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ORIGINAL RESEARCH

Production of pectin methyl esterase (PME) from *aspergillus niger* grown on pineapple peel (*Ananas comosus*) in solid state fermentation.

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Abstract:

Introduction: Pectinases are today one of the upcoming enzymes of the commercial sector. A lot of this enzymes are commercially available, due to their all-embracing applications in bio scouring of cotton, degumming of plant fibers, with several industrial uses

Aims: The aim of the present study is to purify and characterize Pectin methyl esterase (PME) from *Aspergillus niger* (*A. niger*) grown on pineapple peel residue in a solid-state fermentation process

Materials and Methods: *Aspergillus niger* was grown on basal medium containing pectin as an inducer at a pH of 5.5, temperature of 30 °C, and fermentation periods of 96 h to provide the best conditions for pectinase synthesis. The optimum pectinase activities were performed at various pH (4.0-9.0), and temperatures (25 to 90^{o}C), The influence of metals ions was conducted using Zn^{2+}, Mg^{2+}, K^{+}, Na^{+}, Ca^{2+}; EDTA and Mercaptoethanol were used as inhibitors at range concentrations from 10 to 50 mM, respectively.

Results: The isolated pectin methyl esterase from *A. niger*, Ion exchange purification gave about 4.2-fold with specific activity of 7.14 U/mg, and 9.5% enzyme recovery the optimum PME activity was at pH 7.5, and temperature of 45^{o}C; the maximum substrate concentration was 4mg/ml with the Km value of 1.321 and Vmax of 5mMol/min; The present of Ca^{2+}, K^{+} Mg^{2+}, Na^{+}, and Zn^{2+}, ions significantly activated the enzyme activity, however EDTA and Mercaptoethanol inhibits the PME activity greatly

Conclusion: PME from *A. niger* is a good microbial enzyme source and pineapple residue a neglected agro-waste is an alternative source of PME production that can be exploited industrially.

Keywords: Pectin methyl esterase, *Aspergillus niger,* pineapple peel, microbial enzymes, solid state fermentation.

All co-authors agreed to have their names listed as authors.

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1. INTRODUCTION

Pectin is de-esterified by the enzyme pectin methyl esterase (PME; E.C. 3.1.1.11), resulting in the breakdown of carboxyl groups and the formation of methanol. All higher plants, notably citrus fruits, possess pectin methyl esterase [1-3]. Plant PME totally transforms pectin when combined with other pectinases. An excellent substrate for polygalacturonase and other degradation enzymes that totally break down pectin and soften plant tissues by loosening cell walls is PME-demethylated pectin. The loss of appealing cloud stability in citrus juices is the primary problem related to pectin methyl esterase action. This kind of softening, mediated by PME, is an important problem during the processing of different fruits and vegetables. Citrus juices undergo a loss of cloud cover due to reactions among positively charged divalent ions (Ca^{2+} and Mg^{2+}) and negatively charged sites on demethylated pectin, along with the production of insoluble salts of pectate that destabilize cloud particles [4,5].

However, it has become crucial to heat-treat citrus juices to disable the enzymes [6]. Although there were some successful experimental studies on the use of packaged or extracted orange skin or the pulp pectin methyl esterase for the enzymatic firming of vegetables and fruits, and alteration of pectin for use as a functional food ingredient, plant Pectin methyl esterase currently has no application in the food industry.

To improve the processes of maceration, extraction, filtration, liquefaction, and clarifying as well as to aid in the disintegration of pectin, several fungal PME have been extensively used in the commercial manufacturing of fruits and vegetables [7]. The use of PME in the proteolytic tightening of fruits and vegetables treated with $CaCl_2$ and in the enzymatic peeling of fruits treated with a suitable mixture of pectinases is also increasing in acceptance [8]. As a consequence, its activation causes cloud loss in juice, and nectars enhance the feel of fruit and vegetable products [9]. It also significantly boosts the yield of juices extracted using conventional methods or promotes the elimination of water from tissues while drying. Therefore, the PME investigation from numerous sources has been quite important. Solid state fermentation (SSF) is a desirable choice because it provides higher productivity per reactor volume, fewer capital and operating costs, lower space requirements, more straightforward equipment, and easier downstream processing compared to that submerged fermentation (SmF), also referred to as liquid state fermentation, also known as the standard approach for microbial enzyme production.

