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SCREENING OF ASPERGILLUS NIGER ISOLATED FROM SOIL FOR PECTINASE PRODUCTION

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ABSTRACT

This study was conducted to screen for pectinase production by *Aspergillus niger* isolated from soil samples collected from three different locations within Ahmadu Bello University, Zaria, (botanical garden (BG), refuse dump (RD) and sheep pen (SP) sites). Fifteen (15) soil samples were collected from different locations and used for isolation by cultural method. Isolates suspected to be *Aspergillus niger* were further identified by microscopic examination using lactophenol cotton blue stained-preparation and slide culture technique. The isolates were then screened in a pectin-containing medium for their pectinase activity. The isolates were further subjected to pectinase production using citrus pectin as the substrate under submerged fermentation conditions. Seven (7) isolates were confirmed to be *Aspergillus niger* with percentage occurrence of 60% each from sheep pen, refuse dumpsites and 20% from botanical garden. *Aspergillus niger* RD3 produced the highest zone of pectin hydrolysis (53 ± 1.10 mm) while isolate RD5 produced the lowest (35 ± 3.10 mm). Under submerged fermentation conditions, *Aspergillus niger* SP5 had the highest pectinase activity of 2.92 U/mL while isolate RD4 had the lowest pectinase activity of 1.29 U/mL. *Aspergillus niger* can be readily isolated from various soil types with the highest frequency in soils from sheep pen and refuse dumpsites. All the *Aspergillus niger* isolates demonstrated the potential for pectinase production. The study reveals the potential of various A*spergillus niger* isolates from different soil in the production of pectinase.

Keywords: *Aspergillus niger*, Pectinase, Production, Soil, Screening

INTRODUCTION

Pectin is a polymeric material having carbohydrate group esterified with methanol. It is an important component of plant cell wall with highest concentration in the middle lamella (Rohit *et al.,* 2013). This acts as a connecting substance between adjacent cells.

Pectinases consist of a mixture of complex enzymes that specifically catalyzes the hydrolysis of pectin-containing substrates (Torimiro and Okonji, 2013). These enzymes are divided into three main classes that catalyzes depolymerization, demethylation and de-esterification reactions (Hatice and Filiz, 2016). Polygalacturonase (Enzyme Commission [EC] 3.2.1) is a depolymerizing enzyme that catalyzes the breakdown of 1,4-glycosidic bonds between two galacturonic acid residues in the smooth region of the pectin molecule. Whereas, pectin lyase (EC 4.2.2) and pectin esterase (EC 3.1.1) are demethylating and de-esterifying enzymes respectively, these acts as the elimination reaction between two methylated residues and splitting of methoxyl groups releasing methanol (Hatice and Filiz, 2016). Pectinases are derived from different sources such as bacteria, fungi, plants, insects, protozoans and nematodes (Raju and Divakar, 2013). Several bacterial and fungi species have for long been known to produce pectinolytic enzymes and it is widely believed that the production of these enzymes is a major means by which these microorganisms invade the host tissue (Ramachandran and Kurup, 2013). Even though the occurrence of pectinolytic enzymes have been reported in a large number of bacteria and fungi, almost all the commercial preparations of pectinases are produced from fungal sources, with *Aspergillus niger* being the most commonly used fungi species for industrial production of pectinolytic enzymes (Oliyad and Dawit, 2018). *Aspergillus niger* are widely distributed geographically and have been observed in broad range of habitats because they can colonize wide variety of substances. They are commonly found as saprophytes growing on dead leaves, stored grains, compost piles and other decaying vegetation. The spores are widely spread and are often associated with organic materials and soil (Karunakaran *et al*., 2014).

Pectinase are of great significance and have a number of biotechnological applications ranging from fruit juice extraction and clarification, degumming of plant fibres, waste water treatment, scouring of cotton, vegetable oil extraction, paper bleaching, tea and coffee fermentation as additives in poultry fodders, and alcoholic beverages and food industries (Abdel-Moshen *et al*., 2016).

