BIO-UTILIZATION OF APPLE PEEL BY *Aspergillus niger* AND *Aspergillus fumigatus* FOR PECTINASE PRODUCTION USING SOLID STATE FERMENTATION

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Abstract: *Aspergillus niger* and *Aspergillus fumigatus* isolated from agricultural dump site, Castor Research farm and serene environment were screened for their ability to produce pectinase using apple peel by solid state fermentation technique. The test fungi were screened for their ability to produce pectinase using modified Czapek–Dox liquid medium incubated at 37ºC for 24 h, plates showing zones of clearing around the colonies were selected for secondary screening and their crude extracts used for quantitative polygalaturonase assay. The results obtained showed that pectinase production (mg/ml) by *A. niger* and *A. fumigatus* were higher (0.018 and 0.012, respectively) while that of *Mucor spp* was the least (0.0001). Furthermore, the optimal pectinase activities were highest at the pH of 6.5 for both fungi, while *A. niger* and *A. fumigatus* showed optimal pectinase activities (mg/ml) of 0.014 and 0.013, respectively at 40ºC. The result obtained in this study has shown that both *Aspergillus niger* and *Aspergillus fumigatus* could be used industrially for pectinase production using apple peel.

Keywords: Pectinase, apple peel, *Aspergillus niger*, *Aspergillus fumigatus*, fermentation

Introduction

Pectinases which are also known as pectinolytic enzymes are a part of the group of enzymes involved in pectin degradation (Gautam *et al*., 2020) and are today one of the future enzymes of the commercial sector (Oliyad and Dawit, 2018). Pectinase is an enzyme of high molecular weight with negatively charged acidic glycosidic macromolecules that breakdown compound polysaccharides in plant tissues into simpler molecules with extraordinary specificity, catalytic power and substrate specificity (Approvi and Vuppu, 2012; Nisha and Padmaja, 2014). Recently, agro-industrial wastes have been used as carbon, hydrogen, and oxygen sources to produce ethanol, proteins, and microbial enzymes (Oliveira *et al*., 2006). These microbial enzymes are inducible, manufactured only when needed and they contribute to the natural carbon cycle (Hoondal *et al*., 2002; Oliyad, 2017).

Microbial pectinases has been reported to account for 25% of the global food enzymes sales (Oliyad, 2017; Thangaratham and Manimegalai, 2014). These enzymes chiefly, are a heterogeneous group of related enzymes that hydrolyze the pectic substances which are mostly present in plants (Oliyad, 2017). These pectinolytic enzymes are widely distributed in higher plants and microorganisms (Murad and Azzaz, 2011). They are of paramount importance for plants as they help in cell wall extension and softening of some plant tissues during maturation and storage (Gyan *et al.*, 2014; Shet *et al*., 2018). They also help in maintaining ecological balance by causing decomposition and recycling of waste plant materials (Gyan *et al.*, 2014). Microbial production of pectinase has been studied extensively over the years (Murad and Azzaz, 2011). Production of pectinase has been reported from bacteria including actinomycetes, yeast and fungi (Shet *et al.*, 2018; Murad and Azzaz, 2011).

Different *Aspergillus spp* have been found to produce extracellular enzymes (Gaston *et al.*, 2016; Oyeleke *et al.*, 2010). Nevertheless, fungal produced almost all the commercial preparations of pectinases (Murad and Azzaz, 2011). Amid fungi, *Aspergillus spp.* have many advantages as enzyme producers because they are considered suitable host organisms for industrial enzyme production attributable to their high secretion potential considering that they are recognized as Generally Regarded as Safe (GRAS); strains and yield extracellular products which can be recovered easily with rapid growth from fermented inexpensive media (Amit, 2020; Ivana and Mauricio, 2018). *Aspergillus niger* is the most typical fungal species used for industrial production of pectinolytic enzymes (Shet *et al.*, 2018; Murad and Azzaz, 2011; Oliyad, 2017; Ivana and Mauricio, 2018). Thermophilic fungi e.g. *Rhizomucor pusillus* (Salar and Aneja, 2007) are attainable sources of various industrially important thermostable enzymes such as lipases, xylanases, proteases, amylases and pectinases. There are few reports about the production of pectinase by thermophilic fungi even though a number of pectinases have been studied (Mrudula and Anitharaj, 2011).

