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Isolation, Purification and Characterization of a Xylanase from Aspergillus Flavus for Possible use in Paper Industry

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ABSTRACT

Aspergillus flavus was grown in basal mineral salt medium (MSM) with pretreated composite sawdust as the only carbon source under submerged fermentation conditions. The fermentation lasted for 216 hours (9 days) in an incubator shaker 150rpm at 37^{o}C. Maximum xylanase production was at 72 hour incubation, with specific activity of $1.12x10^{-3}$umol/min/mg protein. Crude sample was precipitated with 20-90% ammonium sulphate. The highest specific activity was at 70% fractionation. The purification of xylanase from Aspergillus flavus included ammonium sulphate precipitation, Gel molecular exclusion (G-75) and DEAE-Cellulose (DE-52) chromatography. The specific activity increased from $1.47x10^{-3}$umol/min/mg to $5.31x10^{-2}$umol/min/mg with a purification fold of 36.12 and yield of 6.83%. Aspergillus flavus had optimum activity at pH5 and temperature of 50^{O} C. The xylanase also had activation energy of 7.73kJ/mol, K_M of 0.625mg/ml and V_{max} of 0.00429µmol/min. The purified xylanase was homogenous by SDS-polyacrylamide gel electrophoresis with molecular weight estimated to be 28 KDa. The partially purified enzyme was strongly inhibited by 50µM of Hg^{2+} and Pb^{2+}, while the same concentration of Cu^{2+}, Mg^{2+} and Zn^{2+} has slight inhibition. Ca^{2+} and Mn^{2+} had no effects, but Fe^{2+} had strong stimulatory effects and Co^{2+} had stimulatory effects. From the study, the partially purified xylanase from Aspergillus flavus may not be good for applications in industries, where high temperature and alkaline pH is required.

Keywords: *Aspergillus flavus, Xylanase, Sawdust*

1. INTRODUCTION

Hemicellulose is the second most abundant plant fraction available in nature next to cellulose, as a storage polymer in seeds, and being also a structural component of cell walls in plants. Agricultural residues contain up to 40% hemicellulose formed mainly by pentose sugars primarily xylans (Magge and Kosaric, 1985). Hemicelluloses are of particular industrial interest since they are a readily available bulk source of xylose from which xylitol and furfural can be derived.

Xylan is the most abundant of the hemicelluloses. Hydrolysis of xylan is an important step towards the proper utilization of lignocellulose materials especially hemicellulose in nature (Poorna and Prema, 2007). The yield of xylans by chemical means is only about 50-60% making xylitol production very expensive. Various bioconversion methods have been explored for the production of xylitol from hemicellulose using microorganisms or their enzymes (Nigam and Singh, 1995). Biodegradation of xylan requires action of several enzymes among which xylanases (EC3.2.1.8) play a key role (Blanco et al., 1999). Xylanase is the enzyme that depolymerises xylan backbone producing xylooligomers and subsequently xylose. The great interest in the enzymatic hydrolysis of

xylan is due to possible applications in feedstock, chemical production and paper manufacturing (Coughland and Hazlewood, 1997). A wide variety of microorganisms are known to produce xylanases that are involved in the hydrolysis of xylan. Several microorganisms have been reported as xylanolytic, and most of the bacteria, fungi and yeasts producing xylanases secrete the enzymes extracellularly.

Many filamentous fungi are particularly interesting producers of xylanases from industrial point of view due to the fact that they excrete xylan degrading enzymes into the medium, eliminating the need for cell disruption prior to purification (Sunna and Antranikian, 1997, Polizeli et al., 2005). Furthermore, xylanase levels from fungal cultures are typically much higher than those from yeast or bacteria. Moreover, in addition to xylanases, fungi produce several auxiliary enzymes required for the degradation of the substituted xylan.

To use xylanase for pulp treatment purposes, it is preferable not to have any accompanying cellulolytic activity since the cellulase may adversely affect the quality of paper pulp. Most xylanases used industrially are associated with some setbacks in their properties for example thermostability, broad pH stability and cellulase –free xylanase. These properties are very important to the overall actions of the enzyme and as such hinder its output. Therefore, there is the need for sourcing a better cellulase –free xylanase for use in biobleaching and allied industries in view of the fact that the presence of cellulase will reduce the strength of the product. Various reports on the production of xylanases by Aspergillus species, such as A.niger, A. ochraceus and A. ustus using solid state fermentation (SSF) have been documented but reports on xylanase production using natural substrates like sawdust by Aspergillus species via submerged fermentation is scanty. This study was therefore intended to isolate, partially purify and characterize xylanase from a

filamentous Aspergillus species-Aspergillus flavus using a cheap and inexpensive waste, sawdust as a carbon source under submerged fermentation conditions.

2. MATERIALS AND METHODS

All chemical used are of analytical grades.

2.1 Sources of the Filamentous Aspergillus Fungus

The Aspergillus species used for the study was obtained from Crop Pathology laboratory of the Crop Protection Departments, University of Maiduguri and Ahmadu Bello University, Zaria, Nigeria. They were identified and authenticated by a mycologist at the Crop protection Department, ABU, Zaria**,** Nigeria

2.2 Source of the Sawdust

The sawdust was collected from sawmills within Zaria. The sawdust was dried in the oven at 65°C for 2 hours, ground and passed through a sieve (about 0.5mm pore size) to obtain the fine powder that was used for the study**.**

2.3 Pretreatment of the Sawdust

Pretreatment of sawdust was done as described by Ali et al., 1991**.** This was done as follows: a gram of sawdust was soaked in 1% sulphuric acid solution in the ratio of 1:20 (sawdust: H_2SO_4) for two hours at room temperature. After which it was neutralized with 0.1M NaOH and the pH was brought down to pH 5. It was then filtered with muslin cloth and then washed with distilled water to be free of chemicals until the wash water became clear. It was autoclaved at 121^o C for one hour.

2.4 Inoculation and Culture Medium of the Fungus

2.4.1 Medium Preparation.

The medium was prepared as described by Kim et al., (1985) for growth and enzyme production. It contained (g/100ml) 0.05 proteous peptone, 0.03 urea, 0.02 KH_2PO_4, 0.03 $CaCl_2$, 0.14 $NaNO_3$. 0.03$MgSO_4.7H_2O$. In 250ml Erlenmeyer flasks, 100ml of distilled water was used to dissolve the salts. This was then sterilized in autoclave at 121^{o}C for 15min. After sterilization, trace elements made up of 0.005g $FeSO_4$, 0.0014g $ZnSO_4.7H_2O$, 0.002g $CoCl_2$, 0.0016g $MnSO_4$ making 0.1% concentration and 1g of pretreated sawdust as carbon source were added

2.4.2 Fermentation

One ml of 0.2% sterilized Tween 80 was used to wash spores from 4-day old fungus. The washed spores placed on slide were counted under microscope. One mililitre spore suspensions containing approximately $1.5x10^6$ spores was inoculated into the growth medium. The medium was incubated at 37ºC for 9 days (216 hours).

