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Production, Purification and Characterization of Polygalacturonase from Aspergillus flavus Grown on Orange Peel

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Abstract

Polygalacturonase is a pectinolytic enzyme that catalyzes the hydrolytic cleavage of the polygalacturonic backbone linkage chain. In this study, polygalacturonase was produced from *Aspergillus flavus* isolated from an orange waste dump site. Production was carried out in solid state fermentation. Optimum production of polygalacturonase was found at incubation period of 96 h, pH 4.5 and at 35°C using ammonium sulphate, orange peel as best nitrogen and carbon sources, respectively. The enzyme was precipitated with 60% ethanol resulting in 3.54 fold purification, and purification of the enzyme with Sephadex G-75 resulted in 9.93 fold. The purified enzyme showed maximum activity in the presence of polygalacturonic acid at 35°C and pH 4.5, while for sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) analysis, molecular weight obtained was 66 KDa. The K_m and V_{max} value of the enzyme were found to be 0.705 mg/mL and 1.0508 µmoL/min, respectively. The addition of metal chlorides and inhibitors reduced the enzyme activity. Based on the physicochemical properties of the purified enzyme, this enzyme possesses great potential for industrial and biotechnological applications such as fruit clarifications and oil extraction.

Keywords: *Aspergillus flavus*; Hydrolytic cleavage; Inhibitors; Metal chlorides; Pectinolytic; Polygalacturonase

Introduction

Enzyme technology, a subfield of biotechnology is associated with the application of enzymes as tools of industry, medicine and agriculture. It offers an increasing potential for the production of goods to meet various human needs [1,2]. The industrial enzymes include pectinases, cellulases, amylases and proteases. The majority of these enzymes are derived from fungi (e.g., *Aspergillus*) and bacteria (e.g., *Bacillus*). In the food industry, enzymes are used in extraction to increase the yield of fruit and vegetable juices, to control clarity of juices, to enzymatically peel fruits, to improve the texture of fruits and vegetables, to produce wine; and to extract pigments and food colorings [3]. They have also been applied in the textile industry as well as in coffee and tea fermentation processes [4,5].

The biological decomposition of pectinase is an important process in biomass degradation. Pectins are negatively charged acidic complex heterogeneous polysaccharides found in cell walls (middle lamella) of higher plants. They consist of a backbone of α-1, 4-linked D-galaturonic acid residues in which the carboxyl group at C-6 is esterified with a methyl group in some residues. Most pectin contains smooth linear homogalacturonan regions, and ramified hairy regions. They build up the plant cell wall and contribute to the large quantities of plant fibers with cellulose and hemicelluloses. Pectin has high molecular weight typically in the range 60,000-130,000 g mol^{-1} [6,7]. In plant cells, it exists mainly in the form of calcium pectate and magnesium pectate [5]. Pectin is a gelling agent used to impart a gelled texture to mainly fruit based foods. In the pharmaceutical industry, it is used to delay rise in blood glucose level and reduce blood cholesterol levels and gastrointestinal disorders [8,9]. Other applications of pectin include their use in edible films, as paper substitute, as foams and as plasticizers. Pectin polysaccharides have been used as bioactive food ingredients and as detoxifying agents [9].

Pectinases are enzymes that degrade pectin. They are mainly divided into esterase or polymerases based on their actions. One of the depolymerase enzyme is polygalacturonase (PG). Polygalacturonase catalyzes the hydrolytic cleavage of polygalacturonic acid linkage (1, 4-α-D-galacturonic acid) with introduction of water across the oxygen bridge in the smooth region of pectin. Among the family of Pectinolytic enzymes, polygalacturonase are the most extensively studied. They are found in plant tissues particularly in ripening fruits and are also produced by microorganisms such as fungi and bacteria. Mixtures of polygalacturonase, pectin esterase and pectate lyse are available as commercial enzyme preparations and are used in processing food industries [10]. Polygalacturonases exists in two forms; Endopolygalacturonase and Exopolygalacturonases. Endopolygalacturonase act randomly on the α-1, 4-polygalacturonic backbone chain. Exopolygalacturonases act at the non-reducing end of the α-1, 4-polygalacturonic backbone chain. Polygalacturonases (PGs) are used in industries for many applications. In agro-industries they are used for the removal of settled or suspended solids (clarification) that presents a cloudy appearance of juices especially the acidic ones. The enzymes are also employed in coffee fermentation, and extraction of oils or retting of fibers [11]. Furthermore, PGs are used in food industries for production of jams, jellies, frozen foods, and more recently in low calorie foods as a fat and/or sugar replacement(s) [12-14]. Generally, the applications of polygalacturonase to food and fruit industries have solved several problems that have arisen during extraction, filtration, and clarification of fruit juice and also in biotechnological experiments.

