Characterization and Partial Purification of Pectinase Produced by *Aspergillus niger* Using Banana Peel as Carbon Source

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Abstract: Pectinases are group of enzymes which causes the degradation of pectin. Pectinases are of environmental and industrial importance due to their biotechnological and commercial potentials in waste water treatment, 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. Fungi were isolated from fruit wastes dump sites using standard microbiological technique. The isolates were characterized morphologically and microscopically before subjecting them to screening for pectinolytic ability using standard methods. Molecular identifications were carried out on the isolate with the best pectinolytic ability. 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 isolate with the best pectinolytic ability was identified as *Aspergillus niger* strain AH1 with GenBank accession number MK811422. The crude pectinase obtained were partially purified by ammonium sulphate precipitation, dialysis and gel filtration chromatography methods. 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 *Aspergillus 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 *Aspergillus niger* strain AH1 can produced maximum pectinase at optimized fermentation parameters and could be explored for pectinase production.

Keywords: Fruit wastes; *Aspergillus niger*; optimization; pectinase activity; purification

INTRODUCTION

Enzymes are bio-active compounds or organic catalyst that moderates many biochemical reactions in living tissues and cells (Ruiz *et al*., 2011). They are highly selective catalysts which can greatly accelerate or decelerate both the rate and specificity of metabolic reactions, ranging from biosynthesis of macromolecules to various activities of functional system of living things. The use of chemical catalysts has been pursued for a very long time, though widely used was very cumbersome. The disadvantages of this method includes 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; Azzaz *et al.,* 2020). 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, pectinases 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 and environmental processes (Tewari and Tewari, 2005; Zhong and Cen, 2005). 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).

Agricultural and food wastes constitute the major sources of pollution in many countries according to earlier report (Koubala *et al*., 2006). This is being controlled biologically using microorganisms for the biosynthesis of valuable compounds such as proteins, polysaccharides, oligosaccharides, vitamins, hormones, enzymes and others as raw materials for environmental and 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 of pectinolytic 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). 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 (*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; Zehra *et al.,* 2020). 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). Previous studies reported that fruit peel including banana is rich in pectin and applicable in pectinases production through solid state and submerged fermentation (Zehra *et al.,* 2020). Due to the increasing demand for enzymes in many environmental and industrial processes, this research is designed to study the potential of banana peel as a cheap raw material and carbon source in fermentation system to produce pectinase that meet environmental and industrial needs by locally employing fungal isolate obtained from fruit wastes dumping site, thereby adding value to banana while reducing the postharvest waste and environmental pollution.

MATERIALS AND METHODS

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.

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.

Collection of Soil samples

Soil samples were collected from three different locations of fruit wastes dump 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 Microbiology laboratory within 3 hours of collection for immediate analyses in accordance with the method of Venkata and Divakar (2013). All materials and surfaces were sterilized according to the procedure described by Ugoh and Ijigbade (2013).

Isolation of Pectinolytic Fungi

Fungi were isolated from soil samples using pour plate technique. One gram of soil sample was suspended in 9 ml sterile distilled water. Serial dilutions were made before inoculating into already prepared sterile Potato dextrose agar-streptomycin plates. The inoculated plates were incubated at ambient temperature (28 ± 2 ^{0}C) for 2 days. Colony development were observed after incubation period (Ratnasri *et al*., 2014). The young fungal hyphae were aseptically picked up and transferred to fresh sterile Potato dextrose agar-streptomycin plates repeatedly to obtain pure cultures. The pure cultures were maintained on Potato dextrose agar-streptomycin slants to inhibit contaminants growth until further use (Ugoh and Ijigbade, 2013).

Fungal Identification

Macroscopic and Microscopy Characterization

Isolates obtained were characterized and identified using their morphological characteristics which include macroscopic and microscopic examinations. The characteristics used were 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 (2010). Further molecular identifications were carried out to confirm the phylogeny and evolutionary relationship of the isolate with the best pectinolytic ability after screening.

Molecular Characterization

Deoxyribonucleic acid was isolated from *A. niger* isolate using the Fungal Genomic DNA isolation kit (Qiagen). The primer used was consensus primers, 18S rRNA, ITS1, 5.8S rRNA, ITS2. PCR amplification was done on 28S rRNA gene fragment. Also the forward primer (5'–3') GGAAGTAAAAGTCGTAACAAGG and reverse primer (5'–3') GGTCCGTGTTTCAAGACGG were used for DNA amplification. Sequencing of PCR products were conducted by excising the bands from the agarose, purified and sequenced. After sequencing of the PCR products with the 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).

