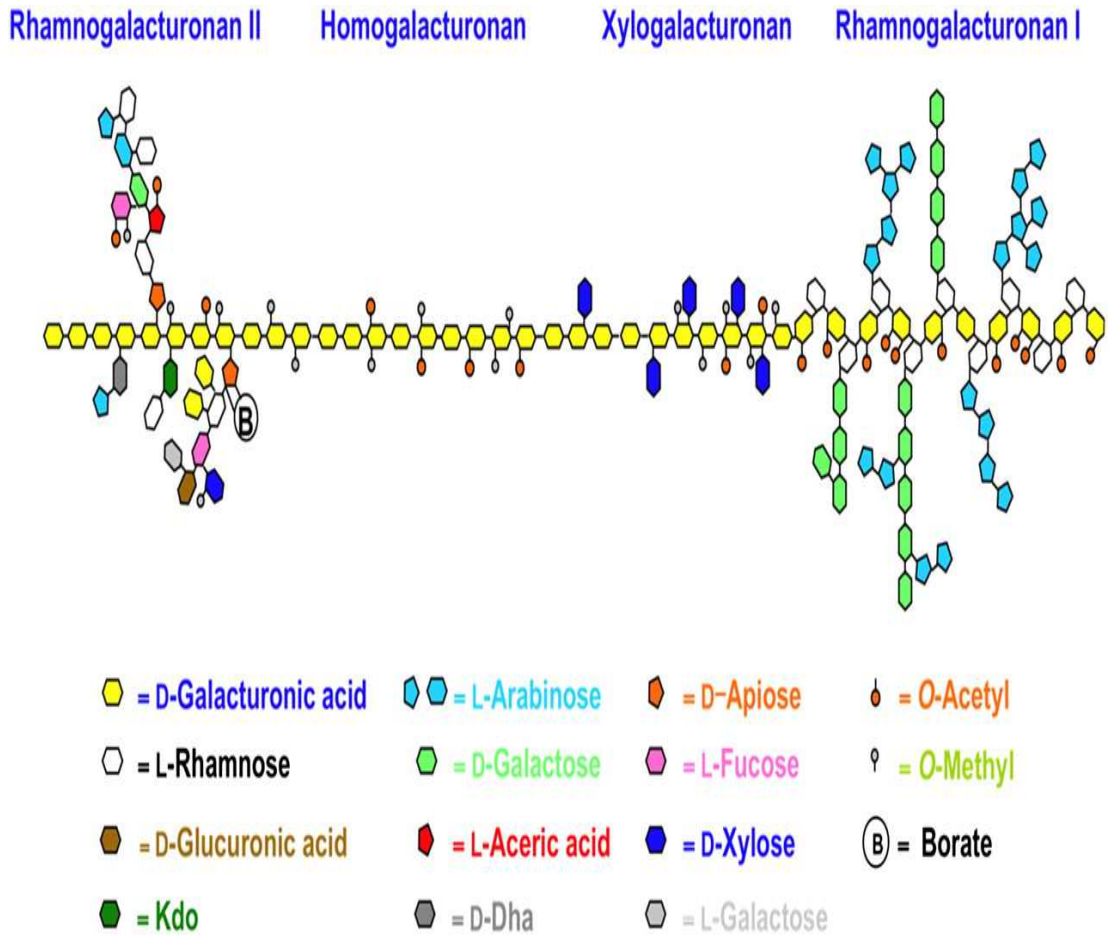

Figure 2: Secondary structure of pectin (Yadav *et al*., 2009)

2.5 Microorganisms for Pectinases Production

2.5.1 Bacteria Pectinases

Bacterial pectinases is produced mainly by bacteria belonging to genera *Bacillus* and *Erwinia*. Bacterial pectinases biosynthesis has been extensively studied in phytopathogens, especially in the soft-rotting *Erwinia* species, *E. carotovora* and *E. chrysanthemi* which are reported to produce a set of pectinolytic enzymes (Matsumoto *et al.*, 2003; Agyei *et al*., 2016). Other bacterial genera reported to have pectinolytic properties include species of *Pseudomonas* (Sohail and Latif, 2016), *Streptomyces* (Ramirez-Tapias *et al.*, 2015). The genus Bacillus and Cocci comprises several varieties of industrially important species contributing approximately half of the existing commercial production of bulk enzymes (Aaisha and Barate, 2016). *Bacillus* species have been the imperative industrial enzyme producers with roles in applied microbiology for over a millennium. Because of several reasons *Bacillus* species continueto be the predominant bacterial enzymes producers in microbial fermentations (Schallmey *et al.*, 2004; Satyanarayana *et al.*, 2005; Ali *et al*., 2016). It is evident from many research works that, among different bacterial isolates screened for pectinolytic properties, *Bacillus* strains were selected as the most potent enzyme producers (Jayani *et al.*, 2010; Rehman *et al.*, 2012; Kavuthodi *et al.*, 2015; Sohail and Latif, 2016). Alkaline pectinases have a wide variety of industrial applications and bacteria mainly *Bacillus* spp. are the prominent producers. They produce large quantities of biologically active molecules generating a high potential for biotechnological and biopharmaceutical applications (Stein, 2005; Ariyaei *et al*., 2019). Another major feature that makes these groups predominant is that most of them are environmental friendly, don't require complex nutritional requirements and are easy to grow and handle (Sohail and Latif, 2016; Poonam, and Nivedita, 2018). *Bacillus* species such as *B. subtilis* and *B. licheniformis* are on the

Food and DrugAdministration's GRAS (generally regarded as safe) status (Schallmey *et al.*, 2004; Mohandas *et al*., 2018). Various properties of *Bacillus* strains are reported which make them superior in industrial biotechnology, including their high growth rates leading to short fermentation cycle times, ability to secrete proteins into the extracellular medium (Barros *et al.*, 2013; Mohandas *et al*., 2018) and their ability to adapt with changing environmental and nutritional conditions (Buescher *et al.*, 2012; Nicolas *et al.*, 2012). Pectinases production from *Bacillus* spp. can also be achieved economically by utilizing different agro-waste as substrate. It is proved that orange peel waste can be used as a sole carbon source for pectinase production by various strains of *Bacillus* (Embaby *et al.*, 2014; Tepe and Dursun, 2014; Kaur and Gupta, 2017; Obafemi *et al*., 2019). Various other agro-industrial wastes such as; rice bran, cassava bagasse, sugar beet pulp, carrot peels etc. are also exploited for pectinase production by *Bacillus* spp. (Ghazala *et al.*, 2015; Nawawi *et al.*, 2017). The time required for the production of pectinases by *Bacillus* spp. are remarkably less when compared to other microbial pectinases earlier reported (Kashyap *et al.*, 2001; Adetunji and Adejumo, 2018). Previous reports indicated that maximum pectinases production through many strains of *Bacillus* can be attained within 48 hours of fermentation period. The optimum condition for pectinase production by *B. firmus* was recorded at a fermentation period of 18 hours and the enzyme production was declined after 30 hours of incubation (Roosdiana *et al*., 2013). Whereas, pectinases production by *B. subtilis* through solid state fermentation started after 18 hours of incubation and the production reached highest at 24 hours (Ahlawat *et al.*, 2008). Pectinases production using *Bacillus* sp. can be attained by either solid state fermentation (SSF), submerged fermentation (SmF) or by both. Medium optimization was found to be effective in both SSF and Smf for pectinases production using *Bacillus* strains (Ghazala *et al.*, 2015; Bibi *et al.*, 2016; Kaur and Gupta, 2017). Zou *et al*.

(2014) developed a new Fed-Batch fermentation for enhanced production of alkaline pectinases using *B. subtilis*. The process combines the enzymatic pretreatment of the carbon source with controlled pH of the fermentative broth to enhance enzyme production in a cheap manner (Zou *et al.*, 2014).

2.5.2 Fungi Pectinases

Filamentous fungi have been used for more than 50 years in the production of industrial enzymes (Frinkler *et* al., 2017). Many extracellular enzymes are produced by fungi which are capable of decomposing organic matter and one of such enzyme is pectinolytic enzymes (Mario *et al*., 2019). Filamentous fungi are considered as one of the most potent producers of pectinases and they can be employed extensively in SSF for the economic production process. Various types of fungal species have been reported to be employed for the production of pectinases. *Aspergillus niger* is the most commonly used fungal species for industrial production ofpectinolytic enzymes (Gummadi and Panda, 2003). Findings on the isolation, characterization, selection, properties and fermentation of *A. niger* strains for the production of pectinolytic enzymes using different substrates was made by different workers (Finkler *et al.*, 2017). Other species of *Aspergillus* were also reported to produce pectinase including *A. oryzae, A. fumigatus, A. terreus, A. soje, A. awamori* etc (Pedrolli *et al.*, 2009; Garg *et al.*, 2016). Production of endo-polygalacturonase was firstly reported in *A. giganteus* (Pedrolliand Carmona, 2014). Further, species of *Penicillium, Fusarium, Mucor, Neurospora crassa*, etc also have role in pectinase production (Pedrolli *et al.*, 2009; Pedrolli and Carmona, 2014; Garg *et al.*, 2016). The fungi produces these enzymes to break down the middle lamella in plants so that it can extract nutrients from theplant tissues and insert fungal hyphae. Nwodo-Chinedu *et al*. (2005) isolated some cellulolytic fungi from wood-wastes in Lagos, Nigeria. The strains of *A. niger* (ANL 301) and *P. chrysogenum* (PCL501)

isolated by the group were reported to produce cellulases (Chinedu*et al*., 2008a) and xylanases (Chinedu*et al*., 2008b; Okafor *et al*., 2007). Thus, these fungi produce the full complement of enzymes required for the hydrolysis of cellulosic biomass. This explains why the fungi thrive on waste plant matter and are capable of utilizing such waste materials as carbon sources in their culture media (Nwodo-Chinedu *et al*., 2007). Phutela *et al*. (2005) reported an incubation period of 48 hours for optimal pectinases production for some fungal isolates. Castilho *et al*. (2000) reported maximum pectinase production by *A. niger* at the third day of fermentation period. According to Demir and Tari (2014), free water is an essential parameter for maximum enzymatic activity and the higher moisture contents favor the swell of wheat bran particles and increase the surface area. Moreover, the spaces between the swollen particles help vegetative growth and eventually increase the solubility of nutrients present in the wheat bran structure. Abdul *et al*. (2009) performed a multistep evaluation of 52 strains of *A. niger* on the basis of polygalacturonase production. Plate screening method was employed for preliminary screening of isolates. The strains exhibiting relative clear zone with diameter above 1.386 centimeter were selected for final screening. Out of 52 strains, 23 were selected for further screening by using optimized enzyme assay method. The enzyme production by 23 selected strains was studied in submerged fermentation with pectin as the only carbon source. Maximum activity was observed in two isolates of *A.niger* (H12 and H51) showing more than 0.700 units after 96 hours incubation period. Only four isolates (H06, H13, H45, and H46) gave the maximum activities after incubation for 72 hours whereas decline was observed at 96 hours.

