Partial Purification of Pectinases from Mutants of *Aspergillus niger* using *Moringa oleifera*

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Abstract

The constant importation of chemicals with scarce foreign currency for purification of pectinase in developing countries has become a major challenge. Pectinase producing *A. niger* was isolated from soil rich in fruits waste and mutated using NTG (0.3 mg/ml). The pectinase hyper producing wild type and mutants were identified as *A. niger* isolate SUMS0061, *A. niger* strain F7-02 and *A. niger* strain AL-30 using molecular tools. Pectinase from *A. niger* isolate SUMS0061 produced 1057.14 U/ml, *A. niger* strain F7-02 produced 2028.57 U/ml and *A. niger* strain AL-30 had 1242.86 U/ml. Purification with *M. oleifera* seed powder at optimum concentration (0.75 mg), pH (4-6), temperature (4°C) and contact time (4 hours) produced 10.08-fold for pectinase from isolate SUMS0061, 16.01-fold for pectinase from strain F7-02 and 14.19-fold for pectinase from strain AL-30. A stepwise purification using *M. oleifera* seed powder and silica gel gave 45.19-fold, 71.20-fold and 63.18-fold for pectinase from isolate SUMS0061, strain F7-02 and strain AL-30 respectively. Molecular weight of each pectinase was 40 kDa. TLC showed that galaturonic acid was the end product of each pectinase hydrolysis. The optimum temperature of pectinase from isolate SUMS0061, strain F7-02 and strain AL-30 were 50 °C, 65 °C and 60 °C respectively. Optimal activity of pectinase from isolate SUMS0061, strain F7-02 and strain AL-30 were at pH 6, 4 and 5 respectively. Each pectinase was stable between pH 3-6. The three pectinases indicated high activities in the presence Ca^{2+}, while Cu^{2+}, Zn^{2+}, Mg^{2+} and Al^{3+} led to reduction in pectinase activity.

Keywords: *Moringa oleifera*, mutants, purification steps, column chromatography

1.0 Introduction

Pectin is a complex polysaccharide consisting mainly of esterified D-galacturonic acid resided in α(1-4)-chain. The acid groups along the chain are largely esterified with methoxy groups in natural products. There can also be acetyl groups present on the free hydroxyl groups. The main chain galacturonic acid also has the occasional rhamnose group which disrupts chain helix formation. Pectin is also known to contain other neutral sugars which are present in side chains. The most common side chain sugars are xylose, galactose and arabinose [1].

Pectinases are a group of at least seven different enzymatic activities that contribute to the breakdown of pectin which is a structural polysaccharide found in primary cell wall and middle lamina of fruits and vegetables. Pectolysis is one of the most important processes for plant, as it plays a role in cell elongation and growth as well as fruit ripening. Microbial pectolysis is important in plant pathogenesis, symbiosis and decomposition of plant deposits [2]. Pectic enzymes have two classes namely pectin esterases and pectin depolymerases. Pectin esterases have the ability to de-esterify pectin by the removal of methoxy residues while pectin depolymerases readily split the main chain and they were further classified as polygalacturonase (PG) and pectin lyases (PL). Thus on the whole, pectinases are hydrolytic enzymes, which hydrolyze pectin molecules and are readily soluble in water [3].

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The main sources of the microorganisms that produce pectinolytic enzymes are yeast, bacteria and large varieties of fungi. Particularly, *Asperigillus species* endopolygalacturonase production was first reported in 1951 using *Saccharomyces fragilis* [4]. Microbial pectinase is most preferred as they are fast growing, more diverse and their environmental and genetic manipulation is easier, needed for generation of hyper producers in reduced time period. Considering genetic modification of microorganisms to improve enzyme production, this investigation was conducted with the objective of obtaining pectinases hyper producing mutant of *Aspergillus niger* using nitrosoguanidine. Effort was also made to investigate the purification potential of *Moringa oleifera* seed powder on the crude pectinases produced.

2.0 Materials and Methods

2.1 Sample Collection

In order to isolate efficient pectin degrading *Aspergillus niger*, soil rich in fruit waste was collected from the popular Ketu Market, in Lagos State, Nigeria. The soil samples were collected in sterile containers and kept under refrigeration condition for further studies.

2.2 Isolation of Mold Strains

Ten-fold serial dilutions of the soil sample were carried out followed by inoculation of prepared potato dextrose agar medium with 1ml of appropriate diluent in duplicates. The inoculated agar medium plates were incubated at 30°C for 3 days.

2.3 Identification of Mold Isolates

A drop of lactophenol cotton blue stain was placed on a clean, grease-free microscopic slide. Speck of each fungal mycelium from pure culture plate was transferred into the drop of stain on the slide. The stain was covered with a cover slide and observed under microscope using ×10 and ×40 objective lens. The structure, spore color, type of hypae and fruiting body of the fungi was observed for the identification of *A. niger.*

2.4 Molecular Characterization of Fungi

DNA extraction and purification was done with QIAamp DNA Mini Kit from Qiagen with catalogue number 51304, the Extracted Nucleic Acid was then treated with RNase. The extracted DNA (3 µl) was loaded on 1% agarose and ran at 96-100 volts using 1xTBE for 1h. Polymerase chain reaction (PCR) technique was used to sequence extracted DNA using primers ITS-4 (5′- TCCTCCGCTTAATTGATATGC -3′) and ITS-86 to generate the forward and backward gene sequences respectively (5′- TGAATCATCGAATCTTTGAA -3′). PCR reactions were carried out in one cycle of heat treatment at 94 °C for 5 minutes, a total of 30 cycles of denaturing at 94°C for 30 seconds; annealing at 56°C for 30 seconds and extension at 72°C for 45 seconds; final extension at 72°C for 5 mins. The purified PCR product (2.5 µl) was used for sequencing. Analysis of the nucleotide sequences generated by the PCR was carried out using the Basic Local Alignment Search Tool (BLAST-N) at the National Centre for Biotechnology Information (NCBI) database to determine strain type of isolates.

2.5 Screening for Pectinolytic Activity

The screening methodology involved the use of pectinase screening agar medium (PSAM) containing: 1 g pectin, 0.3 g Diammonium orthophosphate, 0.2 g KH_2PO_4, 0.3 g K_2HPO_4, 0.01 g $MgSO_4$ and 2.5 g agar in 100 ml of distilled water. The initial pH of medium was adjusted to 4.5. The medium was sterilized and distributed aseptically in petri dishes. The petri dishes containing PSAM was inoculated with *A. niger* and incubated at 30°C for 24 hrs. At the end of the incubation period, plates were stained with 50 mM iodine for result [5].