Pectinolytic enzymes have become vital in the current biotechnological era given their wide range of uses in the bio scouring of cotton, degumming of plant fibers, wastewater treatment, vegetable oil extraction, tea and coffee fermentation, fruit juice extraction and its clarification, the bleaching of paper, poultry feed additives, alcoholic beverages, and food industries. One of the latest commercial enzymes is pectinases. Pectic enzymes can exist in a range of liquid and solid forms and are readily available for purchase [10].

Orange peel is a renowned source of PME in accordance with Cameron et al. [11]. However, the enzyme market only provides lyophilized pure commercially available forms of orange peel PME, which are too costly and rich in ammonium sulphate to be employed in food processing.

Many investigators have attempted to characterize PME from a range of sources, including soil fungus, food spoilage bacteria, and many more [12-18]. In order to reduce waste and find a way to turn waste into wealth, pineapple peel, one of the most neglected waste products of agriculture and a common fruit produced all year, was centered on as a means of producing pectin methyl esterase from *Aspergillus niger*. The negative environmental impact of many used for farming wastes is of major concern due to the fact that are lot of garbage littered all over the state. This work is aimed at purification and characterization of Pectin methyl esterase produced by *Aspergillus niger* using agro waste pineapple peel residue in a solid-state fermentation process.

2. MATERIAL AND METHODS

2.1 PREPARATION OF PINEAPPLE RESIDUE

At Lagos State University, Ojo, ripe pineapple was bought from a fruit seller. The lower part of the pineapple was peeled and utilized as the inducer substrate. It was then chopped up into tiny fragments

and roasted at 60°C till it had lost 5% of its initial mass. Prior to use, the pineapple peels were chopped, ground to a powder and autoclaved for 15 minutes at 121 °C [18].

2.2 MICROORGANISM

The Federal Institute of Industrial Research, Oshodi (FIIRO), Lagos supplied the *Aspergillus niger* utilized in the present study. This strain of A niger, designated strain #A1, was found in the soil of the Federal Institute of Industrial Research, Oshodi (FIIRO). On Potato Dextrose Agar (PDA) slants, pure stock cultures of the Aspergillus niger strains were cultured and grown at 40°C. On brand-new PDA slopes, subcultures emerged every month [18,19].

2.3 PREPARATION OF INOCULUM MEDIUM.

Aspergillus niger subcultures on potato dextrose agar for five days was used to prepare the spore suspension. The spore crop from each slant was scrapped into 5ml of sterile distiller water. The spore suspension two 2 ml was used as a source of inoculum.

2.4 PRODUCTION OF FERMENTATION MEDIUM

The basal medium used for the fermentation and production of pectin methyl esterase contained (g/l); $(NH_4)_2SO_4$, 2.0; K_2HPO_4, 2.0; Yeast extract, 3.0 and carbon source (Pineapple residue), 5.0 at pH5.5. The flask containing the basal medium was sterilized at 121^oC in autoclave for 15 minutes to avoid pectin degradation [18]

2.5 PRODUCTION OF PECTIN METHYL ESTERASE

The solid-state fermentation was carried out in three (3) 250ml Erlenmeyer flask with 100ml of production medium having composition (g/l): $(NH_4)_2SO_4$, 2.0; K_2HPO_4, 2.0; Yeast extract, 3.0 and carbon source (pineapple residue). Final moisture was adjusted to 70% and the pH of the media was adjusted to 5.5–6.0 by using1.0N HCl/1.0N NaOH. The flasks were sterilized at 121^oC for 15 minutes and were allowed to cool to room temperature. Two milliliters conidial suspension was added to the medium, mixed well and incubated at 30^oC for 96 hours. After cooling, the flasks were inoculated with 2ml spore suspension containing106 spores ml^{-1}, which was obtained from a five-day agar slant. The flasks were well mixed and incubated at incubated at 30^0C for 96 hours [18].

