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Purification, Characterization and Application of Polygalacturonase from *Aspergillus niger* CSTRF

Arotupin Daniel Juwon*, Akinyosoye F. A. and Onifade Anthony Kayode

Department of Microbiology, Federal University of Technology, PMB 704, Akure, Nigeria
E.mail: daniel_juwon@yahoo.com

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ABSTRACT

Aims: The research was carried out to study the purification, characterization and application of polygalacturonase from *Aspergillus niger* CSTRF.
Methodology and Results: The polygalacturonase (PG) from the fungus was purified by ammonium sulphate precipitation and dialysed. The resulting fraction of the enzyme was further separated by molecular exclusion and ion exchange chromatography. The enzyme was purified 28.19 fold with a yield of approximately 69 % following purification with SP C-50. It has a relative molecular weight of 79,430 daltons and markedly influenced by temperature, pH and substrate concentrations of reactions with optimum activity at 35 °C, pH 4.0 and 8 mg/mL respectively. The PG was heat stable over a broad range of temperatures. Line weaver-Burk plot for the apparent hydrolysis of pectin showed approximately Km value of 2.7 mg/mL. The activity of the enzyme was enhanced by Na^{+}, Ca^{2+}, Mg^{2+} and Zn^{2+}, while EDTA, $PbCl_2$, $HgCl_2$ and IAA were inhibitory. The ability of the purified enzyme to clarify fruit juice was also investigated.
Conclusion, significance and impact of the study: This study revealed that polygalacturonase possesses properties for clarification of fruit juice and by extension bioprocessing applications.

Keywords: Polygalacturonase, molecular exclusion, dialysed, bioprocessing, purification

INTRODUCTION

Polygalacturonases otherwise referred to as pectic hydrolyases are pectic enzymes that hydrolyse pectic substances into their monomeric units. They are known to be responsible for the hydrolytic cleavage of the B-1, 4-glycosidic of the galacturonan, moiety of pectic substances (Rose, 1980; Forgarty and Kelly, 1983). However, extracellular polygalacturonases catalyses the hydrolytic cleavage of terminal X-1, 4-glycosidic bonds (pectic acid) releasing galacturonic acid as the main product (Kluskeus *et al*., 2005).

Polygalactuonases appeared to be the most frequently encountered pectic enzymes. They are formed in the majority of plant tissues particularly in ripening fruits. Also many plant pathogenic and saprophytic microorganisms produced polygalacturonases (Parvateesam and Verma, 1992; Ajayi *et al*., 2003).Most fungi that exhibited pectolytic activity produce polygalacturonases either as sole pectic enzymes as observed in *Sacchromyces fragilis* (Phaff, 1966), or in association with either or both pectinmethylesterases and pectinlyase (Odutola and Ikenebomeh, 1997). The critical role of these enzymes in the degradation of the host middle lamella and cell walls, leading to plant tissues merceration and cellular death had been documented (Peter *et al*., 1990; Ajayi *et al*., 2003; Chuku *et al*., 2005). The infection of cotton seedlings by *Rhizoctonia solani*, soft rot yam and sweet potato, brown rot of apple by *Monilinia* species and deterioration of tomato by *Botryoplodia theobromae* Pat have been reported to produce polygalacturonases and other cell wall degrading enzymes (Bateman and Miller, 1966; Olutiola *et al*., 1982; Weeransinghe and Naqui, 1985; Shamar and Kaul, 1985; Odutola and Ikenebomeh, 1997; Ajayi et al., 2003). The culture filterates of *Collectotrichum lagnarum* exhibited polgalactuonase activity (Mills and Wood, 1985).

In addition, the culture filterates of *Collectotrichum lindermathianum* in vitro (two constitutive),*Phytophthora infestans, Fusarium solani, F. oxysporium* and *Cochlobus carbonum* were reported to contained polygalacturonases (Barth *et al.,* 1981; Jarvis *et al*., 1982; Perez-Artes and Tena, 1989; Walton and Cerone, 1990). Other fungi reported to have produced polygalacturonases in-vitro included *F. moniliforme* (Caprari *et al*., 1993; Niture and Pant, 2004; Fontana *et al*., 2005), Coryphonetia parasitica (Gaoand Shain 1994), *Collectotrichum lindermathianum* (Hugouuvienx *et al*., 1995), *Aspergillus* species (Solis *et al*., 1996; Fontana *et al*., 2005; Debing *et al*., 2006) and *Phoma sorghina* (Akiyosoye and Oboh, 2004).

