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PECTINOLYTIC ACTIVITY OF ASPERGILLUS NIGER AND ASPERGILLUS FLAVUS GROWN ON GRAPEFRUIT (CITRUS PARASIDIS) PEEL IN SOLID STATE FERMENTATION

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ABSTRACT

The present study was aimed at studying pectinolytic activity of resident fungi isolated from decomposing grapefruit (Citrus parasidis) peels in solid state fermentation. Grape fruit peel was subjected to natural fermentation and the fermenting fungi were isolated, characterized and identified using standard microbiological methods. The isolated fungi were in turn used for fermentation to determine their pectinolytic activity through solid state fermentation technique. Culture parameters such as incubation period, temperature, moisture content and addition of salts supplements were optimized during the research for five days. The identified fungi were Aspergillus Niger and Aspergillus flavus. The peak of pectinolytic activity was at day three of fermentation when the highest pectinase activity of 13.32 µmol/mg/min was recorded for A. Niger and 11.32 µmol/mg/min for A. flavus. Optimum temperature and pH for pectinase activity by A. Niger and A. flavus was at 40 ^{0}C and pH 7.5 and 7.7 respectively. The use of salt supplemented substrate did not alter enzyme activity. In conclusion, the isolated fungi could be promising organisms for pectinolytic enzyme production on grape peel as substrate.

KEYWORDS: Grapefruit, Pectinolytic Activity, Fungi, Fermentation, Aspergillus

INTRODUCTION

Pectinase is a major enzyme whose demand has currently increased globally in the industries (Jayani et al., 2005; Oumer, 2017). They are involved in breaking down pectin or oligo-D-galacturonate in metabolic reactions such as hydrolysis (Jayani et al., 2005; Favela-Torres et al., 2006; Adeleke et al., 2012). In nature, pectinase occur naturally in plant to enhance ripening of fruit. The enzyme also assists biodegradation of plant in carbon cycle and energy transfer within the ecosystem. Pathogenic microorganisms produce pectinase to enhance their attack on primary cell wall of host plant whose major component is pectin (Whitaker, 2000; Garg et al., 2016). Pectinase is widely applicable in the industrial sector for several purposes. It is used in textile, food and feed, pharmaceuticals, waste water treatment plants and other manufacturing companies (Saito et al., 2004; Sharma and Satyanarayana, 2006; Kubra et al.,2018; Satapathy et al., 2020). It is also utilized in the extraction of plant bio-compounds from fruit skin (Neagu et al., 2012; Carrasco et al., 2019).

In order to meet the increase demand for this enzyme, production has been accomplished through solid state (SSF) or submerged fermentation (SmF) (Neagu et al., 2012). Report has it that it can be produced from pectic material as major carbon substrate (Reddy and Sreeramulu, 2012). During solid state fermentation, microorganisms grow directly on the substrate in low water activity as against submerged fermentation, where they grow under liquid (Subramaniyam and Vimala, 2012). Fungi are more preferable in solid state

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fermentation while bacteria do better in submerged fermentation. Solid state fermentation provides a close to natural condition for fungi to thrive and produce metabolites since their mycelia needed to be in direct contact with the substrate, the low availability of water also serves as a deterrent to bacterial contaminants. Earlier reports on enzyme production also supports that solid state fermentation yields more enzyme than submerged fermentation (Favela-Torres et al., 2006). Several advantages of solid state fermentation has also been documented.

Microorganisms have been used for enzyme production, moulds, yeasts and bacteria has been exploited for their ability to produce pectinase. An organism for enzyme production however, must not be pathogenic or toxic, it must meet the standard of Generally Regarded as Safe (GRAS). Some commonly used organisms are Aspergillus, Penicillium, Rhizopus, Saccharomyces, Zymomonas, Bacillus and others (Reddy and Sreeramulu, 2012).

Fungi are organoheterotroph hence they derived their growth requirements from breaking down complex organic molecules into simpler monomers. This is accomplished though secretion of extra cellular enzymes onto the substrate. Hence fungi are good sources of extra cellular enzymes, the enzymes are easily extracted without breaking the cells of the organism **(**Hankin and Anagnostakis, 2007**).** Fungi stand out as a major source of industrially applicable and stable enzymes (Sudeep et al., 2020).

Agricultural waste with pectic substance is generated annually and constitute nuisance, disposal of which is capital intensive. The use of agro wastes as the main carbon source in solid substrate fermentation for production of enzymes and organic acids is an effective solution in solving detrimental problem arising due to the waste disposal management. Various substrates have been exploited for enzyme production previously **(**Smith and Aidoo, 2005**).** Grape fruit (Citrus parasidis) is an annual fruit produced globally. Several tons of peel are generated yearly all over the world. Citrus fruit peels and skins, essentially grape peel is very rich in pectic substances. The peels are mostly left to rotten in public markets and dumping sites constituting environmental pollution. Environmental management is usually complex and capital consuming. Such waste could be exploited for enzyme production through microbial metabolism. Literature abound on the use of orange peel as substrate for enzyme production but seems to be scarce on the use of grape peel. Pectinase is an enzyme applicable to many areas of life significant to the survival of human race though, production is not yet localized and indigenous industries import this enzyme with huge amount of money. There is the need to exploit this waste for local enzyme production, hence, this study is aimed at using grape (Citrus parasidis) peel as indigenous substrate for the production of pectinase through the activity of resident fungi in solid state fermentation.

MATERIALS AND METHODS

Substrate Preparation

Fresh grapefruits were purchased from Sawmill, Ilorin, Kwara State, washed with running water to remove dirt as well as soluble pigments. The fruits were then peeled and the peels were dried in an oven (TT 9053) at 60 ^{0}C for 4 days. This dried material was then milled using a warring blender (Century) and stored in a tight container as the substrate for the production of pectinase enzyme.

