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Production, Purification and Characterization of Polygalacturonase from *Aspergillus niger* in Solid State and Submerged Fermentation Using Banana Peels

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Authors' contributions

This work was carried out in collaboration between both authors. Author FSI designed the study, performed the statistical analysis and proofread the manuscript. Author EGV wrote the protocol, managed the analysis of the study and wrote the first draft of the manuscript. Both authors managed the literature searches. Both authors read and approved the final manuscript.

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ABSTRACT

Aims: Pectinases are extracellular enzymes produced by microorganisms which break down pectic polysaccharides of plant tissues into simpler molecules like galacturonic acids. Polygalacturonase catalyzes hydrolysis of α-1,4-glycosidic linkages in polygalacturonic acid producing D-galacturonate. Polygalacturonases are hydrolytic depolymerases with endo and exo activities that are widely used in food and chemical industries. This present study aimed to isolate fungal strain capable of producing polygalacturonase, compare its production in SSF and SmF, optimize the cultural conditions as well as purify and characterize the enzyme.
Study Design: The design adopted to evaluate the influence of cultural conditions on the enzyme production and physicochemical parameters on purified enzyme activity was One-Factor-at-a-Time approach (OFAT).

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Place and Duration of Study: Department of Microbiology, Faculty of Science, University of Port Harcourt, Nigeria, between November 2014 and December 2015.

Methodology: A total of 12 fungal strains were isolated from fresh banana peels. The isolates were screened for polygalacturonase producing ability using conventional methods and the isolate with the highest zone of inhibition was selected for further studies. The production of polygalacturonase by *Aspergillus niger* in solid state and submerged fermentation using banana peels as carbon source were compared and the best was used for further production studies. The effect of cultural conditions on polygalacturonase production by the fungus was evaluated. The crude enzyme was purified using ammonium sulphate precipitation and gel filtration on Sephadex G100 and effect of some physicochemical parameters on purified enzyme activity was also determined using standard methods.

Results: A total of 5 out of 12 fungi isolated from fresh banana peels were found to be pectin degraders. The most efficient isolate was identified as *Aspergillus niger* based on its colonial, morphological and microscopic examination. Solid state fermentation (SSF) was found to be more suitable (60.20%) for polygalacturonase production by *Aspergillus niger* compared to the submerged fermentation (SmF) (39.80%). Optimization of process parameters revealed that 48 h of incubation was the optimum for polygalacturonase production in solid state fermentation, while 72 h was observed for submerged fermentation. Supplementation of fructose to the fermentation medium led to increased enzyme production. KNO_3 was the best nitrogen source for polygalacturonase production. The enzyme was purified by ammonium sulphate precipitation (75%) and gel filtration on Sephadex G100. The purified polygalacturonase showed a specific activity of 166.67U/mg with 8.59% yield and purification fold of approximately 42. The optimum temperature for polygalacturonase activity was 40℃. T he purified enzyme was stable within 20-50℃ for 1 h. The enzyme has an optimum pH activity a t 5.0 and was stable within the pH range of 4-6. Co^{2+} strongly stimulated the enzyme activity while Ba^{2+} showed the highest inhibitory effect on the enzyme activity.

Conclusion: This study has revealed an enhanced production of polygalacturonase by *A. niger* under solid state fermentation using cost effective agricultural waste (banana peels). Thus, the re-utilization of banana peels as substrate for production of the enzyme by the fungus will minimize the pollution problems their presence may pose to the environment. In addition, the results obtained in this study indicated that the polygalacturonase from *A. niger* could found immense potential application in industrial sectors and biotechnology.

Keywords: Aspergillus niger; banana peels; characterization; polygalacturonase; solid state fermentation; submerged fermentation.

1. INTRODUCTION

Despite the remarkable progress made in increasing food production at the global level, approximately half of the fruits and vegetables produced in Nigeria becomes waste due to lack of proper retailing and adequate storage facilities. Abundant amount of waste materials is produced by agricultural and fruit processing industries, which pose considerable disposal problems and ultimately leads to pollution. Therefore, in recent years considerable attention is being paid by the scientific community to exploit commercially useful products from some of these agricultural wastes. A number of literature shows that enzymes such as pectinolytic enzymes which has wider industrial application can be easily obtained from vegetable wastes like carrot, pineapple, cabbage, tomatoes and beet, through simple extraction processes [1]. Pectinases are a group of enzymes involved in de-polymerization of pectic polymers. This group of enzymes consist pectin esterase, PE (E.C 3.1.1.11), polygalacturonase, PG (E.C 3.2.1.15), pectate lyase, PAL (E.C 4.2.2.2) and pectin lyase, PL (E.C 4.2.2.10) [2]. Pectin is a structural polysaccharide present in primary cell wall and middle lamella of fruits and vegetables. Pectin or pectic substances are heterogeneous group of high molecular weight, complex, acidic structural polysaccharides with a backbone of galacturonic acid residues linked by α-(1-4) linkages [3-7].

Pectin is one of the most widely available polysaccharide in nature after cellulose, starch and chitin. The basic unit of pectin is α, D-galacturonate which is linked through α-1, 4-glucosidic linkages. The side chains of the pectin molecule consist of rhamnose, galactose,

arabinose and xylose. The carboxyl groups of galacturonate are esterified with methanol and based on the degree of esterification, the pectic substances were differentiated into protopectin, pectin, polygalacturonic acid and pectinic acid [8]. These enzymes are used extensively for fruit juice clarification, juice extraction, manufacture of pectin free starch, refinement of vegetable fibers, degumming of natural fibers, waste-water treatment, curing of coffee, cocoa and tobacco and as an analytical tool in the assessment of plant products [5,9]. Pectinolytic Enzymes are also applied in fruit juices, jams, jellies, sauces, ketchups, flavoured syrups, desserts and other food industries [10-12]. In Dairy Industries, these are used to prevent the formation of casein clumps. In wine industry pectinases are mainly used for decreasing astringency by solubilizing anthocyanins without leaching out procyadin polyphenols and pectinases also increase pigmentation by extracting more anthocyanins [13]. In Textile Industries, pectinases are used for the lower discharge of waste chemicals and to improve safety of working conditions for textile workers and the quality of the fabric.

