STUDIES ON THE MICROBIAL PRODUCTION OF XYLANASE BY *ASPERGILLUS NIGER*

A THESIS

Submitted in fulfilment of the requirements for the award of the degree of

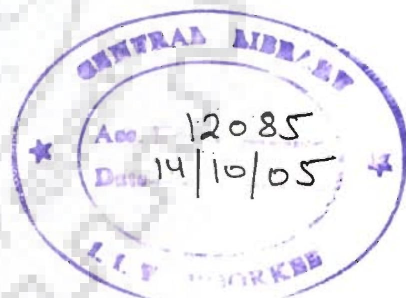
DOCTOR OF PHILOSOPHY

in

BIOTECHNOLOGY

By

NIDHI KAPUR



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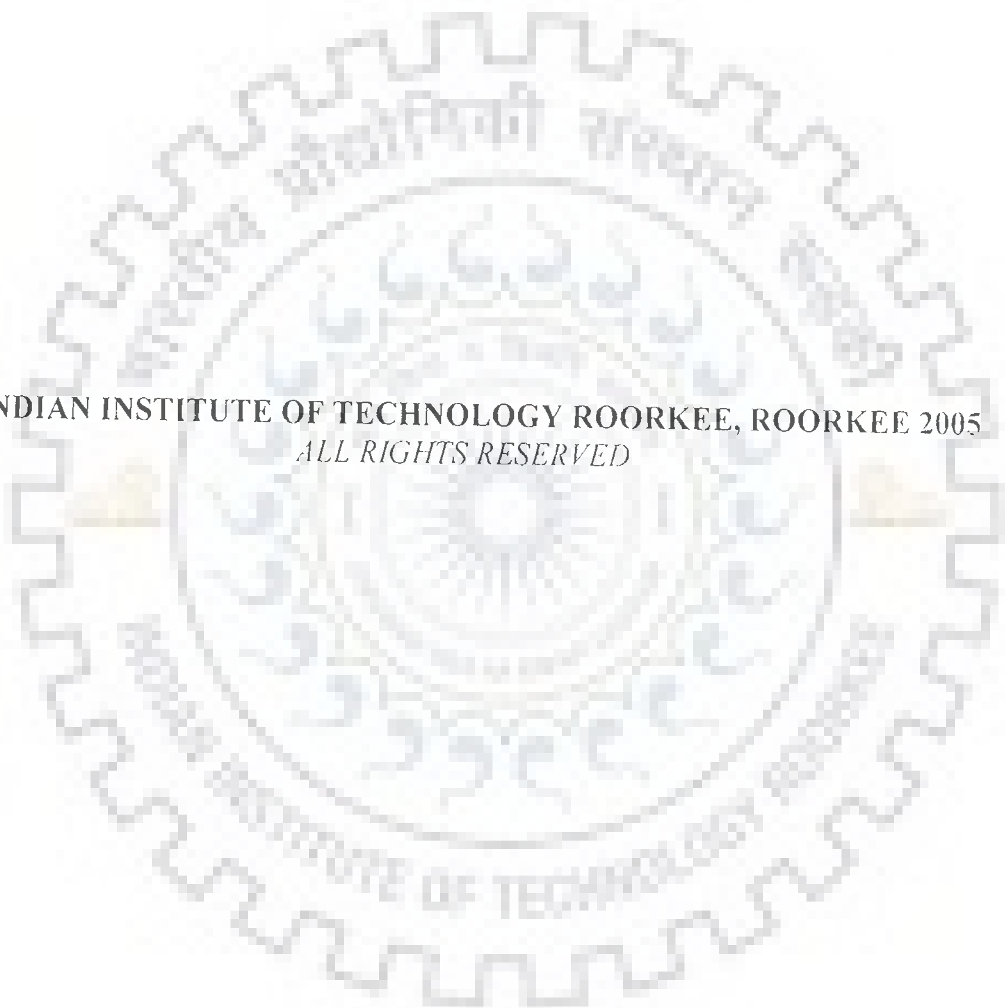

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CANDIDATE'S DECLARATION

I hereby certify that the work which is being presented in the thesis entitled **"STUDIES ON THE MICROBIAL PRODUCTION OF XYLANASE BY *ASPERGILLUS NIGER*"** in fulfillment of the requirement for the award of the **Degree of Doctor of Philosophy** and submitted in the **Department of Biotechnology** of the **Indian Institute of Technology Roorkee**, **Roorkee** is an authentic record of my own work carried out during the period July , 2001 to May, 2005 under the supervision of Dr R P Singh.

The matter presented in this thesis has not been submitted by me for the award of any other degree of this or any other Institute.

Dated: 25th May, 2005

Signature of Candidate

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This is to certify that the above statement made by the candidate is correct to the best of my knowledge.

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ABSTRACT

The present investigation was undertaken to isolate a persuasive microorganism having potential ability to secrete xylanase. An attempt to scale up and economize the process was made by employing the cheaper agro-residues.

A thorough survey of various sites led to isolation of a potent xylanase producing fungal strain. The strain having notably higher levels of xylanase and identified to be *Aspergillus niger* from Indian Agricultural Research Institute, New Delhi was designated as NK-23. In order to improve the levels of enzymatic production, the isolated strain was subjected to both single step as well as mixed mutagenesis. UV mutagenesis followed by N-methyl-n'-nitro-N-nitrosoguanidine (NTG or MNNG) treatment resulted into a mutant strain designated as $NKUC_N$-3.40, which had remarkably higher expression ability of xylanase and β-xylosidase and interestingly had lower levels of cellulase expression. Mutagenesis of the wild type *A. niger* NK-23 had not only improved it's xylanase production ability but also resulted into the distinct morphological features of the mutagenized strain. *A. niger* $NKUC_N$-3.40 had dirty white coloured mycelia that appeared compact and thicker as compared to the yellow coloured and thinner mycelia of the wild type *A. niger* NK-23 strain. Xylanase production from the mutant $NKUC_N$-3.40 strain was evaluated in surface, submerged and solid-state fermentation conditions; of these, the level of production observed was higher in solid-state fermentation. Among the ten different solid supports viz., wheat residue, wheat bran, coconut coir, cotton hull, bagasse, rice bran, loofa sponge, oil cake, polyurethane sponge and glass beads; cotton hull led to higher levels of xylanase production. The amount of substrate, moisture level, pH,

temperature, inoculum and incubation period were also derived to achieve the maximum levels of enzymatic secretion. In order to further enhance the production level different additives were evaluated. Oil cake was found to be the best stimulator for xylanase production. These observations were utilized to scale up and develop a semi-continuous process for xylanase production. The designed bioreactor was used for recycling of the fungal biomass for multiple fermentation cycles. The cotton hull used for this process had yielded better results; since the fungi could anchor effectively and the presence of additional nutrients appeared supporting growth and enzymatic production.

Applicability of the xylanase for pulp and paper processing was analyzed by using XCEHH and CEHH sequences. This had resulted into decrease in the chlorine consumption, which in turn reduces the pollution load on the environment. The enzyme pretreatment led to decrease in kappa number, BOD, COD and AOX. Additionally, it also had affected the increase in the mechanical and optical properties of the paper.