Isolation and identification of fungi

Grapefruit was left to deteriorate in a clear packaging bag for five days, visible fungal mycelium was inoculated on Potatoes Dextrose (PDA) Streptomycin Agar, and incubated at 28±2 ^{0}C for 3 days. The observed growth was further sub-cultured until pure cultures of the organisms were obtained. Identification of isolates was based on cultural and microscopic characteristics. Cultural characteristics include the rate of growth (during incubation), the colony colour, hyphae and spore (Singh et al., 1991; Samson et al., 2004).

Preliminary Screening of Isolates for Pectinolytic Activity

Pure cultures were inoculated on modified Yeast extract pectin (YEP) medium plates containing 5.0 g of yeast extract agar, 2.5 g of pectin, and 10% streptomycin to prevent bacteria growth plate were incubated for 72 hours at 28 ±2 ^{0}C. After incubation iodine-potassium iodide solution (1.0g iodine, 5.0g potassium iodide and 330ml H_2O) was added to detect clearance zones (Phutela et al., 2005)

Confirmatory Screening of Isolates for Pectinolytic Activity

Secondary screening was carried out by the modified method of Banakar and Thippeswamy (2012). Screening was done for the selection of more potent colonies for the production of extracellular pectinase by culturing on pectin agar plates containing 5.0 g of yeast extract agar, 2.5 g of pectin, and 10% streptomycin supplemented with Congo (Methyl) red solution at pH 7.0. Actively growing mycelium's (3 days old) were removed from the growing edge of the fungal isolates by using sterile cork borer of 6 mm diameter, the discs were inoculated to the pre-welled pectin agar plates and incubated at 28 ± 2 ^{0}C for 5 days, after the incubation, plates were observed for the zone of clearance around the colony. Pectinase producing colonies were surrounded by pale orange to clear zone against the dark red background.

Inoculum Preparation

Three mililitre (3ml) of sterilized distilled water was added to a 7 days old PDA slant culture that has fully sporulated. 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, 1ml of suspension (consisting of about 1×10^7 spores/ml) was used as inoculum (Dhillon et al., 2004)

Solid State Fermentation

Ten grams (10g) of the substrate was added to 20 ml of distilled water to moisten the substrate and sterilized in an autoclave at 121 ^{0}C for 15 minutes. This was followed by cooling at room temperature. Inoculation was done aseptically with 1 ml of each of the fungal spore suspension respectively and the flasks were

incubated at 28±2 ^{0}C for 5 days (Kayode and Sani, 2008).

Enzyme Extraction

Crude enzyme extraction was carried out by addition of 100ml of sterile distilled water to 1g of fermented substrate and shake for 30 minutes at 120 revolutions per minute on a rotary shaker. The fermentation mixtures were filtered through cheesecloth (folded 16 times) and the filtrate was collected as the crude enzyme (Sangeeta and Shastri, 2007)

Enzyme Assay

Based on Martin et al. (2004) polygalacturonase activity was determined by measuring the releasing sugar group from citrus pectin using 3, 5-dinitrosalicylic acid (DNSA) reagent assay. The reaction mixture contained 2 ml of crude enzyme and 2 ml of citrus pectin in phosphate buffer in a test tube, the mixture was incubated at 50 ^{0}C for 30 minutes. After incubation the mixture was then filtered, to 2 ml of the filtrate, 2 ml of DNSA reagent was added to stop the reaction and the mixture was kept in boiling water bath at 100 ^{0}C for 10 minutes until the yellow colour developed. Then the tubes were cooled under running tap. The optical density of the resulting colored solution was measured at 540 nm using spectrophotometer. One unit of pectinase activity (U) was defined as the amount of enzyme which released 1 µmol of galacturonic acid per minute (Tadakittisarn et al., 2009).

Determination of pH

One gram (1g) of fermenting substrate was mixed with 100ml of distilled water and shaken on a rotary shaker at 120rpm for 30 minutes. The suspension was filtered with cheesecloth (folded 16 times). The pH meter (Starter 2000) was standardized before use. The pH of the samples were read and recorded.

Optimization of Culture Parameter for Pectinase Production

The parameters considered for optimum pectinase activity were incubation period (1-5 days), incubation temperature (20, 30 and 40^{0}C), moisture content (50, 100 and 150 %) and addition of salt supplement.

Effect of Incubation Period on Pectinase Activity

The optimum incubation period of pectinase was determined by weighing 10g of the substrate into 20 ml of distilled water in conical flasks and sterilized in the autoclave at 121 ^{0}C for 15 minutes followed by cooling. Inoculation was done aseptically with 1ml of each of the fungal spore suspension respectively in an inoculating chamber and the flasks were incubated at 28±2 ^{0}C for 5 days. One gram was taken each day from the fermented substrate for pectinase assay.

Effect of Temperature on Pectinase Production

The previously described procedure for fermentation was followed. The flasks were incubated at 20, 30 and 40 ^{0}C respectively for 5 days. One gram was taken each day from the fermenting substrate for pectinase assay.

Effect of Moisture Content

The effect of moisture content on pectinase activity was studied by varying the amount of water in the substrate. Ten grams of the substrate mixed with 50,100 and 150 % of distilled water. The flasks were treated as previously described. One gram was taken each day from the fermenting substrate for pectinase assay.

Addition of Salt Supplements

Salt solution composed of $MgSO_4$ 0.05 g, KCL 0.05 g, $FeSO_4$0.01 g, $ZnSO_4$0.01 g, $CuSO_4$0.01 g were dissolved in 100 mls of distilled water. Twenty mls of the salt solution was added to 10 g of the substrate. The flasks were incubated at 28±2 ^{0}C respectively for 5 days. One gram was taken each day from the fermenting substrate for pectinase assay.

