PRODUCTION AND CHARACTERIZATION OF PECTINASES OBTAINED FROM ASPERGILLUS FUMIGATUS IN SUBMERGED FERMENTATION SYSTEM USING PECTIN EXTRACTED FROM MANGO PEELS AS CARBON SOURCE

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ABSTRACT
Pectinase was obtained from Aspergillus fumigatus in a submerged fermentation system using pectin extracted mango peels as a carbon source and subjected to a two step purification system of 80% ammonium sulphate precipitation and dialysis. Pectin was also extracted from mango peels and its extraction yield was found to be 17% at pH 2.2, temperature of 70°C and extraction time of 1h. The protein concentration in the crude enzyme was found to be 1.372mg/ml. The specific activity of crude enzyme is 11.20µmole/min/mg. After 80% ammonium sulphate precipitation and dialysis, the specific activities were 11.78µmole/min/mg and 34.60µmole/min/mg respectively. The maximal activity of A. fumigatus pectinase was at 40°C, pH 5.5 and was heat stable up to 40°C. Vmax and Km values were found to be of 625µmole/min and 45.5mg/ml respectively. The pectinase was used to degrade pectins extracted from orange and pineapple peels and its specific activities were found to be 30U/mg and 28.39U/mg respectively as compared to 34.60U/mg which is the specific activity obtained when pectin extracted from dry mango peels was used.

Key words: Mango peels, pectinase, pectin, Aspergillus fumigatus, submerged fermentation.

INTRODUCTION
Mango (Mangifera indica) fruits have been part of human diet for ages due to its nutritional and medicinal values. The frequent use of mango fruits for production of juices, nectars, concentrates, jams, jelly powders and flakes generate lots of mango peels and seed kernels which could be considered as wastes and bring about environmental pollution (Bali, 2003; Ian, 2006; Fowomola, 2010; Ashoush and Gadallah, 2011). These wastes could be harnessed into pectin, oil, biogas and dietary fibres (Hussain et al.1991; Bali et al.2003). Pectins are high molecular weight acid polysaccharides, primarily made up of α-(1-4) linked D-galacturonic acid residues with a small number of rhamnose residues in the main chain and arabinose, galactose and xylose on its side chain (Galiotou-Panayotou et al. 1993). Pectin is found in the plant cell wall where it contributes to cell wall rigidity (Steven et al. 2000; Zykwinska et al. 2005). Three major pectic polysaccharide groups are recognized, all containing D-galacturonic acid to a greater or a lesser extent (Rouse and Crandall, 1976). They are homogalacturonan (HG) which is a linear polymer formed by D-galacturonic acid which can be acetylated and/or methyl esterified, rhamnogalacturonan I (RG I) which is composed of the repeating disaccharide rhamnose- galacturonic acid and rhamnogalacturonan II (RGII) which is a homogalacturonan chain with complex side chains attached to the galacturonic residues. Industrially extracted pectins are used for production of Jams, preservatives and can be used as a substrate for pectinase production by submerged fermentation system.

Pectinase is a generic name for a family of enzymes that catalyze hydrolysis of the glycosidic bonds in the pectic polymers (Yogesh et al. 2009; Vivek et al.2010). Pectinases are classified into Pectin Methyl Esterases (PME) or pectinesterases, Polymethylgalacturonases (PMG), Polygalacturonases (PG), Pectate Lyases (PGL) and Pectin Lyases (PL) (McCready, 1970, Daniel, 2009, Eleonora et al. 2009). Pectinase is sourced from bacteria, yeasts and moulds. The choice for microbial source for pectinase production depends on some features such as the type of culture, (solid-state or submerged fermentation), number and type of the produced pectinases (esterases, hydrolytic depolymerases and eliminative depolymerases), pH and thermal stability of the enzymes, and genotypic characteristic of the strain (wild type, mutagenized strain, and homologous or heterologous recombination) (Chadha et al., 2005; Ernesto et al., 2006; Reda et al., 2008; Eleonora et al., 2009; Pilane et al., 2010; Bhaskara et al., 2011). Pectinases from these microorganisms are used in several conventional industrial processes, such as textile, plant fiber processing, tea, oil extraction, treatment of industrial wastewater etc. Pectinases have also been reported to be essential in purification of viruses and in making of paper (Reena et al., 2005). Submerged fermentation
(SmF) and solid-state fermentation (SSF) processes have been widely used for pectinase production by different types of microorganisms (Ernesto et al., 2006). Although solid state fermentation can be used in pectinase production, submerged fermentation culture is more advantageous because of the ease of sterilization and process control is easier to engineer in these systems. Several studies have been done on the production and characterization of partially purified pectinase produced by microorganisms using agro-waste (such as ground peels) as the carbon source but this current study is focused on optimizing pectinase production from Aspergillus fumigatus using pectin extracted from mango peels as carbon source.

