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CHARACTERISATION OF PARTIALLY PURIFIED CELL WALL-DEGRADING ENZYMES: POLYGALACTURONASE AND CELLULASE FROM TOMATO FRUITS DEGRADED BY *ASPERGILLUS NIGER*

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ABSTRACT

Aspergillus niger is a soil saprobe and produces a wide array of hydrolytic and oxidative enzymes and cell wall degrading enzymes. An investigation on the various properties of partially purified polygalacturonase and cellulase enzymes extracted from tomato fruits deteriorated by *Aspergillus niger* was carried out in this study. The results obtained shows that temperature, pH and substrate concentration have a profound effect on enzyme activity. The molecular weights of the enzymes extracted also suggest that it may be species dependent.

INTRODUCTION

Plant cell walls are difficult to penetrate and degrade because of their complex chemical composition and physical structure. Several polymers, including cellulose and protein, are embedded in a matrix of highly branched polysaccharides which make up the plant cell walls (McNeil *et al*., 1984; Selvendran and O'Neill, 1987). A range of enzymes capable of degrading plant cell wall components are produced by plant pathogens (Riou *et al*., 1991). *Aspergillus niger* is a fungal pathogen and one of the most common species of the genus *Aspergillus*. *Aspergillus niger* (black mold), is a filamentous ascomycete and has the ability of fast growth and pH tolerance. It is most important cosmopolitan fungi associated with postharvest decay of different substrates (Pitt and Hocking, 1997; Perrone *et al*., 2007). This organism is a soil saprobe and it produces a wide array of hydrolytic, oxidative enzymes and cell wall degrading enzymes (Ajayi *et al*., 2007; Giovane *et al*., 1994) involved in the breakdown of plant lignocelluloses. These features of *Aspergillus niger* enables them to cause decay of various organic substances including fruits, vegetables, nuts, beans, cereals, herbs, wood and herbal drugs.

This study was therefore designed to extract cell wall degrading enzymes: polygalacturonase and cellulase from tomato fruits deteriorated by *Aspergillus niger*.

MATERIALS AND METHODS

Organism and culture

The isolate *Aspergillus niger* employed for this research work was from the culture collection of the Federal Institute for Industrial Research Oshodi (FIIRO). The organism was routinely grown and inoculated on potato dextrose agar slants. The organism was sub-cultured from the stock culture. A 96-hr-old culture of *Aspergillus niger* was used whenever it was needed.

Collection of Tomato Fruits

Two different types of tomato fruits were used for this research work, the Roma vf and the Ibadan local variety. Freshly ripe tomato fruits (the Roma vf and the Ibadan local variety) were surface sterilised with 10% v/v sodium hypochlorite solution for 15mins. The tomato fruits were properly rinsed with five changes of sterile distilled water to remove the residual effect of the sodium hypochlorite solution. The tomato fruits were bored with a cork borer (4mm) and the fungus introduced into them. Discs (4mm) obtained from the edge of a 96-hr-old culture of the organism served as the inoculums. The point of inoculation was sealed with molten wax and kept in a polythene bag to avoid contamination with other organisms. The control fruits were similarly treated except that sterile potato dextrose agar discs served as the inoculum. The tomato fruits were then transferred into sterilised bell jars. The experimental and control fruits were kept in different sterilised bell jars and were examined daily for deterioration and pH. The pH values were taken using the Jenway pH meter for ten days of incubation. The rims of the bell jars were sealed with Vaseline. Both the experimental and control tomato fruits were incubated at room temperature.

Extraction of the enzyme from the tomato fruits

The enzymes were extracted after ten days of incubation. This is because within ten days of incubation, the

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inoculated tomato fruits had collapsed extensively. The collapsed tomato fruits were weighed prior to enzyme extraction. The tomato fruits were ground to pulp and homogenised with liquid extractant (1:1 w/v) for 2min at 30sec intervals each. The extractant was 0.5M NaCl in 0.01M citrate phosphate buffer (pH 4.5) containing 5mM NaN_3 to prevent microbial contamination. The homogenate from each jar was clarified by passing it through filter paper (Whatman No.1). Each extract was analysed for cellulase and polygalacturonase. In addition to this, the pH, total reducing sugars and the protein content of the enzymes were determined.