Polygalacturonase have been produced by two methods: solid state and submerged fermentation and extracted by centrifugation and filtration [15]. Purification methods include; ion exchange chromatography, affinity chromatography and gel filtration [5,16-18].

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Benkova and Slezarik developed a purification strategy for the isolation of extracellular polygalacturonase [19]. In their procedure, the enzyme was salted out with ammonium sulphate and precipitated with ethanol after gel filtration through Sephadex G-25. Polygalacturonase activities have been also determined using dinitrosalicyclic acid. Blandino et al. used this method to determine the polygalacturonase activity produced by *Aspergillus awamori* on wheat in solid-state fermentation, coupled with the activity of alkaline polygalacturonase from Streptomyces species RCK-SC [20]. Viscosity reduction measurements have also found widespread use in determining endopolygacturonase activity [21]. The cup-plate method described by Cabeza et al. has been also employed [22]. According to Rasheedha et al., fungi (*Penicillium chrysogenum*) exhibited maximum polygalacturonase production at a range of an initial pH of 6.5 to more alkaline pH levels [23]. SDS-PAGE has been used to determine the purity and molecular weight of the purified polygalacturonase sample [24]. Purification of polygalacturonase is necessary as the crude enzyme may contain different stabilizing components. These components account for the great variations in physical and chemical properties of these enzymes depending on their sources. However, further characterization of the enzymes will lead to a better understanding of their contribution to the various industrial processes.

Materials and Methods

Sample collection

Samples were collected from Orange waste dumpsite from 'Kasuwan Gwari' along 'Jimeta' By-Pass in Adamawa State Nigeria. Soil samples (2 g) obtained from this site was collected and transferred into sterile MacCartney bottles. The soil samples were transported to the Laboratory of the Department of Natural Sciences of the American University of Nigeria Yola for analysis.

Isolation of the microorganisms

Isolation of the fungi was carried out by weighing 1 g of the soil sample aseptically into 10 mL of distilled water in a conical flask. The suspension was mechanically shaken for 10 min at room temperature. One milliliter (1 mL) of the suspension was diluted with 9 mL distilled water. This dilution was repeated four times in series resulting in a 10^{-4} final dilution. Aliquots (100 µL) of each appropriately diluted sample were inoculated using spread plate method onto 100 plates containing sterilized potato dextrose agar (PDA). Streptomycin (100 mg/L) was added in order to prevent bacterial growth. The culture plates were incubated at 30°C for 7 days. After incubation, the plates were examined for the presence of growing fungi. 50 plates were further purified by sub-culturing three to four times to obtain pure fungi isolates which were then maintained on PDA slants at 4°C [25].

Fungal identification

The 3 days old pure culture was used in preparing microscopic slides. A little bit of the mycelia was dropped on the slide and a drop of Lactophenol blue was added to it. A cover slip was placed over it and examination was performed under the light microscope at X400 magnification. Identification was carried out by relating features and the micrographs to "Atlas of mycology" by Barnett and Hunter [26].

Screening of fungal isolates for polygalacturonase production

The isolates were inoculated onto Petri dishes of modified Czapek-Dox Agar (MCDA) in which sucrose was replaced with citrus pectin as the only carbon source and incubated at 30°C for 24-96 h. After incubation, the plates were flooded with 50 mM Iodine to view the clear zones around the colonies [27]. The fungal strains which showed considerable clearing zones in pectin containing agar were selected as possible polygalacturonase producers [28].

Molecular characterization of the screened isolates

Molecular identification was carried out following the DNA extraction procedure described elsewhere [29]. The extracted Aspergillus DNA was amplified using the universal primers ITS1 (5' TCC GTA GGT GAA CCT GCG G 3') and ITS4 (5' TCC TCC GCT TAT TGA TAT GC 3') as adopted primers. The amplified fragments were purified in order to remove the PCR reagents. The integrity of the amplified product was checked on a 1.5% Agarose gel to confirm amplification (Figure 1). The polymerase chain reaction product was sent to a third party company (inquaba) for sequencing.