2.6 Induction of Mutation

The crude spores of hyper producing *A. niger* was prepared in Vogel's medium using 250 ml Erlenmeyer flasks with working volume of 50 ml in rotary shaker operating at 220 rpm and at 30°C for 30 mins [6]. N-methyl-N-nitro-N-nitrosoguanidine solution (MNNG; 0.3mg/ml of buffer saline) was used to induce mutagenesis. MNNG solution (1 ml) and 9 ml of Vogel's medium containing spores of *A. niger* were added in a flask and kept in a water bath at 37°C. After intervals of 30, 60, 90, 120, 150 and 180 mins, 1 ml sample was drawn and wash thrice for 15 mins at 10,000 rpm [7]. Serial dilution was carried out to the express cells and plated on potato dextrose agar plates. Selection or screening of pectinase hyper producer was done according to Yogesh *et al.* [5].

2.7 Enzyme Production by Submerged Fermentation

The hyper producing mutant of *A. niger* was used to produce pectinase using liquid medium containing 10 g, citrus pectin; 1.4 g, $(NH_4)_2SO_4$; 6 g, K_2HPO_4; 2 g, KH_2PO_4; 0.1 g, $MgSO_4.7H_2O$; pH, 6 in 1L of distilled water. Fermentation was carried out in 500 ml Erlenmeyer flask containing 250 ml of growth medium and incubated at 30°C under shaking conditions (175 rpm) for 5 days [8]. The biomass was separated by centrifugation at 10,000 rpm for 15 min at 4°C.

2.8 Pectinase Assay and Determination of Protein Content

Pectinase activity was determined using citrus pectin as substrate. The reaction mixture, containing equal amounts of 1% pectin prepared in sodium acetate buffer (0.05 M; pH4.5) and crude enzyme, was incubated at 50°C in water bath for 30 min. The reaction was stopped with 1.0 ml dinitrosalycyclic acid solution [9] after which the mixture was boiled for 10 min and cooled. The absorbance was read at 540 nm using a spectrophotometer. The amount of reducing sugar released was quantified using galacturonic acid as standard. Protein content of the pectinase was determined by the method of Lowry *et al.* [10], using Bovine Serum Albumin (BSA) as standard.

2.9 Precipitation with Moringa oleifera Seed Powder

Crude pectinase was treated with *M. oleifera* seed powder at different concentration (0.25 - 2.0 mg/10 ml). Turbidity of the supernatant in each tube was determined after 1 h with spectrophotometer preset at 620 nm. Pectinase activity was estimated in terms of reducing sugar produced by 3, 5 dinitrosalicyclic acid [9]. Protein content was determined by the method of Lowry *et al.* [10], using Bovine Serum Albumin (BSA) as standard. Effect of pH, temperature and contact time on the purification potential of *M. oleifera* seed powder was estimated.

2.10 Combined Purification and Gel Filtration Chromatography (second step purification)

Crude pectinase was treated with *M. oleifera* seed powder and chitosan at optimum precipitation condition (concentration, pH, temperature, and contact time). The pectinase activity, turbidity and protein concentration of the enzyme was determined as described earlier. Glass column was packed with silica gel 60. The partially purified pectinase sample was loaded onto the column and elution of the proteins was done using acetate buffer (0.05 M, pH 4.5). Fractions were collected. The absorbance of the samples for protein content and pectinase activity was done by the methods described earlier. The pectinase-positive fractions were pooled together for further analysis.

2.11 Thin Layer Chromatography

A thin layer chromatography (TLC) overlay method [11] was used to analyze component of the pectinase. About 40 µl of pectinase was applied on pre-coated aluminium silica gel 60 plates. The plates were developed with propanol, water and diethyl ether (6:3:1 v/v) and run in duplicate. Each plate was visualized under UV light to see if the separated spots were UV active after which it was sprayed with aniline phosphoric acid reagent and incubated at 105°C for 10 min. Individual retention factors (*R*f) for each spot were recorded by comparing them with the standard.

$$Rf = \frac{\text{Individual distance travel by the sample}}{\text{Distance travel by the running solvent}}$$

2.12 Optimum Temperature and pH of Pectinases

The optimum temperature of the pectinase activity was measured using pectin substrate within a temperature range from 40 to 70°C. Determination of the residual pectinase activity was done using the method of Lia *et al.* [12]. The optimum pH of pectinase was studied by incubation of the enzyme with 50 mM of sodium acetate buffers (pH 3 to 5), sodium phosphate buffer (pH 6 to 7) and Tris-HCl buffer (pH 8.0) in the presence of pectin and then residual activity of pectinase was measured.

2.13 Thermal and pH Stability of Pectinase

Pectinase thermostability was studied by incubating it in a 50 mM sodium acetate buffer at optimum temperature for 5 h. At the interval of 1 hour, samples were withdrawn and the residual pectinase activity was determined [13]. The determination of the pectinase stability was made through measurement of the residual activity following maintenance for 24 h at 4°C in various pH range from 2.0 to 7.0 [14].

2.14 Effect of Metal Ions on Pectinase Activity

Effect of metal ions on pectinase activity was investigated using the following chemicals in concentrations of 10 mM: Zn^{2+}, Ca^{+2}, Cu^{2+}, Mn^{+2}, Mg^{+2} and Al^{3+}. The samples in the presence of various metal ions were incubated for 30 min at 50°C and pH 5, then the residual pectinase activity was tested with pectin [15].

2.15 Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The purity and molecular weight of pectinase was estimated by SDS-PAGE using 12% gels. After migration, protein bands were stained with Coomassie Brilliant Blue (CBB) [16].

3.0 Results

3.1 Isolation of Fungi

The total colony forming unit of mold in the soil sample was 12×10^6 (cfu/g) with *Aspergillus niger* accounting for 5×10^6 (cfu/g). *Aspergillus niger* (A1) demonstrated the largest zone of hydrolysis around the colony (diameter of zone of clearance was 14 mm) on pectin agar medium followed by A2 (Table 1). Isolates A3 to A5 were not pectinase producers.

Table 1: Zones of Pectin Hydrolysis of *Aspergillus niger* on PSAM

Isolates (*A. niger*)	Zones of Pectin Hydrolysis (mm) ± SD
A1	14±0.28
A2	11±0.42
A3	-
A4	-
A5	-

Values are Means ± Standard deviation (SD) of duplicate determinations

3.2 Induction of Mutation

The estimated survival of *Aspergillus niger* (A1) when treated with 0.3 mg of N-methyl-N-nitro-N-nitrosoguanidine at various time intervals of 30 minutes to 180 minutes is presented in Table 2. After 120 min of nitrosoguanidine treatment, no survival was observed.