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).

Fermentation for Pectinase Production

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. From the resulting suspension, 1 ml of the suspension (which contains about 1×10^6 spores ml^{-1}) was used as inoculum (Dhillon *et al*., 2004).

Fermentation Process

The fermentation process was carried out in 250 ml capacity fermenting flasks containing 90 ml of mineral solution and 10 g of banana peel powder under specific fermentation condition. The fermentation 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 to 45 ^{0} C 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).

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 which is the crude enzyme was kept for further use (Jacob *et al*., 2009).

Assay for Pectinase Activity

Two milliliters of the crude enzyme were 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 dinitrosalicylic acid (DNSA) 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 were diluted with 4 ml of distilled water and absorbance were 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).

Optimization Parameters for Pectinases Production

Effect of Incubation Period on Pectinases Production

The fermentation process was carried out in 250ml capacity fermenting flasks containing 10 g of substrate and 90 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 was 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).

Effect of Salt Supplements on Pectinase Production

The fermentation process was carried out in two 250 ml capacity fermenting flasks. Ten gram of substrate was introduced into two 250 ml Erlenmeyer flasks containing 90 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).

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 capacity fermenting flasks containing 10 g of substrate and 90 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 was 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).

Effect of Inoculum Size on Pectinase Production

The fermentation process was carried out in 250 ml capacity fermenting flasks containing 10 g of substrate and 90 ml of mineral solution with sucrose 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 was mixed thoroughly and then incubated at 28 ± 2 ^{0}C for 7 days. Pectinase activity determination was carried out daily as previously described (Adeleke *et al*., 2012; Venkata and Divakar, 2013).

Effect of pH on Pectinase Production

The fermentation process was carried out in 250 ml capacity fermenting flasks containing 90 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 grams 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. Pectinase activity determination was carried out daily as previously described (Adeleke *et al*., 2012; Venkata and Divakar, 2013).

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 250 ml of mineral solution which was introduced into 500 ml capacity fermenting flasks containing 10 g of substrate and sucrose as additional carbon source. The pH of the medium was adjusted to 6. 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 was mixed thoroughly and incubated at 28 ± 2 ^{0}C for 7 days. Pectinase activity determination was carried out daily as previously described.(Adeleke *et al*., 2012; Venkata and Divakar, 2013).

Effect of Temperature on Pectinase Production

The fermentation process was carried out in 250 ml capacity fermenting flasks containing 10 g of substrate and 90 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 was mixed thoroughly and then incubated at different temperature of 30, 40, 50 and 60 ^{0}C for 7 days. Pectinase activity determination was carried out daily as previously described.(Adeleke *et al*., 2012; Venkata and Divakar, 2013).

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.

Purification of Pectinase

Ammonium Sulphate Precipitation

Sixty milliliters 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).

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).

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).

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.

RESULTS AND DISCUSSION

Identification of the Fungal Isolates

A total of five filamentous fungi were isolated from the soil samples obtained from fruit wastes dump sites and their characteristics are presented in Table 1.

Fungal Isolates with 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.

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 1.

Pectinase Activity

Effect of Incubation Period

The effect of incubation period on pectinase production is presented in Figure 2. Pectinase production started at day 1 (0-24 hours) 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.

Effect of Salt Supplements

The effect of addition of salt supplements on pectinase production is presented in Figure 3. Higher pectinase activity was obtained when salts were added to the fermentation medium compared to when there were no salts. Maximum pectinase activity of 281.69±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.

Effect of Additional Carbon Source

The effect of additional carbon source on pectinase activity is presented in Figure 4. 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.

Effect of Inoculum Size

The effect of varying inoculum size on pectinase production was shown in Figure 5. Different inoculum size showed varying pectinase activity on day 1 of incubation. There was no significant difference 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.

Effect of pH

The effect of pH on the activity of pectinase was shown in Figure 6. pH 6 showed significant pectinase activity on 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.

Effect of Moisture Contents

The effect of varying moisture contents on pectinase activity is presented in Figure 7. Pectinase activity 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.

3.4.7 Effect of Temperature

The effect of different temperature ranges on pectinase activity is presented in Figure 8. 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.