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(NIDHI KAPUR)

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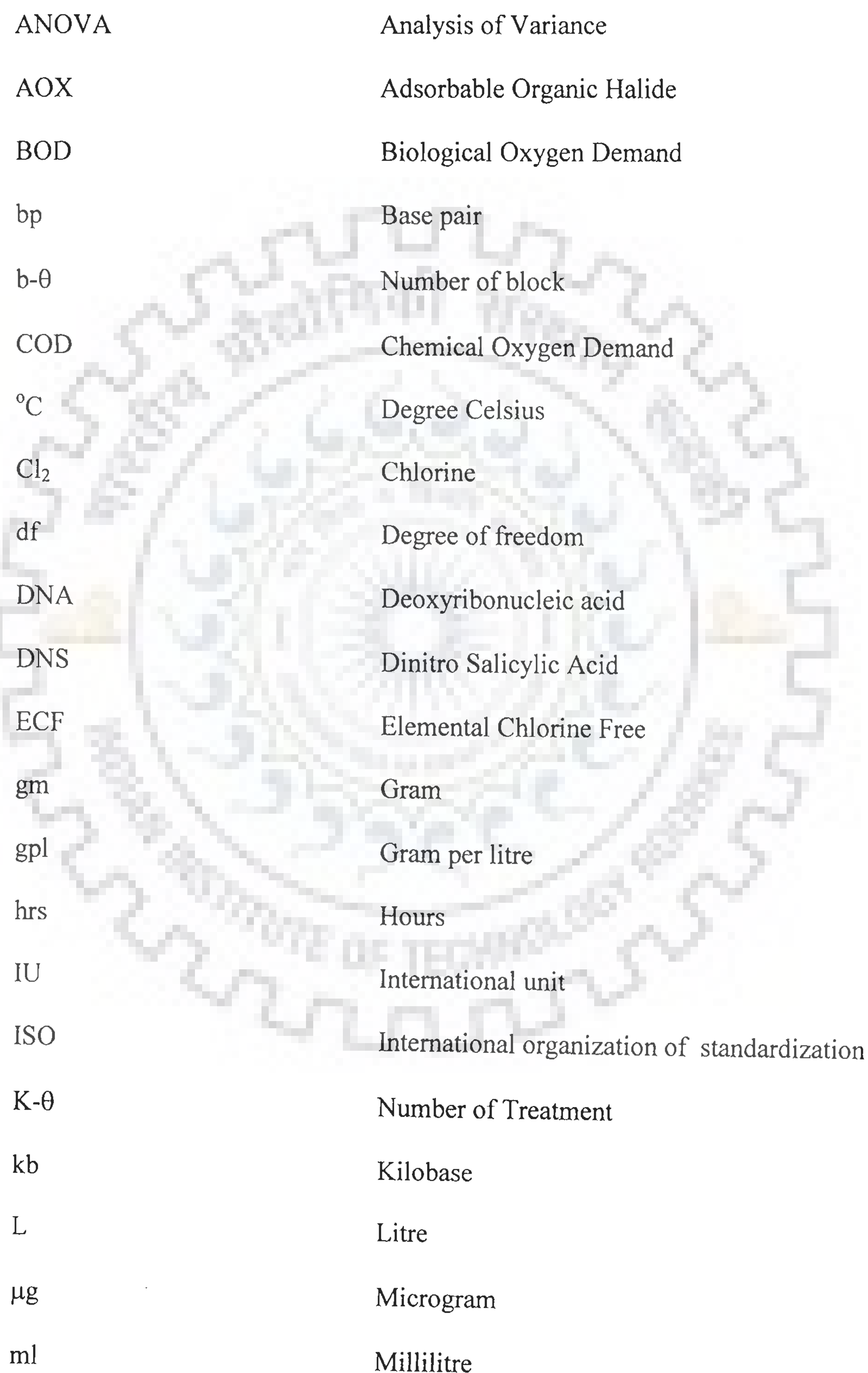


ANOVA	Analysis of Variance
AOX	Adsorbable Organic Halide
BOD	Biological Oxygen Demand
bp	Base pair
b-θ	Number of block
COD	Chemical Oxygen Demand
°C	Degree Celsius
Cl_2	Chlorine
df	Degree of freedom
DNA	Deoxyribonucleic acid
DNS	Dinitro Salicylic Acid
ECF	Elemental Chlorine Free
gm	Gram
gpl	Gram per litre
hrs	Hours
IU	International unit
ISO	International organization of standardization
K-θ	Number of Treatment
kb	Kilobase
L	Litre
μg	Microgram
ml	Millilitre

mm	Millimeter
N	Number of replicates
NTG	N-methyl-N'-nitro-N-nitrosoguanidine
PDA	Potato Dextrose Agar
rpm	Revolution per minute
SEM	Scanning Electron Microscope
SS	Sum of Square
SSE	Error sum of square
SST	Treatment sum of square
TPA	Tons per annum
TCF	Total Chlorine Free
TOX	Total Organic Halide
UV	Ultra Violet
v/v	Volume per Volume
w/v	Weight per Volume

CHAPTER-1

INTRODUCTION

Chapter - 1
INTRODUCTION

The pulp and paper industry is one of the oldest industries and is identified as one of the seventeen industries causing considerable pollution and damage to the environment. Chlorine based bleaching process is commonly used in most of these industries to affect the release of the hemicellulosic and lignin constituents. During various steps of the processing, various chloroaromatic derivatives are released from the lignocellulosic biomass that are ecotoxic and cause considerable damage to the environment. The need for the removal of hemicellulosic and lignin component is essential because these are the major chromophoric substances present in the plant cell wall and removal of these is a prerequisite for the processing of the cellulosic constituent of plant cell wall for pulp and paper industries.

Xylanases have been denoted as a major group of enzymes, having significant application in the paper and pulp industry. Reduction of the ecotoxicity and serious damaging effects caused due to paper mill effluents have been the major concern for the pulp and paper industry in recent years. Xylanases are of great importance to the pulp and paper industries, because the hydrolysis of xylan facilitates the release of lignin from paper and reduces the level of usage of chlorine as the bleaching agent. Vikari *et al.* (1986) was the first to demonstrate that xylanases are applicable for delignification in the bleaching process. The applicability of xylanases are not only limited to the pulp and paper industry but have found wide-spread applications in the baking and textile industry, fruit and vegetable processing, brewing, wine production,

starch-gluten separation, bioconversion and bioremediation of agro-residues.

The major enzyme needed to effect the delignification of pulp is endo-β-1,4-D-xylanase but enrichment with other hemicellulolytic enzymatic components has shown to improve the effect of enzymatic treatment. Xylanases act predominantly on relocated, reprecipitated xylan on the surface of the pulp fibers. Enzymatic hydrolysis of this xylan layer renders the structure of the fiber more permeable, allowing lignin and lignin carbohydrates to diffuse more easily into the bleaching liquor. Xylanases in particular attack the covalent bond existing between xylan and lignin (Fig.1), thus, releasing the lignin, which can then diffuse more easily by using bleach liquor. Although, the details for commercial production of xylanase has not been disclosed, but the process has largely been worked by the major companies like Nova-Nordisk, Promalco Biotech, Danisco Ingredients, etc.

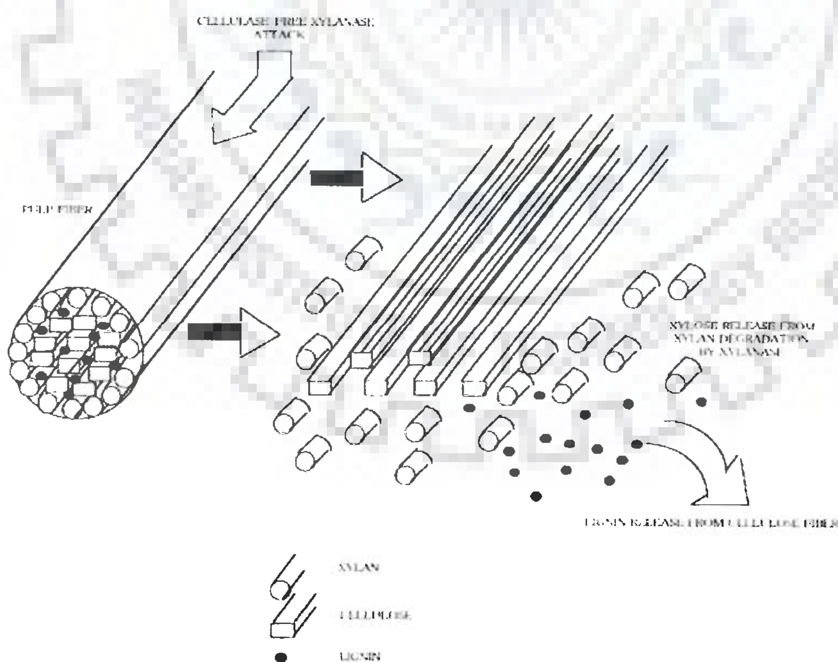


Fig. 1 Removal of lignin associated hemicellulose from pulp by xylanase (Techapun *et al.*, 2003).