Table 2: Colony Count of Mutants of *Aspergillus niger*

Mutants	cfu/ml
M30	3×10^6
M60	2×10^6
M90	2×10^6
M120	1×10^6
M150	-
M180	-

3.3 Pectinase Qualitative and Quantitative Assay

The pectinase activities of both the wild type and mutants are presented in Table 3. Two hyper producing mutants (M33 and M61) were observed to have the largest zone of pectin hydrolysis which were 25 mm (2028.571 U/ml) and 22 mm (1242.857 U/ml) respectively. The two mutants and the wild type were then used for further pectinase production by submerged fermentation. Figure 1 shows zone of pectin hydrolysis produced by mutant of *Aspergillus niger* (M33) on PSAM.

Table 3: Pectinase Activities of Wild Type and Mutants

Isolate Codes	Zone of Pectin Hydrolysis (mm ± SD)	Pectinase Activity (U/ml ± SD)
Wild type	14±0.283	1057.143±30.514
M31	15±1.000	1085.714±49.508
M32	18±0.571	1142.857±39.601
M33	25±0.000	2028.571±10.910
M61	22±0.714	1242.857±31.117
M62	19±1.000	1157.143±40.413
M91	17±0.714	1128.571±50.301
M92	16±0.283	1100.000±30.514
M121	11±0.571	1000.000±60.212

Values are Means ± Standard deviation (SD) of duplicate determinations

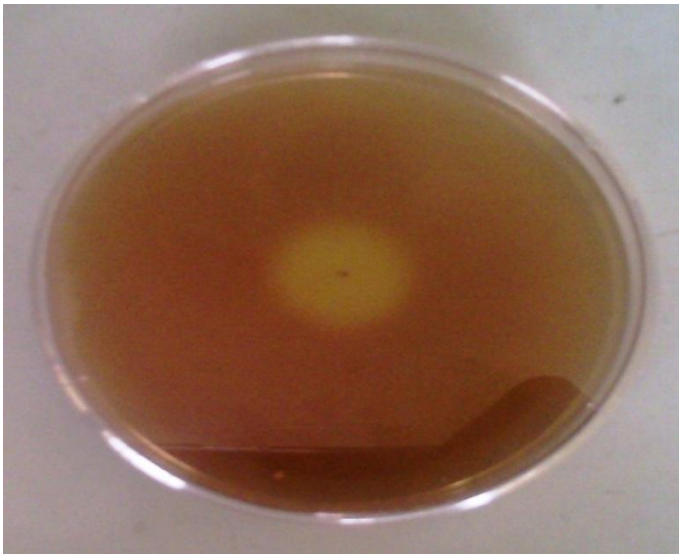

Figure 1: Clear Zone of Pectin Hydrolysis of *Aspergillus niger* (M33) on PSAM

3.4 Molecular Identification of Fungi

3.4.1 Blasting Result of Aspergillus niger Strain SUMS0061 (Wild Type)

The wild type *Aspergillus niger* isolate SUMS0061 gave a nucleic acid of a total of 579 nucleotide bases long and BLAST on NCBI Genbank database at 78% query cover, 84% Ident and maximum score of 448 with a final result as *Aspergillus niger* Accession number FJ011541.1

3.4.2 Blasting Result of Aspergillus niger Strain F7-02 (M33)

Aspergillus niger strain F7-02 (M33) gave a nucleic acid of a total of 601 nucleotide bases long and BLAST on NCBI Genbank database at 95% query cover, 99% Ident and maximum score of 549 with a final result as *Aspergillus niger* Accession number JN561274.1

3.4.3 Blasting Result of Aspergillus niger Strain AL-30 (M61)

Aspergillus niger strain AL-30 gave a nucleic acid of a total of 767 nucleotide bases long and BLAST on NCBI Genbank database at 37% query cover, 94% Ident and maximum score of 320 with a final result as *Aspergillus niger* Accession number KC341975.1

3.5 Effect of Moringa oleifera Seed Powder on Pectinase

Pectinase treatment with *Moringa oleifera* seed powder using 0.25 – 2.0 mg/10 ml incubated for 1 hour at 28°C (room temperature) resulted in increase in specific activity as the concentration of *M. oleifera* seed powder was raised but declined immediately after 0.75 mg. Highest specific activity was 31.942 U/mg/ml (0.75 mg/10 ml) for pectinase from *A. niger* isolate SUMS0061, 91.256 U/mg/ml (0.75 mg/10 ml) for pectinase from *A. niger* strain F7-02 and 50.191 U/mg/ml (0.75 mg/10 ml) for pectinase from *A. niger* strain AL-30 as indicated in Figure 2.

Adjustment of the pH of crude pectinases to pH 3, 4, 5, 6, 7 and 8 before treatment with *M. oleifera* seed powder using 0.75 mg/10 ml of crude pectinase resulted in increase in specific activity as the pH was raised. Maximum specific activity of 34.520 U/mg/ml at pH 6 was obtained for pectinase from *A. niger* isolate SUMS0061, 105.918 U/mg/ml at pH 4 for pectinase from *A. niger* strain F7-02 and 65.955 U/mg/ml at pH 5 for pectinase from *A. niger* strain AL-30 (Figure 3).

Maximum pectinases precipitation was observed at 4ºC using *M. oleifera* at optimum concentration and pH as shown in Figure 4. Specific activity of pectinase from *A. niger* isolate SUMS0061 at 4°C was 49.519 U/mg/ml, pectinase from *A. niger* strain F7-02 at 4°C gave 198.857 U/mg/ml and pectinase from *A. niger* strain AL-30 at 4°C had 96.423 U/mg/ml.

At optimum precipitation temperature, pH and concentration of *M. oleifera* seed powder (0.75 mg), the crude pectinases were incubated at different time interval from 1 hour to 5 hours. Optimum contact time specific activity was achieved at 4 hours with *M. oleifera* seed powder as shown in Figure 5. Specific activity for pectinase from *A. niger* isolate SUMS0061 at 4 hours was 73.181 U/mg/ml, pectinase from *A. niger* strain F7-02 at 4 hours produced 252.742 U/mg/ml and pectinase from *A. niger* strain AL-30 had 142.857 U/mg/ml. Table 4 indicated the specific activities of the pectinases after *M. oleifera* seed powder treatment.