Lali*et al*. (2009) investigated the physiology and some biochemical characteristics of the selected strains. The nutrient medium for each particular strain was optimized and conditions of growth were established. The strain *Penicillium canescens* I-85 reveals the highest pectinase activity at

27 ^{0}C and pH 4.0; the strain *A. niger* at 40 ^{0}C, pH 6; *Trichoderma viride* Ts-2 at 30 ^{0}C, pH 7.5. As a result of optimization of the Nutrient media, the activity of pectinase increased by 122, 28 and 98 %, respectively. Saranraj and Naidu (2014) reported that under solid state fermentation exo and endo pectinases activities of *A. niger* increased with increase in the concentration of carbon source and also reported that pectinase activities by *A. niger* sharply decreased when glucose was added to the pectin containing culture medium. Urmila *et al.* (2005) reported that the fungus *A. fumigatus* exhibited maximum activity of polygalcturonase enzyme in medium containing pectin as substrate. Although an increased level in the production of pectinases was noticed when the agro wastes were supplemented with additional carbon and nitrogen sources and supplementation of sucrose was more effective than glucose in solid state fermentation (Patil and Dayanand, 2006). According to Shoichi *et al*. (2001) the initial pH of the medium has a great effect on the growth of the organism, on the membrane permeability, also on the biosynthesis and stability of the enzyme.

2.6 Fermentation Conditions

Fermentation is the technique of biological conversion of complex substrates into simple compounds through the action of enzymes. Microbial fermentation is the enzymatic decomposition or utilization of substrates into simple sugars by microorganisms such bacteria, yeast and fungi. In the course of this metabolic breakdown, they also release several additional compounds apart from the usual products of fermentation, such as carbon dioxide and alcohol. These additional compounds are called secondary metabolites. Secondary metabolites range from several antibiotics to peptides, enzymes and growth factors (Robinson *et al*., 2001; Machado *et al*., 2004). Pectinases are constitutive or inducible enzymes that can be produced either by submerged (Aquilar and Guitron, 1999) or solid state fermentation (Robinson *et al*.,

2001). Various factors related to environment affect the production of pectinases. Some of them are concentration of nutrients, pH, temperature, moisture content, influence of extraction parameters on recovery of pectinases and the effects played by the inducers. Both carbon and nitrogen sources show overall effect on the productivity of pectinases (Catarina *et al*., 2003; Almeida and Huber, 2011). Pectin, glucose and sucrose when added to the media in higher concentration have a repression effect on the studied enzyme activity (Maria *et al*., 2000) of the various nitrogenous matters that can be used. Optimum sources are $(NH_4)_2SO_4$, yeast extract, soya bean pulp powder, soya peptone.

Temperature and pH are also important parameters to be taken note of. The pH is regulated using a mixture of sources of nitrogen salts but when *A. niger* is being used, pH tends to be acidic. Besides, the nature of the substance also plays a vital role in the pH maintenance. Moisture content in the substrate also plays a significant role (Martin *et al*., 2004). Previous studies have shown that generally,moisture maintained around 50-55 % is good for the production of pectinases by microbial means (Leda *et al*., 2000). Two types of fermentations can be carried out for pectinases production, they are solid state fermentation and submerged fermentation. The growth of organisms is very high with large quantities of enzyme being produced in solid- state fermentation (Demir *et al*., 2012). However in the production of extracellular pectinases, submerged fermentation is preferable as the extracellular pectinases are easier and cheaper to use in great quantities. Most important applications of these enzymes are in juice and wine making, and in the processing of vegetables. Submerged or solid state mediums are used for production of pectinolitic enzymes by fungi (Leda *et al*., 2000).

2.6.1 Solid State Fermentation

This involves the cultivation of microorganisms on moist solid supports, either on inert carriers or on insoluble substrates that can be used as carbon and energy source. This process occurs in the absence or near absence of free water in the space between substrate particles. In this system, water is present in the solid substrate whose capacity for liquid retention varies with the type of material (Pandey *et al*., 2000). However, SSF process possess several advantages over submerged fermentation (SmF) process, even though this process present only at laboratory level (Vidyalakshmi *et al*., 2009). This is also supported by Wang and Chen (2009), who stated that the surface adhesion-based culture are productive than SmF. Several advantageous of SSF are higher fermentation productivity, higher end concentration of products and stability, lower catabolic repression, cultivation of microorganism specifically for insoluble substrate and also lower demand on sterility due to low water activity used (Holker *et al*., 2004).

2.6.2 Submerged Fermentation

This is the cultivation of microorganisms in liquid nutrient broth. Industrial enzymes can be produced using this process. This involves growing carefully selected microorganisms (bacteria and fungi) in closed vessels containing a rich broth of nutrients (the fermentation medium) and a high concentration of oxygen (Kashyap *et al*., 2001). The bioactive compounds are secreted into the fermentation broth. The substrates are utilized quite rapidly; hence need to be constantly replaced/supplemented with nutrients (Vidyalakshmi *et al*., 2009).

However, there are also several disadvantages of SSF which have discouraged the use of this technique for industrial production and therefore have made SmF more applicable in the production of enzymes. These include: the buildup of gradients of temperature, pH, moisture,

substrate concentration or CO_2 during cultivation which are difficult to control under limited water availability (Holker *et al*., 2004).

2.7 History and Description of Banana

Banana has been described as a familiar tropical fruit. From its native South western Pacific home, the banana plant spread into the Islands of the Pacific and to the West Coast of Africa as early as 200-300 BC (Agarwal *et al*., 2009). *Musa sapientum*is a herbaceous plant (up to 9 m long) with a robust treelike pseudostem, a crown of large elongated oval deep-green leaves (up to 365 cm in length and 61 cm in width), with a prominent midrib. Each plant produces a single inflorescence like drooping spike, and large bracts opening in succession, ovate, 15-20 cm long, concave, dark red in color and somewhat fleshy (Gilbert *et al*., 2009).Fruits are oblong, fleshy, 5-7cm long in wild form and longer in the cultivated varieties. The inflorescence is big with a reddish brown bract and is eaten as vegetables. The ripe fruits are sweet, juicy and full of seeds and the peel is thicker than other banana (Agarwal *et al*., 2009). Banana plants are propagated vegetatively by rhizome cuttings. They need temperatures ranges of 25 to 30 ^{0}C. They can grow in hotter temperatures, providing they are given extra water, and they can survive in temperatures down to 14 ^{0}C (Mokbel and Hashinaga, 2005). They also need lots of water, high humidity, and rich, fertile soil. They prefer a more sheltered environment to protect them from high winds and prevent them from getting tattered leaves. Depending on the location, bananas can grow all year round (Mokbel and Hashinaga, 2005).

Table 1: Comparing Solid State and Submerged Fermentation Techniques

Factor	Submerged fermentation	Solid state fermentation
Water	High volumes of water is required and effluents discarded	Low or very little amount of water is required and no effluent
pH control	Easy control of pH	Very difficult to control
Temperature	Easy to monitor and control	Not easy to control
Aseptic conditions	Heat sterilization and aseptic control	Vapour treatment and non-sterile conditions
Subtrates	Soluble substrates	Polymer insoluble substrates
Harvesting	Less time consuming as it can be done either through filtration (fungi) or centrifugation (bacteria and yeast)	It takes a longer time as it require another step of sonication or grinding of the mycelium to release the enzyme
Industrial application	Regularly used	Rarely use
Yield	Moderate	Very high
Labour	Less labour required	More labour required

(Vidyalakshmi *et al*., 2009)

2.7.1 Taxonomical Classification of Banana

Kingdom: Plantae

Division: Magnoliophyta

Class: Liliopsida

Order: Zingiberales

Family: Musaceae

Genus: *Musa*

Species: *Musa sapientum*

Source: (Gilbert *et al*., 2009)

2.8 Uses of Banana

2.8.1 Textile Industry

Banana fibre is reportedly used as blending material in textile industry in countries like Philippines, Malaysia, Japan and Korea. It can be easily blended with other fibres such as jute and mesta being natural fibres(Mohapatra *et al*., 2010). Therefore, lots of industrial products like gunny bags, door mats, carpets, yarn, ropes, geo-textiles, trivialities, luggage carriers and interior decorative crafts paper, tissue paper, paper bag, etc. can be made from this fibre, where great strength is required. It has also some other industrial uses such as natural sorbent, as a base material for bioremediation and recycling and as a natural water purifier (Mohapatra *et al*., 2010).

2.8.2 Paper Industry

Banana fibre can be an alternative raw material of paper industries like writing paper, anti-grease paper, cheque paper as well as hard board industries (Mokbel and Hashinaga, 2005). At first, raw paper materials are collected from banana plants and fibres are collected afterwards. The

collected fibres are soaked in water prior to make pulp. Later, the extracted fibre is bleached by microbial treatment using *Trichoderma* and *Pythium* for 3 to 5 days (Mokbel and Hashinaga, 2005). These fungi acts on cellulose and breaks the bonds between lingo cellulosic complex structures and lignin and hemicelluloses are broken down and leached out. It enhances the brightness of the paper and helps to soften the fibre as well as do pulping process easier (Mokbel and Hashinaga, 2005; Mohapatra *et al*., 2010).

2.8.3 Drinks

A kind of soft drink could also be prepared from sugar syrup that is left out during candy preparation from banana as well as directly from central core sap. These prepared drinks are fortified with flavours which have been standardized for human consumption (Mohapatra *et al.*, 2010).