There is generally an increasing global demand for enzymes in food industries, and about 25% of the overall demand is for pectinase (Osuigwe *et al.,* 2014). To meet this high demand, the discovery of new microbial sources capable of producing higher yields is imperative. This study isolates *Aspergillus niger* from soil samples of different locations and screen isolates for pectin-degrading enzymes.

MATERIALS AND METHODS

Collection of Soil Samples

Fifteen (15) soil samples, five (5) each were collected from three different locations within Ahmadu Bello University, Main Campus, Zaria, namely; botanical garden, refuse dump and sheep pen sites. Ten grams (10g) of each soil sample was separately collected from the locations from a depth of 5-10 cm using hand shovel into clean polythene bags. The soil samples were labelled appropriately and transported to the Main Teaching Laboratory, Department of Microbiology, Faculty of Life Sciences, Ahmadu Bello University, Zaria for further analysis.

Isolation and Characterization of *Aspergillus niger*

Preparation of media

Potato dextrose agar (PDA) used in this study was of analytical grade (Oxoid^{TM}), and was prepared according to the manufacturers instructions.

Isolation of *Aspergillus niger* from soil

This was carried out according to the method described by Kamalambigeswari *et al*., (2018) with slight modifications. Ten grams (10g) of each soil sample was separately suspended in 90 ml distilled water. Tenfold serial dilution was carried out to a dilution of 10^{-4}. Aliquots of 0.1 ml from the 10^{-1} and 10^{-3} dilutions were separately inoculated on freshly prepared potato dextrose agar plates by spread plate technique. The inoculated plates were incubated at room temperature for 3-7 days and observed for appearance of colonies. The isolates exhibiting cultural characteristics of *Aspergillus niger* were sub-cultured onto fresh PDA plates to obtain pure isolates and then preserved on PDA slants at 4^{o} C. Isolates obtained were coded according to the samples location sites namely; BG = Isolates obtained from botanical garden samples, SP = Isolates obtained from sheep pen site samples, RD = Isolates obtained from refuse dump site samples

Identification of *Aspergillus niger*

Cultural identification

The isolated fungi were identified after growth on PDA plates by observing their macroscopic characteristics such as colour, texture, appearance and diameter of colonies (Mohammed, 2013).

Microscopic identification

Wet mount technique (Mohammed, 2013) and slide culture technique (Patrick *et al*., 2010) were employed according to standard procedures for morphological identification of isolates.

Screening of *Aspergillus niger* Isolates for Pectinase Production

Aspergillus niger isolates obtained were screened for their ability to produce extracellular pectinase using pectin-containing agar medium with the following compositions (g/litre); 10 pectin, 3.0 $(NH_4)_2HPO_4$, 2.0 KH_2PO_4, 3.0 K_2HPO_4, 0.1 $MgSO_4$ and 25 bacteriological agar (Famotemi *et al*., 2015). The medium was autoclaved at 121^{o} C for 15 mins and poured into sterile petri dishes after cooling and allowed to solidify. Isolates were then point-inoculated at the centre of the plate with a pinch of the fungal mycelia. The inoculated plates were incubated for 5-7 days at room temperature. After incubation, the cultured plates were flooded with a Lugol's iodine for 1h and rinsed with deionised water. Isolates expressing pectinase activity exhibited a clear zone of hydrolysis around the margins of the colony while isolates without a clear zone exhibited a ring of intense staining around the colony (Rohit *et al*., 2013). The diameter of the zone of hydrolysis was measured using a ruler in millimetres (mm). The screening was carried out in two (2) duplicates and results expressed as means.

Pectinase Production by Submerged Fermentation

Preparation of inoculum

Spore suspension of the isolates was prepared by adding 10 ml of 0.1% Tween 80 to a sporulated five (5) days old slant culture of the *Aspergillus niger* isolates. A sterilized wire-loop was used to dislodge the fungal spores aseptically and then shaken vigorously to obtain a homogenized spore suspension. The spores in the suspension were counted with a haemocytometer to obtain a concentration of 1.0 x 10^6 spore/ml (Olaoluwa *et al*., 2018).