2.6 EXTRACTION OF ENZYME

The fermented media were extracted with 30ml of distilled water. The flasks were shaken vigorously and kept for one hour and filtered through cheese cloth. The crude enzyme was extracted by adding sodium phosphate buffer (0.01M, pH7.5) to each flask. The mixture was centrifuged at 10,000 rpm for 15 min at 4^oC and the supernatant was filtered through Whatman No.1 filter paper to remove spores completely. The clear supernatant obtained after filtration was analyzed for pectin methyl esterase activity [20].

2.7 ENZYME PURIFICATION.

2.7.1 AMMONIUM SULPHATE PRECIPITATION AND DIALYSIS

The crude PME extract was precipitated by the addition of saturated ammonium sulphate in other to achieve maximal precipitation several ammonia contents were used (35-85%). In the second step 6.75mg was added to 15ml of the crude enzyme under constant stirring. The precipitate collected was dissolved in 0.01M sodium phosphate buffer, pH7.5. The solution was dialyzed against 0.01M sodium phosphate buffer, pH7.5 for 24hrs [18-19].

2.7.2 SEPARATION OF ENZYME ON SEPHADEX G-75 CHROMATOGRAPHY COLUMN

A vertical glass tube chromatography column of 1.5 × 70 cm was used. Sephadex G-75was employed for the fractionation of the enzyme. The column was eluted with 0.05 M Phosphate buffer (pH 7.5), containing NaN_3. The void volume was determined with blue dextran. Fractions of 5 ml per tube will be collected and protein content of each eluted fraction was measured at 280 nm on a UV/Vis spectrophotometer (Beckman DU-64) as described by Olutiola and Ayres (1973) and the PME assay was carried out [21].

2.7.3 FRACTIONATION BY ION-EXCHANGE CHROMATOGRAPHY

Dialyzed enzyme was added to 0.02M sodium phosphate buffer, pH7.5 pre-equilibrated DEAE Sephadex column. Elution was done with step-wise gradient from 0-1M NaCl in equilibrating buffer. Ten mililitre (10 ml) of the dialyzed enzyme was applied to the column of DEAE Sephadex. Fraction of 5 ml per tube was collected and the protein content measured as earlier described. Pectin methyl esterase activity of each of the fractions was determined as described under enzyme assay [20].

2.8 CHARACTERIZATION OF ENZYME

2.8.1 EFFECT OF pH ON PME ACTIVITY

Optimal pH of PME activity was determined using the PH range of 4.0-9.0, at an interval of 0.5, this was done by adjusting the pH concentrations using 0.1M NaOH and).1N HCl. Enzyme activity was assayed using citrus pectin solutions of (0.5%). This was carried out using the constant time of 30mins after which the activity is determined spectrophotometrically at each pH [18].

2.8.2 EFFECT OF PHYSICO-CHEMICAL FACTORS ON PURIFIED ENZYMES

The effect of temperature on the enzyme activity was determined by incubating the reaction mixture at different temperatures ranging from 25-90ºC at 5ºC interval for 60mins. The residual enzyme activities were determined for each temperature of incubation and expressed as enzyme unit activity [18].

2.8.3 DETERMINATION OF K_M AND V_{MAX} OF PME ENZYME

This was carried out by measuring the enzyme activities at different substrate concentrations. Using the percentage pectin concentration range of (0.5-5.0mg/ml) for 30mins, then activity is measured spectrophotometrically as aforementioned [18].

2.8.4 EFFECT OF METALS ON ENZYME ACTIVITY

The effect of some cations and chemical inhibitor compounds at different molar concentration on the enzyme activity was determined. In this case, concentrations of 10 to 50mM of K^+, Na^+, Ca^{2+}, Mg^{2+} and Zn^{2+}were employed for the study. The concentrations of 10 to 50mM of ethylene-diamine-tetraaceticacid (EDTA), and mecarptoethanol was employed for chemical inhibitor. The pectin-substrate was initially incubated with each test chemicals at 4ºC for 35 min before being used for the enzyme assay [18-19].