A variety of carbohydrates viz. oat-spelt xylan, birch wood xylan and xylose have been proposed and used for the production of the enzyme. Process of enzyme production can be further economized by replacing conventional synthetic carbohydrate substrates with cheaper substrates and by evaluating the applicability of cheaper materials like agro residues, available in plenty in this country for their usage in fermentation system for production. Over the past several years the production of agro-residues has been increasing continuously. Large quantities of these residues are burnt-off every year causing the environmental pollution. Utilization of these agro-residues not only can lead to the economization of the process but also may result into substantial economic gains to the rural population of the country. Further, proper utilization of these residues and prevention of burning of the same will largely help into curbing the environmental pollution caused due to burning of these residues.

In India, various agro-residues like wheat and rice straw, sugarcane bagasse, wheat and rice bran, wheat residue and cotton-hulls, etc are generated in huge quantities every year, which are used in an uneconomical way either as animal feed or discarded as waste without proper use. Serious attempts have so far not been made to utilize the agro-waste residues and decipher their suitability in developing an indigenous technology for xylanase production.

The present study was undertaken to critically review the parameters which are crucial for developing an indigenous technology for economical production using cheaper agro-residues. These residual materials are available in plenty and are without a proper application programme. The major outlines of the work are therefore defined as below:

a. Isolation of microbial strains from paper industry wastes; decomposing manure, dumpsites of sugarcane industry and from

forest soils. Screening and evaluation of the strains for xylanase production.

b. To attempt improvement in the xylanase production ability of the wild type isolate by physical, chemical and mixed mutagenic treatments.

c. Analysis of the xylanase production under surface, submerged and solid-state fermentation. Selection of fermentation system yielding higher levels of production and evaluation of critical parameters for achieving maximum production levels.

d. To derive and design a process for scaling up the production of the xylanases.

e. To analyze and evaluate the xylanase preparation for its application to the pulp and paper industry.

Details of the investigations on the above defined aspects of xylanase production have been enumerated in the following chapters of the present investigation.

CHAPTER-2

LITERATURE REVIEW

Chapter - 2
LITERATURE REVIEW

2.1 HISTORICAL BACKGROUND

Schulze (1891) introduced the term hemicellulose for the fractions isolated or extracted from plant materials with dilute alkali. The classification of these hemicellulosic fractions depends on the types of sugar moieties present. Various microorganisms are actively involved in the degradation of hemicellulose. The presence of microorganisms that degrade hemicellulose, particularly xylan, which is a major constituent of hemicellulose, was reported over 100 years ago by Hoppe-Seyler (1889) who described a gas production process using wood xylan suspension and river mud microbes. Xylanases for xylan hydrolysis are produced by a number of organisms like bacteria, algae, fungi, protozoa, gastropods and arthropods. Filamentous fungi are particularly interesting, since these secrete xylanases in the medium and their enzyme levels are much higher than those of yeast and bacteria.

The use of xylanases to enhance the bleaching of kraft pulp was first discovered in 1986 (Vikari *et al.*) and has advanced to the point of ongoing mill usage today. Xylanase enzymes are now produced for the pulp and paper industry by some companies around the world. A number of mills worldwide have started using the enzymatic bleaching and a survey had shown that as of early 1995, about one million tones per year or 10% of the total bleached kraft pulp produced in Canada is subjected for enzyme based bleaching (Tolan, 1995).

2.2 HEMICELLULOSE

Lignocellulose are the most abundant renewable natural material in the biosphere, contributing approximately 50% of the total biomass generated every year (10-50 x 10^9 tons) and occur mainly as the agricultural, forestry, fruit and vegetable processing wastes. According to Kuhad and Singh (1993) lignocellulosic biomass comprises of cellulose (30-55%), hemicellulose (10-39%) and lignin (11-29%). Xylan, a hemicellulose, is the second most abundant polysaccharide on the earth and contributes as a major component to the secondary cell wall. The plant cell wall is a composite material in which cellulose, hemicellulose (mainly xylan), and lignin are closely associated. The amount of xylan varies in different plants i.e. 20-35% of total dry weight is present as xylan in hardwood and annual plants, and approximately 8% of total dry weight constitutes as xylan in softwood plants and it's amount varies from cell wall of one plant to the other (Karni *et al.*, 1993) e.g. the birch wood has 35% of the xylan whereas gymnosperms have only 7% in their cell walls. In plants, xylans or hemicelluloses are situated between the lignin and the cellulosic fibers present underneath. Due to substitutions, xylan appears to be interspersed, intertwined and covalently linked at various points with the overlying lignin layer, while producing a covering around the cellulose via hydrogen bonding (Uffen, 1997; Beg *et al.*, 2001). It also provides a physical barrier to the oxidizing chemicals or to enzymatic attack during their usage to strip off the lignin layer.

Hemicellulose are composed of both hexoses and pentoses, mainly D-xylose, D-mannose, D-glucose, D-galactose, 4-O-methyl-D-glucuronic acid and to lesser extent L-rhamnose, L-fucose and various O-methylated sugars. D-xylose and L-arabinose are the major constituents of the pentosans (xylans), while D-glucose, D-galactose and D-mannose are the constituents of the hexosans (mannans) (Eriksson *et*

al., 1990; Bastawde, 1992; Kuhad *et al*., 1997). However many combinations of these residues in nature with or without branched chains, give a multiplicity of different chemical structures. The major hemicellulosic component present in softwood are mannan-based and that in hardwood are xylan-based.

The basic structure of xylan consists of a core chain containing 1,4-linked β-D-xylopyranosyl residues. Xylan is a heteropolysaccharide containing the substituents groups of acetyl, 4-O-methyl-D-glucuronosyl and α-arabinofuranosyl residues linked to backbone of β-1-4-linked xylopyranose units. Lignin is bound to xylans by an ester linkage to 4-O-methyl-D-glucuronic residues. Due to structural heterogeneity of the xylans, xylan degrading enzyme systems include several hydrolytic enzymes.

The enzymes that are responsible for the hydrolysis of hemicellulose are referred to as 'hemicellulases' and are well defined and classified according to the substrate on which they act. These are collectively grouped as glycan hydrolases (E.C. 3.2.1.**) (Bastawde, 1992). Microbial hemicellulolytic systems include a number of enzymes like endo-β-1,4-xylanases, arabinofuranosidase, xylosidase, glucuronidase and acetyl esterase (Poutanen *et al*., 1991; Coughlan & Hazlewood; 1993; Kuhad *et al*., 1997; Kulkarni *et al*., 1999; Beg *et al*., 2001; Collins *et al*., 2005). The best known of these are endo-β-1,4-xylanases, which attack the core chain of xylans and β-xylosidases, which hydrolyze xylooligosaccharides to D-xylose. In addition to these two enzymes, several accessory enzyme activities are necessary for debranching the substituted xylans (Poutanen *et al*., 1991).

Though hemicellulases have been studied extensively they still remain less recognized than cellulolytic systems due to the comparatively complex and variable structure of hemicellulose, which essentially comprises of xylose as monomers. Hemicellulolytic enzyme system, however, deserves the similar attention as the

cellulolytic system due to their immense biotechnological potential (Biely, 1985; Wong *et al.*, 1988; Kuhad & Singh; 1993; Beg *et al.*, 2001). The applicability of xylanases increase day-by-day; rayon, cellophane and several chemicals such as cellulose esters (acetates, nitrates, propionates and butyrates) and cellulose ethers (carboxymethylcellulose, methyl and ethyl cellulose), are all produced from dissolving the pulp, that is, pure form of cotton fiber free from all other carbohydrates. The importance of xylanases is not bound to the paper industry alone but these are equally employed for other industries as well (Table 1).