Polygalacturonase is an important pectin degrading enzyme. Polygalacturonases (PGases) catalyze the random hydrolysis of 1,4 α-D galacturonic acid linkages in smooth region of pectin. They have numerous applications in various types of industries such as juice and food industries. Polygalacturonases have industrial importance since they help to decrease the viscosity, increase the fruit juice yield and help to determine the crystalline structure of fibers [14]. Pectinase production by microorganisms is affected by various environmental parameters such as growth medium, pH, temperature, aeration, agitation, presence of metal ions and incubation time [15]. Both solid state fermentation and submerged fermentation has been used for the production of polygalacturonases [15-17]. This present study aimed to isolate fungal strain capable of producing polygalacturonase, compare its production by *A. niger* in SSF and SmF, optimize the cultural conditions as well as purify and characterize the enzyme.

2. MATERIALS AND METHODS

2.1 Sample Collection

Banana peels were collected from D-line fruit market Port Harcourt, Nigeria and were transported to the Food Microbiology Research Laboratory University of Port Harcourt where they were used for the study.

2.2 Isolation, Characterization and Identification of Isolates

Ten grams of fresh spoilt banana peels was weighed into 90 ml of sterile distilled water and shaken properly with the aid of an orbital shaker to obtain the stock solution. Serial dilutions of 10-1 to 10-6 were prepared. Potato Dextrose Agar (PDA) was sterilized by autoclaving at 121℃ for 15min. Streptomycin was added to prevent bacterial contamination. The above dilutions were plated in duplicates on PDA using the spread plate method. The inoculated medium was incubated at 30℃ for 48 h. Pure cultures were obtained by repeated sub culturing on PDA plates and maintained on PDA slants.

2.3 Screening of Isolates for Pectin Utilization

The isolates were screened for pectinase producing ability by inoculating them in a sterile medium containing 1% citrus pectin, 0.14% $(NH_4)_2SO_4$, 0.20% K_2HPO_4, 0.02% $MgSO_4.7H_20$, 0.1% Nutrient solution (5 mg/L $FeSO_4.7H_2O$, 1.6 mg/L $MnSO_4$, 1.4 mg/L $ZnSO_4.7H_20$, 2.0 mg/L $CoCl_2$), 3% agar pH 5.0 [18]. The medium was thereafter sterilized and dispensed aseptically on petri dishes. The plates were inoculated with a loopful of the isolates to form an oval shaped colony and incubated for 48 h. After incubation plates were stained with iodine solution. Clear zones were formed around the pectinase producing isolates. The isolate with the highest zone of clearance was identified based on cultural, morphological and microscopic examination.

2.4 Preparation of Banana Peel Powder

Fresh banana peels were dried at room temperature (30℃), ground and sieved to obtain smaller substrate particle which provides a larger surface area for microbial attack.

2.5 Inoculum Preparation

The inoculum was prepared by harvesting one slant in 20 ml sterile saline solution under aseptic conditions, the suspension was shaken using an orbital shaker incubator for 10 min for proper dispersion of spores. Thereafter, 1 ml was withdrawn and inoculated into each fermentation flask.

2.6 Production of Polygalacturonase in Submerged Fermentation

The liquid basal medium containing 0.6% $(NH_4)_2SO_4$, 0.6% KH_2PO_4, and $MgSO_4.7H_20$ 0.01% with 2% dry banana peels powder (w/w) as the sole carbon source was added to the basal medium. The medium was sterilized at 121℃ for 15min. The pectinolytic isolate identified was used for inoculation. The flasks were plugged properly and incubated for 5 days at 30℃. Aliquots of the crude enzyme extract were withdrawn at an interval of 24, 48, 72, 96 and 120 h. Whatman No.1 filter paper was used to filter the aliquots which were used for enzyme assay.

2.7 Production of Polygalacturonase Using Solid State Fermentation

Solid state fermentation was carried out in a set of five flask, each containing 15 g of ground dried banana peels and 10 ml of the mineral salt solution of 0.6% $(NH_4)_2SO_4$, 0.6% K_2HPO_4, and $MgSO_4.7H_20$ 0.01%. The medium was sterilized at 121℃ for 40 min [18]. The flasks were inoculated with the test isolate and incubated at 30℃ for 24, 48, 72, 96 and 120 h. Fifty milliliters of sterile distilled water was added to one of the flak at an interval of 24, 48, 72, 96 and 120 h and then filtered. The filtrate obtained was used for conducting polygalacturonase assay.

2.8 Polygalacturonase Assay

Polygalacturonase (PG) activity was determined by measuring the release of reducing groups from citrus pectin using 3,5 dinitrosalicylic acid (DNS) reagent [19]. The reaction mixture containing 2 ml of 1% citrus pectin in 0.2M phosphate citrate buffer pH (5.5) and 0.5 ml of the crude enzyme solution was incubated at 40℃ for 10 min. Thereafter 2.5 ml of DNS reagent was added to stop the reaction and boiled in water bath for 15 min. After cooling, colour absorbance was read at 540 nm using a spectrophotometer. Polygalacturonase activity was defined as the amount of enzyme required to release 1µmol equivalent of galacturonic acid per min.