Pectinase are used by winery and fruit processing industries for extraction and clarification of wine and fruit juice, to remove cloudiness, enhance fruit juice yield, intensifying flavor and color of wine also are of immediate application in maceration, liquefaction, extraction, fermentation and degumming processes etc (Kubra *et al.*, 2018; Oliyad, 2017; Palagiri *et al.*, 2019; Shet *et al.*, 2018; Sudeep *et al.*, 2020; Thangaratham and Manimegalai, 2014). Agricultural wastes like wheat bran, rice bran, potato peels, cassava waste, saw dust, pineapple peel, lemon peel, citrus peel, orange peel, and orange bagasse have been used for production of pectinase enzyme in solid state fermentation (SSF) (Bayoumi *et al.*, 2008; Simran and Vijay, 2017; Thangaratham and Mamimegalai, 2014). Solid state fermentation (SSF) technique is defined as the act of cultivating microorganisms on moist solid supports, either on passive carriers or on insoluble substrates employ as a natural raw material which can be used as carbon and energy source (Murad and Azzaz, 2011; Oliyad, 2018; Ivana and Mauricio, 2018).

Therefore, solid-state fermentation is an attractive method for fungal enzymes production because it fosters the natural growth of microorganisms on a moist insoluble substrate in the absence or near absence of free liquid (Lizardi-Jimenez and Hernandez-Martinez, 2017; Palagiri *et al.*, 2019; Rahul *et al.*, 2007). Among the most substrates previously used, apple peel contains an appreciable amount of pectin and can be used as the substrate and inducer for the production of polygalacturonase by microorganisms. The objective of this study is to produce pectinase by fermentation using two species of aspergili on apple peels by solid state fermentation.

Materials and Methods

Collection and preparation of samples

Top layer of the soil samples were collected from agricultural dump sites, Castor Research Farm and Serene Environment of National Cereal Research Institute (NCRI) Baddegi, Niger State. The soil samples were collected in triplicates by

scooping from each of the sites using hand trowel, following which they were transferred to sterile polyethene bags and transported to the microbiology Laboratory, Federal University of Technology, Minna.

Apple peels were collected from fruit sellers at Ultra Modern Market Minna Metropolis, Niger State, Nigeria. These were spread to air dry at room temperature (30± 2ºC) in the Microbiology Laboratory of Federal University of Technology Minna for two weeks following which they were oven dried (Memmert Universal Heating Oven, India) and ground using electric Wayfair Blender following which they were packed in air-tight polyethene bags and stored.

Isolation of fungi

Twenty gram (20 g) of soil sample each was weighed aseptically into 180 ml of sterile peptone water for serial dilution (1:10) and were made up to 10^{-3}. 1 ml each of the diluted soil samples were inoculated into 18 ml of molten Sabouraud Dextrose Agar (SDA) using pour plating technique. The plates were incubated for 72 h at 25ºC and observed for fungal growth. Pure cultures of the colonies were obtained by further sub-culturing onto the fresh SDA plates by streaking and then incubated for 72 h at 25ºC. Pure cultures of the further isolates were maintained on SDA slants stored in Refrigerator at 4ºC.

Characterization and identification of fungal isolates

The purified isolates were identified on the basis of standard cultural and morphological characteristics as described by Barnet and Hunter (1972).

Primary screening for pectinolysis

The media used for screening for pectinase was the modified Czapek-Dox Agar as described by Nwodo-Chinedu *et al*. (2010) in which sucrose was replaced with equal quantity of Malus pectin (apple peel, 1:1 w/v) as the only carbon source for pectinase producing enzyme All isolates were inoculated on Petri dishes containing Malus pectin and incubated at 37ºC for 24 h. After incubation period, the plates were stained with 50mM iodine to view the clear zones round the colonies (Yogesh *et al*., 2009).

Secondary screening

The *Aspergillus niger* and *Aspergillus fumigatus* which showed considerable zones of clearing in pectin containing agar were selected for secondary screening for pectinase production. The media used was modified Czapek-Dox liquid medium. The selected *Aspergillus niger* and *Aspergillus fumigatus* were each inoculated into Malus pectin containing 100 ml of Czapek-Dox liquid medium in 250ml Erlenmeyer flasks. The flasks were covered with sterile cotton wool and incubated in the dark at 30ºC for 5 days. After incubation, the cultures were harvested by filtration using Whatman No. 1 filter paper and the filtrates stored at 4ºC. These crude enzymes were used for quantitative polygalacuronase assay. Isolates with the highest enzyme activities were selected for further experiments.

Preparation of spore suspension

The selected *Aspergillus niger* and *Aspergillus fumigatus* sub-cultured on SDA slants were used to prepare the spore suspension. The spore from each slant was scrapped into 5ml of sterile water using sterile swab stick and inoculating needle. This suspension was filtered through a Whatman No. 1 filter paper to remove the hyphal filaments. The spore suspension was used as a source of inoculums.

Production of pectinase in solid state fermentation

Solid state fermentation (SSF) was carried out in 250 ml Erlenmeyer flasks that contained 5 g of apple peel and 5 ml of distilled water (1:1 w/v). The flasks were sterilized at 121ºC for 15 min and cooled to room temperature; 1 ml of conidial suspension of the fungi (*A. niger* and *A. fumigatus*) were added separately, mixed well and incubated at 30ºC for 96 h. At the end of the incubation period, the flasks were removed from the incubator and their contents (i.e. crude enzymes) extracted using 50 ml of sterile distilled water.

