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Full Length Research Paper

Partially purified polygalacturonase from *Aspergillus niger* (SA6)

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Polygalacturonase (PG) was isolated from *Aspergillus niger (A. niger)* (SA6), partially purified and characterized. The PG showed two bands on SDS-PAGE suggesting an "endo and exo PG with apparent molecular weights of 35 and 40 KDa, respectively. It was purified 9-fold with a yield of 0.18% and specific activity of 246 µmole/ml/mg. The K_M and V_{max} of the enzyme were 2.74 mg/ml and 0.78 µmole/min/mg, respectively. The optimum temperature and optimum pH of the enzyme were 40°C and 4.5, respectively. The PG was found to be more stable to temperature changes than to pH changes.

Key word: Polygalacturonase, *Aspergillus niger.*

INTRODUCTION

Pectinases (EC. 3.2.1.15 and EC. 3.2.1.67), endo and exopolygalacturonase, (EC. 4.2.2.10), polymethyl-galacturonate lyase and pectin esterase (EC. 3.1.1.11), are a group of enzymes that can degrade pectin containing substrate or modify it during fruit ripening. Pectinases are produced by fungi, yeast, bacteria, protozoa, insects, nematodes and plants (Ahmed et al., 1997; Solis et al., 1997; Whitaker, 1991).

Pectinases are used in the food industry to improve cloud stability in fruits and vegetable nectars (Baker and Bruemmer, 1972; Gupta et al., 1993). Production of pectinases can be done by both solid state cultures and submerged fermentation techniques (Akinola and Olatunji, 2000; Meyrath and Volavsek, 1975; Ronald et al., 1997; Sebastian et al., 1996; Tuttobello and Mill, 1961). However, production of pectinase by *Aspergillus* strains was observed to be higher in solid state fermentation than in sub-merged process (Acuna-Arguelles et al., 1995; Maldonado et al., 1998; Solis-pereira et al., 1996). *Aspergillus niger* is the main micro organism used for the production of pectinases in the food industry and has been found to be safe owing to its wide use (http://www.epa.gov/biotech-rule/pubs/fra/fra006.htm.).

Pectin, the natural substrate of pectinase, is synthesized in the golgi bodies of plants and forms a matrix in which the hemicellulose polysaccharides of the plant cell are embedded. It is broken down by pectinase to pectinic acid and finally pectic acid. During this chemical breakdown the fruit gets softer (ripens) as the cell wall degenerates (McGraw, 1987).

Despite its potentials as input for fruit processing industries, production and evaluation of the characteristics of polygalacturonase from a local strain of *Aspergillu*s (SA6) has not been attempted.

It is therefore, desirable to characterize the locally isolated polygalacturonase since commercial pectinases are often mixtures of cellulases, hemicellulases and proteases hence, they are poorly characterized. This would provide empirical data for future research. We report in this work for the first time the characteristics of polygalacturonase isolated from *Aspergillus niger* (SA6).

MATERIALS AND METHODS

All the reagents used in this study were of analytical grade purchased from Sigma chemical company representatives in Germany.

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Abbreviations: PG, Polygalacturonase; **K_m,** Michaelis-Menten constant; **V_{max},** maximum velocity; **SDS-PAGE,** sodium dodecyl sulphate polyacrylamide gel electrophoresis; **Ea,** energy of activation.

Microorganism

A. niger (SA6) strain was collected from the culture bank of crop protection department of the institute for agricultural research (IAR), Ahmadu Bello University, Zaria, Nigeria. The culture was maintained on potato dextrose agar and sub-cultured periodically throughout the duration of the research.

Preparation of media and cultivation of *A. niger (SA6)*

Exactly 200 g of potato was sliced, cut and boiled. It was filtered with muslin cloth and the volume made up to 1000 ml. Exactly 20 g each of dextrose and agar were added, boiled and autoclaved at 121°C for 15 min. After cooling about 5 g of streptomycin powder was added to prevent bacterial contamination (http: en.wikibooks. org/Potato Dextrose Media Preparation).

Cultivation of the *Aspergillus* was carried out in pre-weighed 250 ml Erlenmeyer flasks covered with aluminum foil containing 5 g of wheat germ and 7.5 ml of 0.4 M HCl. The flasks were autoclaved at 121°C for 15 min. After cooling each flask was weighed and water loss during autoclaving was corrected (Sebastian et al., 1996).

Inocculation of *A. niger (SA6)*

Ten (10) ml of conidial suspension (approximately 10^7 spores/g of dry substrate, which was obtained from a 7–day old agar slant suspended in autoclaved Tween 80 solution was used to inoculate the substrate in a sterile chamber.

After inoculation the contents were carefully mixed and the flasks placed in a humid cultivation room at 30°C under static condition for 3 days according to the method of Sebastian et al. (1996).

Preparation of crude polygalacturonase

After cultivation, cultures were suspended in 40 ml of distilled water stirred for 10 min and the pH measured. This was followed by the addition of 40 ml of 0.05 M acetate buffer pH 5.0 and the mixture left to stand for 10 min. The mixture was filtered through a muslin cloth and Whatman No 1 filter paper and the solid material retained on the filter paper was extracted again with 20 ml of 0.05 M acetate buffer pH 5.0.

Both extracts were pooled, clarified by centrifugation at 5000 x g for 30 min and brought to a total volume of 100 ml with 0.05 M acetate buffer. The extract (crude enzyme) was kept at 4°C until used (Sebastian et al., 1996).

Ultrafiltration of crude polygalacturonase

The crude polygalacturonase was concentrated using Amicon equipment with cellulose acetate membrane in order to remove low molecular weight proteins of 10,000 KDa. and below.

Ammonium sulphate precipitation

The crude polygalacturonase was precipitated with gentle stirring at 40 - 80% saturation of solid ammonium sulphate at interval of 10%. It was centrifuged at 10,000 x g for 15 min. The precipitate was re-dissolved in 0.05 M acetate buffer pH 5.0 and dialyzed over-night against the same buffer to remove low molecular weight substances and other ions that may interfere with the enzyme activity (Dixon and Webb, 1964).

Determination of protein content

Twenty micro litre(µl) of sample enzyme was mixed with 1 ml of Bio-rad dye and vortexed. The mixture was incubated at room temperature for 5 min. Absorbance was read at 595 nm using spectrophotometer. Absorbance values were converted to protein concentration by extrapolation from the standard curve. 1 ml of Bio-rad dye and 1 ml of buffer were used as blank (Bradford, (1976).

Assay of polygalacturonase activity

The reaction mixture contained 0.1 ml of the enzyme, 0.8 ml of citrus pectin substrate and 0.1 ml of buffer. The mixture was incubated for 20 min at 40°C. After incubation 1 ml of DNS was added and the mixture heated at 90°C for 5 min to develop the color.

After cooling the absorbance was read at 575 nm using a spectrophotometer. A blank DNS and buffer were used to zero the spectrophotometer. Absorbance values were used to calculate the activity of the enzyme using the standard curve. One unit of enzyme activity was defined as the amount of enzyme that produced one µmole of D-galacturonic acid per minute at 40°C and pH 5.0 (Soares et al., 1999).

Ion-exchange chromatography (FPLC)

Fifteen ml of the crude enzyme was loaded onto the column (15.5 x 1 cm). Fifty six fractions of 5 ml each were collected at a flow rate of 3 ml/ min. Each fraction was assayed for activity and protein content. An elution profile was plotted to show active peaks.