Pectolytic enzymes of fungal origin (Alves *et al*., 2002; Akinola and Onaolapo, 2003) have long been used for the extraction, clarification and depectinization of juices (Alkorta *et al*., 1998; Silva *et al*., 2002). Most potential sources of polygalacturonases are usually from

*Corresponding author

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pathogenic fungi of plants and fruits. Therefore, sourcing for polygalacturonases from non-pathogenic microorganisms could ease off the pathogenic action of the polygalacturonase from its prime origin. Also, the adoption of the beneficial aspect of polygalacturonases in Nigerian industries is relatively very low. In view of these scenario, this work focused on the purification, characterization and application of polygalacturonase from *Aspergillus niger* CSTRF.

MATERIALS AND METHODS

Sources of organisms

The *Aspergillus niger* CSTRF used in this study was isolated from cultivated soils of Teaching and Research Farm of the Federal University of Technology, Akure, Nigeria. The organism was routinely grown and maintained according to Arotupin *et al*. (2008). The prelimnary screening of the organism for elaboration of polygalcturonase (PG) was carried out with the method of Kimural *et al*. (1993).

Preparation of medium and inoculation of fungus for the production of PG

The medium consisted of 50 mL basal medium (Olutiola and Akintunde, 1979), in 250 mL conical flasks. Zero-point-five milliter spores of 48 h old culture of the fungus (Akinyosoye and Akinyanju, 1989), was used to inoculate the basal medium.

Polygalacturonase assay

Polygalacturonase activity of the culture filterates resulting from the fungal growth was assayed by the method of Miller (1959). The reaction mixture consisted of 1 mL of 1.2 %w/v pectin in 1 mL of 0.1M citrate –phosphate buffer of pH 5.0 and 1 mL of culture filterate (crude enzyme solution). Control experimental tubes contained the same amount of substrate and 1 mL of culture filterates and the mixture was boiled for 20minutes. Both the experimental and control tubes were subsequently incubated at 35 °C for 3 h. The amount of reducing sugars released into the reaction mixture was determined by using 3, 5-dinitrosalicylic acid (DNSA) reagent. One unit of PG activity was defined as the amount of enzyme in 1 mL that would liberate reducing sugars equivalent to 1 mg galacturonic acid per minute under the specific conditions of reaction.

Precipitation and dialysis of enzyme

The crude enzyme was subjected to ammonium sulphate precipitation (Olutiola and Akintunde, (1979) and dialysed using acetylated cellophane tubing from Visking tubing (Gallenkamp) (Whitaker *et al*., 1963). The protein content was determined according to the method of Lowry *et al*. (1951).

Caliberation of Sepadex G-100 chromatography

A vertical glass tube chromatography column 1.5x70 cm (Pharmacia Sweeden) was used. Sephadex G-100 was employed for the fractionation of the enzyme. The packedcolumn was eluted and caliberated with proteins of known molecular weights (Arotupin *et al*., 2008).

Application of the enzyme to sephadex G-100 column

The dialysate enzyme concentration (10 mL) was applied to the eluted column as described by Olutiola and Ayres (1973). Five milliters fractions were collected and protein content determined at 280nm. Fractions with proteins were subsequently assayed for PG and protein as earlier described.

Fractionation of enzyme by ion-exchange chromatography

The Sephadex C-50 column was prepared like Sephadex g-100 column and packed in a shorter column (2.5x40 cm Pharmacia Sweeden). Ten-milliters of the pooled enzyme concentrate was applied to the eluted column. Five-milliters fractions was collected and assayed for PG and protein content.