**MATERIALS AND METHODS**

**Chemicals:** 3, 5-dinitrosalicylic acid (DNS) was a product of Sigma chemical company (USA). All other chemicals used in this work were of analytical grade and were products of Merck (Germany), BDH chemical limited (England), May and Baker limited (England).

**Collection of mango fruits:** The mango (*Mangifera indica*) fruits were obtained from Obollo Afor Market, Enugu State of Nigeria.

**Collection of Microorganism:** Aspergillus fumigatus, Aspergillus flavus and Aspergillus niger were recently isolated from soil on which mango fruits and vegetables where already deacaying using the method described by Martin et al. (2004). The soil samples were collected in clean dry plastic containers and transported to the laboratory.

**Ground Mango Peels:** The fruit peels were peeled, cut into pieces and treated with 96% ethanol to disinfect the peels. The treated peels were then sun dried for seven days. The dried peels were finally ground into powder.

**Extraction of Pectin:** Pectin was extracted using the method described by McCready (1970).

**Media for Isolation of Microorganisms:** Soil samples collected from the site of decaying mango peels where were pooled together and homogenized in sterile medium containing 1% mango pectin; 0.14% of (NH₄)₂SO₄, 0.2% of K₂HPO₄, 0.02% of MgSO₄.7H₂O, 0.1% of nutrient solution containing; 5g/l FeSO₄.7 H₂O, 1.6mg/l MnSO₄.H₂O, 1.4mg/l ZnSO₄.7H₂O, 2.0mg/l CoCl₂. The mixture was incubated at 30°C for 24 hr. A solid medium of 1% mango pectin; 0.14% of (NH₄)₂SO₄, 0.2% of K₂HPO₄, 0.02% of MgSO₄.7H₂O, 0.1% of nutrient solution containing; 5g/l FeSO₄.7 H₂O, 1.6mg/l MnSO₄.H₂O, 1.4mg/l ZnSO₄.7H₂O, 2.0mg/l CoCl₂ and 3% agar-agar (the gelling agent) (w/v) was also prepared and used to enhance physical identification of the Aspergillus strains present and to fully aclimatize the organisms to the use of pectin as carbon source. The medium was autoclaved at 121°C for 15min. It was allowed to cool to about 45°C and then poured into Petri dishes and allowed to gel. The plates were then incubated in a B & T Trimline incubator at 37°C overnight to check for sterility. A loop of homogenized extract from the liquid medium was streaked onto the solid medium under the flame of Bunsen burner. The plates were thereafter incubated at 35°C till visible colonies were observed. All morphological contrasting colonies were purified by repeated streaking and sub-culturing on separate plates. This process was continued till pure fungal cultures were obtained. Pure fungal isolates were then maintained on potato dextrose agar (PDA) slopes or slants as stock cultures. PDA media were prepared according to the manufacture’s description. Three days old pure cultures were examined. The color, texture, nature of mycelia or spores and growth patterns were also observed. The three days old pure cultures were used in preparing microscopic slides. Identification was carried out by relating features and the micrographs to “Atlas of mycology” by Barnett and Hunter (1972). Submerged fermentation (SmF) technique was also employed using a 250ml Erlenmeyer flask containing 100ml of sterile cultivation medium optimized for pectinase with 0.1% NH₄NO₃, 0.1% NH₄H₂PO₄, 0.1% MgSO₄.7H₂O and 1% mango pectin. The flask was stoppered with aluminum foil and autoclaved at 121°C for 15mins From the PDA slants, fresh plates were prepared and three days old cultures were used to inoculate the flasks. In every sterile flask, two discs of the respective fungal isolates were added using a cork borer of diameter 10mm and then plugged properly. The culture was incubated for 7 days at room temperature (30°C). At each day of harvest, flasks were selected from the respective groups and mycelia biomass separated by filtration. Each day, the filtrate was analyzed for pectinase activity and extracellular protein concentration till the 7th day of fermentation. After the 7 days pilot submerged fermentation (SmF) studies, the day of peak pectinase activity was chosen for mass production of enzyme from the respective fungal isolates (Oyeleke et al., 2010; Vivek et al., 2010). Several 250ml Erlenmeyer flasks were used to produce 2.5 liters of the enzyme. Harvesting was carried out on the respective peak days of enzyme activity.