Submerged fermentation

Obtained strains were further used for pectinase production in mineral salt medium comprising the following composition in g/L; $NaNO_3$, 1.4g; KCl, 0.35g; $MgSO_4.7H_2O$, 0.35g; K_2HPO_4, 0.7g; $FeSO_4.7H_2O$, 0.007g; citrus pectin, 7.0g; distilled water, 700ml. Hundred millilitres (100 ml) of the mineral salt medium was transferred into 250 ml conical flasks and initial pH of the medium was adjusted to 6.0 using aliquots of HCl and NaOH. The medium was autoclaved at 121^{o} C for 15 mins and allowed to cool before inoculating with 10 ml (10% of production medium) of the prepared spore suspension containing 1.0 x 10^6 spores/ml. The flasks were incubated at room temperature for six (6) days (Olaoluwa *et al.*, 2018).

Extraction of pectinase enzyme

After incubation, the whole contents of the flasks were separately filtered with Whatman filter paper No.1. Each filtrate was then centrifuged at 5000 RPM for 10 mins at 4^{o} C. The supernatant was used as the crude enzyme suspension for pectinase assay (Olaoluwa *et al*., 2018).

Pectinase assay

Pectinolytic activity was determined according to the method described by Ramachandran and Kurup (2013). This was determined by measuring the release of reducing sugar in a mixture of 0.8 ml of 1% pectin and 0.2 ml of the culture supernatant in 0.2 M citrate phosphate buffer (pH 5.0) incubated at 40^{o} C for 10 mins. The reaction was stopped by adding 2 ml of dinitrosalicylic acid (DNS) reagent. The tubes were further incubated for 5 mins in a boiling water bath and then allowed to cool. The absorbance of the reaction mixture was measured spectrophotometrically at 540nm. One unit of pectinase activity is defined as the amount of enzyme that releases 1µ mol of galacturonic acid per min under assay conditions. Pectinase activity = concentration of galacturonic acid / incubation time x volume of enzyme used.

RESULTS AND DISCUSSION

Isolation and Identification of *Aspergillus niger*

Fifteen soil samples from three different locations were used in the isolation of *Aspergillus niger*, of which seven (7) isolates were confirmed to be *Aspergillus niger* based on cultural and microscopic characteristics as shown in Table 1. The percentage occurrence of the *Aspergillus niger* isolates in the three different soil samples is presented in Table 2 with soil samples from botanical garden having the least isolation rate of 20%, while soils from refuse dump and sheep pen sites had isolation rate of 60% each. *Aspergillus niger* are known to be ubiquitous in the soil, however, the observed differences in the percentage occurrence might be due to the richness in organic matter content of the humic soil from sheep pen and refuse dump sites, whereas botanical garden might have little organic matter as nutrient to the organism. This is similar to the findings of Rakesh and Kavita (2014) who reported the isolation of different fungal isolates with varying percentages of occurrence in which *Aspergillus niger* had the highest percentage of 18.46% while *Aspergillus oryzae* had the lowest percentage of 1.53%.

Pectinase Production by *Aspergillus niger* Isolates

All the seven (7) *Aspergillus niger* isolates screened were found to be capable of producing pectinase which was identified by zone of clearance signifying hydrolysis of pectin on agar plate. *Aspergillus niger* RD3 had the highest (53 ± 1.10 mm) zone of pectin hydrolysis while the least zone of pectin hydrolysis (35 ± 3.10 mm) was observed with RD5 as indicated in Table 3. This is similar to the findings of Kamalambigeswari *et al*., (2018) who screened three different *Aspergillus niger* strains F-3, F-4, F-P and reported isolate F-4 produced the maximum (9 mm) zone of pectin hydrolysis.