Purification of Pectinase

The result of the purification of pectinase produced by *Aspergillus niger* is summarized in Table 2. 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 1. Macroscopic and Microscopy Identification of Fungal Isolates Obtained from Fruit Wastes Dump 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

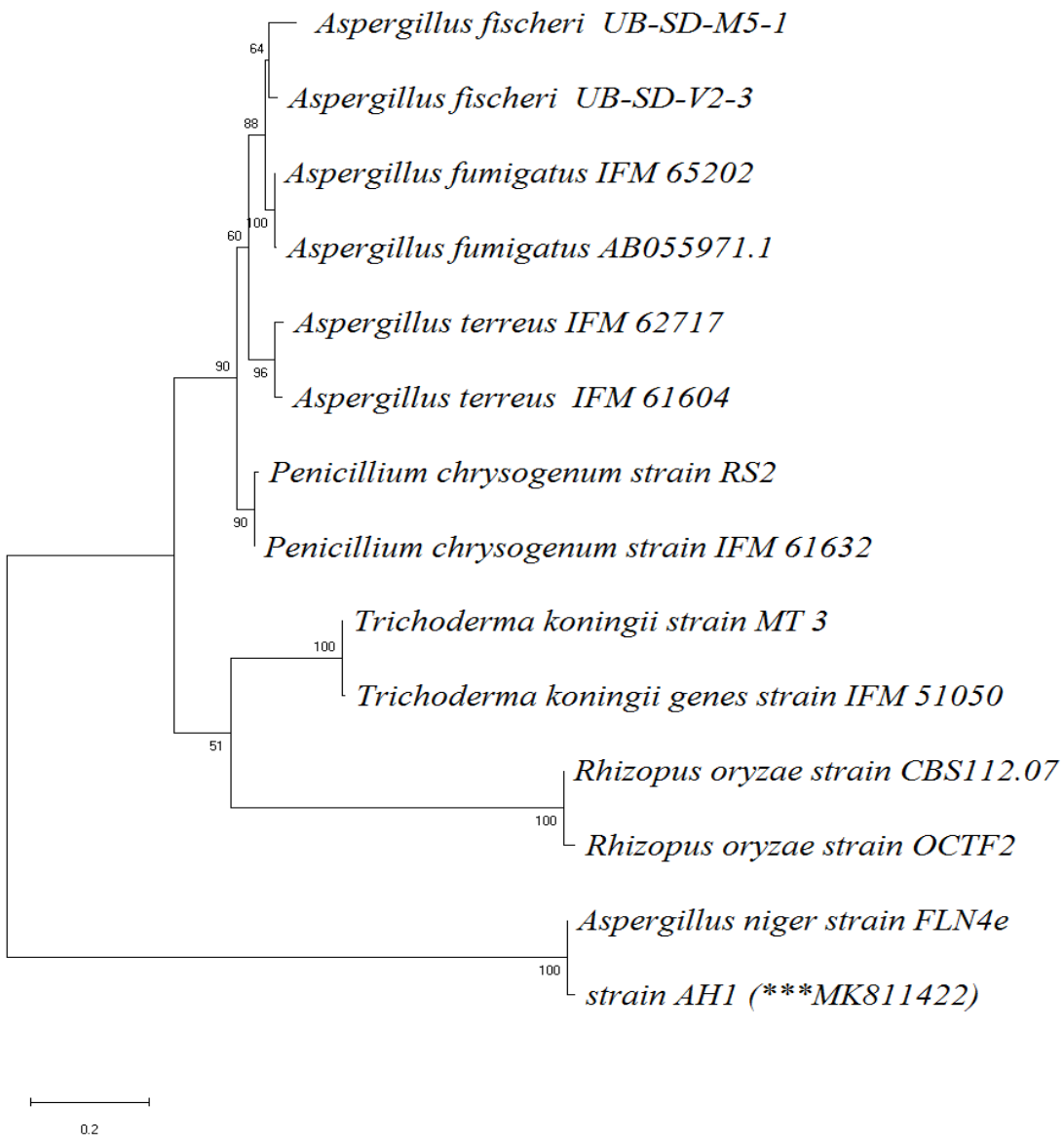


Figure 1: Phylogenetic tree constructed by Neighbor-Joining method derived from analysis of the 18S rRNA gene sequences of native isolates and related sequences obtained from NCBI. Scale bar, 0.2 substitutions per nucleotide position and numbers in parenthesis and asterisks represent GenBank accession numbers.