Gel filtration (FPLC)

One ml of the enzyme sample from ion-exchange was gradually injected into the pre-equilibrated column. Filtration was carried out for 2½ h. Fifty six fractions were collected and protein content and activity were determined. The fractions with high activity were pooled and used for characterization and electrophoresis.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE of the partially purified polygalacturonase was carried out as described by Laemli (1970). 20 µL of the crude enzyme, partially purified enzyme and molecular weight markers were each treated with sample buffer (125 mM Tris-HCl buffer, pH 6.8, 4% SDS, 10% (V/V) Glycerin, 10% (V/V) (β-Mercaptoethanol, 0.25% Bromophenol blue) and incubated at 95°C for 5 min in a Thermo-mixer.

The prepared samples were loaded onto the gel (12.5%). The electrophoresis was performed at 150 volts for 1½ h. After the electrophoresis, the gel was stained overnight and then de-stained several times with 7% acetic acid until clear bands appeared. The apparent molecular weight of the enzyme was estimated from the standard marker proteins.

Initial velocity studies

The effect of substrate concentration on the activity of poly-galacturonase was determined by incubating the enzyme with 1, 3, 5, 7, 9, 13 and 15 mg/ml of citrus pectin. The V_{max} and K_M of the enzyme were determined using double reciprocal plot (Lineweaver and Burk, 1934).

Effect of temperature change on the activity of polygalacturonase

The optimum temperature of the enzyme was determined by incubating the enzyme with citrus pectin at 30 - 60°C at interval of 5°C for 20 min and pH 5.0. The activity was then assayed as per the method of Miller (1959). A plot of temperature versus activity was plotted to obtain the optimum temperature of the enzyme by the method of Stauffer and Etson, (1969).

Effect of pH change on the activity of polygalacturonase

The optimum pH of the enzyme was determined using acetate buffer of pHs-, 3.5 - 5.5 at interval of 0.5 by dispersing the enzyme in 0.05 M acetate buffer in the various pHs and then taking 0.1 ml of the dispersed enzyme for assay as per the method of Miller (1959). A plot of enzyme activity versus pH was plotted to determine the optimum pH of the enzyme.

Determination of pH stability of polygalacturonase

Enzyme solution was dispersed in 0.05 M acetate buffer pH 3.5 - 5.5; phosphate buffer pH 6.0 - 7.5 and Tris-HCl pH 7.5 - 9.0 at interval of 0.5 and maintained at room temperature for 24 h. An aliquot (0.1 ml) of the enzyme was used to determine the residual activity of the enzyme at optimum conditions as per the method of Miller (1959).

Determination of temperature stability of polygalacturonase

Enzyme solution was incubated with citrus pectin at different temperatures 30 - 70°C at interval of 10°C for 1 h. An aliquot of the enzyme was withdrawn and assayed for residual activity at optimum conditions.

Effect of pH on K_M AND V_{max} of polygalacturonase

The activity of the enzyme was determined at varying substrate concentration of 1, 3, 5, 7, 9, 13 and 15 mg/ml at pH values 3.5 - 5.5 using acetate buffer. The kinetic constants V_{max} and K_M were determined at each pH value using double reciprocal plot. The pKa values were determined using the log V_{max}/K_M plot (Dixon, 1953). These values were used to predict the possible amino acids at the active site.

Determination of energy of activation (Ea) of polygalacturonase

The enzyme was incubated at 30 -70°C in a water bath at interval of 10°C as described by Stauffer and Etson, (1969). The various test tubes were cooled to room temperature and the activity of the enzyme determined as per the method of Miller (1959). An Arrhenius plot of log V_o (enzyme activity) versus reciprocal of Kelvin temperature (°K) was done to determine the energy of activation of the enzyme.

RESULTS

The isolated polygalacturonase was precipitated at 70% ammonium sulphate saturation as shown in Figure 1 and purified nine fold with specific activity of 246 µmole/ min/mg and a yield of 0.18% (Table 1).

The ion exchange elution profile of the enzyme on DEAE- sepharose at 0 - 100% NaCl gradient revealed one active peak with highest activity of 1.1 µmole/min/mg as presented in Figure 2.

Gel filtration profile of the enzyme gave one active peak at fractions 3 and 4. Both fractions contained protein and activity as presented in Figure 3.

SDS-PAGE electrophoregram of the enzyme revealed two distinct bands with apparent molecular weights of 35 and 40 KDa suggesting a dimeric protein (endo and exo) polygalacturonase as presented in Figures 4 and 5. The apparent molecular weights of the enzyme were extrapolated from the standard marker proteins.

The K_M and V_{max} of the enzyme as calculated from double reciprocal plot were 2.74 mg of citrus pectin/ml and 0.78 µmoles /min/mg respectively as shown in Figure 6.

The optimum pH and optimum temperature of the enzyme were 4.5 and 40°C respectively as presented in Figures 7 and 8.

Stability studies of the enzyme at different pH's after 24 h of incubation at room temperature gave the highest percentage residual activities as follows: 23.01% at pH 4.5, in acetate, 28.32% at pH 7.0 in phosphate, and 22.13% at pH 7.5 in Tris-HCl. The lowest percentage residual activity was obtained at pH 7.5 in Tris-HCl buffer as presented in Table 2.

Temperature stability studies show that the enzyme was more stable at 40°C as presented in Figure 9.

Plot of Log Vmax/Km the enzyme showed one peak at pH 5.5 and when tangent was drawn on the curve the pKa values deduced were 4.9 and 5.6 as presented in Figure 10.

The activation energy (Ea) of the polygalacturonase was found to be 259.19 cal/mole as presented in Figure 11.

DISCUSSION

The polygalacturonase was precipitated at 40 - 80% ammonium sulphate saturation with the highest activity of 0.78 µmole/min/mg obtained at 70% saturation.

Studies have shown that polygalacturonase can be precipitated between 0 - 90% of ammonium sulphate depending on the source of the enzyme (8, 9, 42). This is consistent with the findings of this study.

Ion-exchange chromatography profile of PG on DEAE-sepharose showed that the enzyme was eluted in the flow through. This could be attributed to the fact that at pH 5 both the enzyme and DEAE- sepharose carried the same charge, hence the protein did not bind the column and the enzyme was therefore eluted.

The gel filtration profile of the PG revealed one active peak with the highest activity of 0.51µmole/min/mg. The SDS-PAGE of the active fractions showed two bands that moved distinctly but closely on the gel with apparent