RESULTS

Identification of fungal Isolates

Isolate A: Colonies were fast growing and covered the agar surface with a dense mycelium that was at first white becoming grey or yellowish brown and reverse was pale yellow. There were clear thin and short hyphae, mycelia were greenish. Spore bearing heads were large, globular and tightly packed. Chains of greenish conidia were also seen. The organism was identified as Aspergillus flavus.

Isolate B: Growth on agar plate was fluffy white which turned black producing large black coloured spores after 3 days of growth. The reverse was pale yellow, mycelium was wrinkled with clear thick and long aerial hyphae, unbranched conidia were present, the organism was identified as Aspergillus niger.

SCREENING FOR PECTINASE ACTIVITY

Aspergillus flavus and Aspergillus Niger gave a zone of hydrolysis of 5.5and 7.0 mm respectively.

Effect of Incubation Period on Pectinase Activity

The highest pectinase activity for A. Niger and A. flavus was at day 3 of fermentation with pectinase activity of 13.32 and 11.32 µmol/mg/min respectively and the lowest pectinase activity was at day 1 with activity of 4.66 and of 3.99 µmol/mg/min respectively (Figure 1).

Effect of Temperature on Pectinase Activity

Optimal pectinase activity with both organisms was achieved at a temperature of 40 ^{0} C. The highest enzyme activity was at 40 ^{0}Cand at day 4 of incubation (Figures 2 and 3)

Effect of Moisture Content on Pectinase Activity

The maximum amount of pectinase was produced at 150 % of moisture for A. niger and A. flavus. At 150 % moisture content, optimum pectinase activity by A .niger and A. flavus was recorded at day 3 with enzyme activity of 12.98 and 11.98 µmol/mg/min respectively (Figures 4 and 5).

Effect of Addition of Salt Supplement on Pectinase Activity

Addition of salt supplement did not affect pectinase activity. The result is presented in Figure 6. The highest pectinase activity for A. Niger and A. flavus was at day 3 of fermentation with enzyme activity of 11.32 and 10.65 µmol/mg/min and the lowest activity was at day.

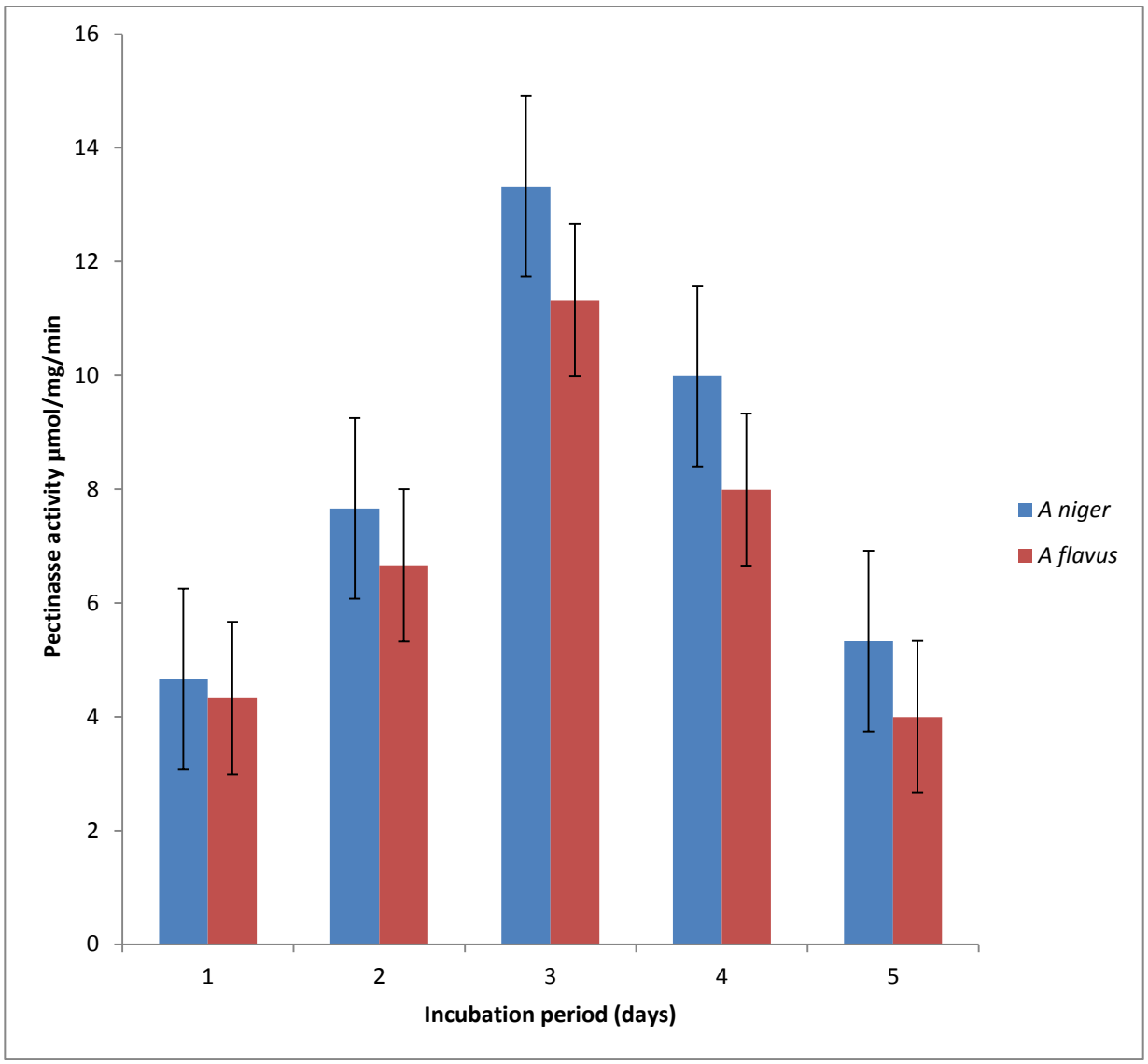

Figure 1: Effect of Incubation Period on Pectinase Activity by Aspergillus niger and Aspergillus flavus