**Pectinase Assay:** Pectinase activity was evaluated by assaying for polygalacturonase (PG) activity of the enzyme. This was achieved by measuring the release of reducing groups from mango pectin using a modification of the 3, 5-dinitrosalicylic acid reagent. The assay consisted of 1 ml of 0.1 M citrate buffer pH 5.0 mixed with 1 ml of a 0.5% (w/v) pectin solution and 0.5 ml of the enzyme solution. The mixture was incubated at 30°C for 1 hr. The optical density at 540 nm of the reaction mixture was measured. The amount of reducing sugar released per unit time was calculated by reference to a standard curve prepared from D-galacturonic acid. The result showed that the enzyme produced by *A. fumigatus* had a specific activity of 4.5 units/mg of protein.

**Purification of Pectinase:** The purified enzyme was obtained by repeated streaking and sub-culturing on separate plates. This process was continued till pure fungal cultures were obtained. Pure fungal isolates were then maintained on potato dextrose agar (PDA) slopes or slants as stock cultures. PDA media were prepared according to the manufacture’s description. Three days old pure cultures were examined. The color, texture, nature of mycelia or spores and growth patterns were also observed. The three days old pure cultures were used in preparing microscopic slides. Identification was carried out by relating features and the micrographs to “Atlas of mycology” by Barnett and Hunter (1972). Submerged fermentation (SmF) technique was also employed using a 250ml Erlenmeyer flask containing 100ml of sterile cultivation medium optimized for pectinase with 0.1% NH₄NO₃, 0.1% NH₄H₂PO₄, 0.1% MgSO₄.7H₂O and 1% mango pectin. The flask was stoppered with aluminum foil and autoclaved at 121°C for 15mins From the PDA slants, fresh plates were prepared and three days old cultures were used to inoculate the flasks. In every sterile flask, two discs of the respective fungal isolates were added using a cork borer of diameter 10mm and then plugged properly. The culture was incubated for 7 days at room temperature (30°C). At each day of harvest, flasks were selected from the respective groups and mycelia biomass separated by filtration. Each day, the filtrate was analyzed for pectinase activity and extracellular protein concentration till the 7th day of fermentation. After the 7 days pilot submerged fermentation (SmF) studies, the day of peak pectinase activity was chosen for mass production of enzyme from the respective fungal isolates (Oyeleke et al., 2010; Vivek et al., 2010). Several 250ml Erlenmeyer flasks were used to produce 2.5 liters of the enzyme. Harvesting was carried out on the respective peak days of enzyme activity.
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\textbf{Protein Determination:} Protein was determined by the method of Lowry \textit{et al.} (1951)