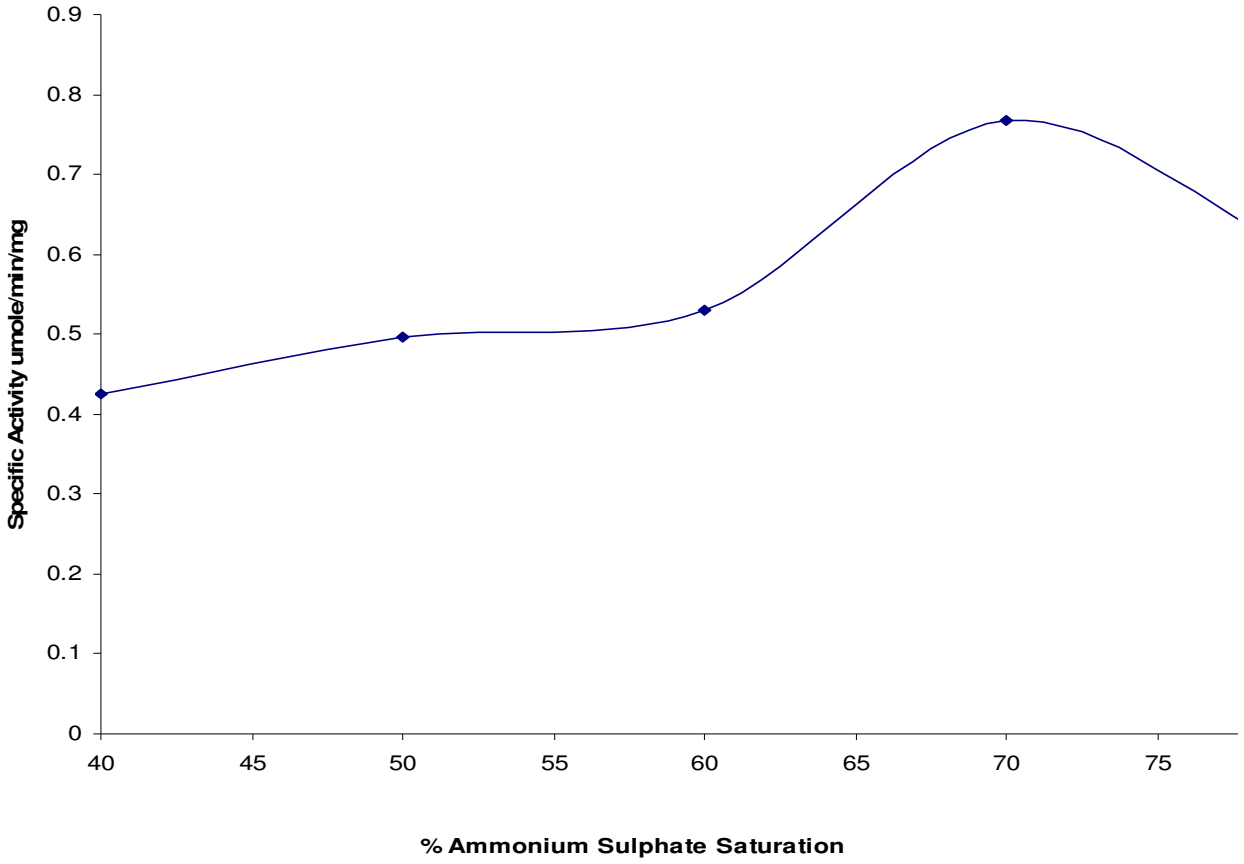

Figure 1. Ammonium sulphate precipitation profile of polygalacturonase.

Table 1. Purification data of polygalacturonase from *Aspergillus niger* (SA6)

Purification steps	Vol. of sample (ml)	Total protein (µg)	Total activity (µmole of D-galacturonic acid/min)	Specific activity (µmole of D-galacturonic acid/mg/min)	Purification fold	% Recovery
Crude extract	500.00	8335.00	225.00	26.99	1.00	100.00
Ammonium sulphate precipitation	25.00	3415.00	17.25	5.10	0.19	7.70
Ion-exchange chromatography (FPLC) on DEAE sepharose	15.00	181.95	18.30	100.58	3.72	8.10
Gel filtration on sephadex G-75	1.00	1.67	0.41	246.00	9.10	0.18

molecular weights of 35 KDa and 40 KDa which suggests the existence of a dimeric protein consisting of both 'endo and exo' polygalacturonase activities. Kester et al. (1996) reported a polygalacturonase with a molecular mass of 20 KDa while DeVries and Visser (2001) reported a range of 35 - 80 KDa. This range is in agreement with the apparent molecular masses of the polygalacturonase isolated in this study. Previous studies have shown that some fungal polygalacturonases exists in iso forms (De Vries and Visser, 2001).

The Michaelis Menten constant (K_M) and the maximum velocity (V_{max}) of the polygalacturonase were 2.74 mg/ml and 0.78 µmole/min/mg, respectively, as calculated from the Lineweaver-Burk plot. Studies have shown that, although polygalacturonases share high degree of sequence homology, their rate of pectin hydrolysis, hence their kinetic constants can differ (Laemmli, 1970; Deviet et al., 2004; http://www.epa.gov/biotech-rule/pubs/fra/fra006.htm.; irpjit and Hoodal, 1996).

Suryakant et al., (2001) reported a K_M of 0.12 mg/ml and V_{max} of 111.1µM/min/mg for polygalacturonase from *Fusarium moniliforme* NCIM 1276. Lucie (2000) also

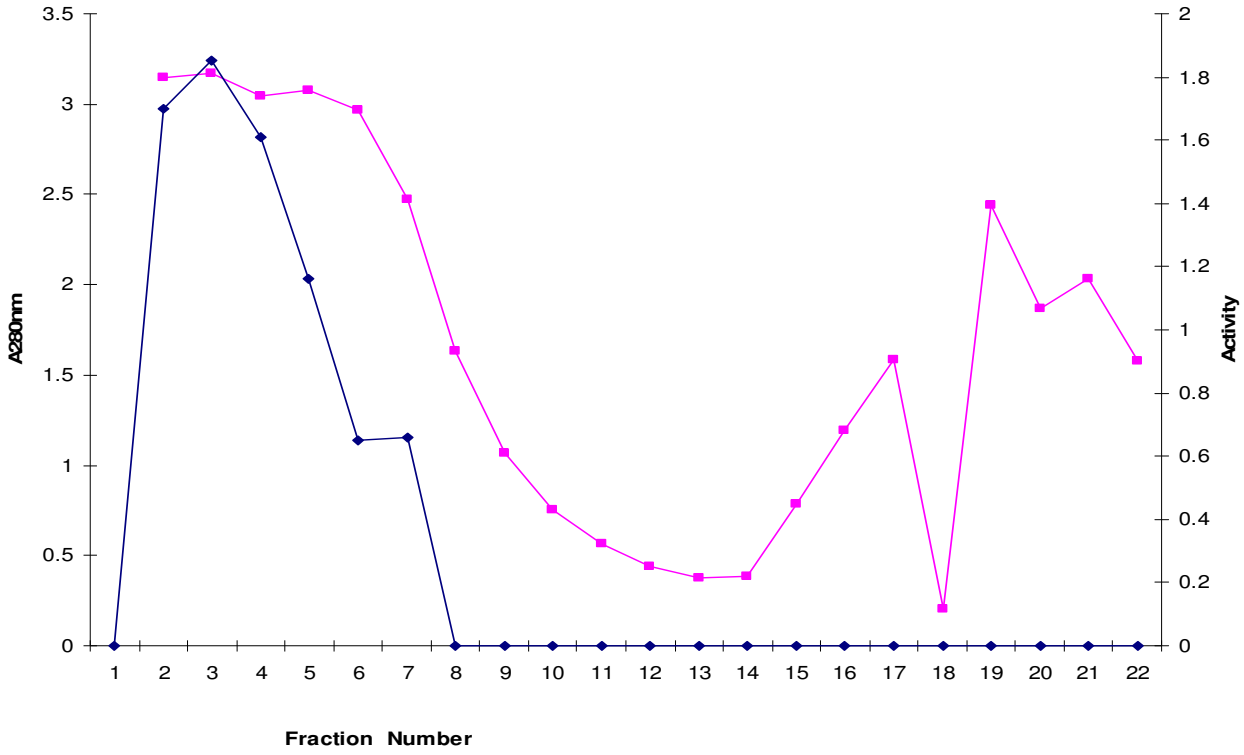

Figure 2. Ion –exchange chromatography profile of polygalacturonase.