Ammonium sulphate Precipitation

Ammonium sulphate (Analytical grade) was added to crude enzyme preparation to 90% saturation according to the method described in Encor biotechnology Inc (2012) and Wingfield (2001). The solution was kept at 4^{o}C for 24hr and the resulting precipitate was removed by centrifugation at 4000rpm for 15min. The precipitate was re-dissolved in a small volume of 0.05M citrate phosphate buffer, pH 4.5. The resulting solution was dialysed overnight against two changes of the same buffer. Dialysis was performed in acetylated cellophane tubing prepared from Visking dialysis tubing (Gallenkamp) as described by Whitaker *et al*. (1963).

Enzyme Assay

Polygalacturonase

Polygalacturonase activity was obtained by estimating the amount of reducing sugars released in the reaction mixture. The reaction mixture consisted of 1ml of 0.1%w/v pectin (Sigma) in 0.1M citrate phosphate buffer pH 4.5 and 0.5ml of the enzyme solution. The control tube contained the same amount of substrate and 0.5ml of the enzyme solution heated at 100^{o}C for 15min. Both the experimental and control tubes were incubated at 35^{o}C for 3hr. The amount of the reducing sugars released during the reaction was measured by the modified dinitrosalicylic acid reagent method of Miller (1959). One unit of polygalacturonase activity was defined as the amount of enzyme in 1ml of the reaction that liberated reducing sugar equivalent to 1µg glucose per minute under the specified conditions of the reaction.

Cellulase

Cellulase enzyme activity was obtained by measuring the amount of reducing sugar released in reaction mixtures the reaction mixture contained 1ml of 0.6% w/v carboxymethyl cellulase (Sigma) in 0.1M citrate phosphate buffer pH 4.5 and 0.5ml of the enzyme solution. The control tube contained the same amount of substrate and 0.5ml of enzyme solution boiled at 100^{0}C for 15min. Both experimental and control tubes were incubated at 35^{o}C for 3hr. The reducing sugar released into the reaction mixture was estimated by the modified dinitrosalicylic acid reagent of Miller (1959). One unit of cellulase activity was defined as the amount of enzyme in 1ml of the reaction that liberated reducing sugar equivalent to 1µg galacturonic acid per minute under the specified conditions of reaction.

The influence of temperature on the activity of enzymes was investigated using temperatures ranging from 20^{o}C-45^{o}C for 3 hours for the enzymes under investigation. The influence of pH on the activities of the enzymes was examined using substrates with pH ranging from 3.0 to 5.5. Citrate phosphate buffer (0.01M) was used to prepare substrate of pH 3.0, 3.5, 4.0, 4.5, 5.0, 5.5. Carboxymethyl cellulose (0.6%) and pectin (0.1%) of pH ranging between 3.0 and 5.5 were employed as substrates for the assay of cellulase and polygalacturonase enzymes respectively. The effect of various concentrations of substrate on the enzyme activities was determined. Carboxymethyl cellulose (pH 4.5) and pectin (pH 4.5) at concentrations of 0.02, 0.05, 0.10, 0.15, 0.20, 0.25 for pectin and 0.1, 0.2, 0.4, 0.6, and 0.8 for Carboxymethyl were prepared and used for polygalacturonase and cellulase assays respectively.

RESULTS AND DISCUSSION

Deterioration of tomato fruits

Ten days after the inoculation of freshly ripe tomato fruits with *A. niger*, the tomato fruits had deteriorated extensively and the infected fruits exhibited appreciable cellulase and polygalacturonase activities while the uninfected tomato fruits possessed only traces of polygalacturonase activity but lacked cellulase activity. The pH of the fresh tomato fruits were 4.41 and 4.21 for the Roma vf and the Ibadan local variety respectively while those of the infected tomato fruits were 4.06 and 4.11 for the Roma vf and the Ibadan local variety respectively. The pH values of the tomato fruits therefore decreased with days of incubation (Table 1).

Production of cell wall degrading enzymes

Extracts obtained from the deteriorated tomato fruits showed favourable cellulase and polygalacturonase activities. The extracts from the uninoculated tomato fruits showed no detectable enzyme activity showing that the enzymes obtained were of fungal origin.