2.8.5 DETERMINATION OF TOTAL PROTEIN

Protein was determined by the Lowry method by using bovine serum albumin as standard (Harris, 1987). For the assay 0.2 ml of sample, was mixed with 2.1 ml of alkaline copper reagent prepared by mixing 1 % $CuSO_4.5H_2O$ and 1% sodium potassium tartrate.$4H_2O$ in a 1:1 ratio and diluting to 100 ml with 2 % Na_2CO_3 (in 0.1 M NaOH). After 10 minutes incubation, 0.2 ml commercial folin Ciocalteau (Folin-C) reagent diluted 1:1 with distilled water was added to medium and the mixture was incubated 30ºC. At the end of the incubation period the absorbance of samples was read at 750 nm against blanks prepared by adding 0.2 ml water to reaction mixture in place of samples [21].

2.8.6 DETERMINATION OF PECTIN METHYL ESTERASE ACTIVITY

For the determination of PME enzyme activity, the Hagerman and Austin (1986) spectrophotometric method for enzyme assay was used with slight modifications. One Unit is defined as that amount of enzyme that caused 0.001 changes in absorbance in 1 min [21]

3. RESULTS AND DISCUSSION

Table 1: The Purification Table of pectin methyl esterase from *A. niger* grown on pineapple residue in a solid-state fermentation.

Purification steps	Volume (ml)	Total PME (U)	Total Protein (mg/ml)	Specific Activity (U/mg)	Purification Fold	Yield (%)
Crude	75	5250	3060	1.72	1	100
$(NH4)_2SO_4$	15	1200	318	3.77	2.2	22.86
Sephadex 75	10	760	144	5.28	3.1	14.5
DEAE Sephadex	5	500	70	7.14	4.2	9.5

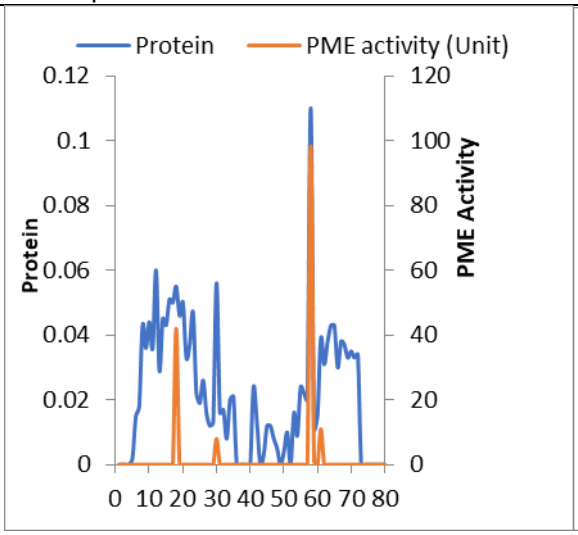


Figure 1a: Elution profile of Pectin methyl-esterase on Sephadex G 75

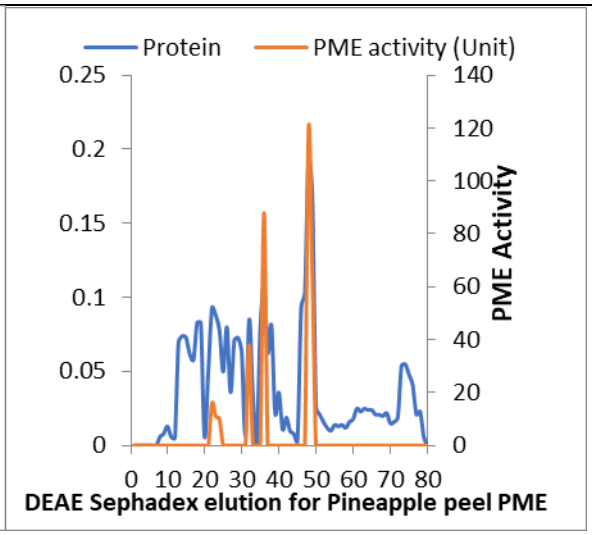


Figure 1b: Ion exchange chromatography of protein and Pectin methyl esterase activity from *Aspergillus niger*