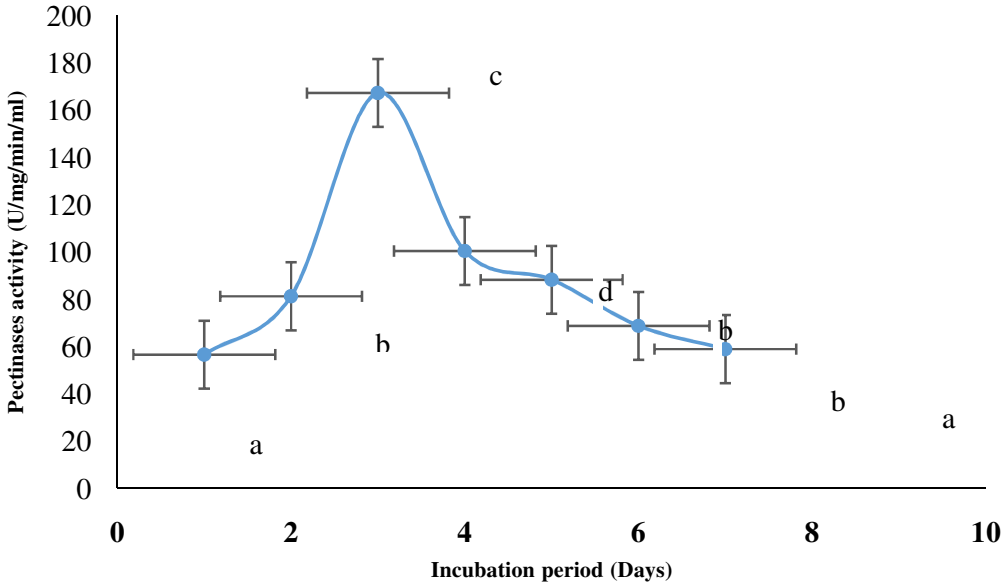


Figure 2: Effect of incubation period on pectinase produced by *Aspergillus niger* using banana peel as carbon source.

Key:

Values are means of replicates ± SEM

Values carrying different superscripts are significantly different at P≤0.05

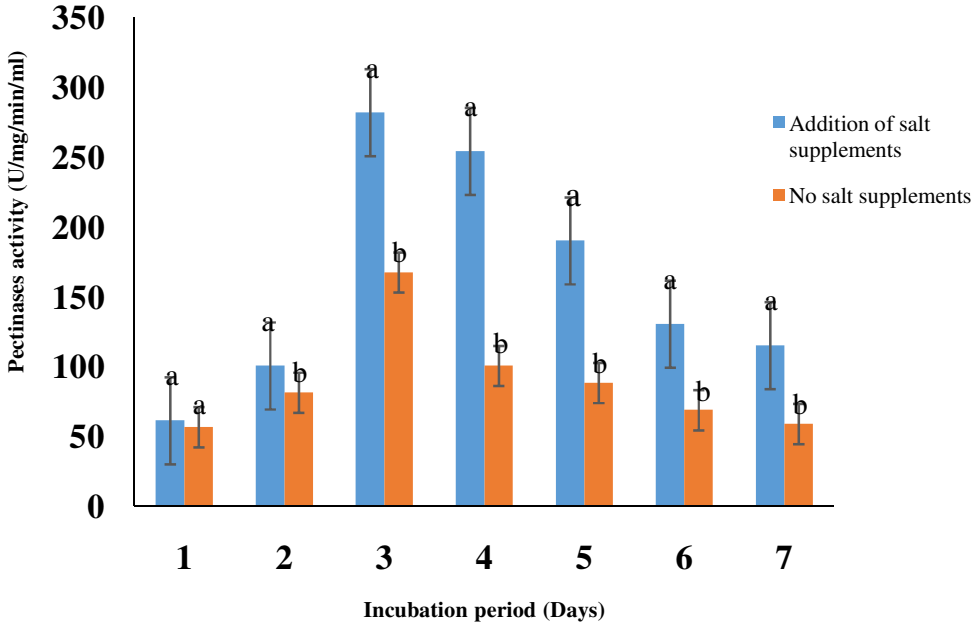


Figure 3: Effect of salt supplements on pectinase produced by *Aspergillus niger* using banana peel as carbon source.

