Production and Characterization of Xylanase from *Aspergillus* sp. and its Mutant

By

Muhammad Faheem

160-FBAS/MSBT/F-14

Department of Bioinformatics and Biotechnology

Faculty of Basic and Applied Sciences

International Islamic University, Islamabad

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1. Biochemistry

Production and Characterization of Xylanase from *Aspergillus* sp. and its Mutant

Researcher
Muhammad Faheem
160-FBAS/MSBT/F-14

Supervisor
Dr. Syed Ali Imran Bokhari
Assistant Professor

Department of Bioinformatics and Biotechnology
Faculty of Basic and Applied Sciences
International Islamic University, Islamabad

(2016)

"In the name of ALLAH The Most Gracious and The Most Beneficial"

Department of Bioinformatics and Biotechnology

International Islamic University, Islamabad

Dated: 23/06/2016

FINAL APPROVAL

It is certified that we have read the final thesis entitled **"Production and Characterization of Xylanase from *Aspergillus* sp. and its Mutant"** submitted by **Mr. Muhammad Faheem** and it is our Judgment that this project is of sufficient standard to warrant its acceptance by the International Islamic University, Islamabad for the MS Degree in Biotechnology.

COMMITTEE

External Examiner

Dr. Saleem A. Bokhari
Assistant Professor
Biosciences Department,
COMSATS Institute of Information Technology,
Islamabad, Pakistan

Internal Examiner

Professor Dr. Zafar Mahmood Khalid
Department of Bioinformatics and Biotechnology
International Islamic University, Islamabad

Department of Bioinformatics and Biotechnology

International Islamic University, Islamabad

Supervisor

Dr. Syed Ali Imran Bokhari

Assistant Professor

Department of Bioinformatics and Biotechnology

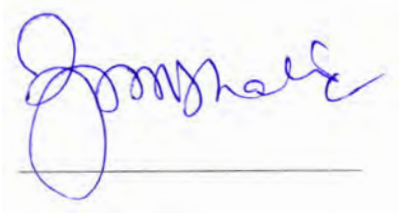
International Islamic University, Islamabad

Chairman

Professor Dr. Zafar Mahmood Khalid

Department of Bioinformatics and Biotechnology

International Islamic University, Islamabad



Dean, FBAS

Professor Dr. Muhammad Sher

International Islamic University, Islamabad

A thesis submitted to Department of Bioinformatics and Biotechnology,
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Master in Sciences of Biotechnology
(MSBT)

This humble effort is

Dedicated

To

My beloved respected

Parents,

Brothers and

Sister

Who inspired me for higher ideals of Life

DECLARATION

I hereby solemnly declare that the work **"Production and Characterization of Xylanase from *Aspergillus* sp. and its Mutant"** presented in the following thesis is my own effort, except where otherwise acknowledged and that the thesis is my own composition. No part of the thesis has been previously presented for any other degree.

Dated: 23/06/2016

Muhammad Faheem

160-FBAS/MSBT/F-14

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ABBREVIATIONS

Abbreviations	**Definitions**
α	Alpha
β	Beta
A. niger	*Aspergillus niger*
BSA	Bovine serum albumin
°C	Degree centigrade
CAZy	Carbohydrate Active enzyme
CDS	Circular dichromism spectroscopy
CFU	Colony forming units
CMCase	Carboxymethylcellulase
DAP	Diammonium phosphate
DG	2-deoxy-D-glucose
E_a	Activation energy
EC	Enzyme code
EDTA	Ethylene diamine tetra acetic acid
FPase	Filterpaperase
g	Gram
g/L	Gram per liter
GH	Glycosidase Hydrolase
h	Hour
IU/g	International Unit per gram
IU protein/mg	International Unit protein per milligram
IU/ml	International Unit per milliliter
IUBMB	International Union of Biochemistry and Molecular Biology
J/K/mol	Joule per Kelvin per mole
K	Kelvin
kb	Kilo base pair
k_d	Denaturation constant
K_m	Michaelis-Menten constant
kDa	Kilo Daltons
kGy	Kilo Grey
kJ/mol	Kilo Joule per mole
mM	Milli molar
mins.	Minutes
Mb	Mega base pair
ml	Milliliter
mg/ml	Milligram per milliliter
nKatal	Nano katal
nkatal/mg	Nano katal per milligram

nKatal/sec	Nano katal per second
NIFA	Nuclear Institute for Food and Agriculture
nm	Nano meter
OD	Optical density
PDA	Potato dextrose agar
pH	Power of hydrogen ion
p*I*	Iso-electric point
psi	Pound per square inch
RSM	Response surface methodology
SDS-PAGE	Sodium dodecyl sulfate poly acryl amide gel electrophoresis
SF	Submerged fermentation
SSF	Solid state fermentation
t_{opt}	Optimum temperature
U/g	Unit per gram
U/mg	Unit per milligram
U/ml	Unit per milliliter
U/ml/min	Unit per milliliter/minute
UV	Ultra violet
µL	Micro liter
µmol/min/ml	Micro mole per minute per milliliter
(w/v)	Weight by volume
(w/w)	Weight by weight
V_{max}	Maximum velocity of enzymatic reaction

ABSTRACT

Aspergillus sp. is a member of the Deuteromycetes fungi, also considered member of the Phylum Ascomycota which can grow in variety of environment producing plant biomass degrading xylanase enzyme. *A. niger* is most commonly found species producing higher xylanase enzyme. Xylanase applications are in the recycling of biomass, paper and pulp industry, in bakery products, for juices clarification, animal feed preparation etc. The current research was conducted for xylanase enzyme production by *Aspergillus niger* and its mutant. For *A. niger* wild type mutagenesis, the γ-rays of 1.0 kilo Gray was found best for enhanced xylanase production. The gamma irradiation was done at Nuclear Institute for Food and Agriculture (NIFA), Peshawar, Pakistan. Mutant was selected by growing irradiated spores on 1 % xylan agar plates. Different growth conditions for both wild and mutant were optimized. It was found that 10 % inoculum size, 2 % corn cob, growth temperature of 30 °C, ammonium nitrate (0.15 %) as nitrogen sources, pH levels 6.0 in Vogel's medium were optimum for xylanase enzyme production through solid state fermentation technique for both wild and mutant. 20-40 % ammonium sulphate precipitation was used for wild and mutant crude enzyme. The overall results showed that 30 % ammonium sulphate concentration was best for *A. niger* wild enzyme precipitation and 20 % ammonium sulphate concentration was best for mutant enzyme precipitation. The partially purified xylanase enzyme from both wild and mutant was used to find out the different kinetics parameters. The results showed that the K_m and V_{max} values for wild xylanase was 23.4 mg/ml and 153.85 nKatal/sec respectively while the K_m and V_{max} values for mutant xylanase was 61.8 mg/ml and 312.5 nKatal/sec, respectively. The results showed that the citrate buffer of pH 6.0 supported the maximum enzyme activity for both wild and mutant. The enzyme activity was observed at different temperatures and 65 °C was optimum for both wild and mutant. The irreversible thermal denaturation was performed from 50 °C to 85 °C, on enzyme activity were determined for wild and mutant. The k_d values was calculated for *A. niger* wild and mutant. When the data was plotted in Arrhenius plot, it was calculated that E_a for denaturation of wild type enzyme was 76.26 kJ/mol and mutant enzyme was 78.08 kJ/mol. The results showed that mutant has no significant effect on production pattern of enzyme but there was significant

variation in kinetic and thermodynamics parameters of wild and mutant enzyme. The higher values of V_{max} showed that mutant has brought various changes in enzyme which resulted in higher activity. The higher value of activation energy (E_a) for mutant enzyme depicted that mutant enzyme requires more activation energy for thermal denaturation as compared to the wild enzyme.

INTRODUCTION

INTRODUCTION

Lignocellulosic wastes are plant biomass like corn cob, wheat bran, wheat straw, rice straw, rice husk, sugarcane bagasse etc. are generated in large quantities through forestry, agricultural practices and industrial processes. These wastes if get accumulated in environment may causes pollution (Abu *et al.* 2000). These wastes can be converted into useful products through biodegradation like substrates for the production of fungal enzymes like xylanases, biofuels, chemicals, fermentation energy sources, animal feeds and human diet (Howard *et al.* 2003).

Lignocelluloses composed of lignin, cellulose and hemicelluloses (xylan) which consists of 20-30 % of plant dry weight (Saha, 2003). The xylan is made up of homopolymeric backbone chain of 1,4-linked β-D-xylopyranosyl units which substituted to varying degrees with glucuronopyranosyl, 4-O-methyl-D-glucuronopyranosyl, α-L-arabinofuranosyl, acetyl, feruloyl and/or p-coumaroyl side-chain groups. In hardwoods wood of O-acetyl-4-O-methyl glucoronoxylan, while in soft woods xylan is composed as arabino-4-O-methylglucorono xylan and in grasses/annual plants xylans are typically arabinoxylans. In hardwood xylan the degree of polymerization is 150-200 β-xylopyranose residues and in softwood xylan the degree of polymerization is 70-130 β-xylopyranose residues (Kulkarni *et al.* 1999). The commonly found structures of xylan in the trees are as follows in figure 1.1 (Ebringerová *et al.* 2005).

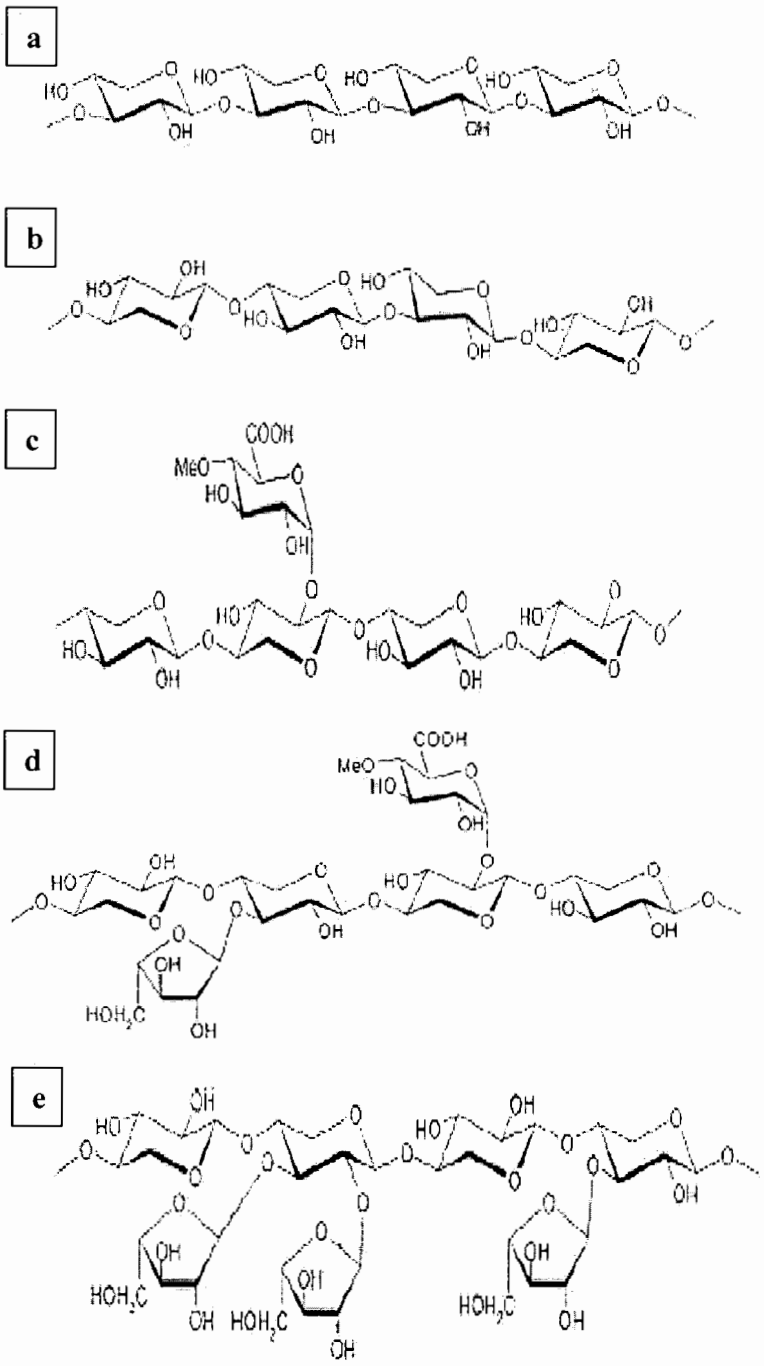


Figure 1.1 Structures of xylan in the trees (a) Homoxylans, Primary structure of X3, β-(1→3), (b) Type Xm, mixed β-(1→3, 1→4)-glycosidic linkages (c) Glucuronoxylans, Primary structure of 4-O-methyl-D-glucurono-D-xylan (d) (Arabino) glucuronoxylan and (Glucurono) arabinoxylan, Primary structure of (L-arabino)-4-O-methyl-D-glucurono-D-xylan (AGX) (e) Arabinoxylans, Primary structure of the water-soluble L-arabino-D-xylan (Ebringerová *et al.* 2005)

Xylans complete degradation is carried out by enzymes known as xylanases (Lee *et al.* 2003) which are diverse. Xylanase enzymes are endo-β-1,4-xylanases (EC 3.2.1.8) and xylosidases (EC 3.2.1.37) which are required for complete hydrolysis of xylan and biomass conversion. The endo-β-1,4-xylanases act on the main chain of xylans and produces short xylo-oligosaccharides while xylosidases produces D-xylose by hydrolyzing xylooligosaccharides (Maciel *et al.* 2008).

Xylanases were first reported in 1955 and originally termed as pentosanases (Whistler and Masek, 1955). In 1961, the "International Union of Biochemistry and Molecular Biology (IUBMB)" recognizes the xylanases and assigned enzyme code EC (3.2.1.8). Different officials names used for xylanase include endoxylanase, endo-1,4-β-xylanase, endo1,4-β-D-xylanase, β-xylanase, β-1,4-xylanase and 1,4-β-D-xylan-xylanohydrolase. The main enzymes involved in catalysis of xylans from different origins include Endo-β-xylanase, 1,4-β-D-xylanxylanohydrolase EC (3.2.1.8) and β-xylosidase, 1,4-β-xylohydrolase, EC (3.2.1.37). Xylanases are single domain proteins having three antiparallel β-sheets and one α-helix. Xylanases from different sources has different characteristics and growth condition like temperature, pH etc. for optimum functioning.

On the basis of reaction catalyzed and products, xylanases are divided into two types depending: debranching enzymes, that hydrolyzes the side branches, releasing arabinose and nondebranching enzymes that do not catalyze the branch points and do not release arabinose (Wong *et al.* 1988). In number of fungi one of these enzymes was found and there are some fungal species which produces both type of xylanases that helps in efficient hydrolysis of xylan. Xylanases on the basis of physio-chemical properties were also classified into two groups: first group of xylanases having low molecular weight (less than 30 kDa) and basic iso-electric point (p*I*) and second group having high molecular weight (greater than 30 kDa) and acidic (p*I*) (Wong *et al.* 1988). More elaborated system named CAZy (Carbohydrate Active enzyme) available now for the classification of enzymes based on the structurally related catalytic and carbohydrates binding domain. According to (CAZy) classification, xylanases comes mainly under glycoside hydrolase families GH 5, GH 7, GH 8, GH 10, GH 11 and GH 43 see (http://www.cazy.org/fam/acc_GH.html).

Different microorganisms like bacteria, yeasts and filamentous fungi such as *Bacillus* sp., *Cryptococcus* sp., *Aureobasidium* sp., *Trichoderma* sp., *Aspergillus* sp., *Penicillium* sp., *Fusarium* sp., *Chaetomium* sp., *Phanerochaete* sp., *Rhizomucor* sp., *Humicola* sp., *Talaromyces* sp. and many more can produces xylanases. Fungi are useful due to diversity for the production of enzymes having novel characteristics (Singh *et al.* 2003). Thermophilic fungi are novel source for the production of thermostable enzymes which are used in many industrial processes (Bruins *et al.* 2001). Among the microorganisms that have xylanolytic activity, *Aspergillus niger* is one of the well known producer (Record *et al.*, 2003; Raana *et al.*, 2004; Maciel *et al.*, 2008). Random mutagenesis can enhance enzyme productivity several fold than the parental strain. These fungi produced xylanase enzymes extracellularly with a wide range of activities from 4-400 IU/ml using various substrates both in submerged and solid state fermentation (SSF) processes (Kapilan, 2015).

Characterization of enzymes are important for their biotechnological applications and different uses. Xylanase enzymes are characterized by different techniques like physiochemical behavior, fluorescence spectrometry, circular dichroism spectroscopy (CDS), matrix-assisted laser desorption ionization-time of flight-mass spectrometry, SDS–PAGE, differential scanning calorimetry (DSC) method (Nath and Rao, 2001). The economics of enzyme production, isolation and purification cost is important. This can be obtained at industrial level by optimizing the fermentation media, using whole cells as sources of enzymes, (Shah and Madamwar, 2005).

Xylanase (EC 3.2.1.8) are important and widely used in the recycling of biomass. Potential applications of xylanase as a bleach boosting agent in paper and pulp industry (Singh *et al.*, 2003; Khandeparkar and Bhosle, 2007). Xylanases usage in pre-bleaching process results in higher brightness ceilings and reduction of bleaching chemicals usage like chlorine upto 20-30 % and organic halogens up to 50 % (Sonia *et al.* 2005).

Xylanases potential applications are: in bakery industry to improve the quality of bakery products like good effects on dough handling, texture, bread volume and stability (Singh *et al.*, 2003; Jiang *et al.*, 2005; Kiachang *et al.*, 2000; Bhat and Hazlewood, 2001). Xylanases can degrades the plant cell wall materials and liberates useful nutrients (Bertichini *et al.* 2009) therefore, used in production of functional food ingredients, in

juices clarification, animal feed and starch separation (Bhat, 2000), in poultry diets for feed utilization and food processing (Walk *et al.* 2011), in agricultural waste conversion and fuel production like ethanol (Damaso *et al.* 2003).

Xylanases are also used in textile industry. Xylanases are applied to treat plant fiber, for example linen and hessian. In plant fiber processing, xylanase free of cellulases can eliminate the strong bleaching step and avoid fiber darkening (Brühlmann *et al.*, 2000; Csiszár *et al.*, 2001).

2.0 AIMS AND OBJECTIVES

The present study is designed to achieve the following objectives:

- To isolate and identify strains of *Aspergillus* sp. from soil samples taken from local vicinity of Islamabad
- Microscopic identification of *Aspergillus niger*
- γ-rays mutagenesis of selected wild-type *Aspergillus* sp. and selection of mutant
- Optimization of xylanase production from *Aspergillus* sp. and its mutant
- Partial purification of xylanase enzymes from *Aspergillus* sp. and its mutant
- Characterization of xylanase enzyme from *Aspergillus* sp. and its mutant

REVIEW OF LITERATURE

REVIEW OF LITERATURE

Kheng and Omer, (2005) isolated and identify USM A1 I as *Aspergillus niger* and selected it for xylanase production on palm kernel cake (PKC) using solid state fermentation system (SSF) technique. Using inoculum size (1×10^4 spores/ml) with moisture ratio of 1:0.75 at ambient temperature (28 ± 3 °C), the xylanase activity was 23.97 U/g PKC. By additional supplementation of carbon source (xylose) at 0.75 % (w/w), the maximum xylanase production obtained were 25.40 U/g and growth of 1.69 mg glucosamine/g PKC. By addition supplementation of nitrogen source ($NaNO_3$) at 0.075 % (w/w), the xylanase production enhanced to 33.99 U/g PKC with growth of 1.67 mg glucosa mine/g PKC. The xylanase production before optimization was 11.72 U/mg glucosamine and after optimization, the xylanase production was 23.12 U/mg glucosamine. The optimized conditions of SSF system increased xylanase production by 157 %.

Krisana *et al.* (2005) isolated fungi strain BCC14405 with thermostable high xylanase production. This was identified as *Aspergillus niger* on the basis of morphological characteristics and internal transcribed spacer (ITS) sequences. The enzyme produced by fungi strain BCC14405 was endo-1,4-β-xylanase due to homology of 20-residue N-terminal protein sequence. Xylanolytic activity by strain BCC14405 was optimum at 55 °C and pH 5.0. The enzyme was purified having molecular mass of 21 kDa. Characterization of enzyme was done using xylan Birchwood, showed *Km* and *Vmax* values of 8.9 mg/ml and 11,100 U/mg, respectively. The activity of enzyme was inhibited by EDTA, $CuSO_4$ and $FeSO_4$. The gene encoding endo-1,4-β-xylanase from BCC14405 was identical to *A. niger* previously identified gene encoding endo-1,4-α-xylanase B.

Corral and Ortega, (2006) studied that plant cell walls composed of polymeric constituents named cellulose, hemicellulose and lignin. Cellulose composed of β-D-glucopyranosyl residues linked by β-1,4-glycosidic bonds: hemicellulose includes xylans, arabinans, glucans and mannans: and lignin, polyphenol complex interconnected with hemicelluloses, forming matrix that surround the cellulose microfibrils. Xylan hydrolysis requires complex enzyme system action including endo-1,4-β-xylanases,

β-1,4-xylosidases and exoxylanases.

Okafor *et al.* (2007) isolated new *Aspergillus niger* ANL301 with high xylanase activity on wood waste. For xylanase production various carbon sources like oat-spelt xylan (Fluka), sawdust, sugarcane pulp and wheat bran were used in the growth media. The xylanase production was monitored after 24 h for a time period of 168 h. The media having oat-spelt xylan gave peak enzyme activity of 0.65 units/ml and specific activity of 3.86 units/mg protein at 120 h. The sawdust media gave 0.80 units/ml of enzyme and specific activity of 3.37 units/mg protein at 120 h. The sugarcane pulp media gave 0.95 units/ml of enzyme and specific activity of 5.69 units/mg protein at 96 h. The media containing wheat bran gave highest xylanase activity of 6.47 units/ml and protein yield of 1.14 mg/ml at 96 h with maximum specific xylanase activity of 9.36 units/mg protein. Wheat bran was found best for low-cost maximum xylanase enzyme production.

Maciel *et al.* (2008) experimented on *Aspergillus niger* LPB 326 for xylanase production on sugarcane bagasse and soybean meal using solid-state fermentation technique. The medium composed of nutrient salt solution (in g/L): $CuSO_4$ 0.4, KH_2PO_4 1.5, $CoSO_4$ 0.0012 with 10 g of sugarcane bagasse and soybean meal in the ratio of 65 % and 35 %, moistened to 85 % of initial water content was incubated for 4 days at 30 °C. The optimum xylanase activity of 3099 IU/g was obtained under these optimized conditions for dry matter.

Bakri *et al.* (2008) isolated newly *Aspergillus niger* S7 and observed xylanase production on different agricultural and industrial wastes using submerged culture technique. The optimum xylanase production of 293.82 IU/ml was obtained on 3 % corn cob hulls (w/v) after 120 h of incubation at pH 7.0.

Ikram-ul-Haq *et al.* (2008) isolated derepressed mutant of *Aspergillus niger* GCBT-35 after treatment with MNNG (N-methyl N-nitro N-nitroso guanidine) and selection of mutant using antimetabolite 2-deoxy D-glucose (2DG). Different growth and cultural conditions was optimized for maximum xylanase production. The growth medium containing (g/L) wheat bran 20.0, $NaNO_3$ 1.0, NH_4Cl 1.0, KH_2PO_4 1.0, $CaCl_2$ 1.0, $MgSO_4.7H_2O$ 0.3, meat extract 5.0 and Tween 80 1.5 ml gave maximum organism growth and enzyme production. After conidial inoculation, 1.0 % (w/v) meat extract as a nitrogen source in the culture medium and 72 h of incubation at initial pH of 5.5, the

xylanase maximum production of 289.86 (U/ml/min) was obtained. Optimization of various growth parameters gave 2.7 fold higher xylanase production from mutant as compared to wild culture.