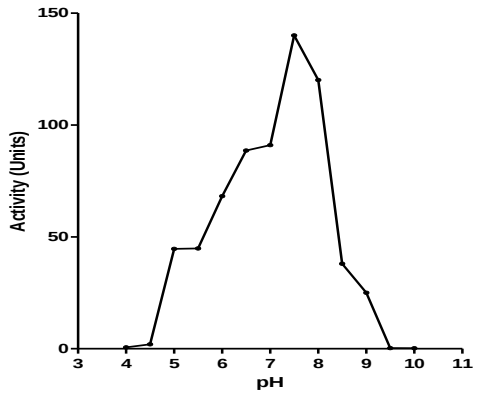


Figure 2a: Effect of pH on the purified pectin. methyl esterase activity of *A. niger*

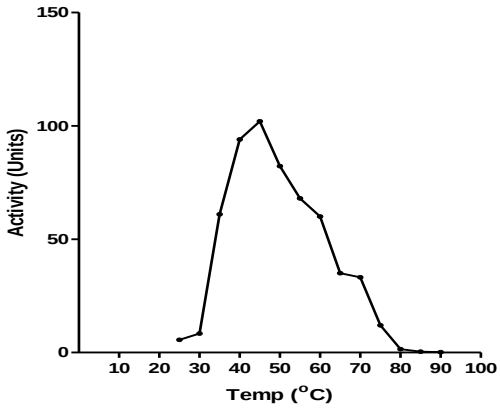


Figure 2b: Effect of temperature on the purified pectin methyl esterase activity of *A. niger*.

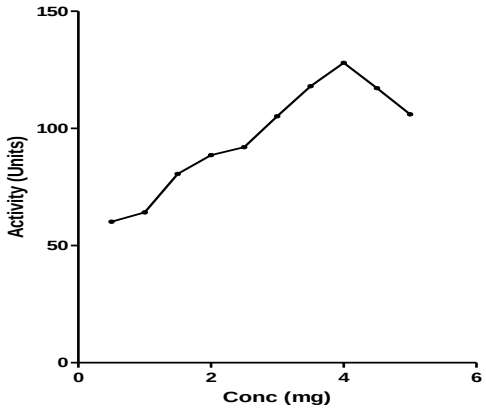
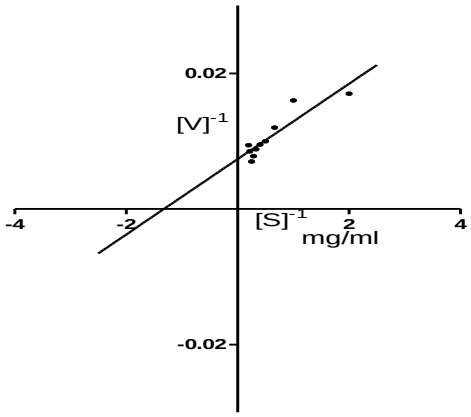


Figure 3a: Effect of substrate concentrations on the purified pectin methyl esterase



Km: 1.321mg/ml; Vmax: 0.005Mol/min

Figure 3b: Lineweaver-burk plot for the hydrolysis activity of pectin by the partially purified pectin methyl esterase activity of *A. niger.*

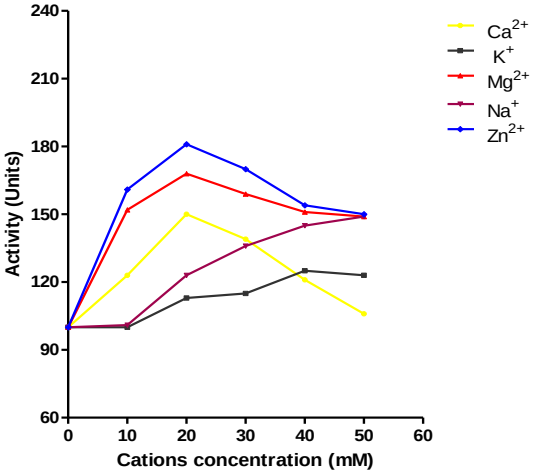


Figure 4a: Effect of Cations on purified pectin methyl esterase activity of *A.niger*