2.8.4 Microcrystalline cellulose (MCC)

Previous reports also confirm that MCC can be extracted from banana fibre and use for industrial purpose. It is used in pharmaceutical industries. It has huge application not only in pharmaceuticals but also in bakery, beverages, animal health products, etc. (Emega *et al*., 2008).

2.9 *Aspergillus niger*

Aspergillus niger is a fungus and one of the most common species of the genus Aspergillus. It causes a disease called black mould on certain fruits and vegetables such as grapes, apricots, onions and peanuts, and is a common contaminant of food (Pel *et al*., 2007). It is ubiquitous in soil and is commonly reported from indoor environments, where its black colonies can be confused with those of *Stachybotrys* (species of which have also been called black mould). Some strains of *A. niger* have been reported to produce potent mycotoxins called ochratoxins; other sources disagree, claiming this report is based upon misidentification of the fungal species.

Recent evidence suggests some true *A. niger* strains do produce ochratoxin A (Mongensen *et al*., 2009).

A. niger is cultured for industrial production of many substances. Various strains of *A. niger* are used in the industrial production of citric acid (E330) and gluconic acid (E574) and have been assessed as acceptable for daily intake by the World Health Organization. *A. niger* fermentation is generally recognized as safe (GRAS) by the United States Food and Drug Administration under the Federal Food, Drug, and Cosmetic Act (Mogensen *et al*., 2009).

Many useful enzymes are produced using industrial fermentation of *A. niger*. For example, *A. niger* glucoamylase is used in the production of high-fructose corn syrup, and pectinases are used in cider and wine clarification. Alpha-galactosidase, an enzyme that breaks down certain complex sugars, is a component of beano and other products that decrease flatulence. Another uses of *A. niger* within the biotechnology industry is in the production of magnetic isotope-containing variants of biological macromolecules for nuclear magnetic resonance analysis (Pel *et al*., 2007).

CHAPTER THREE

3.0 Materials and Methods

3.1 Collection of Banana Samples

A bunch of Cavendish banana used for this study was purchased from Ipata market in Ilorin, Ilorin East Local Government Area of Kwara State. The banana sample was identified in the Herbarium Section of Plant Biology Department, University of Ilorin and a voucher number UILH/001/2019/1249 was given.

3.2 Preparation of Substrate

The ripe banana peel were separated from the fruit pulps and cleaned by washing under tap, and air dried to reduce the moisture content. After air drying, the peel were chopped into small bits of uniform sizes, which were afterwards pulverized into powdery form with the aid of a Binatone blender (model BLG-650), sieved with a very fine mesh of about 0.06mm in diameter and stored in an air-tight container for further use.

3.3 Collection of Soil samples

Soil samples were collected from three different locations of fruits waste dumping sites in Ipata market, Ilorin East Local Government of Kwara State. The soil samples were obtained from 12cm depth below the surface using a sterile hand trowel and kept in sterile sample bottle. The collected soil samples were taken to the laboratory within 3 hours of collection for immediate analyses in accordance with the method of Venkata and Divakar (2013).

3.4 Sterilization of Glass Wares

The sterilization of glass wares such as conical flasks, beaker and test tubes after washing with detergent was carried out in hot air oven at 160 ^{0}C for 1 hour according to the procedure described by Ugoh and Ijigbade (2013).

3.5 Isolation of Pectinolytic Fungi

Fungi were isolated from soil samples using the pour plate technique. One gram of soil sample was dissolved in 9 ml sterilized distilled water. The soil suspension was diluted up to 10^5. The samples were inoculated on already prepared Potato dextrose agar-streptomycin plates. The inoculated plates were incubated at ambient temperature (28 ± 2 ^{0}C) for 2 days. Colonies developments were observed after incubation period (Ratnasri *et al*., 2014).

3.6 Preparation of Pure Cultures of Fungal Isolates

The young fungal hyphae were aseptically picked up and transferred to fresh sterile Potato dextrose agar-streptomycin plates to obtain pure cultures. The pure cultures on Potato dextrose agar-streptomycin plates were grown at 28 ± 2 ^{0}C for 3 days and stored in the refrigerator at 4 ^{0}C until required for further use. The isolates were sub-cultured to obtain young cultures for further studies (Ugoh and Ijigbade, 2013).

3.7 Fungal Identification

3.7.1 Macroscopic and Microscopy Characterization

Isolates obtained were characterized and identified on the basis of their colonial and morphological characteristics which includes macroscopic and microscopic examinations. Among the characteristics used were colonial characteristics such as size, surface appearance, texture, reverse and pigmentation of the colonies (Sharma and Rajak, 2003). In addition, microscopy revealed vegetative mycelium including presence or absence of cross walls, diameter of hyphae, and types of asexual and sexual reproductive structures. Appropriate references were then made using pictorial atlas of soil and seed fungi morphologies in accordance with Watanabe (2002). Further molecular identification was done to confirm the phylogeny and evolutionary relationship of the isolate with the best pectinolytic ability after screening.

3.7.2 Molecular Characterization

1. DNA was isolated from *A. niger* isolate using the Fungal Genomic DNA isolation kit (Qiagen).

2. Using consensus primers, 18S rRNA, ITS1, 5.8S rRNA, ITS2, and 28S rRNA gene fragment were amplified using PCR.

3. The forward primer (5'–3') GGAAGTAAAAGTCGTAACAAGG and reverse primer (5'–3') GGTCCGTGTTTCAAGACGG was used for DNA amplification.

4. PCR amplification was then performed in a 15-µL reaction mixtures containing approximately 1 µL (50 ng/µL) DNA template, 7.5 µL 2× Taq PCR MasterMix, 5.9 µL ddH2O, and 0.3 µL of each primer (10 µmol/µL), performed in PCR cycling conditions of 35 cycles of 94 ^{0}C for 30 s (denaturation), primer annealing at 55 ^{0}C for 50 s, and 72 ^{0}C for 150 s, and a final extension at 72 ^{0}C for 10 minutes.

5. PCR products were then electrophoresed on 1.5 % agarose gels using 1× TAE buffer (40 mMTris acetate; 1 mM EDTA; pH 8), containing 200 ng/mL ethidium bromide.

6. Sequencing of PCR products were conducted by excising the bands from the agarose, purified and sequenced.

7. After sequencing of the PCR products with a known primer, the phylogenetic tree was constructed using Neighbor-joining method derived from analysis of the 18S rRNA gene sequences of native isolates and related sequences obtained from NCBI (Al-najada *et al*., 2012).

3.8 Screening of Fungal Isolates for Pectinase Production

The pure isolates were screened for pectinase production by inoculating the organisms on Czapek-dox-pectin agar medium plates and incubated at 28 ± 2 ^{0}C for 5 days. After incubation, the plates were flooded with iodine solution and then incubated for 15 minutes at room

temperature. A clear zone around the growth of microorganisms indicated pectinases activity (Venkata and Divakar, 2013).

3.9 Fermentation for Pectinase Production

3.9.1 Inoculum Development

The inoculum size was developed by modification of the method described by Dhillon *et* al. (2004). Five milliliter of sterile distilled water was dispensed into fully sporulated *Aspergillus niger* slant. An inoculating needle was used to dislodge the spore clusters under aseptic conditions and then it was shaken thoroughly to prepare homogenized spore suspension. One ml of the suspension contains about 1×10^6 spores ml^{-1}.

3.9.2 Fermentation Process

The fermentation process was carried out in 250 ml Erlenmeyer flasks containing 100 ml of mineral solution and 10 g of banana peel powder under optimal fermentation condition. These optimal conditions are pH of 5 and in the presence of sucrose as the carbon source. After sterilization by autoclaving at 121 ^{0}C for 15 min, the flasks were cooled and inoculated with 2 ml of 1×10^6 spores ml^{-1}. The contents of the flasks were mixed thoroughly to ensure uniform distribution of inoculum and incubated at 50 ^{0}C for 7 days (Ratnasri *et al*., 2014).

3.9.3 Extraction of Crude Enzyme

At every day of fermentation, the contents of the flasks were filtered using Whatsman No 1 filter paper. The resulting filtrate was used as the crude enzyme and kept inside a refrigerator for further use (Jacob *et al*., 2009).

3.9.4 Assay for Pectinase Activity

Two milliliters of the crude enzyme was added to 2 ml of 1 % pectin-phosphate buffer solution in test tubes and incubated for 30 minutes at 40 ^{0}C. After incubation, the content was filtered

using Whatsman No 1 filter paper, 0.5 ml of the resulting filtrate was added to test tubes and 0.5 ml of DNS reagent was added to stop the reaction. The test tubes were placed in a boiling water bath for 5 minutes, after which they were left to cool at room temperature. The content of the test tubes was diluted with 4 ml of distilled water and absorbance was determined at 540 nm using Jenway 6320D Spectrophotometer. The amount of reducing sugar was extrapolated from galacturonic standard curve. One unit of pectinase activity (UI) was defined as the amount of enzyme that released 1 mg of galacturonic acid in 1 minute under standard assay condition (Martins *et al*., 2000; Adeleke *et al*., 2012; Venkata and Divakar, 2013).

3.10 Optimization Parameters for Pectinases Production

3.10.1 Effect of Incubation Period on Pectinases Production

The fermentation process was carried out in 250ml Erlenmeyer flasks containing 10 g of substrate and 100 ml of sterile distilled water. After sterilization by autoclaving at 121 ^{0}C for 15 minutes, the flasks were left to cool and inoculated with 1ml of 1×10^6 spores ml^{-1}. The content of the flasks were mixed thoroughly and incubated at 28 ± 2 ^{0}C for 7 days. Samples were taken every day and pectinase activity was determined as previously described (Adeleke *et al*., 2012; Venkata and Divakar, 2013).

3.10.2 Effect of Salt Supplements on Pectinase Production

The fermentation process was carried out in two 250 ml Erlenmeyer flasks. Ten gram of substrate was introduced into two 250 ml Erlenmeyer flasks containing 100 ml of mineral solution supplemented with salts and the other flask containing 100 ml of sterile distilled water respectively. The flasks were sterilized by autoclaving at 121 ^{0}C for 15 minutes, after which the flasks were left to cool and inoculated with 1ml of 1×10^6 spores ml^{-1}. The content of the flasks were mixed thoroughly and then incubated at 28 ± 2 ^{0}C for 7 days. Samples were taken every day

and pectinase activity was determined as previously described (Adeleke *et al*., 2012; Venkata and Divakar, 2013).