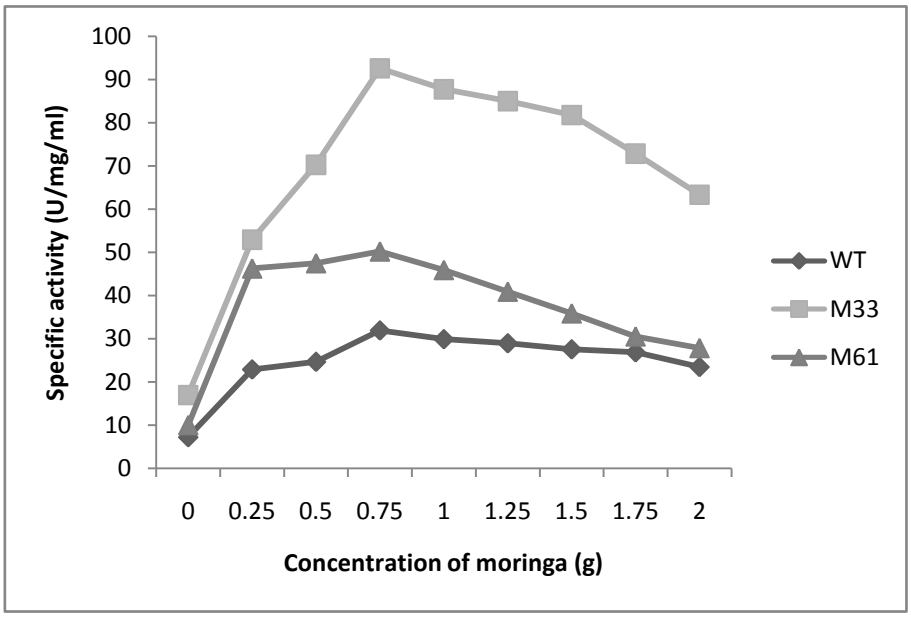

Figure 2: Effect of purification potential of *M. oleifera* seed powder on pectinase activity

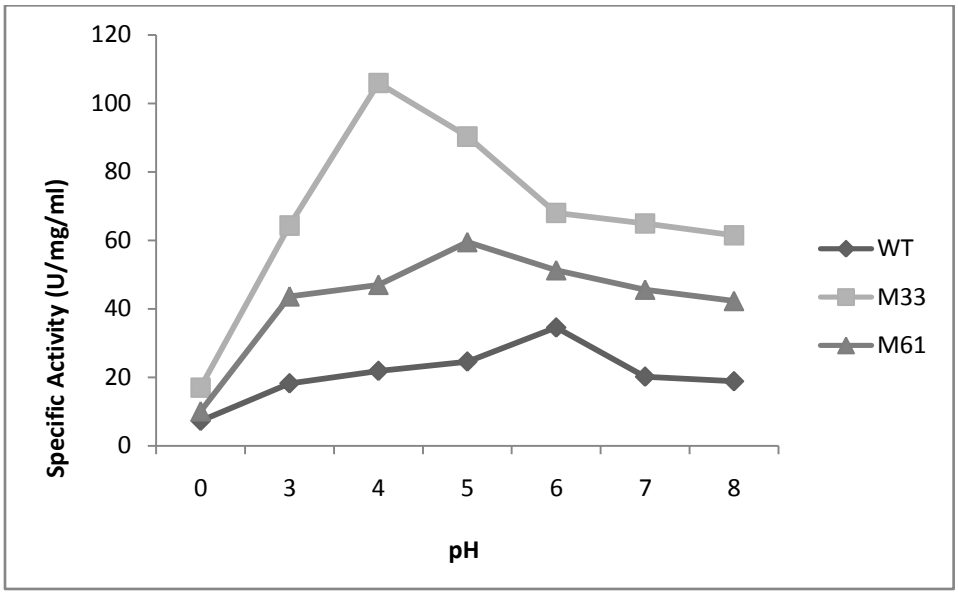

Figure 3: Effect of pectinase pH on the purification potential of *M. oleifera*

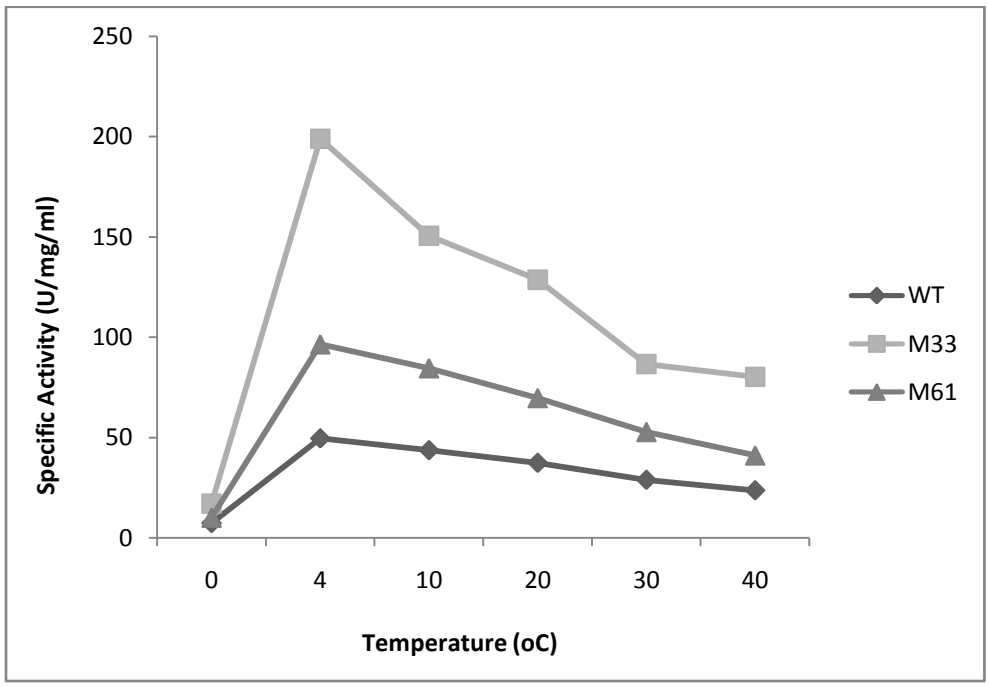

Figure 4: Effect of pectinase temperature on the purification potential of *M. oleifera*