Effect of physicochemical factors on purified enzyme

The effects of temperature, heat (temperature) stability, pH, substrate concentration and chemical agents on enzyme activity were determined by the method of Arotupin *et al*. (2008).

Clarification of pineapple juice

The preparation was carried out with a slight modification of the method of Akinola and Onaolapo (2003). The method involved pulping of 20 kg pineapple fruit in a pulper (240 mm x 820 mm). The pulp was hydrolysed in 500g per batch of pulp using enzyme concentrations of 0 %, 20 %, 40 %, .60 %, .80 %, 1.0 % v/w at 25 °C for 10 min, 30 min, 60 min, 90 min and 120 min. The total solid content (Brix) of resulting juice was determined with hand refractometer, % acidity was determined as anhydrous citric acid and yield of free flow juice was determined on percentage basis (AOAC, 1984).

RESULTS AND DISCUSSION

The fungus *Aspergillus niger* CSTRF, when grown on mineral medium containing pectin (Sigma) produced polygalacturonase. The production of the PG into the culture filterates of the fungus confirmed the hydrolysis of pectin leading to the production of PG. Arotupin *et al*. (2008) had earlier reported the hydrolysis of pectin in the culture medium of *A. repens*. Similarly, the hydrolysis of pectin as the main carbon source in culture media by *A. flavus* and *A. niger* had been documented (Fredrick *et al*., 1990; Casida, 1997). Also, the ability of *A. flavus* to grow

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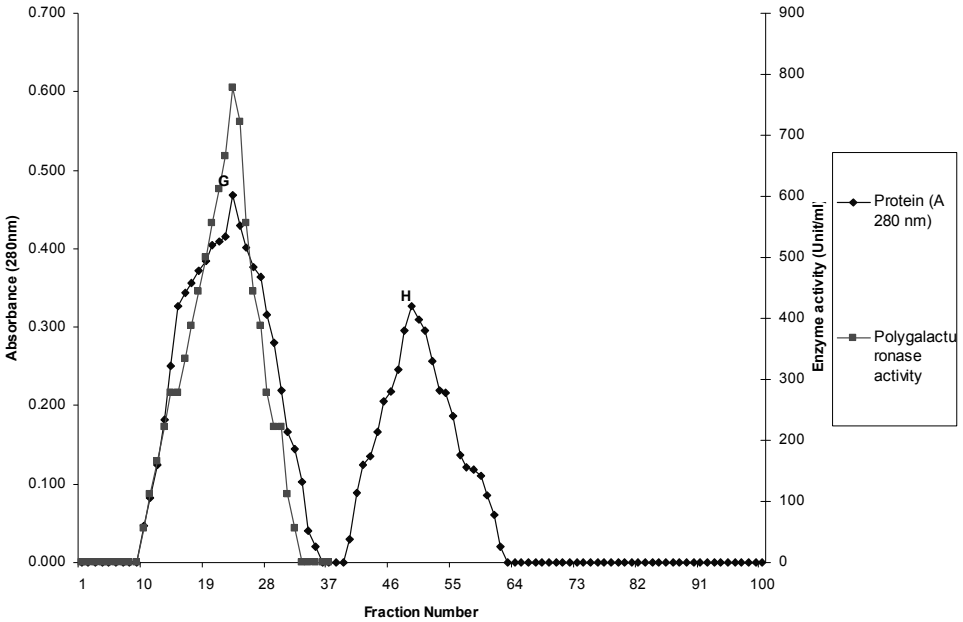

Figure 1: Gel (G-100) separations of proteins in concentrated extract of *A. niger* and enzymatic activity of the fractions