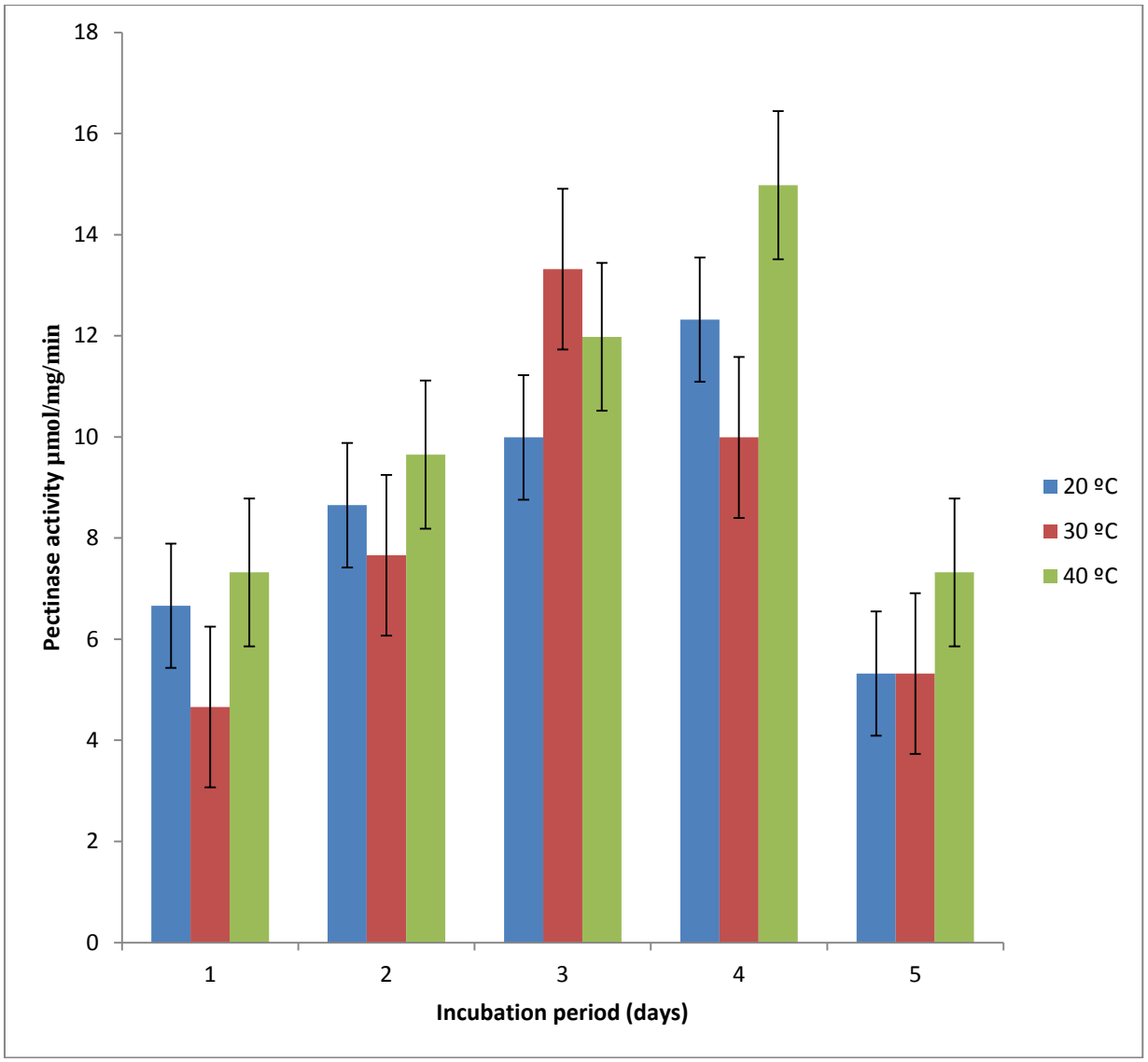

Figure 2: Effect of Temperature on Pectinase Activity by Aspergillus niger

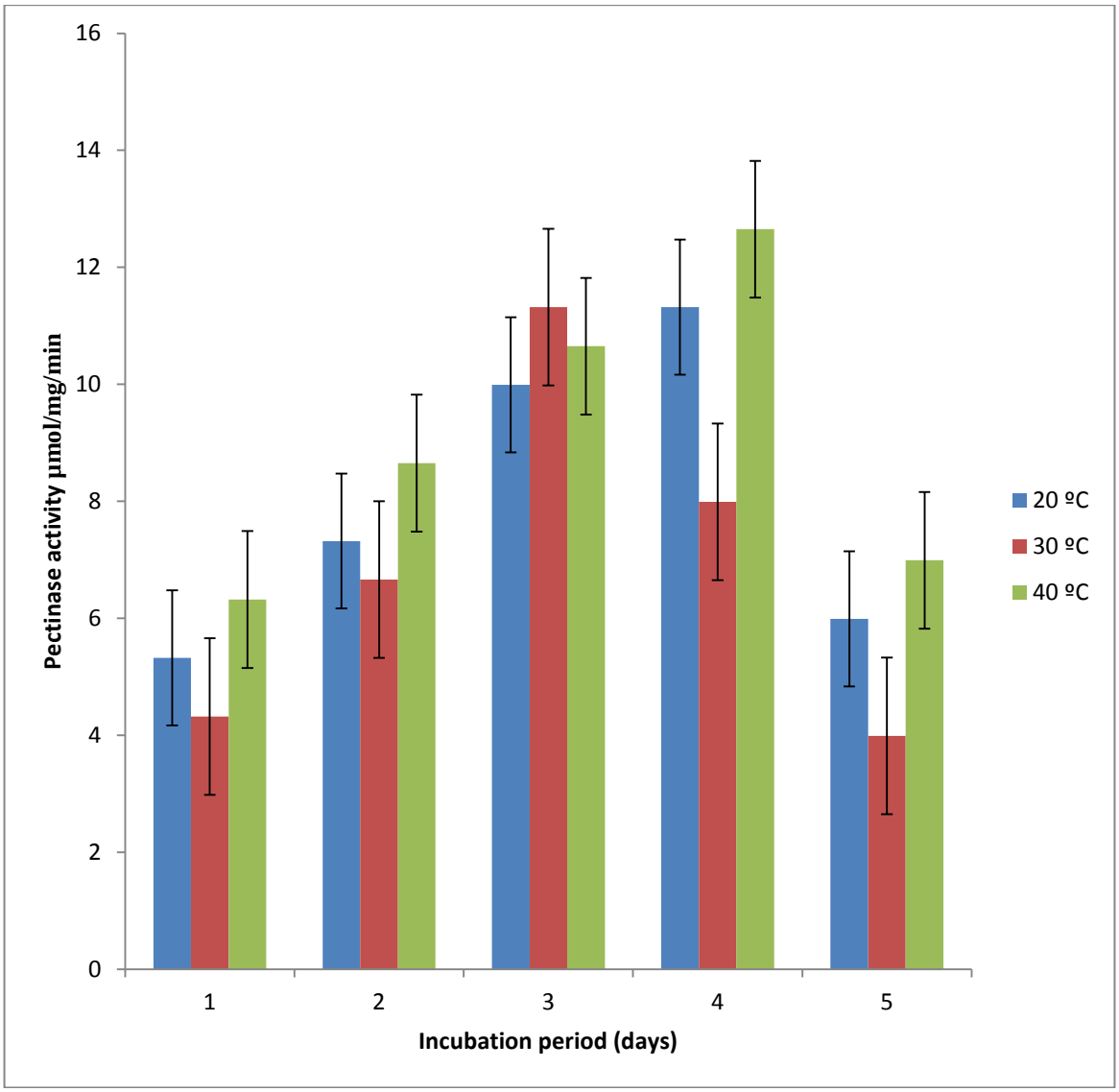

