International Journal of Advanced Research in Biological Sciences
ISSN: 2348-8069
www.ijarbs.com
DOI: 10.22192/ijarbs Coden: IJARQG (USA) Volume 8, Issue 6 -2021

Research Article

DOI: http://dx.doi.org/10.22192/ijarbs.2021.08.06.007

Purification and Characterization of Pectinase from *Aspergillus niger* Strain F7-02 Mutant

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Abstract

This study was aimed at purifying and characterizing thermal stable pectinase produced from mutant strain of *Aspergillus niger*. High pectinase producing *Aspergillus niger* was isolated and mutated with N-methyl-N-nitro-N-nitrosoguanidine 0.3 mg/ml. Mutants obtained were screened for pectinase production using pectinase screening agar medium. Highest producing mutant was further identified using molecular technique. Pectinase produced from this mutant was purified with chitosan and characterized. Molecular identification of the mutant gave 99% identity as *Aspergillus niger* strain F7-02. Qualitative and quantitative assay of the enzyme gave 25 mm zone of pectin hydrolysis and 2028.57 U/ml specific activities respectively. At optimum purification of chitosan concentration (0.75 mg), pH 4, temperature (4 °C) and contact time 4 hours, pectinase from *Aspergillus niger* strain F7-02 produced 19.05 purification fold; A stepwise purification using chitosan and silica gel gave 71.20-fold. Optimum temperature and pH of the pectinase activity was 65 °C and 4.0 respectively. The pectinase was stable between pH 3.0-6.0. The pectinase specifically indicated high activities in the presence of Mn^{2+} and Ca^{2+}. Molecular weight of the pectinase was 40 kDa. Thin layer chromatography showed that galacturonic acid was the end product of pectinase hydrolysis.

Keywords: Mutation; purification; *Aspegillus niger*; pectinase; chitosan.

Introduction

The biotechnological potential of pectinolytic enzymes from microorganisms has drawn a great deal of attention from various researchers worldwide as likely biological catalysts in a variety of industrial processes. Pectinases have crucial roles in food industries. These enzymes are useful for fruit juice extraction and wine clarification; tea, cocoa, and coffee concentration and fermentation; vegetable oil extraction; preparation of jam and jellies; and pickling (1). Furthermore, these enzymes are used in paper and pulp industries, bleaching of paper, bio-scouring of cotton, retting and degumming of plant fibers, oil extraction, wastewater treatment, and poultry feed additives, protoplast fusion technology, and bioenergy production.

Pectin is an important component of the middle lamella and primary cell wall of higherplants. Pectins are high molecular weight acidic heteropolysaccharide primarily made up of α (1-4) linked d-galacturonic acid residues (2). The three major pectic polysaccharides groups are all containing d-galacturonic acid to a greater or a lesser extent. They are homogalacturonan (HG), rhamnogalacturonan I (RGI), and rhamnogalacturonan II (RGII).

Fungi produce numerous extracellular enzymes that possess a special effect in the decomposition of organic matter. These include pectinolytic enzymes which are excreted to break down the middle lamella in plants so that it can insert fungal hyphae and extract nutrients from the plant. In addition to fungi, pectinolytic enzymes are naturally produced by many other organisms like bacteria, insects, nematodes, and protozoans. For the commercial production of pectinases, *Aspergillus spp.*, *Erwinia spp.*, *Bacillus spp.*, and *Penicillium spp.* have been extensively used (3).

Pectic enzymes can be available in dry powdered form as well as in liquid form. Nowadays interest in thermostable liquid enzymes has developed as they are associated with many benefits. They are non-polluting biochemical catalysts which are safe and easy to use. Moreover, enzymatic liquids go into process medium instantly and disperse evenly through the application process, as opposed to powder products (4). Purification is a pre-requisite for pectinase commercialization and application. The cost of enzyme purification chemicals is expensive especially in the developing countries. However, before advocating the usefulness of enzyme, it is very essential to characterize the enzyme produced so that it could be made a commercial product. Keeping these in view, this study aimed at purifying and characterizing thermal stable pectinase from mutated strain of *Aspergillus niger* obtained through chemical mutagenesis.

Materials and Methods

Isolation and identification of fungi

Ten-fold serial dilutions of 1 g soil sample collected from wastefruits degrading site were carried out and inoculation of prepared potato dextrose agar medium with appropriate 1ml of the diluent was done in triplicates. The inoculated Potato Dextrose Agar (Oxoid) medium plates were incubated at 30°C for 5 days. After the period of incubation,the structure, spore color, type of hypae and fruiting body of the fungi was observed under microscope(using ×10 and ×40 objective lens) for the identification of fungi.

