Purification and biochemical characterization of pectinase produced by Aspergillus fumigatus isolated from soil of decomposing plant materials

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ABSTRACT

This study isolated, screened, and identified a pectinase-producing fungus from a decomposing plant material. It also cultured the isolated fungus under optimized conditions to obtain crude pectinase enzyme as well as purified and investigated the biochemical characteristics of the purified enzyme. The fungal strain was isolated on pectinase screening agar medium containing 1% pectin and obtained a clear zone. It was identified as Aspergillus fumigatus and cultivated for enzyme production using banana, plantain, and orange peels as the solid substrate. Under optimized conditions, a maximum of 3.52 U/ml pectinase activity was obtained at 65% moisture content after 144 h (6 days) of incubation period on orange peel, 1.5 ml inoculum, and 3% salt content. A. fumigatus pectinase was purified 4.45-fold and a yield of 26.16% with a specific activity of 38.88 U/mg. The molecular weight determined on sodium dodecyl sulfate (SDS-PAGE) was 31.6 kDa. The pectinase exhibited maximum activity at 60°C, optimum pH of 5.0, and stability at 40–50°C. The enzyme showed a preference for polygalacturonic acid as its primary substrate with a \( K_m \) of 3.08 mg/ml and \( V_{max} \) of 1.61 U/ml. The enzyme was activated by 0.5 mM Na⁺, K⁺, and 1–5% toluene. The enzyme activity was inhibited by metal cations; 20% ethanol, 4.0 mM SDS, and L-cysteine. The obtained results showed that A. fumigatus pectinase could be a candidate for potential industrial and biotechnological application.

1. INTRODUCTION

The use of enzyme technology to meet various human needs is becoming popular. Enzymes are used extensively in industry to improve the production of fruit juices, fruits texture, and enzymatic peeling of fruits [1-3]. It has been used in the textile industry as well as coffee and tea fermentation [4,5]. Pectinases are enzymes that are responsible for the biological degradation of pectin, a large molecular weight polysaccharide found in plants [6,7]. One of the most important processes in biomass degradation is the biological decomposition of pectin [8,9]. In plants, pectinases play a diverse role such as cell-to-cell adhesion, a source of signaling molecules, and in the ripening of fruits [10]. Pectinases are also important in plant pathogenesis and have been reported to be the first enzyme synthesized in certain fungal and bacterial pathogens grown on isolated plant cell walls so as to “prepare” the cell wall components for subsequent degradation by other enzymes [11]. Pectinases are also important industrial enzymes used in the production of fruit juices and wines [5,12]. Vegetable oil extraction can be augmented by applying enzymes such as pectinase [5]. Pectinases are secreted by a wide range of microorganisms such as bacteria and fungi [13,14] and it accounts for 25% of the global food enzyme sales [5]. Microorganisms that are used for the production of commercially important enzymes such as pectinases must be “generally regarded as safe” and able to secrete the enzyme in large quantities to the extracellular environment for easy extraction of the enzyme [15]. The preference for a microbial source is usually due to the low cost of production, enzyme content is more predictable and controllable, availability of raw materials for production, and mass production without ethical clearance [16]. The cost of production can be reduced by optimizing the enzyme production conditions [17]. The high cost of the production, as well as the stability of pectinases in an industrial process, is conceivably the major constraint in the commercialization of the new sources of pectinase. Given the biotechnological potential of pectinolytic enzymes, microbial production of pectinases has been extensively studied in recent years [5,17-19]. Thus, the study of physicochemical properties of pectinase produced by Aspergillus fumigatus may help in the development of products which will intervene in plants diseases caused by pectinase-secreting pathogen organisms and also help in eliminating the use of carcinogenic organic solvents in industrial processes.
2. MATERIALS AND METHODS

2.1. Collection of Samples

Agro wastes such as orange peels, banana peels, and plantain peels were collected from local market and dumpsite in Omole Estate, Ile-Ife. The wastes were oven dried for 36 h at 80°C, pulverized, and stored in an air tight container. The pulverized samples were mixed with soil and left to decay in a container for several weeks. Soil samples around the decomposing peels were collected in sterile bottles and taken to the laboratory for analysis.

2.2. Isolation of Fungi and Screening for Pectinase Production

About 1 g of the collected soil sample was serially diluted and 1 ml of the diluted sample was taken into sterilized Petri plates. Agar solution was then poured into the plates using the pour plate method, allowed to set, and incubated at 37°C for 72 h. The cultures were subsequently subcultured until pure isolates were obtained. The screening for pectinase production was done using pectin agar plate [20]. Briefly, pure cultures were inoculated into sterilized and solidified pectin agar medium. Inoculated plates were incubated at 37°C for 48 h. Thereafter, the plates were flooded with iodine-potassium iodide solutions for 5–10 min and pectinase-producing colonies were detected by the appearance of a clear zone around them. The fungi isolate with the highest value of the clear zone of the hydrolysis of pectinase was selected and stored in the fridge on pectin agar.