Figure 3: Effect of Temperature on Pectinase Activity by Aspergillus flavus

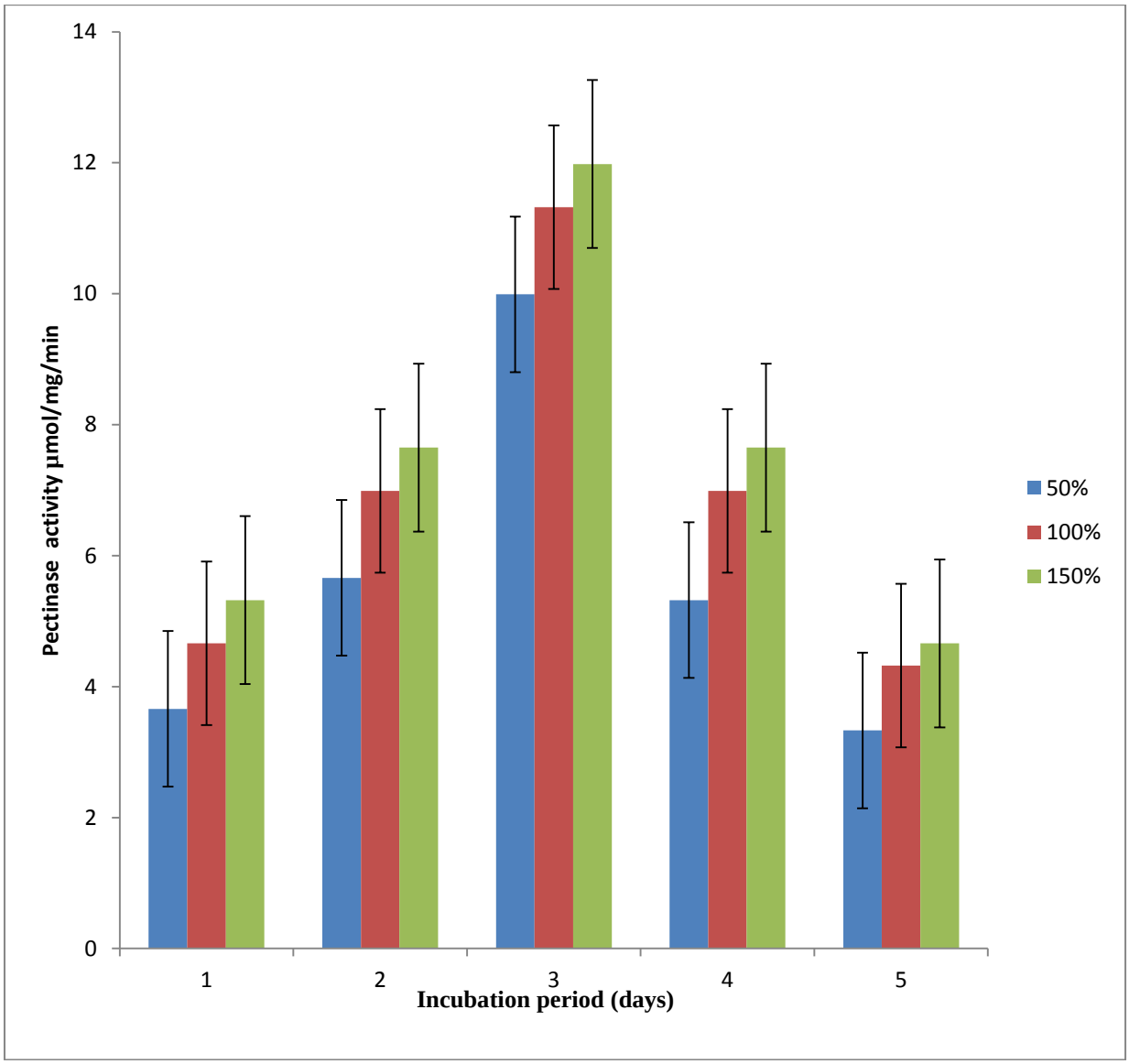

Figure 4: Effect of Moisture Content on