Screening for pectinolytic activity

The screening methodology involves 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 were inoculated with *A. niger* and incubated at 30°C for 24 h. At the end of the incubation period, plates were stained with 50 mM iodine for visualization of clear zone of pectin hydrolysis **(5).**

Mutagenesis

The crude spores of high producing *A. niger* were prepared in Vogel's medium using 250ml Erlenmeyer flasks with working volume of 50ml in rotary shaker operating at 220 rpm and at 30°C for 30 min.**(6).** N-methyl-N-nitro-N-nitrosoguanidine (Sigma Aldrich) solution (NTG; 0.3 mg/ml of buffer saline) was used to induce mutagenesis. One ml of NTG solution and 9ml of Vogel's medium containing spores of *A. niger* was added in flask and kept in water bath at 37°C. After interval of 30, 60, 90, 120, 150 and 180 min, 1ml samples were drawn and washed with sterilized distilled water in triplicate for 15 min at 10,000 rpm**(7).** Serial dilution was carried out to the express cells and plated on potato dextrose agar plates. Selection, based on colonial characteristics, was done and screening for pectinase highest producer among the mutants was carried out as described earlier (**5).**

Molecular characterization of fungi

DNA extraction and purification was done with QIAamp DNA Mini Kit from Qiagen with catalogue number 51304. The extracted DNA was then treated with RNase. 3 µl of the DNA 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 min, 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 min. About 2.5 µl of the purified PCR product 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.

Enzyme production by submerged fermentation

The highest producing mutant of*A.niger* was used to produce pectinase in liquid medium containing, citrus pectin (10g), $(NH_4)_2SO_4$ (1.4g), K_2HPO_4(6g), KH_2PO_4 (2g),$MgSO_4.7H_2O$ (0.1g), in distilled water (1L), at pH 6. The medium was sterilized at 121 ºC for 15 minutes after which it was allowed to cool and inoculated with 1×10^6crude spores of *Aspergillus niger*. Fermentation was carried out in 500 ml Erlenmeyer's flask containing 250 ml of growth medium and incubated at 30°C under shaking conditions (175 rpm) for 5 days (9).The biomass was separated by centrifugation at 10,000 rpm for 15 min at 4°C.

Pectinase assay

Pectinase activity was determined using citrus pectin(British Drug House)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(British Drug House) solution after which the mixture was boiled for 10 min and cooled (9).The color was read at 540 nm using a spectrophotometer UV 754 hospibrand USA). The amount of reducing sugars released was quantified using galacturonic acid as a standard.

Protein content

Protein content of the pectinases was determined by following the method of Lowry *et al.*,**(10)**with bovine serum albumin as a standard.Protein extract, 0.2 ml was measured into tubes and 0.8 ml distilled water was added to it. The standard was prepared with bovine serum albumin (10 mg/ml) in 5 ml alkaline solution, 0.5 ml of Folin and ciocalteu's phenol reagent(J. T. Baker) solution was added to all the test tubes and left for 30 min after which the optical density was read at 600 nm wavelength in a spectrophotometer (T70 PG Instrument UV model).

Chitosan precipitation

About 100 mg of chitosan was dissolved in 1 % of acetic acid (1 ml in 99 ml de-mineralized water) under agitation and hydrated overnight. The solution was maintained at pH 4.0. The chitosan solution was treated with various concentrations (up to 2.0 mg/10 ml) of prepared chitosan solution and allowed to precipitate for 1 h at room temperature. 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 BSA as standard. Effect of pH, temperature and contact time on the purification potential of chitosan was estimated.

Gel filtration chromatography

Glass column was packed with silica gel 60 (70 to 230 mesh, M and B). The partially purified pectinase sample was loaded on to the column and elution of the proteins was done using acetate buffer (0.05 M, pH 4.5). Fractions volume was collected. The assay 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.

Optimum temperature of pectinase

The optimum temperature of the pectinase activity was measured using citrus pectin substrate within a temperature range from 40 to 70°C in a water bath. Determination of the residual pectinase activity was done as described in earlier Lia *et al.,* **(11)**.

Optimum pH of pectinase

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.