Protein purification by SDS gel electrophoresis

The dialysed enzyme using SDS gel electrophoresis contained a single band with an estimated molecular weight of 96KDa for cellulase from both varieties of tomato fruits. A molecular weight of 38 KDa was obtained for polygalacturonase obtained from the Roma vf variety of tomato fruits infected by *Aspergillus niger* while a molecular weight of 52 KDa was obtained from the Ibadan local variety of tomato fruits (Fig. 17).

Table 1. pH of tomato fruits (*Lycopersicon esculentum*) with days of incubation.

Days of incubation	Roma vf variety of tomato fruits	Roma vf control	Ibadan local variety	Ibadan local variety control
0	4.21	4.19	4.23	4.20
1	4.40	4.21	3.94	4.29
2	4.49	4.04	4.23	4.02
3	4.93	4.41	4.99	4.38
4	4.57	4.26	4.49	4.14
5	4.28	4.19	4.54	4.21
6	4.14	3.89	4.21	3.98
7	3.94	3.90	4.46	3.87
8	4.18	3.87	4.26	3.78
9	4.06	3.67	4.11	3.76

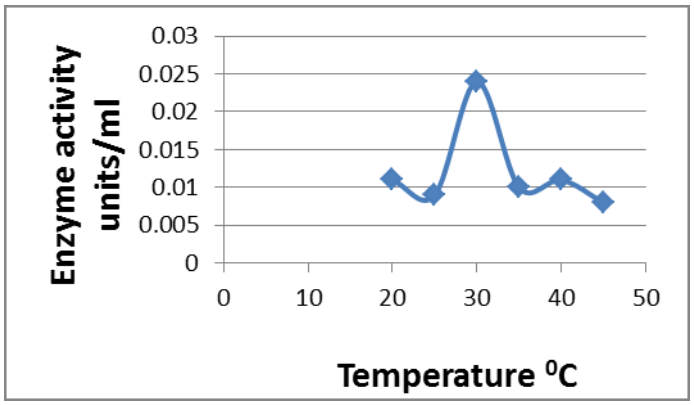

Fig. 1. Effect of temperature on the activity of partially purified polygalacturonase obtained from the Ibadan local variety of tomato fruits infected by *Aspergillus niger*.

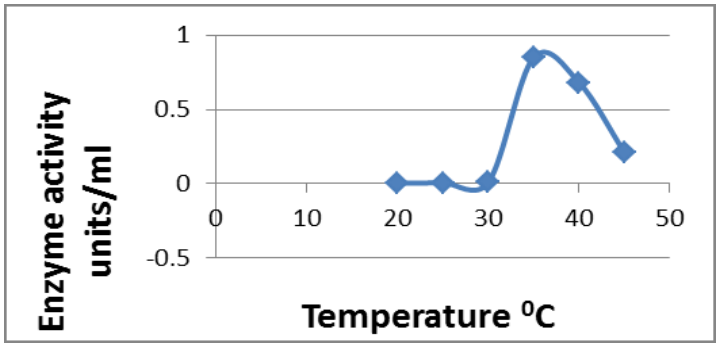

Fig. 2. Effect of temperature on the activity of partially purified cellulase obtained from the Ibadan local variety of tomato fruits infected by *A. niger*.

Properties of partially purified enzymes

The enzymes were partially purified by Ammonium Sulfate Precipitation and SDS PAGE. The polygalacturonase and cellulase were employed to determine the effects of certain factors on the activity of the enzymes.

Effect of Temperature

The effect of the temperature of incubation greatly affected the activities of all the enzymes gotten from the two varieties of tomato fruits. In all cases, the enzyme activity increased with increase in temperature of incubation until an optimum was reached. Subsequent

increase in temperature beyond the optimum led to a fall in enzyme activity (Figs. 1- 4).

Effect of pH on Enzyme Activities

The pH of the reaction mixtures remarkably affected the activities of all enzymes produced from both varieties of tomato fruits. The activities of all enzymes increased with increase in pH and gradually decreased after reaching the optimum ph. Maximum activity of the polygalacturonase produced by both varieties of tomato fruits was pH 3.5 (Fig. 5 and 7) while the optimum pH for the cellulase from both varieties was at pH 3.0 (Fig. 6 and 8).