2.9 Protein Determination

Total proteins were estimated by the Bradford method using bovine serum albumin (BSA) as standard [20].

2.10 Effect of Inoculum Size on Enzyme Production

Different inoculum size of heavy spore suspension of the most potent isolates was prepared by harvesting one slant in 20 ml sterile saline solution under aseptic conditions. The following inoculum sizes were varied: 1, 2, 4, 6, 8 and 10 ml in each fermentation flask. At the end of the incubation period, polygalacturonase produced were determined for each flask.

2.11 Effect of Carbon Sources on Enzyme Production

One percent of glucose, fructose, manittol, starch and cassava peels were separately added to the fermentation medium in order to ascertain the best carbon source for the fermentation. Enzyme activity was measured at the end of each fermentation process.

2.12 Effect of Nitrogen Sources on Enzyme Production

Nitrogen source of 0.6% NH_4Cl, $NaNO_3$, KNO_3, Peptone, Yeast extract and Urea were separately added to the fermentation medium in order to ascertain the best nitrogen source for the fermentation. Enzyme activity was measured at the end of each fermentation process.

2.13 Purification of Crude Enzyme

2.13.1 Ammonium sulphate precipitation

The cell free filtrate (100 ml) was brought to 75% saturation by mixing with ammonium sulphate (Sigma) slowly with gentle agitation and allowed to stand for 24 h at 4℃. After the equilibration, the precipitate was removed by centrifugation (5000 rpm at 4℃ for 15 min). The obtained precipitate was dissolved in 50 ml of 0.2 M sodium acetate buffer.

2.13.2 Desalting by dialysis

According to [21] the precipitate was desalted by dialysis as follows: 10 cm dialysis bag was activated by rinsing in distilled water. One end of the dialysis bag was tied tightly and the formed precipitate was placed into the bag. The other end of the dialysis bag was tightly tied to prevent any leakage. Thereafter, the dialysis bag was suspended in a beaker containing 0.2 M sodium acetate buffer to remove low molecular weight substances and other ions that may interfere with the enzyme activity.

2.13.3 Gel filtration chromatography

The dialyzed enzyme fraction was further purified by gel filtration chromatography. The vertical glass tube chromatography column (2.5×70 cm) of Sephadex G-100 (Particle size, 40-120 µ) was prepared and calibrated as described by Ajayi [22]. Thirty milliliters of the enzyme concentrate was loaded to the Sephadex G-100 column and eluted with 0.2 M sodium-acetate buffer with a flow rate of 20 ml/h. Fractions (each 5 ml) were subsequently collected and absorbance was measured by using spectrophotometer (absorbance at λ280). Each of the fractions was analysed for polygalacturonase activity. The fractions showing higher enzyme activity were pooled together for further characterization.

2.14 Enzyme Characterization

2.14.1 Effect of pH on enzyme activity

The effect of pH on polygalacturonase activity was measured at a fixed assay temperature of 40℃. Various pH values ranging from 4.0 - 9.0 were used. In each case, the following pH buffer solutions were used following the assay method described by [19] using sodium acetate (pH 4.0, 5.0), sodium citrate (pH 6.0, 7.0), and sodium phosphate (pH 8.0, 9.0).

2.14.2 Effect of pH on enzyme stability

The pH stability of the enzyme was determined by exposing the purified enzyme first at varied pH values (4 to 9) using different pH buffer solutions as mentioned previously for 2 h. Thereafter, aliquots of the mixtures were taken to measure the residual enzyme activity (%), under standard assay conditions [23].

2.14.3 Effect of temperature on enzyme activity

The optimum temperature was determined by incubating each reaction mixture at varied temperatures (20-70℃). The relative activities (as percentages) were expressed as the ratio of the purified polygalacturonase obtained at a certain temperature to the maximum activity obtained at the given temperature range [23].

2.14.4 Effect of temperature on enzyme stability

Thermal stability of the enzyme was investigated by measuring the residual activity after exposing the enzyme at various temperatures ranging from 20 to 70℃ for 1 h. The initial relative activity was taken as 100%.

2.14.5 Effect of different metal ions on enzyme activity

To determine the influence of different ions on enzyme activity, the following ions and salts were used: Ca^{+2}, EDTA, Cu^{+2}, $Zn+^{2}$, Mg^{+2}, $Ba+^{2}$ $CoCl_2$ and NaCl. The listed ions and salts were added to the reaction mixture at concentration (1 mM). Each metal ion salt was added into 0.25 ml of 2% pectin in 0.1 M acetate buffer, pH 6.0. The mixture was incubated at 40℃ for 10 min and assayed. The relative activity of the control (without) metal ion was taken as 100%.

2.14.6 Effect of substrate concentrations on enzyme activity

The effect of varying the concentrations of different substrate (banana peels) was studied. The banana peels powder was used in the preparation of 2% solution of the substrate in 0.1 M acetate buffer, pH 6.0. The concentrations were varied between 0.05 and 0.25 mM. The effect of varying the concentrations of substrate followed the assay procedure earlier described [19]. In place of the pectin in the assay procedure, banana peels was used.

2.15 Statistical Analysis

The results obtained from this study were subjected to t-test (pair two samples for means) and analysis of variance ANOVA (two factors without replication).

3. RESULTS AND DISCUSSION

A total of twelve fungal isolates were gotten from fresh banana peels and only five were found to degrade pectin. The most efficient isolate among the five fungal strains was selected for further studies and subsequently identified as *Aspergillus niger* using colonial and microscopic characteristics.