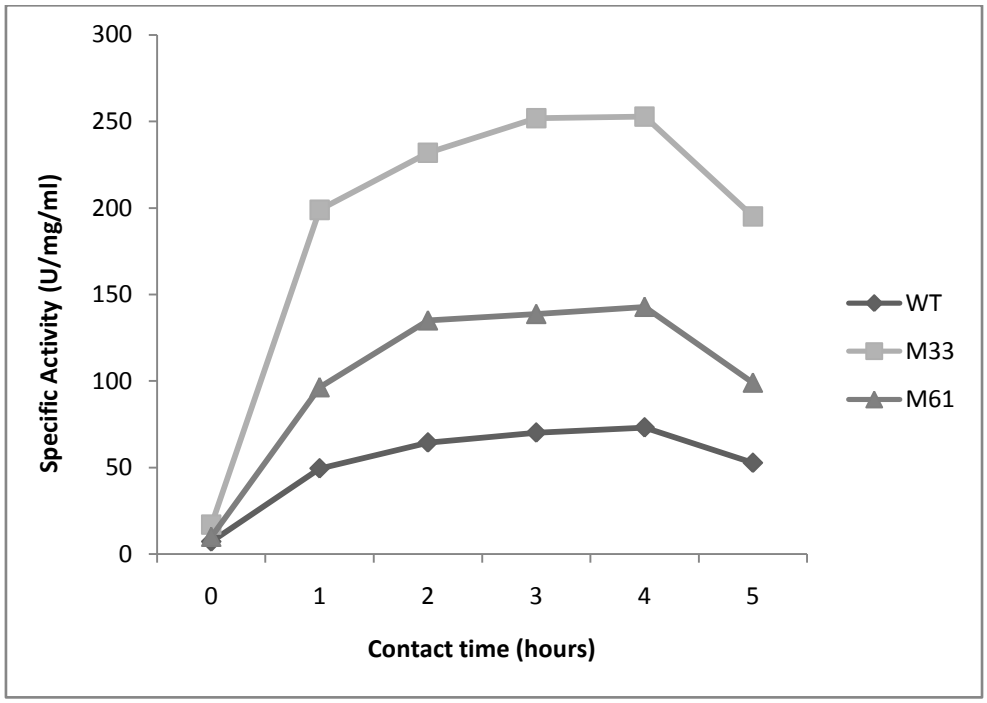

Figure 5: Effect of pectinase contact time on the purification potential of *M. oleifera*

Table 4: Activity of pectinases at optimum *M. oleifera* purification condition

Moringa	Turbidity at 620 nm ± SD	Activity (U/ml) ± SD	Protein concentration (mg/ml) ± SD	Specific activity (U/ml) ± SD
Wild type	0.197±0.013	3228.571±10.910	64.571±13.741	50.000±0.794
M33	0.142±0.017	5214.286±39.601	25.000±7.074	208.571±5.598
M61	0.163±0.023	4071.429±40.413	30.882±7.074	131.838±5.713

Values are Means ± Standard deviation (SD) of duplicate determinations

3.6 Second Step Purification

When the partially purified pectinases were loaded on Silica gel 60 column, fractions showing pectinase activity were pooled together. The second step purification produced 45.194 purification fold for pectinase from *A. niger* isolate SUMS0061, 71.204 purification fold for pectinase from *Aspergillus niger* strain F7-02 and 63.175 purification fold for pectinase from *A. niger* strain AL-30 as presented in Tables 5 to 7. The protein concentrations were indicated in two major peaks as shown in Figures 6 to 8. Figure 9 shows that the pectinases were of high molecular weight. Galaturonic acid was the end product of pectin hydrolysis as indicated by thin layer chromatography analysis presented in Figure 10.

Table 5: Purification fold of gel filtrated pectinase from *A. niger* isolate SUMS0061 (WT)

Wild type pectinase	Activity (U/ml)	Protein concentration (mg/ml)	Specific activity (U/ml)	Purification fold
Crude	1057.143±30.514	145.588±18.392	7.261±1.659	1.0±1.000
Moringa	3228.571±39.601	50.000±15.563	64.571±2.545	8.893±1.534
Silica gel	4342.857±40.413	13.235±4.074	328.134±9.920	45.191±5.980

Values are Means ± Standard deviation of duplicate determinations

Table 6: Purification fold of gel filtrated pectinase from *A. niger* strain F7-02 (M61)

M33 pectinase	Activity (U/ml)	Protein concentration (mg/ml)	Specific activity (U/ml)	Purification fold
Crude	2028.571±10.910	119.118±15.563	17.030±0.701	1.0±1.000
Moringa	5214.286±40.413	25.000±12.728	208.571±3.175	12.247±4.529
Silica gel	10700.000±50.301	8.824±4.283	1212.602±11.744	71.204±16.753

Values are Means ± Standard deviation of duplicate determinations

Table 7: Purification fold of gel filtrated pectinase from *A. niger* strainAL-30 (M61)

M61 pectinase	Activity (U/ml)	Protein concentration (mg/ml)	Specific activity (U/ml)	Purification fold
Crude	1242.857±31.117	123.529±12.728	10.061±2.445	1.0±1.000
Moringa	4071.429±49.508	30.882±15.563	131.838±3.181	13.104±1.301
Silica gel	6542.857±50.301	10.294±4.283	635.599±11.744	63.175±4.803

Values are Means ± Standard deviation of duplicate determinations

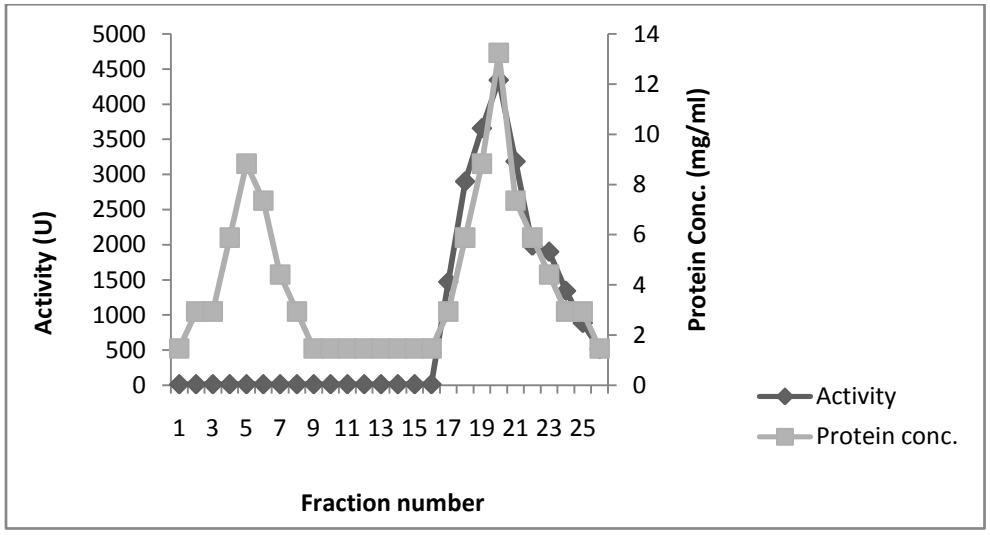

Figure 6: Elution profile for pectinase activity and protein concentration from *A. niger* isolate SUMS0061