Ahmad *et al.* (2009) used *Aspergillus niger* for optimum xylanase production on wheat bran using submerged fermentation technique. For optimum xylanase activity, wheat bran concentration (2.5, 3.0 and 3.5 %), fermentation temperatures (25.0, 27.5, 30.0 and 32.5 °C) and initial pH levels (5.0, 5.5, 6.0 and 6.5) and incubation period of 96 h was used. The 3.0 % of wheat bran gave maximum enzyme activity of 78.03 ± 2.73 IU/ml at 30 °C, pH 5.5 and 72 h incubation period. The enzyme activity of 66.07 ± 1.94 IU/ml was obtained on 3.5 % of wheat bran concentration at 30 °C, pH 5.5 and after 72 h incubation period. The maximum enzyme activity at 2.5 % of wheat bran concentration obtained was 64.47 ± 2.75 IU/ml at 30 °C, 5.5 pH after a period 72 h of incubation.

Kavya and Padmavathi, (2009) isolated the *Aspergillus niger* from garden soil for xylanase enzyme production on cheaper sources like wheat bran, rice bran, soya bran, ragi bran and saw dust using solid state fermentation. The enzyme production of (9.87 U/ml) was obtained on wheat bran with unoptimized condition. Different growth parameters were optimized and maximum enzyme production of (12.65 U/ml) was obtained by using wheat bran 10 g per 250 ml conical flasks, with a mineral solution as a moisturizing agent and moisture ratio 1:0.7 at 28 °C after 6 days of incubation. The study results proved that *A. niger* used is useful for high xylanase production.

Irfan *et al.* (2010) observed the enzyme production from *Aspergillus niger* on Mendel and Weber media having sugarcane bagasse using submerged fermentation technique. Sugarcane bagasse was treated with the various concentrations of Na_2SO_4 (0.5-10 % w/v), Na_2SO_3, H_2O_2 (1-5 % v/v) and H_2O_2 + 2 % NaOH. Maximum enzyme production 65.8 IU/ml was obtained on sugarcane bagasse treated with H_2O_2 + 2 % NaOH after 72 h of incubation period. Enzyme production with 1 % w/v Na_2SO_3 treated bagasse was CMCase 26.4 IU/ml, FPase 15.7 IU/ml , respectively.

Yang *et al.* (2010) experimented xylanase production from *Aspergillus niger* C3486 on medium containing D-xylose. The enzyme was purified by disc acrylamide gel electrophoresis using DEAE-Sephadex A-50 and Sephadex G-100 column chromatography having molecular mass of about 25 kDa. The xylanase was purified

14.79 fold with 29.88 % recovery. The enzyme activity was optimal at temperature 55 °C and pH 5.5. The xylanase enzyme was characterised, showed the K_m value and V_{max} of 0.104 mg/ml and 24.8 µmol/min/mg, respectively. The effect of various metal ions was checked on enzyme activity. The Hg^{2+} and Cu^{2+} showed inhibitory effects, while Mg^{2+} and Cr^{3+} had small stimulating effects on the enzyme activity. The zymogram analysis proved this enzyme an active xylanase having activity only on the xylan.

Dai *et al.* (2011) used *Aspergillus niger* JL-15 for optimized production of xylanase XylA on orange peel using solid-state fermentation (SSF) by the response surface methodology (RSM). The growth conditions like various glycerin and ammonium sulphate concentrations, moisture content, fermentation time was optimized which gave significant effects on the XylA production ($P < 0.05$). The maximum xylanase enzyme production of 917.7 U/g (dry fermentation product) was obtained on 4.2 % glycerin, 3.1 % $(NH_4)_2SO_4$, 61 % moisture content, at 55 °C and pH 5.0 for 73.4 h fermentation period, respectively. This enzyme production was 3.2 folds higher than that of the basic medium enzyme production of 218.5 U/g. The XylA enzyme was purified by SDS-PAGE having molecular mass of about 30.0 kDa. XylA was characterized showed the K_m and V_{max} values of 9.24 mg/ml and 54.05 µmol/min/ml, respectively.

Tallapragada and Venkatesh, (2011) isolated xylanase producing *Aspergillus niger* in pure form from garden soil sample and further selected by congo red test. The xylanase enzyme production was obtained in czapekdox medium using different carbon and nitrogen sources. The optimal xylanase activity was obtained on xylan (as a carbon source), yeast extract (as a nitrogen source), at pH of 8.0 and temperature of 28 °C, respectively. The study results proved that *A. niger* used was useful for xylanase production.

Soliman *et al.* (2012) observed the production of xylanase (E.C 3.2.1.8) from *Aspergillus niger* and *Trichoderma viride* on different lignocellulosic substrates using Solid State Fermentation (SSF). Farametric optimization of xylanase by *A. niger* and *T.viride* gave 11.1 and 6.9 fold increase on barely bran as compared to rice straw as initial solid substrate .Maximum xylanase production from *A. niger* and *Trichoderma viride* was obtained on Barley Bran (BB) as compared to other substrates. When *A. niger*

and *T. viride* grown on 1.0 g barley bran in absence of additive nitrogen source with moisture levels of 1:3 (75 %), at pH 5.5 and 35 °C after 2.0 days incubation, the xylanase production by *A. niger* was higher as compared to *T. viride.*

Jin *et al.* (2012) experimented the xylanase production from thermophilic *Aspergillus niger* strains. The xylanase production was optimal at pH of 5.0 and temperature of 55 °C, respectively. The highest xylanase activity of 2762 U/g dry matter was obtained with inoculum concentration of 1.5 x 10^5 CFU/g dry matter with culture medium containing 30 % wheat bran, 70 % corncob, 1 % NH_4NO_3 with solid medium and water ratio of was 1:1.2. The enzyme was characterized and enzyme activity was stable at a pH range of 3.0 to 9.0. The xylanase enzyme activity was stable at 50 °C for 30 min, when it was kept at 85 °C for 30 min 55 % enzyme activity left.

Dobrev & Zhekova, (2012) isolated and purified endo-xylanase using ultra filtration, anion exchange chromatography and gel filtration from *Aspergillus niger* B03. The enzyme is nonglycosylated protein having molecular weight of 20,000 Da determined by SDS-PAGE and 21,000 Da as determined by gel filtration. The enzyme activity was optimum at pH of 5.0 and temperature of 55 °C. The effect of various metals ions on enzyme activity showed that Mn^{2+} and Co^{2+} activated the enzyme activity while Ag^{+}, Cu^{2+}, Fe^{3+}, Fe^{2+} and Pb^{2+} inhibited the enzyme activity. The enzyme stability was improved significantly by presence of glycerol and sorbitol. The enzyme substrate specificity and xylan hydrolysis suggested it an endoxylanase.

Ahmad *et al.* (2013a) observed xylanase enzyme production from *Aspergillus niger* using sugar cane bagasse through submerged fermentation technique. Different concentration of sugar cane bagasse (2.5, 3.0 and 3.5 %), fermentation temperatures (i.e. 25.0, 27.5, 30.0 and 32.5 °C) and initial pH levels (5.0, 5.5, 6.0 and 6.5) and incubation time period of 24, 48, 72, 96 h were used for maximum production of xylanase enzyme. The maximum enzyme activity of (44.00 ± 1.45) was obtained on medium having 3.0 % sugarcane bagasse, pH 5.5, at 30 °C with 72 h of incubation period. The maximum enzyme production of (42.00 ± 0.29) was obtained on 3.5 % at 30 °C, 5.5 pH after incubation period of 72 h while on 2.5 % concentration maximum enzyme production was 39.07 ± 0.55 at 30 °C, 5.5 pH after incubation period of 72 h.

Ahmad *et al.* (2013b) investigated the xylanase production by *Aspergillus*

niger in submerged fermentation. The enzyme purification and characterization was determined under different conditions like pH, temperature and heat stability. The molecular mass of enzyme was determined through SDS-PAGE and found 30 kDa. Two partial purification steps comprising of ammonium sulphate precipitation and Sephadex gel filtration column chromatography raised the specific activity of the enzyme from 41.85 to 613.13 with 48.63 % yield. The partially purified enzyme activity was found optimum at temperature of 60 °C and pH of 7.5.

Izidoro and Knob, (2014) evaluated the xylanase production by *Aspergillus niger* strain using various agro-industrial wastes. Xylanase activity was found best on brewer's spent grain in liquid Vogel's medium with pH of 5.0, at 30 °C of fermentation temperature during 5 days of incubation. The enzyme was characterized and enzyme activity was found optimum at 50 °C, with a half-life of 240 mins. The enzyme activity was optimum at pH of 5.0 and remain stable at pH ranging from 4.5 to 7.0. The study proved that brewer's spent grain is abundantly available low-cost substrate which can be used for xylanase production.

Subbulakshmi and Iyer, (2014) isolated xylanase producing *Aspergillus niger* from soil sample collected from the garden. The maximum release of the sugar into the medium was analyzed under different conditions like different temperature, pH, substrate and agitation. Heavy growth and high enzyme activity were found at pH 8.0, incubated for three days at 30 °C in minimal broth kept under agitation. The enzyme stability at different temperature and pH was observed, by analyzing the hydrolytic action of xylanase on xylan. The xylanase enzyme activity was found stable at temperature of 40 °C and pH of 8.0.

Kanimozhi and Nagalakshmi, (2014) investigated the xylanase production by *Aspergillus niger* using various substrates such as wheat bran, paddy straw, sugarcane bagasse and saw dust via solid state fermentation technique. The maximum xylanase production of 1.4 U/ml was obtained on wheat bran in growth medium containing xylose (as carbon source) and $NaNO_3$ (as nitrogen source) at 30 °C and initial pH 6.

Sharma *et al.* (2015) isolated 90 fungal strains from different sources like compost, decaying wood, agricultural and other lignocellulosic wastes etc., for xylanase production. Xylanolytic activity was observed from 15 fungal strains during submerged

fermentation. The maximum xylanase production of (59.5 U/ml) was obtained from fungal strain S9, followed by S20 (49.75 U/ml), S75 (40.75 U/ml) and S58 (40.5 U/ml). The isolated fungal strain S9 was identified as *Aspergillus* sp. (*Aspergillus* sp. *S9*). The xylanase enzyme from *Aspergillus* sp. *S9* was characterized for different kinetic parameters. The xylanase production and activity was observed under different temperatures ranging (50 to 90 °C) and broad pH range (6.0 to 11.0). The xylanase production and activity was optimum at temperature of 80 °C, pH of 9.0 after fermentation time period of 144 h, respectively. Conclusively, the *Aspergillus sp.* S9 xylanase enzyme have potential for industrial applications.

MATERIALS AND METHODS

MATERIALS AND METHODS

The major part of this work was performed at Biotechnology Lab, International Islamic University, Islamabad, Pakistan.

3.1 (a) Chemicals and glassware

Sigma Aldrich Company USA, Bio world company, Daejung company Korea, Lab M UK chemicals were obtained and used in the studies. Chemicals such as agar, dextrose, antibiotic (tetracycline), ethanol etc. were used. Glassware included spreader, beaker, flask, pipettes, tips, test tubes, petri dishes, microscopic slides and cover slips etc.

3.1 (b) Instruments

Instruments such as laminar flow hood, autoclave, spectrophotometer, incubator, centrifuge, water bath and microscope were used.

3.1.2 Collection of sample

The soil samples were collected from different places (H-10, I-10 and G-10) in Islamabad, Pakistan. During sampling pre-sterilized sample bottles and sterile spatula were used for sample collection. Precautionary measures was taken to minimize the contamination.

3.1.3 Fungus isolation

The soil samples collected were further used for isolation of xylanase producing *Aspergillus* sp. fungi by using following techniques.

3.1.4 Potato dextrose agar (PDA) preparation

Potato dextrose agar was used for the cultivation of fungi. Potato dextrose agar medium encourages luxuriant fungal growth. The contents of PDA medium are shown in table 3.1. PDA medium was prepared from potatoes, dextrose and agar. 200 grams of scrapped and sliced potato were boiled and the potato extract was obtained. Afterwards, it was filtered through cheese cloth and stored in bottle. To make 200 ml of PDA medium, 100 ml of potato infusion, 1.5 grams of agar and 2 grams of dextrose were added into 100 ml of distilled water in flask.

Table 3.1 Contents of potato dextrose agar medium

Ingredients (for 200 ml PDA medium)	**Concentration**
Agar	1.5 g
Dextrose	2.0 g
Potato infusion	100 ml
Distilled water	100 ml

3.1.5 Sterilization

Petri dishes, pipette tips, glass spreader, loop, 0.9 % saline solution and flask (containing PDA medium) were sterilized by autoclaving at temperature of 121 °C under 15 psi pressure for 15 minutes.

3.1.6 Petri plates preparation

Antibiotic (Tetracycline 0.25 mg/ml of medium) was added in the autoclaved medium to prevent bacterial growth. Afterwards, autoclaved medium in flask was poured into sterilized petri plates and allowed to cool and solidify in laminar flow hood.

3.1.7 Serial dilution

In this technique six test tubes were taken and 9 ml of sterilized 0.9 % saline solution was added in each test tube. One gram of soil sample was added in first test tube (10^0) containing 9 ml of sterilized 0.9 % saline solution. After mixing thoroughly, 1 ml from first test tube was transferred to second tube (10^{-1}) containing 9 ml of sterilized 0.9 % saline solution and this process was carried up to 6^{th} test tube (10^5) which formed 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} times dilutions.

3.1.8 Plating

0.1 ml portion from each dilution (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5}) was pipetted on PDA solidified medium on a separate petri dish labeled with dilution factor and spread evenly throughout by sterilized glass spreader to get desired number of colonies.

3.1.9 Incubation

The petri plates inoculated with sample were then placed into incubator at 30 °C in an inverted position. All plates were examined on daily basis.

3.2 Purification

In microbiology, this technique is used to isolate a pure strain (colony) from mixture of colonies of different fungi. Using a sterilized loop single colony (showing same coloured spore) from previously incubated petri plates showing growth were picked and streaked on a new PDA plate and then placed into incubator at 30 °C. This process was repeated again and again until uniform colonies of fungus were obtained.

3.2.1 Identification

On the basis of cultural and microscopic characteristics reported by Watanabe, (2002), the fungus identification was done.

Microscopic slides preparation

One drop of sterilized distilled water was dropped on microscopic glass slide by micro pipette. A small portion of specimen from uniform fungal culture was taken with help of sterilized loop and then placed on microscopic slide and covered with a cover slip very carefully. The slide was examined under microscope at 4x, 10x and 40x magnification. These steps were repeated for all fungal cultures isolated. Potato dextrose agar (PDA) plates and slants were used to maintain pure cultures of *Aspergillus* sp. at 4 °C. Culture of *Aspergillus* sp. was refreshed fortnightly. The culture purity after growth was checked under the microscope.

3.3 Mutagenesis and selection

Spore suspension of *Aspergillus* sp. wild type was maintained at approximately (3.0×10^6 spores/ml) and transferred to eppendorf tubes of 2 ml vials for gamma irradiation. The prepared spore suspension were treated with different doses of γ-rays (0.8, 1.0 and 1.2 kGray) in γ-cell radiation chamber from NIFA (Nuclear Institute for Food and Agriculture), Peshawar, Pakistan. The irradiated spores were plated and plates with 3 log kill were used for screening of potential mutant. The potential mutant were grown on 1 % xylan agar plate and the colony which formed larger hallow was selected for further studies (Rajoka *et al.* 1997). The mutant appeared on the 1 % xylan plate were stained with 2.5 % saferanin in order to see the hallow formation (Gohel, 2014). The *Aspergillus* sp. mutant selected was properly labelled. Mutant culture of *Aspergillus* sp. was refreshed fortnightly. The culture purity after growth was checked under the microscope. The selected mutant was grown on PDA plates and slants for preparation of

spore suspension. Inoculum preparation for mutant was done in similar way as described below for the *Aspergillus* sp. wild type organism.

3.4 Preparation of inoculum

For the preparation of inoculum, the Vogel's medium was used. The Vogel's medium contents are given below in table 3.2.

Table 3.2 Contents of Vogel's medium per 100 ml

Sr. No.	Chemicals/Reagents	Quantity
1	Distilled water	100 ml
2	Trisodium citrate	0.5 g
3	Potassium Di-hydrogen phosphate	0.5 g
4	Ammonium sulphate	0.4 g
5	Ammonium nitrate	0.2 g
6	Magnesium sulphate	0.02 g
7	Yeast extract	0.1 g

The medium contents: 0.5 % trisodium citrate , 0.5 % KH_2PO_4, 0.4 % $(NH_4)_2SO_4$, 0.2 % NH_4NO_3, 0.02 % $MgSO_4.7H_2O$ and 0.1 % yeast extract were added one by one in 100 ml distilled water in a conical flask to prevent any precipitation. The Vogel's medium flasks were sterilized by autoclaving at 121 °C, 15 psi pressure for 15 minutes. After sterilization, Vogel's medium flasks were transferred inside laminar flow hood for cooling at the room temperature. Spore suspension of *Aspergillus* sp. and mutant was prepared from 5-7 days-old slants. 10 ml of 0.9 % sterilized saline water was added into 5-7 days-old slants tubes with growth. The growth surface of slants was scratched with sterilized wire loop followed by shaking of spores. From this tube, 1 ml of spore suspension (containing almost 10^7 spores/ml determined with haemocytometer) was used for inoculum preparation. The spore suspension was aseptically transferred to each 150 ml flasks containing 10 ml of Vogel's medium. These flasks were then incubated

inside incubator at 30 °C for 48 h.

3.5 Substrates

Carbon sources like wheat bran, rice husk, corncob and dab grass was utilized as substrates for the biosynthesis of xylanase enzyme by wild and mutant strains. The substrates was purchased and collected locally. The substrates were dried and ground. The prepared samples were stored in air tight containers separately, for their further utilization in the xylanase synthesis. Carbon sources were added at the rate of 2 % (w/v) into 150 ml flasks containing 10 ml of Vogel's medium. These flasks were autoclaved and after sterilization, 1 ml of uniform spore suspension (determined through haemocytometer) from wild or mutant culture inoculum was inoculated separately inside laminar flow. These flasks were then incubated inside incubator at 30 ± 1.0 °C for 48 h.

3.5.1 Production of xylanase

The *Aspergillus* sp. wild type and mutant batches were grown on different carbon sources for xylanase production using solid state fermentation technique according to the modified Kang *et al.,* (2004) method. Erlenmeyer flasks (150 ml) with a working volume of 10 ml was used for the production of xylanase from both wild type and mutant organism. Briefly, 2 g substrate was added in 150 ml Erlenmeyer flask having 10 ml of Vogel's medium (0.5 % trisodium citrate, 0.2 % NH_4NO_3, 0.5 % KH_2PO_4, 0.4 % $(NH_4)_2SO_4$, 0.02 % $MgSO_4.7H_2O$, 0.1 % yeast extract) to maintain the desired moisture level (i.e. 65 %). The flasks were then autoclaved at 121 °C for 15 minutes and transferred inside laminar flow. 1 ml of spore suspension (3.0×10^6 spores/ml) from both wild and mutant was added into each sterilized flask, mixed and incubated at 30 °C for 5 days under static condition. After incubation and growth, 50 ml of citrate buffer (pH 6.0) was added to the flask and extract was taken by filtering the crude solution through cheese cloth. Afterwards, enzyme and protein assays were performed for both *Aspergillus* sp. wild and mutant extracts.

3.5.2 Optimization of inoculum size

Spore suspension of (3.0×10^6 spores/ml) of *Aspergillus* sp. from both wild and mutant was used as inoculums. To optimize inoculum size 5 %, 10 %, 15 % and 20 % of the inoculum was used. The spore suspension showing maximum yield of xylanase was selected for wild and mutant separately.

3.5.3 Selection of carbon source

Different substrates including wheat bran, rice husk, corn cobs and dab grass was used as substrate. The substrate showing maximum xylanase production was selected for wild and mutant separately.

3.5.4 Optimization of carbon source concentration

Different concentrations including (2.0, 3.0 and 3.5 %) of selected carbon source was used to optimize the production of xylanase for both wild and mutant. The substrate concentration giving maximum xylanase production was selected for wild and mutant.

3.5.5 Optimization of nitrogen source

Various nitrogen sources including organic and inorganic were used to enhance the xylanase production for both wild and mutant. Urea, DAP, Ammonium nitrate and Ammonium sulphate was used as a nitrogen source. Each nitrogen source was used in such a way to maintain 0.15 % nitrogen concentration in the Vogel's medium replacing standard nitrogen sources i.e. NH_4NO_3 and $(NH_4)_2SO_4$ source. The nitrogen source supporting maximum xylanase yield was selected for further studies.

3.5.6 Optimization of temperature

For temperature optimization, both wild and mutant were grown on different temperatures (25, 28, 30, 32 and 35 °C) for maximum xylanase production on optimized carbon and nitrogen sources. The temperature supporting maximum xylanase yield was selected for further studies.

3.5.7 Optimization of pH

After optimization of carbon, nitrogen sources and temperature, the xylanase production for both wild and mutant was carried out in different pH (4.0 to 7.0) citrate buffers for optimum enzyme production. The pH buffer supporting maximum xylanase yield was selected for further studies.

3.6 Protein estimation

The total protein estimation was done as described by the Bradford method microassay. 100 µl of appropriately sample was added to 1 ml Bradford reagent (Kruger, 1994). The reactions time was given by incubating the blank and test samples at 45 °C for 15 minutes inside the incubator. After reaction time, the OD from

spectrophotometer was taken for blank and each test sample of wild and mutant. The estimation of protein in samples was done against standard curve (See Appendix I).

3.6.1 Preparation of Bradford reagent

The Bradford reagent for protein assay was prepared by different ingredients (Kruger, 1994) were as follows in table3.3

Table 3.3 Contents of Bradford reagent

Sr. No.	Chemicals/Reagents	Quantity
1	Distilled water	1000 ml
2	Comassie Blue G 250	100 mg
3	Ethanol (95 %)	50 ml
4	Phosphoric acid (85 %)	100 ml

The above ingredients were added into distilled water one by one and then followed by continuous stirring until a clear solution was obtained. The prepared Bradford solution was stored in an amber color bottle inside refrigerator at 4 °C.

3.7 Xylanase assay

Xylanase assay was performed according to Bailey *et al.* (1992) by taking 0.5 ml of enzyme solution, 0.5 ml of substrate solution of 1 % (w/v) xylan and 1 ml of 10 mM phosphate buffer, pH 6.0 in a 5 ml glass test tube. The mixture solution test tube was incubated at 45 °C for 15 mins. inside the incubator. After incubation, 1 ml of 3,5-dinitrosalicylic acid was added in reaction mixture tube in order to assayed the xylose released. The blank was prepared in similar way as like test sample with a difference that distilled water was added in place of enzyme solution. The blank and sample test tubes were kept in boiling water for 5 minutes. After boiling time, the tubes were allowed to cool at room temperature. The blank and test sample absorbance was taken at 550 nm (Miller, 1959) on spectrophotometer against xylose as standard.

3.7.1 Preparation of DNS (Dinitrosalicylic acid) solution

The DNS solution for enzyme assay was prepared by different ingredients (Miller, 1959) as follows in table 3.4.

Table 3.4 Contents of DNS solution

Sr. No.	Chemicals/Reagents	Quantity
1	Distilled water	1000 ml
2	Rochelle salt (sodium potassium tartrate)	182 g
3	3.5 Dinitrosalicylic acid	10 g
4	NaOH	10 g
5	Phenol (melted at 60 °C)	2 g
6	Sodium meta bisulphate	0.5 g

The above ingredients were dissolved in distilled water one by one and then followed by continuous stirring until a clear solution was obtained. Afterwards the solution was stored in refrigerator in an amber color bottle to avoid photo oxidation. The estimation of xylanase in samples was done against standard curve (See Appendix II).