2.3. Identification of the Pectinase-producing Isolate

The selected fungus with the highest value of pectinase hydrolysis was macro-morphologically characterized by observing colony characteristics such as color, texture, and spore structure according to the handbook for the identification of fungi [21] and micromorphologically by employing conventional lactophenol cotton blue technique (LPCB).

2.4. Pectinase Production using Solid State Fermentation

About 10 g of solid substrates, consisting of 99.7% (9.97 g) each of the powdered plant peels, 0.1% (0.01 g) (NH4)2SO4, 0.1% MgSO4, and 0.1% K2HPO4 were weighed into 250 ml Erlenmeyer’s flasks and then autoclaved at 121°C for 20 min. After cooling, 1 ml of the standard inoculum was introduced and the final moisture content adjusted to 70% using distilled water. The flasks were incubated for 14 days at 37°C. Following fermentation, 60 ml of citrate buffer (0.1 M, pH 5.0) was added to the flasks and stirred for 30 min under ice. The mixture was filtered and centrifuged at 12,000 rpm for 20 min. The resulting supernatant was tested for pectinase activity.

2.5. Pectinase Assay Method

The enzyme activity was determined using pectin as a substrate. The assay mixture consists of 0.80 ml of the substrate solution (1.0% w/v citric pectin in 0.1 M citric buffer pH 5.0) and 0.20 ml of enzyme solution. The reaction medium was incubated at 50°C for 20 min and terminated by the addition of 1.5 ml of 3, 5-dinitrosalicylic acid reagent [22]. The absorbance of the reaction medium was taken at 540 nm. One unit of pectinase activity (U) was defined as 1 µmol reducing sugar released per minute using galacturonic acid as standard.

2.6. Protein Concentration Determination

The protein concentration was estimated by the method of Bradford [23]. Bovine serum albumin (BSA) was used as standard.

2.7. Optimization of Pectinase Production

The step-wise optimization of pectinase produced by the isolate was studied for four optimization parameters: Incubation time, moisture content, the volume of inoculum, and (NH4)2SO4 salt concentration.

2.7.1. Effect of incubation time, ammonium salt concentration, and moisture content on pectinase production

Production medium was prepared into seven 250 ml flasks, at intervals of 48 h up to 14 days, a flask was taken and the crude enzyme extracted for enzyme activity and protein concentration [24]. Powdered orange peel was used as the solid substrate. The moisture content was adjusted from 40 to 80% at 5% interval while the inoculum volume was varied from 0.5 ml to 4.0 ml. After 6 days, the crude enzyme was extracted and checked for enzyme activity. The effect of ammonium salt concentration was checked by varying (NH4)2SO4 salt from 0.1% to 4.0%.

2.8. Purification Procedure

2.8.1. Pectinase purification on CM-Sephadex C-50 and Sephacryl S-200

Crude pectinase produced was subjected to 80% ammonium sulfate saturation and left overnight in the refrigerator. The resulting precipitate was dissolved in a small volume of 0.1 M citrate buffer (pH 5.0) and dialyzed extensively in cold 0.1 M citrate buffer (pH 5.0). 5 ml of the concentrated enzyme was layered on CM-Sephadex C-50 column equilibrated with 0.1 M citrate buffer (pH 5.0). Fractions were collected at a rate of 15 ml/h. Unbound protein was removed by citrate buffer (0.1 M, pH 5.0) followed by a step-wise elution with 0.5 M and 1.0 M NaCl in the buffer. Pectinase activity and protein concentration of the fractions were determined. The active fractions were pooled and diazlyzed against 50% glycerol in 0.1 M citrate buffer, pH 5.0. 5 ml of the concentrated enzyme was layered on a Sephacryl S-200 column. The column was eluted with 0.1 M citrate buffer, pH 5.0 at the flow rate of 20 ml/h. Pectinase activity was checked while protein concentration was monitored spectrophotometrically at 280 nm.