3.10.3 Effect of Additional Carbon Source on Pectinase Production

Four different carbon sources were used which included: maltose, fructose, glucose and sucrose. The fermentation process was carried out in 250 ml Erlenmeyer flasks containing 10 g of substrate and 100 ml of mineral solution supplemented with respective carbon source. After sterilization by autoclaving at 121 ^{0}C for 15 minutes, the flasks were left to cool and inoculated with 1 ml of 1 × 10^6spores ml^{-1}. The content of the flasks were mixed thoroughly and then incubated at 28 ± 2 ^{0}C for 7 days. Samples were taken every day and pectinase activity was determined as previously described (Adeleke *et al*., 2012; Venkata and Divakar, 2013).

3.10.4 Effect of Inoculum Size on Pectinase Production

The fermentation process was carried out in 250 ml Erlenmeyer flasks containing 10 g of substrate and 100 ml of mineral solution with sucrose (optimum carbon source from 3.10.3) as additional carbon source. After sterilization by autoclaving at 121 ^{0}C for 15 minutes, the flasks were left to cool and inoculated with different inoculum sizes of 1, 1.5, 2 and 2.5 ml of 1 × 10^6 spores ml^{-1}. The content of the flasks were mixed thoroughly and then incubated at 28 ± 2 ^{0}C for 7 days. Samples were taken every day and pectinase activity was determined as previously described (Adeleke *et al*., 2012; Venkata and Divakar, 2013).

3.10.5 Effect of pH on Pectinase Production

The fermentation process was carried out in 250 ml Erlenmeyer flasks containing 100 ml of mineral solution and sucrose as additional carbon source. The pH of the medium was varied and adjusted to 4, 5, 7 and 8 in accordance with the method of Patil *et al*. (2012). Ten gram of the substrate was introduced into the flasks and sterilized at 121^{0}C for 15 minutes. After sterilization,

the flasks were left to cool and inoculated with 2 ml of 1×10^6 spores ml^{-1} (optimum inoculum size from 3.10.4). The content of the flasks were mixed thoroughly and then incubated at 28 ± 2 ^{0}C for 7 days. Samples were taken every day and pectinase activity was determined as previously described (Adeleke *et al*., 2012; Venkata and Divakar, 2013).

3.10.6 Effect of Moisture Content on Pectinase Production

To determine the optimum moisture content for pectinase production under assay conditions, moisture contents was varied at 100, 150, 200, and 250n ml of mineral solution which was introduced into 500 ml Erlenmeyer flasks containing 10 g of substrate and sucrose as additional carbon source. The pH of the medium was adjusted to 6 (optimum pH from 3.10.5). The flasks were sterilized by autoclaving at 121 ^{0}C for 15 minutes, after which the flasks were left to cool and inoculated with 2 ml of 1×10^6 spores ml^{-1}. The content of the flasks were mixed thoroughly and incubated at 28 ± 2 ^{0}C for 7 days. Samples were taken every day and pectinase activity was determined as previously described.

3.10.7 Effect of Temperature on Pectinase Production

The fermentation process was carried out in 250 ml Erlenmeyer flasks containing 10 g of substrate and 100 ml of mineral solution with sucrose additional carbon source. The pH of the medium was adjusted to 6. After sterilization by autoclaving at 121 ^{0}C for 15 minutes, the flasks were left to cool and inoculated with 2ml of 1×10^6 spores ml^{-1}. The content of the flasks were mixed thoroughly and then incubated at different temperature of 30, 40, 50 and 60 ^{0}C for 7 days. Samples were taken every day and pectinase activity was determined as previously described.

3.11 Protein Content Determination

Protein content of the enzyme extract was estimated by following the method described by Mohamed *et al*. (2006) using bovine serum albumin (BSA) as the standard.

3.12 Purification of Pectinase

3.12.1 Ammonium Sulphate Precipitation

Sixty milliliter of the crude enzyme was first brought to 80 % ammonium sulphate saturation and stored at 4 ^{0}C for 24 hours. The precipitated proteins were centrifuged for 20 minutes at 4000 rpm. The resulted precipitate was dissolved in small quantity of phosphate buffer. Both enzyme activity and protein content was determined following the method described by Lukong *et al*. (2007).

3.12.2 Dialysis

The fraction obtained from $(NH_4)_2SO_4$ precipitation was introduced into 12 kb molecular weight cut off dialysis bag and dialyzed against phosphate buffer with three changes of buffer to ensure complete removal of impurities. Thereafter, pectinase activity and protein content were determined (Adejuwon and Olutiola, 2007).

3.12.3 Gel Filtration Chromatography

The partially purified pectinase obtained after dialysis was subjected to gel filtration chromatography. Sephadex G-75 was used to pack the column followed by equilibration with phosphate buffer. Fifteen ml of the enzyme was poured on top of the column and eluted with phosphate buffer without disturbing the gel surface. Twenty five fractions (5 ml each) were collected every 60 seconds in sterile sample bottle. Pectinase activity and protein determination were carried out for each individual fraction. Sharp peaks of fractions obtain were collected and investigated for the partially purified enzyme according to the method described by Lukong *et al*. (2007).

3.13 Statistical Analysis

All experiments were carried out in duplicates and results were expressed as mean ± SEM. All data collected after each experiment was subjected to one-way Analysis of Variance (ANOVA) using IBP SPSS version 20 package and the means were calculated using the Duncan's Multiple Range Test of the same software. Means that were at 0.05 levels were considered significant. Microsoft excel was used to plot all the graphs.

CHAPTER FOUR

4.0 Results

4.1 Identification of the Fungal Isolates

A total of five filamentous fungi were isolated from the soil samples obtained from fruit wastes dumping sites and their characteristics are presented in Table 2.

4.2 Screening of Fungal Isolates for Pectinolytic Ability

All the five filamentous fungi isolates exhibited pectinolytic ability as indicated by various sizes of zones of inhibition after screening. The best pectinolytic ability was observed from *Aspergillus niger* as indicated by widest zones of inhibition after screening and was selected for pectinase production.

4.3 Molecular Identification of Pectinolytic Fungus

The molecular analysis of the best pectinolytic fungus isolate shows that it has 100 % homology with *A. niger* strain FLN4e and was then identified as *A. niger* strain AH1 with GenBank accession number MK811422 as shown in Figure 3.

4.4Pectinase Activity

4.4.1 Effect of Incubation Period

The effect of incubation period on pectinase production is presented in Figure 4. Pectinase production started at day 1 as indicated by the activity and increased progressively as incubation period increased. Pectinase activity was at the peak on day 3 of incubation with activity of 167.35±0.67U/mg/min/ml after which it declined.

Table 2. Macroscopic and microscopy identification of fungal isolates obtained from fruits waste dumping sites.

Isolates	Macroscopic and microscopy characteristics	Probable organism
Isolate A	Growth on agar plate was fluffy white which turned black producing large black coloured spores after 1-2 days of growth. At reverse it was pale yellow with wrinkle mycelium growth with clear thick and long aerial hyphae, and black coloured spores were also found. There is presence of conidia, not branched and thick dense mycelium.	*Aspergillus niger*
Isolate B	Growth on potatoedextrous agar was smooth with colonies appearing as grey-blue-green after 1-2 days of incubation. At reverse, it was white to tan with septate hyphae and hyaline. Conidiphore were short, smooth and colourless or greenish, with round, columnar vesicle head.	*Aspergillus fumigatus*

Isolate C	Colonies were fast growing and covered the agar surface with a dense mycelium that was at first white becoming grey or yellowish brown. At reverse, it was pale yellow with clear thin and short hyphae, mycelia were greenish. Spore bearing heads were seen as large and globular, and were tightly packed. Chains of conidia were also seen. Conidia were greenish in colour.	*Aspergillus flavus*
Isolate D	Woolly to cottony, flat, spreading colonies were seen with 1-2 days of incubation. From the front, the colour of the colonies was white to creamy and yellowish red. At reverse, it was tan to red brown. Cushion-like mat of hyphae bearing conidiophores over its surface which was orange in colour was observed. Hyaline septate and microconidia were also seen microscopically. Microconidia has a distinct basal foot cell and pointed distal ends.	*Fusarium* sp

Isolate E	Colonies grows rapidly and filled the potato dextrose agar plate within 1-2 days of incubation. From the front, the colour of the colony was white initially, which turns grey to yellowish brown over time. At reverse, it was white to pale in colour. Non-septate hyphae, sporangiophores, rhizoids, sporangia and sporangiospores were seen microscopically. Sporangiophores were brown in colour and unbranched. Rhizoids were located at the point where stolons and sporangiohores met. Sporangiospores were round to ovoid in shape, hyaline to brown in colour.	*Rhizopus* sp