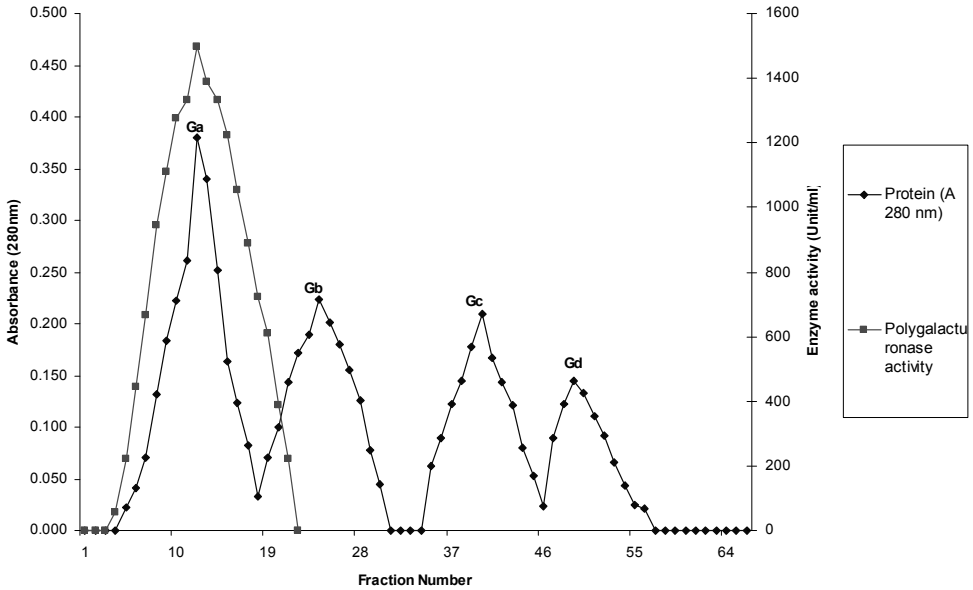

Figure 2: Separation by ion exchange chromatography (Sp Sephadex C-50) of proteins extracted from *A. niger* separated by gel filtration and enzyme activity of the fractions

on carbon substrates including pectin had been reported as an indication of its utilization by the fungus (Arotupin, 2007). The production of PG by *Aspergillus, Fuasrium, Penicillium, Thermoascus, Lentinus* species on various substrates during solid substrate fermentation and submerged fermentation (Favela-Torres *et al*., 2006) are strong evidences of the hydrolysis of pectin and pectin containing materials for the growth of the fungi.

Purification of polygalacturonase from *Aspergillus niger* CSTRF

Figure 1 showed two absorption peaks designated as G and H respectively by the ammonium-dialysate fractions on Sephadex G-100 column. The G component which was the major peak of *A. niger* presented PG activity, while peak H had no PG activity. Furtherance to the

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Table 1: Purification of polygalacturonase (PG) from *Aspergillus niger*

Enzyme	Fraction	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification	Yield
PG	Crude	10800	3670	2.94	1	100
	$(NH_4)_2SO_4$	9200	966	9.52	3.24	85.19
	G-100	7777.8	140	55.56	18.90	72.02
	SP C-50	7500	90.5	82.87	28.19	69.44

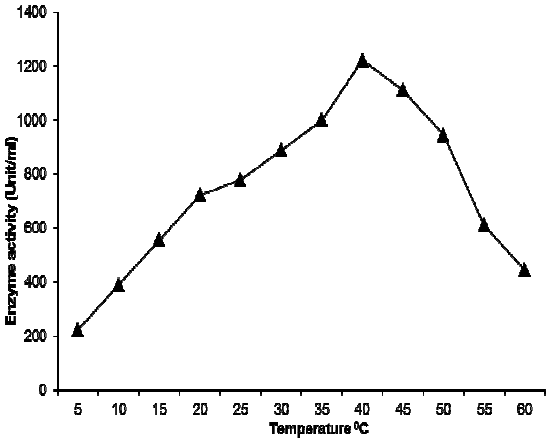

Figure 3: Effects of temperature on the purified (sp C50 fraction) polygalacuronase activity *A. Niger*

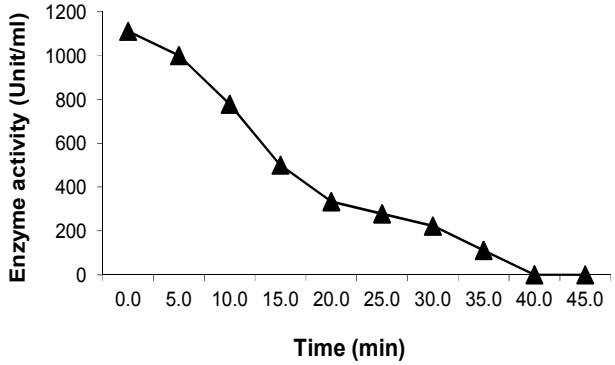

Figure 4: Effect of duration of heating (60 C) on purified (sp C-50 fraction) polygalacturonase activity of *A. niger*.