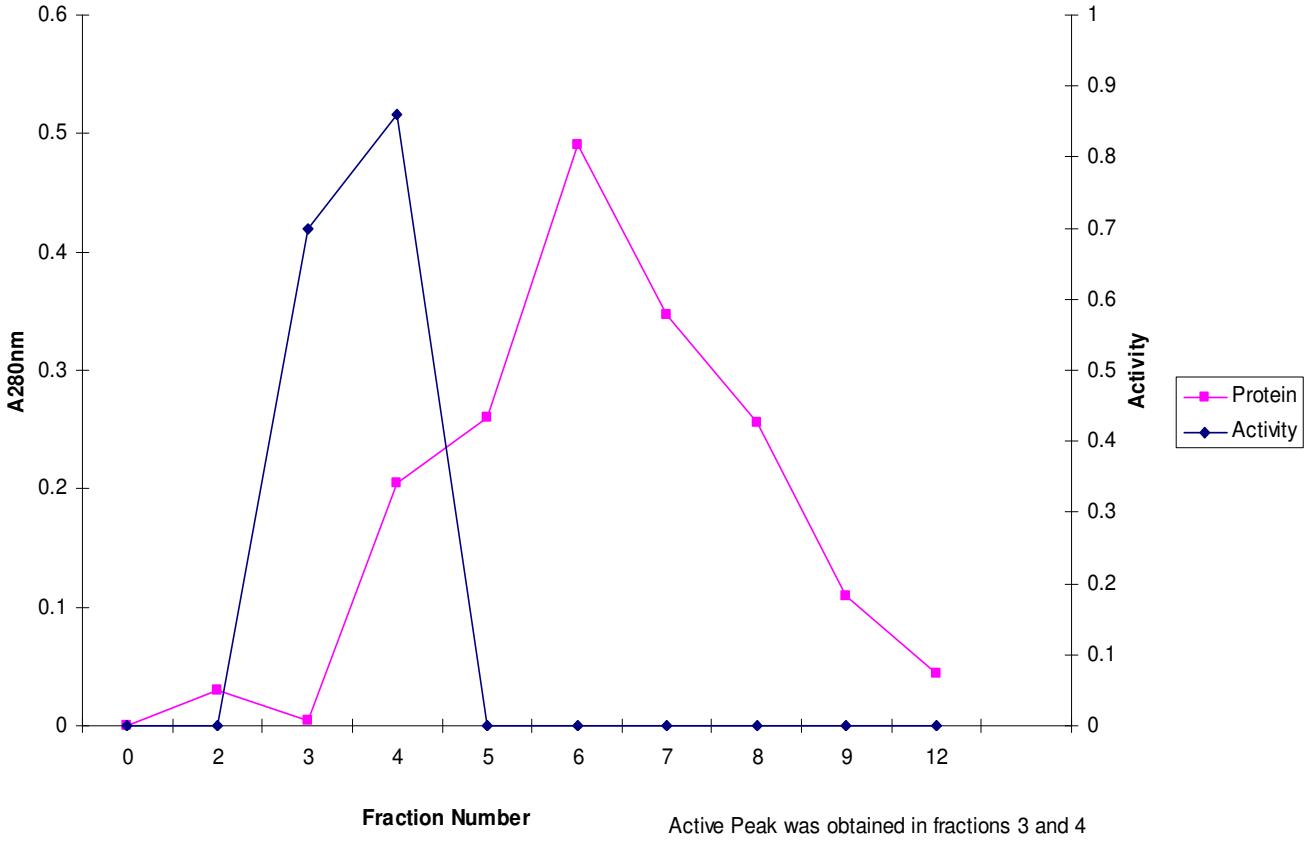

Figure 3. Gel filtration profile of polygalacturonase.

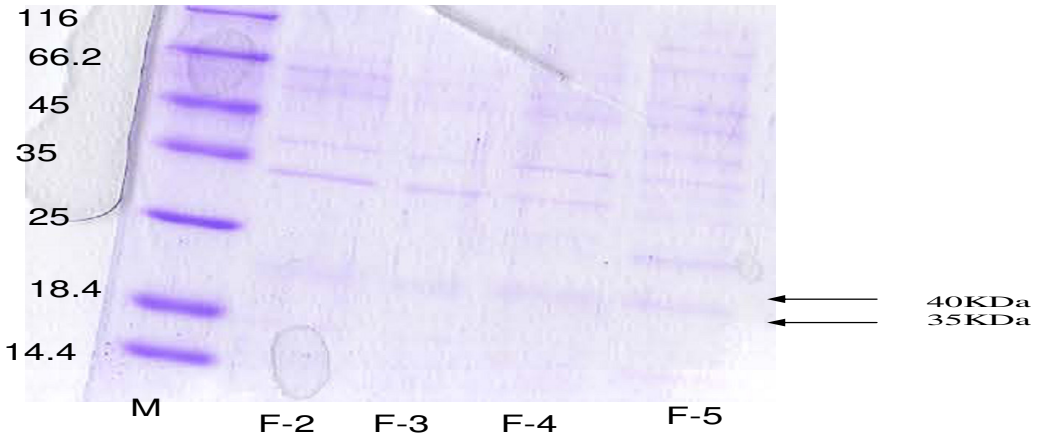

Figure 4. Electrophoregram of partially purified polygalacturonase from ion-exchange chromatography. M = Standard Marker Proteins; F2 = Fraction 2; F3 = Fraction 3; F4 = Fraction 4; F5 = Fraction 5.

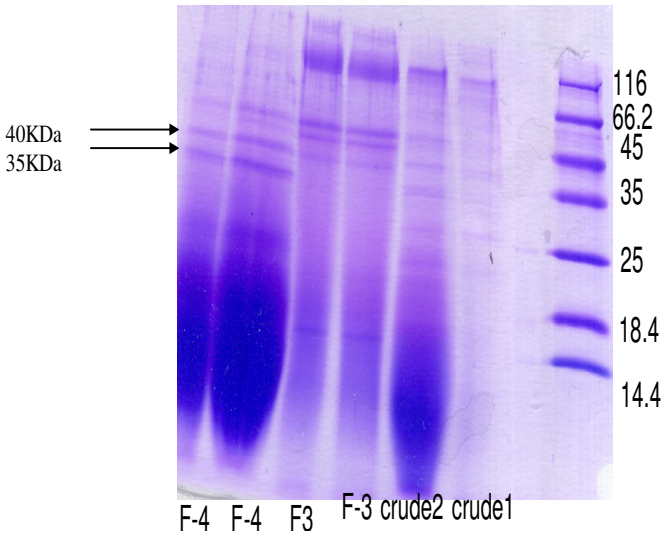

Figure 5. Electrophoregram of partially purified polygalacturonase from gel filtration. M = Standard Marker Proteins; 1 and 2 = Crude Enzyme; F3 = Fraction 3; F4 = Fraction 4.

reported K_M values of <0.15 – 5.0 mg/ml and specific activities of 8.8-7000 U/mg for some fungal poly-galacturonases. The K_M value of the polygalacturonase isolated in this study is comparable to those reported by previous workers.

The plot of log V_{max}/K_M versus pH revealed two ionizable groups (pKa 4.9 and 5.6) associated with catalysis of the enzyme. These pKa vqlues appear to implicate carboxylate and imidazole goups of Glutamic acid and Histidine in the active site of the enzyme. Previous studies have implicated a protonated Histidine and a carboxylate group in the active site of fungal polygalacturonases (Polizeli et al., 1991; Renata et al., 2001; Rexova – Benkova, 1973; Solis et al., 1997).

The polygalacturonase showed optimum activity at pH 4.5. Fungal polygalacturonases are known to operate in mild acidic environment. Sebastian et al., (1996) and De Vries and Visser (2001) reported a range of optimum pH (3.8 - 9.0) for *A. niger* polygalacturonases. Lucie (2000) reported pH optima for 23 polygalacturonases isolated from various species with a range of pH 3.8 - 6.5. The pH optima for 30 fungal polygalacturonases reported by Suryakant et al., (2001) ranged 2.5 - 6.0. Natalia et al. (2004) also reported an optimum pH of 4.5 for polygalacturonase isolated from *Monilla* sp. SB9. The optimum pH and possible amino acids in the active site of poly-galacturonase is in agreement with those reported by previous workers.