Thermal stability of pectinase

Pectinase thermostability was studied by incubating the enzyme in a 50 mM sodium acetate buffer at optimum temperature (obtained above) for 5 h. At the interval of 1 h samples was withdrawn and the residual pectinase activity was determined **(12).**

pH stability of pectinase

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 rangefrom 2.0 to 7.0**(13).**

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 with the presence of various metal ions were incubated for 30 min at 50°C and then the residual pectinase activity was tested with pectin **(14).**

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

The purity and molecular weight of pectinase was estimated by SDS-PAGE using 12% gels. The glass sandwich was filled with separating gel up to the mark. This was then overlaid with water for 30 minutes to make the gel surface straight after polymerization. Thereafter, requisite amount of 4% stacking gel solution was added and comb was inserted for polymerization. Running buffer was added to the glass sandwich up to both cathodic and anodic level in the electrophoretic chamber. The comb was removed. About 20 µl of pectinases samples (denatured in Laemmli buffer) was loaded into the wells **(15).**Standard molecular weight marker protein (Sigma) was also loaded in one lane. This was then run at a constant current of 20 mA. The mobility of sample was tracked with bromophenol blue in the Laemmli buffer. After migration protein bands were stained with Coomassive Brilliant Blue (CBB)**(15).**

Thin Layer Chromatography (TLC)

A thin layer chromatography (TLC) overlay method was used to analyze component of the pectinase reaction. **(16).** About 40 µl of pectinase was applied on pre-coated aluminium silica gel 60 plates (Merck, Art. 5553 DC-Alufolien). The plates were developed with propanol, water and diethyl ether (6:3:1 v/v). The plates were run in duplicate. The plates were 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 galacturonic acid (MERCK).

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

Results and Discussion

Pectinases are increasing in commercial importance. It has been reported that pectinase contribute to more than 25 % of the global enzyme sales (**17).** Pectinase producing *A.niger*was isolated from soil collected from waste fruit dumping site. This *A. niger* subjected to nitrosoguanidine treatment dose at 0.3 mg/ml at different time intervals ranging from 30 to180 min. It was observed that with gradual increase in treatment timethe number of survivors was decreased. Nitrosoguanidine treatment at 150 and 180 minutes gave no survivor as shown in figure 1. Lokeswary and Reddy **(18)** who utilize nitrosoguanidine to mutate fungi cultures also observed that the survival percentage of the cultures afterNTG treatments decreased as the exposure time increased and became nil thereafter.

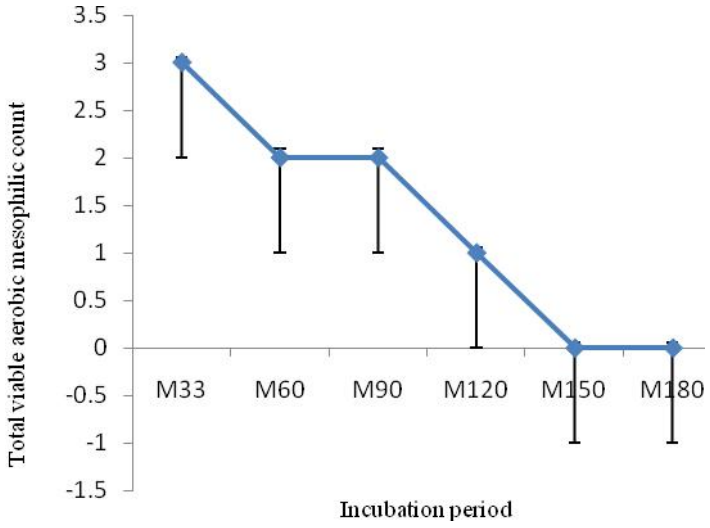
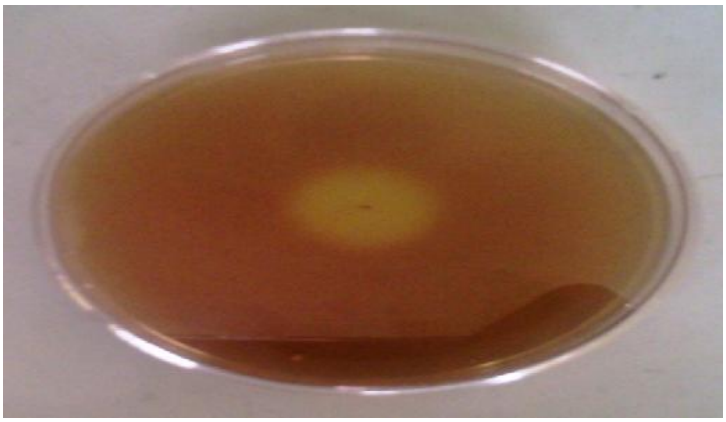


Figure 1: survival rate after NTG treatment

Quantitative and qualitative screening of mutants showed that mutant (M33) was the highest pectinase producing mutant with 2028.571 U/ml and 25 mm as indicated in figure2 and table 1 respectively. Bapiraju *et al.*,[19] reported that nitrosoguanidine is an effective mutagen for fungal strain improvement for enhanced enzymes activity. Agarose gel (1%) electrophoresis for PCR products of the 18S rRNA amplified from mutant (M33) indicated 625 bp shown in figure3. The highest producing mutant (M33) gave a DNA long fragment and BLAST on NCBI Genbank database at 95% query cover, 99% identity and maximum score of 549 with a final result as *Aspergillus niger* strain F7-02 with accession number JN561274.1.