Production of crude polygalacturonase

The Solid State Fermentation (SSF) method was used for polygalacturonase production in 250 mL Erlenmeyer flasks containing the production medium namely orange peel, and basal medium prepared by washing the peel thoroughly with tap water to remove all water-soluble compounds. The peels were cut into pieces and oven dried to constant weight at 50°C for 48 h. The dried peels were then powdered by blending with a Warring electric blender [29,30]. The fermentation medium was then constituted by combining the following: 11.6 g of the dried powdered orange peel, 0.15 g urea, 1.57 g sucrose, 0.68 g $(NH_4)_2SO_4$, 0.33 g KH_2PO_4 and 0.15 g $FeSO_4$). A control medium was also prepared containing; 0.5 g citrus pectin, 0.15 g urea, 1.57 g sucrose, 0.68 g $(NH_4)_2SO_4$, 0.33 g KH_2PO_4 and 0.15 g $FeSO_4$ in 50 mL of distilled water. The final moisture content and pH of the two media were adjusted to 70% and 5.5 respectively and then sterilized at 121°C for 15 min [31]. The flasks were inoculated with 3 days old spore suspension of *A. flavus* containing 1×10^{-4} spores ml/L from the agar slants [32].

Extraction of crude polygalacturonase

The crude polygalacturonase was extracted from the fermentation

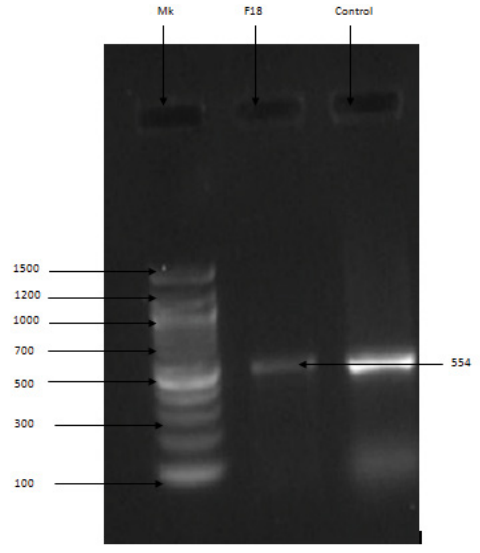

Plate1: Agarose gel electrophoresis of the amplified *Aspergillus flavus* DNA
Lane1: MK DNA marker; **Lane2:** (F18) *Aspergillus flavus*, **Lane 3:** positive control

Figure 1: Gel electrophoresis of amplified Aspergillus DNA.

medium by adding 50 mL of acetate buffer (0.1 M, pH 5.0) to the flask containing the growing fungi. The contents were then filtered. The crude extract was centrifuged at 10,000 rpm for 15 min at 4°C and the supernatant was used as the source of the crude polygalacturonase [33].

Assay of crude enzyme activity

Polygalacturonase activity of the culture filtrate was assayed by measuring the amount of reducing sugar released in the reaction mixture using the modified method of Miller [30] as reported by Wang et al. [32]. The reaction mixture contained 0.8 mL of 0.5% (wt/vol) polygalacturonic acid in 100 mM Na-acetate buffer (pH 5.0) and 0.2 mL of the crude enzyme solution. Calibration of the spectrophotometer was done using the enzyme blank (positive control) and reagent blank (negative control). The enzyme blank was prepared by mixing 0.8 mL of substrate and 0.2 mL of the crude enzyme solution and boiling for 10 min, while the reagent blank was prepared by boiling a mixture of 0.2 mL distilled water and 0.8 mL for 10 min. Both the experimental and control tubes ware incubated at 40°C for 30 min and the reaction was terminated by the addition of 1.5 mL of 3,5-dinitrosalicyclic acid and placed in a boiling water for 10 min. The reaction mixture was allowed to cool after which absorbance was measured at 540 nm. A standard calibration curve of galacturonic acid was extrapolated and used for the estimation of the polygalacturonic acid (µmol/mL). One unit of Polygalacturonase activity is defined as the amount of enzyme that would liberate reducing sugar equivalent to 1 µmol galacturonic acid per min under the specified assay conditions such as pH and temperature [34].

Protein determination

The protein concentration was determined using Bradford's method [33]. Protein concentration was extrapolated from standard curve using bovine serum albumin (BSA) as Standard.

Optimization of Polygalacturonase Production

The isolate was grown under different conditions to test the influence of growth medium on polygalacturonase production. The effects of carbon, nitrogen, incubation period, temperature and pH were determined according to the methods described by Thakur et al. [8].

