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Purification and characterization of pectinase by *Aspergillus niger* isolated from vegetable oil effluents using submerged fermentation

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Abstract
Pectinases are useful industrial enzymes that catalyse the degradation of pectin into monogalacturonic acid by opening glycosidic linkages. This study isolated pectinase producing fungi from vegetable oil factory effluent, purify and characterize the pectinase produced. Enzyme production was carried out by submerged fermentation and purification carried out by ammonium sulphate precipitation, dialysis and gel chromatography. Enzyme characterization was also carried out for various physiochemical parameters and the molecular weight determined via sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). The fungus was identified as *Aspergillus niger* LMBPL1B9. The study showed that the purification fold increased from 1 to 1.1698 using Sephadex G-100 column chromatography. The enzyme maintained relative stability between 25 °C - 55 °C. The enzyme was relatively stable between pH 4.5 -11 peaking at pH 8.0. Metal ions such as K^+ stimulated enzyme production. Pectinase activity increased with increasing substrate concentration peaking at 3.0 mg/ml. While the K_m value of pectinase was 0.32 mg/ml.

Keywords: pectinase, chromatography, thermostable, kinetics, molecular weight

Introduction
Enzymes are biocatalysts produced by living organisms to increase the rate of a set of chemical reactions required for various life processes. They are responsible for the catalysis of all essential life processes such as DNA replication and transcription, protein synthesis, metabolism, and signal transduction. Their ability to perform very specific chemical transformations has made them increasingly useful in numerous industrial processes (Li *et al*., 2012) [14].
Microorganisms possess numerous abilities, producing an array of enzymes that have been studied and utilized over a long time now. Microorganisms are preferred as enzyme sources because of their short life span, high productivity rate, cost-effectiveness, and are free of harmful chemicals that are found in enzymes from plant and animal sources (Khatri *et al*., 2015; Smith, 2013) [12, 24]. Microbial enzymes are produced by fungi, bacteria and actinomycetes. Microorganisms of interest are subjected to varying genetic manipulations and hence provide the opportunities for strain improvement and further investigation (Oumer & Abate, 2018) [19]. The increasing demand for eco-friendly biotechnological products has resulted in microbial enzymes being recognized as very efficient industrial tools. Thus increasing the need to assess various microbial sources for enzyme production (Mishra. Suneetha Vuppu. and Bishwambhar., 2011) [17].
Pectinase enzymes comprise a group of enzymes that are involved in the degradation of pectin into monogalacturonic acid by opening glycosidic linkages. Pectins are the high molecular weight polysaccharides, primarily composed 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 (Khatri *et al*., 2015) [12]. Pectin provides rigidity to plants and occurs in the middle lamella and primary cell wall of higher plants in the form of calcium pectate and magnesium pectate (Khatri *et al*., 2015) [12].
Pectinases from fungi possess appreciable thermal stability and activity over a range of pH 4 - 7 and may be suitable for various industrial applications that require the usage of elevated temperatures, extremes of pH and ionic concentrations. Hence the necessity to assess a range of unique habitats and study the indigenous flora with the better enzymatic ability (Akbar & Gyana Prasuna, 2012; Buga *et al*., 2010). [1, 5].
The sludge from vegetable oil factories offers a suitable niche for various fungi due to the abundance of moisture and organic debris of plants and animals. There are also several parameters influencing enzyme production in submerged fermentation, usually, it consists of physical and chemical parameters.
The present investigation focuses on the isolation and screening of pectinolytic fungi, production followed by its purification and characterization.