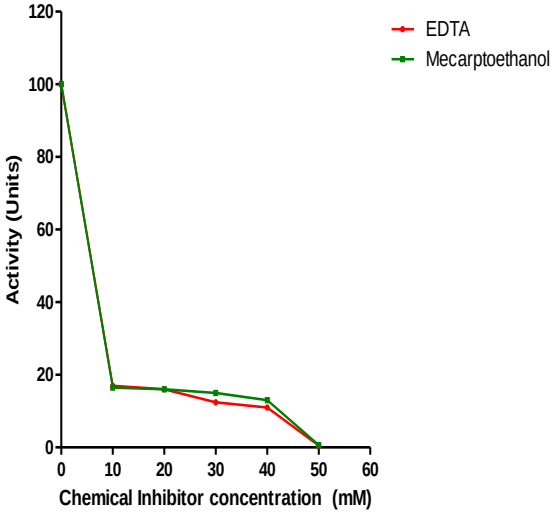


Figure 4b: Effect of chemical (inhibitors) on pectin methyl esterase activity of *A. niger*

There has been several scholarly information about the innate ability of *Aspergillus flavus* and *Aspergillus niger* and *Fusarium oxysporium* [22] Pectin methylesterases (PMEs: EC 3.1.1.11) which hydrolyzes pectin by demethylesterification of GalA C-6 producing methanol, protons, and polygalacturonate. The reaction helps in virulence factors which enhance pathogen invasion and spread through plant tissues [23-24]. PMEs along with other pectinolytic enzymes are widely used in the food and beverage industries and paper and fiber industries, among others.

PMEs work in concert with other pectinolytic enzymes, pectate lyases (EC 4.2.2.2), and pectate glycohydrolases (EC 3.2.1.15), among others, to depolymerize pectin. Highly esterified pectin is largely resistant to depolymerization [25]. In this present study the ion exchange chromatography of the purified enzyme has about 4.2-fold with a relatively high enzyme recovery of 9.5% as compared to the 1.5 and 14.8 % in that of the work of Lo and Perera, 2014. However, the optimization of the fermentation is expected to produce a better yield, Moreover the difference may be associated with the strain of microorganism used, the substrate employed as well as the analytical method used [26-27]. The ammonium sulphate-dialysate fraction on Sephadex G-75 gave four absorption peaks (Figure 1a). The major peak was tube number 58 component of A. niger has a high pectin methyl esterase activity, while

other peaks had no PME activity. Further fractionation of the components of peak in tube number 48 of *A. niger* from the gel filtration on DEAE Sephadex also has similar number of distinct peaks of absorption shown as observed in (Figure 1b). Only the components of the second peak in tube number 48 exhibited pectin methyl esterase activity, while those other peaks lack such enzymatic activity.
There is an optimum pH at which enzyme catalyzes reaction at its maximum. Deviations or changes in pH from the optimum value led to decreased activity due to changes in the ionization of groups at the active site of the enzyme. Wide deviations in pH lead to the denaturation of the enzyme protein itself, due to interference with the many weak non-covalent bonds maintaining its three-dimensional structure. Enzyme activities are mostly dependent on changes with the pH of the reaction mixture which not only influence the enzyme activity, but also affect the Km and Vmax [27].