Key:

Values are means of replicates ± SEM

Values carrying different superscripts are significantly different at P≤0.05

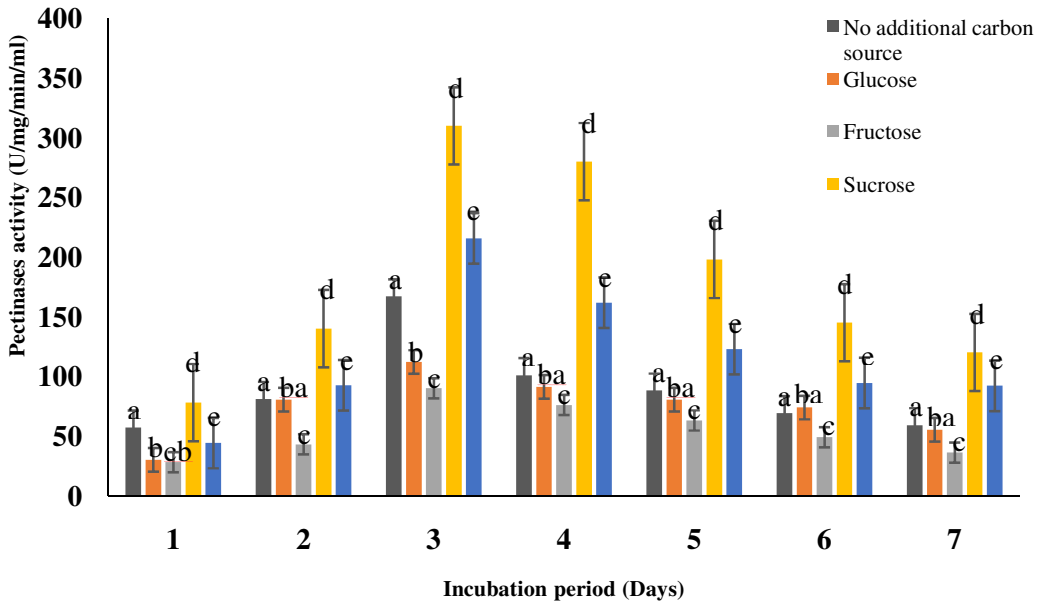


Figure 4: Effect of additional carbon source on pectinase produced by *Aspergillus niger* using banana peel as carbon source.

Key:

Values are means of replicates ± SEM, Values carrying different superscripts are significantly different at P≤0.05

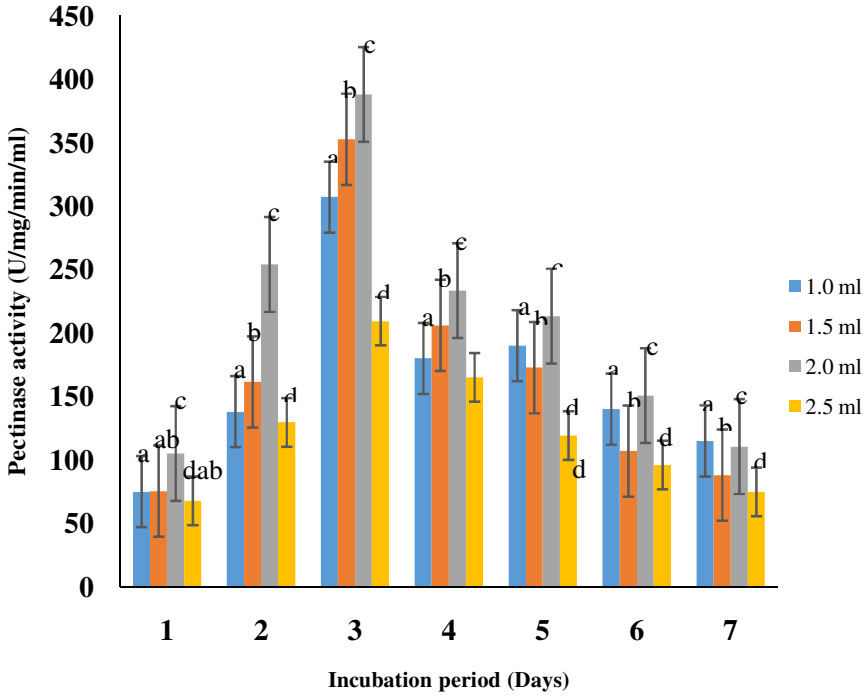


Figure 5: Effect of inoculum sizes on pectinase produced by *Aspergillus niger* using banana peel as carbon source.