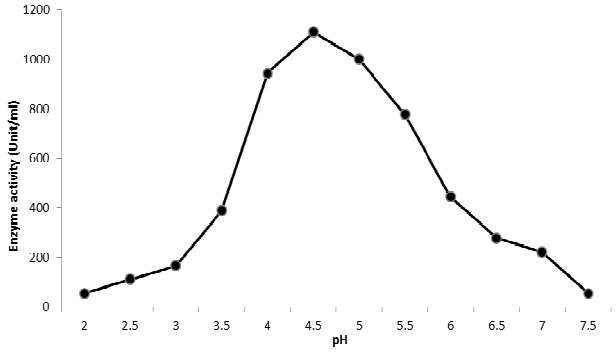

Figure 5: Effect of pH on the purified (sp C-50 fraction) polygalacturonase activity of *A. niger*

fractionation of the components of peak G of *A. niger* on Sp sephadex C-50, four peaks of absorption were displayed as Ga, Gb, Gc and Gd respectively. However, only component Ga exhibited PG activity, while other peaks lack enzyme activity (**Figure 2**). **Table 1** showed approximately 28 purification fold and 69 % yield of the enzyme fractions on ion exchange chromatography. The fractionation of the ammonium sulphate-dialysate fractions on Sephadex G-100 tend to point to the pattern of synthesis of PG. The position of the enzyme on the chromatography suggested that only PG1 was produced during growth.

However, Silva et *al*. (2002) suggested several peaks of PG activity to be PGI, PGII and PGIII in order of production. In this study, the production pattern of PG could be described as non sequential. Sequential production of pectinases-PG had been reported by various authors for different microorganisms (Crotti *et al*., 1999; Soares *et al*., 1999; Silva *et al*., 2002). The PG molecular weight from *A. niger* was approximately 79,430 daltons. Jayani *et al*. (2005) reported low molecular weights PGases ranging from 35 to 496 daltons for different species of fungi. Of all the reasons adduced by Oyede (1998) for the differences in molecular weights, nature and type of organisms used, substrates employed and analytical methods in addition to the monomeric units of the polypeptides could justify the differences.

Characteristics of purified enzyme (Temperature optimum)

The highest PG activity was observed at 40 °C (**Figure 2**). Also, *A. niger* PG lost approximately 10 % of activity when heated for 5 min. However, the enzyme retained approximately 10 % of activity on heated for 35minutes (**Figure 3**). The results indicated that maximum quantity of PG activity was considerably influenced by temperature. The optimum temperature for PG activity of *A. niger* was 40 °C after which there was a decline in the enzyme activity. This clearly showed that higher temperatures affected the activity of the enzyme. Similar observation had been reported by Palaniyappan *et al*.(2009). Also, PG produced by *A. flavus, A. fumigatus* and *A. repens* exhibited maximum activity at 35 °C, 40 °C and 45 °C respectively (Arotupin, 2007). Olutiola (1982) observed temperature of 35 °C as optimum for PG from *Penicillium citrinum*. Barthe *et al*. (1981) and Yoon *et al*. (1994) documented temperature of 40 °C for the maximum PG activity from *Collectotrichum inidermathianum* and *Gernoderma incidum*. This same temperature optimum was implicated for the PG obtained from *A. niger, Botryodiplodia theobromae* and *Penicillium variabile*