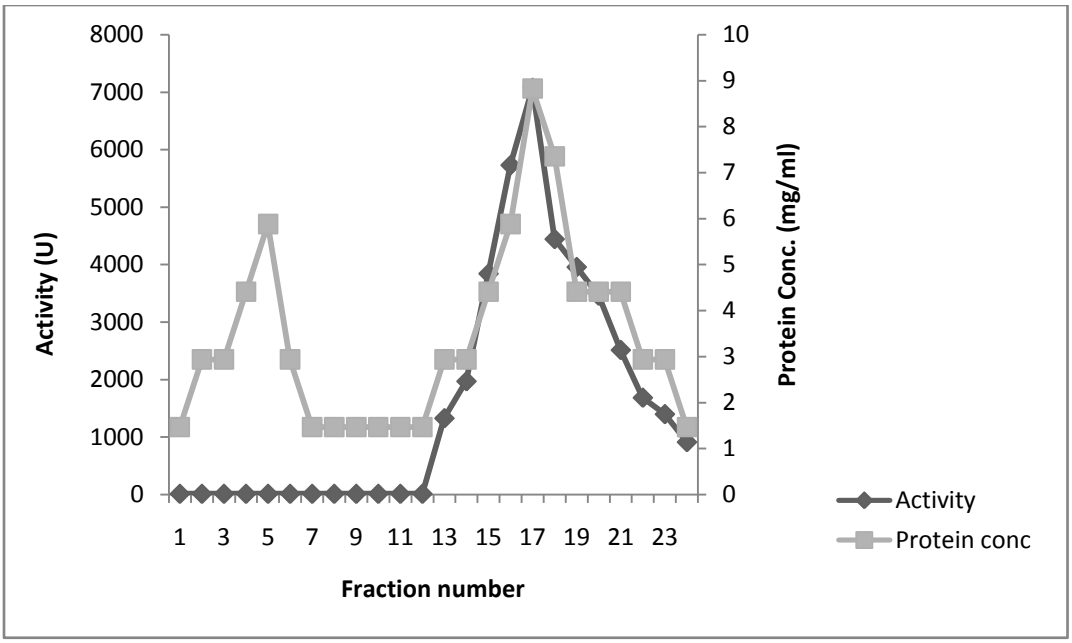

Figure 7: Elution profile for pectinase activity and protein concentration from *A. niger* strain F7-02

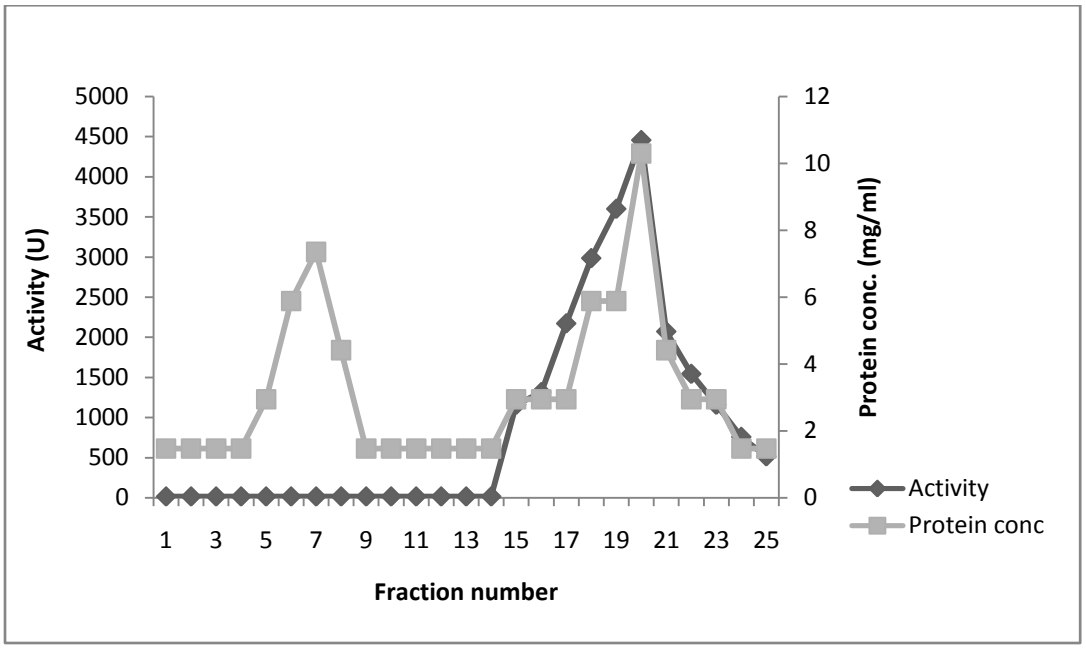

Figure 8: Elution profile for pectinase activity and protein concentration from *A. niger* strain AL-30

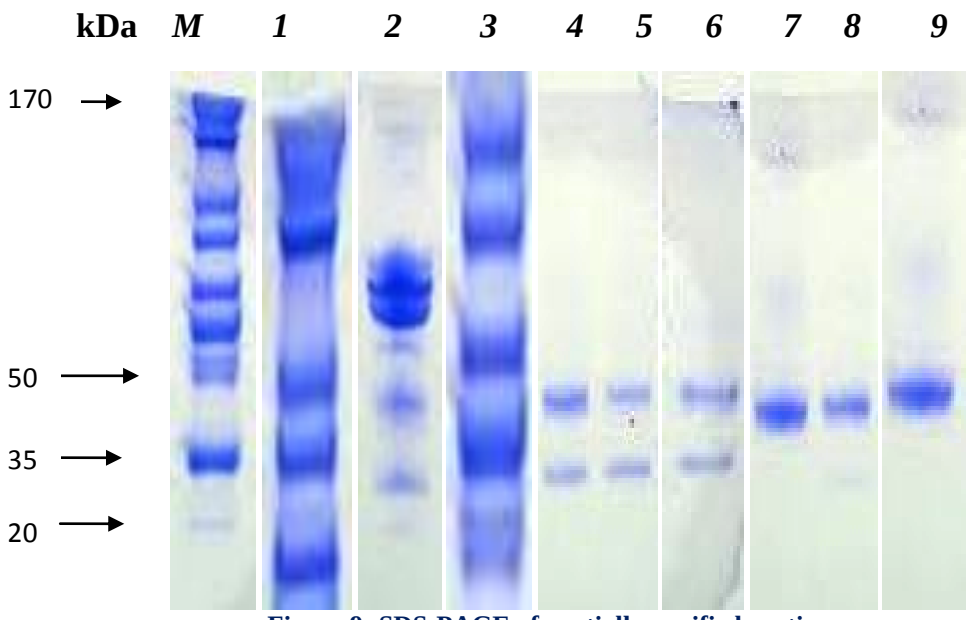

Figure 9: SDS-PAGE of partially purified pectinases

Lane M, molecular mass makers;
Lane 1, Lane 2 and Lane 3, crude pectinase from *A. niger* isolate SUMS0061, *A. niger* strain F7-02, *A. niger* strain AL-30;
Lane 4, Lane 5 and Lane 6, partially purified pectinase from *A. niger* isolate SUMS0061, *A. niger* strain F7-02; *A. niger* strain AL-30;
Lane 7, Lane 8 and Lane 9, silica gel filtration of partially purified pectinase from *A. niger* isolate SUMS0061, *A. niger* strain F7-02, *A. niger* strain AL-30.