3.1 Identification of Pectin Degrading Fungal Isolate

Table 1 presents colonial and microscopic identification of the most active pectin degrading

fungus. The result shows that, Colonies grow and spread within 2-3 days of incubation period with fluffy and slightly velvety texture. The hyphae were Septed and branch with round conidial heads (globose), large and also radiate or as they grow splitting into loose columns of conidia chains with age.

3.2 Polygalacturonase Production by *Aspergillus niger*

Fig. 1 shows polygalacturonase production by *Aspergillus niger* in solid state and submerged fermentation using banana peels as carbon source. The result indicated that, the peak of enzyme production was observed at 48 h of incubation in solid state fermentation (60.20%) and 72 h for submerged fermentation (39.80%). Similarly, 120 h of fermentation was found to produce the least enzyme for both solid state and submerged fermentation.

3.3 Effect of Carbon Source on Enzyme Production

Fig. 2 shows the effect of carbon source on polygalacturonase production using banana peels as substrates for fermentation. *Aspergillus niger* had showed highest polygalacturonase production in the presence of fructose (17.23 U/ml) followed by glucose (15.28 U/ml). The result revealed that cassava peels and starch were poor polygalacturonase inducers. The fungus produced the lowest amount of the enzyme when starch was incorporated in the medium.

Table 1. Colonial and microscopic identification of the most active pectin degrading fungus

Isolate code	Colonial Morphology on agar	Microscopy	Inference
PDF3	Growth: Colonies growth spreading within 2-3 days of incubation period with fluffy and slightly velvety texture. The aerial mycelium is white at first subsequently developing in to dark brown to black conidial heads with no reverse colour.	Septed and branch hyphae with round conidial heads (globose), large and also radiate or as they grow splitting into loose columns of conidia chains with age. Conidiophores arising from the substratum, mostly colourless to brown, smooth, splitting when crushed like pieces of cane. Vesicles globose while phialides are borne directly on the vesicle, metulae and foot cells are usually present.	*Aspergillus niger*

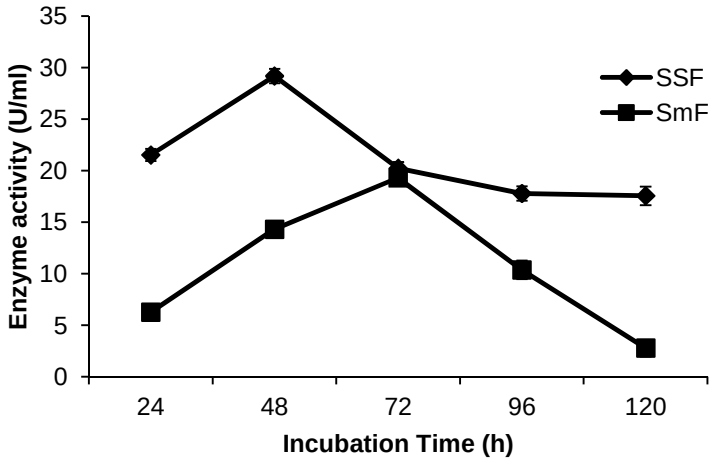

Fig. 1. Production of polygalacturonase by *Aspergillus niger* in solid state fermentation (SSF) and submerged fermentation (SmF) using babana peels as carbon source

3.4 Effect of Nitrogen Sources on Enzyme Production

Fig. 3 show the effect of nitrogen source on polygalacturonase production by *A. niger* using banana peels as substrates for fermentation. Our result indicated that among the various nitrogen sources investigated ammonium nitrate (KNO_3) was the best nitrogen for the production of the enzyme by the fungus, followed by peptone and urea in the decreasing order. The lowest production of polygalacturonase was observed in the presence of NH_4Cl.

3.5 Effect of Inoculum Size on Enzyme Production

The effect of inoculum size on enzyme production is depicted in Fig. 4. The result revealed that polygalacturonase production increased with increase in inoculum volume up to 4 ml after which it decreased steadily. The result shows that optimum production of the enzyme occurred when the inoculum size was 4 ml.

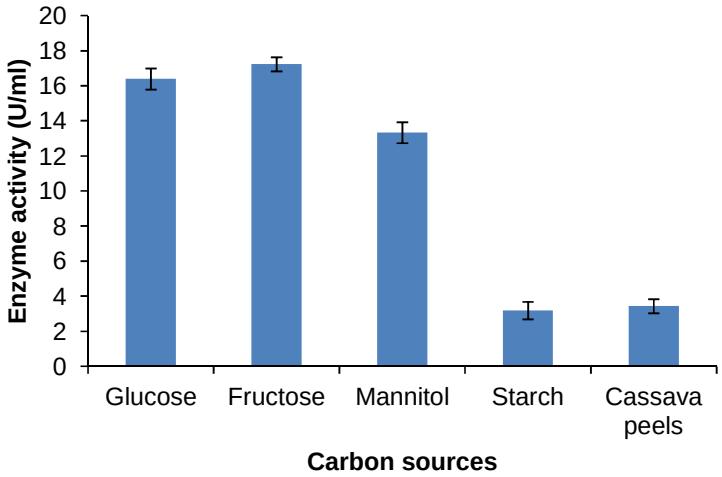

Fig. 2. Effect of carbon sources polygalacturonase production by *Aspergillus niger* using banana peels as substrates