Materials and methods
Isolation of pectinase producing fungi
Fungi used was isolated from vegetable oil effluents in Agbado-Ekiti, Nigeria. The fungi were isolated by carrying out serial dilution on a Potato Dextrose Agar plate at 28 ℃ for 7 days.
Pure colonies were picked and purified by repeated streaking (Sreenivasulu *et al*., 2017) [25]. Fungi were screened on pectinase screening agar medium (PSAM) containing ($NaNO_3$ 2g, KCl 0.5g, $MgSO_4$ 0.5g, K_2HPO_4 1g, peptone 0.5g, agar 20g and pectin 10g in 1000 mL) (Arijit *et al*., 2013) [2]. at 28 ℃ for 7 days.
The plates were and flooded with 1% cetrimide solution for

primary screening. The pectinolytic fungi produced a clearing zone when exposed to cetrimide. The identification of the fungi was done using colony morphology and gene sequencing.

Pectinase production

The fungi which showed higher pectinase production ability on PSAM plates was selected for fermentation. Pectinase production medium was prepared according to the method of (Sethi *et al*., 2016) [22]. modified using submerged fermentation. Uninoculated fermentation broth was used as control. Fermentation broth was withdrawn and centrifuged at 10,000 rpm at 4 ˚C for 10 minutes to recover the cell free supernatant containing crude enzyme.

Pectinase assay

Pectinase assay was done by measuring the amount of reducing sugars expressed as galacturonic acid units liberated from pectin using 3,5-dinitrosalicylic acid (Miller, 1959). The reaction mixture containing 450 µL of 1 % (w/v) pectin (Sigma) as substrate and 50 µL of enzyme extract was incubated at 50 ℃ for 10 minutes. The reaction was then terminated by adding 1.5 mL of 3,5-dinitrosalicylic acid reagent. A control was run simultaneously that contained all the reagents but the reaction was terminated before the addition of enzyme (Nagar *et al*., 2012) [18]. The contents were placed in a boiling water bath (100 ℃) for 15 minutes and then cooled in ice-cold water. The absorbance of the resulting colour was measured against the control at 540 nm using a spectrophotometer. Glucose was used as a standard reducing sugar. For the calculation of the reducing sugars released by the enzymatic activity, a standard curve was prepared by using a standard glucose solution in different concentrations. One unit of pectinase activity is defined as the amount of enzyme that catalyzed the release of 1 µmol of reducing sugar as galacturonic acid equivalent per minute under specific assay conditions (Silva D, Martins ES, 2002) [23].

All experiments were conducted in triplicate and the mean values of all the sets of observations were taken for evaluation of results.

Protein assay

Protein assay in the enzyme preparation was carried out by Lowry's method (Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, 1951) [15]. using bovine serum albumin (BSA) as standard.

Purification of the pectinase enzyme

The crude enzyme was subjected to precipitation in a salting-out process (Dixon, M. and Webb, 1971) [9]. The precipitated fractions was dialyzed and separated using Sephadex G100 column chromatography (Ding, Z., Peng, L., Chen, Y., Zhang, L., Gu, Z., Shi, G. and Zhang, 2012; Wakil & Osesusi, 2018) [8, 27]. The fractions obtained were pooled together and analyzed for pectinase activity and protein content, they (fractions) were then used for further study.

Characterization of the purified pectinase enzyme

Effect of pH

The effect of pH on the stability of the purified pectinase was determined by incubating the pectinase-substrate mixture in appropriate buffers (Citrate-phosphate for pH 4, 5, 6, 7 and Tris-HCl for pH 8, 9, 10, 11) for one hour. The enzyme assay was carried out for each enzyme according to the standard method for pectinase assay. Relative activity was carried out by using maximum activity as a standard reference (Parashar & Kumar, 2018) [20].

Effect of temperature

The effect of temperature on the stability of the purified pectinase was determined by incubating the pectinase-substrate mixtures at varying temperatures (20 - 70 ℃) at 5 ℃ intervals in a water bath for one hour. Pectinase assay was carried out for each enzyme according to the standard method for pectinase assay. Relative activity was carried out by using maximum activity as a standard reference.

Effect of metal ions

The effect of metal ions on the stability of the purified pectinase was determined by pre-incubating the pectinase-substrate mixtures with metal salts (Ca^{2+}, Cu^{2+}, Mn^{2+}, Mg^{2+}, Ba^{2+}, Zn^{2+}, K^{+}, Na^{+}) for one hour. The metal salts concentrations of 5 mM were used. Pectinase assay was carried out for each enzyme according to standard method for pectinase assay. Relative activity was carried out by using the control as standard reference.