2.4.3 Xylanase Activity Assay

Xylanase (E.C.3.2.1.8) activity was assayed by the reducing sugar method of Miller (1959) as described by Khan (1980), Khanna and Gauri (1993) using birch wood xylan (Sigma-Aldrich) as enzyme substrate. The reaction mixture contained 0.1ml of 1% xylan solution (w/v), in 0.1M sodium acetate buffer (pH5), and 0.2mLof dilute enzyme sample and incubated at 37^o C for 30minutes. After 30 min incubation, 2.0ml of DNS solution was added to stop the reaction and then boiled for 10min. After cooling, absorbance was measured at 540nm against a reagent blank and D-xylose as the standard. One unit of xylanase activity was defined as the amount of enzyme required to liberate 1µmol of xylose equivalent per minute under the assay conditions.

2.4.4 Determination of Biomass

The fungal biomass concentration were measured daily as described by Withers et al.,(1998). The biomass concentration in the culture media was measured by collecting the biomass on pre-dried Whatman no.1 filter paper, washing the biomass with distilled water and drying to constant weight at 70^o C. The biomass concentration was calculated as follows:

Biomass concentration (mg/L) =A-B/V

Where A= weight of the dry cells +filter paper
B= weight of the filter paper
V= volume of culture medium.

2.4.5 Enzyme Time Course

Enzyme production by the selected Aspergillus flavus was determined by assaying for xylanase activity as above from inoculation time to the terminal hour (0-216 hour) to evaluate the time at which the enzyme was produced maximally. The production was monitored at 6 hour interval.

2.4.6 Protein Determination

The amount of protein in crude samples and other fractions was determined by the method of Bradford (1976) using bovine serum albumin (BSA) as the standard protein.

2.4.7 Ammonium sulphate precipitation

Culture from the seventy (72 h) incubation was used for the ammonium sulphate precipitation.

Solid ammonium sulphate salt was added slowly to 10ml crude sample to an initial concentration of 35%. After standing for 15 min, the mixture was centrifuged for 20 min at 10000xg. More ammonium sulphate was added to the supernatant to a concentration of 90%, and the mixture was centrifuged for 20 min at 10000xg. Xylanase activities were determined in all supernatant and precipitate fractions at each addition of ammonium sulphate salt. The fraction with the highest specific activity was reconstituted with 0.1M sodium acetate buffer (pH5) and kept overnight at 4ºC.

2.5 Gel Filtration (Molecular Exclusion Chromatography)

Five (5g) of sephadex G-75 was swollen in 50ml of 0.05M sodium acetate for 2hours and loaded into a column (2.0x6 50cm) equilibrated with 50mM sodium acetate buffer (pH5). Then1.5ml of enzyme sample was loaded and eluants were collected in 5ml fractions. Flow rate was 0.8ml/min.Xylanase activity and protein concentrations were determined.

2.6 Ion exchange Chromatography

The xylanase active fractions from Gel filtration purification step (4.0ml) were applied to DEAE-cellulose column (2.0x50cm) preequilibrated with 50 mM sodium acetate buffer (pH5). The flow through was collected and then the column was eluted with NaCl gradient (0.0-0.5M).The flow rate was 0.3ml/min. Xylanase activity and protein concentration were determined in the fractions eluted

2.7 Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis was carried out at all purification steps to ascertain the level of purity of the xylanase. The SDS-PAGE was done as described by Laemmli (1970).

2.8 Estimation of Molecular Weight

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) of the purified xylanase was carried out using standard protein markers. The markers are lysozyme (18Kda), soybean trypsin inhibitor (28Kda), carbonic anhydrase (39Kda), ovalbumin (60Kda), BSA (84Kda), phosphorylase B (120Kda) and myosin (215Kda). The markers called Pierce Blue Prestained MW marker mix was obtained from thermoscientific USA. Relative mobility value of the protein standards was determined along side the purified xylanase and log of MW of these markers was plotted against their corresponding relative mobility values. The molecular weight of the purified xylanase was estimated from this plot.

2.9 Initial Velocity Studies (Determination of K_M and V_{Max})

Initial velocity studies of xylanase were determined by assaying xylanase activity at various concentrations of the birch wood xylan (the substrate). The concentrations ranged from 1-30mg/ml. A plot of reciprocal of xylanase activities against reciprocal of substrate concentrations used was plotted (Lineweaver-Burk plot) where K_M and Vmax were obtained from the plot.

2.10 Effect of temperature on xylanase activity

Incubating the enzyme, purified xylanase and birth wood xylan for 30 min at 25ºC, 30ºC, 40ºC, 50ºC, 60 ºC 70ºC, and 80ºC . After cooling to room temperature, the activity of xylanase at different temperatures was determined. Activities of xylanase were plotted against varying temperature to obtain the optimum temperature. From the effect of temperature on the enzyme activity, activation energy (Ea) of the purified xylanase was determined from a slope of Arrhenius plot obtained by plotting Log v_O against the reciprocal of the temperature (1/Tº K) in Kelvin.

2.11 Effect of pH on Xylanase Activity

The optimum pH for purified xylanase was obtained by assaying the activity of xylanase at varying pH using sodium acetate buffer pH 4-5.0; phosphate buffers pH 6-7.0 and Tris-HCl buffer pH 8-9

2.12 Effect of Metal ions on Xylanase Activity

Activity of the purified xylanase was determined in the presence of 50mM final concentration of the following cations $Ca^{2+}Mg^{2+}$, Fe^{3+},Pb^{2+},Hg^{2+}, Mn^{2+},Co^{2+},$Cu^{2+}$and Zn^{2+}. The concentration of the cations was arrived at after preliminary studies. Previous studies had reported the use of 25 mM of cations. Fifty (50ul) microlitres of each cation solution was used to deduce the effects on the purified xylanase.

3. RESULT AND DISCUSSION

Biomass or fungal load of Aspergillus flavus were monitored after 24 hours on incubation. The biomass was few in the range of 0-72hrs being the lag phase but was most produced in the range of 96-120 hours being the exponential phase. The explanation could be due to new environment, and only few enzymes may be available to degrade the carbon source. Xiao et al., (2004) reported that in the initial step of induction, a small amount of constitutive xylanase and enzymes production to degrade the carbon source to xylose and glucose for maximum mycelium biomass production. Raimbault and Alazard (1980) reported that maximum enzyme production and declining was achieved much faster due to the rapid degradation of substrate as a consequence of rapid growth. The degradable products formed, were used up by the biomass to enhance xylanase production in the fermentation process. The time course of xylanase production by the Aspergillus flavus was at 72 hrs with a specific activity of $1.47x10^{-3}$µmol/mg. Although, the yield is low, it is well documented that each microbial strain's metabolism, growth and enzyme production pattern differ with the fermentation environment (Prakasham et al., 2007).