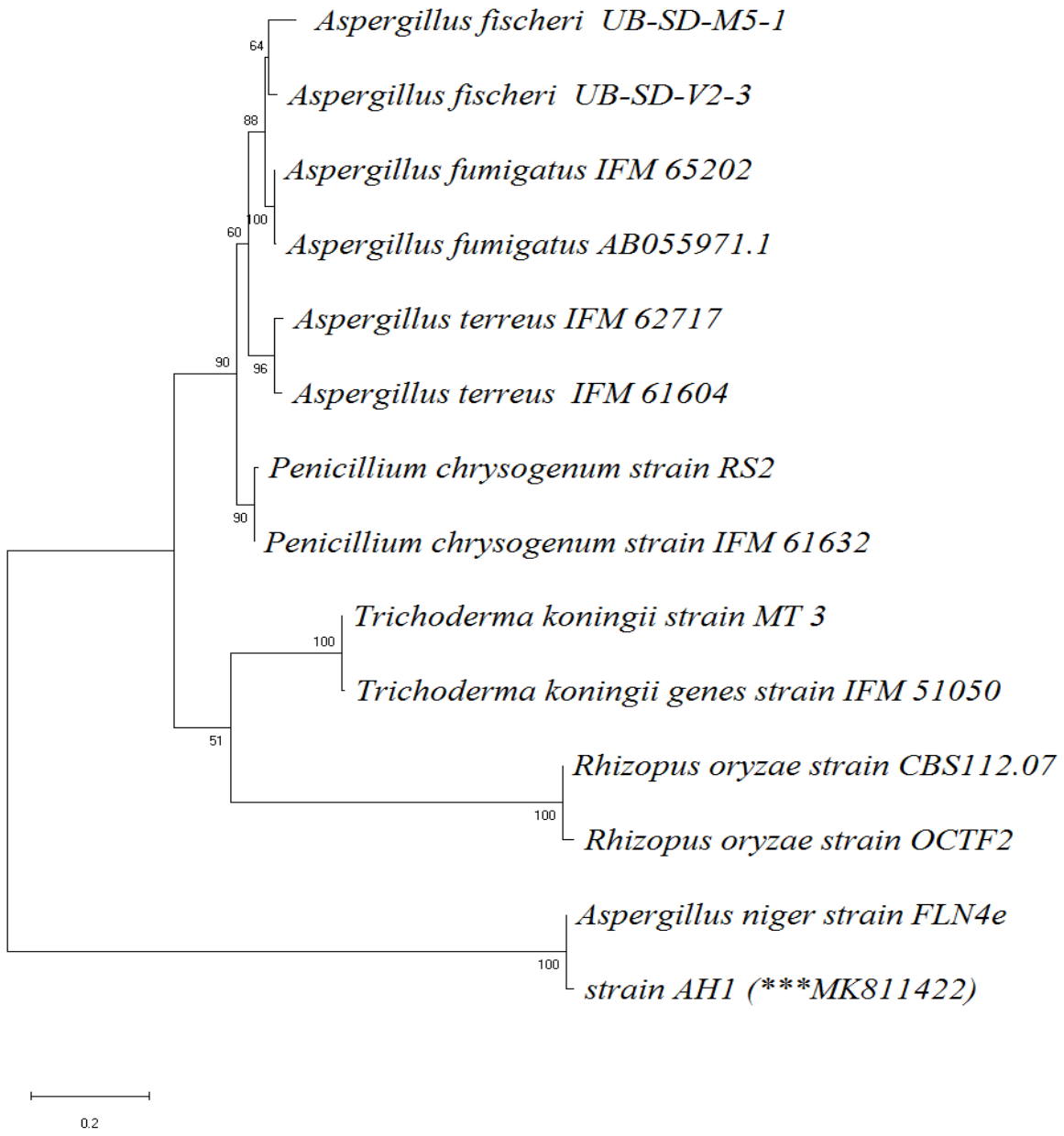

Figure 3: Phylogenetic tree constructed by Neighbor-Joining method derived from analysis of the 18S rRNA gene sequences of native isolates and related sequences obtained from NCBI. Scale bar, 0.2 substitutions per nucleotide position and numbers in parenthesis and asterisks represent GenBank accession numbers.

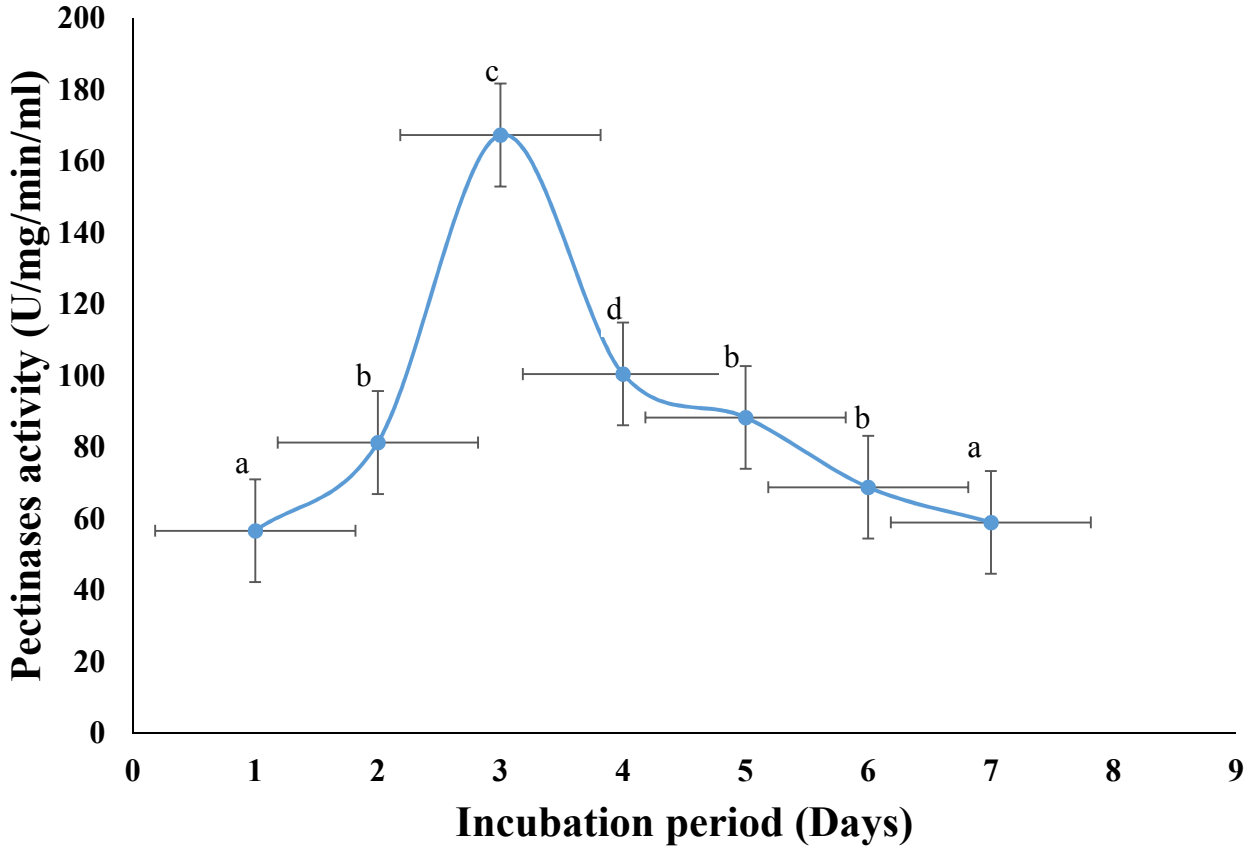

Figure 4: Effect of incubation period on pectinase produced by *Aspergillus niger* using banana peel as carbon source.

Key:

Values are means of replicates ± SEM

Values carrying different superscripts are significantly different at P≤0.05

4.4.2 Effect of Salt Supplements

The effect of addition of salt supplements on pectinase production is presented in Figure 5. Higher pectinase activity was obtained when salts were added to the fermentation medium compared to when there was no salts. Maximum pectinase activity of281.69±0.99 U/mg/min/ml was obtained from the medium supplemented with salts on day 3 of incubation and activity of 167.35±0.67 U/mg/min/ml was obtained from the medium without salts on day 3 of incubation. In both cases, pectinase activity decreased after day 3.

4.4.3 Effect of Additional Carbon Source

The effect of additional carbon source on pectinase activity is presented in Figure 6. Maximum pectinase activity was obtained when sucrose was used as additional carbon source with highest activity of 315.22±0.67 U/mg/min/ml on day 3 of incubation. Next to sucrose is maltose with activity of 216.44±0.29 U/mg/min/ml on day 3.There was no significant difference between glucose and fructose as they both decreased the enzyme activity. Lowest pectinase activity of 90.02±0.27 U/mg/min/ml was obtained on day 3 of incubation when fructose was used at additional carbon source. In all cases, activity declined after day 3.

4.4.4 Effect of Inoculum Size

The effect of varying inoculum size on pectinase production was shown in Figure 7. Different inoculum size showed varying pectinase activity on day 1 of incubation. There was no significant differences between 1 ml and 1.5 ml of 1×10^6 spores ml^{-1} inoculum sizes on pectinase activity at day 1. Pectinase activity increased significantly with inoculum size 2 ml of 1×10^6spores ml^{-1} on day 3 of incubation with maximum activity of 388.02±1.30U/mg/min/ml. Minimum pectinase activity of 209.37±0.30 U/mg/min/ml was obtained on day 3 when inoculum size 2.5 ml of 1×10^6 spores ml^{-1} was used. In all cases, pectinase activity declined after day 3.