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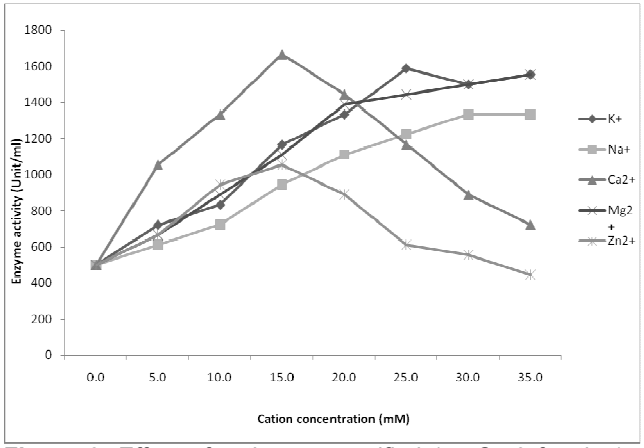

Figure 6: Effect of cations on purified (sp C-50 fraction) polygalacturonase activity of *A. niger*

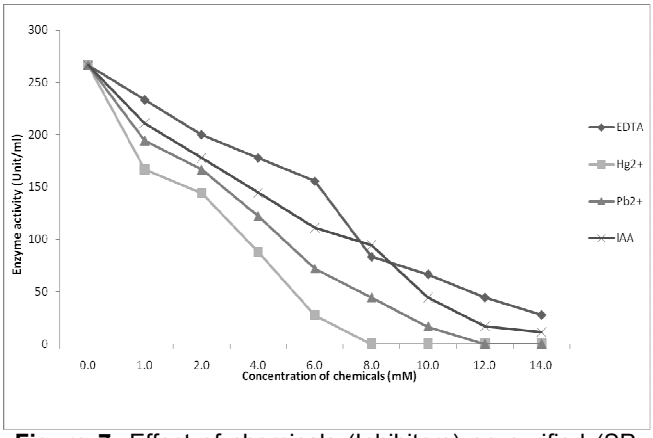

Figure 7: Effect of chemicals (Inhibitors) on purified (SP C-50 fraction polygalacturonase activity of A*. niger*

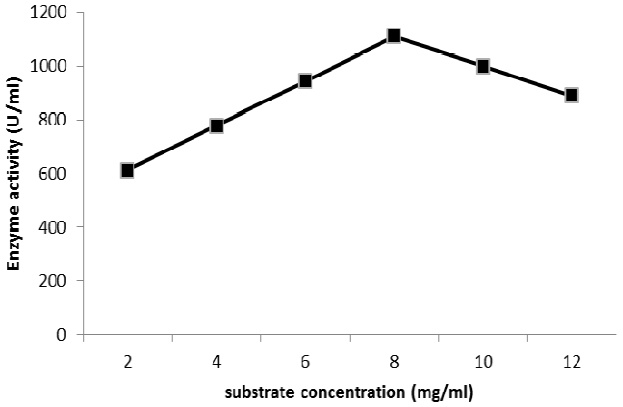

Figure 8: Effect of substrate concentration on purified (SP C-50 fraction) polygalacturonase activity of *A.niger*

(Oyede, 1990), *Botryodiplodia theobromae* Pat (Ajayi *et al*. 2003). A slightly higher temperature of 42 °C was reported for *Rhizoctonia solani* PG, while temperature optima of45 °C to 50 °C were reported for PG from *Ventaria inequalis* and *A.alliaceus*. The variations in the temperature optimum of PG of these fungi suggest a broad range of temperature tolerable by the enzyme. In addition, the nature, sources and differences in the physiological activities of the fungi may be responsible for these observations (Arotupin, 1991).

Temperature stability

The fairly stable nature or the PG from *A. niger* at 60 °C for 35 mins is of great significance in biotechnological processes (Favela Torres *et al*., 2006). Percentage loss of activity of PG obtained from *A. niger, Botryodiplodia theobromae* and *Penicillium variabile* heated at 70 °C have been reported (Oyede, 1998; Ajayi *et al*., 2003). Daniel *et al*. (1996) also reported thermal inactivation of enzymes at high temperature. In addition, extremely high temperature could lead to deamination, hydrolysis of the peptide bonds, interchange, and destruction of disulphide bonds and oxidation of the amino acids side chains of the enzyme protein molecules (Creighton, 1990; Daniel *et al*., 1996).