Effect of carbon sources on polygalacturonase production: Agro wastes such as banana peels, orange peel, yam peels and plantain peels and pure chemicals such as glucose, sucrose and pectin were used in the production medium at a concentration of 1% w/v to check the effect of carbon source on enzyme production. The culture supernatants were assayed for polygalacturonase activity.

Effects of nitrogen sources on polygalacturonase production: The following substances: peptone, urea, casein, yeast extract, and $(NH4)_2SO_4$ were used as nitrogen sources in the basal medium individually to test their effect on polygalacturonase production. However, urea and ammonium sulphate were substituted with other nitrogen sources. The culture supernatants were assayed for polygalacturonase activity.

Effect of incubation time on the production of polygalacturonase: The production media containing fungal isolate was incubated for 48, 60, 72, 96, 108, 120, 132 h at 30°C and pH 3.0 to determine the time optima for production.

Effect of temperature on the production polygalacturonase: The production media containing the fungal isolate was incubated at selected temperatures: room temperature (25 ± 2), 35, 40, 45 and 50°C, in order to determine the temperature optima for production of the enzyme.

Effect of pH on production of polygalacturonase: The production media containing the fungal isolate was incubated using selected 0.1 M buffers of different buffering capacity; namely citrate buffer (3.0-5.0) and phosphate buffer (6.0-8.0).

Enzyme purification

The supernatant obtained was precipitated using 60% (v/v) of ethanol by the gradual addition of chilled ethanol with continuous stirring using a magnetic stirrer after which it was kept in a refrigerator at 4°C for 1 hour. The resulting precipitate was collected by centrifugation (12,000 rpm) at 4°C for 20 min. The resulting pellets were dissolved in minimum amount of 0.1 M citrate buffer (pH 7.5) and the enzyme was dialyzed against 0.1 M citrate buffer (pH 7.5). Polygalacturonase was further purified using gel filtration Sephadex G-75 column described by Siddiqui et al. [31]. Fractions of 1.5 mL were collected at a flow rate of 10 mL/h and assayed for polygalacturonase activity and protein concentration. The active fractions was pooled and dialyzed against 50% glycerol in 0.1 M phosphate buffer (pH 7.5).

The relative molecular weight of purified enzyme was estimated using 12% SDS-PAGE as described by Lemmli [34] against medium-range protein molecular weight markers (Merck Biosciences, UK). The proteins were stained with Boomassie Brilliant Blue R-250 (Sigma-Aldrich) [35].

Characterization of the Purified Polygalacturonase

Effect of pH on *Aspergillus flavus* polygalacturonase: 1 mM of different buffers (from pH 4-10.5) using polygalacturonic acid as substrate and the activity of polygalacturonase was measured as earlier described in section 3.6. The following buffer systems were employed in this study: citrate (4.0-5.5), sodium phosphate (6.0-8.0), Tris (8.5) and sodium carbonate (9.0-10) [34].

Effect of temperature on polygalacturonase: Effect of temperature was determined using the method described by Tari et al. [35]. The enzyme was pre-incubated in a 0.1 M sodium citrate buffer within a temperature range of 40-60°C. At intervals of 15, 30, 45, 60, 75 and 90 min, aliquots of samples were withdrawn and the residual polygalactronase activity assayed using polygalacturonic acid as earlier described [28].

Effect of metal chlorides and Ethylenediaminetetraacetic acid (EDTA) on *Aspergillus flavus* polygalacturonase: The assay was performed in the presence of various metal chlorides at final concentrations of 1 mM, 5 mM using the following salts: $CaCl_2$, NaCl, KCl, $MgCl_2$ and EDTA Mixture of the enzyme solution (0.2 mL) and the various salt concentrations (1 mM, 5 mM) was pre-incubated for 10 min at 40°C, before initiating the reaction with polygalacturonic acid. Residual polygalacturonase activity was then evaluated as previously described in section 3.6. The enzyme samples without the salt solution were taken as control with 100% activity [28].

Effect of inhibitors on *Aspergillus flavus* polygalacturonase activity: The assay was performed using chemical inhibitors at final concentrations of 1 mM, 5mM and using the following acids: benzoic acid, oxalic acid, citric acid and ascorbic acid. A mixture of the enzyme solution (0.2 mL) and the various acid concentrations (1 mM and 5 mM) was pre-incubated for 10 min at 40°C, before initiating the reaction with polygalacturonic acid. Residual polygalaturonase activity was then evaluated as previously described in section 3.6. The enzyme samples without the acid solution were taken as control with 100% residual activity [36].