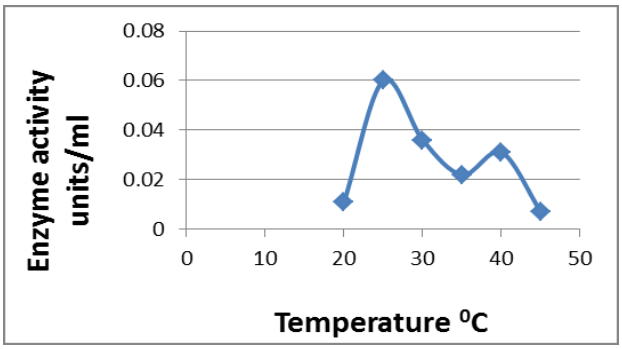

Fig. 3. Effect of temperature on the activity of partially purified polygalacturonase obtained from the Roma vf variety of tomato fruits infected by *A. niger*.

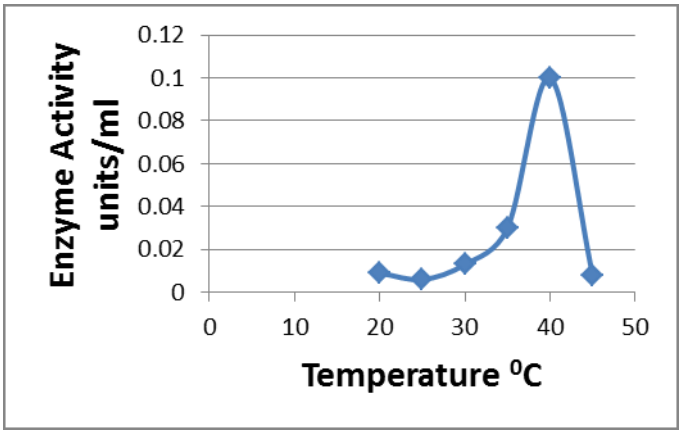

Fig. 4. Effect of temperature on the activity of partially purified cellulase obtained from the Roma vf variety of tomato fruits infected by *A. niger*.

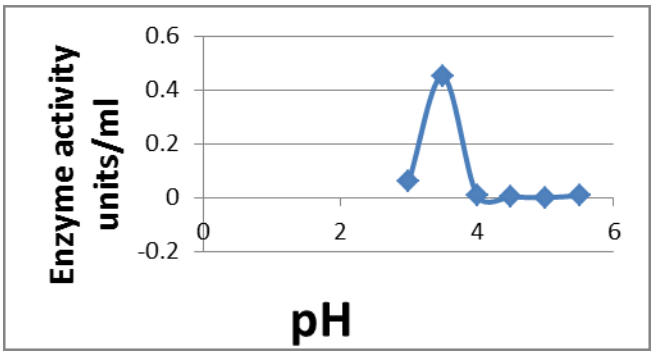

Fig. 5. Effect of pH on the activity of partially purified polygalacturonase obtained from the Ibadan local variety of tomato fruits infected by *A. niger*.

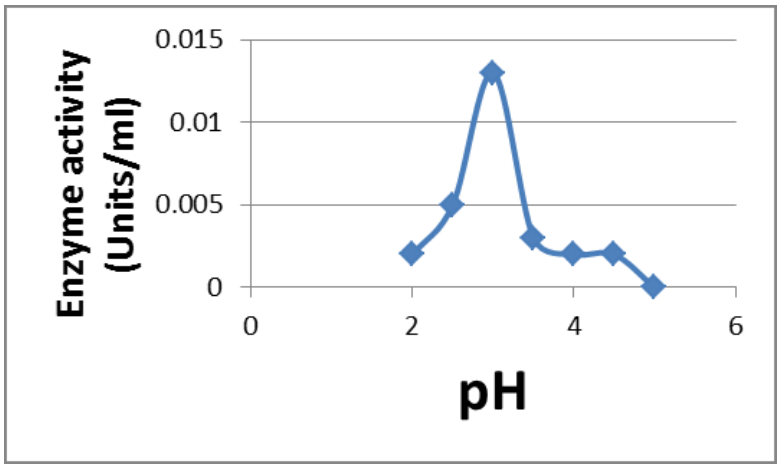

Fig. 6. Effect of pH on the activity of partially purified cellulase obtained from the Ibadan local variety of tomato fruits infected by *A. niger*.