Figue 2: Clear zone of pectin hydrolysis of *A.niger* strain F7-02 (M33) on PSAM.

Table 1: Crude pectinase activity, pH and zone of pectin hydrolysis of the wild type and mutants

Isolate codes	Zone of pectin hydrolysis (mm)± S.D	Pectinase activity (U/ml) ± S.D
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 of duplicate determinations

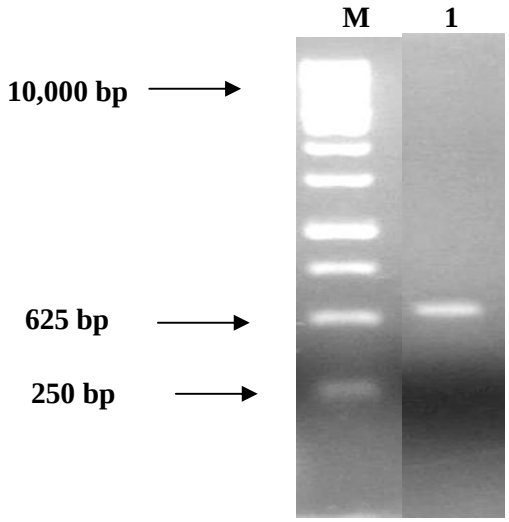


Figure 3: Gel electrophoretogram of the isolates polymerase chain reaction purification product

Specific activity of the treated pectinase, from *A. niger* strain F7-02, increased as the chitosan concentration increases (Figure 4) up to 0.75 mg/ml and further increase in concentration did not cause increase in the specific activity. Purification of pectinase from *A. niger* strain F7-02 produced 6.75 degree of purification. Chitosan's positive charge allows it to have many electrostatic interactions with negatively charged molecules such as proteins, fats, metal ions e.t.c. in enzymes **(5)**

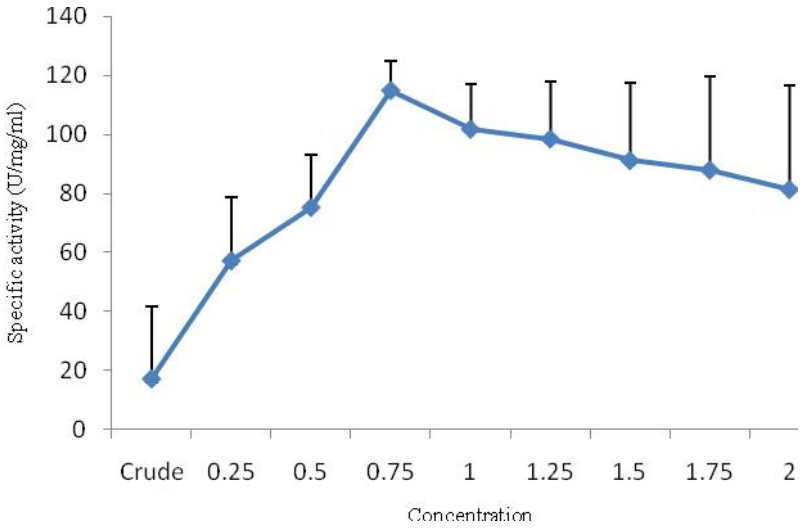


Figure 4: Effect of chitosan concentration on pectinase

High activity of pectinase from *A.niger* strain F7-02 was obtained at pH 4.0 which produced up to 9.22 degree of purification (Figure 5). These results support Celestino *et al.*, **(20)** who worked on pectinase from *Acrophialophora nainiana*. At optimum precipitation temperature (4 °C), the pectinase from *A.niger* strain F7-02 produced up to 12.92 degree of purification as shown in Figure 6. Crude pectinase was found to be stable up to 32 days whereas partially purified pectinase remained stable up to 2 months at 4°C (**21).**Optimum contact time was observed to be 4 hours (Figure 7) with an increased yield of 23.5 degree of purification.