The crude xylanase by A. flavus at 72 hr incubation precipitated with ammonium sulphate salt had maximum specific activity at 70% saturation. Andre' et al., (2006) precipitated crude xylanase from Penicillium expansum with ammonium sulphate at 60% saturation under submerged culture. The gel filtration step using sephadex G-75 eluted xylanase at fraction 11 which was not in the void volume. The fraction has a total activity of $2.56x10^{-3}$µmol/min and specific activity of $1.22x10^{-2}$ µmol/min/mg. However, xylanase activity was distributed across most of the elution profile which probably could

have been due to the expression of a wide spectrum of xylanases by A. flavus as was observed with A.niger strains (Berrin et al., 2000). A combination of this broad elution of xylanase and may be loss in specific activity, possibly due to removal of some stabilizing elements in the culture supernatant on purification of the enzyme, may be largely accountable for the low yields of activity in the selected peak which is in agreement with the report of Silas et al.,(2008). The ion-exchange chromatography using DEAE-cellulose DE-52 of the xylanase active fraction was eluted in fraction 25 with NaCl salt gradient of 0.15M.The fraction has total activity of $3.45x10^{-3}$µmol/min and specific activity of $5.31x10^{-2}$µmol/min/mg. The low recovery yields of 4.83%, though low this was an efficient step to purify the xylanase under this culture conditions, resulting in appreciable specific activity, which increased from $1.47x10^{-3}$µmol/min/mg in the crude to $5.31x10^{-2}$µmol/mg, equivalent to purification fold of 36.12. The low recovery could be attributed to weak interaction between the protein and eluting salt gradient. Wong and Saddler (1992) reported that xylanases can either be classified based on molecular weight or pI. They have either a high (basic) or low (acidic) pI. Based on this, the interaction between the protein and the eluting salt might be weak. Andre' et al., (2006) reported a xylanase yield from Penicillium expansum using anionic exchange column DEAE-sephadex A-50 of 1.3% and fold of 7.4. Julio et al., (2006) reported yield of 5.7% and a fold of 427.83 of xylanase from Bacillus circulans under solid-state cultivation.

The SDS-PAGE of the partially purified xylanase from A. flavus revealed a single band, which may be suggestive of a monomeric polypeptide with an estimated molecular weight of 28kDa. Although the native protein did not reveal a much clear resolution as to have accurately estimated its molecular weight but from the molecular weight and the absence of cellulase activity, the xylanase isolated under this culture conditions may be a family 11 xylanase. It has been reported that, the group of xylanases that is characterized by a small molecular weight and lack of cellulase activity has been classified under glycoside hydrolases (GH) family 11 of xylanases, a group viewed favourably in the pulp and paper industry (Henrissat, 1991; Henrissat and Bairoch, 1993; Henrissat and Davies 1997). Silas et al., (2008) isolated a single band subunits xylanase of 22kDa with absence of cellulase activity from A. terreus.

The effect of substrate concentration, or rate dependence of enzymic reaction; of birch wood xylan ranging from 1.0—30mg/ml on xylanase activity was evaluated under the assay conditions (50^0C and pH5.0). The substrate concentration could not be higher than this because xylan presents low solubility. The kinetic parameters of Michealis-Menten constant, K_M and maximal reaction velocity estimated according to the Lineweaver-Burk double reciprocal plot were K_M and Vmax values of 0.629mg/ml and $4.29x10^{-3}$µmol/min respectively for the purified xylanase from A.flavus. The low K_M value is a good indication that the xylanase may be desirable in pulp and paper and allied industries. Low K_M means high affinity for the substrate; therefore the enzyme can pick up the smallest available xylan in the reaction medium. The values obtained are consistent with the reported range of K_M values for microbial xylanase. Beg et al.,(2001) reported the K_M values for microbial xylanases as 0.27—14mg/ml. Julio et al.,(2006) reported K_M and Vmax values of 9.9mg/ml and 25.25µmol/min respectively for xylanase from Bacillus circulans BL53 on solid-state cultivation. Silas et al., (2008) reported K_M and Vmax values of 3.57mg/ml and 55.5µmol/mg protein/min respectively for cellulase-free xylanase from Aspergillus terreus UL4029 in submerged culture. Andre' et al., (2006) also reported K_M and Vmax values of xylanase from Penicillium expansum in shake flask to be 3.03mM and 0.027µmol/min/µg of protein using wheat bran as carbon source.

As for the characterization of the xylanase under study, the optimum pH of the free enzyme was 5.0. The enzyme was most active at this pH and it may therefore be concluded that this enzyme is slightly acidic in nature. The low pH and may be acidic nature of the protein may not be a desirable quality in biobleaching industry. If one uses a xylanase with an acidic pH optimum, it is possible to wash most of the alkali out of the pulp (Vikari and Srinivastan, 1999) Silas et al., (2008) reported the optimum pH for xylanase from Aspergillus terreus UL4209 at 6.0. Only in rare cases, fungi are reported to produce xylanase at initial pH lower than 7.0 as observed by Subramaniyan and Prema 2002; Shah and Madamwar (2005). Other reports have shown that the initial pH range of 4-7 is optimal for xylanase production by Aspergillus species (Fernandez-Espinar et al., 1992; Gosh et al., 1993; Raj and Chandra 1995; Carmona et al., 1997). Dekker, (1993) reported that the xylanases of fungal origin are more active at pH that oscillates between 3.5 and 5.5 but are quite stable over a wide range (3-10). The optimal pH of bacterial xylanases oscillates between 5.0-7.5. The temperature at which the xylanase from Aspergillus flavus in this study was most active was 50^0C and as the temperature increased, the activity decreased. This suggests that Aspergillus flavus may be a mesophilic fungus. A number of reports have indicated that the optimal temperature for production of most fungal xylanases ranged between 30 and 50^0C (Haltrich et al., 1996; Subramaniyan and Prema 2002). Angayarkanni et al.(2006) reported the optimum temperature of 50^oC for xylanases produced by Aspergillus niveus ,Aspergillus indicus and Aspergillus flavus. Most Aspergillus species are regarded as mesophilic fungi. For example, the optimal temperature for the production of xylanase by A. terreus and A. niger was reported as 35^0C (Gawande and Kamat, 1999), 30^oC for A. ochraceus and A. versicolor (Biswas et al., 1990). The changes induced by high temperatures during enzyme production may not be very clear, but it has been suggested that at high temperatures, microorganisms may synthesize reduced number of proteins that are probably essential for growth and other

physiological processes including enzyme production (Gawande and Kamat, 1999). The activation energy of the purified xylanase was 7.73KJ/mol. The low K_M value, optimum temperature and pH of 50^{0}C and 5.0 respectively and the low activation energy facilitates the reaction spontaneously. It also implies that minimal energy is required to overcome the transition state. Chandralata et al., (1999) reported a marine fungal isolate of Aspergillus species, at 50^{0}C optimum temperature, the activation energy was only about 16KJ/mol. Faulet et al.,(2006) reported a higher activation energy of 49.8KJ/mol for a thermostable xylanase from a symbiotic fungus, Termitomyces species at a temperature optimum of 65-70^{0}C.