Determination of Kinetic Parameters of *Aspergillus flavus* polygalacturonase

The kinetic parameters (V_{max} and K_m) of the enzyme were determined by varying the concentration of polygalacturonic acid (0.1-10 mg/mL) and measuring the initial reaction velocities. The data was plotted according to the method of Lineweaver and Burk [37].

Statistical analysis

Data obtained from both characterization and effect of physiochemical factors (pH, temperature, carbon, incubation period, nitrogen salt and inhibitors) on production and activity of polygalacturonase were expressed as SEM, and calculated using GraphPad Prism five software.

Results

Of the three fungal isolates obtained from natural sources in this study, *Aspergillus flavus*, produced relatively higher polygalacturonase activity on a selective medium containing pectin. Microscopic and molecular characterization confirmed the isolate as *Aspergillus flavus* with genetic profile size of 554 bases (Plate 1). The type-derived sequence of the *Aspergillus flavus* isolate inspected for quality matched the standard NCBI strain (https://www.ncbi.nlm.nih.gov/nuccore/597900376). Among the raw carbon substrates used, orange peel had the maximal production of polygalacturonase (3.7 U/mL) followed by yam peel, and plantain peel. The least production of enzyme was observed when glucose was used as carbon source (Figure 2). For the organic and inorganic nutrition sources used for polygalacturonase production, results showed that maximal production of polygalacturonase by *Aspergillus flavus* was obtained in the presence of ammonium sulphate (Figure 3). Results also showed that inorganic nitrogen sources were more suitable for polygalacturonase production than organic nitrogen sources. Effect of incubation period also showed that the production of polygalacturonase increased with duration of incubation with the maximal production recorded on day 4 (96 h) of fermentation after which there was a decline (Figure 4). Maximum production temperature of polygalacturonase was 35°C. (Figure 5). Maximum production of polygalacturonase was attained at pH 4.5 (Figure 6). The purified polygalacturonase showed a single band on 12% SDS-PAGE with a molecular weight of 66 kDa which indicated that *Aspergillus flavus* polygalacturonase is homogeneous and probably monomer. The purified enzyme showed maximum activity at 35°C and pH of 4.5 (Figures 7 and 8). Effect of metal chlorides showed that chloride salts reduced the activity of the enzyme at 1 mM and 5 mM concentrations (Table 1). Inhibitory effect of acid on purified polygalacturonase showed ascorbic acid was the strongest inhibitor of enzyme. At 1 mM ascorbic acid, there was a residual activity of 19.54%. Citric acid, benzoic acid and oxalic acid also inhibited enzymatic activity (Table 2). Oxalic acid however, showed lower inhibitory activity of 30.57% at 5 mM concentration followed by benzoic acid, while citric acid exhibited the highest relative activity (Table 3). Results of kinetics of the enzyme activity revealed the K_m and V_{max} values of 0.705 mg/mL and 1.0508 U/mL, respectively for the polygalacturonase of *Aspergillus flavus* (Figure 9).

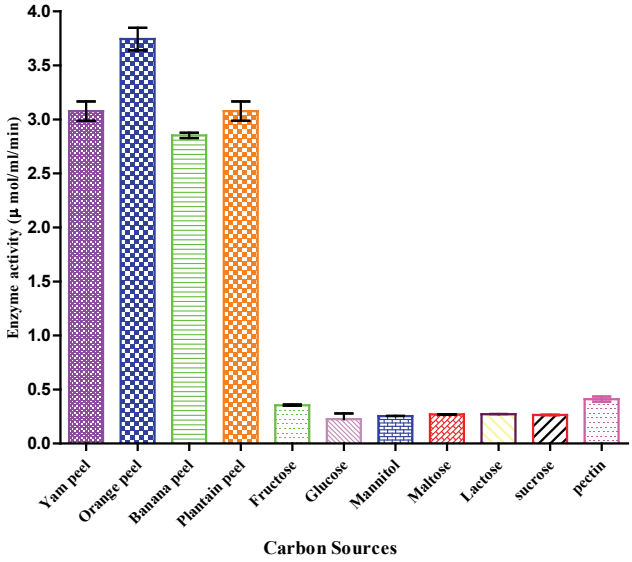

Figure 2: Effect of carbon source on crude polygalacturonase production from *Aspergillus flavus*.