Table 1. Applications of xylanase (Beg *et al.*, 2001; Collins *et al.*, 2005)

Field	Applications
Bioconversion	Hydrolysis of the polymeric sugars to monomeric sugars, which can be fermented to ethanol, xylitol and other chemicals and for the production of single cell proteins
Pulp and Paper	Enzymatic pre-bleaching, deinking, de-barking, beating, pulp fiber refining and production of dissolving pulps
Feed	Feed supplementation to improve nutritional properties of agricultural silage
Food	Extraction of coffee and plant oils, improving starch recovery, processing cereal flour, producing food thickness, clarification of fruit juices, wines and xylooligosaccharides production
Textile	Retting of flax

2.3 ENZYMATIC DEGRADATION OF XYLAN

The use of microbial enzymes for the hydrolysis of xylan is advantageous because of the high specificity of the reactions catalyzed, the mildness of the

conditions of the reaction and the lack of substrate losses due to the chemical modifications (Wong *et al.*, 1988). Xylanases are fast becoming a major group of industrial enzymes, finding significant application in the paper and pulp industry. The main enzyme needed to enhance the delignification of pulp is endo-β-1,4-xylanase but enrichment with other hemicellulolytic components has been shown to improve the effect of enzymatic treatment. Xylanases by attacking the bonds existing between xylan and lignin lead to the release of the lignin, which can then diffuse more easily into the bleaching liquor. Xylanase treatment has been shown to result into the reduced requirement of the chlorine for bleaching while still achieving the higher brightness and good pulp properties. Use of xylanases represents a successful and newer technology that may remarkably reduce the chlorine usage for bleaching.

2.3.1 Xylanolytic System

Xylanases are widespread in nature and are reported from marine and terrestrial bacteria, fungi, marine algae, protozoa, snails, crustaceans, insects and seeds of terrestrial plants (Dekker & Richards, 1976; Bastawde, 1992; Kulkarni *et al.*, 1999; Beg *et al.*, 2001; Techapun *et al.*, 2003; Collins *et al.*, 2005). The xylanolytic system represent the repertoire of hydrolytic enzymes mainly composed of endo-β-1,4-xylanases (β-1,4-D-xylanxylanohydrolase; E.C. 3.2.1.8) and β-1,4-xylosidases (β-1,4-D-xyloside xylohydrolase; E.C. 3.2.1.37); along with α-glucuronidase, acetylxylan esterase and phenolic acid esterase that act co-operatively to convert xylan to its constituent sugars as shown in figure 2 (Gilbert & Hazlewood, 1993; Sunna & Antranikien, 1997).

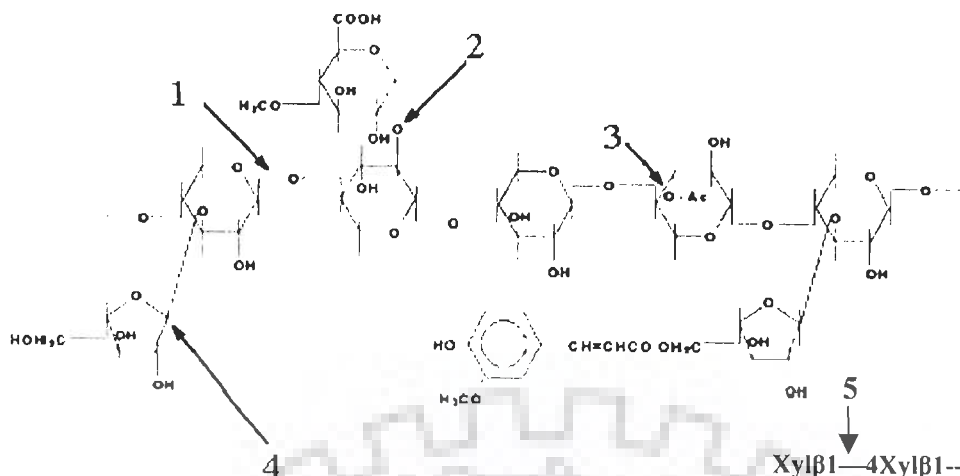


Fig. 2 Enzymes involved in xylan degradation
1, Endo-β-1,4-xylanase (EC 3.2.1.8); 2, α-glucuronidase (EC 3.2.1.1);
3, Acetylesterase (EC 3.1.1.6); 4, α -L-arabinofuranosidase (EC 3.2.1.55);
5, β-xylosidase (EC 3.2.1.37)

2.3.1.1 Endoxylanase (β-1,4-D-xylanxylanohydrolase; E.C. 3.2.1.8)

The endoxylanases are the most widely studied and characterized hemicellulolytic enzyme. Endoxylanases cleave the internal glycosidic linkages of the heteroxylans backbone, resulting in a decreased degree of polymerization of the substrate. The attack on the substrate isn't random and the bonds hydrolyzed depend on the nature of the substrate or the presence of substituents. During the early course of hydrolysis, the pre-dominant products include xylo-oligosaccharides, which hydrolyze to xylotriose, xylobiose and xylose (Dekker & Richards, 1976; Wong *et al.*, 1988). The endo-acting xylanases have been differentiated according to the end products released from the hydrolysis of xylan (e.g. xylose, xylobiose and xylotriose, and/or arabinose) as non-debranching (arabinose non-liberating) or debranching (arabinose liberating) enzymes (Dekker & Richards, 1976). Wong *et al.* (1988) suggested that endoxylanases could be grouped into those that are low molecular weight (below 30 kD) basic protein with high pI values and high molecular weight

(above 30 kD) acidic protein with low pI values. Endoxylanases have been identified in *Aspergillus fischeri* (Senthilkumar *et al.*, 2005); *Aspergillus foetidus* (Shah & Madamwar, 2005); *Aspergillus* sp. (Biely *et al.*, 1991; Gawand & Kamat, 1999), *Bacillus* sp. (Lopez-Fernandez *et al.*, 1998; Gupta *et al.*, 2000), *Cellulomonas* NCIM 2353 (Chaudhary & Deobagker; 1997), *Cryptococcus albidus* (Morosouli, 1986), *Streptomyces* sp. (Beg *et al.*, 2000), *Staphylococcus* sp. SG-13 (Gupta *et al.*, 2000), *Trichoderma reesei* (Tenkanen *et al.*, 1992; Lappalainen *et al.*, 2000) and many other microorganisms (Béra-Maillet *et al.*, 2005; Kolenova *et al.*, 2005; Lin *et al.*, 2005). In 1989, International Energy Agency published a standardized endoxylanase assay using oat-spelt arabinoglucuronoxylan as substrate and stepwise instructions to quantify the reducing sugars released by the enzyme action (Bailey *et al.*, 1992). Alternative procedures for assessment of endoxylanase activity are available especially when the background reducing sugar concentration is high. These methods include measurement of the decrease in viscosity, increase in fluidity of solution of xylan (Fincher & Stone; 1974) or carboxymethyl derivatives of xylan (Gorbocheva & Redonova, 1977; Sengupta *et al.*, 1987), monitoring the decrease in turbidity of stable suspension of suitable insoluble xylans (Wong & Broda, 1992) and the release of dye from covalently linked soluble xylans (Mc Cleary, 1992; Biely *et al.*, 1997).