Key:

Values are means of replicates ± SEM

Values carrying different superscripts are significantly different at P≤0.05

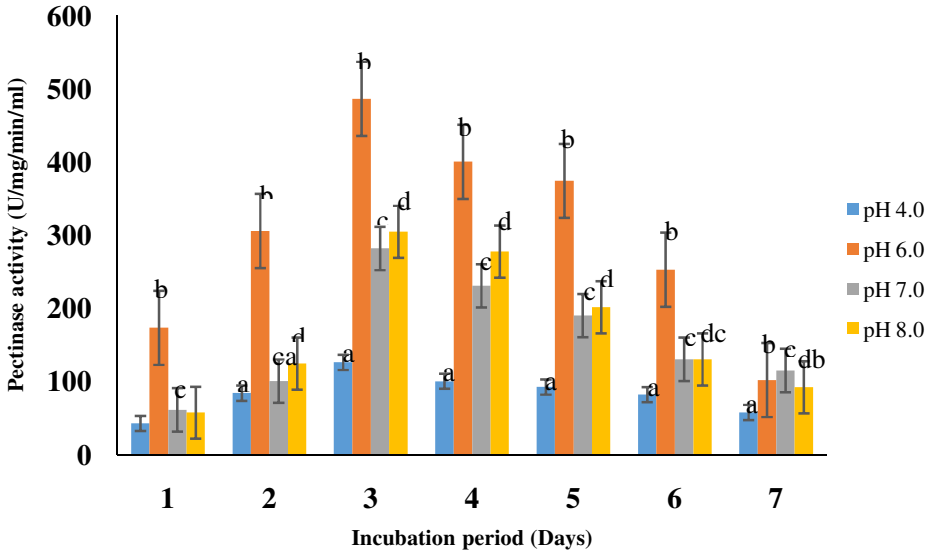


Figure 6: Effect of pH on pectinase produced by *Aspergillus niger* using banana peel as carbon source.

Key:

Values are means of replicates ± SEM

Values carrying different superscripts are significantly different at P≤0.05

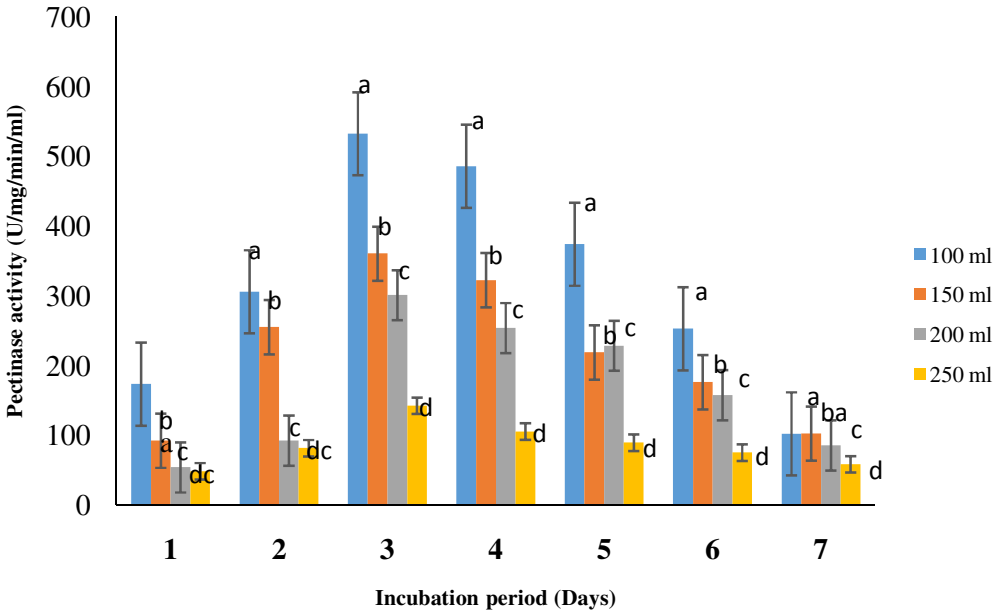


Figure 7: Effect of moisture contents on pectinase produced by *Aspergillus niger* using banana peel as carbon source.