Pectinase Activity by Aspergillus niger

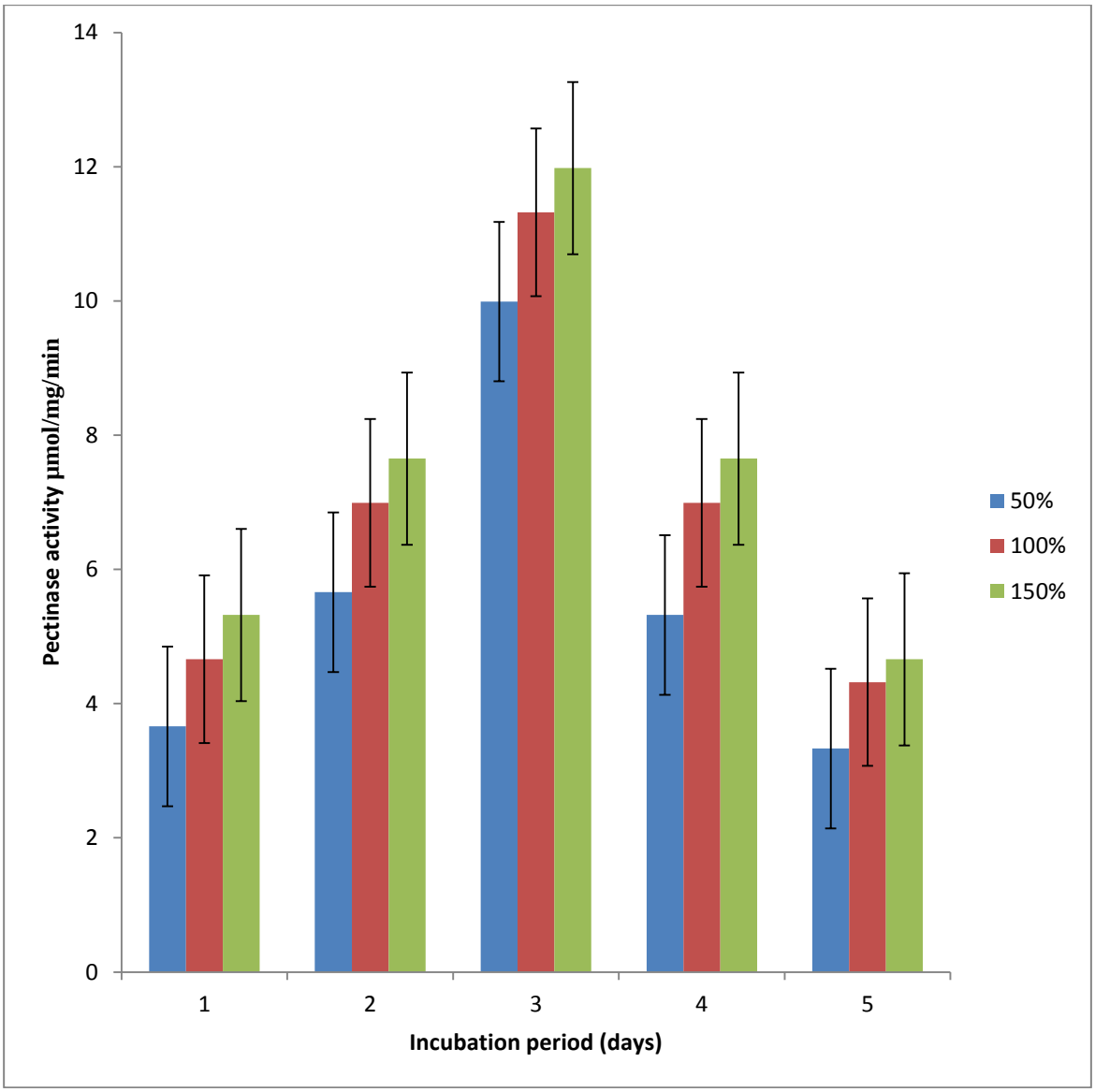

Figure 5: Effect of Moisture Content on Pectinase Activity by Aspergillus flavus