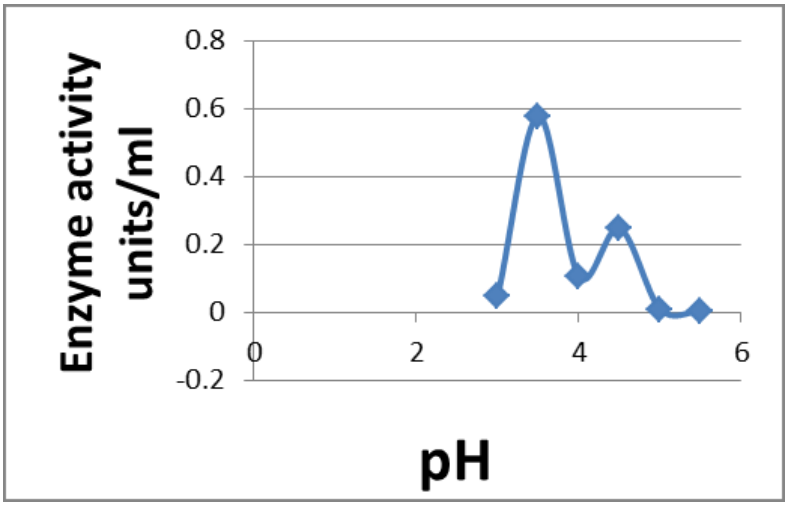

Fig. 7. Effect of pH on the activity of partially purified polygalacturonase obtained from the Roma vf variety of tomato fruits infected by *A. niger*.

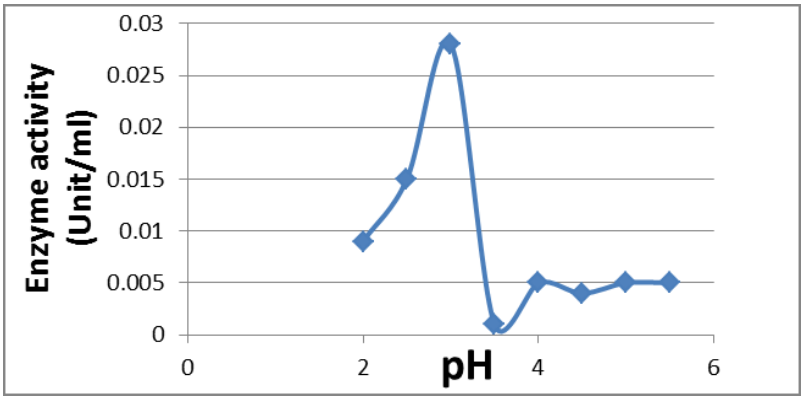

Fig. 8. Effect of pH on the activity of partially purified cellulase obtained from the Roma vf variety of tomato fruits infected by *A. niger*.

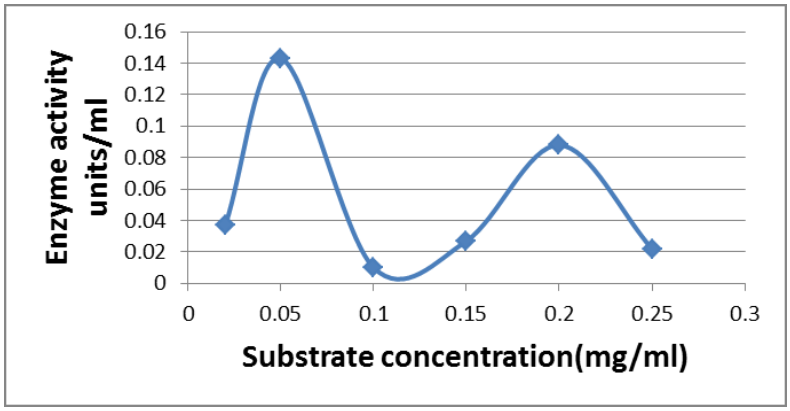

Fig. 9. Effect of substrate concentration (0.1% pectin) on the activity of partially purified polygalacturonase obtained from the Ibadan local variety of tomato fruits infected by *A. niger*.