The production of pectinase under submerged fermentation using citrus pectin as substrate is shown in Table 4, with crude enzyme from isolate SP5 having the highest pectinase activity of 2.92 U/ml, whereas isolate RD4 had the lowest pectinase activity of 1.29 U/ml. This observed variation might be due to the differences in the metabolic potentials of the different isolates, and this might be due to differences in the nature of the soil from which they were isolated. In a similar study by Islam *et al*. (2013) twelve (12) *Aspergillus niger* strains isolated from decomposed apple were screened for pectinase production under solid state fermentation in which P-8 produced the highest pectinase activity (405 U/g) whereas P-2 produced the lowest pectinase activity (324 U/g). Similar findings reported by Pramod *et al*. (2014) shows that out of 82 *Aspergillus niger* strains isolated from soil and spoiled fruits, 18 strains were positive for pectinase production using citrus fruit peel as substrate under solid state fermentation with strain PSV23 having maximum pectinase activity (5.411 U/ml).

Table 1: Cultural and Microscopic Characteristics of the *Aspergillus niger* Isolates

Isolate's Code	Cultural Characteristics			Microscopic Characteristics		Identity
	Colour Change	Colony Texture	Colour of Reserve	Conidiophore	Phialides	
BG1	White-Black	Floc/comp	White-Yellow	Long smooth	Biseriate cover entire vesicle	*Aspergillus niger*
SP1	White-Black	Floc/comp	White-Yellow	Long smooth	Biseriate cover entire vesicle	*Aspergillus niger*
SP3	White-Black	Floc/comp	White-Yellow	Long smooth	Biseriate cover entire vesicle	*Aspergillus niger*
SP5	White-Black	Floc/comp	White-Yellow	Long smooth	Biseriate cover entire vesicle	*Aspergillus niger*
RD3	White-Black	Floc/comp	White-Yellow	Long smooth	Biseriate cover entire vesicle	*Aspergillus niger*
RD4	White-Black	Floc/comp	White-Yellow	Long smooth	Biseriate cover entire vesicle	*Aspergillus niger*
RD5	White-Black	Floc/comp	White-Yellow	Long smooth	Biseriate cover entire vesicle	*Aspergillus niger*

Key: BG = Isolates obtained from botanical garden samples, **SP** = Isolates obtained from sheep pen site samples, **RD** = Isolates obtained from refuse dump site samples

Table 2: Percentage Occurrence of *Aspergillus niger* in Various Soil Samples Analysed

n= 15

Sample Location	Number of Samples Collected	Number Positive (%)
Botanical garden	5	1 (20)
Sheep pen	5	3 (60)
Refuse dump site	5	3 (60)
Total	15	7 (47)

Key: **n=**Total number of samples collected

Table 3: Pectinase Production by *Aspergillus niger* Isolates on Solid Medium

Isolate's Code	*Diameter of Zone of Pectin Hydrolysis (mm)
BG1	40.5 ± 3.54
SP1	39.5± 2.12
SP3	41.5± 2.12
SP5	52± 2.83
RD3	53 ± 1.10
RD4	36.5 ± 2.12
RD5	35 ± 3.10

Key: **BG**= Botanical garden, **SP**= Sheep pen, **RD**= Refuse dump. * Values are mean ± standard deviation of two replicates

Table 4: Pectinase Production by *A. niger* Isolates via Submerged Fermentation

Isolate's code	Pectinase Activity (U/mL)
BG1	2.37
SP1	2.18
SP3	2.12
SP5	2.92
RD3	1.51
RD4	1.29
RD5	2.09

Key: **BG**= Botanical garden, **SP**= Sheep pen, **RD**= Refuse dump

CONCLUSIONS

This study shows that *Aspergillus niger* can be readily isolated from different soil types with the highest frequency of 60% in soil samples from sheep pen and refuse dump sites. All the *Aspergillus niger* isolates were found to have ability for pectinase production, with *Aspergillus niger* SP5 having the highest pectinase production of 2.92 U/ml under submerged fermentation. *Aspergillus niger* SP5 can be used for further studies to produce pectinase by submerged fermentation using agricultural residues as substrate.

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