Enzyme preparation

In SSF, the enzyme was extracted from the fermented mycelia substrates by homogenously mixing the entire substrates with distilled water (1:1 w/v) which was agitated on a rotary shaker (Benchmark Scientific) at 100 rpm with a contact of 1 h at 30ºC separated by filtration using a Whatman No. 1 filter paper. Pooled extracts were centrifuged at 6000 rpm for 20min. at 4ºC and the clear supernatant was used as the source of extracellular enzyme.

Pectinase assay

Pectinase activity was determined using Malus pectin as substrate. The reaction mixture, containing equal amount of 1% (0.8 ml) pectin prepared in sodium acetate buffer (0.05M, pH 5.5) and suitably diluted crude enzyme (0.2 ml), was incubated at 50ºC in water bath for 30 min. Pectinase activity was stopped using Malus pectin as substrate. The reaction mixture, containing equal amount of 0.8 ml of 1% pectin prepared in sodium acetate buffer (0.05M, pH 5.5) and suitably diluted with 0.2 ml of crude enzyme obtained in this study, was incubated at 50ºC in water bath (MH Enterprises, India) for 30 min. The reaction was ceased with 1.0 ml dinitrosalycyclic acid solution (Miller, 1959 as cited by Adebare *et al*. (2012) and Okafor *et al*. (2010) after which the mixture was boiled for 10 min and cooled. The colour was read at 540nM using a spectrophotometer (Labtronics, India). A standard graph was obtained using standard glucose solution. One unit of pectinase activity was defined as the amount of pectinase enzyme which liberated 1 µm glucose per minute.

Results and Discussion

Pectinase production from isolated fungi

Table 1 show that the preliminary pectinase production by three test organisms (*Aspergillus niger*, *Aspergillus fumigatus* and *Mucor spp*). The pectinase activity as shown in the Table 1 produced by *Aspergillus niger* was 0.018 mg/ml followed by 0.012 mg/ml produced by *Aspergillus fumigatus*. However, the 0.0001 mg/ml produced by *Mucor spp*. was the least.

Table 1: Pectinase activities of *Aspergillius niger*, *Aspergillius fumigates* and *Mucor spp* on apple peel

Test organism	Optical density (540nM)	Pectinase activity (mg/ml)
Aspergillus niger	1.64	0.018
Aspergillus niger	1.16	0.012
Mucor spp.	0.16	0.0001

Each data is the mean value of triplicate determinations

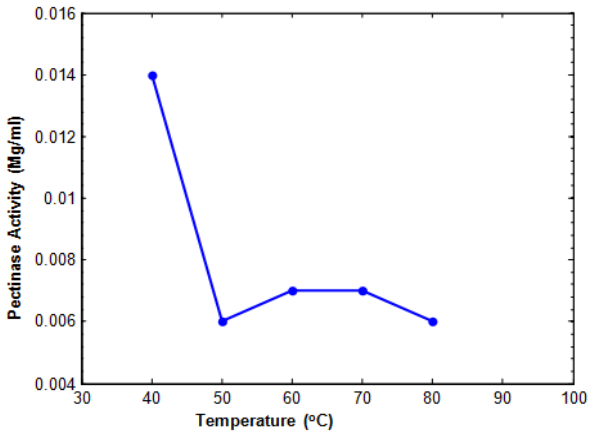

Fig. 1: Effect of temperatures on pectinase production by *Aspergillus niger*

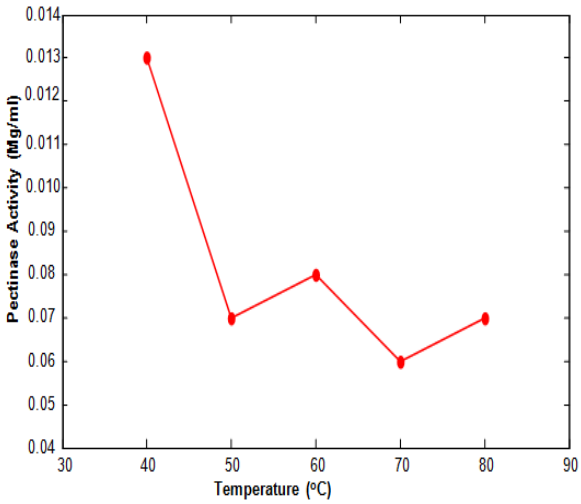

Fig. 2: Effect of temperatures on pectinase production by *Aspergillus fumigatus*

Pectinase activities of Aspergillus species at different temperatures

The effect of temperatures on pectinase activity of *Aspergillus niger* and *Aspergillus fumigatus* are shown in Figs. 1 and 2. Generally, the optimal pectinase activities were highest at 40ºC for both organisms (*Aspergillus niger*-0.014 mg/ml and *Aspergillus fumigatus*-0.013 mg/ml). However, the least pectinase activity for both organisms were observed at 50ºC (0.006 mg/ml-*Aspergillus niger*) and 70ºC (0.007 mg/ml-*Aspergillus fumigatus*).