In this present study the optimal pH was found to be 7.5, however, the enzyme (PME) show catalysis at about pH 5.0 to 8.0, this study is in line with that of Arotupin et al., 2008 [18] which has the Optimum pH of 6.5. The difference in the pH may be due to the microorganism used in this work which is quite different from that used by Arotupin and his coworkers in 2008. In the work of Lo and Perera [28] the Pectin methyl esterase was found to be at its optimal activity at pH 8, compare to pH 7.5-8.0 from Arbaisah et al, [29]. This present study conforms to the work of Arbaisah *et al.,*[29] which purifies Pectin methyl esterase from soursop.
The rate of enzyme-catalyzed reactions is affected in two ways; a rise in temperature increases the thermal energy of the substrate molecules which leads to increases the rate of the reaction. Increasing the thermal energy of the molecules that make up the protein structure of the enzyme will increase the chances of breaking the multiple weak, non-covalent interactions that stabilizes three-dimensional structure of the enzyme. However, in this study the optimum temperature that has the highest activity was at 45^{o}C, whereas, 30^{o}C optimum temperature was reported by Arotupin *et al.,*[18] using *A. repens*, however the temperature of 50^{o}C is recorded in the work of Silva *et al*., [30] which uses *Penicillum viridactum* as well as other that *A. niger* was used. This result suggests that PME from *A. niger* in this present study which is able to work stably at temperature of temperature range of 35^{o}C to 60^{o}C which makes it a very good source of PME for wide range of industrial application.
Substrate concentration has a great effect on the activity of PME as observed in this study. However, maximum substrate concentration was observed at 4mg/ml and it became saturated and decline at a higher substrate concentration. The work of other researchers [30-32] confirms this with an increase in activity of both polygalacturonase and pectin methyl esterase upon increase in concentration of pectin, this increased activity with increase in substrate concentration may be attributed to the effective binding of the substrate to the active site [32]. This agrees with the work of Arotupin *et al* [18]. The apparent Km value pectin hydrolysis fall within the range reported by Ajayi *et al.* [31]. The Km is very low with the value of 1.321 mg/ml with Vmax of 0.005mol/min which indicates that the enzyme has strong affinity for the pectin substrates.

Metal ion form an integral part of the active enzyme most of the time or it may combine with the substrate to give the true substrate of enzyme, it however act as a co factor for enzyme catalysis [33-35] the present study shows that PME is activated by metal ions. The concentrations of cations used in this study exerted their effects on pectin methyl esterase activity. In this work, specific concentrations of cations were activator, while others were inhibitory to enzyme activity. It has been reported that cations such as Ca^{2+} ions do activate the PME enzyme [35]. Other reports also, have shown that activation of PME by cations is related to the competitive displacement of PME bound to blocks of carboxylic groups on pectin. Thereby PME becomes free for further catalysis of pectin [34-37]
However, the activation of pectin lyases activities by K^{+}, Mg^{2+}, and Ca^{2+} have been documented [37] where calcium and magnesium acts as activator of PME which can be seen in the rapid increase in enzyme activity on addition of the metals at different concentrations. The enzyme is more active at a higher metal concentration compared with a lower concentration. It is noteworthy that the concentrations of EDTA, and Mecarptoethanol employed in this study were inhibitory to the activity of Pectin methyl esterase (PME) produced by *A. niger* it has been documented by Obineme *et al,* [38] which reported that EDTA may act by chelating Ca^{2+} with a resultant loss of catalytic activity of the enzyme.
In this present study, *A. niger* Pectin methyl esterase (PME) grown on pineapple residue in solid state fermentation has shown activity that makes it a tool to be used in beverages like coffee mix production,

production of tea, processing of textile fibers and in the treatment of paper pulp and as well as pretreatment of waste from vegetable food processing.

CONCLUSION

The study shows that PME activity is higher in the purified enzyme sample with an optima pH and temperature of 7.5 and 45^{o}C respectively. The enzyme has a low Km value of 1.321 and Vmax of 0.001mol/min with high affinity for the substrate pectin. The enzyme activated by Mg^{2+} and Ca^{2+}, Zn^{2+} Na^{+}, K^{+} (cations) at different concentration dependent manner and inhibited by EDTA and Mecarptoethanol. Hence the PME from *A. niger* is a reliable source for industrial exploration.

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COMPETING INTERESTS

No competing interest.

AUTHORS' CONTRIBUTIONS

'FOO designed the study and performed the experiment with OSN, OOO proof read the draft manuscript and perform the statistical analysis AOB reviewed the initial and final manuscript while author EBO conceptualize the research. All authors read and approved the final manuscript."

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