\textbf{Enzyme Purification}

\textbf{Ammonium Sulphate Precipitation of Pectinase:}

\begin{tabular}{|c|c|c|c|c|c|c|}
\hline
Purification Step & Volume (ml) & Protein conc. (mg/ml) & Activity (U/ml) & Specific Activity (U/mg) & Total activity (U) & Purification Fold \\
\hline
Crude enzyme filtrate & 1000 & 1.372 & 15.36 & 11.20 & 15360.00 & 1 \\
80\% Ammonium sulphate precipitation & 65.00 & 6.206 & 73.08 & 11.78 & 4750.20 & 1.1 \\
Dialyzed enzyme & 72.20 & 4.030 & 139.47 & 34.60 & 10032.00 & 2.9 \\
\hline
\end{tabular}

The crude extract was brought to 80\% ammonium sulphate saturation. The precipitate was redissolved in 40ml of 0.05M acetate buffer pH 5.0 after centrifugation and stored in a refrigerator.

\textbf{Dialysis:} Ammonium sulphate precipitated protein was dialyzed overnight against 0.05M sodium acetate buffer pH 5.0 with change of buffer every 6hr and was stored frozen at 4°C.

\textbf{Enzyme Characterization}

\textbf{Effect of pH and Temperature:} Pectin extract was dissolved (1:10) in different buffers (pH 3.5-10.0, 0.05M). After incubation at 40°C, the pectinase activity was measured for the value of pH optimum. For the determination of optimum temperature, the reaction mixture (enzyme and its substrate) was incubated at temperature from 30 to 70°C for 1 hour with pectinase activity determined.

\textbf{Effect of Substrate Concentration:} The effect of substrate concentration on the activity of pectinase was determined by incubating the enzyme with 5, 15, 20, 25, 30, 35, 40 and 45mg/ml of mango pectin at pH 5.5 and 40°C. The \(V_{\text{max}}\) and \(K_m\) values of the enzyme were determined using the double reciprocal plot.

\textbf{RESULTS AND DISCUSSION}

\textbf{Pectin Extraction:} The extraction yield of pectin extract was 17\% at extraction conditions of 70°C at pH 2.2 and extraction time of 1 hour. This is in agreement with works on pectin from different sources (Rouse and Crandall, 1976; Hussain \textit{et al.}, 1991; Rehman \textit{et al.}, 2004; Zia-ur-Rehman and Abdul, 2005; Sharma \textit{et al.}, 2006; Pranati and Rishabha, 2011). The differences in the yields could be as a result of disparities in the sources of pectin and other factors such as extraction technique, changes in pH, temperature, extraction time and environmental factors (Kertesz, 1951, Rehman, \textit{et al.}, 2004). This result proves mango peel a good source of pectin for biotechnology.

\textbf{Pectinase production:} A volume of 2.5 liters of crude pectinase was produced from \textit{Aspergillus fumigatus} in submerged fermentation. Total protein and total activity of mass-produced crude enzyme were determined and found to be 1372mg/ml and 15360.00 \(\mu\)mole/min/mg, respectively (Table 1). These values are relatively high compared to the work of Baladhandayutham and Thangavelu, (2010) from \textit{Aspergillus niger}.

Table 1: The table below shows the purification table for partially purified pectinase

Ammonium sulphate precipitation profile: Pectinase in the crude extract was purified between 20-80\% of ammonium sulfate (Figure 1). As the ammonium sulphate concentration was raised from 20 to 80\%, the pectinase activity (\(\mu\)mole/min) increased from 7.10 to 46.04\(\mu\)mole/min making 80\% ammonium sulphate saturation suitable for pectinase precipitation. The enzyme being protein can be precipitated by the addition of ammonium sulphate, thus increasing the purity of the enzyme in the precipitates. More purification could not be achieved with this method. Other techniques such as sephadex gel filtration, ion exchange chromatography have been attempted by other workers (Sakellaris \textit{et al.}, 1989; Joshi \textit{et al.}, 2011).
**Pectinase Characterization:** The crude pectinase when stored at 4°C was found to be stable up to 45 days whereas, partially purified pectinase remained stable up to 3 months. It was suggested by Joshi et al. (2011) that some of the inhibitors that might be present in the crude extract could have been removed during purification, thus increasing the stability of the enzyme. The effect of temperature on the pectinase enzyme production was studied by conducting experiments at different temperatures namely 25°C, 30°C, 35°C, 40°C, 45°C, 50°C, 55°C, 60°C, 65°C and 70°C by keeping all other conditions constant for the incubation period of 60mins. The result shows that increase in temperature from 25°C to 40°C was accompanied by an increase in pectinase activity. The temperature increases up to 40°C after which, the enzyme activity decreased steadily making 40°C the optimal temperature for pectinase activity as shown in Figure 3. The enzyme exhibited highest activity of 172.25U/ml at temperature of 40°C. The decrease in enzyme activity is as a result of enzyme denaturation.