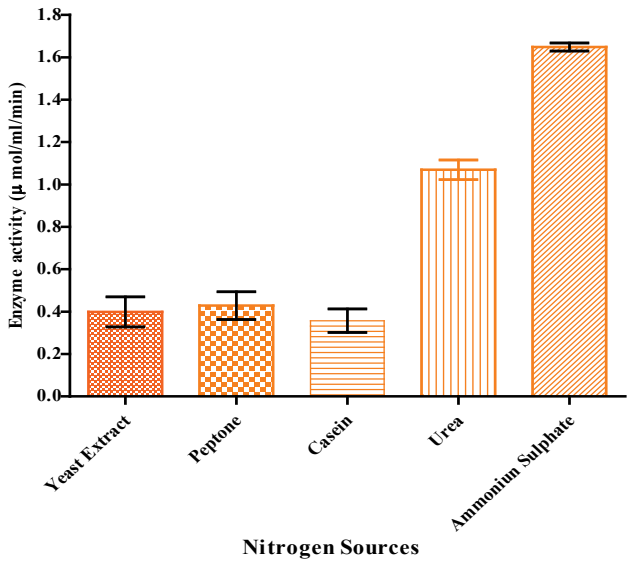

Figure 3: Effect of different nitrogen sources on crude polygalacturonase production from *Aspergillus flavus*.

Discussion

The ability of the *Aspergillus flavus* strain to degrade pectin demonstrated by the production of polygalacturonase in this study forms the bases for estimation of its polygalacturase activity. The differences in yield of enzyme between the raw and commercial carbon sources used as substrates for polygalacturonase production in this study may be due to the differences in proportion of amylase to amylopectin present in the different substrates used. It has been reported that the proportion of amylase to amylopectin usually tend to vary from one starch/polysaccharides source to another [38]. Furthermore, may be the concentration of commercial carbon were high since low concentration of glucose 0.5% stimulates enzyme production as reported by Phutela et al. [38]. Maller et al. reported orange peel as the best carbon source for polygalacturonase production activity on solid state fermentation with *Aspergillus niveus* [39]. Yeast extracts have also been reported as potential inducers of polygalacturonase [40]. Result indicated that maximum production of polygalacturonase occurred due to sufficient nutrient and decrease in the production may be due to the depletion of nutrients. Similar observation was also obtained during pectinase production in submerged fermentation

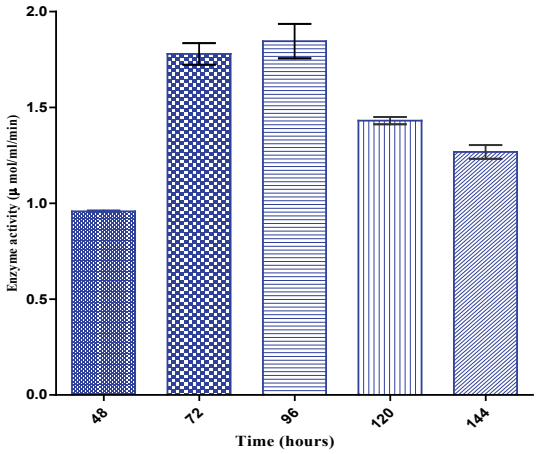

Figure 4: Effect incubation period on crude polygalacturonase production from *Aspergillus flavus*.

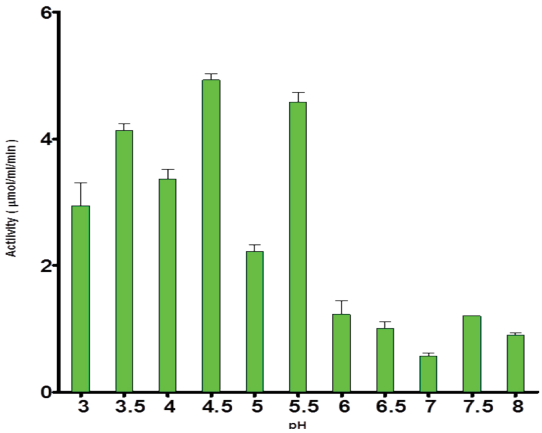

Figure 5: Effect of pH on crude polygalacturonase production from *Aspergillus flavus*.