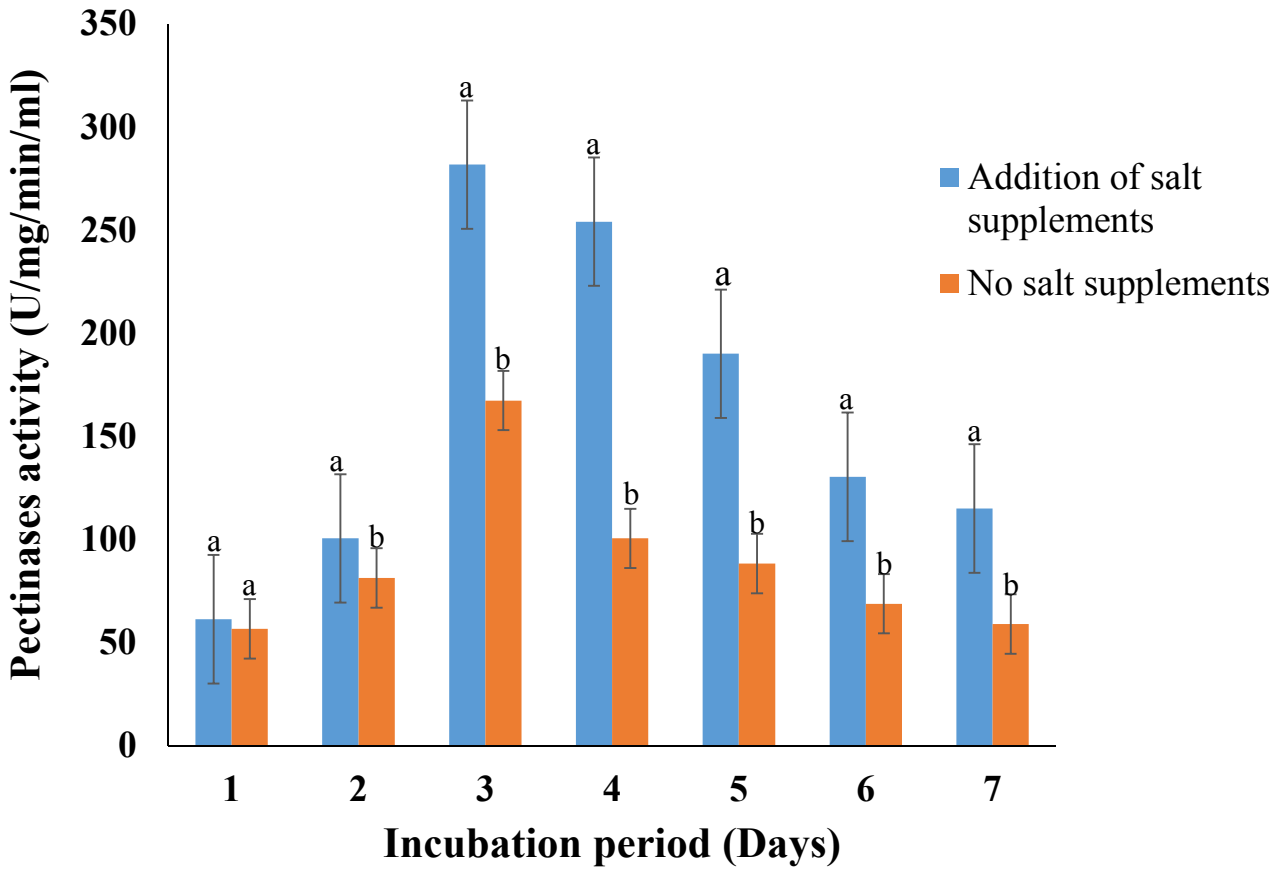

Figure 5: Effect of salt supplements on pectinase produced by *Aspergillus niger* using banana peel as carbon source.

Key:

Values are means of replicates ± SEM

Values carrying different superscripts are significantly different at P≤0.05

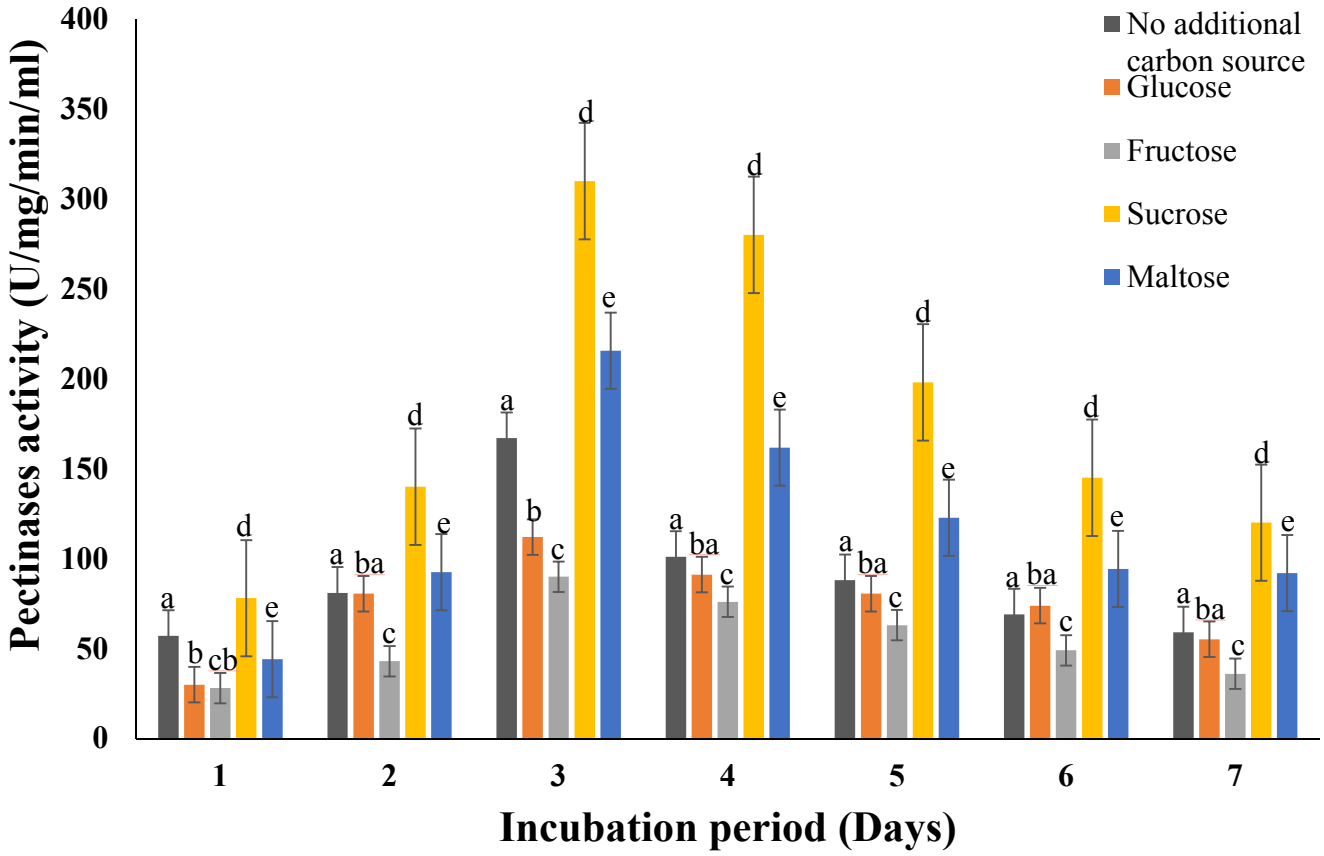

Figure 6: Effect of additional carbon source on pectinase produced by *Aspergillus niger* using banana peel as carbon source.

Key:

Values are means of replicates ± SEM

Values carrying different superscripts are significantly different at P≤0.05

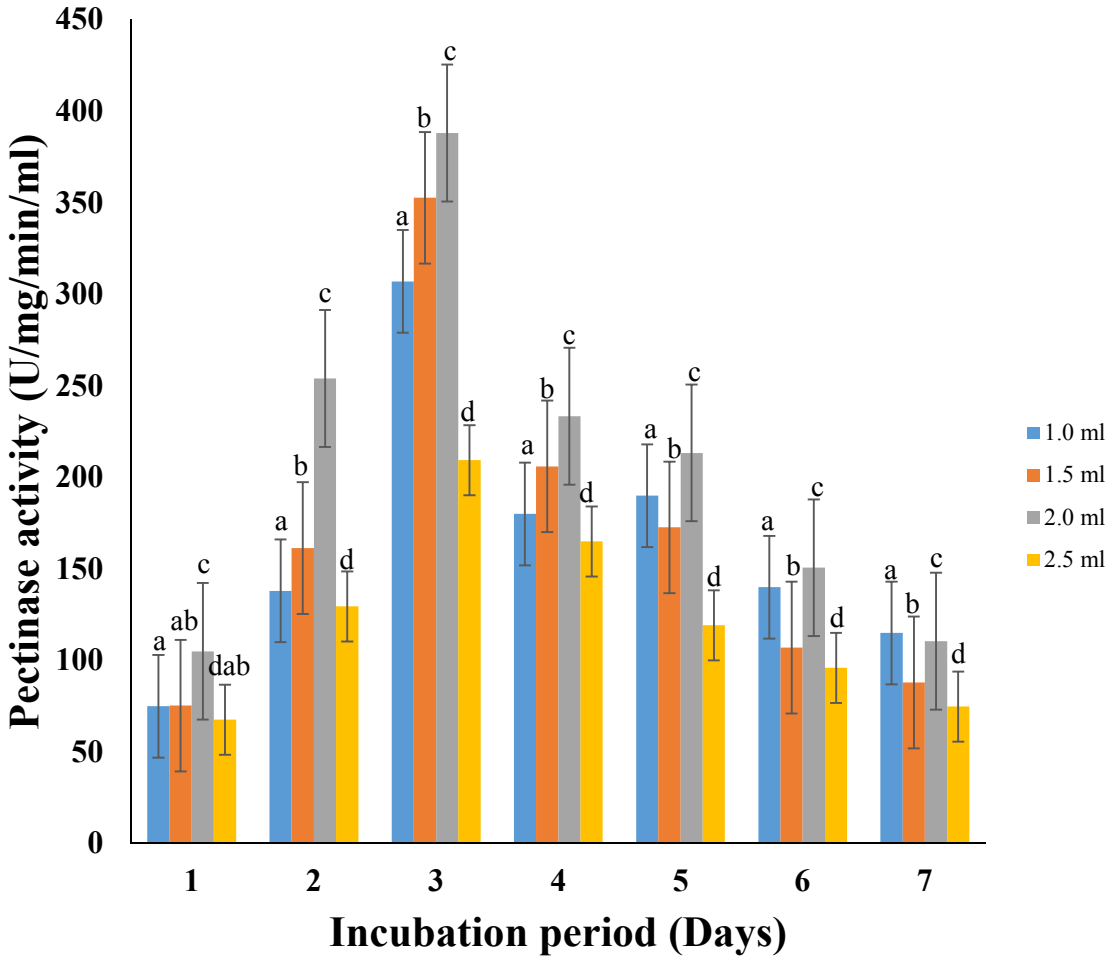

Figure 7: Effect of inoculum sizes on pectinase produced by *Aspergillus niger* using banana peel as carbon source.

Key:

Values are means of replicates ± SEM

Values carrying different superscripts are significantly different at P≤0.05

4.4.5 Effect of pH

The effect of pH on the activity of pectinase was shown in Figure 8. pH 6 showed significant pectinase activity among the used pH ranges at day 1 of incubation. There were no significant differences between the pH values of 4, 7 and 8 on pectinase activity on day 1. The optimal pH of 6 was obtained at day 3 of incubation with activity of 486.07±0.20 U/mg/min/ml. Next to pH 6 is pH 8 with pectinase activity of 304±0.42 U/mg/min/ml on day 3 of incubation. Lowest pectinase activity of 126.01±0.10 U/mg/min/ml was obtained from pH 4 on day 3 of incubation. In all cases, pectinase activity decreased after day 3.