3.7.2 Unit of activity

According to the International Union of Biochemistry, the amount of enzyme required to release 1 nanomole of reducing sugar (xylose) in 1 second is termed as one Nano Katal (1 nKatal) of xylanase (Pillai, 2010). One unit of enzyme (U, micromoles/ml) is equivalent to 16.67 nKatals (katal is equal to moles/sec.).

3.7.3 Calculation of enzyme activity

The enzyme activity was calculated by the following formula

$$\text{Activity} = \frac{\text{Standard factor} \times \text{Absorbance}}{\text{Time (min) of incubation}} \times \text{Dilution}$$

$$\text{Standard factor} = \frac{\text{Concentration (µmol /ml) of standard solution}}{\text{Absorbance at 550 nm}}$$

3.8 Partial purification of xylanase

The crude enzyme extract (500 ml) from wild and mutant strains was collected carefully and 20-40 % ammonium sulphate precipitation was applied (See appendix III). Afterwards, the flasks containing crude extract along with ammonium sulphate was placed for overnight at 4 °C inside refrigerator. After refrigeration, the crude extracts was centrifuged at 4000 g for 5 mins. (Bokhari *et al.*, 2009). The resulting precipitates obtained was collected and resuspended in 10 mM citrate buffer (pH 6.0) minimum volume and stored inside refrigerator at 4 °C.

3.8.1 Desalting (Dialysis)

The dialysis tubing of 6 inches were cut and boiled inside beaker containing 2 mMol EDTA (200 ml) and sodium bicarbonate (2 % w/v) for 30 minutes. Afterwards, the dialysis tubing's were boiled in distilled water for 10 minutes. The prepared tubing was finally washed with distilled water thoroughly. The ammonium sulphate precipitates extracts from both wild and mutant strains were taken into dialysing tubing and placed in 2 liter of 10 mmol citrate buffer solution (pH 6.0) at 4 °C for 24 h inside refrigerator. After dialysing, the extracts of wild and mutant from dialysing tubing's were transferred into labelled falcon tubes and stored inside refrigerator. Afterwards, the enzyme and protein assays were performed for dialysed samples of wild type and mutant.

3.9 Characterization of xylanase

The partially purified xylanase enzyme from wild and mutant was used to find out the different kinetics parameters as follows

3.9.1 Effect of substrate concentrations on enzyme activity

The partially purified xylanase enzyme from wild and mutant was used to find out the K_m and V_{max}. For this the enzyme from wild and mutant were used and enzyme assay were performed at 45 °C in the reaction mixtures containing 1 ml of citrate buffer of pH 6.0, 0.5 ml of dialysed enzyme and 0.5 ml of different concentrations of xylan substrate (oat-spelt-xylan; 0.1 to 0.6 mg/ml) in different tubes. The data obtained were plotted in the form of Lineweaver-Burk plot to find out the kinetics parameters (K_m and V_{max}) value for both wild and mutant xylanase (Bokhari *et al.* 2009).

3.9.2 Effect of pH on the activity of crude xylanase

The partially purified xylanase enzyme from wild and mutant were assayed under various pH values (4.0 to 7.0) at 45 °C. The citrate buffers of different pH ranging: pH 4.0, 4.4, 4.8, 5.0, 5.2, 5.4, 5.6, 6.0, 6.4, 6.8 and 7.0 were used. The dialyzed 0.5 ml enzyme from wild and mutant were taken in different tubes containing 1 ml of different pH citrate buffer, 0.5 ml of 1 % xylan substrate. The enzyme assay was performed at 45 °C.

3.9.3 Effect of temperature on the xylanase activity

The dialyzed enzyme from wild and mutant were assayed at various temperatures from 45 °C to 95 °C. For this the enzyme from wild and mutant were used and enzyme assay were performed at different temperatures ranging from 45 °C to 95 °C in the reaction mixtures containing 1 ml of citrate buffer pH 6.0, 0.5 ml of dialyzed enzyme and 0.5 ml of 1 % xylan substrate in different tubes.

3.9.4 Irreversible thermal denaturation of enzyme

The effect of different temperature on enzyme activity for wild and mutant were determined. The enzyme was incubated at different temperatures ranging from 50 °C to 85 °C for up to 30 minutes. Aliquots were prepared out in 6 different eppendorf tubes and each tube was taken out from incubator sequentially after 5 minutes. The tubes containing enzyme after incubation were kept in ice for three hours (Violet and Meunier, 1989) and then enzyme assay was performed for each sample. The data were fitted into the 1st order plot and then examined as explained previously (Siddiqui *et al.* 1999). The k_d was calculated from the slope of residual percent activity against time while activation energy (E_a) was calculated from a plot of k_d verses reciprocal of temperature in Kelvin.

RESULTS

4. RESULTS

4.1 Isolation of fungus

Different methods and techniques were employed in isolation of fungus. Soil samples were collected from different places in Islamabad, Pakistan. Serial dilution method was used for fungus isolation. Number of dilutions was made, ranging from (10^{-1} to 10^{-5} dilutions). From each dilution, 0.1 ml was spreaded on separate PDA plates labeled with dilution factor. After spreading, plates were placed inside incubator overnight for incubation at 30 °C. After 48 h incubation, growth became visible and there were colonies of fungi on each plate (Figure 4.1).

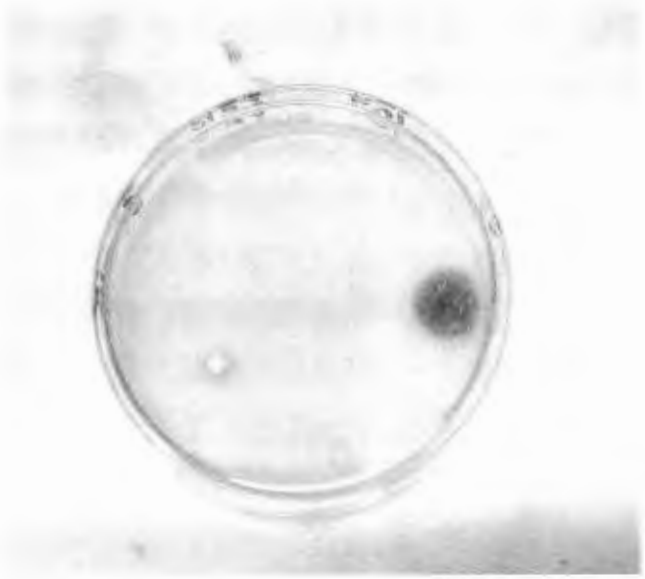


Figure 4.1 Growth of fungus after serial dilution on PDA plate

Streaking method was performed to purify single colony of fungus from mixed culture of fungi. Many desired single colonies were purified from mixture of colonies present on various plates. On the basis of cultural characteristics and microscopic analysis, the isolate was identified as *A. niger* (Figure 4.2). The colonies were initially white, quickly becoming black, reverse was pale yellow. Hyphae were septate and hyaline.

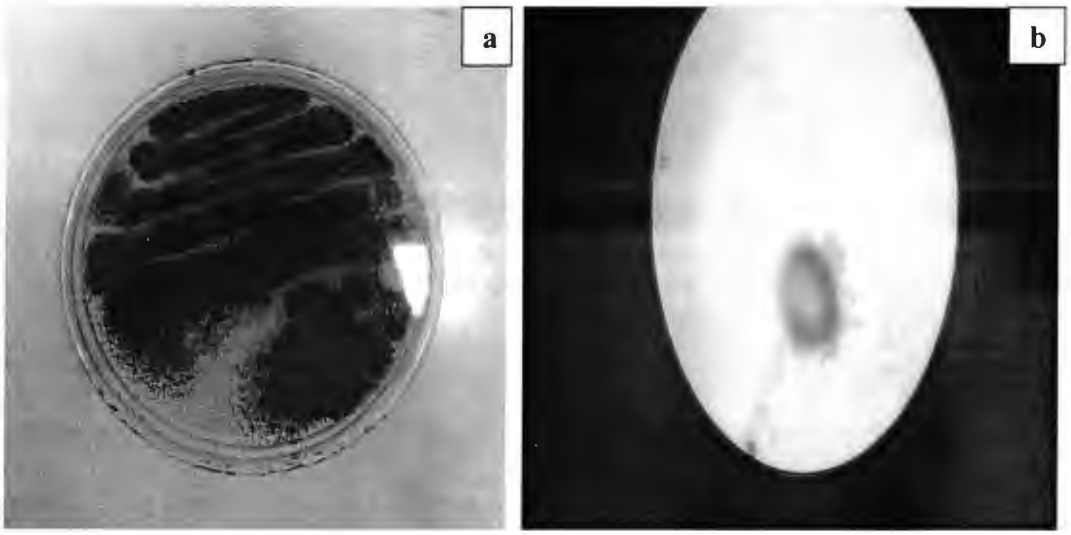


Figure 4.2 (a) Growth of *A. niger* on PDA plate (left) after 48 h incubation (b) Microscopic view of single colony, spores and hyphae of *A. niger* (right)

4.2 Mutagenesis and selection

The spore suspension of wild type *Aspergillus niger* was prepared (containing approximately 3.0 × 10^6 spores/ml determined by haemocytometer) for gamma irradiation as shown in figure 4.3.

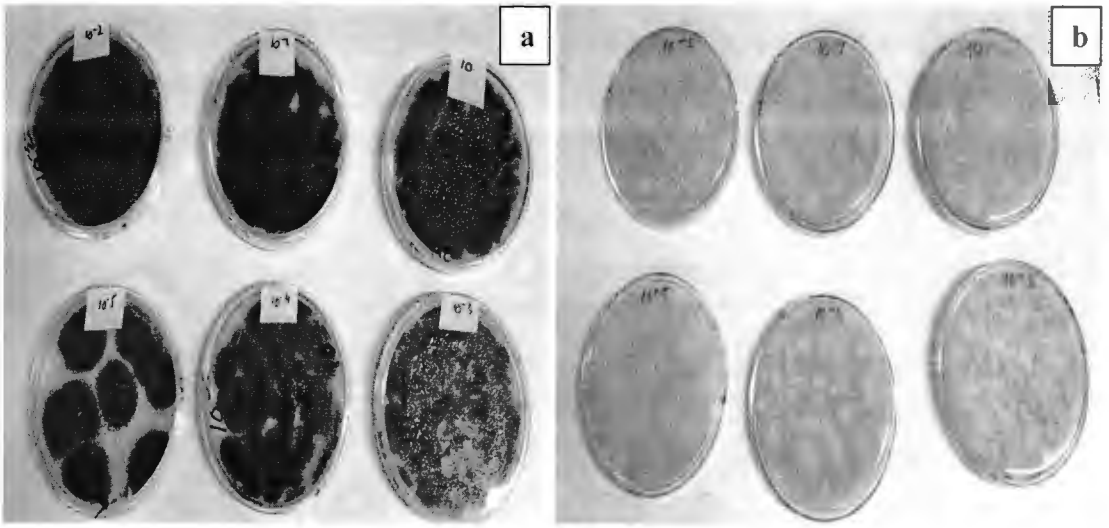


Figure 4.3 Growth of *A. niger* spores on agar plates after 48 h incubation (a) Plates of *A. niger* front view (b) Plates of *A. niger* back view

The spore suspension containing (approximately 3 × 10^6 spores/ml) was transferred to eppendorf tubes of 2 ml vials and treated with different doses of γ-rays

(0.8, 1.0 and 1.2 kGray) in γ-cell radiation chamber from Nuclear Institute for Food and Agriculture (NIFA) Institute, Peshawar, to isolate xylanase hyper-producing mutant of *Aspergillus niger*. The irradiated spores were grown on 1 % xylan agar plate and the colony which formed larger hallow as shown in figure 4.4 was selected for further studies. The dose of 1.0 kGy was found to be optimal and killed 99.9 %. The selected mutant was properly labelled and further grown on PDA plates and slants. Afterwards, the mutant spore suspension and inoculum were prepared by similar method as used for the wild type organism.

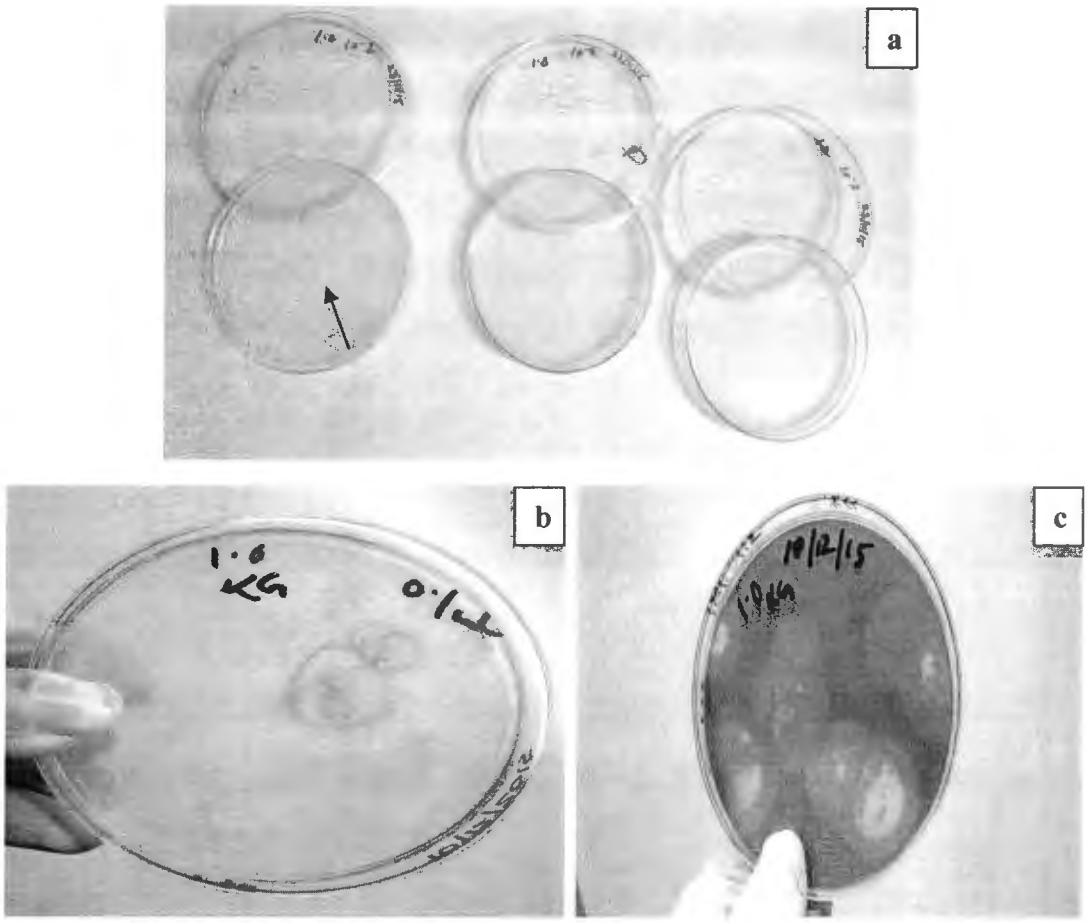


Figure 4.4 Growth of *A. niger* mutant (a) 1.0 kGy irradiated spores on 1 % xylan agar plates with 3 log kill after 48 h incubation. (b) Unstained Plate of *A. niger* mutant 1.0 kGy irradiated spores on 1 % xylan agar plates (c) 2.5 % Safranin stained plate of *A. niger* mutant (Gohel, 2014)

4.3 Optimization of inoculum size

Spore suspension of (3.0×10^6 spores/ml) of wild type and mutant of *Aspergillus niger* was used as inoculums. For optimization, inoculum size of 5 %, 10 %, 15 % and 20 % of *Aspergillus niger* wild type and mutant were used. The overall results presented in table 4.1 showed that maximum xylanase production was obtained at 36 h on 10 % spore suspension for both wild and mutant separately. Figure 4.5 shows production pattern of maximum enzyme production for wild was 42.65 nKatal and for mutant production pattern of maximum enzyme production was 45.47 nKatal while Figure 4.6 shows production pattern of the maximum protein production for wild was 365.47 µg/ml with a specific activity of 116.69 (nkatal/mg) and for mutant protein production was 382.94 µg/ml with a specific activity of 118.73 (nkatal/mg).

Table 4.1 Production of xylanase from *A. niger* wild type and mutant on various inoculum (spore suspensions) concentrations

Inoculum Concentration	Time (h)	Wild			Mutant		
		Enzyme nKatal	Protein µg/ml	Specific Activity (nkatal/mg)	Enzyme nKatal	Protein µg/ml	Specific Activity (nkatal/mg)
5 %	12	5.85	106.21	55.07	7.44	115.36	64.49
	24	20.38	235.64	86.48	23.11	250.61	92.21
	36	28.64	295.39	96.95	30.82	310.72	99.18
	48	24.49	260.83	93.89	26.76	275.19	97.25
10 %	12	14.85	182.94	81.17	20.63	225.29	91.57
	24	27.36	273.23	100.13	32.97	319.41	103.22
	36	**42.65**	**365.47**	**116.69**	**45.47**	**382.94**	**118.73**
	48	35.32	322.58	109.49	37.31	335.05	111.35
15 %	12	10.97	155.29	70.64	14.58	185.43	78.62
	24	22.77	246.79	92.26	28.44	306.91	92.66
	36	34.86	320.11	108.9	38.91	341.75	113.85
	48	30.75	312.89	98.27	33.11	316.39	104.64
20 %	12	11.59	170.73	67.88	15.71	190.69	82.38
	24	23.96	252.91	94.73	25.48	258.16	98.69
	36	30.41	301.78	100.76	35.19	324.81	108.34
	48	26.71	270.67	98.68	28.52	308.66	92.46

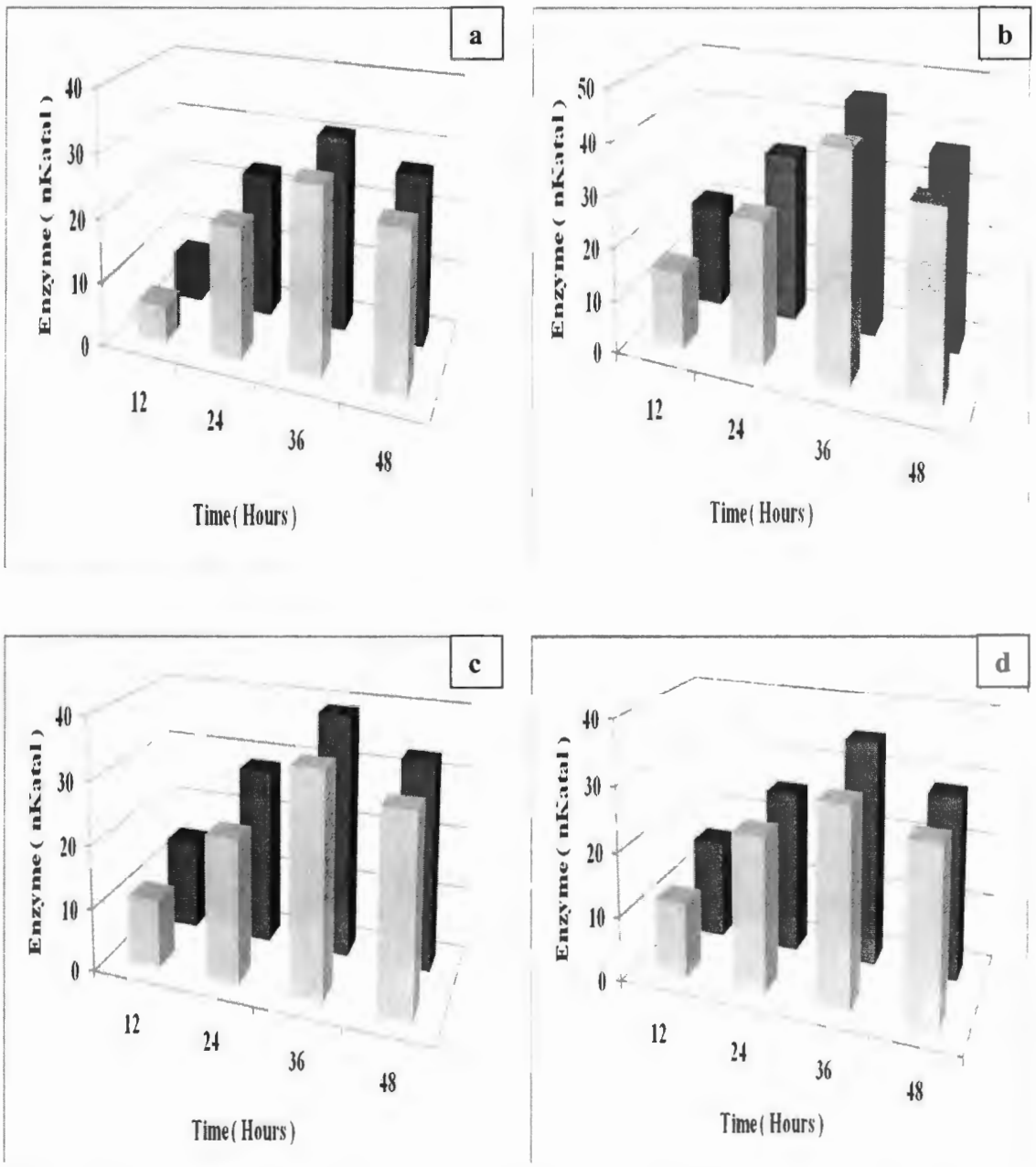


Figure 4.5 Production pattern of xylanase enzyme from *A. niger* wild type and mutant on various inoculum concentrations (a) 5 % (b) 10 % (c) 15 % (d) 20 %. Grey bars present *A. niger* wild type and black bars present *A. niger* mutant

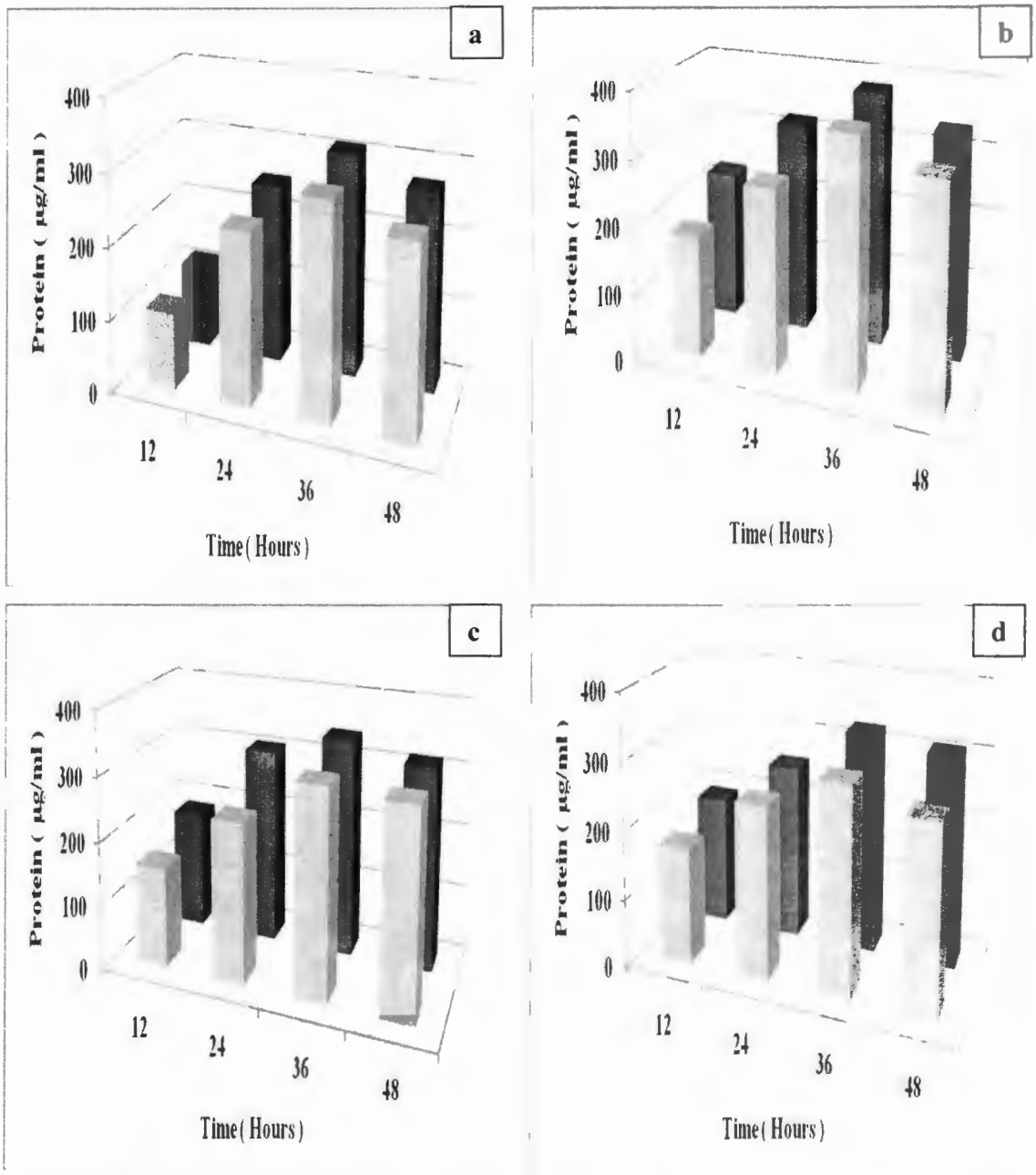


Figure 4.6 Production pattern of protein from *A. niger* wild type and mutant on various inoculum concentrations (a) 5 % (b) 10 % (c) 15 % (d) 20 %. Grey bars present *A. niger* wild type and black bars present *A. niger* mutant

4.3.1 Selection of carbon source

The wild type and mutant of *A. niger* were grown on different carbon substrates including wheat bran, rice husk, corn cobs and dab grass for 48 h at 30 °C. After every 12 h, a flask for wild type and mutant was removed from incubator to study the enzyme and protein production in time course. The overall results presented in table 4.2 showed that maximum xylanase production was obtained at 36 h on corn cobs for both wild and mutant separately. Figure 4.7 shows production pattern of maximum enzyme production for wild was 47.15 nKatal and for mutant maximum enzyme production was 49.07 nKatal while figure 4.8 shows production pattern of the maximum protein production for wild was 380.47 µg/ml with a specific activity of 123.92 (nkatal/mg) and for mutant protein production was 392.94 µg/ml with a specific activity of 124.87 (nkatal/mg).