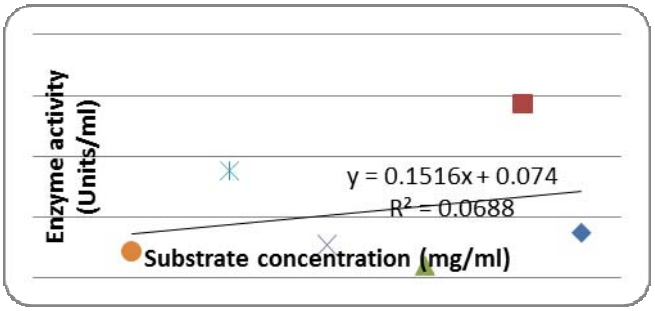

Fig. 10. Lineweaver-Burk plot for the hydrolysis of pectin by the partially purified polygalacturonase obtained from the Ibadan local variety of tomato fruits infected by *Aspergillus niger*

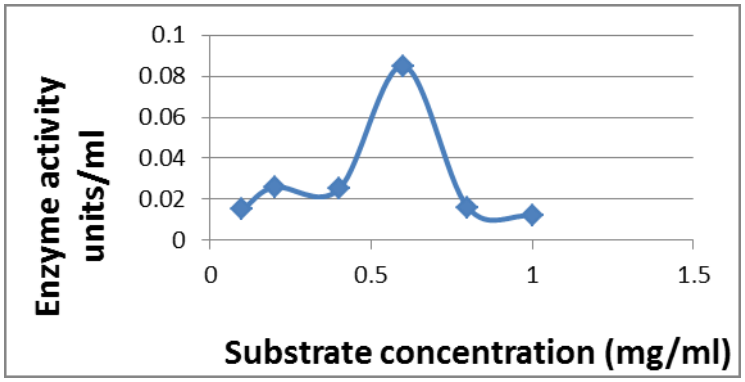

Fig. 11. Effect of substrate concentration (0.6% carboxymethyl cellulase) on the activity of partially purified cellulase obtained from the Ibadan local variety of tomato fruits infected by *A. niger*.

Effect of Substrate Concentration

The activities of all enzymes produced from the two varieties of tomato fruits increased with increase in substrate concentration until a maximum was reached above which there was no further increase in enzyme activity (Figs. 9, 11, 13 and 15). The Lineweaver-Burk plot for the hydrolysis of pectin by the partially purified polygalacturonase obtained from the Ibadan local variety of tomato fruits and the Roma vf variety (Fig. 10 and 14). The Lineweaver-Burk plot for the hydrolysis of carboxy methyl cellulose by the partially purified cellulase obtained from the Ibadan local variety of tomato fruits and the Roma vf variety (Fig. 12 and 16).

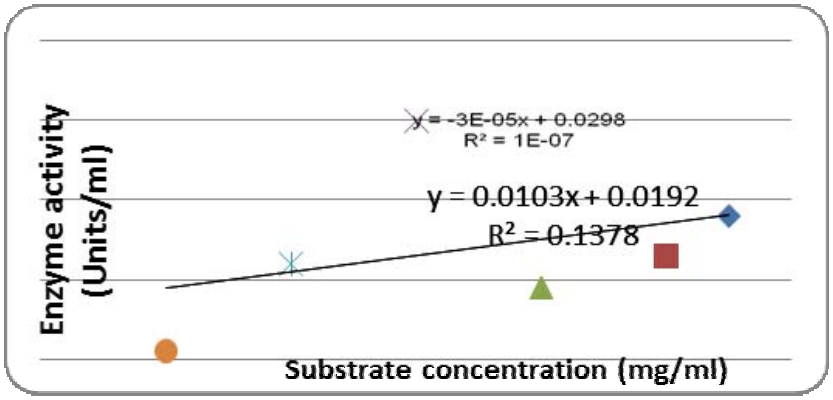

Fig. 12. Lineweaver-Burk plot for the hydrolysis of carboxy methyl cellulose by the partially purified cellulase obtained from the Ibadan local variety of tomato fruits infected by *Aspergillus niger*.