Effect of substrate concentration

The effect of substrate concentrations on the pectinase activity was determined by incubating various concentrations of substrate (pectin) from 0.2 to 1.2 mg/ml with pectinase for one hour under appropriate conditions. Pectinase activity was carried out according to standard methods as described earlier. The enzyme kinetics was also carried out.

Effect of inhibitors and surfactants

The effect of surfactants and inhibitors Tween 20, Triton X, Sodium Lauryl Sulfate (SLS), Sodium Dodecyl Sulfate (SDS), 2-mercaptoethanol (BME), Dithiothreitol (DTT), and Ethylenediaminetetraacetic acid (EDTA) on the stability of the purified pectinase was determined by pre-incubating the enzymes substrate mixtures with the surfactants and inhibitors at various concentrations for one hour at a concentration of 1mM. Pectinase activity was carried out according to standard methods as described earlier.

Molecular weight determination

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out by the modified method of Laemmli (Laemmli, 1970) [13]. Acrylamide-bis-acrylamide solution, resolving gel buffer, stacking gel buffer, ammonium sulphate, staining and destaining solutions, and bromophenol blue were the same as used for Native- PAGE. SDS (10 % w/v) was prepared.

Results and Discussion

Isolation of pectinase producing fungi bacteria

The fungal isolates that showed observable zone of hydrolysis upon the cetrimide treatment of the PSAM media were selected for further production and pectinase assay, the highest pectinase producer was used for further studies. The strain was identified as *Aspergillus niger* LMBPL1B9 upon blasting of the obtained gene sequence. *Aspergillus niger* has been reported as a notable pectinase producer and subject to further optimization and characterization of the

production parameters can be used for scale up of pectinase production.

Summary of the purification profile of *Aspergillus niger* pectinase showed a reduction in the total pectinase activity and total protein content across the purification steps, with a purification fold of 1 to 1.1698 using Sephadex G-100 column chromatography Table I. This is similar to reports by (Irshad *et al*., 2014) and (Khatri *et al*., 2015) [11, 12]. who reported a decrease in total activity and protein content with a reduction in the purification fold and a recovery yield. Although the results differs from (Arijit *et al*., 2013) [2]. who reported an increase in total protein activity across the purification steps.

Table 1: The purification profile of *A. Niger* pectinase

Purification step	Total activity (U/mL)	Total Protein (U/mL)	Specific activity (U/mg)	Purification fold	Recovery yield %
Crude extract	705.127	28.598	24.657	1	100
80% Ammonium sulphate precipitation	598.95	25.512	23.477	0.952	85
Dialysis	322.14	18.607	17.313	0.702	46
Gel chromatography	312.239	7.456	41.878	1.698	44

The findings showed that the pectinase maintained relative stability after one hour incubation, although the activity reduced with decreasing temperature as *A. niger* had its highest activity at 55 °C (99.99 %) (Figure I). This observation is similar to findings obtained by (Akbar & Gyana Prasuna, 2012; De Freitas *et al*., 2006; Irshad *et al*., 2014) [1, 6, 11]. Who reported relative pectinase stability at 55 °C but differs from reports obtained by (Buga *et al*., 2010; Meena *et al*., 2015) [5, 16]. Who reported maximum pectinase activity after one hour at 40 °C. This suggests that this pectinase could be thermostable.

The pectinase was also observed to be relatively stable across yarying pH regimes after with the highest activity at pH 8.0 (Figure II). This agrees with the discovery of (Khatri *et al*., 2015) [12]. Who reported maximal pectinase stability at pH 8.2. This differs from findings of (Irshad *et al*., 2014; Oumer & Abate, 2018) [11, 19]. Who reported maximum pectinase activity at pH 6.