2.3.1.2 β-D-xylosidase (xylobiase, β-1, 4-D-xyloside xylohydrolase, EC 3.2.1.37)

β-D-xylosidases are exoglycosidases that hydrolyze smaller xylo oligosaccharides and xylobiose from the non-reducing ends to liberate monomeric xylose (Wong *et al.*, 1988). These are the larger enzymes whose molecular weight lie between 60 to 360 kD and may be monomeric or dimeric proteins. β-xylosidase

appears to be mainly cell associated in bacteria and yeast but is extra-cellular in moulds (Biely, 1985). Among the xylo-oligomers, xylobiose is usually the best substrate. The affinity of the enzyme towards xylo-oligosaccharides decreases with increasing degree of polymerization of the substrate. β-xylosidase have been reported in bacteria and fungi that include *Humicola grisea* var. *thermoidea* (Almeida *et al.*, 1995), *Cellulomonas flavigena* (Avalos *et al.*, 1996), *Bacillus thermantarcticus* (Lama *et al.*, 2004), *Melanocarpus albomyces* IIS 68 (Saraswat & Bisaria, 1997), *Thermoascus* sp. (Matsuo *et al.*, 1998), *Trichoderma koningii* G-39 (Li *et al.*, 2000), *Aspergillus phoenicis* (Rizzatti *et al.*, 2001). These enzymes are routinely assayed colorimetrically or flourimetrically by their ability to release *p*-nitrophenol or methylumbeliferone respectively, from the corresponding β-D-xylosides (Couglan & Hazlewood, 1993).

2.3.1.3 α-L-Arabinofuranosidase (EC 3.2.1.55)

The cell wall of graminaceous plants consists of arabinoxylans, it consist of β-1,4-linked D-xylopyranose backbone, which may be substituted at the O-2 and or O-3 positions with L-arabinofuranoside residues (Luonteri *et al.*, 1999). Arabinosidases (EC 3.2.1.55) are exo-acting glycoside hydrolases which generally catalyse the hydrolysis of α-L-arabinofuranosidic moieties or α(1→2) or/and α-(1→3) bonds linking a L-arabinofuranose to a D-xylose in polymers such as arabinoxylan respectively. Arabinosidases belongs to family 51 of the glycoside hydrolases (Remond *et al.*, 2004).

2.3.1.4 α-D-Glucuronidase (EC 3.2.1.131)

The backbones of xylans are substituted with 4-*O*-methyl-α-D-glucopyranosyl, or α-D-glucopyranosyl acid groups. α-Glucuronidases (EC 3.2.1.131)

are enzymes, which are able to hydrolyze the α-1,2-linkage between 4-*O*-methylglucuronic acid and xylose. α-Glucuronidase (EC 3.2.1.139) is one of the accessory enzymes which affect the removal of α-D-glucuronosyl or 4-O-methyl-α-D-glucuronosyl (MeGlcA) residues from acidic xylooligosaccharides generated after the action of endo-β-1,4-xylanase. α-Glucuronidase activity has been detected in the culture filtrates of both fungi and bacteria (Smith & Forsberg, 1991; Puls and Schuseil, 1993; Bronnenmeier *et al*., 1995; de Vries *et al*., 2000). However, most of organisms secrete only low levels of α -glucuronidases. The purified α-glucuronidases have been rather large proteins with molecular masses of around 100 kD. The first α-glucuronidase was purified from *Thermoascus aurantiacus* (Khandke *et al*., 1989). Thus, most α-glucuronidases need to act synergistically with backbone-hydrolyzing xylanase in order to liberate substantial amounts of 4-*O*-methylglucuronic acid from xylans. The enzymes of *P. chrysosporium*, *Thermoanaerobacterium* sp. and *Aspergillus tubingensis* are reported to exhibit also low activity towards polymeric xylan (Shao *et al*., 1995; de Vries *et al*., 1998).

2.3.1.5 Acetyl xylan esterase (EC 3.2.1.72)

Acetyl xylan esterases are responsible for removal of acetate residues from the main chain of xylan, which is a complex heteropolysacchride and requires the concerted action of a number of glycanases and esterases (Chavez *et al*., 2004). Acetyl xylan esterases of particular interest are those, which are obtained from fungi of the genera *Aspergillus, Trichoderma, Schizophyllum.* Preferred species are *Trichoderma reesei*, *Aspergillus niger* and *Schizophyllum commune*. As described above, acetyl xylan esterase can be used to deacetylate xylan. Since it was observed that the activity of acetyl xylan esterase as a single enzyme might lead to precipitation

of the obtained polymer, therefore, it is preferable to use the enzyme in conjunction with other xylan degrading enzymes such as xylanases, arabinofuranosidases, xylosidases and glucuronidases. Acetyl xylan esterases can preferably be used in processes wherein xylan has to be degraded. As a consequence of the deacetylate reaction, xylan becomes better accessible for xylanases. Specific applications of acetyl xylan esterases or combinations of this enzyme with other xylan degrading enzymes include the pretreatment of animal feed to increase the digestibility, *in situ* treatment of fruit juices and beer to improve rheological characteristics and clarity and enzyme added during pulp and paper processing improves the process of bleaching and de-watering.

2.3.1.6 Ferulic acid esterase (EC 3.1.1.73)

Feruloyl esterases or Ferulic acid esterase (FAE, E.C.3.1.1.73) responsible for cleaving the ester-linkage between the main chain of xylan and monomeric or dimeric ferulic acid have been purified and partially characterized (Faulds & Williamson, 1991, McCrae *et al.*, 1994; Williamson *et al.*, 1998b; Topakas *et al.*, 2003a,b). These enzymes act synergistically with xylanases to hydrolyze ester-linked ferulic acid (FA) from cell wall material (Faulds & Williamson, 1991; Topakas *et al.*, 2003a,b). Reports had suggested that microorganisms, such as *Aspergillus*, *Penicillium*, *Fusarium* and *Talaromyces*, produce several types of feruloyl esterases that differ in affinity for 5-*O*- and 2-*O*-feruloylated α-L-arabinofuranosyl residues (Williamson *et al.*, 1998a,b; Ralet *et al.*, 1994; Kroon *et al.*, 2000; Topakas *et al.*, 2003a,b; Garcia-Conesa *et al.*, 2004). Feruloyl esterases have now been classified into four types (A–D) based on their specificity towards mono- and di-ferulates, for substitutions on the phenolic ring, and on their amino acid sequence identity (Crepin *et al.*, 2004). The

nomenclature of feruloyl esterases follows both, the source of the enzyme and the type of feruloyl esterases (e.g. the type-C feruloyl esterase from *Sporotrichum thermophile* is termed StFaeC). Carbohydrate esters of ferulic acid can also be involved in ether linkages with lignin components providing thus the connection between lignin and hemicellulose. An understanding of the co-production of xylanases and esterases by microorganisms and synergistic interactions between the enzymes is of applied interest to food industry (Maat *et al.*, 1992), animal nutrition (Chen *et al.*, 1995a,b), the drink industry (Grassim & Fauquembergue, 1996) and to the pulp and paper industry (Nissen *et al.*, 1992; Record *et al.*, 2003).

2.4 SOURCES OF XYLANASE

Xylanases are widely distributed. They occur in both prokaryotes and eukaryotes (Dekker & Richards, 1976) and have been demonstrated in higher eukaryotes, including protozoa, insects, snails and germinating plant seeds (Taiz & Hoigman, 1976). Amongst the prokaryotes, bacteria and cyanobacteria from marine environments produce xylanases (Dekker, 1985). Intracellular and extracellular xylanases from various bacterial and fungal sources have been studied extensively. Intracellular xylanases occur in rumen bacteria and protozoa (Dekker & Richards 1976).

Xylanase and β-xylosidase are widely distributed in nature. These enzymes are secreted by cells into the surrounding medium (Biely, 1981; Biely & Petrakova, 1984) but some xylanases are cell bound (Dekker & Richards 1976). In almost all bacteria and yeast, β-xylosidase is cell associated (Godden *et al.*, 1989), however, in some fungi, it is secreted in the medium (Wong & Saddler, 1992). Filamentous fungi on the other hand are particularly useful producers since they secrete the enzyme into the medium and their enzyme levels are much higher than those of yeast and bacteria.