In the pH stability studies, results obtained showed that, the enzyme retained 23.01% of its initial activity at pH 4.5 after 24 h incubation with citrus pectin at ambient temperature in acetate buffer, 28.32% at pH 7.0 in phosphate buffer and 22.13% at pH 7.5 in Tris-HCl buffer. The results indicate that, though the enzyme exhibits some level of stability in different pH environments, the stability is low. The reason for this rather low stability could be attributed to marked changes in the charge distribution on the enzyme after 24 h incubation leading to possible denaturation and eventual reduction of activity.

Temperature studies of polygalacturonase revealed optimum activity at 40°C and activation energy calculated from Arrhenius plot of 259.19cal/mole. According to collision theory, reactants must collide to form products after absorbing sufficient energy. Deeviet et al. (2004) reported activation energy of 4.39 Kcal/mole for a commercial pectinase. In a similar study, Sartoglu et al. (2001) reported a value of 9.424 Kcal/mole for a commercial pectinase. These values are comparable to the activation energy of the polygalacturonase isolated in this study. The optimum temperature of the poly-galacturonase is also comparable to the optimum

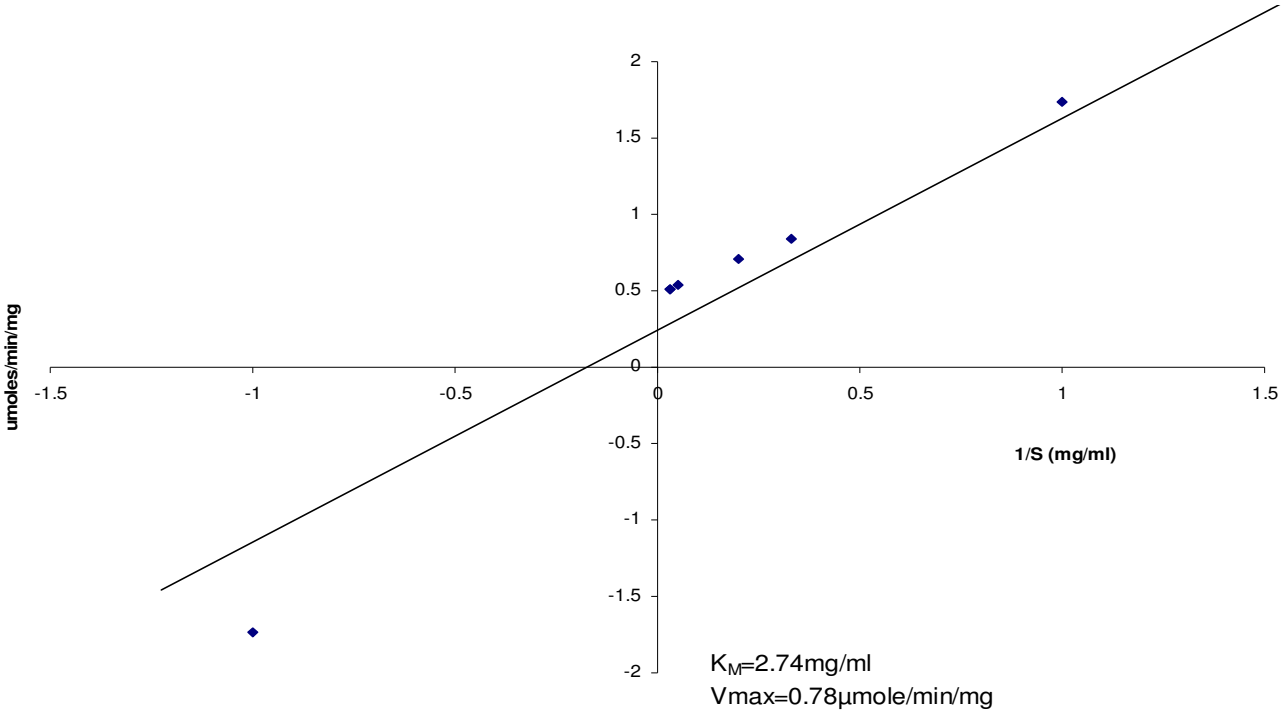

Figure 6. LineWeaver-Burk plot of polygalacturonase.

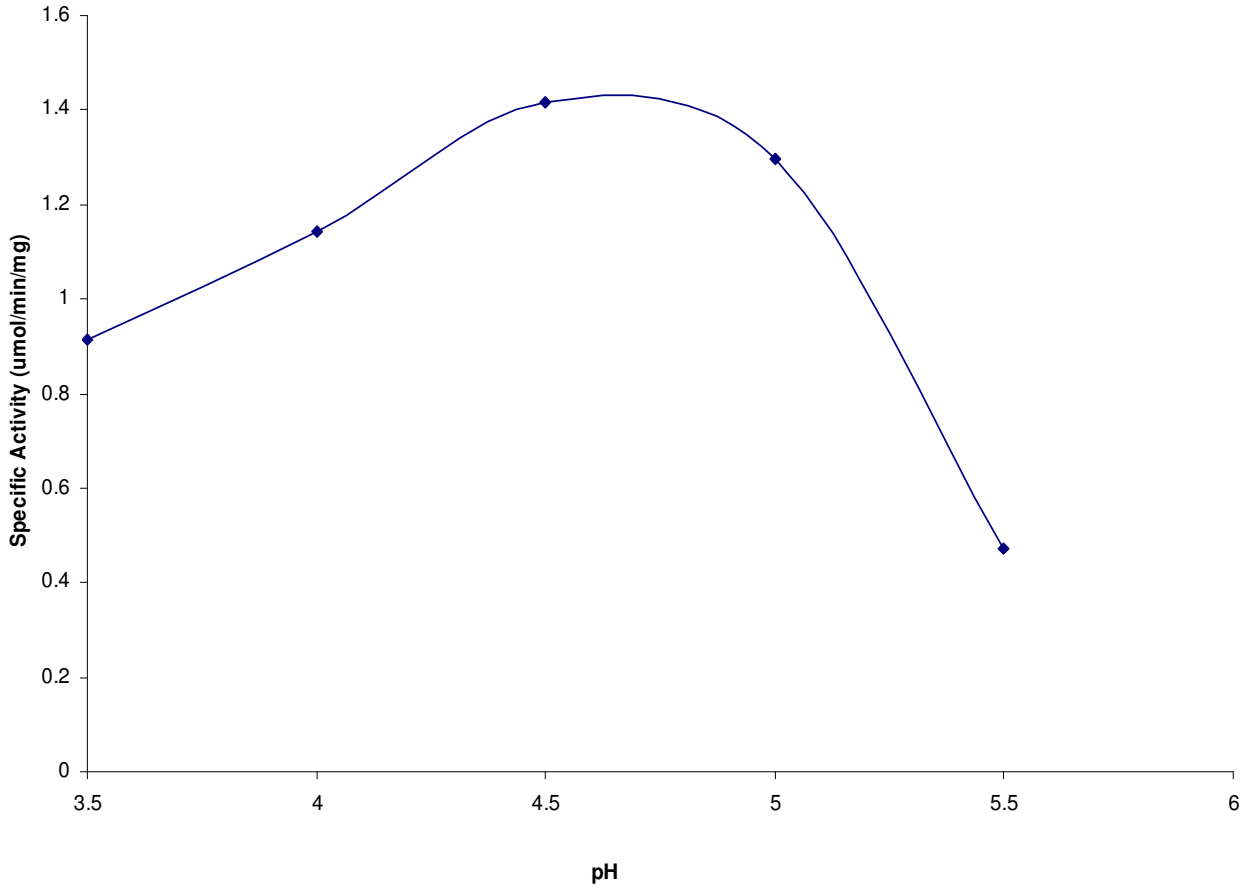

Figure 7. Effect of pH change on the activity of polygalacturonase.