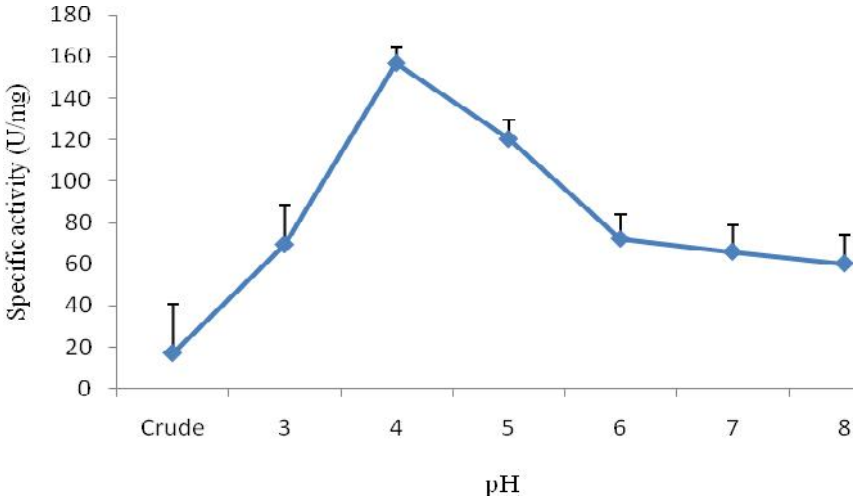


Figure 5: Effect of pectinase pH on chitosan

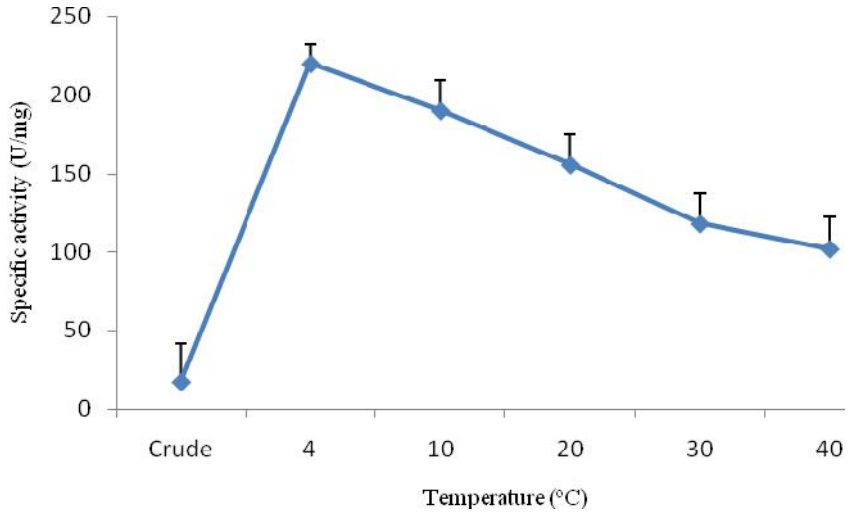


Figure 6: Effect of pectinase incubation temperature with chitosan

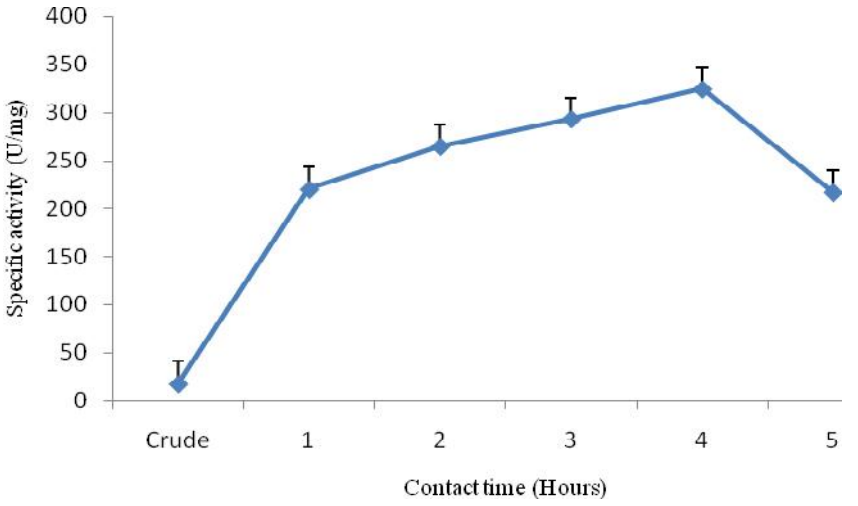


Figure 7: Effect of pectinase contact time with chitosan

Fractions that showed pectinase activities after gel filtration were pooled together (Figure 8). This purification step produced up to three times purification fold (Table 2). Impurities that can be present in the crude pectinase were removed during gel filtration **(22).**