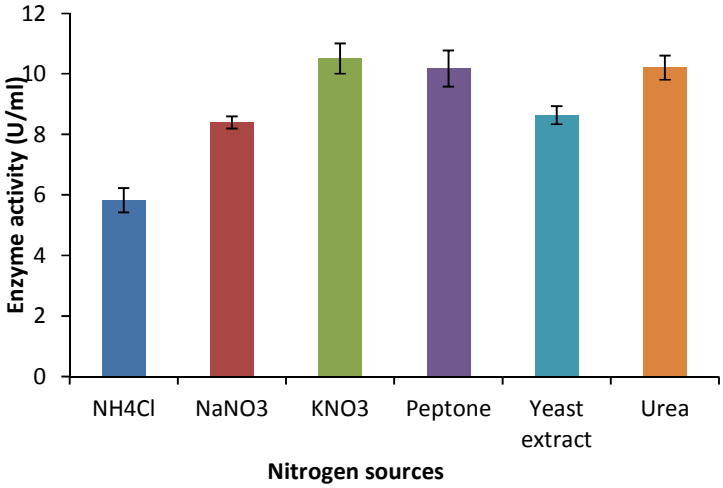

Fig. 3. Effect of nitrogen sources polygalacturonase production by *Aspergillus niger* using banana peels as substrates

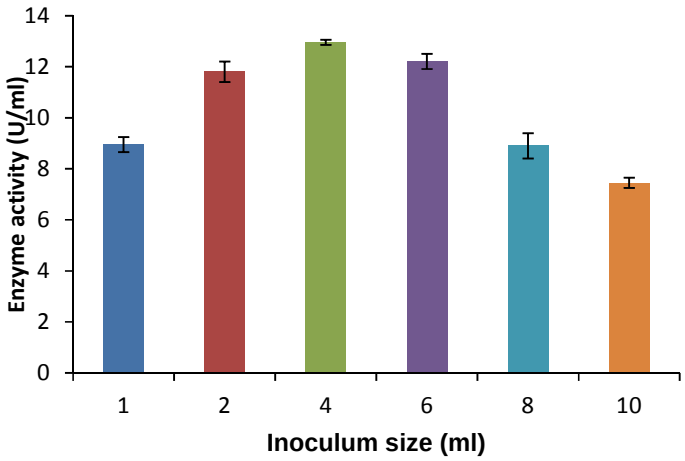

Fig. 4. Effect of inoculum size on polygalacturonase production by *Aspergillus niger*

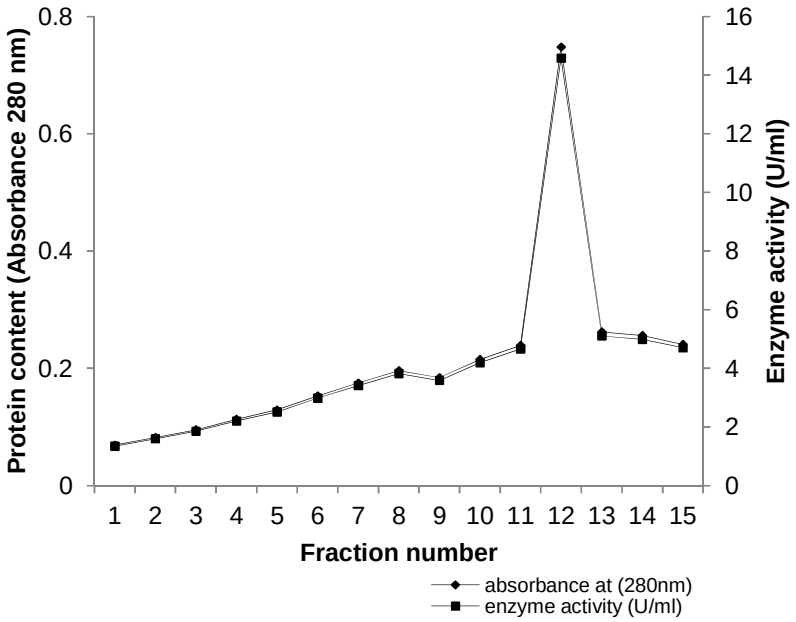

Fig. 5. Elution profile of polygalacturonase of *Aspergillus niger* on Sephadex G100

3.6 Purification of Polygalacturonase from *A. niger*

Fig. 5 (above) represent the elution profile of pectinase by *Aspergillus niger* using banana peels as substrate. The result indicated that, the active fraction was observed at fraction number 12 for enzyme activity and protein content on Sephadex G100.

The result of the purification procedures are summarized in Table 2. The result indicated that polygalacturonase of *Aspergillus niger* was purified to homogeneity by ammonium sulphate precipitation and one step gel filtration on Sephadex G100. The specific activity in ammonium sulphate salting out (75% saturation) was 18.98 U/mg protein and 31% recovered in precipitation proteins with a purification fold of 4.80. Sephadex G100 gel filtration (Fig. 5) provided a purification fold of 42 with a yield of 8.59%. An overall purification of up to 42-fold with an 8.9% recovery and specific activity of 166.67 U/mg protein was achieved (Table 2).

Table 2. Summary of the purification of polygalacturonase from *A. niger*

Purification step	Protein volume (ml)	Total protein (mg)	Total activity (U)	Specific activity (U/mg protein)	Yield (%)	Purification fold
Crude extract	100	442	1747	3.95	100	1.0
Ammonium sulphate	68	28.56	541.96	18.98	31.02	4.80
Sephadex G100	30	0.9	150	166.67	8.59	42.17

3.7 Effect of Temperature on Purified Polygalacturonase Activity and Stability

Fig. 6 shows dependence of polygalacturonase activity and stability on temperature. The purified enzyme activity increased with increase in temperature of incubation until an optimum was reached. Further increase in temperature beyond the optimum led to a decline in enzyme activity. The enzyme activity was maximal at 40℃. In the case of thermal stability profile, the initial activity was taken as 100% before incubation at different temperatures for 1 h. The result indicated that the purified polygalacturonase activity from *A. niger* retained considerably activity over a relatively broad range of storage temperatures (20 – 50℃) for 1 h (Fig. 6). The polygalacturonase retained over 90% of the initial activity between 20℃ and 50℃, while about 39% and 61% of residual activity was lost after pre-incubation at 60℃ and 70℃, respectively for 1 h. The purified enzyme was most stable at 20℃ (100%).