The *A. niger* pectinase was stimulated by K^+, Ba^{2+}, Cu^{2+}, Mn^{2+}, Zn^{2+} and Ca^{2+} as shown in Figure III, indicating that the enzyme was metallo-dependent. This is similar to results obtained by (Sethi *et al*., 2016; Torimiro & Okonji, 2013) [22, 26]. who reported a three-fold stimulation of pectinase biosynthesis by *Aspergillus terreus* due to the addition of K^+. While the pectinase enzyme was inhibited by Mg^{2+} and Ca^{2+}, which agrees with reports that Ca^{2+}, Zn^{2+} and Mg^{2+} inhibited the pectinase activity of *Aspergillus flavus* MTCC7589 at 1.0 mmol/l concentration (Yadav *et al*., 2008) [28]. This suggests that the requirement of metal ions for pectinase activity varies depending upon their sources and enzyme structure (Khatri *et al*., 2015) [12].

The study showed that tween 20, triton X, sodium lauryl sulfate (SLS), sodium dodecyl sulfate (SDS), enhanced the activity of *A. niger* pectinase, while2-Mercaptoethanol (BME), dithiothreitol (DTT), and ethylenediamine tetraacetic acid (EDTA) had varying effects on *A. niger* pectinase Figure IV. This observation is similar to results obtained by (Arijit *et al*., 2013; Ramkumar *et al*., 2018) [2, 21]. Who reported improved pectinase stability in the presence of surfactants SDS, tween oils and triton X. (Garg *et al*., 2016) [10]. Also reported that the effect off surfactants and agitation was dependent on enzyme structure.

It was observed that increasing substrate concentration resulted in increasing activity for *A. niger* pectinase, with the highest pectinase activity obtained at 2.0 mg/ml after which increasing substrate concentration resulted in decreasing pectinase activity (Figure V). The apparent K_m value of this pectinase (0.32 mg/ml) is indicative of the affinity of substrate to fill half the active sites of an enzyme. This is similar to the K_m values by *A. niger* (Arotupin *et al*., 2008; Dhembare *et al*., 2015) Dhembare *et al*., 2015) [3, 7].

The molecular weight was determined using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) Figure VI. The molecular weight was determined as 39.12 kDa. This is in close agreement with reports by some authors who reported the molecular weight of *Aspergillus niger* as 38 ± 0.1 kDa (Yadav *et al*., 2008) [28]. But this differs from reports by (Arotupin & Akinyosoye F. A. Onifade, 2012; Khatri *et al*., 2015) [4, 12]. who reported the approximate molecular weight of *Aspergillus niger* strain MCAS2 to be 66 kDa as observed from SDS-PAGE.