2.8.2. Native molecular weight determination

The native molecular weight was determined on Sephacryl S-200 column (1.5 cm × 65.5 cm). The standard proteins were lysozyme (14,000 Da; 15 mg/ml), trypsin (24,000 Da; 10 mg/ml), pepsin (35,000 Da; 10 mg/ml), ovalbumin (45,000 Da; 10 mg/ml), and BSA (66,000; 10 mg/ml). Fractions of 5.0 ml were collected and monitored spectrophotometrically and the elution volume of each protein was estimated. Void volume (V0) was determined using blue dextran (2 mg/ml). The pure enzyme (5.0 ml) was then passed through the same column and the elution volume of the pectinase was estimated. The method of Laemmli [25] was used to determine the subunit molecular weight.

2.9. Characterization of the Enzyme

2.9.1. Kinetic parameter determination

The kinetic parameters (Vmax and Km) of the enzyme were determined for three substrates: 67% methoxylated citric pectin, 7.8% methoxylated apple pectin, and polygalacturonic acid (PGA). The concentrations were varied from 1 to 8 mg/ml and the initial reaction velocities were determined. The data were plotted according to the method of Lineweaver and Burk [26].

2.9.2. Effect of Temperature and pH

The optimum temperature of the enzyme was assayed using the method of Tari et al. at temperatures between 30°C and 90°C. Citric pectin was
used as the substrate. Effect of pH was studied using citrate buffer (4.0–5.0), phosphate buffer (6.0–8.0), and borate buffer (9.0–10.0). Citric pectin was used as substrate.

2.9.3. Thermal Stability of A. fumigatus Pectinase Activity
The enzyme was pre-incubated in a 0.1 M citrate buffer within a temperature range of 40–70°C. For a time span of 120 min at intervals of 15 min, aliquots of enzyme samples were withdrawn and the residual activity was determined.

2.9.4. Effect of Salts on A. fumigatus Pectinase Activity
Influence of various salts at a different concentration on the enzyme activity was checked. The tested salts were NaCl, KCl, NH₄Cl, SnCl₂, CaCl₂, BaCl₂, MnCl₂, MgCl₂, and AlCl₃. The enzyme was pre-incubated for 10 min at 60°C with the various salts, before the addition of pectin; then, the residual pectinase activity was evaluated. Assay mixture without salts was taken as control.

2.9.5. Effect of organic solvents on A. fumigatus pectinase activity
Pectinase assay was performed in the presence of 1%, 5%, 10%, and 20% of various organic solvents. The organic solvents used include hexane, methanol, ethanediol, acetone, toluene, and diethyl ether (DEE). The enzyme activity was checked as previously described.

2.9.6. Inhibition of pectinase activity
The effects of known enzyme inhibitors such as ethylenediaminetetraacetic acid (EDTA), beta-mercaptoethanol (BME), Triton X-100, sodium dodecyl sulfate (SDS) and Tween-80, and L-cysteine on the pectinase activity were studied. The enzyme was pre-incubated with 0.5 mM and 1.0 mM, 2.0 mM, 3.0 mM, and 4.0 mM of these inhibitors before the addition of substrate. Reactions in the absence of these inhibitors were used as the control with 100% enzyme activity. All chemicals were solubilized in distilled water.

3. RESULTS AND DISCUSSION

3.1. Isolation, Screening, and Optimization of A. fumigatus
This study made an attempt to isolate the fungus capable of producing pectinase, from the soil of decomposing plant materials. Among all the isolates, A. fumigatus showed the maximum pectinase activity based on carbohydrase hydrolysis of pectin in pectin-agar plate assay. A. fumigatus was identified based on microscopic and macroscopic examinations on LPCB. A. fumigatus is a saprothrophic fungus that is found widespread in nature. This organism has been found to reproduce not only asexually but also has a functional cycle of sexual reproductive [27]. Furthermore, the fungus can grow at a temperature range of 37–50°C [28].

The clear zone of hydrolysis around the selected strain is shown in Figure 1. When subjected to pectinase production, orange peel produced the highest pectinase activity as well as total protein concentration (Figure 2). Maximum pectinase activity (1.75 U/ml) was observed at 6 days of incubation with a steady decline of activity after 6 days (Figure 3a). The best moisture content for A. fumigatus (65%) produced a pectinase activity of 2.3 U/ml (Figure 3b). Furthermore, highest pectinase production was observed at 1.5 ml inoculum volume (Figure 3c) and 3% salt concentration (Figure 3d), respectively. Similar observations on the orange peel as an excellent carbon source for pectinase production have been reported [29,30]. The reason for the highest pectinase activity using orange peel as solid substrate could be the high percentage of pectin found in the cell wall of the peel. Several works have been carried out to show that pectinolytic fungi produce pectinase maximally between 3 and 6 days incubation period. The ideal substrate humidity for pectinase production by Aspergillus foetidus was reported to be between 80% and 90%.