3.8 Effect of pH on Purified Polygalacturonase Activity and Stability

The dependence of polygalacturonase activity and stability on pH is depicted in Fig. 7. The pH activity profile of polygalacturonase from *A. niger* exhibited maximum value at pH 5.0. The result indicated that at 9.0, the activity decreased to 57%. The purified enzyme displayed wide range of pH stability (3-7) after 2 h of pre-incubation. The initial activity before the pre-incubation of purified enzyme for 2 h was taken as 100%. Result revealed higher stability at pH 5.0 (100%) and lower stability after the optimal pH was exceeded. About 43% of the original activity was lost when the enzyme was stored at pH 9.0 for 2 h.

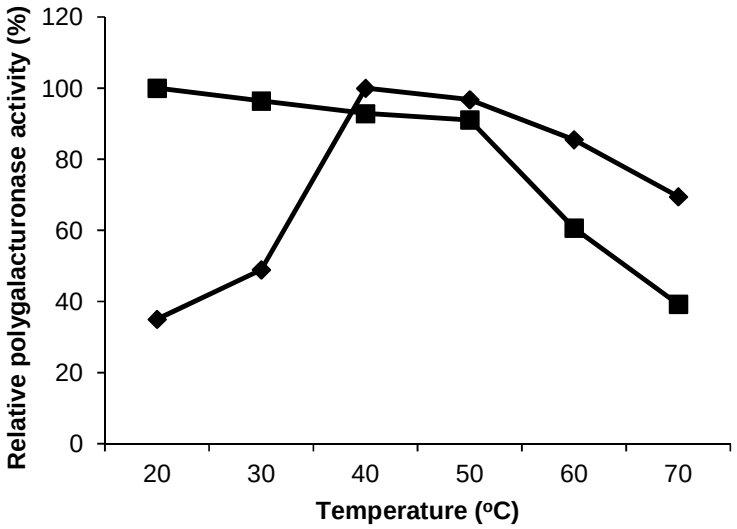

Fig. 6. Dependence of polygalacturonase activity (♦) and stability (■) on temperature

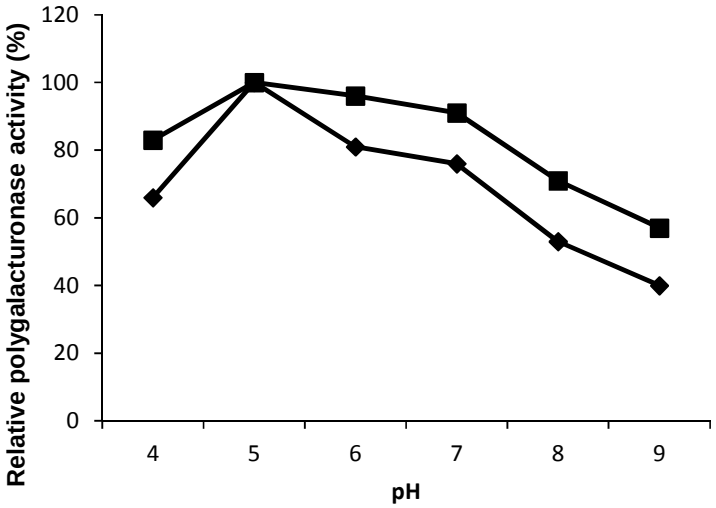

Fig. 7. Dependence of polygalacturonase activity (♦) and stability (■) on pH

3.9 Effect of Metal Ions and Some Inhibitors on Purified Polygalacturonase Activity

The effect of metal ions and some inhibitors on purified polygalacturonase activity is depicted in Table 3. As shown in Table 3, all the metal ions evaluated either as chloride or sulphate ions with the exception of Co^{2+}, Mg^{2+} and Zn^{2+}, caused considerable inactivation of the enzyme activity at 1 mM level. Ba^{2+} elicited 45% inactivation of the purified polygalacturonase activity. The enzyme activity did not show any requirement for monovalent Na^{+} or divalent Ca^{+}. In fact, while Na^{+} seemed to have any effect on the enzyme activity, the presence of Ca^{2+} elicited 16% inhibition of the enzyme activity. Co^{2+} at 126% relative activity strongly stimulated the enzyme activity, while Mg^{2+} and Zn^{2+} at 117% and 112% enhancement of the enzyme activity respectively was moderately stimulatory. EDTA showed only 12% inactivation of the purified polygalacturonase activity.

3.10 Effect of Substrate Concentration on Enzyme Activity

Fig. 8 shows the dependence of polygalacturonase activity on of different substrate (banana peels powder) concentration. The enzyme activity increased with increase in substrate concentration until the optimum activity was observed after which the activity declined. The result indicated that the optimum enzyme activity was obtained at a concentration of 0.2% while the least activity was found at concentration of 0.05%.