pH optimum

Maximum activity at pH 4.50 was attained by PG of *A. niger* (**Figure 4**). The moderately acidic nature of the *A. niger* PGase in this study corroborated the influence of pH on PGases activity. At certain pH, the activity of PGase was at maximum and changes in pH either to higher or lower values cause significant lowering of the enzyme activity. However, since catalytic power is often confined to a relatively small range of pH, it is probable not unlikely that only one of the ionic forms or rather the active site of the enzyme is catalytically active (Dixon and Webbs, 1971). Dixon and Webbs (1971) and Conn and Stump (1989) separately reported that changes in pH have an effect on the affinity of the enzyme for the substrate.

Effect of cations (salts)

The maximum PG activity occurred at concentration 35 mM of K^+ and Mg^{2+} and at 30 mM of Na^+, while concentration of 15 mM Ca^{2+} and Zn^{2+} accounted for maximum PG activity of *A. niger* (**Figure 5**). In this study, certain concentrations of cations were stimulatory, while others were inhibitory to the PG. The concentrations of Ca^{2+} and Zn^{2+} beyond 15 mm were inhibitory. Notably, concentrations of K^+, Na^+ and Mg^{2+} were stimulatory to PG activity of *A. niger*. Oyede (1998) reported the stimulatory role of K^+, Na^+ and Mg^{2+} on PG activity from *Penicillium variabile*, while concentrations of ca+ beyond 15mM inhibited the enzyme activity. Also Ajayi *et al*. (2003) reported the stimulatory action of the increased concentrations of Ca^{2+}, Mg^{2+}, Zn^{2+} and K^+ on PG activity produced by *Botryodiplodia theobromae* Pat with Zn^{2+} concentration above 10 mM being inhibitory. These degrees of stimulation and inhibition could be a function of the sources of enzyme from different mould genera. Also in a situation where concentrations employed stimulated enzyme activities, the highest concentrations are not

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Table 2: Effect of PG concentrations on pineapple juice characteristics

Juice characteristics	Time (mins)	Concentration of PG (%)				
		0.2	0.4	0.6	0.8	1.0
Brix(TSS)	0	4.90a	4.90a	4.90a	4.90a	4.90a
	10	4.90a	6.60b	7.00b	7.60b	9.60b
	30	7.00b	7.90c	8.60c	10.00c	11.00c
	60	8.60c	10.20d	10.60d	11.6d	12.80d
	90	10.00d	10.50d	11.70de	12.90e	13.30d
	120	10.60d	11.90e	12.20e	13.20e	13.50d
% Acidity	0	0.24a	0.24a	0.24a	0.24a	0.24a
	10	0.24a	0.38b	0.56b	0.62b	0.63b
	30	0.44b	0.59c	0.69c	0.74bc	0.76c
	60	0.58c	0.66cd	0.78cd	0.80cd	0.76c
	90	0.66cd	0.78de	0.83d	0.89d	0.88c
	120	0.72d	0.83e	0.86d	0.92d	0.88c
pH	0	3.07a	3.07a	3.07a	3.07a	3.07a
	10	3.11ab	3.49b	3.69b	3.71b	3.72b
	30	3.52b	4.27c	4.67c	4.87c	5.12c
	60	4.07c	4.97d	5.07c	5.32d	5.49c
	90	4.67d	5.32de	5.67d	5.87e	5.97d
	120	5.47e	5.69e	5.97d	6.07e	6.08d
Juice yield	0	67.67a	67.67a	67.67a	67.67a	67.67a
	10	67.67a	69.67a	79.67b	81.67b	81.67b
	30	75.67b	77.67b	87.67c	89.67c	87.67bc
	60	77.67b	79.67b	93.67cd	93.67c	87.67bc
	90	77.67b	79.67b	95.67d	95.67c	89.67c
	120	79.67b	79.67b	95.67d	95.67c	89.67c
Pectin content	0	0.28b	0.28d	0.28d	0.28d	0.28c
	10	0.31b	0.22c	0.17c	0.15c	0.13b
	30	0.29b	0.19bc	0.12b	0.10b	0.09a
	60	0.23a	0.15ab	0.09b	0.06ab	0.04a
	90	0.21a	0.11a	0.03a	0.02a	0.03a
	120	0.19a	0.11a	0.03a	0.02a	0.03a