The effects of cations on the purified xylanase revealed that Fe^{2+} and Co^{2+} were stimulators or activators of the enzyme, while Hg^{2+} and Pb^{2+} were strong inhibitors of the enzyme. Approximately, a third of known enzymes possess metal as part of their structures (Conn et al.,, 1987). However, literature on the requirements of any of these cations by xylanases is scarce, but a number of reports have implicated these cations. Julio et al.,(2006) reported that Hg^{2+} and SDS strongly inhibited xylanase activity from Bacillus circulans , while Pb^{2+}, Na+, Cu^{2+}, Fe^{2+}, Zn^{2+}, Mg^{2+} and Ca^{2+} had a slight inhibition. Co^{2+} enhanced the xylanase activity. Isil and Nulifer (2005) reported that Mn^{2+}, Zn^{2+} and Ca^{2+} enhanced the xylanase activity from Trichoderma harzianum 1073 D3, while Mg^{2+} and Cu^{2+} had no significant effect on the activity. Khandeparker and Bhosle (2001) reported that in the presence of Co^{2+}, Zn^{2+}, Fe^{2+}, Mg^{2+} and Ca^{2+} and the metal chelator EDTA, the activity of xylanase from Arthrobacter sp. MTCC 5214 increased, whereas strong inhibition of the enzyme activity was observed in the presence of Hg^{2+}. From the study, the inhibition of the xylanase from A.flavus by Hg^{2+} and Pb^{2+} suggested that there is a sulphydryl group in or close to the active site. Although no dialysis was carried out, but from the effects of cations on the enzyme, one may draw such conclusion. Chivero et al.,(2001) reported that ions that reacted with sulphydryl groups such as Hg^{2+} suggests that there was an important cysteine residue in or close to the active site of the enzyme. Inactivation of xylanases by Hg^{2+} had also been reported by other authors (Saba, 2002; Wang et al., 2003)

3.1 Results

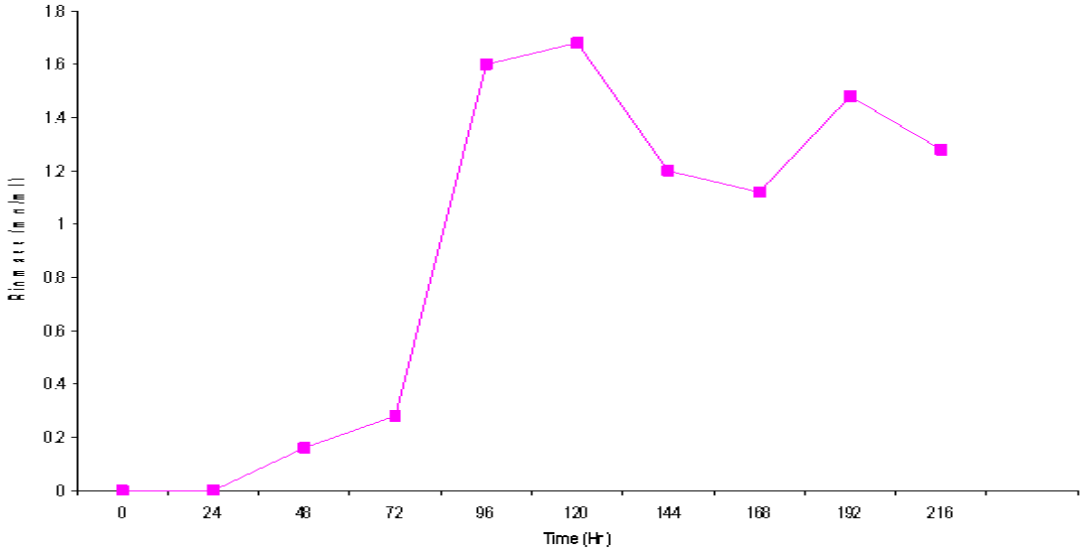

Fig 1: Biomass/ fungal load of Aspergill flawus with sawdust as a carbon source

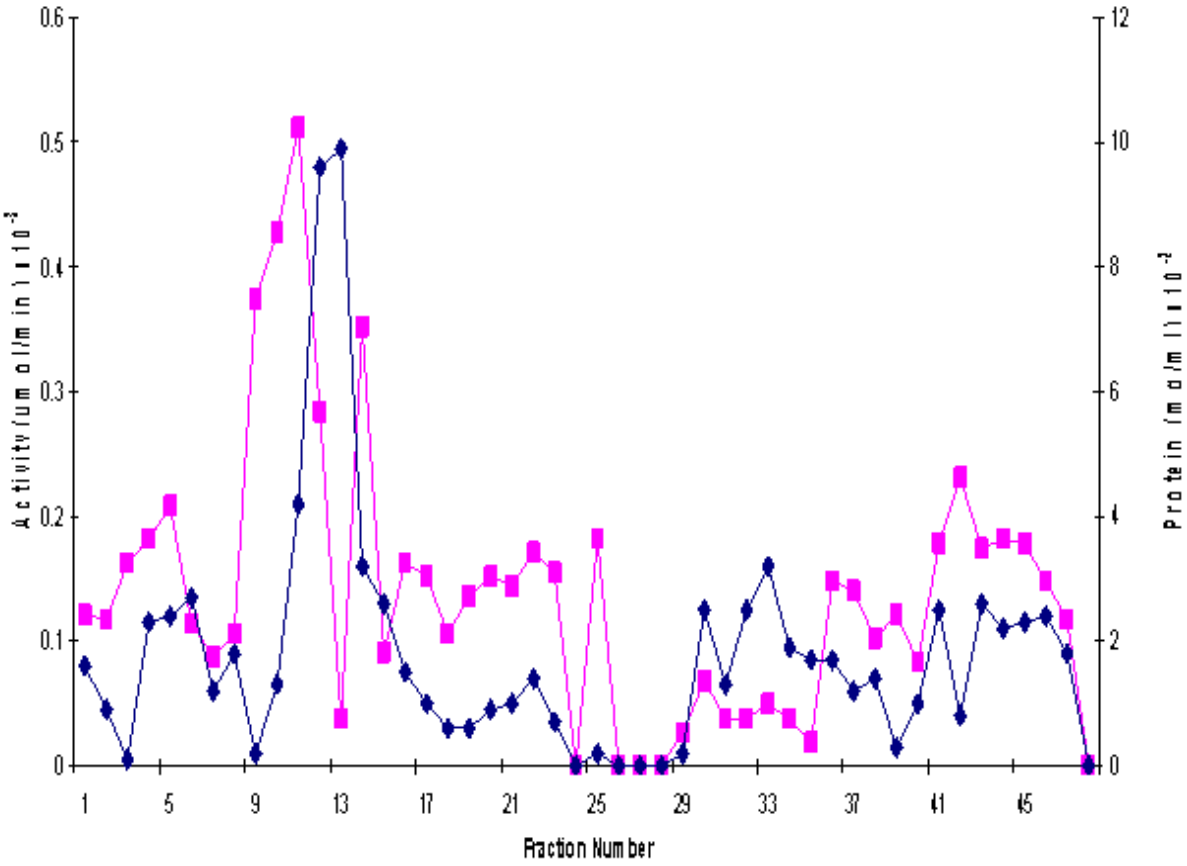

Fig 2: Elution profile of protein and xylanase activity produce by a falvus is sephadex G-75. The (20x50) equabrated with 50mM sodium aoutate buffer (pH5). 5 ml fraction at a flow rate of 0.8 ml/min collected.