Table 3. Effect of metal ions and some inhibitors on purified polygalacturonase activity

Metal salt/ inhibitor	Concentration (mM)	Relative activity (%)
None	0	100
Calcium chloride	1	84
Copper sulphate	1	87
Zinc sulphate	1	113
Magnesium sulphate	1	117
Barium chloride	1	54
Cobalt chloride	1	126
Sodium chloride	1	101
Ethylene diamine tetraacetic acid (EDTA)	1	88

3.11 DISCUSSION

The aim of this study was to isolate, screen, produce and purify polygalacturonase from fungi using cheaper agricultural waste (banana peels). Twelve fungal isolates were isolated from banana and were screened for pectinase production using method described by [18]. Five fungal isolates were found to be pectinolytic in nature with maximum of 27 mm zone of

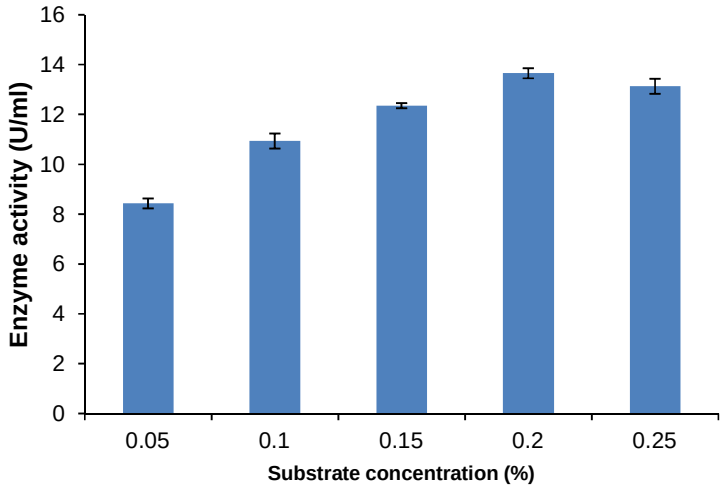

Fig. 8. Dependence of polygalacturonase activity on substrate concentration

clearance and 5.2 mm enzymatic index. The fungus with the highest zone of clearance was identified as *Aspergillus niger* based on colony morphology on agar and Microscopy. This organism has been widely reported as pectin degraders by several researchers. However, comparative study of their pectinases producing potentials in respect to different fermentation methods is scanty.

Results obtained from this study shows that solid state fermentation tends to favour polygalacturonase production than submerged fermentation with *A. niger*. It is evident from the result that *A. niger* is highly favoured in solid state fermentation than submerged fermentation with progressive increase in enzyme production from 24 h of incubation to an optimum of 48 h, after which continuous decrease was observed. This result shows significant increase ($p<0.05$) in enzyme production (polygalacturonase) when *A. niger* was compared in solid state and submerged fermentation using banana peels as substrate. The optimum enzyme production was observed after 48 h of incubation in solid state fermentation, while 72 h was observed as the optimum incubation period in submerged fermentation. The least of enzyme production was observed at 120 h of incubation. This result is in conformity with the report of Ramachandran and Kurup [24] that compared solid state and submerged fermentation in the production of polygalacturonase and pectin lyase from *Penicillium citrinum* using orange waste as substrate. They found solid state fermentation to have higher enzyme activity both for polygalacturonase (94.03%) and pectin lyase (5.64%) than submerged fermentation with (0.18%) and (0.14%) for polygalacturonase and pectin lyase, respectively. This result also agrees with the finding of Shruti and Sudev [25] who fermented dried pineapple residue with mixed culture of *Aspergillus fumigates* and *Aspergillus sydowii* through solid state fermentation. The peak of enzyme activity in their research was found at 48 h of fermentation for polygalacturonase (340 U/g) and pectin lyase (80 U/ml). The similarity in the results is partly due to similarity in type of substrates used in the fermentation. Similarly, Nitinkumar and Bhushan [26] reported similar incubation time of 72 h in submerged fermentation with *Penicillium* spp. while Niketan et al. [27] reported 96 h of incubation time for *A. niger* which is contrary to the finding of this study.

The optimization of carbon source on polygalacturonase production revealed that glucose and fructose were better sources of carbon than starch and cassava peels, which suggest that pectinolytic microorganisms readily utilize simple sugars (glucose and fructose) than complex polysaccharides such as starch and cassava peels. This is because simple sugars are excellent carbon source for pectinolytic microorganisms' proliferation which in turn, is responsible for the high yield of galacturonic acid generation from their substrates. Ramachandran and Kurup [24] reported that, carbon source other than pectin induced pectinase production

like, galacturonic acid, fructose, and mannose induced polygalacturonase production in *Geotrichum candidum*.

The organisms' preference to nitrogen source shows that, KNO_3, peptone and urea gave high polygalacturonase production followed by $NaNO_3$, yeast extract while the least polygalacturonase production was observed with NH_4Cl. This observation is supported by [28] who reported KNO_3, urea and peptone as the best polygalacturonase inducer for *Bacillus licheniformis*. In the same vein, Kashyap et al. [29] reported that, when various nitrogen sources were supplemented in wheat bran medium, yeast extract (YE), peptone and ammonium chloride were found to enhance pectinase production up to 24%.

An investigation was carried out to ascertain the best inoculum size for optimum enzyme production. It was revealed that, there was a steady increment in enzyme production from 1ml inoculum size to 4 ml after which a decrease was noticed. Four millimeter (4 ml) was the optimum inoculum size for polygalacturonase production which falls within the range of 2 to 10 ml inoculum size reported in the production of bacteria pectinase from agro-industrial wastes [30].