**Effect of pH:** The effect of pH on the pectinase activity was studied by conducting experiments at different pH namely pH 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, 9.5, 10.0, 10.5 keeping temperature at 40°C. The result shows that increase in pH was accompanied by an increase in enzyme activity up to pH 5.5 after which, the enzyme activity decreased steadily making pH 5.5 the optimum pH for pectinase activity (Figure 2). The enzyme exhibited highest activity of 195.50U/ml at pH 5.5. The decrease in enzyme activity at higher pH may be due to preference of fungi (*A. fumigatus*) to lower pH for its growth and metabolism (Baladhandayutham and Thangavelu, 2010). Also, enzymes are amphoteric molecules containing a large number of acid and basic groups, mainly situated on their surface. The charges on these groups will vary, according to their acid dissociation constants, with the pH of their environment. The increase in pH will affect the total net charge of the enzymes and the distribution of charge on their exterior surfaces, in addition to the reactivity of the catalytically active groups. This effect of pH on charge distribution on the ionizable groups interrupts the tertiary structure of the enzyme and thus courses its denaturation (Reena et al., 2005, Eleonora et al., 2009). A maximum pectinase activity of 195.50 U/ml was obtained with mango pectin extract for incubation period of 60mins hours at temperature 40°C and at pH value of 5.5. Hence optimum pH value was chosen as pH 5.5 for the rest of the experiment.

**Kinetic studies:** Pectinase showed a maximum velocity, Vmax, of 625μmole/min and Michaelis-Menten constant, Km, of 45.5mg/ml pectin (Figure 4). The kinetic study of pectinase from *A. fumigatus* using pectin extracted from mango peels
as a carbon source showed an interesting result when compared to that from other sources. The results for Vmax and Km show that the enzyme has higher affinity for pectin extracted from dried mango peels. This could be that active site of pectinases are more exposed to their respective specific substrates. Banu et al. (2010) reported a lower \( K_m \) of 1.0mg/ml and \( V_{max} \) of 85U/mg for pectinase isolated from Penicillium chrysogenum. Baladhandayutham and Thangavelu, (2010) reported \( K_m \) and \( V_{max} \) of 294.12 and 2.33 hr.ml/U respectively for pectinase from Aspergillus niger. Baladhandayutham and Thangavelu, (2011) reported \( K_m \) and \( V_{max} \) to be 79.3 and 1.724 hr.ml/U respectively for pectinase from Aspergillus awamori. A comparative study was performed using pectinase obtained in this work to degrade pectins extracted from orange and pineapple peels. The specific activities on them were found to be 30U/mg and 28.39U/mg respectively when compared with 34.60U/mg obtained when pectin from dry mango peels was used (Figure 5). The comparative study of the effect of different pectins on the pectinase activity indicated why it is advisable to use pectinase produced (using mango pectin as a substrate) to clarify mango juice, and that from orange and pineapple for clarification of orange and pineapple juices respectively. This result has established suitable conditions for maximizing the production of pectin and pectinase for production and clarification of mango juice.

**Conclusion**

In conclusion, as most work on pectinases has been from microbial sources utilizing fruit peels as the only carbon sources, this work contributes to new and alternative sources of pectinase. Pectinase produced from Aspergillus fumigatus using pecting extracted from dried mango peels as the only carbon source, if purified to homogeneity and harnessed properly could be a better and cheaper source of pectinase enzyme for clarification of juice and for other industrial purposes such as in paper industries, textile and laundry industries, as well as detergent industries.

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