*Numbers with the same letter among the juice characteristics of each concentration of PG were not significantly different at $p<0.05$ and $n=3$.

necessarily the best for maximum activation as earlier reported to cause reduction in enzyme activation and even lead to enzyme inhibition (Ng and Peikus, 1981; Dudchenko, 1986). Murray *et al*. (1990) showed the formation of a chelate compound between the substrate and metal ions could form a more stable metal-enzyme-substrate complex and stabilizing the catalytically active protein conformation. Added to this could be that metal ions which could not activate directly could act indirectly by ensuring that the true activating metal ions combine only with the active centre. Brown and Kelly (1993) affirmed the ability of metal ions often acting as salt or ion bridges between two adjacent amino acids.

All the concentrations of EDTA, Hg^{2+}, Pb^{2+} and IAA were inhibitory to the activity of polygalacturonase and even lead to complete loss of activity (**Figure 6**). This study is inconsonance with the reports of Whitaker (1972),

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Torchinsky (1981) and Ajayi *et al*. (2003) who reported that Hg^{2+} and Pb^{2+} reacted with protein sulphydryl group converting it to marcaptides as well as imidazole, carboxyl and peptide group. The resultant effects of which lead to the denaturation of disulphide by catalytic acid of Hg^{2+} and Pb^{2+}. Famurewa *et al*. (1993) and Sakamoto *et al*. (1994) attested the inhibitory activity of ETDA on enzyme. The metal building reagent like EDTA can inactivate enzyme either by removing the metal ions from the enzyme forming coordination complex, or by building inside enzyme as a ligand (Schmid, 1979). The changes in the reaction mixture occasioned by the IAA toward acidity could probably be responsible for the PG inhibition. Arotupin (1991) observed that changes in pH either to higher or lower values significantly inhibited enzyme activity.

Enzyme kinetics

The PG of *A.niger* attained maximum at 8 mg/mL and further increase in substrate concentration resulted in decline of the enzyme activity (**Figure 7**). The PG had an apparent Km value of approximately 2.7 mg/mL. The initial increase in the PG activity with increased substrate concentration until optimum was reached may be as a result of the effective binding of the substrate to the active site (Arotupin *et al*., 2008). This could be explained in term of the enzyme-substrate affinity cum the rate of the breakdown of the enzyme-substrate complex to form the products. The observed decline in the PG activity after the optimum could be due to the enzyme saturation and that, further increase in substrate concentrations resulted in over saturation of the enzyme without binding. In this situation, the substrate molecules crowd enzyme leading to the formation of ineffective enzyme-substrate complexes. A monomolecular competitive inhibition may occur leading to decline in activity. The apparent Km value of approximately 2.7 mg/mL indicated that the half active sites of the PG from *A. niger* could easily be filled. Therefore, this low Km value signified strong affinity of this enzyme for the substrate.

Clarification potentials of the purified enzyme

The effects of purified PG on the characteristics of pineapple juice are illustrated in **Table 2**. The highest yield of juice was observed at PG of 0.6 to 0.8 % v/w equivalents to 200 U/mL and 220 U/mL with total solid content of 12.20 and 13.20. The hydrolysis time was 90 mins and 120 mins with pH 6.10 and 6.20 respectively. However, the PG activity increased with time and concentration. The increase in the juice flow as a result of the treatment with the purified PG attested to the breakdown of tissues holding the juice. Enzymic activity strongly affected the polyphenols in the fruit, resulting in the discolouration of the fruit, but the flavour tends to improve with enzyme processing (Akinola and Onaolapo, 2003).

In conclusion, the *Aspergillus niger* CSTRF produced copious polygalacturonase whose characteristics could be employed in fruits and juice industries. Also, the juice clarification could be attained with the appropriate enzyme dosage.

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