3.2. Purification of A. fumigatus Pectinase
A. fumigatus pectinase was purified with a fold of 4.45, a yield of 26.14%, and specific activity of 38.88 U/mg of protein (Table 1). The elution profiles after CM Sephadex C-50 ion-exchange chromatography and Sephacryl S-200 are shown in Figure 4 and 5, respectively. The molecular weight of native and denatured pectinase was determined using the marker proteins lysozyme (14.5 kDa), trypsin (24 kDa), pepsin (35 kDa), ovalbumin (45 kDa), and BSA (66 kDa) to be about 32.5 kDa by gel filtration and SDS PAGE, respectively, indicating that the enzyme is of monomer in nature (Figure 6). Microbial pectinases have been reported to be monomeric in structure and generally fall within the size range of 30–70 kDa [31-35]. Two endopolygalacturonases PGI and PGII purified from A. japonicas showed the molecular weight of 38 and 65 kDa, respectively [36]. Polygalacturonase isolated from A. awomori showed a molecular weight of 41 kDa [37]. Furthermore, pectinase isolated from Bacillus licheniformis showed a molecular weight of 38 kDa [38].
3.3. Kinetic Parameters of *A. fumigatus* Pectinase

The kinetic parameters ($K_m$ and $V_{max}$) of *A. fumigatus* pectinase for citrus pectin, apple pectin, and PGA as substrate are 7.11, 6.01, and 3.08 mg/ml, respectively. Kinetic data are summarized in Table 2. *A. fumigatus* has a higher affinity for PGA. Several $K_m$ values in the range of 1–9 mg/ml have been reported for polygalacturonases [39-48].

Exo-polygalacturonase from *Penicillium frequentans* was shown to have a $K_m$ of 1.6 mg/ml [41]. A value of 0.22 mg/ml was reported for *Rhizomucor pusillus* pectinase using PGA as substrate [42]. It can be said that the kinetic parameters of pectinases vary with the enzyme source and nature substrate used for the assay. In most cases, the $K_m$ value is low, which is in agreement with the result of the present study.

3.4. Effect of pH and Temperature on *A. fumigatus* Pectinase Activity

*A. fumigatus* pectinase activity was optimum at 60°C as shown in Figure 7. The thermal stability of the pectinase showed that at 60°C, the enzyme retained 100% activity for 45 min and losing about 50% activity at the end of 120 min of incubation (Figure 8). Similar results were obtained for pectinase from other sources [9,43], as well as from *Aspergillus* spp. [5]. These results indicate that for industrial application of the pectinase from *A. fumigatus*, the suitable temperature range could be 40–50°C. *A. fumigatus* pectinase has an optimum pH at 5.0 and decreases significantly below and above this value (Figure 9).

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**Table 1: Summary of the purification process for *Aspergillus fumigatus* pectinase**

| Purification step                  | Total protein (mg) | Total activity (U) | Specific activity (U/mg) | Yield (%) | Purification fold |
|-----------------------------------|--------------------|--------------------|--------------------------|-----------|-------------------|
| Crude sample                      | 21.63              | 188.74             | 8.73                     | 100.00    | 1.00              |
| 0–80% (NH$_4$)$_2$SO$_4$ precipitation | 12.29              | 128.53             | 10.66                    | 68.09     | 1.22              |
| Ion-exchange chromatography       | 3.40               | 51.64              | 15.19                    | 27.36     | 1.74              |
| Sephacyl-200 gel filtration       | 1.21               | 49.38              | 38.88                    | 26.16     | 4.45              |

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**Figure 3:** Effect of optimization parameters on *Aspergillus fumigatus* pectinase production. (a) Effect of incubation time on the pectinase production, (b) effect of moisture content on the pectinase production at 6 days of the incubation, (c) effect of inoculum volume on pectinase production at 6 days of incubation period and 65% moisture content, (d) effect of ammonium sulfate concentration at 6 days incubation period, 65% moisture content, and 1.5 ml inoculum.

**Figure 4:** CM-Sephadex C-50 ion-exchange chromatography.
Similar results have been reported from previous work on *Aspergillus* spp. [5]. Acidic pectinases are produced generally by fungi, especially *Aspergillus* spp. [44]. Therefore, *A. fumigatus* pectinase can be one of the candidates of acid pectinases, which can be employed in the extraction of pectin in fruit juices, in winemaking, and in maceration of vegetables.

The values shown represent the average of duplicate experiments. Error bars represent the standard deviation.