2.4.1 Xylanase from Bacterial Origin

The first report of xylanase from alkaliphilic bacteria was published in 1973 by Horikoshi and Atsukawa (1973). Gram-positive, spore-forming bacteria either grow aerobically, or under anaerobic conditions and ferment xylan with production of volatile fatty acid and gas products. Among aerobic, or facultative anaerobic species, xylanolytic activity has been reported in *Bacillus subtilis*, *Bacillus circulans*, *Bacillus pumilus, Bacillus polymyxa* and *Bacillus coagulans* (Wong *et al*., 1988; Gosalbes *et al*., 1991; Virupakshi *et al*., 2005; Yuan *et al*., 2005). *Streptomyces* species includes *Streptomyces exfoliates*, *Streptomyces flavogriseus*, *Streptomyces lividans*, *Streptomyces xylophagus* and *Streptomyces halstedii* JM8 (Wong *et al*., 1988; Ruiz-Arribas, 1995; Díaz et al., 2004). Strictly anaerobic, fermenting microbes, which grow under mesophilic conditions, have also been reported, such as *Clostridium acetobutylicum*, *Clostridium stercorarium* and *Clostridium papyrosolvens* C7 (Wong *et al*., 1988; Nielsen *et al*., 1993).

The gram negative, aerobic, non-spore forming soil microbe, *Pseudomonas fluorescens* subsp. *cellulosa*, has also been shown to degrade xylans (Gilkes *et al*., 1991; Uffen, 1997). Other *Pseudomonas* type xylanolytic gram-negative bacteria seem to reside principally in ruminant animals. These cells represent a large, fastidiously anaerobic group of cells that include *Bacteriodes ruminicola*, *Butyrivibrio fibrisolvens*, *Butyrivibrio succinogenes*, *Butyrivibrio ovatus*, and *Ruminococcus albus* (Whitehead and Hespell, 1990; Gilkes *et al*., 1991; Utt, 1991). Thermophilic bacterial strains include *Bacillus stearothermophilus*, *Clostridium thermocellum*, *Clostridium thermohydrosulfuricum* and *Clostridium thermosaccharolyticum* (Biely, 1985). An additional anaerobic cell that grows and thrives at high temperature includes

Thermoanaerobacter acetigenum, *Thermoanaerobacter ethanolicus*, *Thermoanaerobacterium* sp. strain JW/SL-YS485, *Thermoanaerobium brockii* and *Thermobacteroides* species (Biely, 1985; Shao *et al.*, 1995). Hyperthermophilic bacteria include *Caldocellum saccharolyticum*, *Dictyoglomus* species, *Thermotoga maritima Fj*SB8, *Rhodothermus marinus* and *Thermotoga* sp strain FjSS3-B.1 (Luthi, 1990; Mathrani & Ahring, 1991; Dalhberg *et al.*, 1993; Thomson, 1993; Winterhalter & Liebl, 1995; Subramaniyan & Prema, 2000).

2.4.2 Xylanase from Fungal Origin

Initial selection of fungi of interest was made on the basis of their capacity to produce high titres of xylanase and β-xylosidase, but with minimal protease activity. Fungi of interest included *Aspergillus awamori*, *Aspergillus phoenicis*, *Basidiomyces* sp., *Sporotrichum pulverulentum*, *Trichoderma reesei*. Xylan degradation also occurs by certain fungal strains like *Aspergillus niger, Aspergillus fumigatus, Aspergillus foetidus, Fusarium oxysporum, Neurospora crassa, Penicillium janthinellum, Penicillium wortmanni, Penicillium capsulatum, Trichoderma reesei, Trichoderma harzianum and Trichoderma viride* (Biely, 1985; Wong, 1988; Filho *et al.*, 1996; Seyis & Aksoz, 2005; Shah & Madamwar, 2005). Thermophilic fungi include *Humicola lanuginose, Thermoascus aurantiacus, Talaromyces byssochlamydoides and Sporotrichium thermophile* (Shao *et al.*, 1995).

2.4.3 Xylanase from Actinomycetes

Among the actinomycetes, *Streptomyces raseiscleroticus*, *Saccharo monospora viridis,* thermotolerant *Streptomyces* sp. T7 and *Nocardiopsis dassonvillei*, were reported to secrete xylanase (Keskar, 1992; Srinivasan & Rele, 1999).

2.4.4 Xylanase from Yeasts

Biely (1978) reported 95 strains of yeasts and yeast like organisms resulting into the production of xylanases when grown on xylose, xylan from deciduous trees and hemi-cellulose from conifers. *Cryptococcus albidus* was observed to produce β-xylanase and β-xylosidase simultaneously (Biely *et al.*, 1981). *Trichosporon beigelii* (Stevens & Payne, 1977) and *Aurobasidium pullulans* (Leathers *et al.*, 1984; 1986) have also been reported to produce extra-cellular endoxylanase.

2.5 HETEROGENEITY OF XYLANASES

A greater extent of variation is observed among xylanases, which although perform similar function but differ in their physiochemical properties, such as molecular weight, isoelectric point, etc. The varying xylosidic linkages in lignocellulosic are not equally accessible to xylanolytic enzymes. Moreover, during hydrolysis, the accessibility of some linkages changes. Bacteria and fungi, in order to attack the different xylanolytic linkages, have evolved xylanolytic system consisting of a number of enzymes with different specificities. This is analogous to, but more complex than, the multienzyme cellulolytic systems of bacteria such as *Clostridium thermocellum* and *Trichoderma reesei* as hemicellulose is structurally much more complex than the cellulose.

Different hemicellulolytic microorganisms produce varying levels of the xylanolytic enzymes, many of which have different degrees of specificity. For instance, the zymogram analysis has detected various xylanases in the culture filtrate of *Aspergillus niger* (Biely, 1985) and *Butyrivibrio fibrisolvens* H17c (Lin & Thompson, 1991). However, the detection of multiple bands on zymograms may be due to post-translational modification such as glycosylation or proteolysis or both

leading to a greater degree of heterogeneity of these enzymes. The detection of minor bands with xylanolytic activity may be due to distinct growth and/or purification conditions, or these components may be involved in specialized functions such as the hydrolysis of linkages, which are not found frequently (Wong *et al.*, 1988).

2.6 XYLANASE CLASSIFICATION

Xylanases can be classified into different groups mainly by taking into account the following major criteria:

2.6.1 Molecular Weight and pI

Xylanases that belong to high or low molecular weight constituents and have either a high (basic) or low (acidic) pI (Wong *et al.*, 1988), this classification pattern also takes into consideration of the sequence analysis and can reliably predict the crystal structure.

2.6.2 Kinetic Parameters

Xylanases can be defined as of the following three types (Dekker, 1985; Bastawde, 1992).

Endo-β-(1→4)-D-xylan xylanohydrolase, [E.C. 3.2.1.8]: These enzymatic components act randomly on xylan to produce large amounts of xylo-oligosaccharides of various chain lengths.

Exo-β-(1→4)-D-xylanase [β-(1→4)-D-xylan xylohydrolases]: These enzymes remove single xylose units from the non-reducing terminal of the xylan chain.

β-Xylosidase or Xylobiase [E.C - 3.2.1.37]: These enzymes hydrolyze disaccharides like xylobiose and the higher xylooligosaccharides with decreasing specific affinity.

2.6.3 On the Basis of Crystal Structure

Xylanases are grouped into two families:

Family 11/ Family G: This family of xylanases results into oligosaccharides with lower degree of polymerization. Members of this family act on xylobiose, cellobiose and xylobioside and yield xylose and xylobiose whereas few others yield exclusively xylotriose or higher oligosaccharides (White *et al.*, 1994; Jeffries, 1996).

Family10/FamilyF: These are the true xylanases lacking the cellulase activity and are secreted by both bacteria and fungi. Catalytic domain of this family consists of the β-pleated sheets formed into a bilayered trough that surrounds the catalytic site (Withers *et al.*, 1995; Jefferies, 1996). Many amino acids in the members of this family are positionally conserved.