4.4.6 Effect of Moisture Contents

The effect of varying moisture contents on pectinase activity is presented in Figure 9. Pectinaseactivity decreased as moisture contents increased. Moisture content of 100 ml showed higher pectinase activity on day 1 of incubation. There was no significant difference between moisture content of 200 ml and 250 ml on day 1 as both showed lowest pectinase activity. The optimal moisture content for pectinase activity was 100 ml which was obtained on day 3 of incubation with highest activity of 532.36±0.33 U/mg/min/ml. Lowest pectinase activity of 142±0.32 U/mg/min/ml was obtained on day 3 when moisture content of 250 ml was used. In all cases, pectinase activity decreased after day 3.

4.4.7 Effect of Temperature

The effect of different temperature ranges on pectinase activity is presented in Figure 10. Pectinase activity increased progressively from day 1 to day 3 as temperature increased. The optimal temperature recorded was obtained at 50 ^{0}C on the day 3 of incubation with activity of 575.37±0.67 U/mg/min/ml. Further increase or decrease in temperature resulted in decrease in pectinase activity. Minimum pectinase activity of 353.03±0.67 U/mg/min/ml was obtained on

day 3 of incubation when 30 ^{0}C temperature was used. In all cases, pectinase activity decreased after day 3.

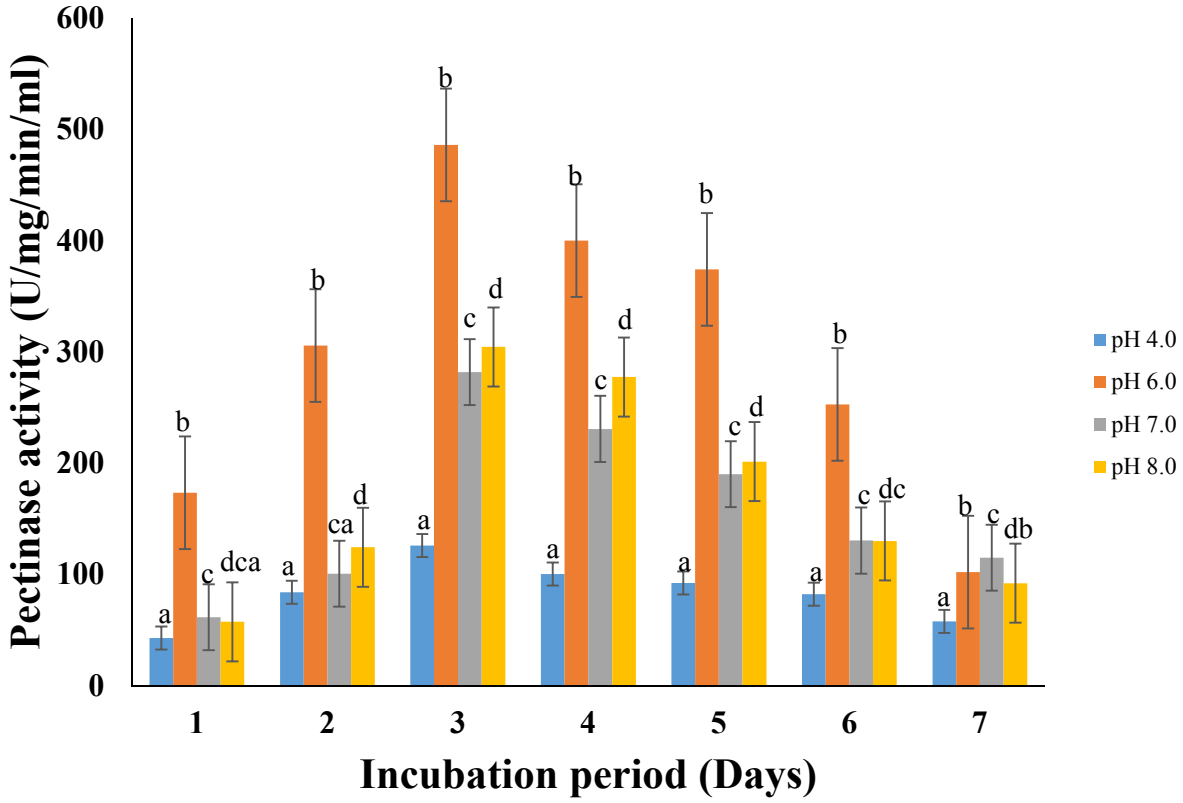

Figure 8: Effect of pH on pectinase produced by *Aspergillus niger* using banana peel as carbon source.

Key:

Values are means of replicates ± SEM

Values carrying different superscripts are significantly different at P≤0.05

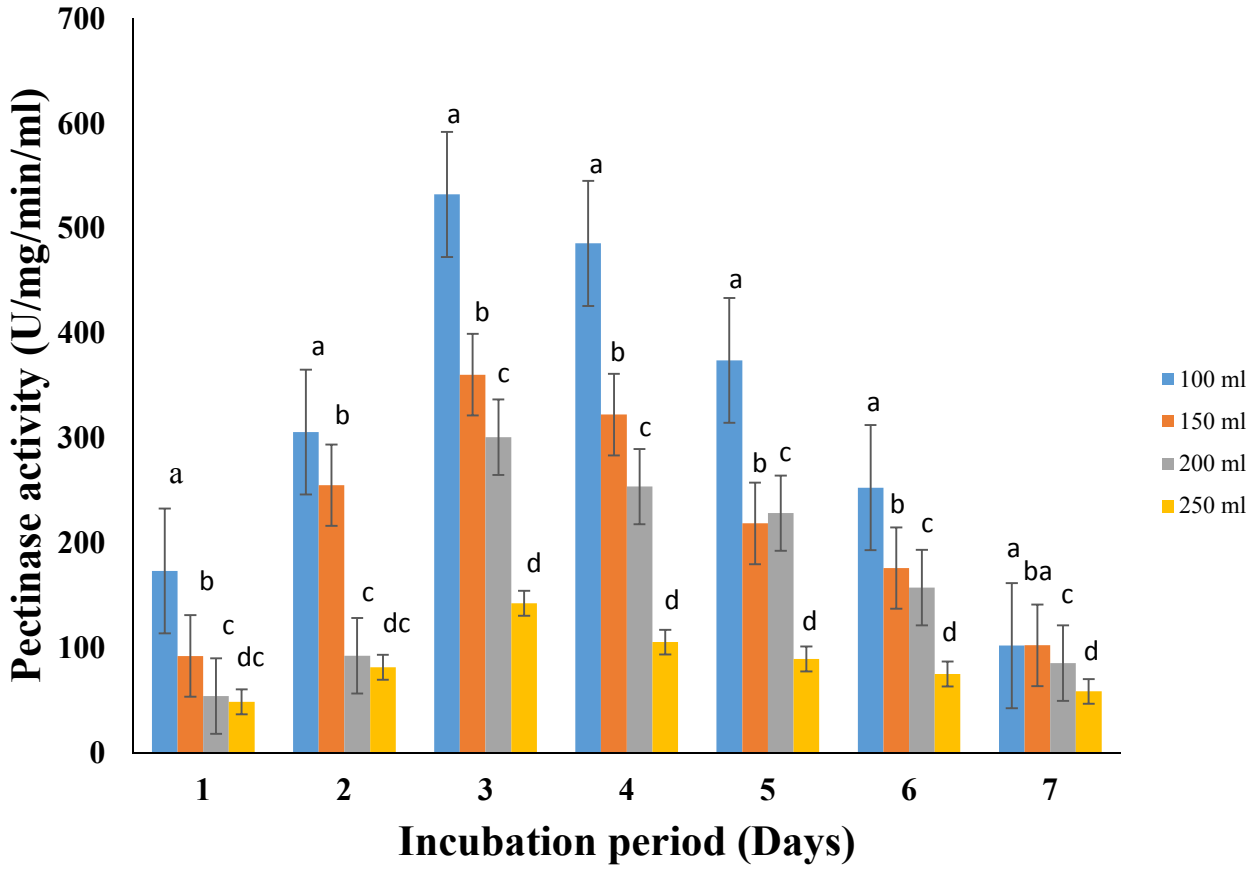

Figure 9: Effect of moisture contents on pectinase produced by *Aspergillus niger* using banana peel as carbon source.

Key:

Values are means of replicates ± SEM

Values carrying different superscripts are significantly different at P≤0.05

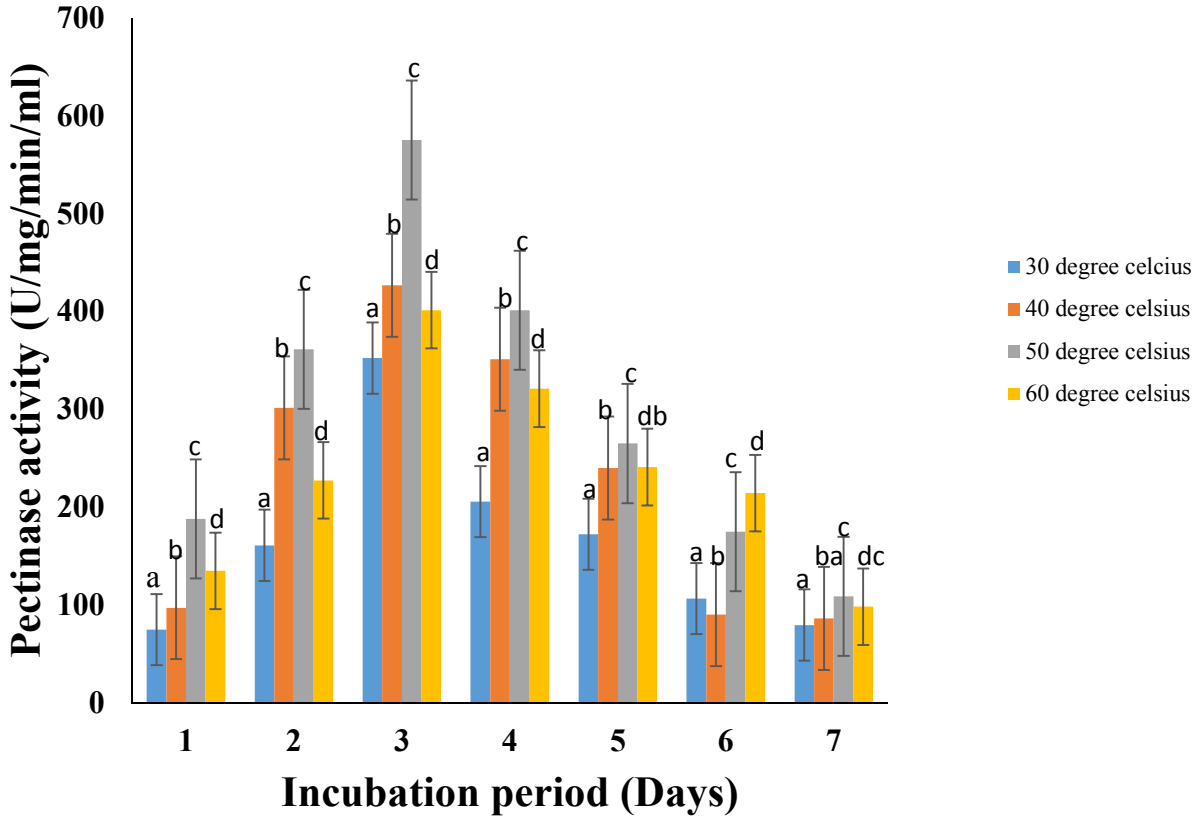

Figure 10: Effect of temperature on pectinase produced by *Aspergillus niger* using banana peel as carbon source.