### 3.5. Effect of Salts, Organic Solvents, and Inhibitors on *A. fumigatus* Pectinase Activity

Table 3 presents the effect of monovalent, bivalent, and trivalent chloride salts on *A. fumigatus* pectinase. The result shows that the pectinase activity increased significantly by the monovalent ions but decreases by bivalent ions and the trivalent ion. Activation of the pectinolytic enzyme has been reported [45]. This increase in the enzyme activity may be due to charge neutralization by Na⁺, K⁺, and NH₄⁺ on the pectin polymer [12], so as to reduce the repulsion between the pectin and the overall negative charge of the enzyme. Inhibition by all salt of divalent cations such as CaCl₂ and MgCl₂ may be due to either the formation of homogalacturonan chain cross-link by the Ca²⁺ and Mg²⁺ cation [46]; thus, decreasing the
Table 2: Kinetic parameters of the Aspergillus fumigatus purified pectinase

| Substrate      | $K_{m}$ (mg/ml) | $V_{max}$ (µmol/min/ml) |
|----------------|-----------------|-------------------------|
| PGA            | 3.08            | 1.61                    |
| Apple pectin   | 6.10            | 3.39                    |
| Citrus pectin  | 7.11            | 4.24                    |

Table 3: The effect of salts on Aspergillus fumigatus pectinase activity

| Residual activity (%) | 0.1 mM | 0.5 mM | 1 mM |
|-----------------------|--------|--------|------|
| Control               | 100.00 | 100.00 | 100.00 |
| NaCl                  | 178.48±5.21 | 203.80±10.76 | 135.87±7.93 |
| KCl                   | 154.01±3.56 | 195.78±6.17 | 145.15±2.99 |
| NH₄Cl                 | 162.02±4.99 | 185.48±9.66 | 127.51±8.27 |
| CaCl₂                 | 96.62±2.44 | 24.89±1.43 | 8.44±1.01 |
| BaCl₂                 | 91.14±5.87 | 54.43±7.13 | 6.33±0.56 |
| HgCl₂                 | 94.51±3.11 | 89.03±5.20 | 19.83±1.39 |
| MgCl₂                 | 92.68±1.65 | 68.01±1.91 | 23.33±2.18 |
| MnCl₂                 | 89.65±5.52 | 55.34±3.89 | 16.01±0.90 |
| SnCl₂                 | 98.66±0.91 | 76.98±2.09 | 56.11±4.41 |
| AlCl₃                 | 89.16±1.92 | 74.23±5.60 | 59.71±3.77 |

Table 4: Effect of organic solvents on Aspergillus fumigatus pectinase activity

| Residual activity (%) | 1% | 5% | 10% | 20% |
|-----------------------|----|----|-----|-----|
| Control               | 100.00 | 100.00 | 100.00 | 100.00 |
| Hexane                | 134.64±2.13 | 138.64±3.40 | 135.20±4.92 | 136.31±1.35 |
| Toluene               | 115.08±1.56 | 115.58±1.23 | 106.53±0.71 | 96.98±1.51 |
| DEE                   | 106.98±1.05 | 105.99±0.75 | 101.73±0.23 | 92.46±0.65 |
| Acetone               | 95.98±2.70 | 94.18±1.70 | 96.36±0.71 | 93.47±3.26 |
| Ethanol               | 96.65±1.06 | 94.41±1.98 | 90.50±2.92 | 58.66±5.24 |
| Methanol              | 91.06±0.65 | 88.27±1.34 | 87.71±4.34 | 35.20±3.13 |

Table 5: Effect of inhibitors on Aspergillus fumigatus pectinase activity

| Residual activity (%) | 0.5 mM | 1.0 mM | 2.0 mM | 3.0 mM | 4.0 mM |
|-----------------------|--------|--------|--------|--------|--------|
| Control               | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 |
| BME                   | 99.59±1.23 | 99.56±0.98 | 99.58±1.21 | 99.54±1.05 | 99.17±0.48 |
| EDTA                  | 90.50±2.42 | 86.36±1.54 | 86.78±1.71 | 88.02±6.29 | 86.78±4.04 |
| Triton X-100          | 83.47±3.26 | 82.75±1.45 | 81.36±2.14 | 80.17±3.91 | 80.58±1.98 |
| SDS                   | 87.19±4.32 | 61.57±3.71 | 47.52±0.33 | 46.69±1.53 | 45.87±0.75 |
| Tween-80              | 96.24±2.11 | 69.83±3.98 | 39.67±0.67 | 38.84±0.16 | 38.02±0.12 |
| L-cysteine            | 80.03±5.04 | 79.34±2.43 | 78.06±3.13 | 55.37±4.10 | 34.30±0.75 |
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