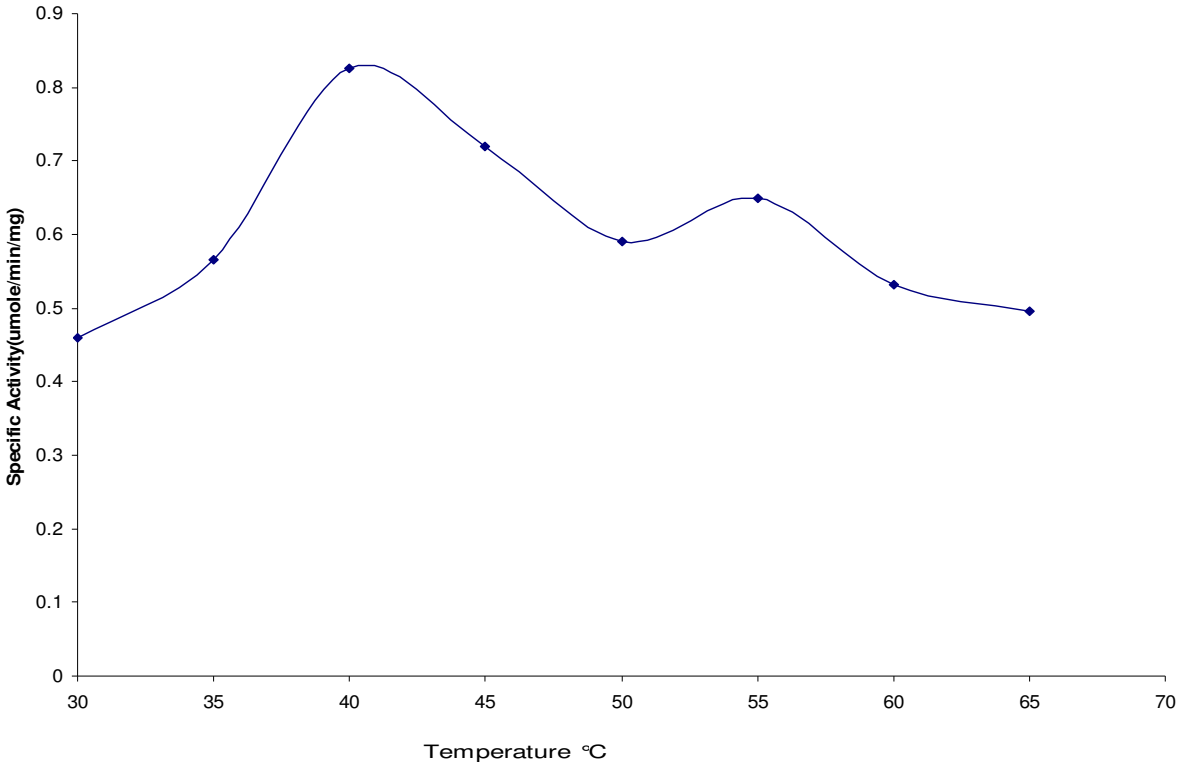

Figure 8. Effect of temperature change on the activity of polygalacturonase.

Table 2. pH stability of polygalacturonase.

pH	Specific activity (umole/min/mg) (initial)	% Residual activity (umole/min/mg) (final)
3.5	0.1239	9.44
4.0	0.1416	11.21
4.5	0.295	23.01
5.0	0.0649	5.31
5.5	0.1711	13.57
6.0	0.2655	20.65
6.5	0.236	18,29
7.0	0.3599	28.32
7.5a	0.00531	0.413
7.5b	0.2832	22.125
8.0	0.0708	5.5342
8.5	0.2006	15.6704
9.0	0.177	13.8296

temperature reported for many polygalacturonases isolated from several fungi, yeast plant and bacteria with a range of 37 - 55°C (Bradford, 1976; Sartoglu et al., 2001; Federici, 1985; Natalla et al., 2004; Ronald et al., 1997; McGraw, 1987).

The polygalacturonase is stable at 40°C and it retained 75.5% of its optimum activity at 50°C. This stability is a good attribute as it makes the enzyme less susceptible to thermal inactivation during fruit processing. The good stability could be due to slow denaturation of the enzyme. Verlent et al. (2004) reported a polygalacturonase that is stable at 60°C. A polygalacturonase with a half life of 18 min at 80°C was reported by Nirpjit and hoondal (1996). Karbassi and Vaughn (1980) reported a polygalacturonase from Bacillus stearothermophillus that was active at 70°C.

Conclusion

The polygalacturonase excreted by *A. niger* (SA6) was partially purified and characterized. The electrophoregram of the enzyme has revealed that polygalacturonase exists as a dimer (endo and exo forms). The enzyme showed optimum activity at pH 4.5 and 40°C, respectively. From the results of this work histidine and glutamic acid are present in the active site of the enzyme.

The data presented in this research would provide reference for future work with *A. niger* (SA6) strain.

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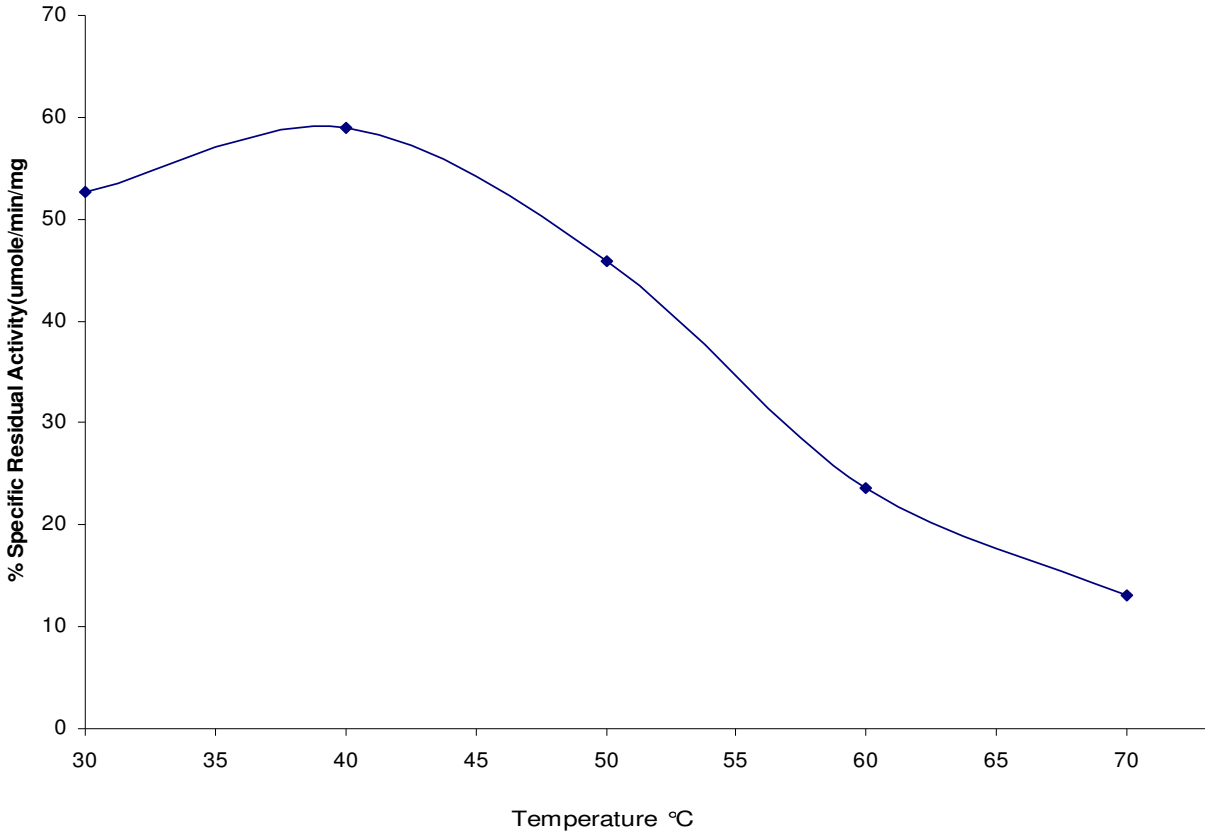

Figure 9. Temperature stability of polygalacturonase.