Key:

Values are means of replicates ± SEM

Values carrying different superscripts are significantly different at P≤0.05

4.5 Purification of pectinase

The result of the purification of pectinase produced by *Aspergillus niger* is summarized in Table 3. The fraction obtained from 80 % ammonium sulphate precipitation revealed enzyme specific activity of 0.611 U/mg, dialysis with specific activity of 0.702 U/mg and gel filtration with 0.791 U/mg. The percentage yield decreased from 100 % crude enzyme to 8.94 % gel filtration but the purification fold increased progressively from 1.00 to 1.38 fold after purification.

Table 3. Purification summary of pectinase produced by *Aspergillus niger* using banana peel as carbon source.

Purification steps	Volume of enzyme (ml)	Total pectinases activity (U/mg/min/ml)	Total protein conc. mg/protein	Specific activity (U/mg)	Purification fold	Percentage (%) yield
Crude enzyme	500	2954.81	5165.00	0.572	1.00	100.00
$(NH_4)_2SO_4$	60	521.96	853.80	0.611	1.07	17.67
Dialysis	15	398.21	567.35	0.702	1.22	13.48
Gel-filtration	5	264.10	334.10	0.791	1.38	8.94

CHAPTER FIVE

Discussion

Five filamentous fungi were isolated from fruit wastes dumping sites and were identified as presented in Table 2. The presence of these isolates in the soil from fruit wastes dumping sites could be attributed to the fact that fungi are known decomposers in any ecosystem. Their degradative ability has been reported by several authors (Patil andDayanand, 2006; Banu *et al*., 2010) after isolating similar organisms from fruits waste dumping sites.

All the isolates were found to exhibit pectinolytic ability based on the screening, which could be as a result of their ability to produce extracellular pectinases to hydrolyze pectic components of plant cell wall. This is similar to the report of Okafor *et al*. (2007) who observed that cellulosic components of fruits wastes appears to be good sources of carbon and energy for fungi with plant cell wall degrading ability. The best pectinolytic ability was obtained from *A.niger* and then selected for pectinase production. The result obtained indicated that banana peel can be used as substrate for pectinase production which is in line with the reports of Finkler *et al*. (2017) and Obafemi *et al*. (2019) who stated that agro residues can be exploited for microbial enzyme production.

Molecular identification of *A. niger* with best pectinolytic ability showed that it has 100 % homology with *A.niger* strain FLN4e and was then identified as *A. niger* strain AH1 with GenBank accession number MK811422 as shown in Figure 3.

Aspergillus niger has been acknowledged as good producer of microbial enzymes according to the earliest report (Oyewole *et al*., 2012). The isolated *A. niger* strain AH1 exhibited the best

pectinolytic ability which could be attributed to its potentiality of using the banana peel as carbon and energy source to produce pectinases enzyme.

The incubation period for achieving maximum enzyme production depend largely the characteristics of the culture and substrate. Pectinase production started at day 1 of incubation and was at the peak on day 3 of incubation, and gradually decreases thereafter. The fact that pectinase activity reached the peak at day 3 could be traceable to organism in its exponential phase with higher metabolic activities and generation of essential nutrients necessary for growth. The decline in pectinase activity at day 4 and thereafter may be due to depletion of essential nutrients required for growth, accumulation of toxic metabolites and could also be as result of the organism in its stationery phase. This is similar to the work of Castilho *et al.* (2000) but contrary to the reports of Martin *et al.* (2004) who also worked on pectinase production. The disparity in findings could be traceable to the use of different fermentation condition, different substrates and different strain of the organism.

Supplementing of medium with salts has been reported to maximize enzyme production by increasing cell permeability resulting in easy release of enzyme into the medium. In this study, maximum pectinase production was obtained when salt supplements were used compared to when the medium was not supplemented with salts. This result is similar to the report of Pedrolli *et al*. (2009).

Media composition plays a significant role in enzyme production. Growth and maintenance of organism are greatly influenced by environmental conditions and available nutrients (Maria *et al*., 2000). In this study, higher pectinase activity was achieved when sucrose was used as additional carbon source. This could be because sucrose is a disaccharide sugar and can be easily metabolize by the organism, breaking it down into its monomeric units compared to the main

carbon source in banana peel which was starch. This result is similar to the report of Rajendra *et al*. (2011b).

Inoculum size is an important factor in enzyme production. Low number of inoculums will require longer time for the cells to multiply sufficiently to utilize the substrate and produce enzyme (Ram *et al*., 2017). In this study, maximum pectinase activity was obtained when 2 ml of 1×10^6 spores ml^{-1} was used. This could be the required number of spores for complete utilization of the substrate by the organisms. Further increase in inoculum size above this optimum resulted in decrease in enzyme activity which may be as a result of competition as the number of spores became greater than the available nutrient. This is in agreement with the work of Maciel *et al*. (2011).

pH is one of the critical factors which strongly influenced the growth of an organism and enzyme yields (Ram *et al*., 2017). Fungi especially *Aspergillus* species have been known to work best at acidic and slightly alkaline pH as reported by earlier authors (Guptal *et al*., 2005). It was observed in this study that the optimum pH for *A. niger* strain AH1 was pH 6. Further increase or decrease in pH resulted in decline of enzyme activity. This pH probably was the optimum for the organism to utilize the substrate and hence, produced acidic pectinase. According to Zohdi and Mehrnoush (2013), the acidification or alkalization of culture medium reflects the substrate consumption. The optimum pH for pectinase enzyme activity has been reported by earlier authors to fall within pH 3-9 (Sivakumar *et al*., 2012).

Moisture content has a very important role in submerged fermentation system. It is involved in biomass development, microbial access to substrate and oxygen availability in the fermentation medium (Finkler *et al*., 2017). Maximum pectinase activity was achieved at 100 ml moisture contents. Further increase in moisture contents resulted in decline of enzyme activity. This may

be due to decrease in the level of available oxygen as the moisture content increases which could inhibit the performances of *A. niger* strain AH1 and could also be as a result of particle agglomeration where the moisture contents greatly exceed the substrate water holding capacity, and may also be attributed to decrease in microbial access to the substrate as the moisture contents increases. This result is similar to the work of Holker *et al.* (2004).

Temperature has been one of the most vital environmental factors affecting the development of biological processes which may lead to protein denaturation, enzymatic inhibition and cell death if not properly managed (Aaisha and Barate, 2016). In this study, maximum pectinase activity was obtained at 50 ^{0}C. This could be the optimum temperature required for growth and product formation by the *A. niger* strain AH1. The decline in enzyme activity at temperature above this optimum could be as a result of excess heat accumulation, protein denaturation and loss of enzymatic structure. This result is similar to the work of Maciel *et al*. (2011); Mrudula and Anitharaj (2011) but contrary to the report of Adeleke *et al*. (2012) who also worked with similar range of temperature. The disparity in findings could be attributed to the use of different substrates, working under different laboratory conditions and probable use of different organism.

The purification of pectinase is very essential as it removed excess impurities from enzyme of interest. The crude pectinase was purified by ammonium sulphate precipitation, dialysis and gel-filtration chromatography. The specific activity of pectinase increased after the crude enzyme was brought to 80 % ammonium sulphate precipitation. This result is similar to the work of Buga *et al*. (2010) and also agrees with the report of Adejuwon and Olutiola (2007).

The precipitated enzyme was further dialyzed against phosphate buffer to remove excess salt and other impurities which might be present. There was decrease in protein concentration as shown in Table 3 after dialysis which could be as a result of the removal of other protein of lower

molecular weight that were not the protein of interest. This is in agreement with the work of Lukong *et al*. (2007).

Further removal of other proteins was also carried out using gel-filtration chromatography method, with fractions collected every 60 seconds. Fractions collected after gel-filtration chromatography had highest specific activity. Although, the specific activity of the enzyme increased along the purification steps as shown in Table 3. This result is in agreement with the finding of Lukong *et al.* (2007). It has been reported that for a purification procedure to be successful, the specific activity of the enzyme of interest must be increasing along the purification steps (Keller *et al*., 2006). The increase in specific activity is a measure of the purification achieved, thus the enzyme had a purification of 1.38 and percentage yield of 8.94 % after gel-filtration chromatography as shown in Table 3.

Conclusion

Aspergillus niger strain AH1 showed higher capability of utilizing the substrate as carbon and energy source to produce pectinase enzyme under submerged fermentation condition. Higher pectinases activity was obtained at day 3 of incubation, temperature of 50 ^{0}C, pH 6.0, 2 ml of 1 × 10^{6} fungal spores ml^{-1}, moisture contents of 100 ml, with salt supplements and in the presence of sucrose as additional carbon source. Therefore, the use of banana peel to produce pectinase under submerged fermentation with *A. niger* can be explored for industrial applications as the process is simple, cheap and can help greatly to reduce environmental garbage from banana peel.

Recommendations

It is highly recommended that:

- More agricultural residues should be explored for enzyme production.
- There is a need for further purification steps to be carried out for complete removal of impurities.