Table 4.2 Production of xylanase from *A. niger* wild type and mutant on various carbon substrates

Carbon Source	Time (h)	Wild			Mutant		
		Enzyme nKatal	Protein µg/ml	Specific Activity (nkatal/mg)	Enzyme nKatal	Protein µg/ml	Specific Activity (nkatal/mg)
Wheat Bran	12	7.59	126.47	60.01	10.5	155.88	67.35
	24	24.84	264.11	94.05	30.26	310.11	97.57
	36	41.96	347.05	120.9	42.07	356.52	118.17
	48	26.6	287.64	92.47	28.31	287.11	98.6
Rice Husk	12	5.59	109.41	51.09	7.53	128.82	58.45
	24	15.69	198.23	79.15	21.2	241.17	87.9
	36	26.95	294.11	91.63	27.89	314.7	88.82
	48	16.67	217.05	76.8	19.45	229.41	84.78
Corn Cob	12	15.45	192.94	80.07	23.23	235.29	94.47
	24	28.06	280.23	100.01	33.77	329.41	102.51
	36	**47.15**	**380.47**	**123.92**	**49.07**	**392.94**	**124.87**
	48	33.42	320.58	104.24	35.61	335.05	106.28
Dab Grass	12	6.01	117.64	51.08	8.34	135.29	61.64
	24	22.83	247.05	92.41	24.72	255.29	96.83
	36	37.03	338.82	109.29	41.25	342.35	120.49
	48	25.47	270.45	94.17	27.26	276.88	98.45

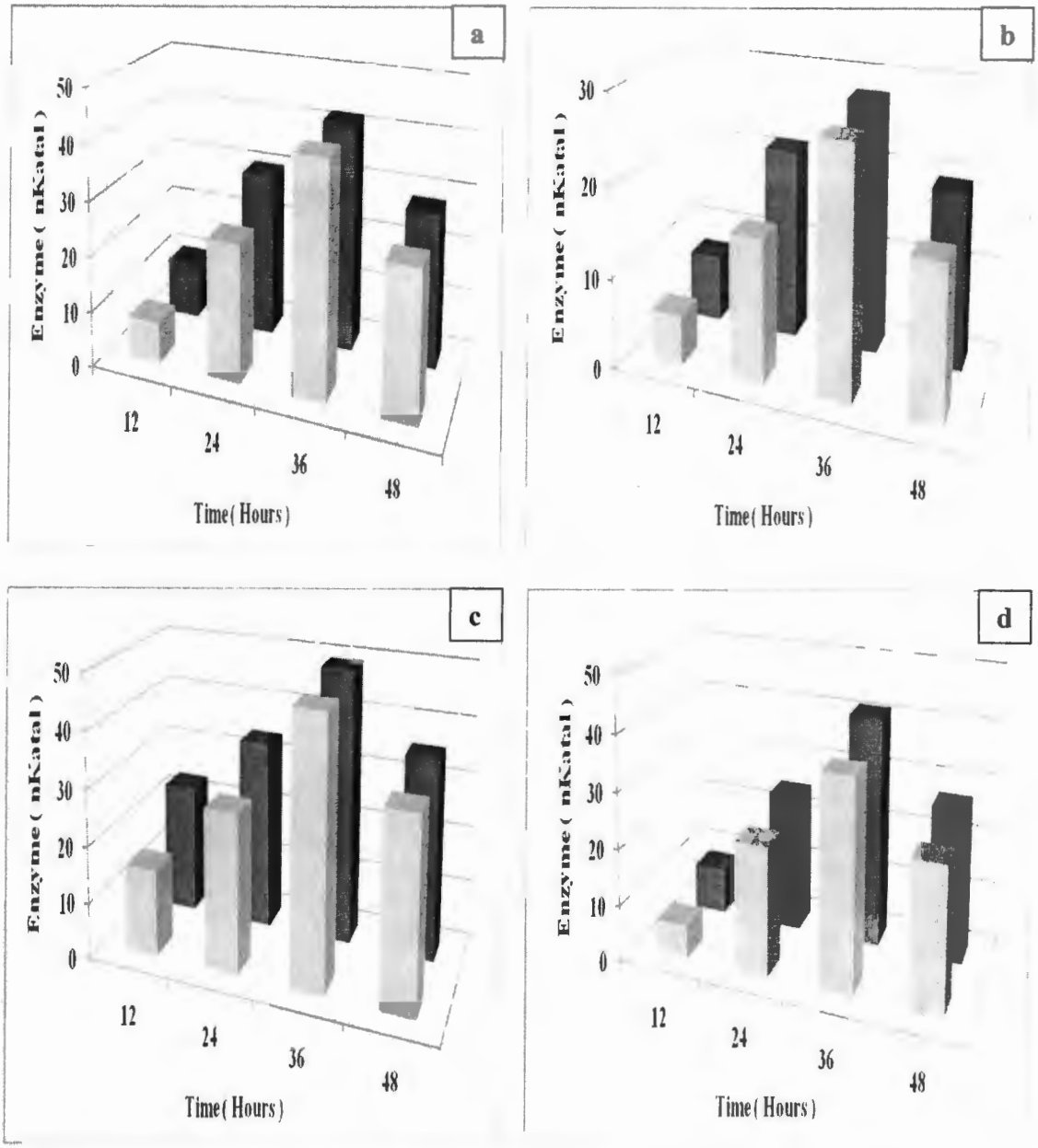


Figure 4.7 Production pattern of xylanase enzyme from *A. niger* wild type and mutant on various carbon substrate (a) Wheat bran (b) Rice husk (c) Corn cob (d) Dab grass. Grey bars present *A. niger* wild type and black bars present *A. niger* mutant

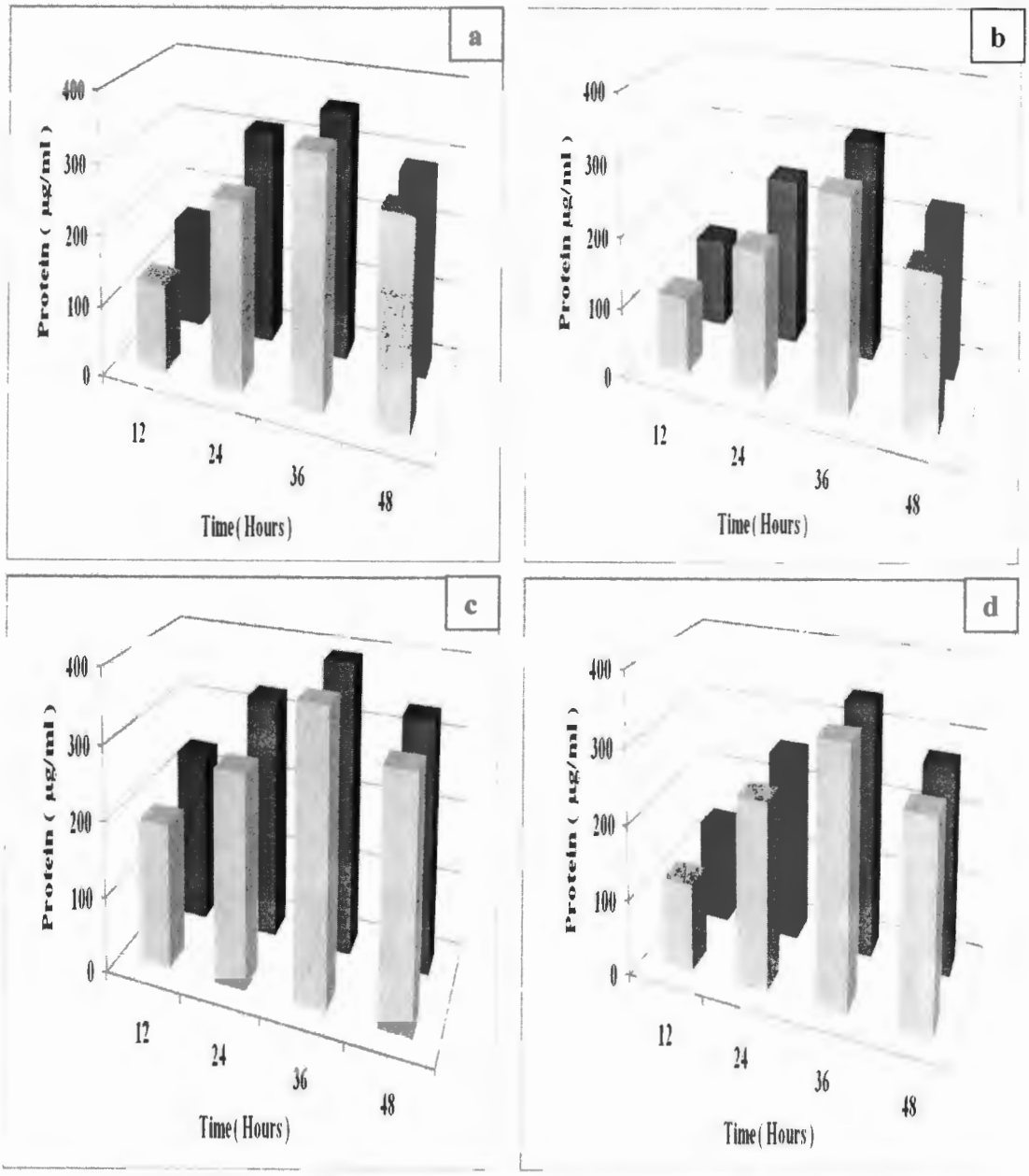


Figure 4.8 Production pattern of protein from *A. niger* wild type and mutant on various carbon substrate (a) Wheat bran (b) Rice husk (c) Corn cob (d) Dab grass. Grey bars present *A. niger* wild type and black bars present *A. niger* mutant

4.3.2 Optimization of carbon source concentration

The wild type and mutant of *A. niger* was grown on the corn cobs different concentrations including (2.0, 3.0, 3.5 %) for 48 h at 30 °C to optimize the production of xylanase. The overall results presented in table 4.3 shows that the maximum xylanase and protein production for wild type and mutant was obtained at 36 h with 2 % corn cob. Figure 4.9 shows production pattern of maximum enzyme production at 2 % corn cob for wild was 46.68 nKatal and enzyme production for mutant was 48.34 nKatal while figure 4.10 shows production pattern of maximum protein production at 2 % corn cob for wild was 405.35 µg/ml with a specific activity of 115.15 (nkatal/mg) and for mutant the maximum protein production was 425.91 µg/ml with a specific activity of 113.49 (nkatal/mg).

Table 4.3 Production of xylanase from *A. niger* wild type and mutant on optimized carbon substrate various concentrations

Carbon Substrate Concentration	Time (h)	Wild			Mutant		
		Enzyme nKatal	Protein µg/ml	Specific Activity (nkatal/mg)	Enzyme nKatal	Protein µg/ml	Specific Activity (nkatal/mg)
2.0 %	12	16.36	218.67	74.81	19.93	228.72	87.13
	24	35.77	332.85	107.46	36.76	329.18	111.16
	36	**46.68**	**405.35**	**115.15**	**48.34**	**425.91**	**113.49**
	48	33.25	324.12	102.58	35.15	335.29	104.83
3.0 %	12	13.66	187.66	72.79	17.94	224.73	79.82
	24	26.54	264.18	100.46	31.95	312.86	102.12
	36	33.19	320.75	103.47	38.43	345.42	111.25
	48	28.66	280.37	102.22	33.72	324.31	103.97
3.5 %	12	11.73	173.11	67.76	15.17	215.44	70.41
	24	23.19	250.76	92.47	28.49	276.83	102.91
	36	30.86	305.49	101.01	35.91	337.12	106.51
	48	26.33	270.83	97.21	31.22	310.14	100.66

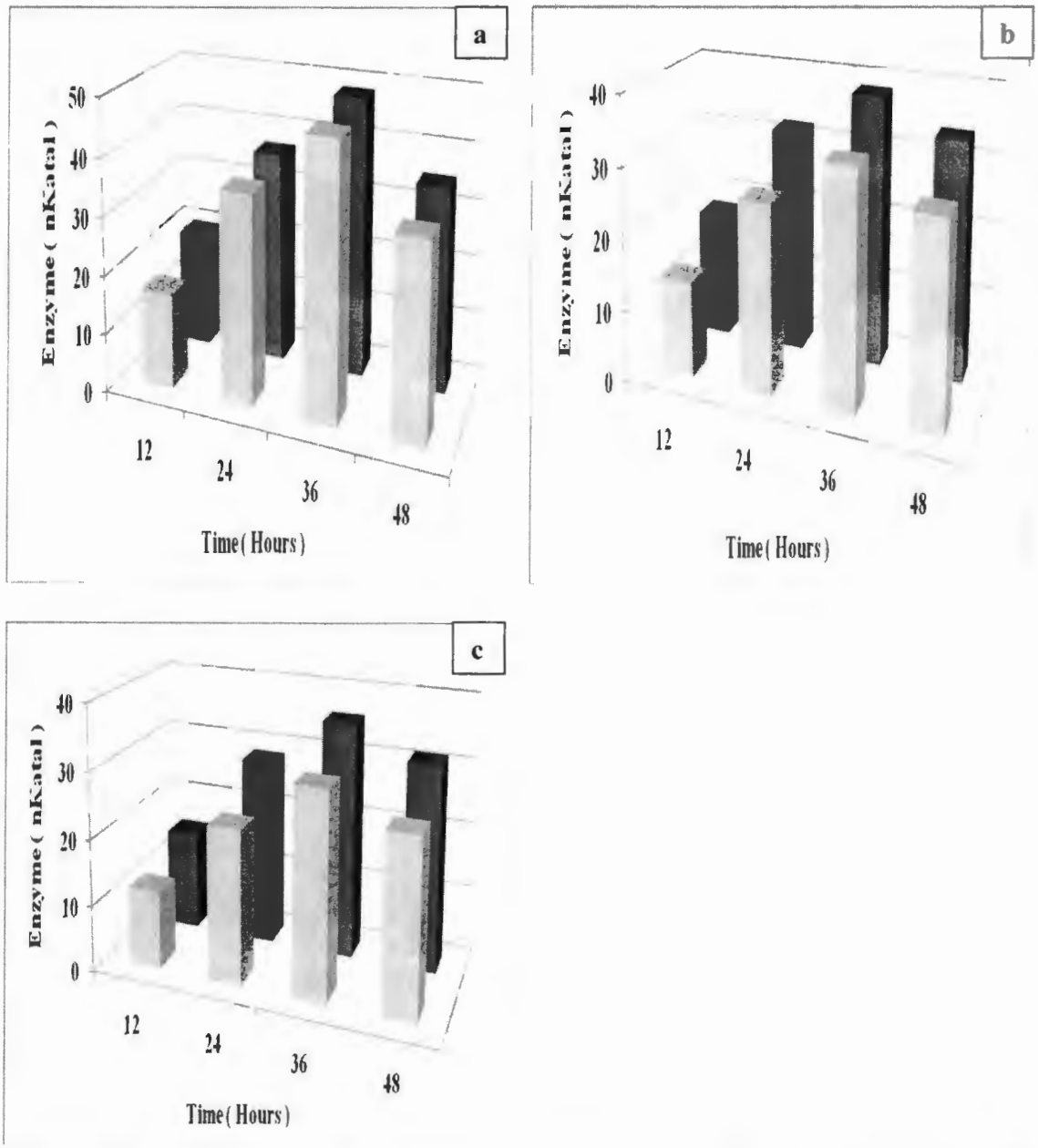


Figure 4.9 Production pattern of xylanase enzyme by *A. niger* wild type and mutant on corn cob various concentrations (a) 2.0 % corn cob (b) 3.0 % corn cob (c) 3.5 % corn cob. Grey bars present *A. niger* wild type and black bars present *A. niger* mutant

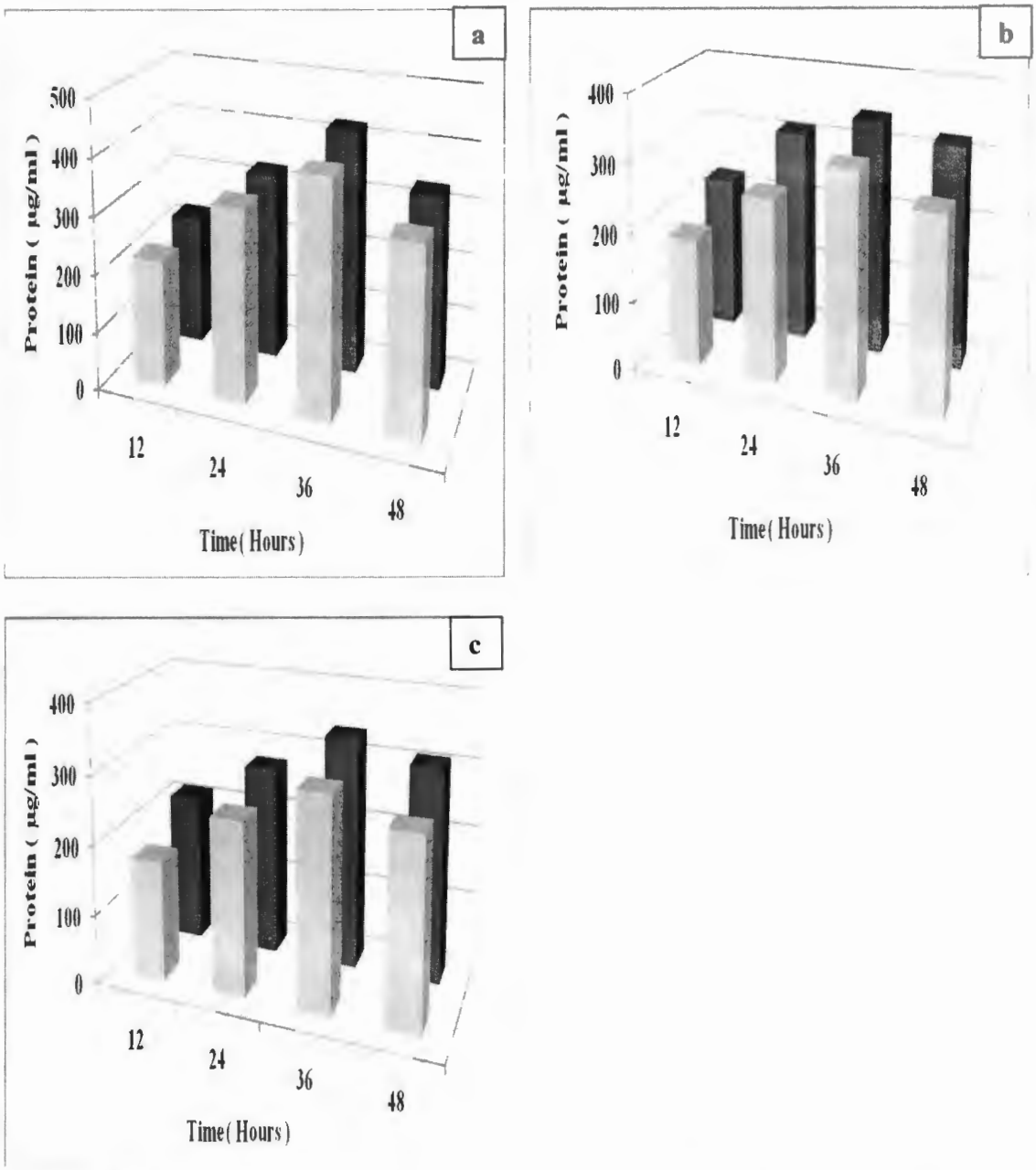


Figure 4.10 Production pattern of protein by *A. niger* wild type and mutant on corn cob various concentrations (a) 2.0 % corn cob (b) 3.0 % corn cob (c) 3.5 % corn cob. Grey bars present *A. niger* wild type and black bars present *A. niger* mutant

4.3.3 Optimization of nitrogen source

The wild type and mutant of *A. niger* with optimized carbon source and concentration was grown for 48 h at 30 °C on various nitrogen sources including organic (Urea, DAP) and inorganic (Ammonium nitrate, Ammonium sulphate) to optimize the production of xylanase. The results presented in table 4.4 showed that NH_4NO_3 showing maximum xylanase production at 36 h for wild and mutant separately. Figure 4.11 shows production pattern of maximum enzyme production for wild on NH_4NO_3 was 54.36 nKatal and enzyme production for mutant was 50.89 nKatal while figure 4.12 shows production pattern of maximum protein production on NH_4NO_3 for wild was 450.23 µg/ml with a specific activity of 120.73 (nkatal/mg) and for mutant the maximum protein production was 430.12 µg/ml with a specific activity of 118.31 (nkatal/mg).