The Polygalacturonase produced by *Aspergillus niger* were purified using ammonium sulphate precipitation, concentrated by dialysis and finally by gel filtration. The result shows decrease in total protein and total activity, whereas specific activity increased from crude extract to Sephadex G100 purified fraction. This decrease in protein activity was attributed to the removal of impurity from the crude which is responsible for the high total protein and enzyme activity. The enzyme yield for polygalacturonase was 8.59% and purification fold of 42.17 on Sephadex G100 fraction. This is consistent with previous observation [31,32]. Both researchers reported decrease in both total protein and total activity, although [31] reported very low yield of 13% and 0.789% for ammonium sulphate precipitation and Sephadex G100 with corresponding purification fold of 1.2 and 1.6, respectively. They attributed the low enzyme yield to working with concentrated protein solution. On the contrary a high yield of 85.19%, 72.02% and 69.44% has been reported for $(NH_4)_2SO_4$, G100 and SP C-50, respectively with corresponding purification fold of 3.24, 18.90 and 28.19 [32].

The effect of temperature on enzyme activity indicated that 40℃ was the optimum temperature for polygalacturonase activity, though there is an appreciable enzyme activity at 30℃ and 50℃. On the other hand, polygalacturonase were more stable at 20 to 40℃ which implies that pectinase are adversely affected by increase in temperature. These results are in accordance with Niketan et al. [27] who reported 40℃ as optimum temperature for *Aspergillus flavus, Aspergillus niger and Aspergillus oryzae*. El-Batal et al. [31] also reported 40℃ as optimum temperature for enzyme activity and 30 to 50℃ for temperature stability when gamma irradiated *Penicillium citrinum* was used. In contrast to our result, Shruti and Sudev [25] reported optimum temperature of 35℃ while Gummadi et al. [33] reported an optimum temperature at 30℃ for pectin lyase activity with *Aspergillus niger* NCIM 548. Arotupin et al. [32] reported a decline in the enzyme activity with a temperature more than 40℃. Aalberto et al. [16] reported optimum temperature of 60℃ for exopolygalacturonase from *Leucoagaricus gongylophorus*. It was reported that extremely high temperature lead to deamination, hydrolysis of the peptide bonds, interchange and destruction of disulphide bonds and oxidation of the amino acid side chains of the enzyme protein molecules.

The result of this study showed that, pH 5 was the optimum pH for polygalacturonase activity. Polygalacturonase activity with respect to pH showed a steady significant increase ($p<0.05$) from pH 4 to pH 7 and significantly ($p<0.05$) decreased from pH 8 to 9 when compared with one another. This finding conformed with several reports that polygalacturonase are acidic pectinases. Similar result has been obtained in previous studies [23]. Several other workers reported optimum polygalacturonase activity at pH 5.0 [34-36]. Our result is consistent with optimum of pH 5.0 reported for exogalacturonase from *Leucoagaricus gongylophorus* [16]. The maximum enzyme activity of *Monascus* spp. and *Aspergillus* spp for exo-polygalacturonase was obtained at pH 5.5. The stability of enzyme in suitable buffer system at 40℃ was equally investigated in this current study. The result revealed that polygalacturonase was stable over a wide range of pH (4 to 7), but was more stable at pH 5. This result is similar to the optimum pH of 5.0 and stability at 4 to 7 reported for *Penicillium citrinum* [31].

On polygalacturonase preference to metal ions, Zn^{2+}, Mg^{2+} and $CoCl_2$ enhance enzyme activity while Ba^{2+} and Cu^{2+} had slight inhibitory effect on enzyme activity. This observation is consistent with [37] who reported enhanced pectinase activity by K^{+}, Ni^{2+}, Mn^{2+} and Zn^{2+} from *Bacillus stearothermophilus, Bacillus cereus* and *Bacillus subtilis* while N^{+} had a slight inhibitory effect on the enzyme. Similarly, Ca^{2+}, Mg^{2+}, Mn^{2+} and Zn^{2+} were observed to activate pectinase from *Penicillium italicum* but was inhibited by Cu^{2+} and Fe^{2+} [38]. Our observation is in contrast with Banu et al. [39] who reported that Ca^{2+} had little effect on pectinase activity from *Penicillium chrysogenum* while Co^{2+} inhibited the enzyme activity. However, our result is similar to their report that Mg^{2+} stimulated the pectinase activity. In another study, Ajayi [22] reported the stimulatory action of the increase in concentration of Ca^{2+}, Mg^{2+}, Zn^{2+} and K^{+} on polygalacturonase activity produced by *Botryodiplodia theobromae*. According to Arotupin et al. [32] the formation of a chelate compound between substrate and metal ions could form a more stable metal-enzyme-substrate complex and stabilizing the catalytically active protein conformation.

4. CONCLUSION

The results from this study show that banana peels is an excellent source of pectin, and pectinolytic microorganisms such as *Aspergillus niger* is associated with their biodegradation in the environment. The result also shows that, the enzyme production efficiency was increased by optimization of cultural conditions, such as carbon source, nitrogen sources and other parameters evaluated in this study. It was also revealed from the study that, solid state fermentation is more favourable in production polygalacturonase by *A. niger* compared to submerged fermentation both in terms of enzyme yield and incubation time. The result of this study indicated that polygalacturonase of *A. niger* can be purified to homogeneity by ammonium sulphate salting out and gel filtration on Sephadex G100 with specific activity of 166.67 U/mg protein, purification fold of approximately 42 and yield of 8.59%. Characterization studies of the purified enzyme showed that the optimum temperature and pH were 40℃ and 5.0 while the enzyme was most stable at 20℃ and 5.0, respectively. This indicated that the fungus is a mesophile with good potential to elaborate an acidic polygalacturonase using cheap carbon sources such as banana peels. However, based on the physicochemical properties of the purified enzyme, this enzyme possesses great potential for industrial and biotechnological applications.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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