2.7 THE "XYLANOSOME"

Xylanosome are the discrete, multifunctional, multienzyme complex found on the surface of several microorganisms (Sunna & Antranikian, 1997). The xylanosome complex B (CB) from *Butyrivibrio fibriso* H17C is analogous to the cellulosome of the same (Lin & Thomson, 1991). The xylanosome complex has the molecular weight of >669 kD and consists of 11 protein bands representing xylanase activities ranging from M_r 45 kD to 180 kD, and out of these, three had shown the endoglucanase activity and are the low molecular weight constituents. All the 11-enzymatic components of the xylanosome complex do not necessarily represent 11 different gene products, as glycosylated or breakdown products could also be active. *Clostridium papyrosolvens* C7 expresses a multi-complex cellulase-xylanase system, which is responsible for hydrolysis of cellulose and xylan. This multiplex system consists of 7 protein complexes whose molecular weight ranges from 500 to 660 kDa (Pohlschroder *et al.*, 1994; Beg *et al.*, 2001).

2.8 XYLANASE BIOSYNTHESIS

Production of xylanolytic enzymes by various bacteria and fungi is found to be inducible. Smaller molecules, which are the degradation product of the complex polysaccharide xylan, act as inducers. A basal level of these hydrolytic enzymes is always present in the cell. Small soluble signal molecules, due to the activity of these enzymes are generated by degradation of polysaccharide substrate. These signal molecules are able to enter the cell and induce the synthesis of the corresponding enzyme. The potent inducer molecules are xylose, xylobiose, xylooligosccharides, heterosaccharides of xylose and glucose and their positional isomers. In some cases xylan itself acts as inducer, but weakly, due to its complex structure and high molecular mass it can't penetrate the cell wall (Kulkarni *et al.*, 1999). The low molecular mass substrates act, as inducers need transferase enzymes for their translocation into the cytoplasm. The most prominent signaling molecule is probably xylobiose, which was found to be an effective inducer in a range of microorganisms, together with other xylooligosaccharides (Pinaga *et al.*, 1994). There is evidence that positional isomers of xylobiose such as 1,2-β-xylobiose and 1,3-β-xylobiose are more potent inducers as compared to the simple cleavage product from xylan. A possible explanation of the weaker induction efficiency of xylobiose is the rapid hydrolysis of 1,4-isomer by β-xylosidase and thus the shorter availability of this inducer. Figure-3 shows the mechanism of xylanase biosynthesis. Induction of xylanase synthesis can also be achieved by β-methyl-D-xylopyranoside. This is a non-metabolizable, structural analogue of xylobiose and can be synthesized comparatively lower cost. It can be successfully employed for the induction of xylanases in fungi.

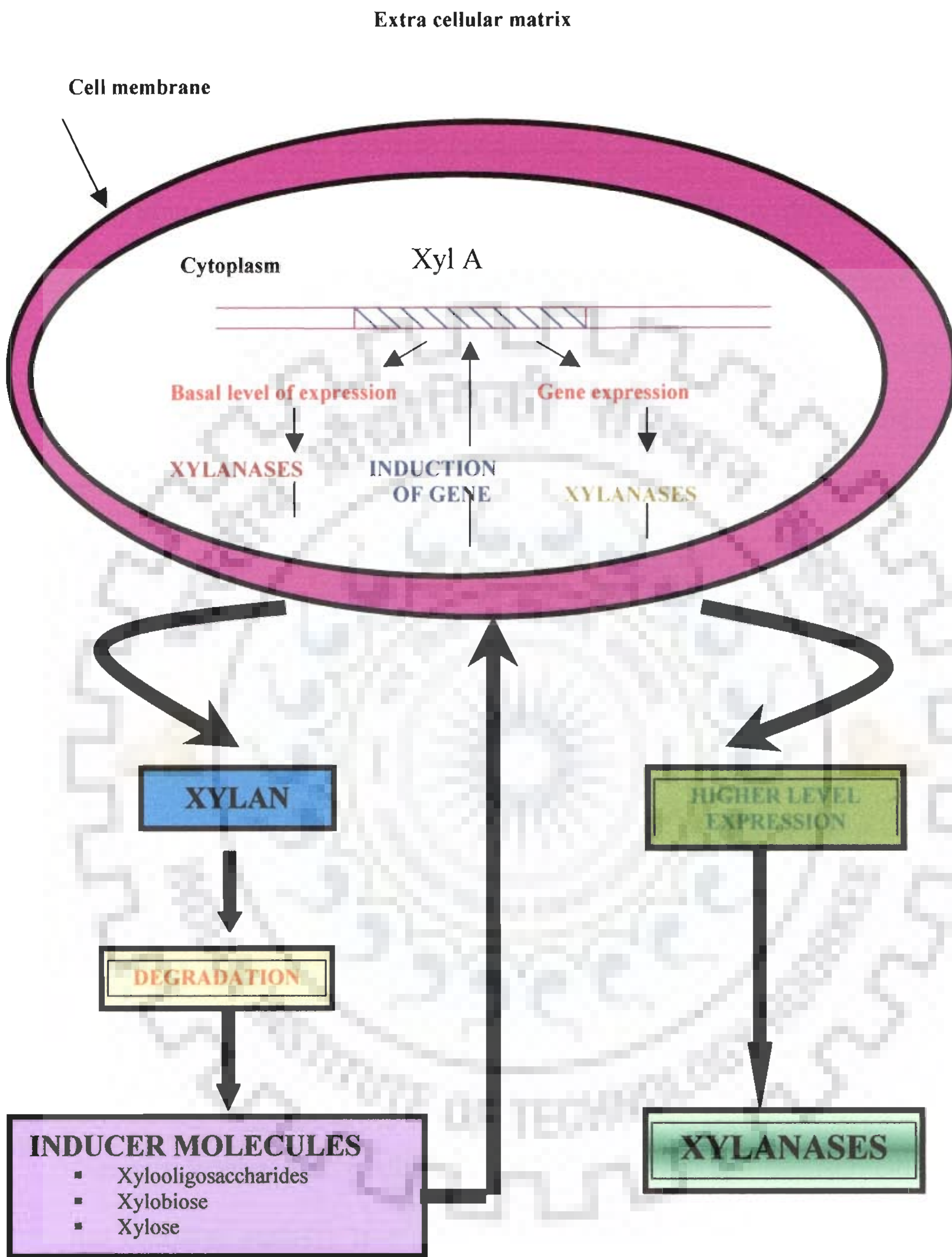


Fig. 3 Mechanism of xylanase biosynthesis

Catabolic repression by glucose and xylose is a common phenomenon observed in xylanase biosynthesis. Catabolic repression of xylanase gene appeared to be controlled at two levels directly by repression of gene transcription and indirectly by repression of transcriptional activator. Catabolic repression means enzyme synthesis goes down when easily metabolizable carbon sources are present in the growth medium. Sequence analysis of T-6 xylanase gene has revealed that AT rich region preceding the promoter is the binding site for a protein that regulates induction. This binding site is found to be 230 bp inside the xylanase structural gene and has a consensus sequence TGT/AAANC//GNTNA/TCA. Underlined letters in this sequence represented the most critical bases, N is any base and the double slash denotes an axis of symmetry.