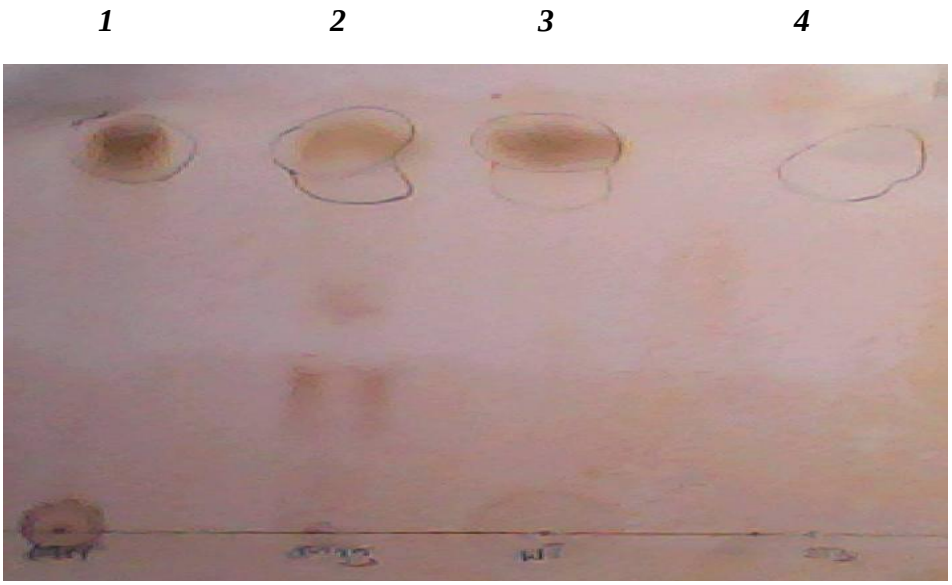

Figure 10: Thin layer chromatography analysis of the products of galaturonic acid hydrolysis by the extracellular pectinases. Lane 1 sample, pectinase from *A. niger* strain AL-30; **Lane 2** sample, pectinase from *A. niger* strain F7-02; **Lane 3** sample, pectinase from *A. niger* isolate SUMS0061; **Lane 4,** galaturonic acid (standard).

3.7 Effect of Temperature on Pectinase Activity

The result presented in Figure 11 depicts how a temperature range of 40°C to 70ºC affected pectinase activity. The activity of pectinase was increased with increase in temperature. The optimum activity was obtained at 50ºC (4342.857 U) for pectinase from *A. niger* isolate SUMS0061, 65°C (14242.857 U) for pectinase from *A. niger* strain F7-02, and 60°C (12300 U) for pectinase from *A. niger* strain AL-30 (Figure 11).

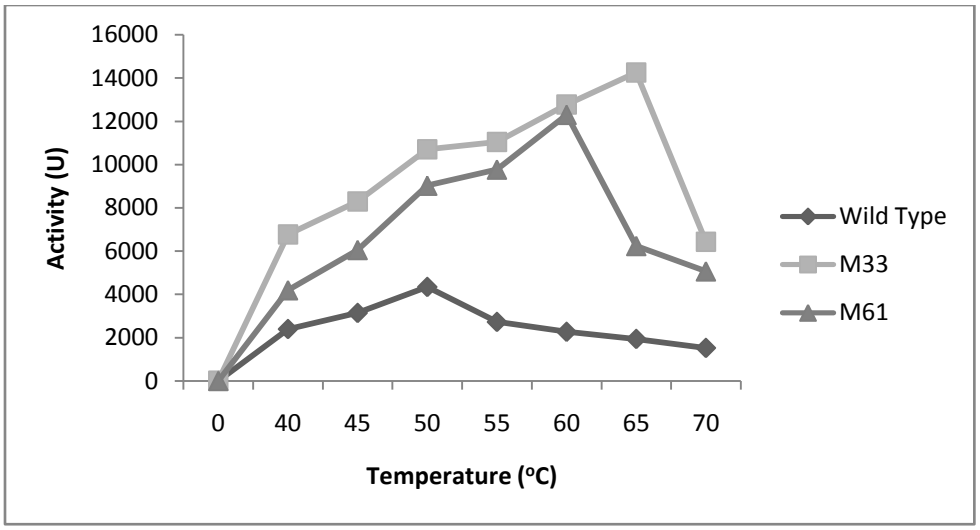

Figure 11: Optimum temperature of the partially purified pectinases

3.8 Effect of pH on Pectinase Activity

Activity was at optimum for pectinase from *A. niger* isolate SUMS0061 at pH 6. For pectinases from *A. niger* strain F7-02 and strain AL-30, the activity was optimum at pH 4 and pH 5respectively (Figure 12).