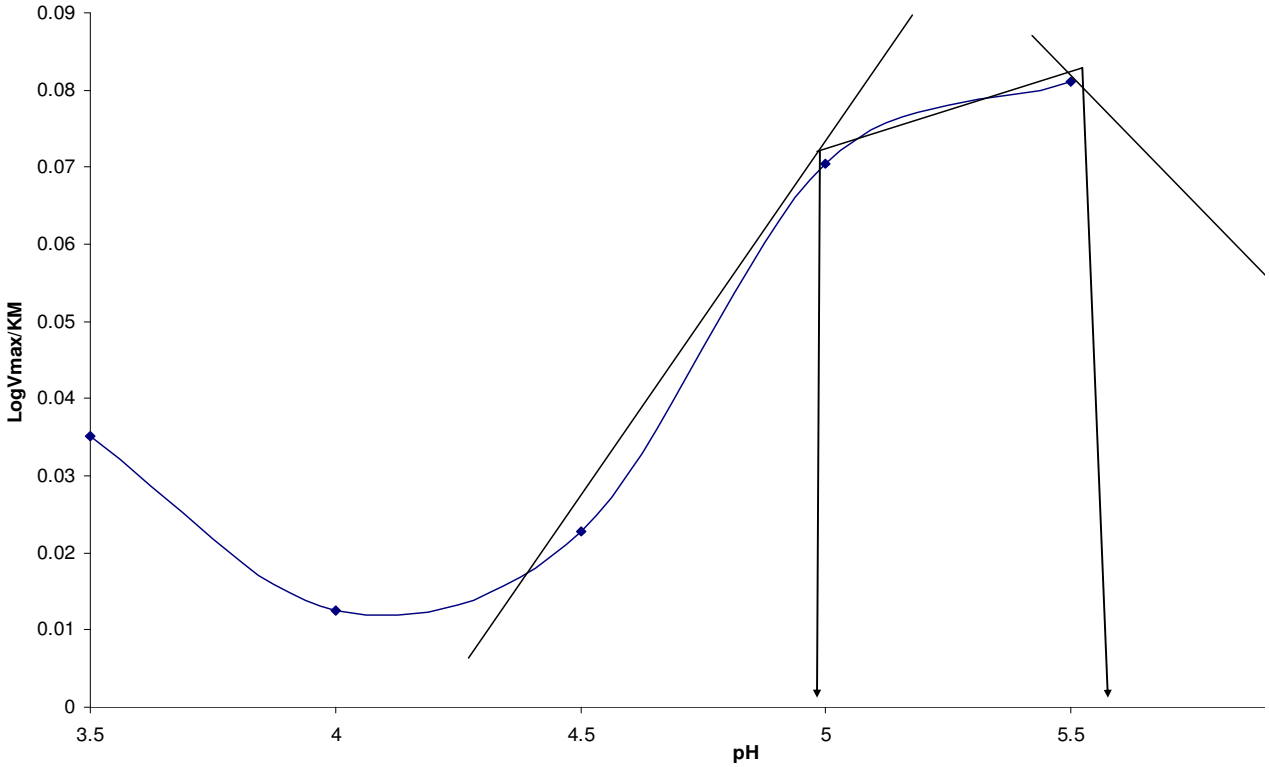

Figure 10. Plot of Log Vmax/Km of polygalacturonase.

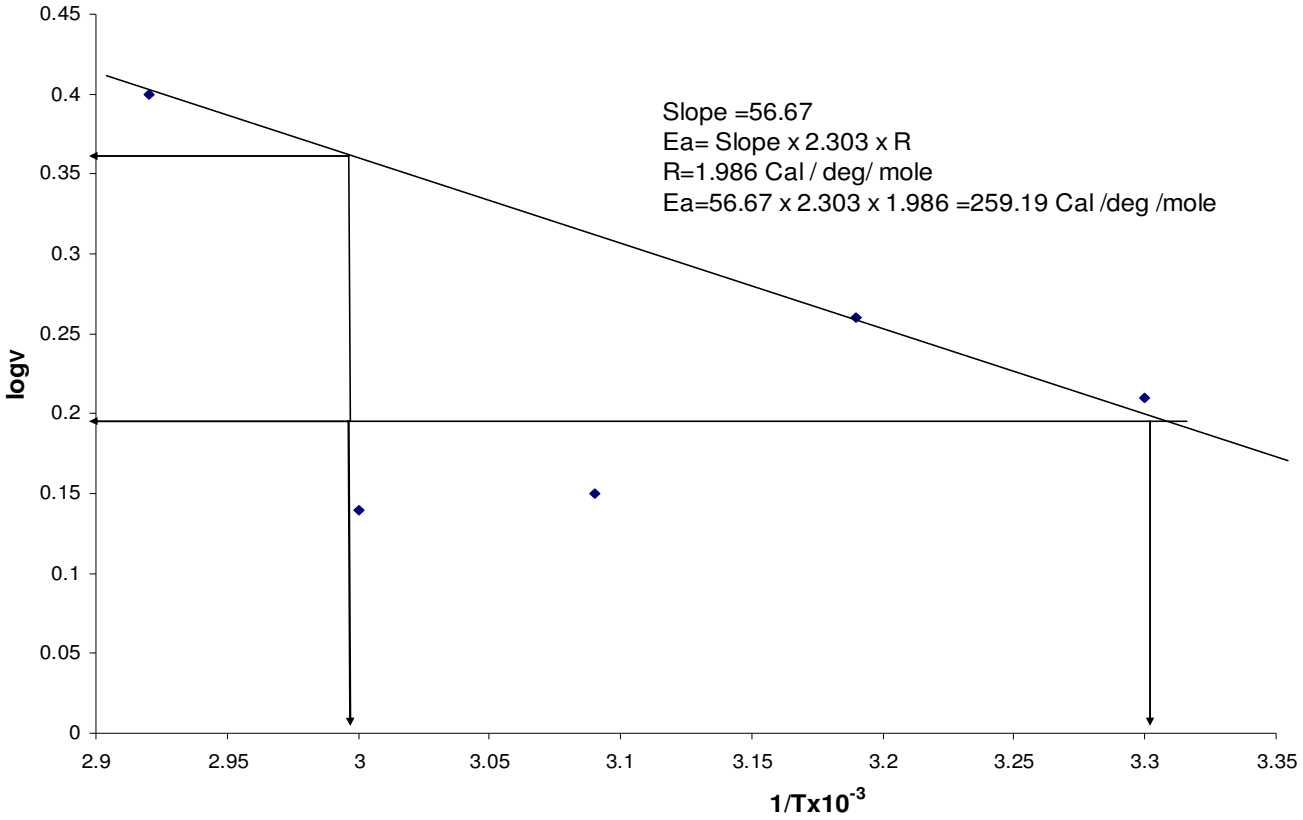

Figure 11. Arrhenius plot of polygalacturonase.

of Microbial Biochemistry, Philipps University, Marburg, Germany.