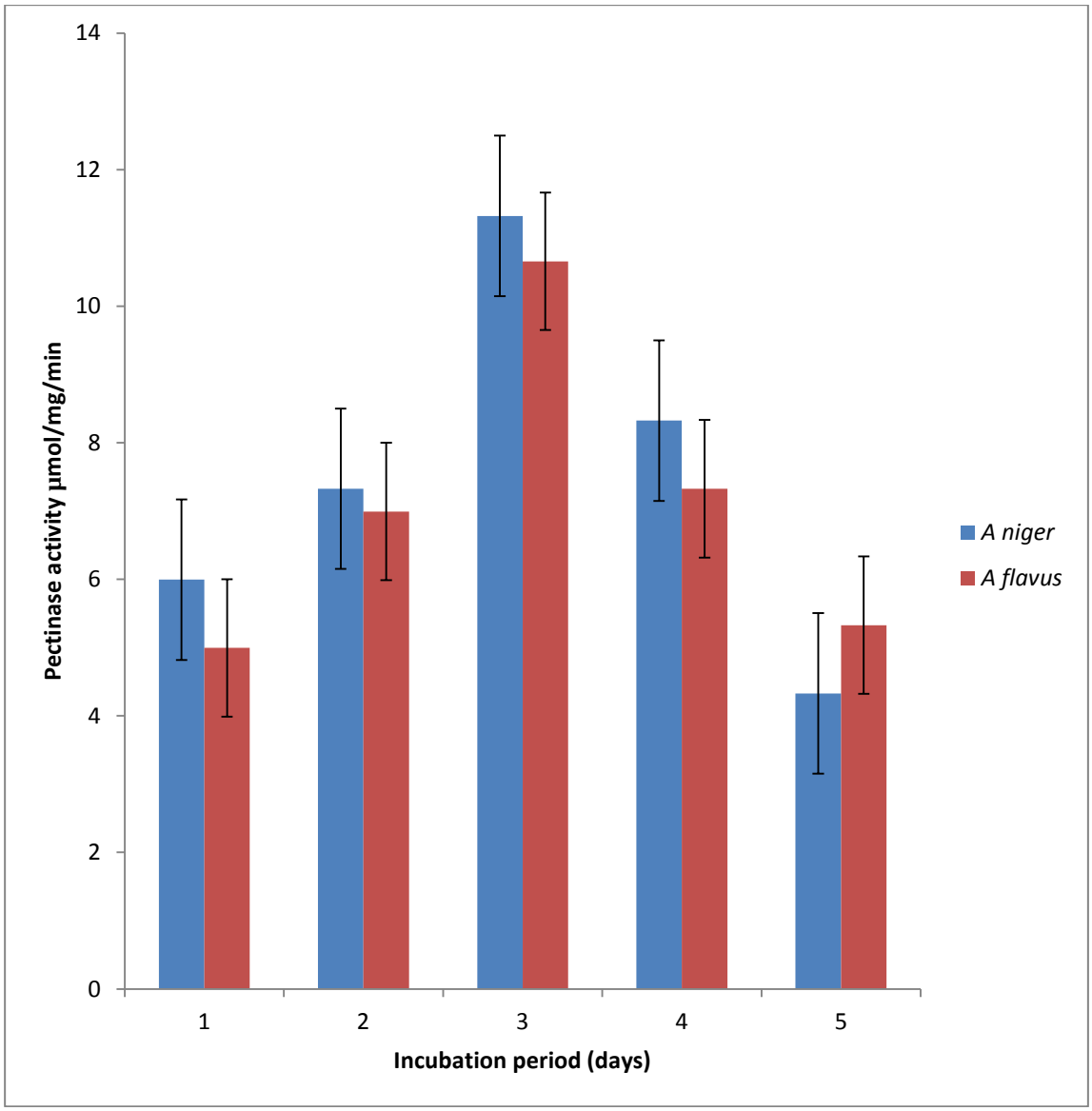

Figure 6: Effect of Addition of Salt Supplement on Pectinase Activity by Aspergillus niger and Aspergillus flavus.