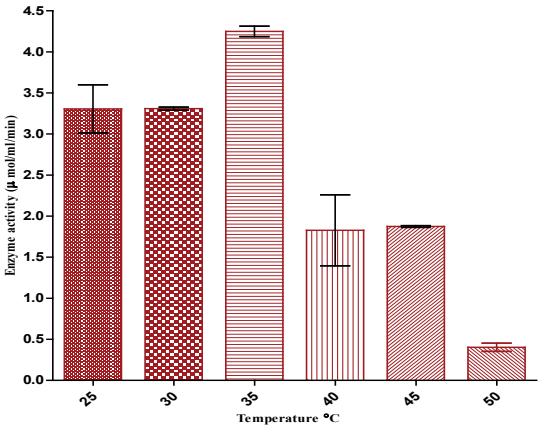

Figure 6: Effect of Temperature on crude polygalacturonase production from *Aspergillus flavus*.

[27,41,42] and solid-state fermentation with *Penicillium chrysogenum* and *Aspergillus brasilensis*. However, Banu et al. reported maximum production of polygalacturonase on day 5 of fermentation [23]. Result also showed that the moderate temperatures (mesophile) of the fermentation system favored the growth of *Aspergillus flavus* for

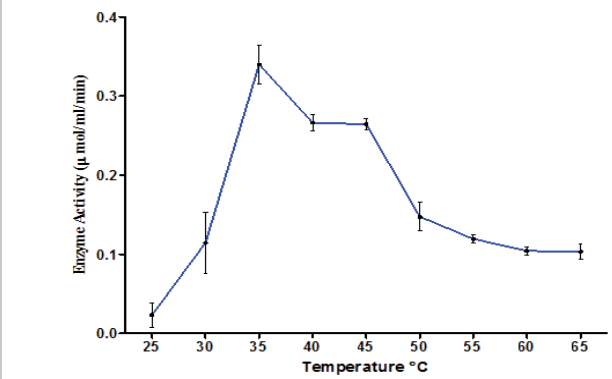

Figure 7: Effect of temperature on activity of *Aspergillus flavus* polygalacturonase.

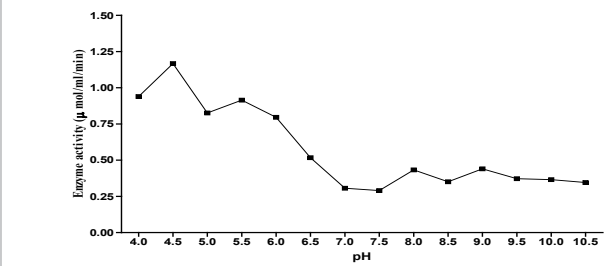

Figure 8: Effect of pH on activity of *Aspergillus flavus* polygalacturonase.

Purification Steps	Volume (ml)	Protein Concentration (mg/mL)	Total Protein (mg)	Activity (U/mL)	Total Activity (U)	Specific Activity (U/mg)	Yield (%)	Purification fold
Crude	200.00	1.92	384.00	1.86	372.00	0.969	100.00	1.00
Ethanol Precipitation	45.00	0.68	30.60	2.33	104.85	3.426	28.19	3.54
Gel Filtration (Sephadex G_{75})	13.00	0.11	1.43	1.06	13.78	9.63	3.70	9.93

Table 1: Summary of purification of *Aspergillus flavus* polygalacturonase.

% Residual Activity	Concentration of Salts /EDTA/Enzyme Activity (U/mL)	
Salts/EDTA	1 mM	5 mM
Control	100.00	100.00
KCl	51.48 ± 0.400	50.12 ± 0.695
NaCl	52.69 ± 2.370	58.26 ± 1.160
$CaCl_2$	67.66 ± 3.335	61.240 ± 0.749
$MgCl_2$	47.175 ± 0.405	59.575 ± 0.055
EDTA	42.09 ± 6.771	50.11 ± 1.997

Table 2: Effect of Salts and EDTA on *Aspergillus flavus* polygalacturonase activity (U/mL).

% Relative Activity	Concentration of Chemical (mM)/Enzyme Activity (U/mL)	
Chemicals	1 mM	5 mM
Control	100.00	100.00
Citric Acid	45.21.± 0.980	56 ± 3.050
Benzoic Acid	45.75 ± 0.753	43.33 ± 1.285
Oxalic acid	46.89 ± 3.970	30.57 ± 0.150
Ascorbic acid	19.54 ± 0.915	ND

Table 3: Effect of Inhibitors on *Aspergillus flavus* polygalacturonase.