REFERENCES

Acuna-Arguelles ME, Gutierrez-Rojas M, Viniegra-Gonzalez G, Favela-Torres E (1995). Production and Properties of Three Pectinolytic Activities Produced by *Aspergillus niger* in Sub-merged and Solid Sate Fermentation. Appl. Microbiol. Biotechnol. 43(5): 808-814.

Ahmed R, Ahmad S, Ighal J,and Baizy MA (1997). Shake Flask Studies for Preliminaries by *Aspergillus foetidus*. Park J. Sci. Res. 49: 36-39.

Akinola SO, Olatunji OO (2000). Pilot Production of Pectinase from Corn Pomace and Production of Clarified FruitJuices from Pulpy Fruits. Book of Abstracts. International Conference on Biotechnology, Sheraton Hotel and Towers, Abuja, October 24-26.

Baker RA, Bruemmer JH (1972). Pectinase Stabilization of Orange Juice Cloud. Agric. Food Chem. 20: 1169-1173.

Bradford MM (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. Biochemistry, 72: 248-254.

De Vries RP, Visser J (2001) *Aspergillus* Enzymes Involved in Degradation of Plant Cell Wall Polysaccharides. Microbiol. Mol. Biol. Rev. 65(4): 497-522.

Deviet D, Ahmet R, Ozdural MM (2004). Performance of Immobilized Pectinex Ultra SP-L on Magnetic Duolite- Polystyrene Composite Particles Part 1: A Batch Reactor Study. 64(4): 417-421.

Dixon M, Webb EC (1964). Enzymes 2nd Edition Longmans, New York. pp. 116-166.

Dixon M (1953). The Determination of Enzyme Inhibitor Constants. Biochem. J. 55: 170-171.

Federici F (1985). Production, Purification and Partial Characterization of an Endopolygalacturonase from *Cryptococcus albidus Var. albidus*. Antonie Van Leeuwenhoek, 51(2): 139-150.

Gupta MN, Guoqing D, Mattiasson B, Pectin L (1993). Production by *Penecillium griseoroseum*; Effect of Tea Extract, Caffeine, Yeast Extract and Pectin. Biotechnol. Appl. Biochem. 18: 321-327.

http://www.epa.gov/biotech-rule/pubs/fra/fra006.htm.

http: en.wikibooks.org/Potato Dextrose Media Preparation

Karbassi A, Vaughn RH (1980). Production of Constitutive Thermostable, Hyper Active Exo-Pectinase from Bacillus GK-8. Biotechnol. Letters, 18(12): 1435-1438.

Kester HCM, Kusters-Van Someren MA, Muller Y, Visser J (1996). Primary Structure and Characterization of Exopolygalacturonase from *Aspergillus tubingensis*. Eur. J. Biochem. 240: 738-746.

Laemmli UK (1970). Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4. Nature, 227: 680-685.

Lineweaver H, Burk D (1934). The Determination of Enzymes Dissociation Constants. J. Am. Chem. Soc. 56: p. 658.

Lucie P (2000). Pectinase of Aspergillus niger: A Molecular and Biochemical Characterization. Thesis, Wageningen University. Netherlands. ISBN 90-5808-203-2 21.

Maldonado MC, Strasser De Saad AM (1998). Production of Pectin Esterase and Polygalcaturonase by Aspergillus niger in Sub-merged and Solid State Systems. J. Ind. Microbiol. Biotechnol. 20: 34-38.

McGraw H (1987). Encyclopedia of Science and Technology 6th ed. 13 PAC-PLAN. pp. 175-176.

Meyrath J, Volavsek G (1975). Production of Microbial Enzymes In: Enzymes in Food Processing (2nd Ed) G. Reed Ed New York Academic Press. pp. 225-300.

Miller GL (1959) The Use of Dinitrosalicylic Acid Reagent for Determination of Reducing sugar. Anal. Chem. 31: 426-428.

Natalla M, Simone RDS, Roberto DS, Eleni G (2004). Pectinase Production by Fungal Strains in Solid State Fermentation Using Agro-industrial Bio-product. Braz. Arch. Biol. Technol. 47(5): p. 5.

Nirpjit S, Hoodal DGS (1996). Production of Constitutive, Thermostable, Hyperactive Exopectinase from *Bacillus CK-8*. Biotechnol. Lett. 18(12): 1435-1438.

Polizeli ML, Jorge JA, Terenzi HF (1991). Pectinase production by *Neurospora Crassa*: Purification and Biochemical Characterization of Extracellular Polygalacturonase. J. Gen. Microbiol. 137(8): 1815-1823.

Renata I, Barense Maria, Angelica dos, Chellegatti SC, Maria Jose V, Fonseca, Surain Said (2001). Partial Purification and Characterization of Exopolygalacturonase II and III of *Penicilium frequentans.* Braz. J. Microbiol. 32: p. 4.

Rexova-Benkova I (1973). The Size of the Substrate Binding Site of an *Aspergillus niger* Extra Cellular Polygalacturonase. Eur. J. Biochem. 39: 109-115.

Ronald PC, Carl WB, Melvin SF (1997). Isolation and Characterization of an Endopolygalacturonase from *Cochliobolus stativus* and a Cytological Study of Fungal Penetration of Barley. Phytophatology, 87: 1148-1159.

Sartoglu K, Demir N, Acar J, Mutlu M (2001). The Use of Commercial Pectinase in the Fruit Industry, part 2: Determination of Kinetic Behavior of Immobilized Commercial Pectinase. J. Food Eng. 47(4): 271-274.

Sebastian F, Cavalitto-Jorge A, Arcas R-AH (1996). Pectinase Production Profile of *Aspergillus foetidus* in Solid State Cultures at Different Acidities. Biotechnol. Lett. 18(3): 251-256.

Soares MMCN, Silva R, Gomes E (1999). Screening of Bacterial Strains for Pectinolytic Activity Characterization of the pgase Produced by *Bacillus species*. Rev. Microbiol. 30: 229-303.

Solis S, Flores ME, Huitron C (1997). Imporvement of Pectinase Production by Interspecific Hydrides of *Aspergillus* strains. Lett. Appl. Microbiol. 24: 77-81.

Solis-pereira S, Favela-Torres E, Gutierrez-Rojas M, Rousso S, Sauccedo-castafieda G, Vinegra-Gonzales G (1996). Production of Pectinases by *Aspergillus niger* in Solid State Fermentation at High Initial Glucose Concentration. World J. Microbiol. Biotechnol. 12: 257-260.

Stauffer CR, Etson D (1969). Enzyme Assay and Kinetic Characterization. J. Biol. Chem. 244: 5333-5338.

Suryakant K, Niture, Aditi Pant, Ameeta R Kumar (2001). Active Site Characterization of the Single Endo-Polygalacturonase Produced by *Fusarium monil*forme NCIM 1276. Eur. J. Biochem. 268: 832-840.

Tuttobello R, Mill PJ (1961). Pectic Enzymes of *Aspergillus* niger. Biochem. J. 79: 57-64

Verlent I, Van loey CA, Smout C, Duvetter T, Hendriclox ME (2004). Purified Tomato Polygalacturonase Activity during Thermal and High Pressure Treatment. Biotechnol. Bioeng. 86(1): 63-71.

Whitaker JR (1991). Microbial Pectolytic Enzymes In: Microbial Enzymes and Biotechnology Forgarty WM and Kelly CT (ed), Elsevier Applied Science, London and New York, pp. 133-175.