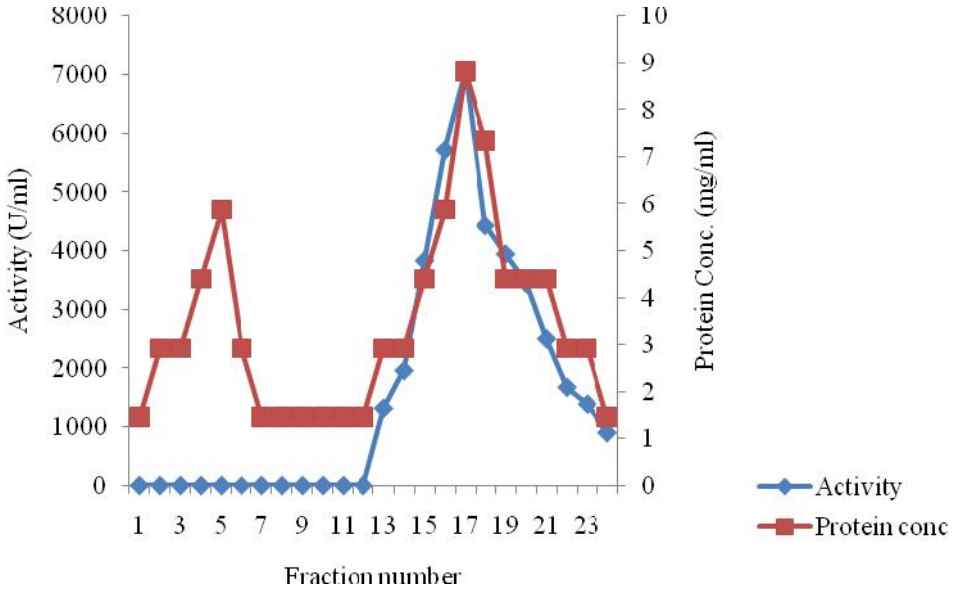


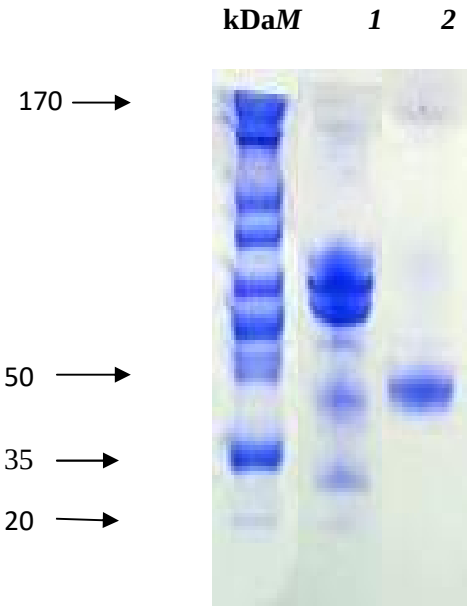
Figure 8: Elution profile for protein and activity of pectinase from *A. niger*strain F7-02

Table 2: Purification of pectinase

A. niger strain F7-02 pectinase	Activity (U/ml) ± S.D	Protein concentration (mg/ml) ± S.D	Specific activity (U/mg) ± S.D	Purification fold± S.D
Crude	2028.571±10.910	119.118±15.563	17.030±0.701	1.0±1.000
Chitosan	7071.429±49.508	17.647±7.074	400.716±7.000	23.530±9.986
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

The detection of pectinase activity in fractions 12 to 16 suggested that the pectinase is of high molecular weight. Result obtained from SDS-PAGE indicated that the molecular weight of the enzyme was 40 kDa (Figure 9). Microbial pectinase produced from *A. niger* by submerged fermentation had molecular weight of 40 kDa (5). Although, Vebhar and Neelam, **(23)** reported the size of the purified pectinase found to be 66 kDa.



Lane M, molecular mass makers;

Lane 1, crude pectinase from *A. niger* strain F7-02.

Lane 2, silica gel filtration of partially purified pectinase from *A. niger* strain F7-02;

Figure 9: SDS-PAGE of partially purified pectinase from *A. niger* strain F7-02.

Pectinase from *A. niger* strain F7-02 showed optimum activity at 65 °C (Figure 10). This result is in agreement with the earlier work of Martins *et al.*,**(22)** who observed optimum temperature for thermostable pectinase to be 65°C. The pectinase was thermal stable between 60 and 240 minutes at 50 °C (Figure 11).