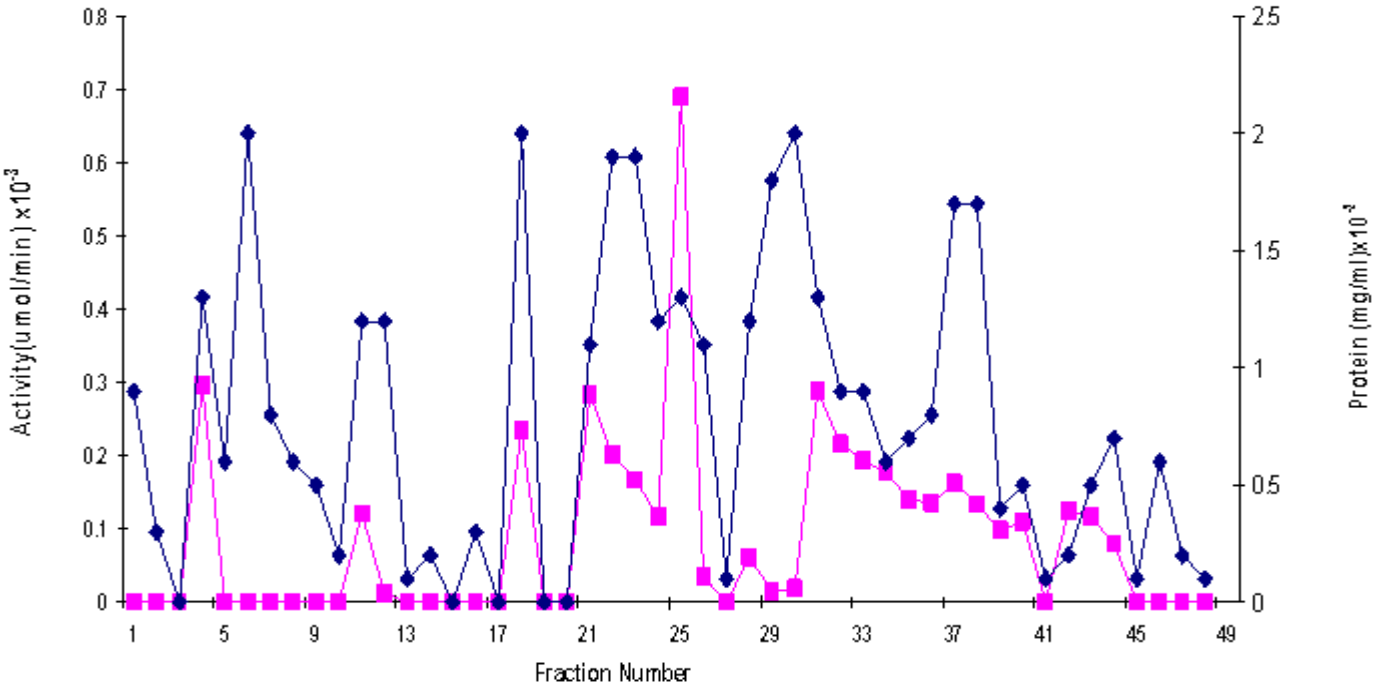

Fig 3: Elution profile of xylanase activity and protein produced by a flavus in DEAE. The colum (2.0x50cm) was prequilibrated with 50mM sodium aoutate buffer (pH5). The column was eluted with NACl gradient (0.0-0.5M)

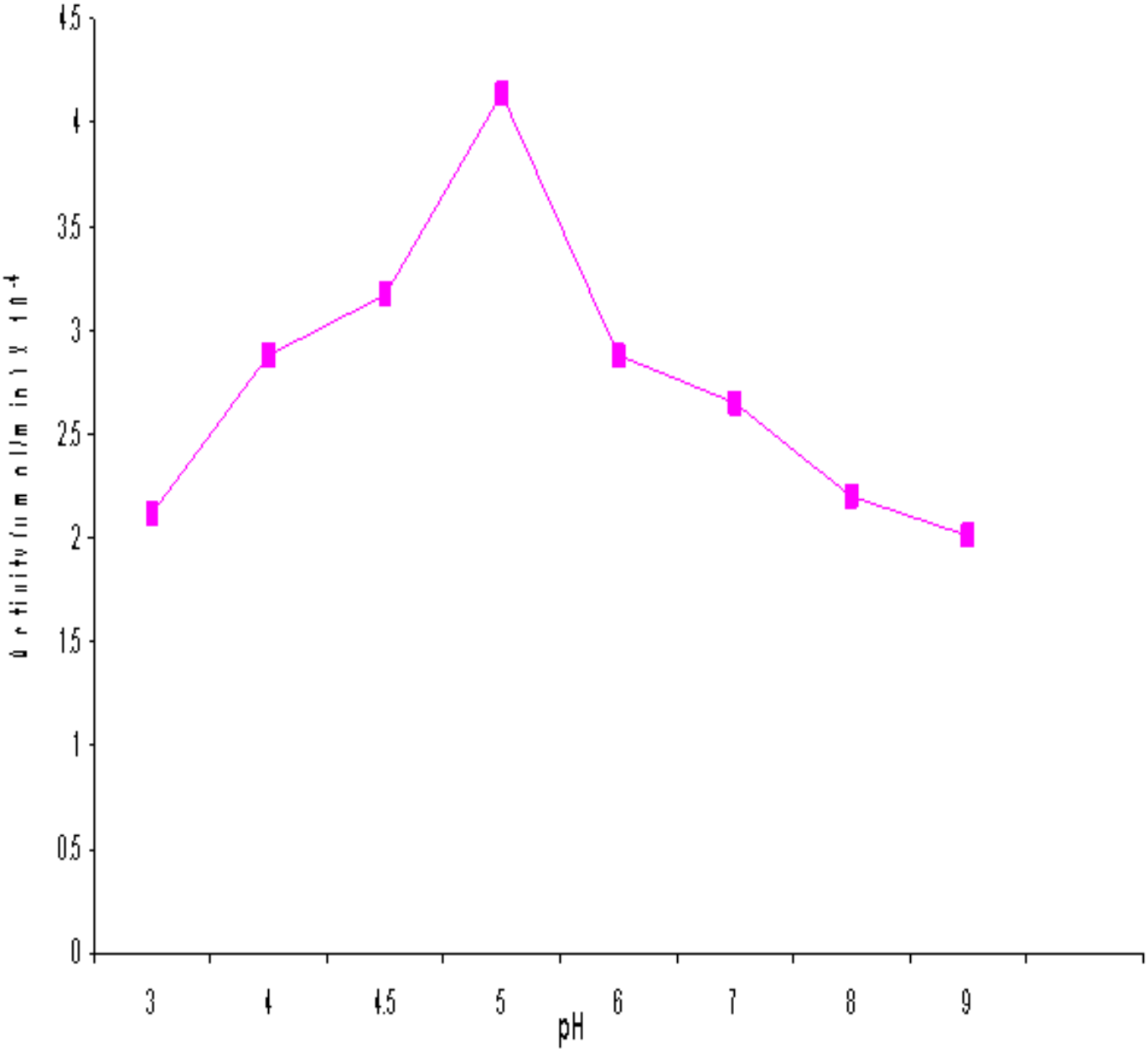

Fig 4: Elect of pH on xylanase produced by A. flavus determined at pH values (pH3-9). The buffers used were 0.1M sodium aoutate (pH3-5), 0.1M sodium phosphate (pH6-7) and 0.1M tris-base (pH8-9)