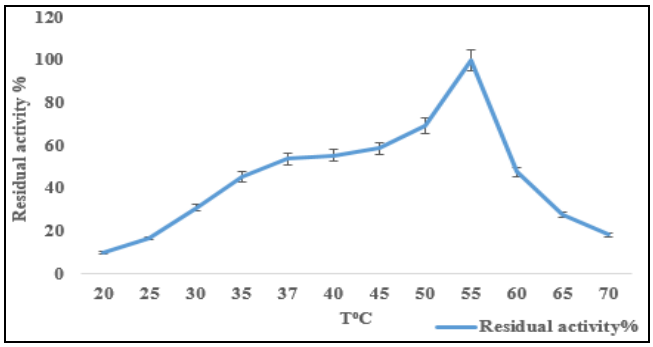

Fig 1: Effect of Temperature (TºC) on the stability of pectinase

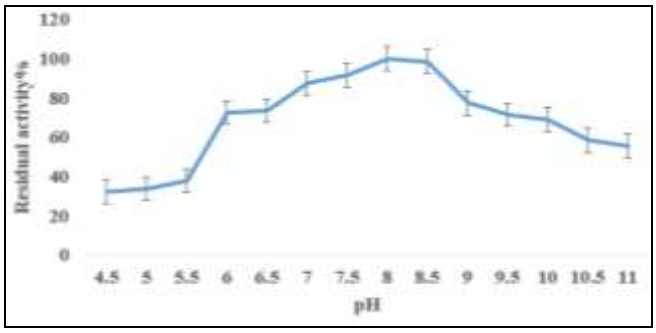

Fig 2: Effect of pH on the stability of pectinase

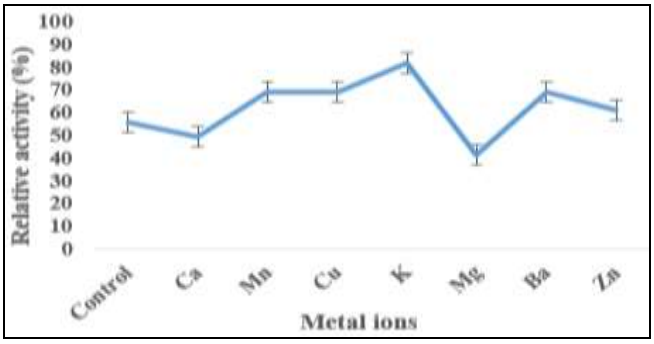

Fig 3: Effect of metal ions on pectinase activity

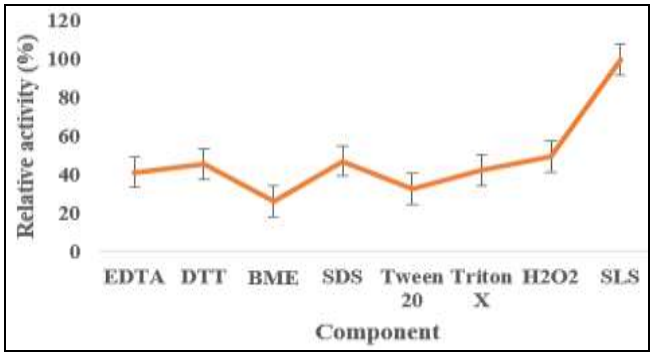

Fig 4: Effect of surfactants, inhibitors and oxidizing agents on pectinase activity

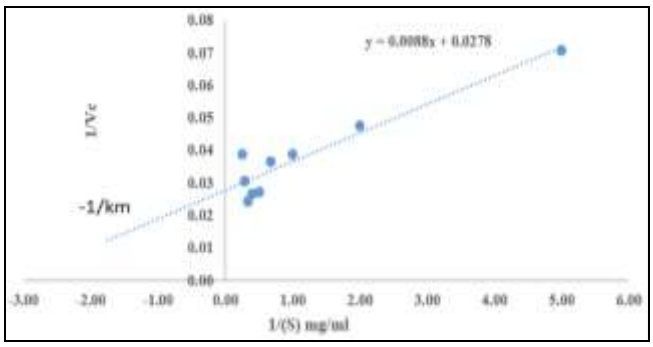

Fig 5: Lineweaver-burk plot for the hydrolysis of pectin by the purified pectinase of *Aspergillus niger*

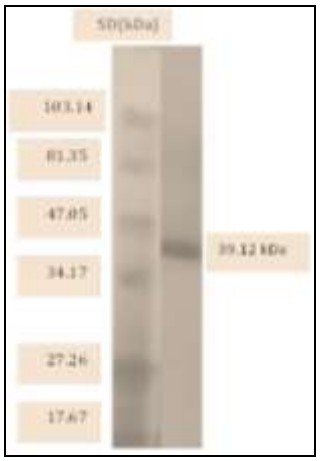

Fig 4: *Aspergillus niger* pectinase

Conclusion

Pectinases are industrial enzymes which possess enormous applications in biotechnology. This *Aspergillus niger* was isolated from vegetable oil mill effluents, used for pectinase production, purified and Characterized. This pectinase was observed to be thermally stable and results indicated its alkaline nature. Thus it could be assessed for other biotechnological applications.

Conflicts of interest/Competing interests

The authors declare no competing interests in the publication of this research article.

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