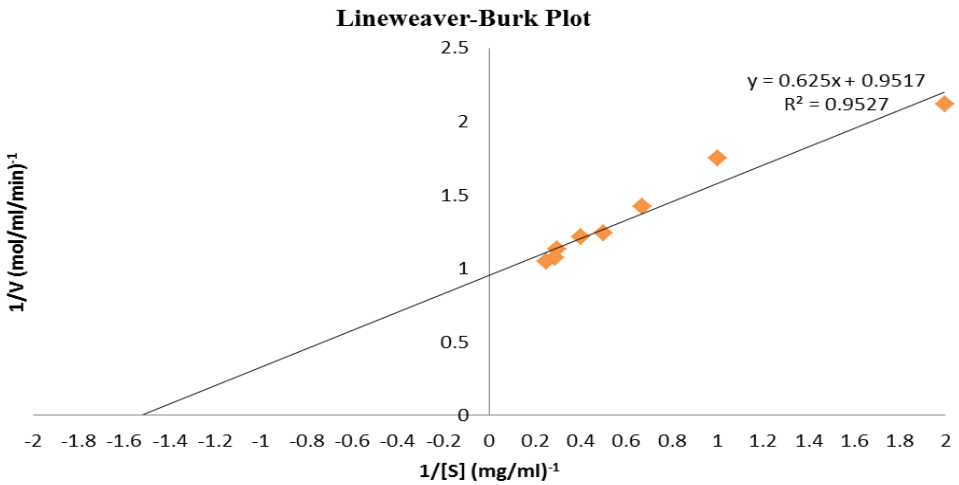

Figure 9: Kinetic parameters *of Aspergillus flavus* polygalacturonase.

polygalacturonase production. This finding is similar to Abbasi et al. who reported production temperature of polygalacturonase between 25 and 35°C [43]. The results indicated that moderate acidic nature of the media favored the growth of *Aspergillus flavus.* A further increase in pH caused a decrease in production of polygalacturonase, which could be due to the fact that as the acidity of fermentation medium increases the production of enzymes decreases probably due to the denaturing of the enzyme proteins. However, Adeleke et al. reported the highest polygalacturonase production at pH 5.5 [25]. The purification result of this study showed that polygalacturonase of *Aspergillus flavus* can be purified to homogeneity by ethanol precipitation and gel filtration on Sephadex G-75. Polygalacturonase isolated from this *Aspergillus flavus* was purified approximately 10 fold with 3% recovery. Similar results were founded by Ngo et al. using a two-step purification process (Ethanol precipitation and Sephadex G-75 gel filtration) for polygalacturonase from filtrate of *Aspergillus awamori* L1 in submerged culture which gave 30.4-fold purification and 68.6% recovery [44]. The reduction of polygalacturonase activity in the presence of calcium chloride may be due to complex formation with enzymes. Polygalacturonase activity was also reduced in the presence of $MgCl_2$ and EDTA, the latter of which has been reported to inactivate enzyme by removing metal ions from it thus forming a coordination complex or by building inside enzyme as a ligand [45]. This finding also indicates that citric acid can still function as food preservative and *Aspergillus flavus* cannot withstand the 5 mM concentration of ascorbic acid. Al-Najada et al. had earlier reported the preservative effect of citric acid on the Pectinolytic fruit spoilage by *Aspergillus tubingensis* [36]. Exhibition of low K_m values is an indication that the enzyme requires only a small amount of substrate to become saturated; hence, the maximum velocity is reached at relatively low substrate concentration. A large K_m on the other hand is an indication for the need for high substrate concentrations by the enzyme to achieve reaction velocity [46]. Similar report by Kant et al. showed K_m and V_{max} values of 0.083 mg/mL and 18.21 U/mL of polygalacturonase respectively from *Aspergillus brasilensis* [47].

Conclusion

Results obtained from this work indicate that orange peels can be used effectively in the production of polygalacturonase under solid state fermentation system using *Aspergillus flavus*. Polygalacturonase produced from this fungal species has an optimum temperature and pH of 35°C and 4.5, respectively. K_m and V_{max} were also found to be 0.705 mg/mL and 1.0508 µmol/min, respectively. Based on the physicochemical properties of the purified enzyme, this enzyme possesses great potential for industrial and biotechnological applications such as fruit juice clarifications, oil extraction. Orange peels used in this process can be considered as a cheap substrate for effective and resourceful production of polygalacturonase using *Aspergillus flavus* because not only do they contain a significant amount of pectin, they are readily obtained with little or no cost. This will minimize the production cost of polygalacturonase and also the amount of pollution of the environment by this waste.

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