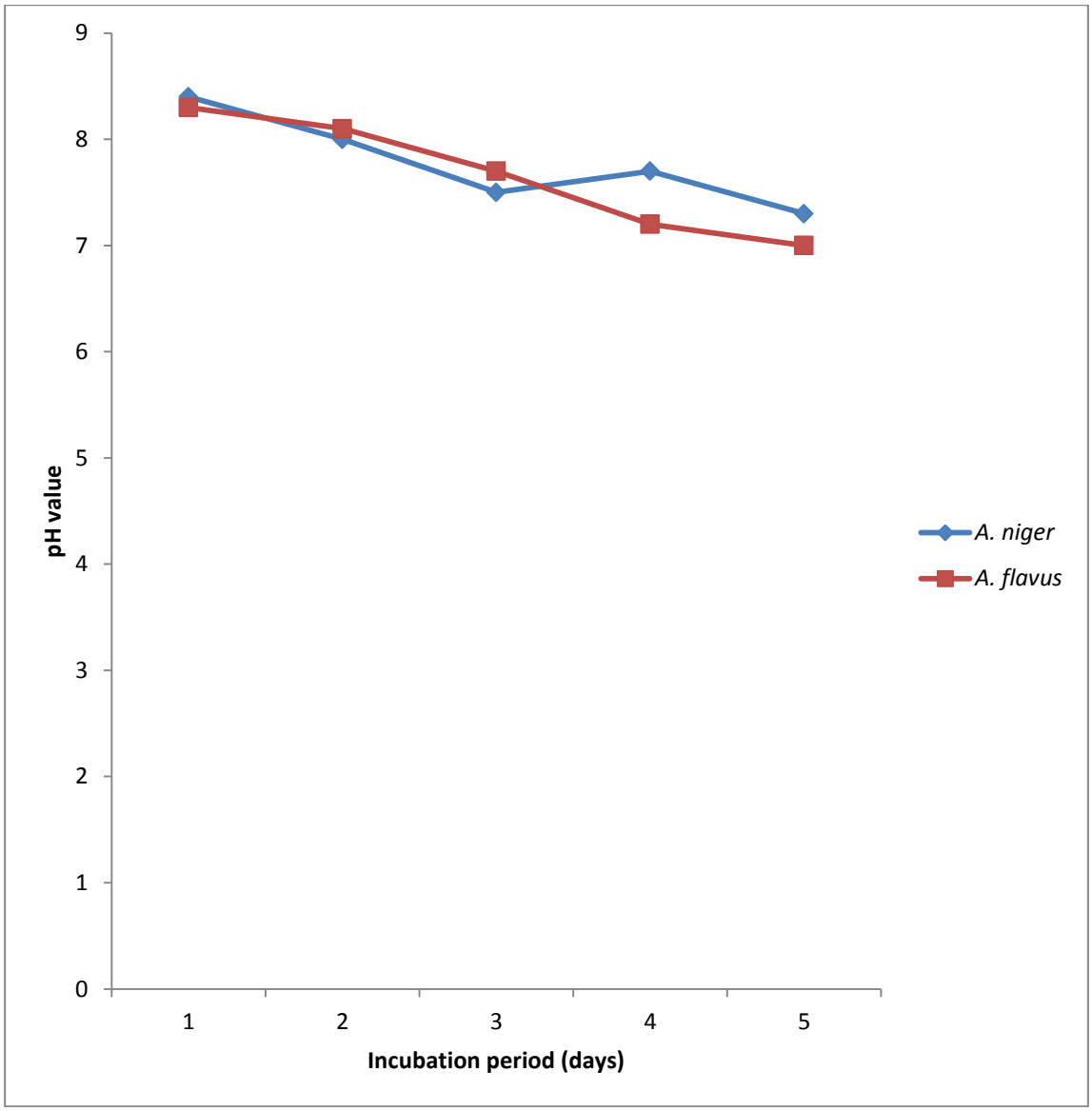

Figure 7: Changes in pH during Fermentation with Aspergillus niger and Aspergillus flavus

DISCUSSION

The identified fungal isolates were Aspergillus Niger and Aspergillus flavus. Fungal isolates have been reported to possess pectinolytic activity and have been successfully used for pectinase production by different researchers (Silva et al., 2002; Banu et al., 2010; Maciel et al., 2011), Aspergillus and Penicillium are reportedly the most used for enzyme production (Sukumaran et al., 2005; Favela-Torres et al., 2006), in fact the most common source of microbial pectinase has been reported to be Aspergillus Niger (Castilho et al.,2000). The two fungi possess high pectinolytic activity as shown from the screening. Time courses of pectinase production by Aspergillus Niger and Aspergillus flavus indicated that the production started from the first day of incubation period, and activities increased progressively until a peak was assumed on day 3 at 30 ^{0}C and day 4 at 40 ^{0}C. However, the pectinolytic activity of these fungi reduces afterwards. A similar trend in result has been previously reported (Castilho et al., 2000). Maximum enzyme activity for Aspergillus Niger has been reported to fall within 48-72 hours (Omojasola and Jilani, 2008; Abdullah et al., 2018). Some other authors however, have observe optimum activity at days 4-7 using Aspergillus Niger (Adeleke et al., 2012). The decrease in activity could be due to the depletion of nutrients in the medium, as mycelia increases and or accumulation of waste produce by the fungi. Generally, incubation period of a microorganism depends majorly on the composition of the substrate and biochemical characteristics of the strain like types of enzyme produced, cell division rate, such as its growth rate, moisture availability, temperature, oxygen concentration and inoculum size **(**Lonsane and Ramesh, 1990**).** The short period of achieving optimum enzyme activity could be of advantage to production industries.

Temperature is another critical parameter that must be controlled to get optimum enzyme activity. It has been found out that temperature is a significant controlling factor for enzyme production **(**Akintobi et al., 2012; Adeleke et al., 2012**)**. Enzyme activity is usually

optimized and highly stabilized at certain temperature, above or below this, the enzyme becomes in active, slow or entirely denatured (Lehninger et al., 1992).Optimum temperature for pectinase activity by Aspergillus niger and Aspergillus flavus in this study was 40 ^{0}C and day 4. There have been variations in optimum temperature for pectinase activity from different species of Aspergillus reported by various authors, 30- 40^{0}C was regarded as the best range (Aguilar and Huitron, 1995; Hours et al., 1999; Khatri et al., 2015). The fairly high temperature obtained in this study is a desirable property for an enzyme preparation that is commonly used in food processing industry.

Optimum pectinase activity was recorded at 150 %, further increase or decrease in moisture content resulted in reduction in the enzyme production. It has been earlier reported that reduction in enzyme activity/production at high moisture content may be due to either substrate particle agglomeration, or decrease in porosity. Low moisture content cause reduction in solubility of nutrients of the substrates and low degree of swelling (Murthy et al., 1999).

According to Shoichi et al. (2001), the initial pH of a medium has profound effect on the growth of the fermenting organisms. The pH affects the stability and activity of enzyme produce by microorganisms as well as their membrane permeability and biosynthesis (Murad and Azzaz, 2001). The pH for optimum pectinolytic ability for both organisms lies around weakly acidic to weakly basic region in this research.

Salt supplement did not show any impact on pectinase activity in this study because there was no pronounce change in enzyme activity when the medium was supplemented with salt. Supplementing the medium with salts especially of Nitrogen has been reported to positively affects enzyme activity (Sudeep et al., 2020). However, salts could serve as growth enhancer or enzyme inhibitor (Ouedraogo et al., 2017). In conclusion, grape peel was found to be a suitable substrate for Aspergillus Niger and flavus isolated to exhibit pectinolytic ability. The pectinolytic activity was found to be optimum at 40 ^{0}C and pH 7.5 and 7.7 for A. niger and A. flavus respectively. The two species of Aspergillus used in this research could be promising strains for pectinase production. However, further research is required to confirm this.

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