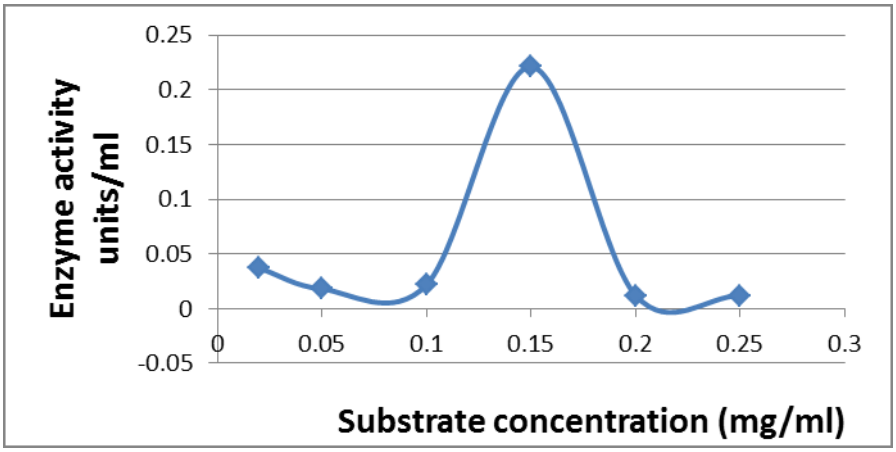

Fig. 13. Effect of substrate concentration (0.1% pectin) on the activity of partially purified polygalacturonase obtained from the Roma vf variety of tomato fruits infected by *A. niger*.

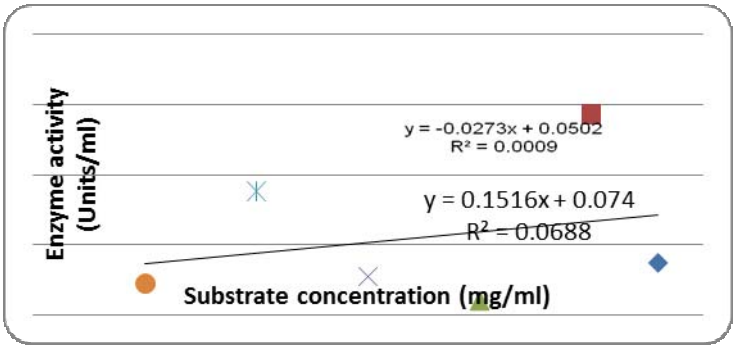

Fig. 14. Lineweaver-Burk plot for the hydrolysis of pectin by the partially purified polygalacturonase obtained from the Roma vf variety of tomato fruits infected by *Aspergillus niger*

In this investigation, *Aspergillus niger* was inoculated into freshly ripe tomato fruits for the production of cellulase and polygalacturonase enzymes. The results showed that appreciable polygalacturonase and cellulase activity occurred in extracts obtained from the two varieties of tomato fruits (he Roma vf and the Ibadan local variety) infected by *Aspergillus niger* are of fungal origin. This investigation agrees with Ajayi and Olasehinde (2009) who had earlier reported the decrease in the pH values of tomato fruits infected with *Aspergillus niger*.

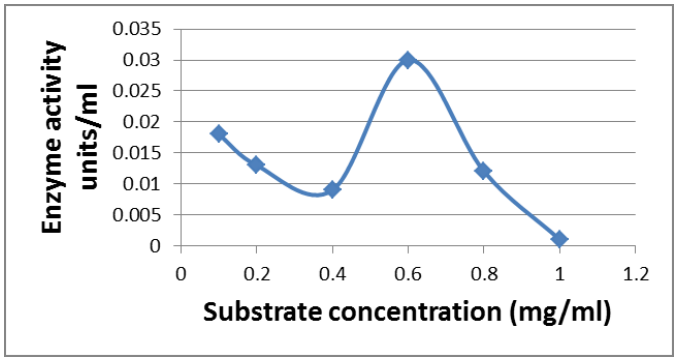

Fig. 15. Effect of substrate concentration (0.6% carboxymethyl cellulase) on the activity of partially purified cellulase obtained from the Roma vf variety of tomato fruits infected by *A. niger*.

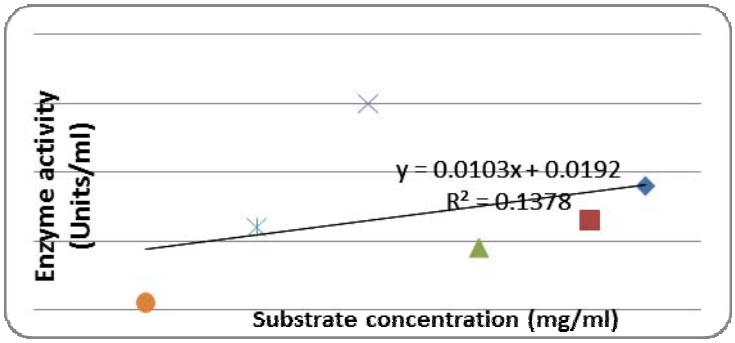

Fig. 16. Lineweaver-Burk plot for the hydrolysis of carboxymethyl cellulose by the partially purified cellulase obtained from the Roma vf variety of tomato fruits infected by *Aspergillus niger*.