Table 4.4 Production of xylanase from *A. niger* wild type and mutant on various nitrogen sources

Nitrogen Source + Carbon Source	Time (h)	Wild			Mutant		
		Enzyme nKatal	Protein µg/ml	Specific Activity (nkatal/mg)	Enzyme nKatal	Protein µg/ml	Specific Activity (nkatal/mg)
Urea + Corn Cob	12	6.4	121.15	52.82	9.08	138.9	65.37
	24	30.71	315.66	97.28	31.47	317.68	99.06
	36	37.68	340.11	110.78	38.37	345.71	110.98
	48	31.2	315.65	98.84	34.56	327.61	105.49
DAP + Corn Cob	12	13.43	185.25	72.49	12.15	172.96	70.24
	24	28.81	284.5	101.26	26.7	270.77	98.6
	36	50.84	432.1	117.65	47.94	420.81	113.92
	48	42.67	380.1	112.25	33.34	323.69	102.99
NH_4NO_3 + Corn Cob	12	29.44	290.46	101.35	24.72	260.55	94.87
	24	37.45	345.23	108.47	32.72	320.11	102.21
	36	**54.36**	**450.23**	**120.73**	**50.89**	**430.12**	**118.31**
	48	48.23	415.19	116.21	46.22	408.77	113.07
$(NH_4)_2SO_4$ + Corn Cob	12	20.58	238.65	86.23	18.94	229.31	82.59
	24	24.23	252.96	95.78	23.46	243.87	96.19
	36	41.12	355.21	115.83	40.08	350.96	114.2
	48	40.37	353.77	114.11	39.31	360.41	109.07

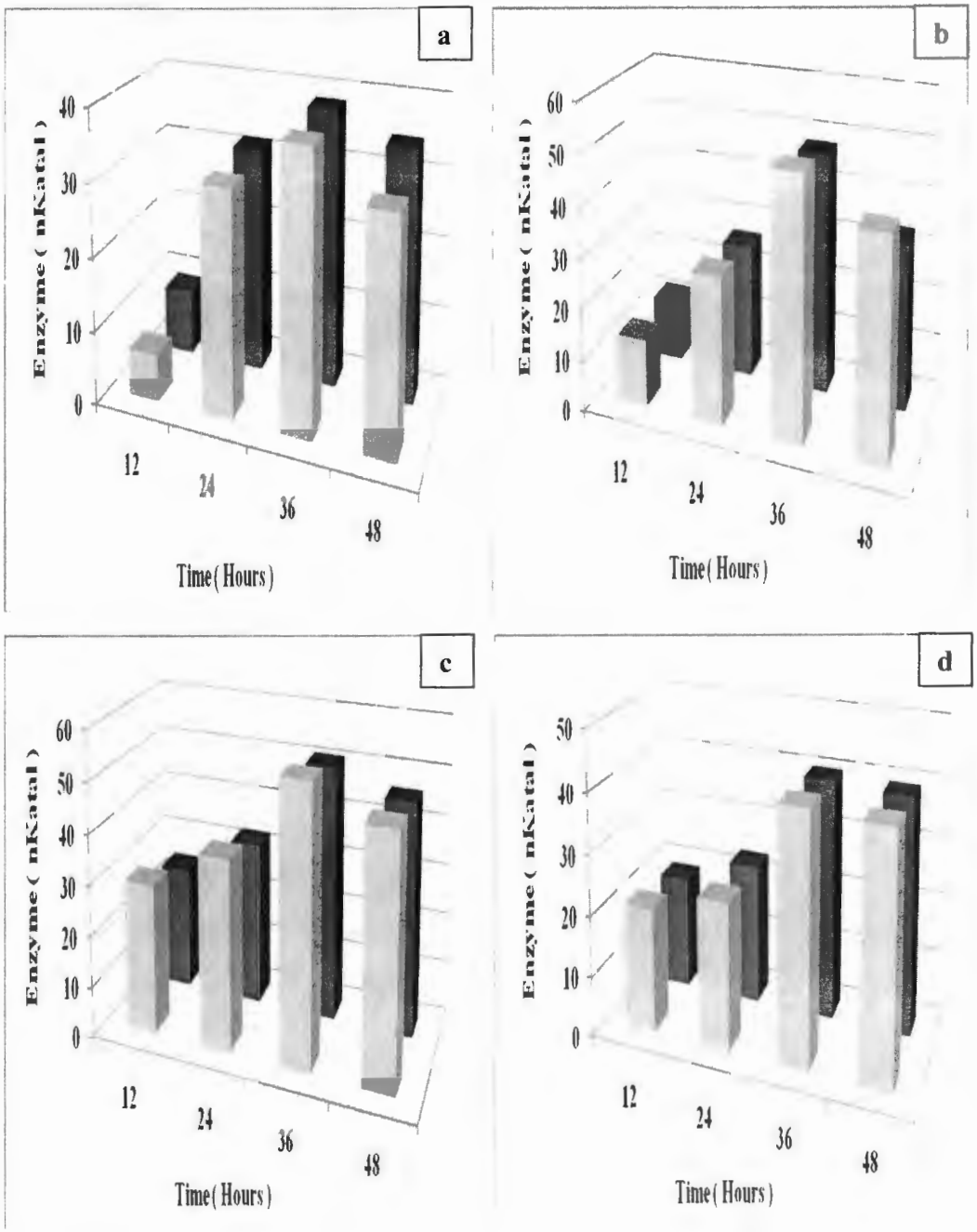


Figure 4.11 Production pattern of xylanase enzyme from *A. niger* wild type and mutant on various nitrogen sources (a) Urea (b) DAP (c) NH_4NO_3 (d) $(NH_4)_2SO_4$. Grey bars present *A. niger* wild type and black bars present *A. niger* mutant

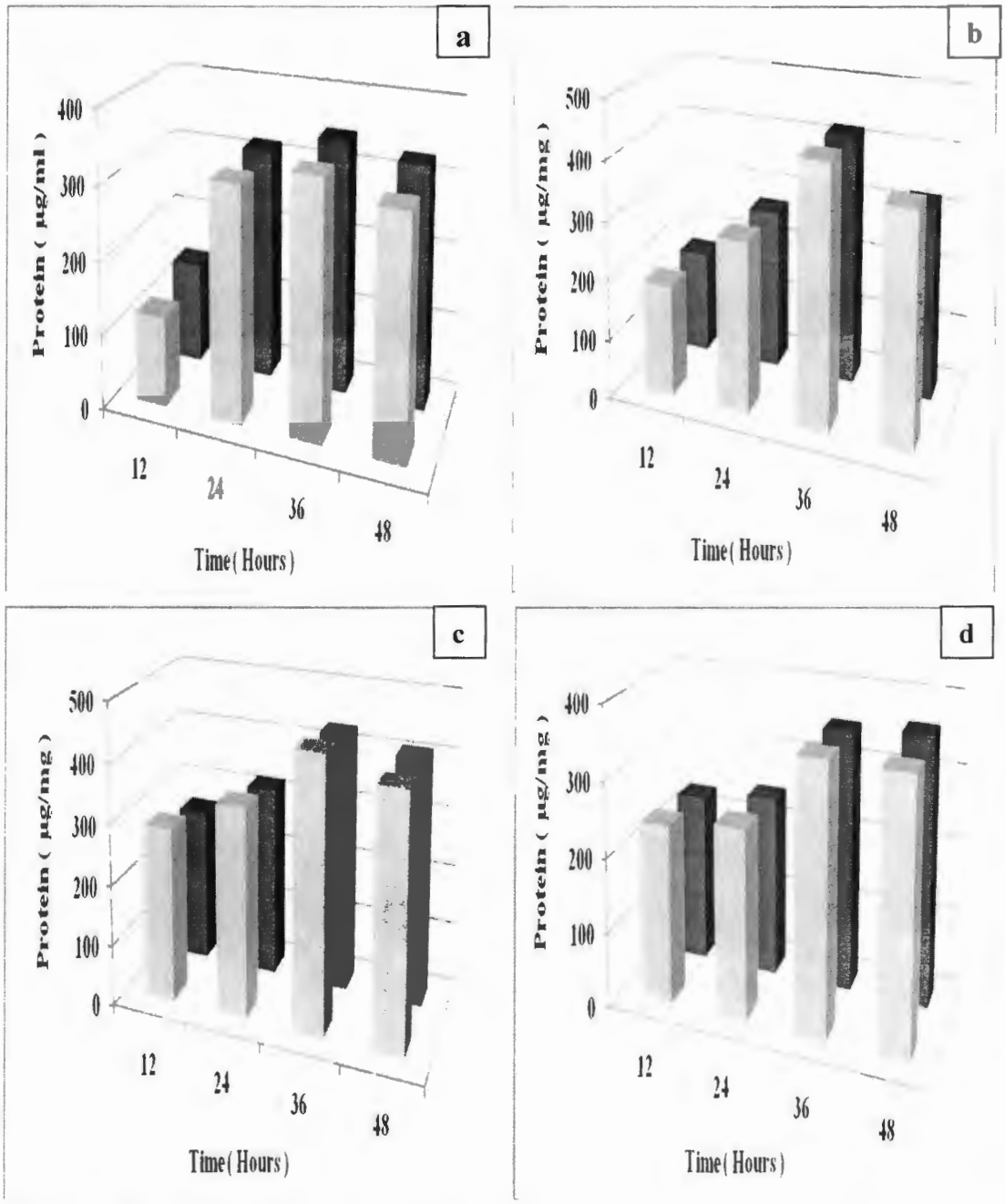


Figure 4.12 Production pattern of protein from *A. niger* wild type and mutant on various nitrogen sources (a) Urea (b) DAP (c) NH_4NO_3 (d) $(NH_4)_2SO_4$. Grey bars present *A. niger* wild type and black bars present *A. niger* mutant

4.3.4 Optimization of temperature

The wild type and mutant of *A. niger* was grown for 48 h at different temperatures ranging from (25, 28, 30, 32 and 35 °C) using optimized carbon and nitrogen source to optimize the production of xylanase. The results presented in table 4.5 showed that 30 °C was optimum temperature for xylanase production from both wild and mutant separately at 36 h. Figure 4.13 shows production pattern of xylanase maximum enzyme production for wild at 30 °C was 47.68 nKatal and enzyme production for mutant was 49.74 nKatal while figure 4.14 shows at 30 °C production pattern of maximum protein production for wild at 30 °C was 410.25 µg/ml with a specific activity of 116.22 (nkatal/mg) and for mutant at 30 °C the maximum protein production was 425.71 µg/ml with a specific activity of 116.84 (nkatal/mg).

Table 4.5 Production of xylanase from *A. niger* wild type and mutant on various temperatures °C

Temperature ° C	Time (h)	Wild			Mutant		
		Enzyme nKatal	Protein µg/ml	Specific Activity (nkatal/mg)	Enzyme nKatal	Protein µg/ml	Specific Activity (nkatal/mg)
25	12	5.25	118.75	44.21	7.16	120.46	59.43
	24	8.30	130.11	63.79	10.35	138.88	74.52
	36	23.29	255.35	91.20	27.33	276.49	98.84
	48	15.75	210.77	74.72	13.27	200.67	66.12
28	12	5.86	120.88	48.47	6.13	115.42	53.11
	24	14.3	205.46	69.59	15.6	212.77	73.31
	36	28.41	286.22	99.25	30.55	310.86	98.27
	48	24.84	260.71	95.27	26.75	280.11	95.49
30	12	17.86	219.91	81.21	18.43	225.67	81.66
	24	34.68	330.11	105.05	33.26	321.88	103.33
	36	**47.68**	**410.25**	**116.22**	**49.74**	**425.71**	**116.84**
	48	32.25	320.52	100.61	32.55	323.22	100.70
32	12	12.11	180.66	67.03	13.43	204.11	65.79
	24	23.69	255.39	92.76	24.63	260.39	94.58
	36	31.86	315.88	100.86	32.13	322.83	99.52
	48	27.85	282.73	98.50	28.71	285.64	100.51
35	12	7.36	122.76	59.95	7.55	123.44	61.16
	24	11.98	160.91	74.45	10.42	140.15	74.34
	36	29.58	305.11	96.94	28.04	281.89	99.47
	48	26.16	275.6	94.92	24.94	263.61	94.60

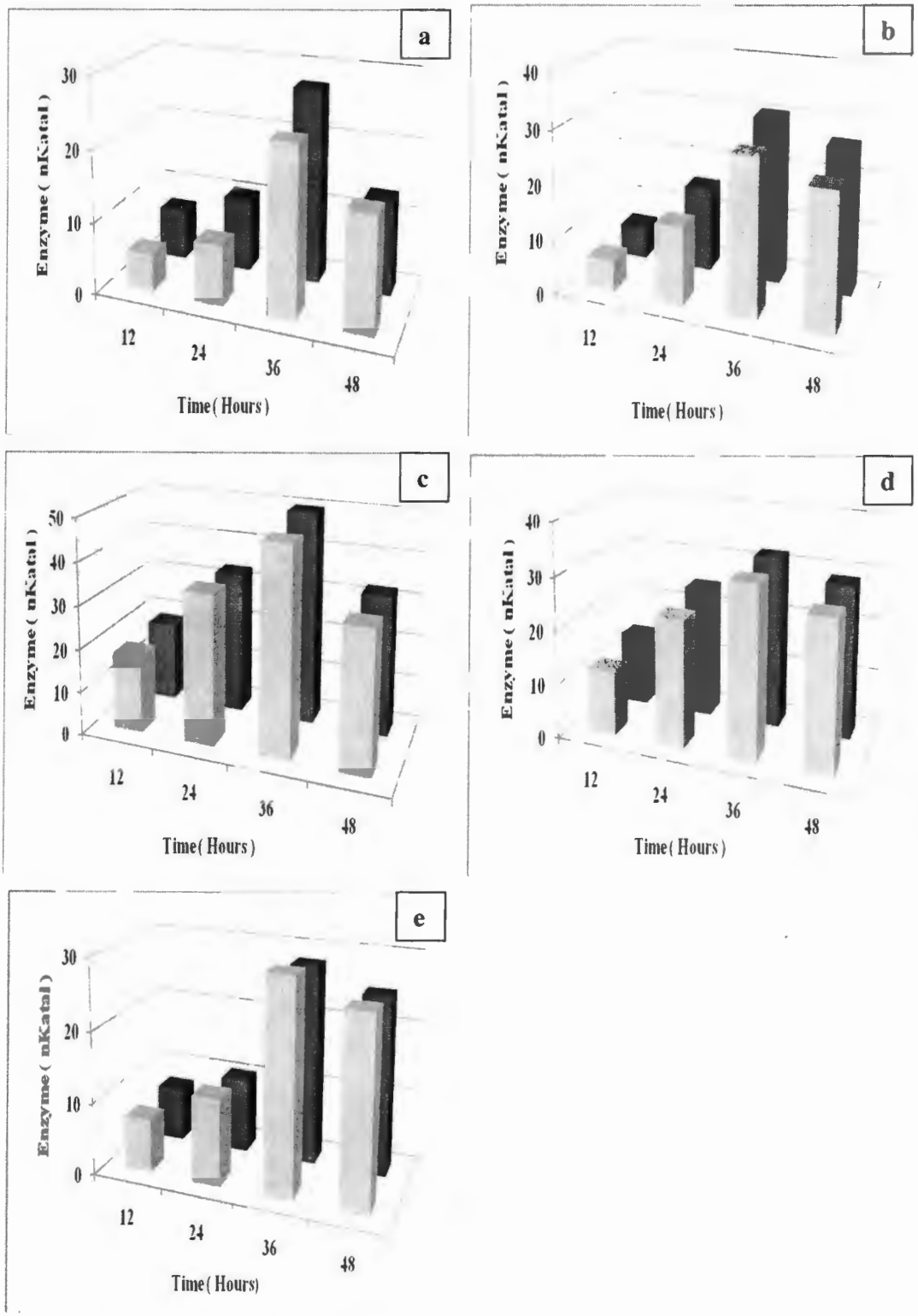


Figure 4.13 Production pattern of xylanase enzyme from *A. niger* wild type and mutant on various temperatures (a) 25 °C (b) 28 °C (c) 30 °C (d) 32 °C (e) 35 °C. Grey bars present *A. niger* wild type and black bars present *A. niger* mutant

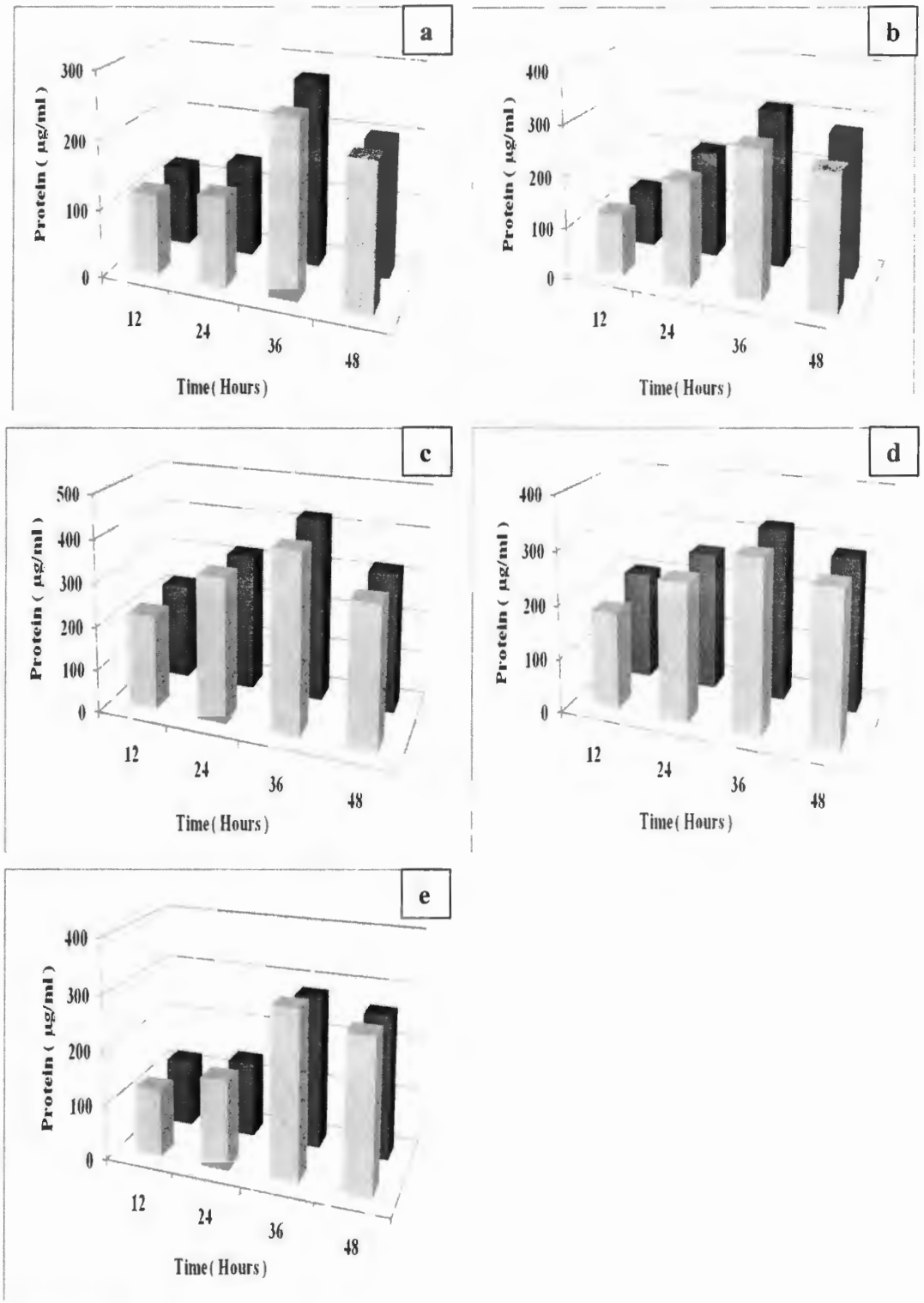


Figure 4.14 Production pattern of protein from *A. niger* wild type and mutant on various temperatures (a) 25 °C (b) 28 °C (c) 30 °C (d) 32 °C (e) 35 °C. Grey bars present *A. niger* wild type and black bars present *A.niger* mutant

4.3.5 Optimization of pH

The wild type and mutant of *A. niger* with optimized temperature, carbon and nitrogen source was grown on different citrate buffers ranging from pH 4.0 to 7.0 up to 48 h for optimum production of xylanase. The results presented in table 4.6 showed that pH buffer 6.0 supported the maximum xylanase production at 36 h for both and wild and mutant separately. Figure 4.15 shows production pattern of maximum enzyme production for wild on pH buffer 6.0 was 41.01 nKatal and enzyme production for mutant was 36.39 nKatal while figure 4.16 shows production pattern of maximum protein production for wild on pH buffer 6.0 was 355.09 µg/ml with a specific activity of 115.49 (nkatal/mg) and for mutant on pH buffer 6.0 the maximum protein production was 328.88 µg/ml with a specific activity of 110.64 (nkatal/mg).

Table 4.6 Production of xylanase from *A. niger* wild type and mutant on various pH (citrate buffers)

pH	Time (h)	Wild			Mutant		
		Enzyme nKatal	Protein µg/ml	Specific Activity (nkatal/mg)	Enzyme nKatal	Protein µg/ml	Specific Activity (nkatal/mg)
4	12	6.05	118.32	51.13	10.71	153.77	69.64
	24	11.54	162.55	70.99	14.61	175.99	83.01
	36	27.87	302.75	92.05	26.87	283.01	94.94
	48	25.8	290.11	88.93	22.88	260.11	87.96
5	12	11.15	155.88	71.52	8.66	143.23	60.46
	24	22.84	265.98	87.51	20.16	230.79	87.35
	36	28.56	305.77	93.4	25.03	268.83	93.10
	48	24.05	280.44	85.75	20.66	239.66	86.20
6	12	13.01	170.33	76.38	11.08	157.23	70.47
	24	29.56	310.49	95.20	24.3	265.11	91.66
	36	**41.01**	**355.09**	**115.49**	**36.39**	**328.88**	**110.64**
	48	26.62	292.67	90.95	22.15	243.19	91.08
7	12	8.53	140.62	60.65	8.01	142.79	56.09
	24	10.79	155.76	69.27	9.06	146.37	61.89
	36	22.08	254.89	86.62	17.56	223.71	78.49
	48	14.66	179.41	81.71	12.46	163.83	76.05

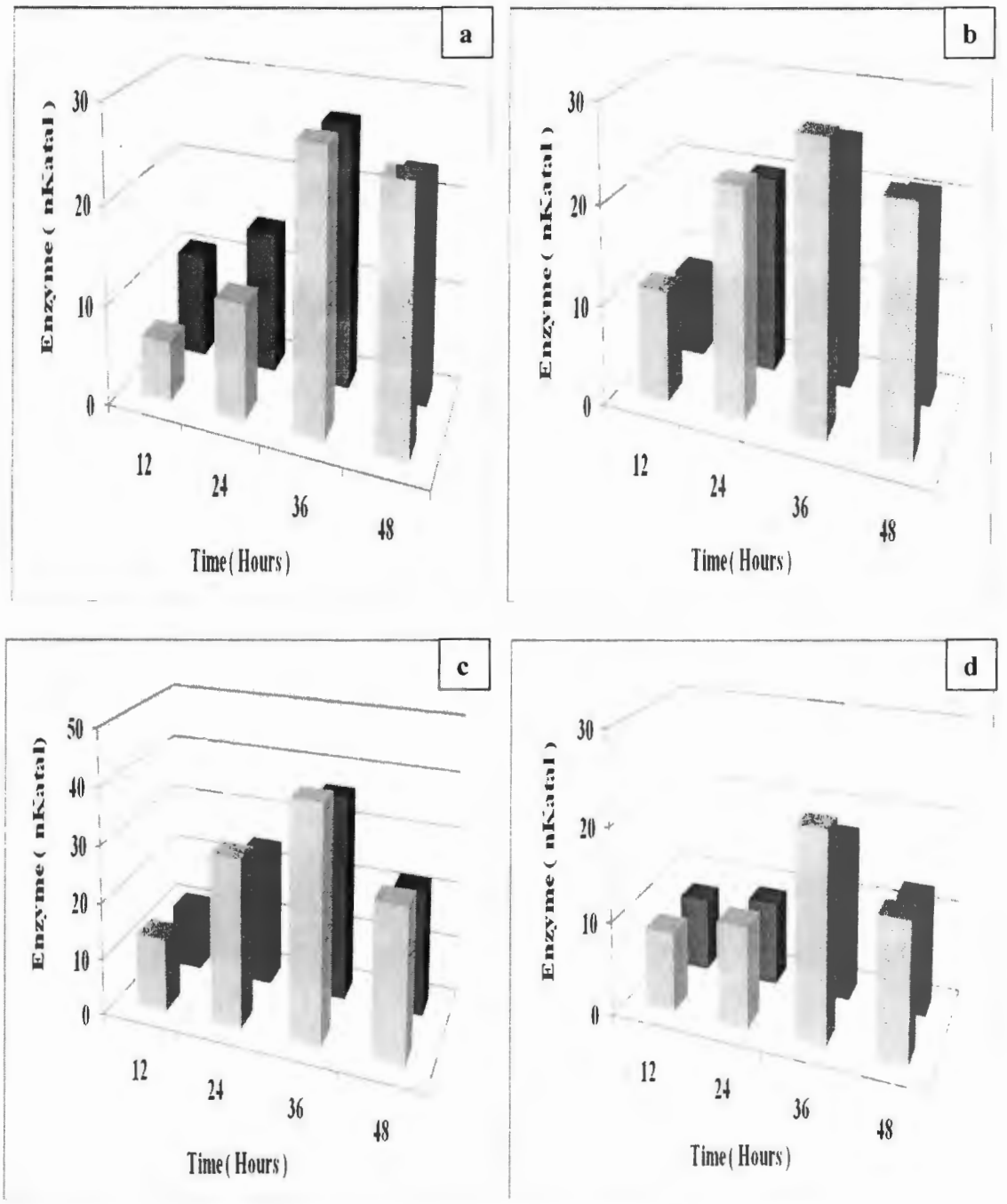


Figure 4.15 Production pattern of xylanase enzyme from *A. niger* wild type and mutant on various pH citrate buffers (a) pH 4 (b) pH 5 (c) pH 6 (d) pH 7. Grey bars present *A. niger* wild type and black bars present *A. niger* mutant