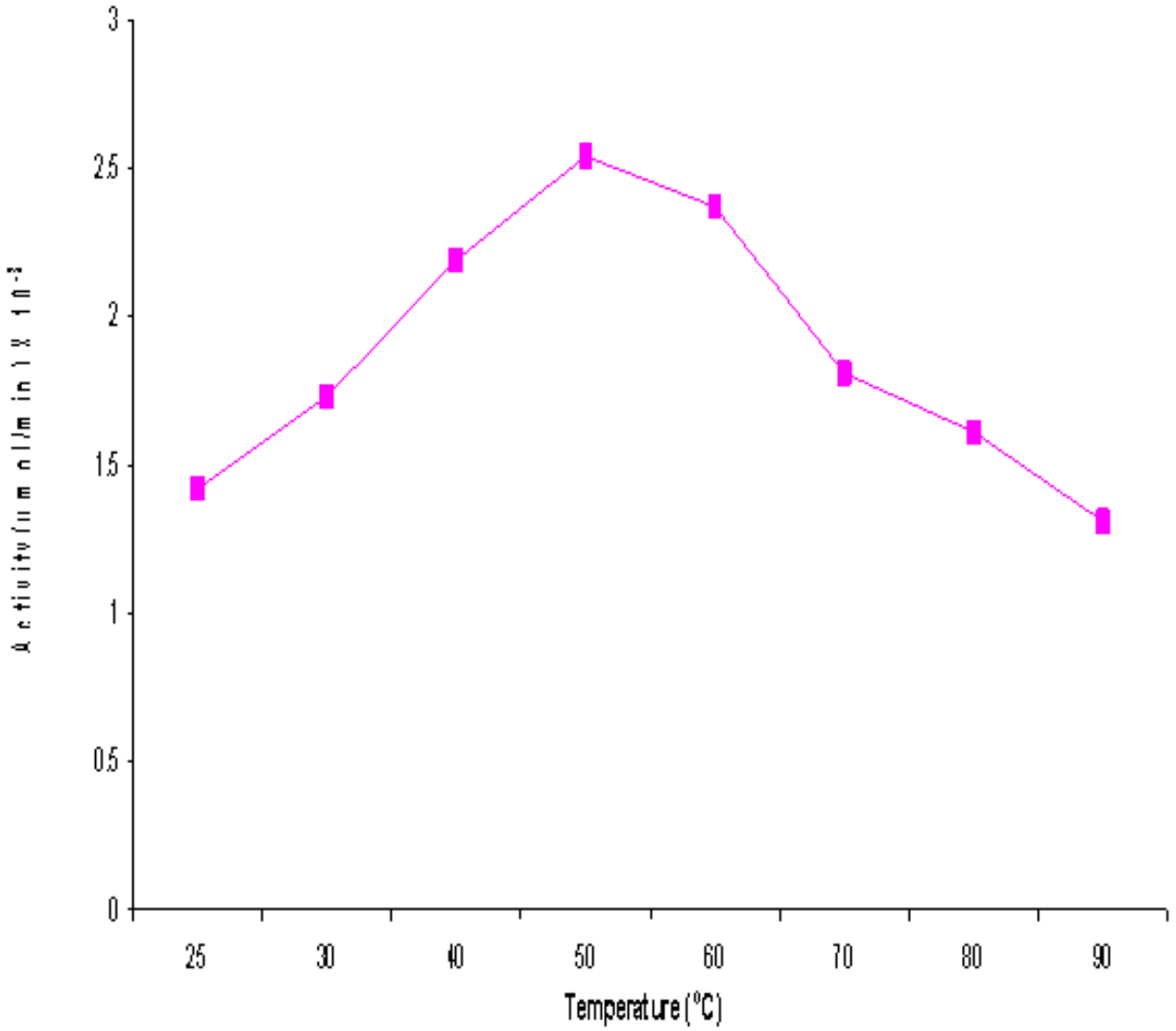

Fig 5: Elect of various temperature ranges on xylanase produced by A. flavus

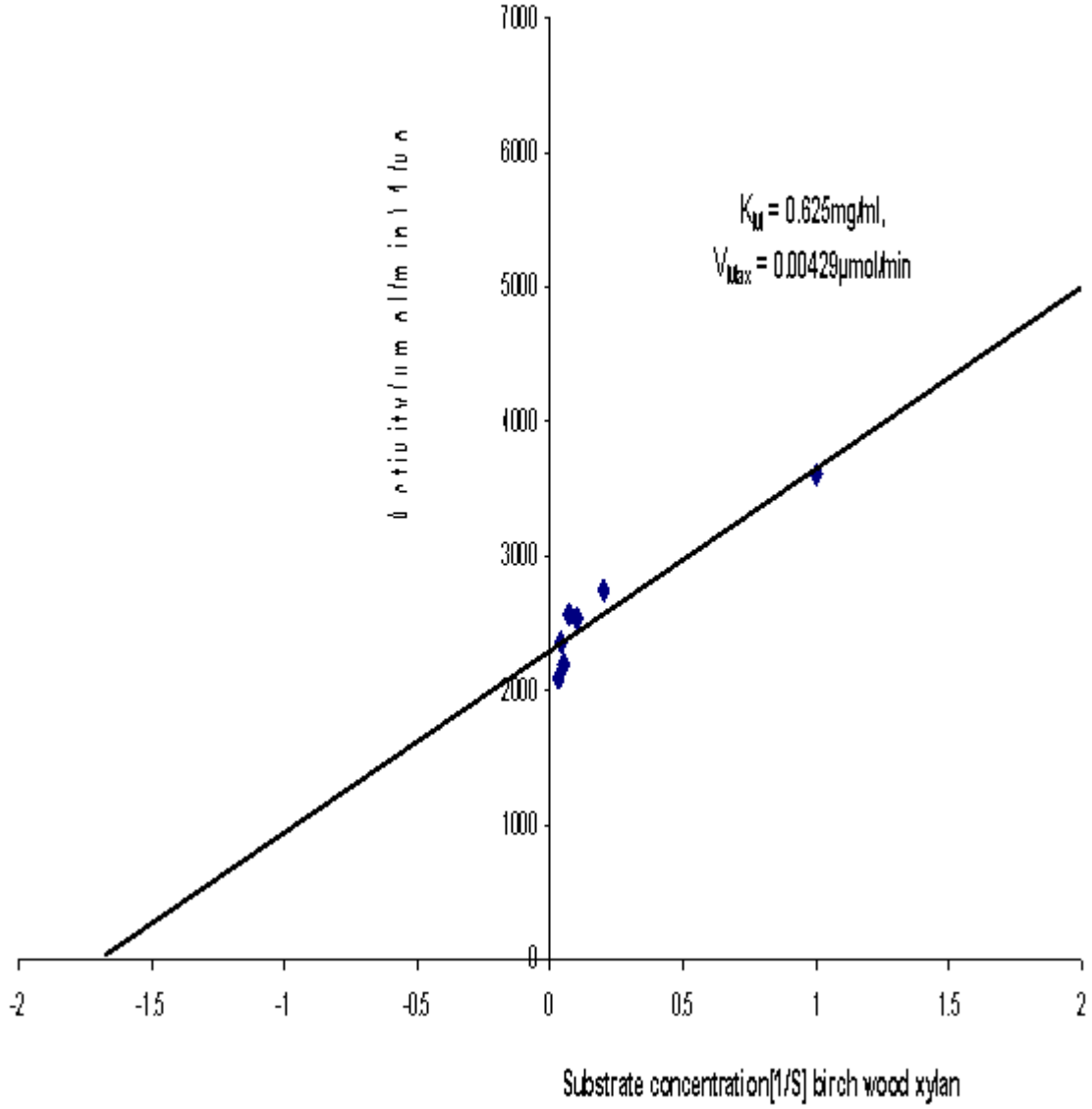

Fig 6: Double reciprocal plot (lineweaver-burk) of purified xylanase from A. flavus