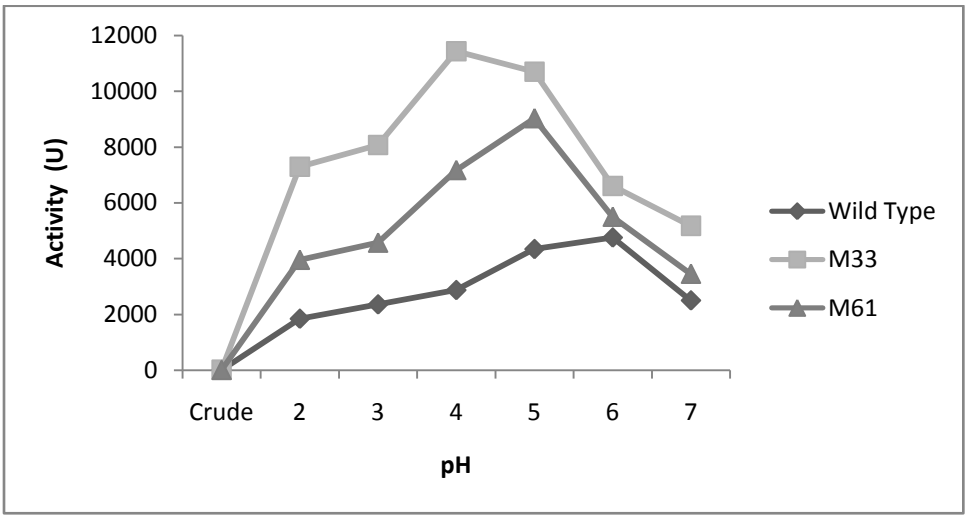

Figure 12: Optimum pH of partially purified pectinase

3.9 Effect of Metal Ions on Pectinase Activity

The activity of pectinase from *A. niger* isolate SUMS0061 was increased in the presence of Ca^{+2} while Cu^{2+}, Zn^{2+}, Mg^{2+} and Al^{3+} led to reduction in the activity of pectinases (Figure 13). Pectinase activity of both *A. niger* strain F7-02 and *A. niger* strain AL-30 increased significantly by 102.8% and 108.7% in the presence of Mn^{2+}.

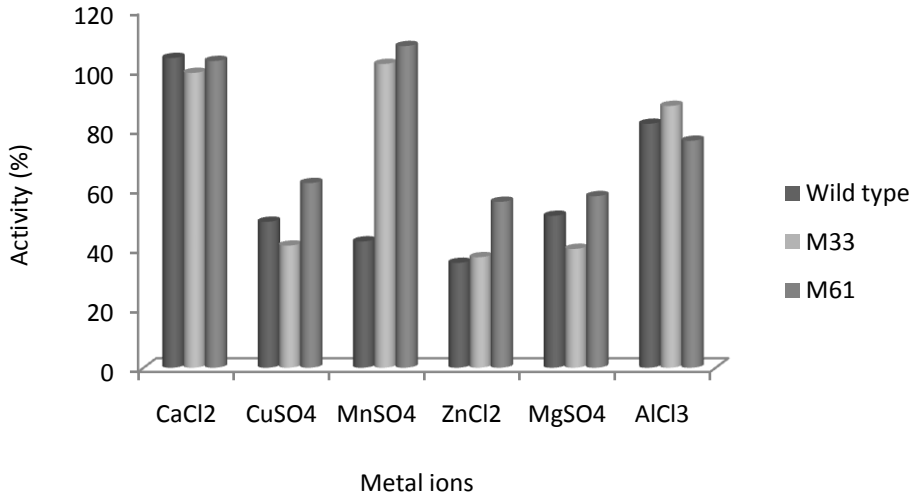

Figure 13: Effect of activators and inhibitors on pectinase activity

4.0 Discussion

The high budget incurred by the government, especially in poor countries, for the importation of enzyme purification chemicals has raised concerns especially in the face of dwindling economic resources. The present study has clearly demonstrated the potential of *M. oleifera* seed powder in purification of pectinase at optimum condition as a cost effective substitute for chemical purification of pectinase.

A reduction in total colony forming units of mold in the soil sample was recorded as the treatment time increased. NTG has been previously reported as an effective mutagen for strain improvement of *Rhizopus speices* for enhanced enzyme activity which often cause reduction in the number of microbial survivor as the treatment time increases [17]. The emergence of *A. niger* (A1) as the fungi with the largest zone of pectin hydrolysis is an indication that the pectin in the clear zone has been degraded by the pectinase produced from the microorganism. The DNA extraction and purification provided a veritable means of characterizing the fungi yielding a wild type (WT), Mutant M33 and Mutant M61 which were subsequently used for pectinase production by submerged fermentation. Although Mutant M33 and M61 had the highest pectinase producing ability, appearance of a clear zone after treatment of the culture plate with iodine solution is an indication of pectinase production by the microorganism [5].

Findings from this study revealed that *M. oleifera* seed powder was able to confer precipitation and give high pectinases activity based on concentration, pH, temperature and contact time. It has been reported that the sulphates of elements found in moringa seeds do confer a purifying property on the seeds as evident in the process of softening of hard water [18]. The activities of partially purified pectinases were high but the corresponding high protein concentration observed after partial purification reduced the specific activity. About 2.66% was estimated for protein obtained from *M. oleifera* seeds [19]. The same author reported a value of 5.7% protein content for *M. oleifera* leaves.

Although the protein contents of the fractions collected after column chromatography were indicated at two major peaks (Figures 6 to 8), the second step purification yielded about three-fold higher specific activity. This may be indicative of the fact that some of the inhibitors which are likely to be present in the crude pectinases could have been removed during purification [20]. The detection of pectinases activity in fractions 12 to 16 suggested that the pectinases are of high molecular weight in the region of 40 kDa (Figure 9).

Vebhar and Neelam [21] reported that when purified pectinase was subjected to electrophoresis on 10% SDS-PAGE, a single band was observed, indicating complete purification of the enzyme. Using standard protein markers, the size of the purified pectinase was found to be 66 kDa. With respect to retention factor (Rf), an individual value at 0.88 was estimated for each of the pectinases and galaturonic acid standard (Figure 10). This indicated that galaturonic acid is the end product of the pectinases hydrolysis and the enzymes released were polygalaturonase.

The optimum temperature of pectinases of *A. niger* was reported to be 50°C [22] with the optimum temperature for thermostable polygalaturonase found to be 65°C [23]. However, findings have indicated highest pectinase activity of 60°C for 30 minutes [24]. The same author also reported increased in pectinase activity by increasing the temperature from 20°C to 60°C.The optimal pH activity for pectinase from *A. niger* isolate SUMS0061 was achieved at pH 6 which is in agreement with previous studies [25]. Pectinase from *A. niger* strain F7-02 and strain AL-30 had optimum activity at pH 4 and 6 respectively, although similar study has estimated optimum activity for pectinase by *A. niger* at pH 3.8 [5]. Generally, most fungal pectinases are stable at low pH [26].With respect to metal ions, pectinase from *A. niger* isolate SUMS0067 gave high activity in the presence of Ca^{2+} which agrees with previous studies that Ca^{2+} has significant influence on the activity and stability of enzymes [27]. However, Mn^{2+} may also enhance pectinase activity as it is the case with *A. niger* strain F7-02 and strain AL-30 as observed in the present study.

5.0 Conclusion

The increased importation of enzyme purification materials/chemicals with scarce foreign currency has become a major challenge in the developing countries. There have been several attempts to reduce the cost of enzyme production using locally available (bio) materials. This study has been able to establish the potential of *M. oleifera* seed powder in the purification of pectinase at optimum condition as a cost effective substitute for chemical purification of pectinase.

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