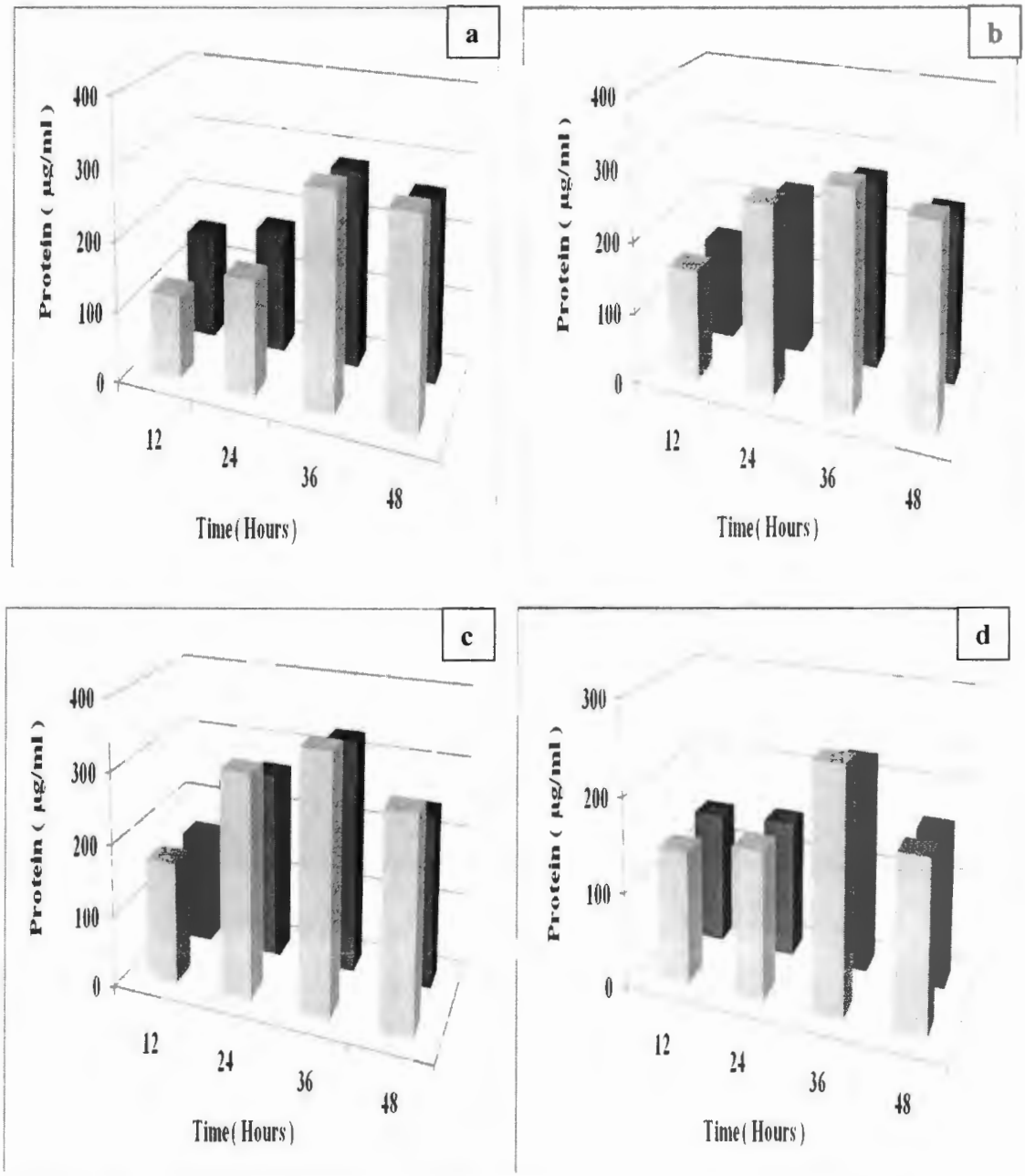


Figure 4.16 Production pattern of protein from *A. niger* wild type and mutant on various pH citrate buffers (a) pH 4 (b) pH 5 (c) pH 6 (d) pH 7. Grey bars present *A. niger* wild type and black bars presenting *A. niger* mutant

4.4 Partial purification of xylanase

The ammonium sulphate concentration of 20-40 % was used for the precipitation of crude enzyme extract from both *A. niger* wild type and mutant (See appendix III). The dialysis was done through suspending dialyzing tubing in 10 mM citrate buffer pH 6.0 and left overnight at 4 °C inside refrigerator. The extracts after ammonium sulphate treatment were centrifuged. The results presented in table 4.7 and figure 4.17 showed that 30 % ammonium sulphate concentration was best for wild type enzyme precipitation and 20 % ammonium sulphate concentration was best for mutant type enzyme precipitation.

Table 4.7 Precipitation of xylanase enzyme from *A. niger* wild type and mutant on various ammonium sulphate concentrations

Salt Concentration	Wild			Mutant		
	Enzyme nkatal	Wild Protein µg/ml	Specific Activity Wild (nkatal/mg)	Enzyme nkatal	Mutant Protein µg/ml	Specific Activity Mutant (nkatal/mg)
20 % Salt	32.35	223.52	144	39.91	111.76	357
30 % Salt	32.16	102.94	312	29.9	94.11	290
40 % Salt	24.3	126.47	192	29.21	111.76	230
40 % Supernatant	1.15	76.47	15	0	58.82	0

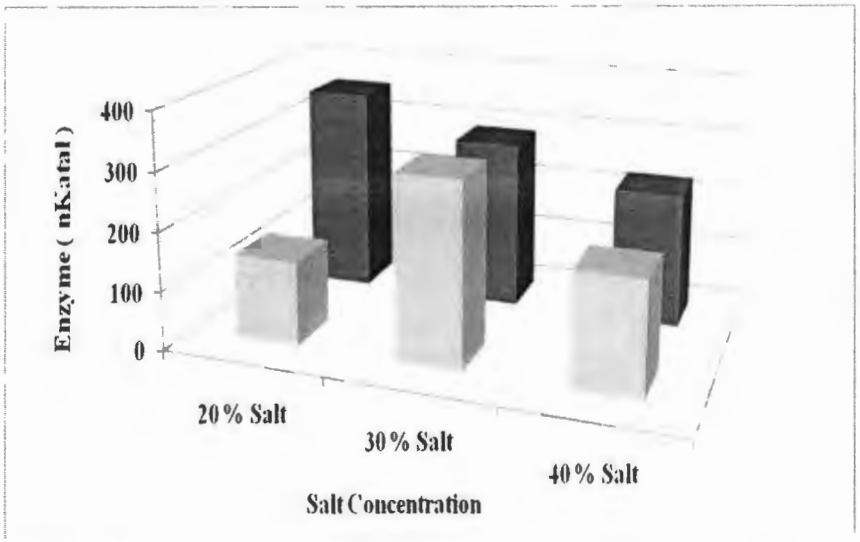


Figure 4.17 20-40 % ammonium sulphate precipitation results. Grey bars present *A. niger* wild type and black bars present mutant enzyme

Precipitates of both wild and mutant enzyme shown in figure 4.18 was then resuspended in a minimum volume of 10 mM phosphate buffer (pH 6.0) for further characterization.

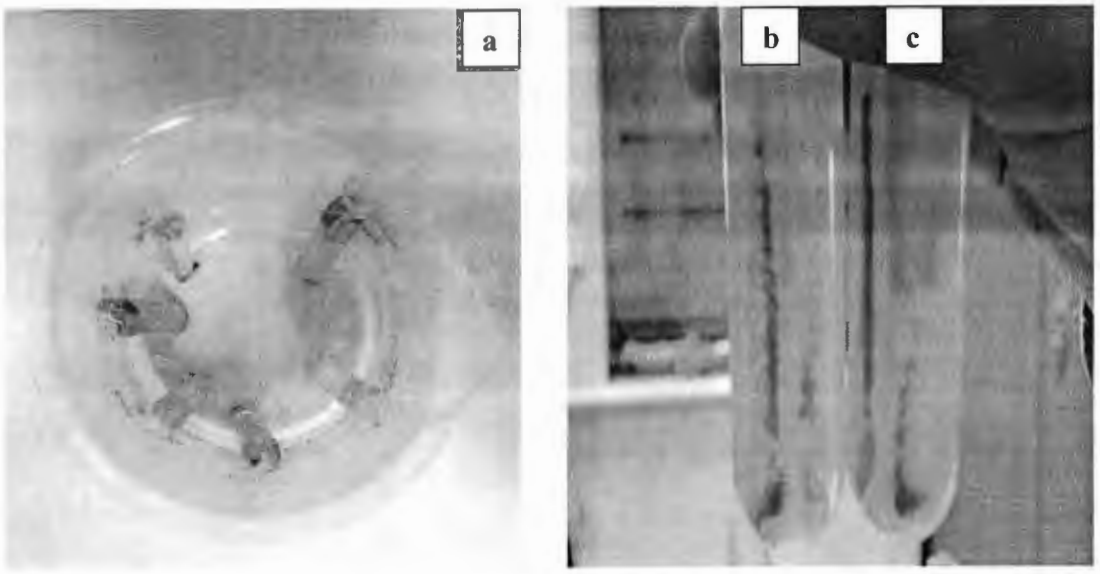


Figure 4.18 20-40 % ammonium sulphate precipitation (a) Dialyzing Tubing (b) Precipitated *A. niger* wild enzyme after centrifugation (c) Precipitated *A. niger* mutant enzyme after centrifugation

4.5 Characterization of xylanase

The partially purified xylanase enzyme from *A. niger* wild and mutant was used to find out the different kinetics parameters as follows.

4.5.1 Effect of substrate concentrations on enzyme activity

The partially purified xylanase enzyme from *A. niger* wild and mutant was used to find out the K_m and V_{max}. For this the enzyme from wild and mutant were used and enzyme assay were performed at 45 °C in the reaction mixtures containing 1 ml of citrate buffer of pH 6.0, 0.5 ml of dialysed enzyme and 0.5 ml of different concentrations of xylan substrate (oat-spelt-xylan; 0.1 to 0.6 mg/ml) in different tubes. The data obtained as shown in table 4.8 was plotted in the form of Lineweaver-Burk plot for wild (Figure 4.19) and for mutant (Figure 4.20) to find out the kinetics parameters. The K_m and V_{max} values for wild xylanase was K_m = 23.4 mg/ml and V_{max} 153.85 nKatal/sec and K_m and V_{max} values for mutant xylanase was K_m = 61.8 mg/ml and V_{max} 312.5 nKatal/sec.

Table 4.8 Effect of enzyme on the different substrate concentrations

Xylan Concentration	Wild Enzyme nkatal	Mutant Enzyme nkatal
0.1 %	2.77	2.95
0.2 %	10.39	9.47
0.3 %	15.93	15.81
0.4 %	23.38	19.74
0.5 %	26.97	21.62
0.6 %	29.94	26.41

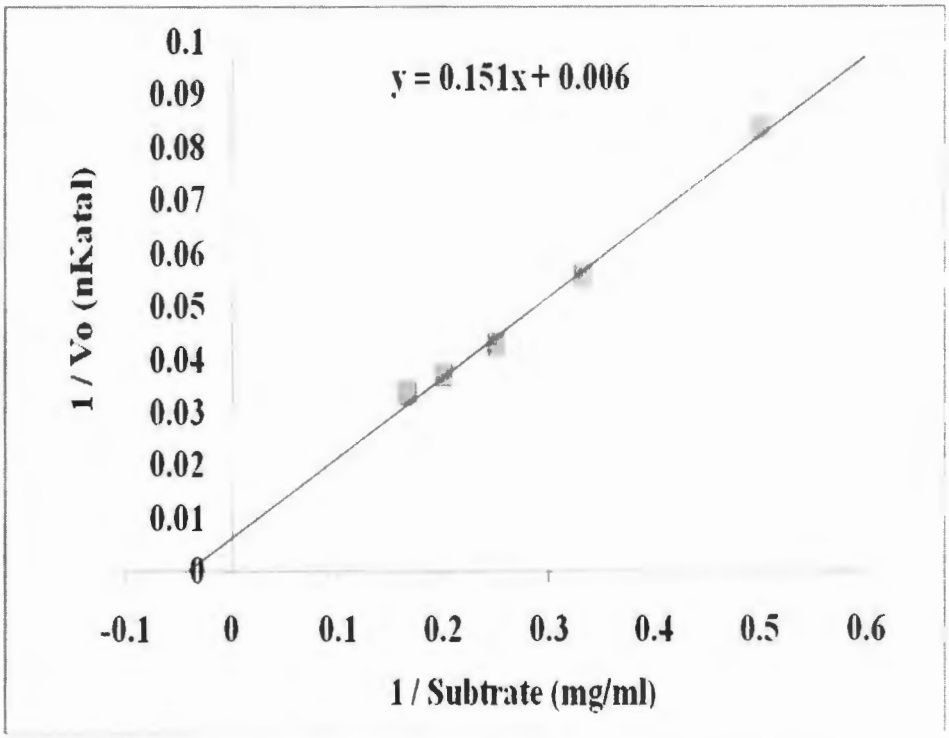


Figure 4.19 Lineweaver-Burk plot for enzyme activity on xylan substrate for wild type *A. niger*

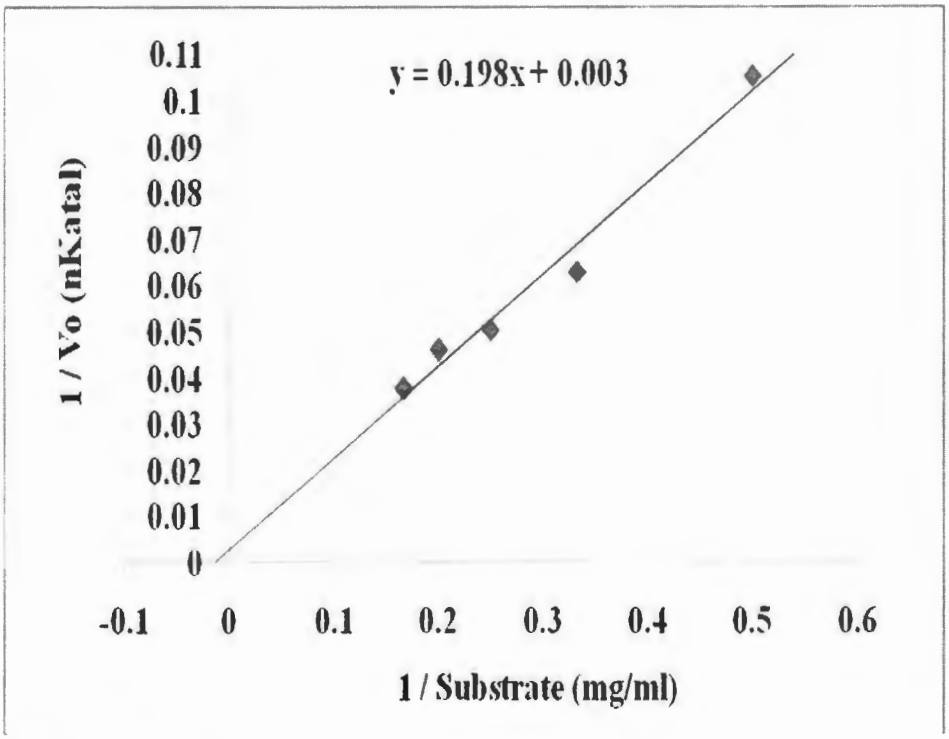


Figure 4.20 Lineweaver-Burk plot for enzyme activity on xylan substrate for mutant *A. niger*

4.5.2 Effect of pH on xylanase activity

Xylanase enzyme from *A. niger* both wild type and mutant were assayed at 45 °C at various pH values (4.0-7.0). The different buffers used were: pH 4.0, 4.4, 4.8, 5.0, 5.2, 5.4, 5.6, 6.0, 6.4, 6.8 and 7.0. The results presented in table 4.9 showed that the pH buffer 6.0 supported the maximum enzyme activity for both wild type and mutant *A. niger*. Figure 4.21 shows that wild has maximum enzyme activity of 40.62 nkatal while figure 4.22 shows that mutant has maximum enzyme activity of 43.4 nkatal.

Table 4.9 Effect of various pH (citrate buffers) on *A. niger* wild type and mutant enzyme activity

pH Buffer Concentration	Wild Enzyme nkatal	Mutant Enzyme nkatal
4.0	11.77	13.13
4.4	13.61	18.25
4.8	21.43	23.36
5.0	24.34	25.95
5.2	24.8	26.49
5.4	29.21	29.96
5.6	35.27	38.03
6.0	40.62	43.40
6.4	36.25	38.75
6.8	32.1	35.2
7.0	29.4	32.49

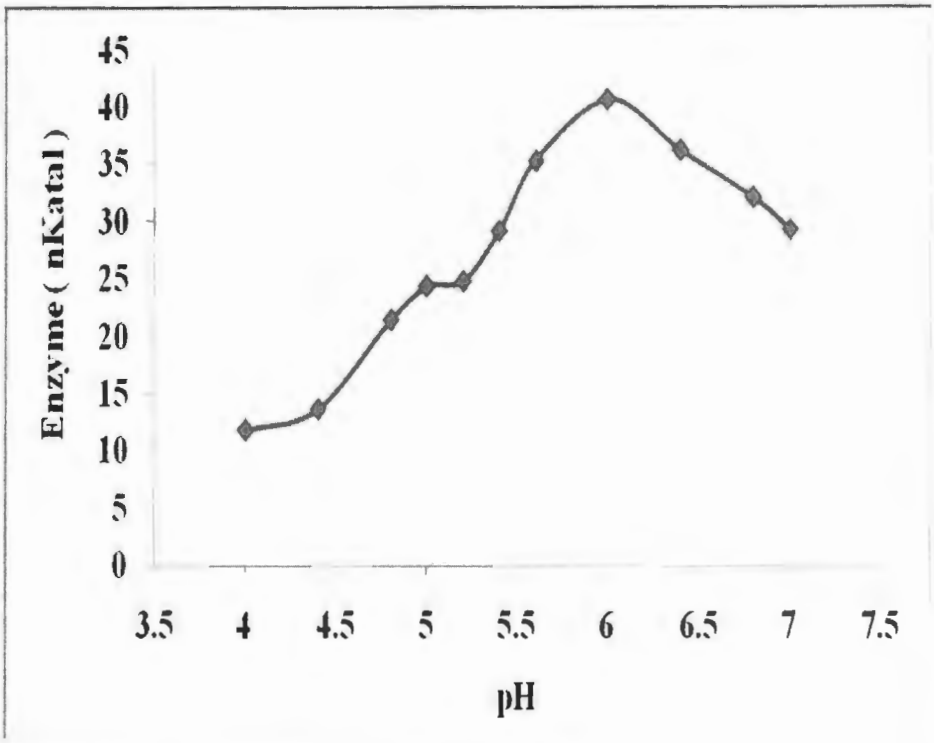


Figure 4.21 Effect of various pH (citrate buffers) on *A. niger* wild type enzyme activity

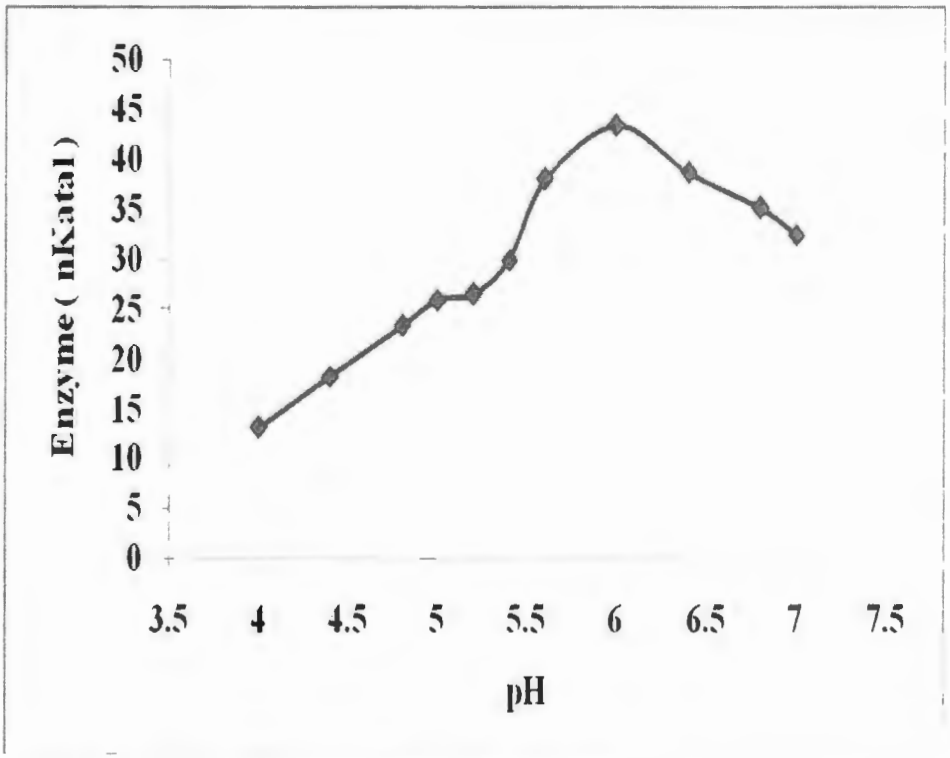


Figure 4.22 Effect of various pH (citrate buffers) on *A. niger* mutant enzyme activity

4.5.3 Effect of temperature on the xylanase activity

Xylanases from *A. niger* wild type and mutant were assayed at different temperatures ranging from 45 °C to 95 °C in the reaction mixtures containing 1 ml of citrate buffer pH 6.0, 0.5 ml of dialysed enzyme and 0.5 ml of 1 % xylan substrate in different tubes. The results presented in table 4.10 showed that the maximum enzyme activity was observed at 65 °C. Figure 4.23 shows that maximum enzyme activity of 43.0 nkatal for wild at 65 °C (t_{opt}) while figure 4.24 shows maximum enzyme activity of 39.52 nkatal for mutant at 65 °C (t_{opt}).