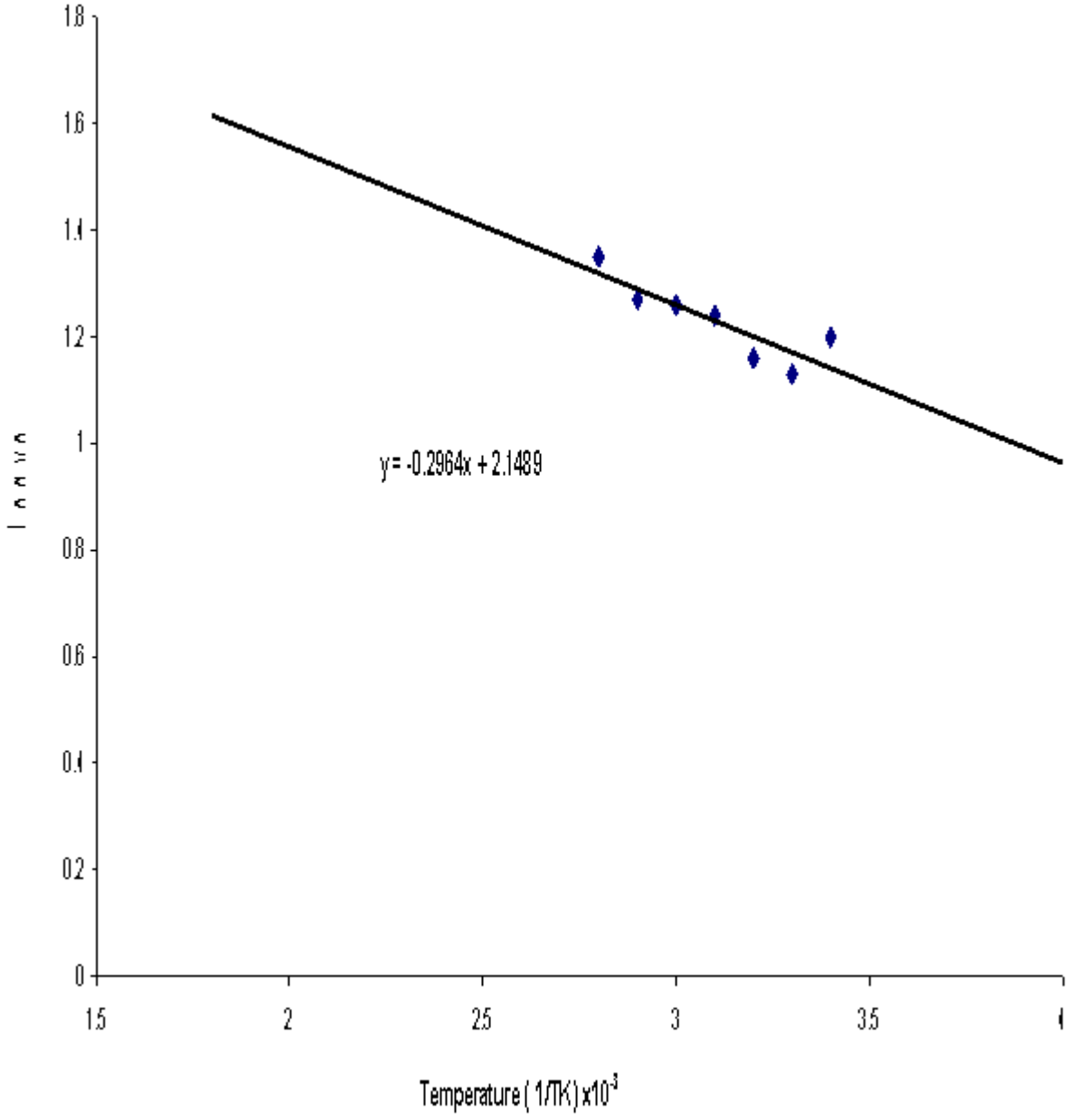

Fig 7: Arthinius plot of partially purified free xylanase produced by A. falvus…

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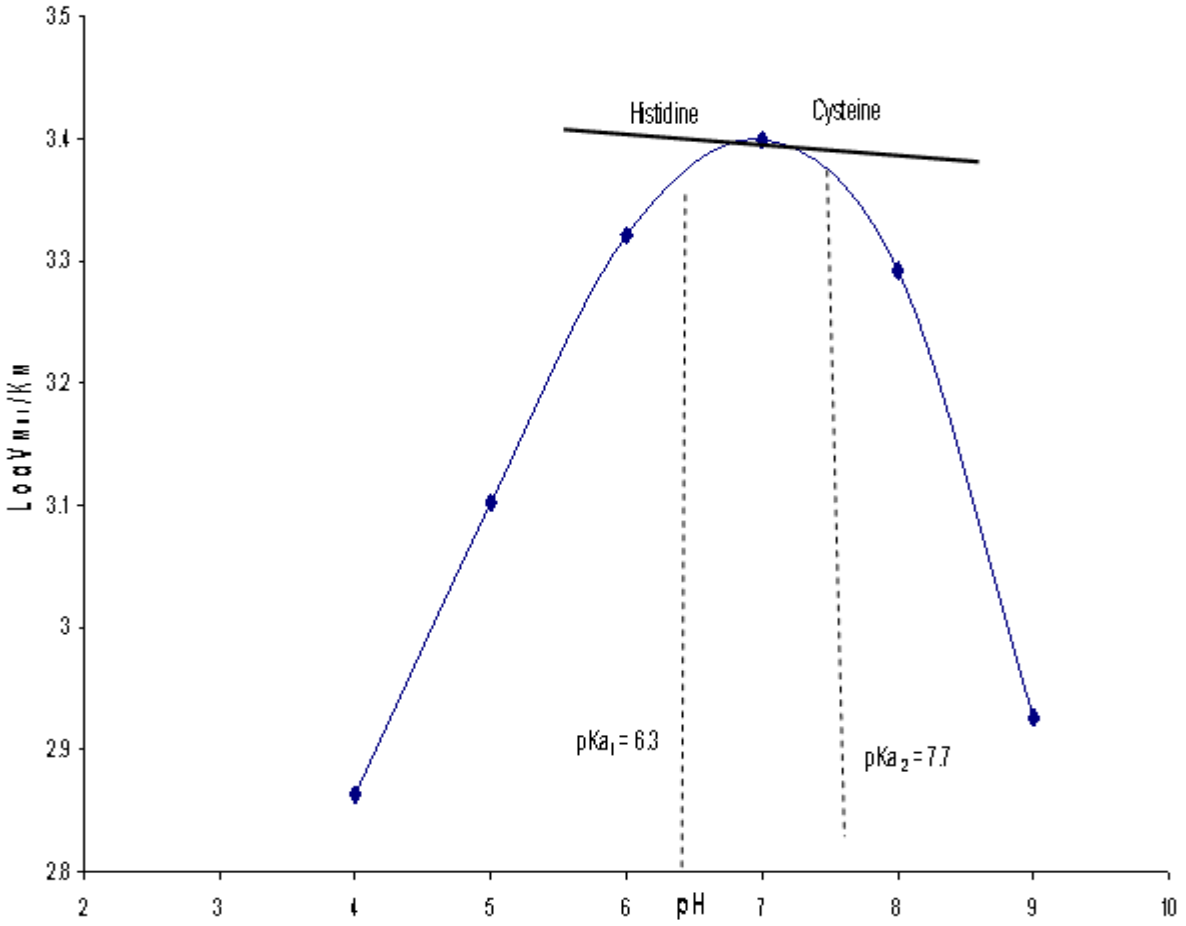


Fig 8: Dixon plot for A flavus xylanase

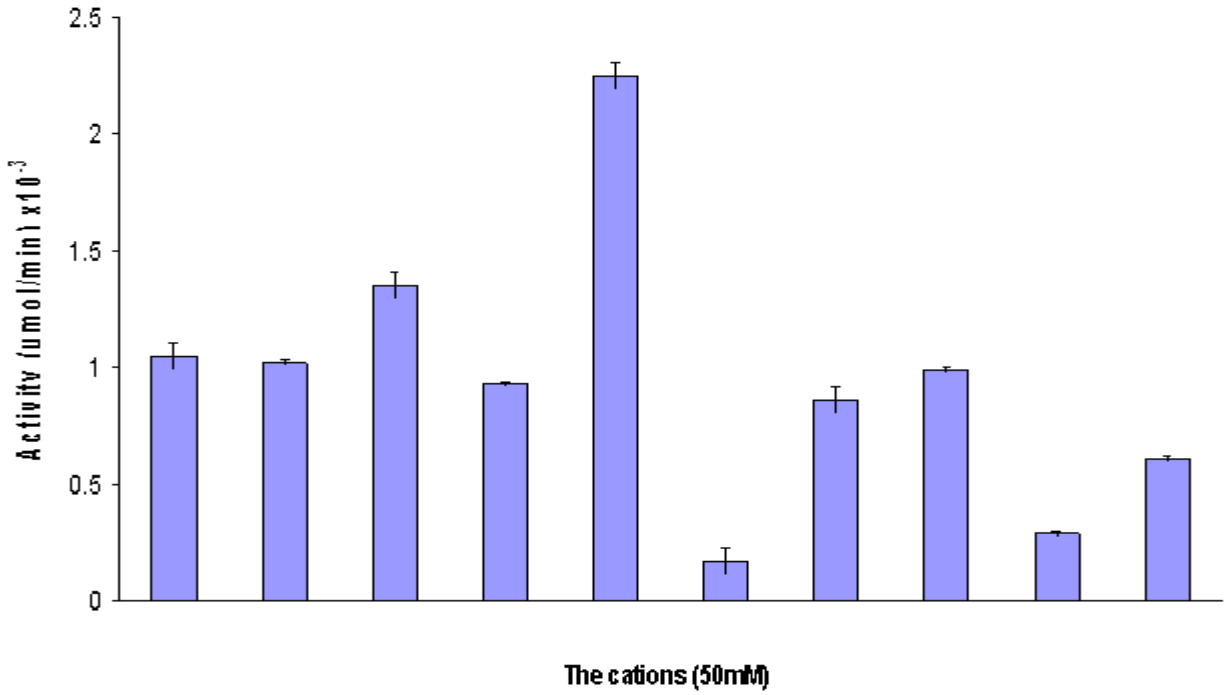
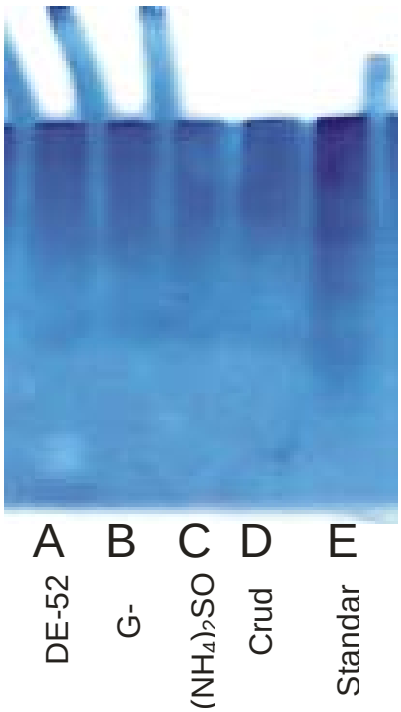


Fig 9: Effect of 50mM of cations on the partially purified xylanase produced by A. flavus. The cations Fe^{2+}, Co^{2+} and Mn^{2+} enhanced the xylanase activity, while Hg^{2+} and Pb^{2+} reduced the activity

Table 1: Summary of purification of xylanase from A. flavus

Steps	Total protein (mg)	Total activity (µmol/min)	Specific activity (µmol/min/mg)	Fold	Yield (%)
Crude	**34.2**	**0.0505**	**0.00147**	**1**	**100**
Ammonium sulphate	**8.1**	**0.0247**	**0.00305**	**2.07**	**48.91**
Protamine Sulphate	**4.88**	**0.0163**	**0.00334**	**2.28**	**32.27**
Gel (G-75)	**0.21**	**0.00256**	**0.0122**	**8.29**	**5.07**
DE-52	**0.065**	**0.00345**	**0.0531**	**36.12**	**4.93**



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