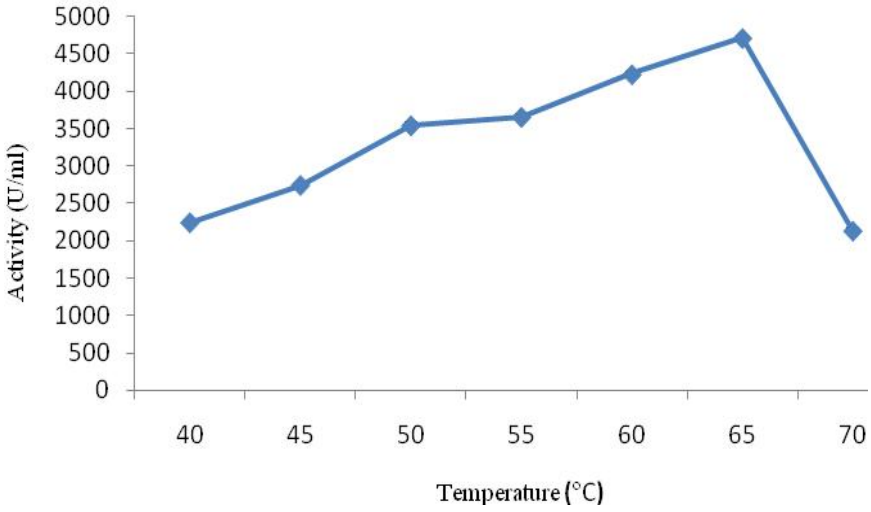


Figure 10: Optimum temperature of partially purified pectinase

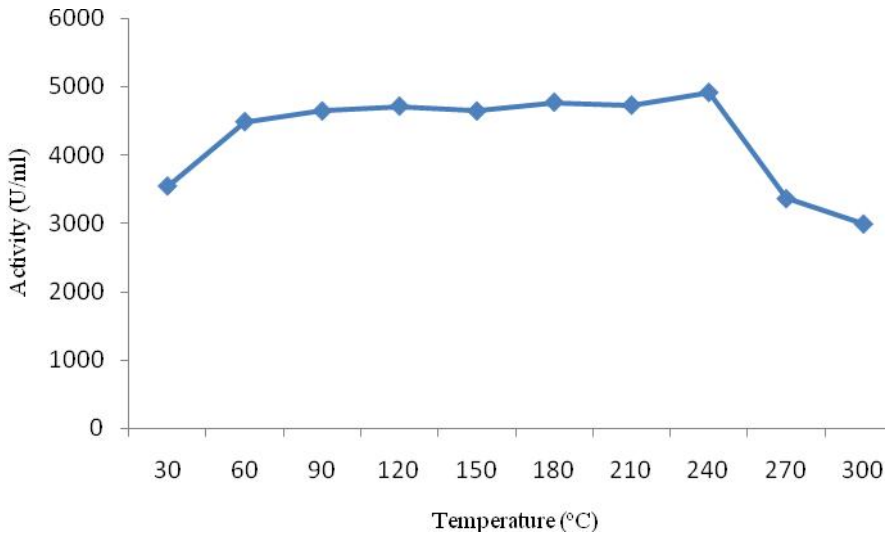


Figure 11: Temperature stability of pectinase

Pectinase from *A.niger* strain F7-02 showed optimum activity at pH 4.0; this is similar to previous study by Yogesh *et al.*,**(5)** who reported that the optimum pH for pectinase by *A.niger* was 3.8. The pectinase activity was observed to be stable at range of pH 3-6 (Figure 12). This result is in agreement with a similar workdone by Maria *et al.*,**(24)**on production of fungal pectinolytic enzymes. The authors reported that the fungal pectinase was stable in the pH range from 3.0 to 6.0. This is similar to the report of Martins *et al.*, **(25)** who explained that polygalaturonase from *Penicillium* spp was stable at the range of pH 3.0 to 6.0. Most fungal pectinases are stable at low pH (**26).**