Table 4.10 Effect of various temperatures on *A. niger* wild type and mutant enzyme activity

Temperature °C	Wild Enzyme nkatal	Mutant Enzyme nkatal
45	36.28	35.88
50	40.06	37.76
60	42.04	38.55
65	**43.0**	**39.52**
70	40.87	35.84
75	38.26	31.24
80	35.31	23.19
85	28.62	20.20
90	25.0	17.63
95	20.93	14.37

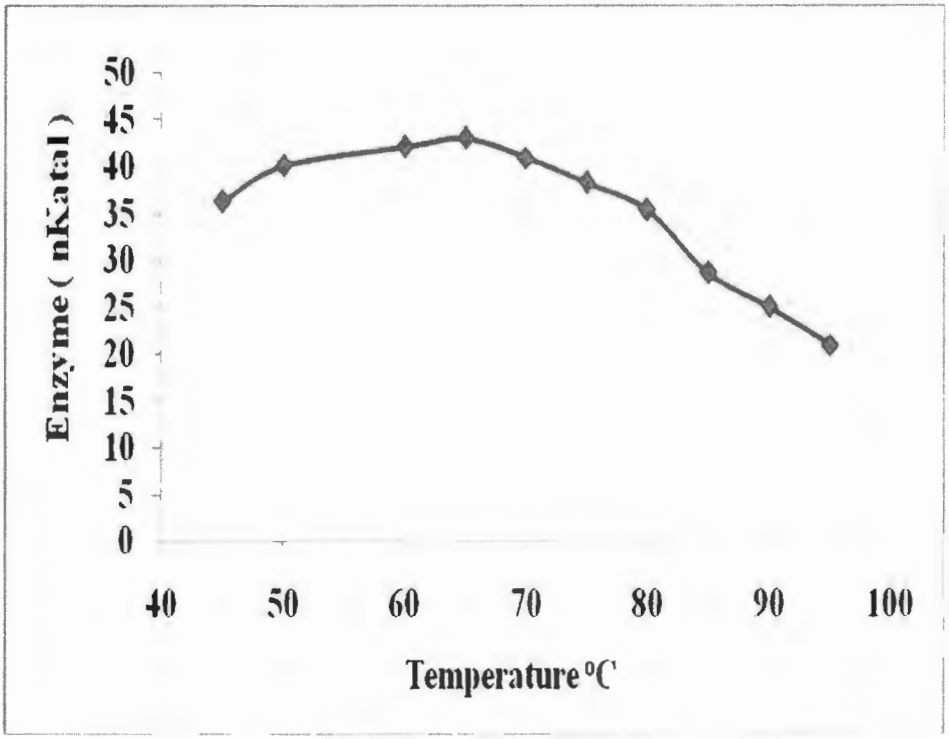


Figure 4.23 Effect of various temperatures °C on *A. niger* wild type enzyme assay activity

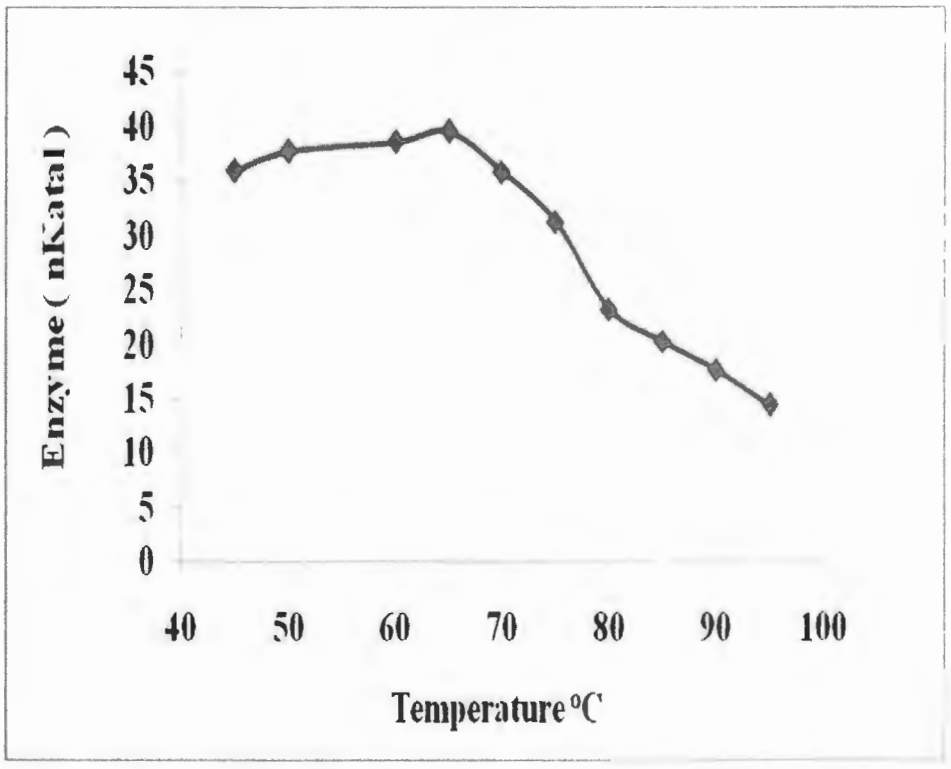


Figure 4.24 Effect of various temperatures °C on *A. niger* mutant enzyme assay activity

4.5.4 Irreversible thermal denaturation of enzyme

The irreversible thermal denaturation effect by temperature on enzyme activity for *A. niger* wild type figure 4.25 and mutant figure 4.26 were determined by incubating the enzyme at different temperatures ranging from 50 °C to 85 °C for 30 minutes. Aliquots were taken out in 6 different eppendorf tubes and each tube was taken out from incubator sequentially after 5 minutes and this action was performed up to 30 minutes and enzyme activity was measured. The data presented in table 4.11 were fitted to the 1st order plot and examined. The k_d values for *A. niger* wild and mutant at various temperatures was calculated as shown in table 4.12. The thermal irreversible denaturation value for *A. niger* wild was E_a = 76.26 kJ/mol which was calculated using Arrhenius plot (Figure 4.27) while thermal irreversible denaturation value for *A. niger* mutant was E_a = 78.08 kJ/mol which was calculated using Arrhenius plot (Figure 4.28).

Table 4.11 Irreversible thermal denaturation of xylanase from *A. niger* wild type and mutant

Temp °C	Wild Enzyme nkatal						Mutant Enzyme nkatal					
	5 mins.	10 mins.	15 mins.	20 mins.	25 mins.	30 mins.	5 mins.	10 mins.	15 mins.	20 mins.	25 mins.	30 mins.
50	45.67	45.33	45.01	44.55	44.32	43.68	46.16	45.81	44.97	44.51	44.13	43.42
60	45.10	44.56	43.76	42.65	41.40	40.53	45.31	44.13	43.52	42.35	40.15	37.95
70	44.59	42.65	41.36	40.44	38.07	36.16	44.39	41.63	40.17	37.99	35.61	32.20
75	61.22	57.03	53.23	49.24	45.09	35.01	60.34	54.16	47.40	41.68	36.73	31.26
80	53.40	47.75	44.37	40.44	36.34	30.87	51.85	44.62	38.89	32.67	27.60	22.46
85	47.39	40.390	36.96	32.42	28.54	24.34	46.28	37.19	30.73	25.93	21.28	17.05

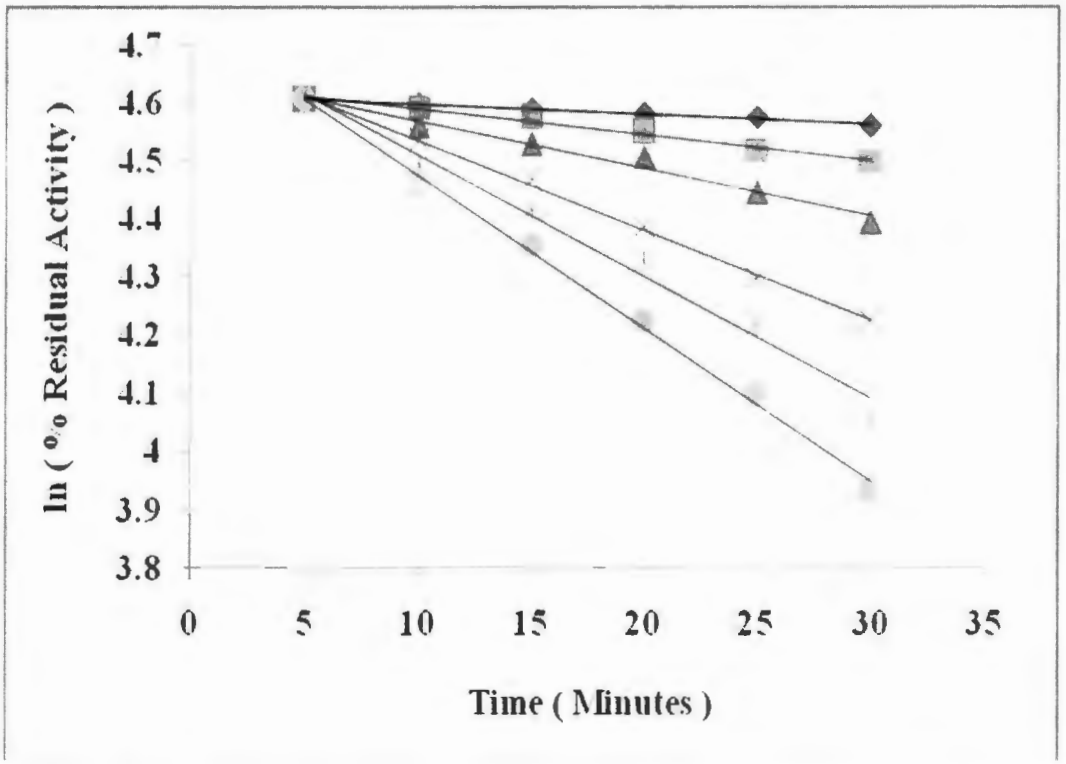


Figure 4.25 Irreversible thermal denaturation of xylanase from *A. niger* wild type

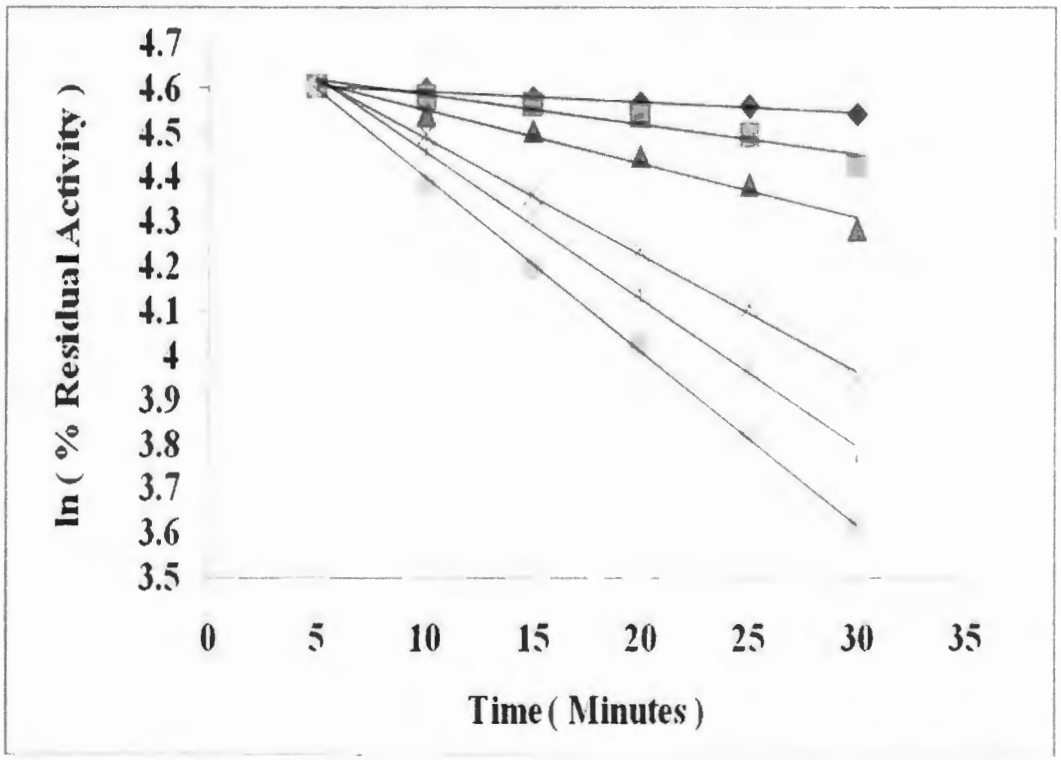


Figure 4.26 Irreversible thermal denaturation of xylanase from *A. niger* mutant

Table 4.12 k_d values for *A. niger* wild type and mutant xylanase at various temperatures

Temperature °C	Temperature (K)	Wild k_d	Mutant k_d
50	323	0.0017	0.0025
60	333	0.0045	0.0067
70	343	0.0081	0.0122
75	348	0.0154	0.0262
80	353	0.0209	0.0331
85	358	0.0262	0.0391

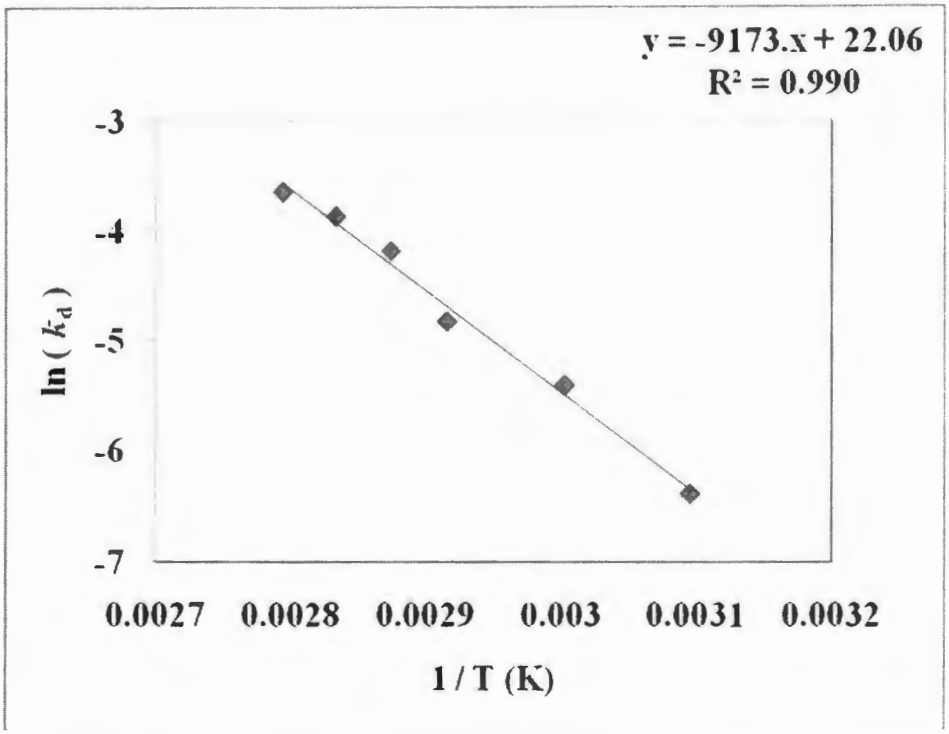


Figure 4.27 Arrhenius plot for calculation of Activation energy (E_a) for *A. niger* wild type xylanase

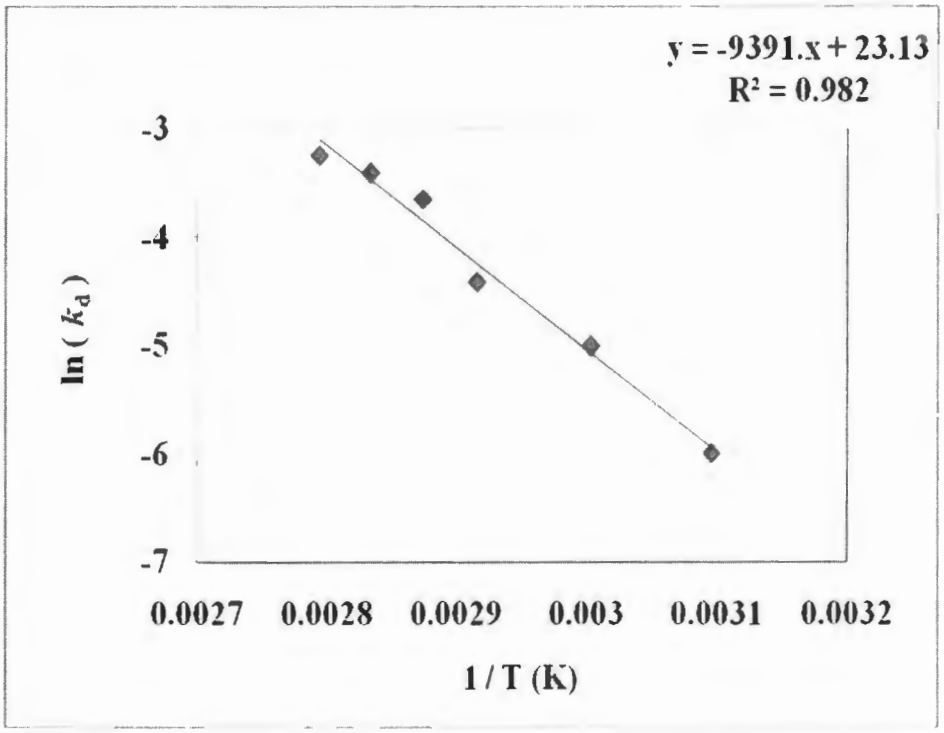


Figure 4.28 Arrhenius plot for calculation of Activation energy (E_a) for *A. niger* mutant xylanase

DISCUSSION

DISCUSSION

Aspergillus sp. is a member of the Deuteromycetes fungi, also considered members of the Phylum Ascomycota. *Aspergillus* sp. found in almost all oxygen rich environments and can grow on carbon-rich substrates like monosaccharides and polysaccharides. *A. niger* has intermediate genome size range at 33.9 Mb. *Aspergillus* sp. are important medically and commercially used for enzymes production like citric acid, xylanase, glucose oxidase, lysozyme. (Geiser, 2009; Bennett, 2010).

Xylanase (EC 3.2.1.8) are important and widely used in the recycling of biomass, as a bleach boosting agent in paper and pulp industry for higher brightness with reduction of bleaching chemicals usage (Singh *et al.,* 2003; Khandeparkar and Bhosle, 2007). In food industry xylanases are used to in bakery products (Bhat and Hazlewood, 2001; Kiachang *et al.*, 2000; Singh *et al.,* 2003; Jiang *et al.,* 2005), in juices clarification, production of functional food ingredients, animal feed and starch separation (Bhat, 2000), in agricultural waste conversion and ethanol production (Damaso *et al.* 2003).

The current research was conducted for xylanase enzyme production by *Aspergillus* sp. and its mutant using different carbon sources for organism growth through solid state fermentation technique. For optimization conditions, inoculum size of 5 %, 10 %,15 % and 20 % of wild type and mutant of *A. niger,*substrates like wheat bran, rice husk, corn cobs and dab grass at three concentrations (2.0, 3.0 and 3.5 %), five incubation temperatures (25, 28, 30, 32.5 and 35 °C), four nitrogen sources including organic (urea, DAP) and inorganic (ammonium nitrate and ammonium sulphate), four pH levels of the culture medium (4.0, 5.0, 6.0 and 7.0) were used to optimize the maximum xylanase enzyme production.

The inoculum size of 10 % spore suspension was optimized for both wild and mutant separately. The results are in accordance with Soliman *et al.* (2012) who also reported 10 % inoculum as optimized for *Aspergillus* sp. Kumar *et al.* (2014) also reported highest xylanase production of 26.1 IU/ml at 10 % inoculum using *Bacillus tequilensis* SH0 from wheat bran. Different carbon sources were used for maximum xylanase production and the overall results showed that maximum xylanase production was obtained on 2 % corn cobs carbon source at 36 h for both wild and mutant separately. The results are in accordance with Bokhari *et al.* (2009) who reported

maximum production of xylanase from thermophilic fungus *Thermomyces lanuginosus* and its mutant derivatives on corn cob.

The effect of various nitrogen sources (both organic and inorganic) on xylanase production was determined in Vogel's medium. The overall results showed that NH_4NO_3 showing maximum xylanase and protein production for wild and mutant separately. Kanimozhi and Nagalakshmi, (2014) obtained maximum xylanase production by *Aspergillus niger* using wheat bran on $NaNO_3$ optimized nitrogen source.

After optimization of inoculum, carbon source, carbon source concentration, nitrogen source, the effect of various incubation temperatures was determined on xylanase production. The overall results showed that 30 °C was optimum temperature for xylanase production from both wild and mutant separately. The results are in accordance with Ahmad *et al.* (2009). They obtained maximum xylanase production at 30 °C by *Aspergillus niger* using wheat bran which confirm our finding. The effect of various pH medium citrate buffers on xylanase production from wild and mutant was determined. The overall results showed that pH buffer 6.0 supported the maximum xylanase production for both and wild and mutant separately. Ahmad *et al.* (2009) reported that maximum xylanase production from medium buffer of pH 5.5.

The ammonium sulphate concentration of 20-40 % was used for precipitation of crude enzyme extract from both wild and mutant. The overall results showed that 30 % ammonium sulphate concentration was best for wild type enzyme precipitation and 20 % ammonium sulphate concentration was best for mutant type enzyme precipitation. The decrease in the requirement of ammonium sulphate for precipitation of mutant enzyme may be due to the change in the surface charges or hydrophobic patches on the surface (Roe, 2001). The results are comparable to those reported by Bokhari *et al.* (2009). They obtained maximum precipitation of xylanase from *Thermomyces lanuginosus* on 30-40 % ammonium sulphate precipitation which confirms our finding about xylanase precipitation by ammonium sulphate.

The partially purified xylanase enzyme from both wild and mutant was used to find out the different kinetics parameters. The effect of various substrate concentrations on enzyme activity from both wild and mutant were observed and K_m and V_{max} were calculated. The results showed that the K_m and V_{max} values for wild xylanase was 23.4

mg/ml and 153.85 nKatal/sec respectively while the K_m and V_{max} values for mutant xylanase was 61.8 mg/ml and 312.5 nKatal/sec, respectively. Chidi *et al.* (2008) isolated *Aspergillus terreus*, UL 4209 strain and reported K_m and V_{max} values were 3.57 mg/ml and 55.5 mol/min/mg protein, respectively using shake flask cultures containing oat spelt and/or birchwood xylans which confirms our finding. Bokhari *et al.* (2009) obtained maximum xylanase K_m and V_{max} values for wild enzyme 14,918 mg/ml and mutant were found 17,897 IU mg/protein, respectively. The K_m and V_{max} values reported by Bokhari *et al.* (2009) are remarkably higher for both wild and mutant xylanase enzyme. The enzyme production variation is due to thermophilic nature of *Thermomyces lanuginosus* and its mutant.

The effect of pH on the activity of crude xylanase enzyme from both wild and mutant was checked. The results showed that the pH buffer 6.0 supported the maximum enzyme activity for both wild type and mutant *A. niger*. The results are in accordance with Ratanachomsri *et al.* (2006). They obtained thermophilic xylanase enzyme from *Marasmius* sp. and found that enzyme activity remained highly stable in acetate buffer of pH 6.0.

The effect of temperature on the xylanase assay activity for wild and mutant were observed at different temperatures ranging from 45 °C to 95 °C. The results showed that the maximum enzyme activity was observed at 65 °C. The results showed that the maximum enzyme activity was observed at 55 to 65 °C. The results are in accordance with Lucena-Neto and Ferreira-Filho, (2004). They obtained thermophilic xylanase enzyme from fungus *Humicolagrisea* var. *Thermoidea* using wheat bran and banana stalk and found that enzyme activity was optimum at 55 to 60 °C which confirms our finding. Sharma and chand, (2012) obtained xylanase enzyme from *Pseudomonas* sp. XPB-6 using seed medium and found that enzyme activity was optimum increase upto at 55 °C and then declined at 65 °C progressively retaining 57 % of the activity which confirms our finding.