Key:

Values are means of replicates ± SEM

Values carrying different superscripts are significantly different at P≤0.05

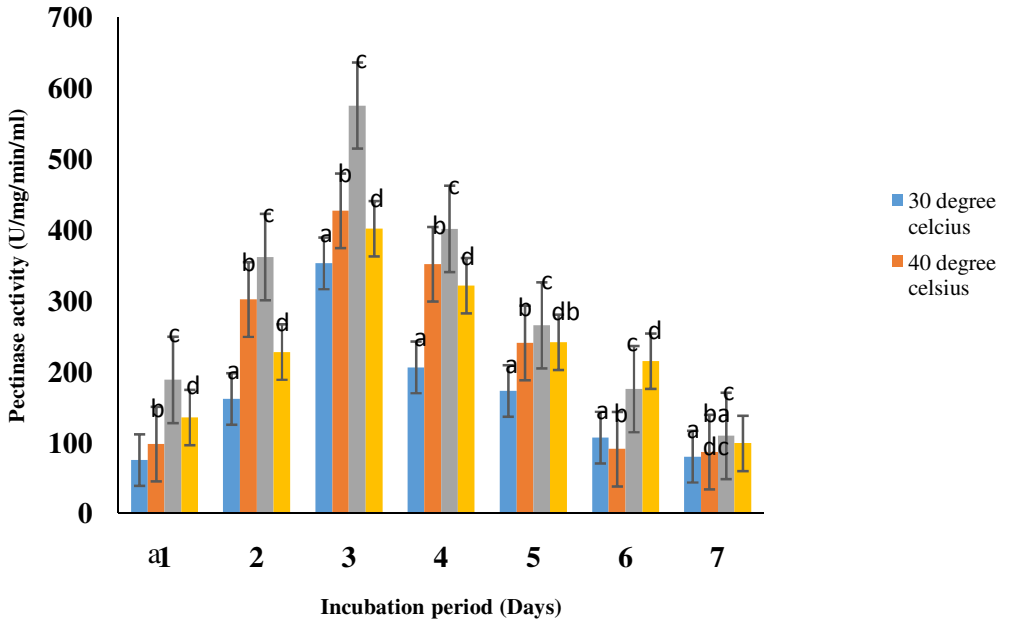


Figure 8: Effect of temperature on pectinase produced by *Aspergillus niger* using banana peel as carbon source.

Key:

Values are means of replicates ± SEM

Values carrying different superscripts are significantly different at P≤0.05

Table 2. 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

DISCUSSION

Five filamentous fungi were isolated from fruit wastes dump sites and were identified as presented in Table 1. 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 and Dayanand, 2006; Banu *et al.*, 2010) after isolating similar organisms from fruit wastes dump sites. All the isolates were found to exhibit pectinolytic ability, this could be as a result of their ability to produce extracellular pectinase to hydrolyze pectic components of plant cell wall. Cellulosic components of fruits wastes have been reported to be good sources of carbon and energy for fungi with plant cell wall degrading ability (Okafor *et al.*, 2007). 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 1. The best pectinolytic ability was obtained from *Aspergillus 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. *Aspergillus niger* has been acknowledged as good producer of microbial enzymes according to the earliest report (Oyeleke *et al*., 2012). The isolated *A. niger* strain AH1 exhibited the best pectinolytic ability by using the banana peel as carbon and energy source to produce pectinase enzyme.

The incubation period for achieving maximum enzyme production depend largely the characteristics of the culture and substrate (Azzaz *et al.,* 2020). Pectinase production started at day 1(0-24 hours) 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 stationary 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 according to Pedrolli *et al.* (2009). In this study, maximum pectinase production was obtained when salt supplements were used compared to when the medium was not supplemented with salts. 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 is starch. This observation is similar to the report of Rajendra *et al.* (2011b) who reported increase in pectinase production when sucrose was used as additional carbon source.

Inoculum size is an important factor in enzyme production. Too few 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 probably be the best required number of cells/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 traceable to competition for growth requirements as the number of spores became greater than the available nutrient. This observation is in agreement with the work of Maciel *et al.* (2011) who observed optimal enzyme productivity at 2ml of 1×10^6 spores ml^{-1}inoculum size.

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 Guptal *et al*. (2003). It was observed in this study that the optimum pH for *A. niger* strain AH1 to produce pectinase 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 finding is similar to the work of Holker *et al.* (2004) who reported maximum enzyme production at 100 ml moisture contents.

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 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 2. 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 2.

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 and environmental 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 local enzyme production. However there is a need for further purification steps to be carried out for complete removal of impurities.

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