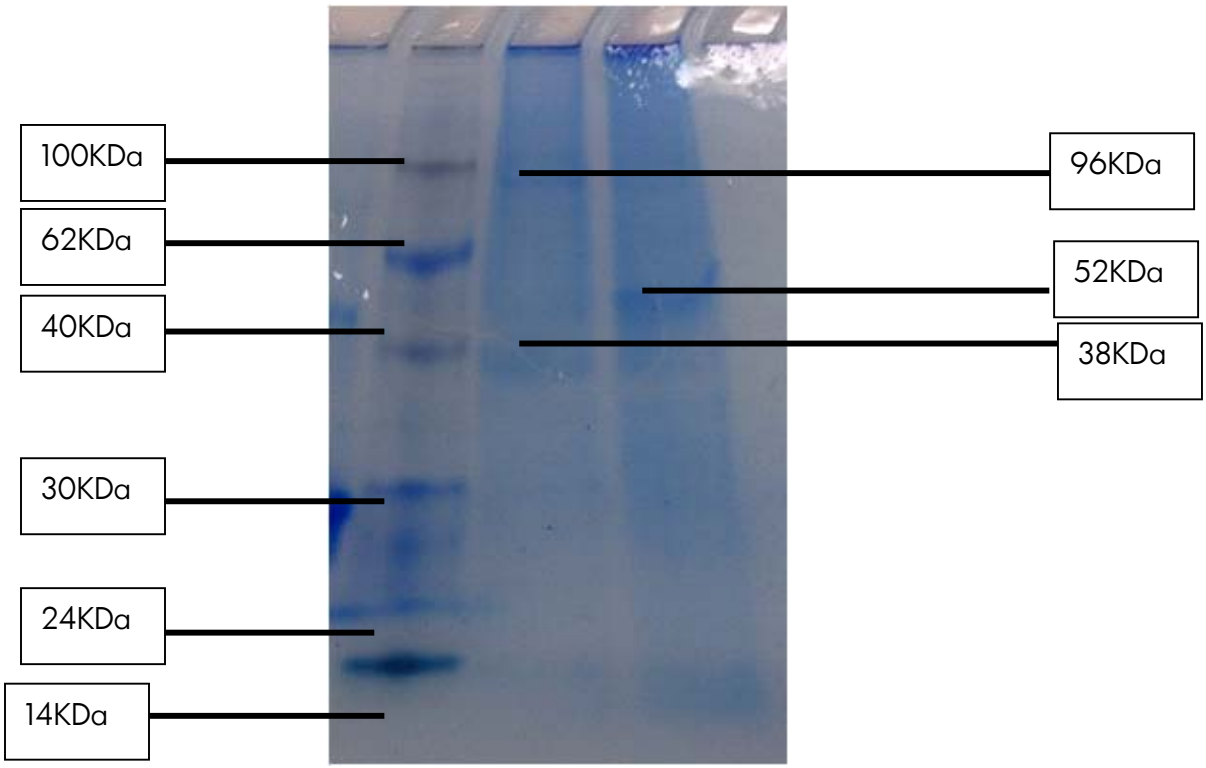

Fig. 17. Molecular weights of partially purified enzymes.

Aspergillus niger caused deterioration of the freshly ripe tomato fruits within ten days of incubation. It has been reported from previous researches that during this period of deterioration, the organism causing the deterioration secretes proteins which exhibited polygalacturonase and cellulase activities (Ajayi *et al*., 2003; Jan and Chen, 2003; Kalogeris *et al*., 2003).

The molecular weights obtained suggest that the different types of polygalacturonase obtained are species dependent. Molecular weights of 30KDa and 56 KDa were earlier reported for polygalacturonases obtained from cocoa beans infected with *Penicillium citrinum* (Olutiola *et al*., 1982). The differences in molecular weights of enzymes have been associated with a number of factors including the type of host cell wall, nature and types of organisms used the type of substrate employed and the analytical methods which tend to vary from one laboratory to the other (Sevillano *et al*., 1997).

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