Pectinase activities of Aspergillus species at different pH

The effect of pH on the pectinase activity of *Aspergillus niger* and *Aspergillus fumigatus* are shown in Figs. 3 and 4. As observed in these figures, the pectinase activities were optimal at the pH of 6.5 for both organisms and lowest at pH 5 (*Aspergillus niger*) and pH8 (*Aspergillus fumigatus*).

The best pectinase activity based on secondary screening was given by *Aspergillus niger* and *Aspergillus fumigatus* with 0.018 mg/ml and 0.012 mg/ml respectively while *Mucor* showed insignificant activity with 0.0001 mg/ml. (Table 1) The optimum pectinase production was obtained after 96 h at temperature of 40°C (Figs. 1 and 2) and pH of 6.5 (Figs. 3 and 4). The result obtained in this study agrees with the findings of Adebare *et al*. (2012). These workers reported pectinase activity of *Aspergillus niger* at 40ºC. However, the result is not in agreement with the findings of Freitas *et al*. (2006) and Rubinder *et al*. (2002). These workers observed that most fungi investigated for pectinase production showed optimum growth range of 45 to 60ºC. The reason may be due to the variation of environmental factors. According to Aminzadeh *et al.* (2007), maximum pectinase production was observed in the medium with the acidic initial pH values ranging from 4 to 6, contrary to the range of pH 6.5 as reported in this study. This could be due to the substrate used. Reda *et al.* (2008) observed that the pectinase productivity by *Bacillus firmus*-I-10104 reached its maximum at initial pH of 6.0 and 6.2, while Debing *et al.* (2005) reported that the pH of 6.5 was the optimal pH for pectinase production from *A. niger* by solid state fermentation, and this agrees with the pH value (6.5) obtained in this present study.

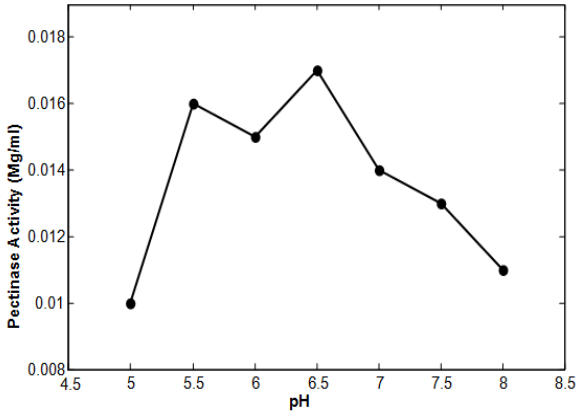

Fig. 3: Effect of pH on pectinase production by *Aspergillus niger*

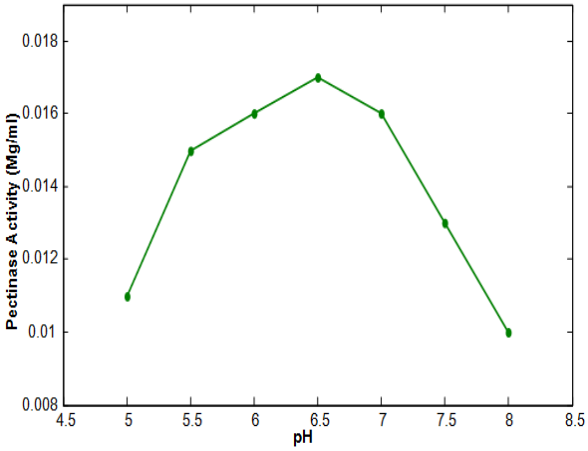

Fig. 4: Effect of pH on pectinase production by *Aspergillus fumigatus*

Furthermore, maximum production of pectinase by *Aspergillus niger* and *Aspergillus fumigatus* was observed on the 5th day. This is similar to the works of Adebare *et al.* (2012) who observed the maximum production by *Aspergillus flavus* and *Aspergillus oryzae* on the 5th day. Moreover, Sarvamangala and Dayanand (2006) observed that pectinase production from sunflower head by *Aspergillus niger* after 96hrs in solid state conditions was similar to those obtained in this present study. Therefore, the result obtained in this study has shown that *Aspergillus niger* and *Aspergillus fumigatus* could be used industrially for pectinase production using apple peel.

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