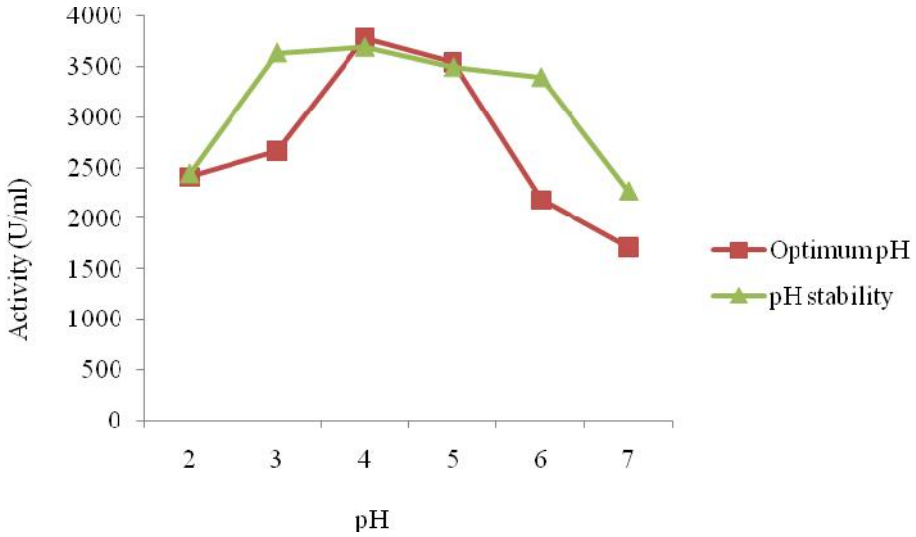


Figure 12: Effect of pH on partially purified pectinase

Ca^{2+} and Mn^{2+} activated pectinase activity. Cu^{2+}, Zn^{2+}, Mg and Al^{3+} inhibited the activity of pectinase (Figure 13). Past research has indicated that Ca^{2+} has significant influence on the activity and stability of enzymes **(27).** The result from thin layer chromatography showed that individual Rf was 0.88 for the pectinase when compared to the standard (Figure14). This showed that galacturonic acid was the end product of the pectinase hydrolysis; this indicated that the enzymes released was polygalacturonase.

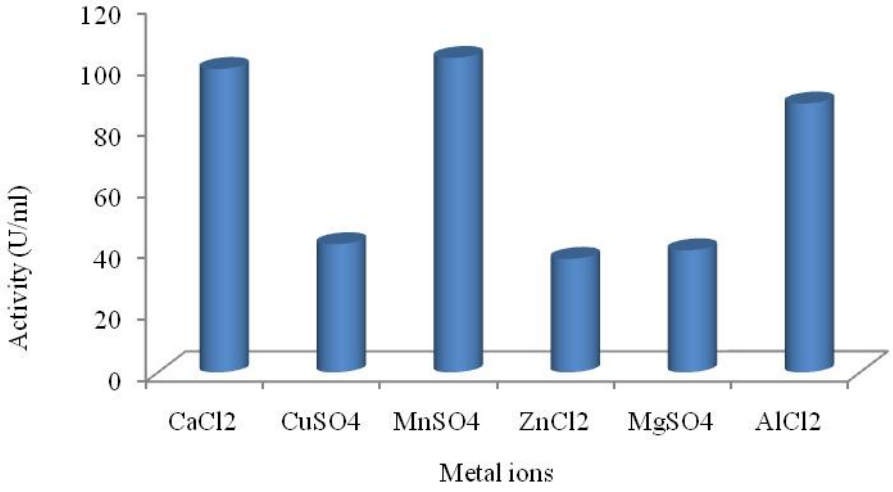
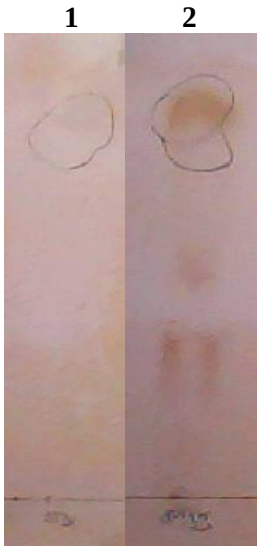


Figure 13: Effect of metal ions on partially purified pectinase



Lane 1, standard (galaturonic acid).

Lane 2 sample, pectinase from *Aspergillus niger*strain F7-02;

Figure 14: Thin layer chromatography analysis of the products of galaturonic acid hydrolysis by pectinase from *A. niger* strain F7-02.

Conclusion

In the present study, pectinase produced from *A. niger* strain F7-02 (mutant) had optimum activity at 65 °C and is relatively thermal stable. This study examines the purification potential of chitosan in pectinase production. It can be concluded that chitosan is a promising bio material for pectinase purification. Therefore utilization of locally available bio materials, such as chitosan, for pectinase purification will help reduce cost of enzyme purification in enzyme technology.

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Quick Response Code

DOI:10.22192/ijarbs.2021.08.06.007

How to cite this article:
Famotemi, A.C;Kareem, S.O.; Oluwafemi, F.; Adewuyi, S. (2021). Purification and Characterization of Pectinase from *Aspergillus niger* Strain F7-02 Mutant. Int. J. Adv. Res. Biol. Sci. 8(6): 45-57.
DOI: http://dx.doi.org/10.22192/ijarbs.2021.08.06.007