The irreversible thermal denaturation effect by temperature on enzyme activity for wild and mutant were determined by incubating the enzyme at different temperatures ranging from 50 °C to 85 °C for 30 minutes. The k_d values for *A. niger* wild and mutant enzyme at various temperatures was calculated. When the data was plotted in Arrhenius

plot, it was calculated that Activation energy (E_a) for denaturation of wild type enzyme was 76.26 kJ/mol and mutant enzyme was 78.08 kJ/mol. The higher value of E_a for mutant enzyme depicted that mutant enzyme requires more activation energy for thermal denaturation as compared to the wild enzyme. Bokhari *et al.* (2009) reported for thermophilic fungus *Thermomyces lanuginosus* and its mutant derivatives on corn cob. They obtained the thermal irreversible denaturation of xylanase value at 55 °C for wild was 167.2 kJ/mol and for mutant was 146.3 kJ/mol. At higher temperatures, the half life of both enzymes decreased sharply, i.e., 219.6 and 13.7 min at 70 °C and just 31.8 and 6.0 min at 80 °C for mutant and wild-type enzyme, respectively.

Conclusion and future prospects

The study results obtained showed that 10 % inoculum size, carbon source corn cobs at 2.0 % concentration, Ammonium nitrate as nitrogen source, initial medium of pH 6.0 and fermentation temperature of 30 °C for 36 h gave maximum xylanase enzyme production. The partial purification of wild type and mutant xylanase was done by 20-40 % ammonium sulphate precipitation. The characterization results of wild type and mutant xylanase showed that the K_m and V_{max} values for wild xylanase was 23.4 mg/ml and 153.85 nKatal/sec respectively while for mutant xylanase the K_m and V_{max} values was 61.8 mg/ml and 312.5 nKatal/sec, respectively. The results showed that the citrate buffer of pH 6.0 supported the maximum enzyme activity for both wild and mutant. The enzyme activity was observed at different temperatures and 65 °C was optimum for both *A. niger* wild and mutant. The irreversible thermal denaturation was performed from 50 °C to 85 °C, on enzyme activity were determined for *A. niger* wild and mutant. The k_d values was calculated for *A. niger* wild and mutant. When the data was plotted in Arrhenius plot, it was calculated that E_a for denaturation of wild type enzyme was 76.26 kJ/mol and mutant enzyme was 78.08 kJ/mol. The results showed that mutant has no significant effect on production pattern of enzyme but there was significant variation in kinetic and thermodynamics parameters of wild and mutant enzyme. The higher values of *Vmax* showed that mutant has brought various changes in enzyme which resulted in higher activity. The higher value of E_a for mutant enzyme depicted that mutant enzyme requires more activation energy for thermal denaturation as compared to the wild enzyme. The *A. niger* wild type and its mutant xylanase enzyme can be produced on industrial large scale using corn cob, found in abundance in Pakistan which will be economically feasible.

REFERENCES

REFERENCES

Abu E.A., Onyenekwe P.C., Ameh D.A., Agbaji A.S. and Ado S.A., (2000) Cellulase (E.C.3.2.1.3) production from sorghum bran by *Aspergillus niger* SL, An assessment of pretreatment methods, Proceedings of the Int. Conf. on Biotechnol., Commercialization and Food Security, Abuja, Nigeria, p. 153-159

Ahmad Z., Butt M.S., Anjum F.M. and Asgher M., (2009) Effect of wheat bran concentration on xylanase biosynthesis by *Aspergillus niger*. Int. J. Agric. Biol. (Pakistan), Vol. 11, No. 5, p. 571-576

Ahmad Z., Butt M.S., Nadeem M.T. and Yasin M., (2013a) Optimization of cultural conditions for xylanase biosynthesis by *Aspergillus niger* using sugarcane bagasse, Pak. J. of Food Sci., 23(2), p. 94-99

Ahmad Z., Butt M.S. and Riaz M., (2013b) Partial purification and characterization of xylanase produced from *Aspergillus niger* using wheat bran, Pak. J. Agri. Sci., 50(3), p. 433-437

Bailey M.J., Biely P. and Poutanen K., (1992) Inter-laboratory testing of methods for assay of xylanase activity, J. Biotechnol., 23, p. 257-270

Bakri Y., Al-Jazairi M. and Al-Kayat G., (2008) Xylanase production by a newly isolated *Aspergillus niger* SS7 in submerged culture, Pol. J. Microbiol., 57(3), p. 249-251

Bertechini A.G., Carvalho J.C.C., Mesquita F.R., Castro S.F., Meneghetti C. and Sorbara J.O.B., (2009) Use of a protease to enhance the utilization of soybean meal amino acids by broilers, Poult. Sci., 88(1), p. 69

Bennett J.W., (2010) An overview of the genus *Aspergillus* in *Aspergillus* Molecular Biology and Genomics (Machida M. and Gomi K.), Caiser Academic Press, Portland, p. 1-17

Bhat M.K., (2000) Cellulase and their related enzymes in biotechnology, Biotechnol. Adv., 18, p. 355-383

Bhat M.K. and Hazlewood G.P., (2001) Enzymology and other characteristics of cellulases and xylanases, In Bedford MR, Partridge CC, (eds.) Enzymes in farm animal nutrition, CAB International, Wallingford, UK, 38, p. 11-60

Bokhari S.A.I., Latif F. and Rajoka M.I., (2009) Purification and characterization of xylanases from *Thermomyces lanuginosus* and its mutant derivative possessing novel kinetic and thermodynamic properties, World J. Microb. Biotechnol., 25(3), p. 493-502

Brühlmann F., Leupin M., Erismann K.H. and Fiechter A., (2000) Enzymatic degumming of ramie bast fibers, J. Biotechnol., 76, p. 43-50

Bruins M.E., Jasen A.E. and Boom R.M., (2001) Thermozymes and their applications, a review of recent literature and patents, Appl. Biochem. Biotechnol., 90, p. 155-186

Chidi S.B., Godana B., Ncube I., Van Rensburg E.J., Cronshaw A. and Abotsi E.K., (2008) Production, purification and characterization of celullase-free xylanase from *Aspergillus terreus* UL 4209, Afr. J. of Biotechnol., 7(21), p. 3939-3948

Corral O.L. and Ortega F.V., (2006) Xylanases advances in agricultural and food biotechnology, Enzyme Biotechnol., p. 305-322

Csiszár E., Urbánszki K. and Szakás G., (2001) Biotreatment of desized cotton fabric by commercial cellulase and xylanase enzymes, J. Mol. Catal B Enzyme., 11, p. 1065-1072

Dai X.J., LiU M.Q., Jin H.X. and Jing M.Y., (2011) Optimization of solid-state fermentation of *Aspergillus niger* JL-15 for xylanase production and xylooligosaccharides preparation, Czech. J. Food Sci., 29(5), p. 557-567

Damaso M.C.T., Almeida M.S., Kurtenbach E., Martins O.B., Pereira N., Andrade C.M.M.C. and Albano R.M., (2003) Optimized expression of a thermostable

xylanase from *Thermomyces lanuginosus* in *Pichiapastoris*, Appl. Environ. Microbiol., 69, p. 6064-6072

Dobrev G. and Zhekova B., (2012) Purification and characterization of endoxylanase Xln-2 from *Aspergillus niger* B03, Turkish J. of Biol., 36(1), p. 7-13

Ebringerová A., Hromádková Z., Heinze T. (2005) Hemicelluloses, Adv. Polym. Sci., 186, p. 1-67

Geiser D.M., (2009) Sexual structures in *Aspergillus*: morphology, importance and genomics, Medical Mycology, 47(sup1), p. 21-26

Gohel H.R., Contractor C.N., Ghosh S.K. and Braganza V.J., (2014) A comparative study of various staining techniques for determination of extra cellular cellulase activity on Carboxy Methyl Cellulose (CMC) agar plates, Int. J. Curr. Microbiol. App. Sci., 3, p. 261-266

Howard R.L., Abotsi E., Jansen van Rensburg E.L. and Howard S., (2003) Lignocellulose biotechnology, issues of bioconversion and enzyme production review, Afr. J. Biotechnol., 2(12), p. 602-619

Irfan M., Nadeem M., Syed Q. and Baig S., (2010) Submerged cultivation of *Aspergillus niger* on pretreated sugarcane bagasse, World J. Agric. Sci., 6(4), p. 466-472

Ikram-ul-Haq., Hussain R., Hameed U. and Javed M., (2008) Selection of *Aspergillus niger* mutant using Antimetabolite 2-Deoxy D-Glucose after N-Methyl N-Nitro N-Nitroso Guanidine (MNNG) treatment, Pak. J. Bot., 40(6), p. 2613-2623

Izidoro S.C. and Knob A., (2014) Production of xylanases by an *Aspergillus niger* strain in wastes grain *Acta Scientiarum*, Biol. Sci., 36(3), p. 313-319

Jiang ZQ., Yang SQ., Tan SS., Li LT. and Li XT., (2005) Characterization of a xylanase from the newly isolated thermophilic *Thermomyces lanuginosus* CAU44 and its application in bread making, Lett. Appl. Microbiol., 41, p. 69-76

Jin N., Shuhan Ma., Liu Y., Yi X., He R., Xu H., Qiao D.R. and Cao Y., (2012) Thermophilic xylanase production by *Aspergillus niger* in solid state fermentation using wheat straw and corn cob, Afr. J. Microb. Res., 6(2), p. 2387-2394

Kang S.W., Park Y.S., Lee J.S., Hong S.I. and Kim S.W., (2004) Production of cellulases and hemicellulases by *Aspergillus niger* KK2 from lignocellulosic biomass, Bioresour. Technol., 91, p. 153-156

Kanimozhi K. and Nagalakshmi P.K., (2014) Xylanase production from *Aspergillus niger* by solid state fermentation using agricultural waste as substrate, Int. J. Curr. Microbiol. App. Sci., 3(3), p. 437-446

Kapilan R., (2015) Solid state fermentation for microbial products, Arch. Appl. Sci. Res., 7(8), p. 21-25

Kavya V. and Padmavathi T., (2009) Optimization of growth conditions for xylanase production by *Aspergillus niger* in solid state fermentation, Polish J. Microb., 58(2), p. 125-130

Khandeparkar R. and Bhosle N.B., (2007) Application of thermoalkalophilic xylanase from *Arthrobacter* sp. MTCC 5214 in biobleaching of kraft pulp, Bioresour. Technol., 98, p. 897-903

Kheng P.P. and Omar I.C., (2005) Xylanase production by a local fungal isolate, *Aspergillus niger* USM AI 1 via solid state fermentation using palm kernel cake (PKC) as substrate, Songklanakarin J. Sci. Technol., 27(2), p. 325-336

Kiachang L., Azadi P., Collins R., Tolan J., Kim J.S. and Eriksson K.E.L., (2000) Relationships between activities of xylanases and xylan structures, Enzyme Microbiol. Technol., 27, p. 89-94

Krisana A., Rutchadaporn S., Jarupan G., Lily E., Sutipa T. and Kanyawim K., (2005) Endo-1,4-β-xylanase B from *Aspergillus niger* BCC14405 isolated in Thailand, Purification, characterization and gene isolation, J. Biochem. Mol. Biol., 38(1), p. 17-23

Kruger N.J., (1994) The Bradford method for protein quantitation, In basic protein and peptide protocols, Humana Press Inc., 32, p. 9-15

Kulkarni N., Shendye A. and Rao M., (1999) Molecular and biotechnological aspects of xylanases, FEMS Microbiol. Rev., 23, p. 411-456

Kumar S., Nivedita S. and Shruti P., (2014) Purification, characterization and gene encoding of xylanase from Bacillus Tequilensis SH0 isolated from compost using low cost wheat bran as substrate, Glob. J. Biol. Agri. And Health Sci., Vol. 3(3), p. 1-15

Lee R.C., Hrmova M. and Burton R.A., (2003) Biofunctional family 3 glycoside hydrolases from barley with alpha-L-arabinofuranosidase and beta-D-xylosidase activity characterization, primary structure and COOH terminal processing, J. Biol. Chem., 278, p. 5377-5388

Lucena-Neto S.D.A. and Ferreira-Filho E.X., (2004) Purification and characterization of a new xylanase from *Humicola grisea var. thermoidea*, Brazil. J. of Microbiol., 35(1-2), p. 86-90

Maciel G.M., Vandenberghe L.P.S., Haminiuk C.W.I., Fendrich R.C., Bianca B.E.D., Brandalize T.Q.S., Pandey A. and Soccol C.R., (2008) Xylanase production by *Aspergillus niger* LPB 326 in solid-state fermentation using statistical experimental designs, Food Technol. Biotechnol., 46(2), p. 183-189

Miller G.L., (1959) Use of Dinitrosalicylic acid (DNS) for determination of reducing sugars, Anal. Chem., 31, p. 426-428

Nath D. and Rao M., (2001) pH dependent conformational and structural changes of xylanase from an alkalophilic thermophilic *Bacillus* sp. (NCIM 59), Enzyme Microb. Technol., 28, p. 397-403

Okafor U.A., Okochi V.I., Onyegeme-okerenta B.M. and Chinedu S.N., (2007) Xylanase production by *Aspergillus niger* ANL 301 using agro-wastes, Afr. J. Biotechnol., 6(14), p. 1710-1714

Pillai S.K.K., (2012) investigations of the bioprocess parameters for the production of hemicellulases by *Thermomyces lanuginosus* strains (Doctoral thesis submitted to Durban university of technology, South Africa), p. 45

Raana K., Tasneem M., Haq I. and Mukhtar H., (2004) Effect of additional carbon and nitrogen on the production of xylanase by a mutant strain of *Aspergillus niger* GCBCX-20, Int. J. Biol. Biotech., 1(4), p. 529-533

Rajoka M.I., Bashir A. and Malik K.A., (1997) Mutagenesis of *Cellulomonas biazotea* for enhanced production of xylanases, Bioresour. Technol., 62, p. 99-108

Ratanachomsri U., Sriprang R., Sornlek, W., Buaban B., Champreda V., Tanapongpipat S. and Eurwilaichitr L., (2006) Thermostable xylanase from *Marasmius* sp. purification and characterization, J. of Biochem. and Mol. Biol., 39(1), p. 105-110

Record E.M., Asther C., Sigoillot S., Paes P.J., Punt M., Delattre M., Haon C.A., Van Den Hondel J.C., Sigoillot L., Lesage-Meessen. and Asther M., (2003) Overproduction of the *Aspergillus niger* feruloyl esterase for pulp bleaching application, Appl. Microbiol. Biotechnol., 62, p. 349-355

Roe S., (2001) Protein purification techniques, Oxford University press, 2nd Eds., p. 134- 142

Saha B.C., (2003) Hemicellulose bioconversion, J. Ind. Microbiol. Biotechnol., 30, p. 279-291

Shah A.R. and Madamwar D., (2005) Xylanase production by newly isolated *Aspergillus foetidus* strain and its characterization, Proc. Biochem., 40, p. 1763-1771

Sharma P.K. and Chand D., (2012) Purification and characterization of thermostable cellulase free xylanase from *Pseudomonas* sp. XPB-6. Adv. in Microbiol., 2, p. 17-25

Sharma S., Vaid S. and Bajaj B.K., (2015) Screening of thermo-alkali stable fungal xylanases for potential industrial applications. Current Research in Microbiology and Biotechnology, 3(1), p. 536-541

Siddiqui K.S., Shamsi A.M., Anwar M.A., Rashid M.H. and Rajoka M.I., (1999) Partial and complete alteration of surface charges of carboxymethylcellulase by chemical modification thermostabilization in water-miscible organic solvent, Enzyme Microb. Technol., 24, p. 599-608

Singh S., Madlala A.M. and Prior B.A., (2003) *Thermomyces lanuginosus*; properties of strains and their hemicellulases, FEMS Microbiol. Rev., 27, p. 3-16

Soliman H.M., Sherief A.D.A. and El-Tanash A.B., (2012) Production of xylanase by *Aspergillus niger* and *Trichoderma viride* using some agriculture residues. J. Agri. Res., 7(1), p. 46-57

Sonia K.G., Chadha B.S. and Saini H.S., (2005) Sorghum straw for xylanase hyper-production by *Thermomyces lanuginosus* (DgWg) under solid state fermentation, Bioresour. Technol., 96, p. 1561-1569

Subbulakshmi S. and Iyer P.R., (2014) Production and purification of enzyme xylanase by *Aspergillus niger*, Int. J. Curr. Microbiol. App. Sci., 3(10), p. 664-668

Tallapragada P. and Venkatesh K., (2011) Isolation, identification and optimization of xylanase enzyme produced by *Aspergillus niger* under submerged fermentation, J. Microbiol. Biotech. Res., 1(4), p. 137-147

Violet M. and Meunier J.C., (1989) Kinetic study of irreversible thermal denaturation of *Bacillus licheniformis* α-amylase, Biochem. J., 263, p. 665-670

Walk C.L., Cowieson A.J., Remus J., Novak C. and McElroy A.P., (2011) Effects of dietary enzymes on performance, goblet cells and intestinal goblet cell number of broilers exposed to a live *Coccidia* oocyst vaccine, Poult. Sci., 90(1), p. 91-98

Watanabe T., (2002) Pictorial atlas of soil and seed fungi, morphologies of cultured fungi and key to species, CRC press, 2nd Eds., p. 193

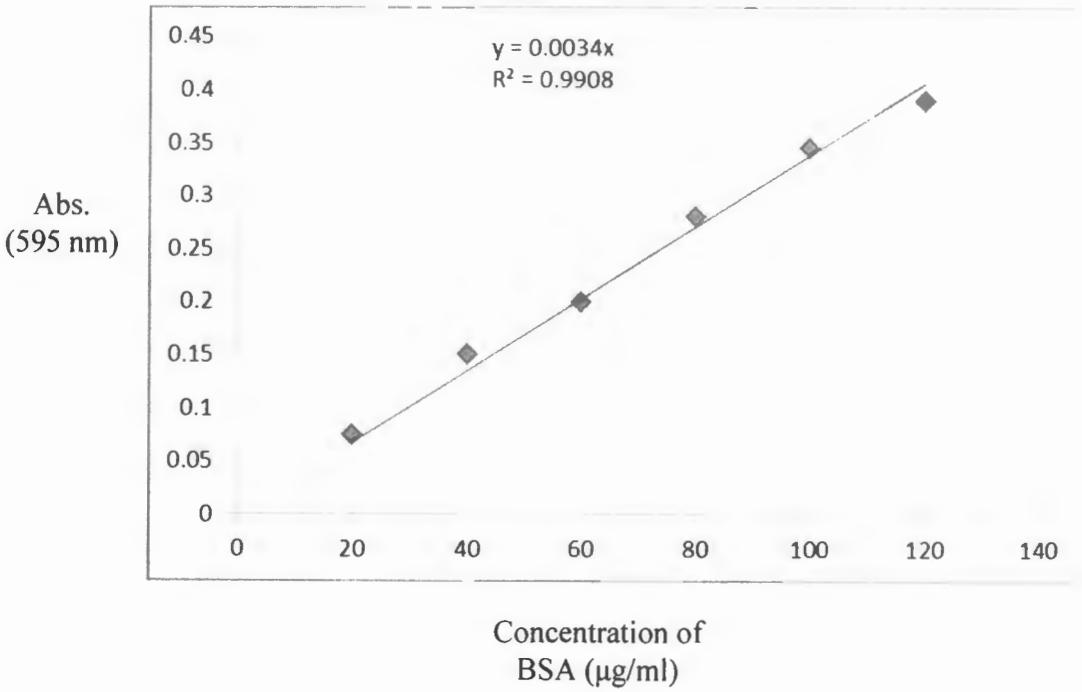
Wong K.K.Y., Tan L.U.L. and Saddler J.N., (1988) Multiplicity of β-1,4-xylanase in microorganisms, functions and applications, Microbiol. Rev., 52, p. 305-317

Yang Y., Zhang W., Huang J., Lin L., Lian H., Lu Y.P., Wu J. and Wang S., (2010) Purification and characterization of an extracellular xylanase from *Aspergillus niger* C3486, Afr. J. Microb. Res., 4(21), p. 2249-2256

APPENDICES

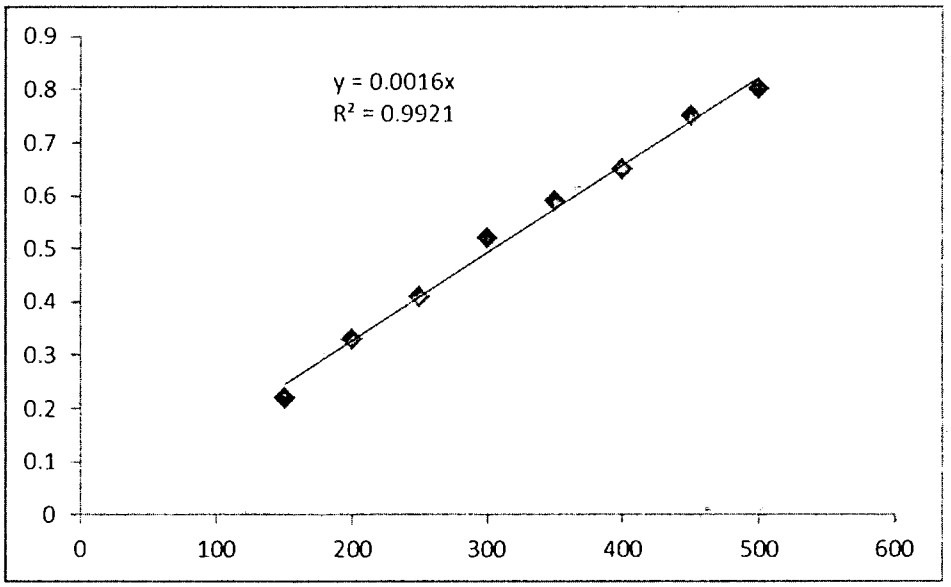
APPENDIX I

BSA Standard Curve for Protein Estimation



APPENDIX II

Xylose Standard Curve for Xylanase Assay



Abs.
(550 nm)

Concentration of
Xylose (µg/ml)

APPENDIX III

CONCENTRATION OF AMMONIUM SULPHATE

Initial concentration of ammonium sulphate (percentage saturation at 0 °C)	Final concentration of ammonium sulphate, % saturation at 0°C																
	20	25	30	35	40	45	50	55	60	65	70	75	80	85	90	95	100
	Solid ammonium sulphate (grams) to be added to 1 liter of solution																
0	107	136	166	197	229	262	295	331	366	404	442	483	523	567	611	659	707
5	80	109	139	168	200	232	266	300	336	373	411	450	491	533	578	624	671
10	54	82	111	141	171	203	236	270	305	342	379	418	458	500	545	589	636
15	26	55	83	113	143	174	207	240	275	310	348	386	426	466	510	555	600
20	0	27	56	84	115	145	177	210	244	280	316	354	392	433	476	519	565
25		0	27	57	85	117	148	182	214	248	284	321	360	401	442	485	529
30			0	28	57	87	119	150	184	217	253	289	328	367	408	451	495
35				0	28	58	88	120	153	187	221	258	295	334	374	416	459
40					0	29	59	90	122	155	190	225	262	300	340	381	424
45						0	29	60	91	125	158	193	229	267	306	347	388
50							0	30	61	93	127	161	197	233	272	312	353
55								0	30	62	94	129	163	200	238	277	317
60									0	31	63	96	131	166	204	242	283
65										0	31	64	98	134	170	208	247
70											0	32	66	100	136	173	212
75												0	32	67	102	139	176
80													0	33	68	104	141
85														0	34	69	106
90															0	34	71
95																0	35
100																	0

Source: Protein purification techniques (A practical approach) by Simon Roe, Second edition (2001), Oxford University Press. Page no: 137.