2.9 GENETIC MANIPULATION OF MICROBIAL STRAINS

2.9.1 Mutagenesis

Xylanases are of vital significance for the paper and food industries and are also used as animal feed additives. Microbial strains producing xylanolytic activity are good sources for isolating the native enzymes (Zorec *et al.*, 2001). Enzyme production can be modulated by different approaches mainly by defining fermentation parameters, mutagenesis and by genetic modifications. Due to the relatively later developments of the genetic modification techniques in filamentous fungi, many of the studies were directed towards specifying fermentation parameters for increasing the enzyme production. Due to the pace of technological development during late 80's many of the present day techniques that were initially oriented for *Saccharomyces cerevisiae* are also practiced for a number of other fungal strains. Hence the strain improvement programmes employ mutagenesis strategies along with the molecular

cloning techniques. Biswas *et al.* (1990) had achieved the enhanced production of xylanase and β-xylosidase from *Aspergillus orchraceous* by multistep mutagenesis. Various other groups had also attempted the mutagenesis for enhanced enzyme production (Kuhad *et al.*, 1998; Butt *et al.*, 2002; Trujillo *et al.*, 2003; Lu *et al.*, 2003). One of the other major objectives of the strain improvement programme includes prevention of the proteolytic enzymes produced, that otherwise result into inactivation of the desired enzymatic components.

2.9.2 Molecular Cloning and Expression of Xylanase Gene

Enzymes of industrial significance are cloned and expressed in suitable microbial hosts for increasing the yields in order to develop a cost effective and market friendly process. According to Bergquist *et al.* (2002) the cost of the enzyme for bleaching purpose should not exceed $2.25 per gram and cost of overall process employing enzyme based technology should not exceed US$ 4.50 per tons to satisfy the economics of both manufacturer and user. Hence, selection of the appropriate host-vector system is critical in order to have the cost effective production of enzymes. The main objective for the molecular cloning of xylanase gene is to produce xylanases without having the cellulases as the contaminant enzymes. Furthermore, the additional advantage is the direct accessibility to the xylan in fermentation could be enhanced by expressing the xylanase and β-xylosidase genes in xylose fermentating organisms. In addition, another advantageous parameter that is worth consideration for selecting the expression system is the extracellular production of enzyme. Purification step is thus easy and economical for the secreted enzyme than that for the product expressed intracellularly which was the prevalent mode of production in most of the cloning systems.

A 1383 bp long DNA fragment from *Vibrio sp.* strain XY-214 was responsible for encoding a 51kD, protein (Araki *et al.*, 2000). *Bacillus circulans* Teri-42 xylanase gene was cloned in *E. coli* DH-5α using plasmid pUC19; however, 87% higher expression was observed when the xylanase gene was cloned in *Bacillus subtilis* using the plasmid pBA7 (Qureshy *et al.*, 2000). Similarly, xylanase genes from *Paenibacillus sp.* and *Bacillus stearothermophilus* T-6 were also cloned in *E.coli* (Gat *et al.*, 1994; Lee *et al.,* 2000). *Clostridium thermocellum* xylanase gene segment (1.6kb) was inserted in pJX-18 vector and expressed in *Bacillus subtilis* (Jung & Pack, 1993). In *Pseudomonas fluorescens sub sp. cellulosa*, xylanase and arabinofuranosidase genes, separated by a 118bp linker DNA were observed to undergo transcription in similar orientation. Further, in *B. polymyxa* also, xylanase and endoglucanase genes were separated by a 155 bp linker DNA (Gosalbes *et al.*, 1991). *Trichoderma reesei* QM6a, XYN2 gene was expressed in yeast *Sacchromyces cerevisiae* under the control of alcohol dehydrogenase (ADH2) and phosphoglycerate kinase (PGK-1) gene promoter and terminator respectively. Resultant recombinant strains produced 1200 and 160 nkat ml^{-1} of xylanase activity respectively under the control of ADH2 and PGK-1 promoters. The recombinant xylanase had an optimum temperature of 60°C at pH 6 and more than 90% activity was retained after treating the enzyme for 60 minutes at 50°C (Kulkarni *et al.*, 1999; La Grange *et al.*, 2000).

2.10 FACTORS AFFECTING ENZYME PRODUCTION

A wide range of environmental parameters in addition to the components of the culture medium affects the growth and production of xylanase by fungi. In addition to using the substrate specific media for the production xylanase by fungi, induction of several mineral salts e.g. (KH_2PO_4, $MgSO_4$), metallic ions and the

nitrogen sources profoundly affect the xylanase production. The effects of individual components on xylanase production by *Sclerotium rolfi* were assayed (Haltrich *et al.*, 1994). Elevated xylanase production in presence of increased amounts of nitrogen source has been reported for *A. fumigatus* (Kitpreschvanich *et al*, 1992).

2.10.1 Carbon Source/Substrate

The choice of an appropriate substrate is of great importance for the successful production of xylanases. The substrate not only serves as carbon and energy source, but also provides the necessary inducing compounds for the organism. Purified xylans can be excellent substrates and frequently used for small-scale experiments. For large-scale production processes, these expensive substrates, however, are not suited. Some organisms interestingly yielded higher levels of xylanases when grown on cellulose than when cultivated on xylan at the similar concentration (Haltrich & Steiner, 1993). Several inexpensive substrates, mainly insoluble lignocellulosic material, such as barley husk, corn cobs, hay, wheat straw, or wheat bran were identified, evaluated and are realistic alternatives to expensive carbon sources for large scale production processes. Xylose and lactose are soluble substrates and have been described as effective inducer of xylanase activity in several organisms.

Higher activities of xylanase have been reported when the cells were grown on a medium containing xylose or xylan as the carbon source (Karni *et al.*, 1993). Other sources, which also have been studied, are glucose, fructose, lactose, maltose, xylan, and xylose. Out of various hemicellulosic agricultural residues e.g. bagasse, rice straw, tamarind seed powder, sawdust, paddy husk, and wheat bran have been reported to yield into maximum production. They may serve as realistic alternatives to costly carbon sources for small-scale fermentors. The presence of xylan as the sole

carbon source in the medium has been observed to induce xylanase production by many workers. Dey *et al.* (1992) found xylose as a poor carbon source for supporting xylanase production. On the contrary, Kluepfel *et al.* (1990) have reported better xylanase production in presence of xylose rather than xylan. (Anthony *et al.*, 2003).

2.10.2 Substrate Pretreatment

Gomes *et al.* (1994) investigated the effects of physical pre-treatment of wheat straw that was used as the inducing substrate for xylanase production by *T. aurantiacus*. Both steam pretreatment (190° C, 10 min) and grinding of the steamed substrate were found to be effective for improving the enzyme production. They suggested that the lower enzyme yields obtained on the untreated substrate was due to its inaccessibility to the enzymatic attack and that the alteration of the substrate caused by the pretreatment process, modifications of the physical features of the lignin, increase in the available surface area and pore sizes, partial decrystallization of the cellulose, removal of acetyl groups from hemicellulose, or depolymerisation of the hemicellulose, were the desired modifications for xylanase production.

2.10.3 Nitrogen

For several fungal organisms, addition of a complex nitrogen supplement was advantageous, resulting in higher xylanase production as compared to the use of inorganic nitrogen sources (Brown *et al.*, 1987; Smith & Wood, 1991; Espinar *et al.*, 1992). Increased xylanase activities were obtained when peptones or yeast extract were used as such with combination in the production medium. Relatively cheaper and complex nitrogen supplements have been successfully used in certain organisms and include corn steep liquor, soybean meal or potato protein (Haltrich *et al.*, 1993;

Purkarthofer *et al.*, 1993; Gomes *et al.*, 1994; Haltrich *et al.*, 1994). Furthermore, the nitrogen source can significantly influence the pH of the medium during the course of fermentation (Haapala *et al.*, 1994). According to Bhatt *et al.* (1994) nitrates, yeast extract, peptone and casein hydrolysate had led to better growth and xylanase production. Defalted rapeseed meal was an excellent nitrogenous source for the production by *Streptomyces* sp. (Elegir *et al.*, 1995). Many workers have also reported the use of inorganic nitrogen source such as sodium nitrate (Biswas *et al.*, 1990; Siedenberg *et al.*, 1998), ammonium sulphate (Rothlisberger *et al.*, 1992; Puchart *et al.*, 1999; Beg *et al.*, 2000), diammonium hydrogen phosphate (Gupta *et al.*, 2001), ammonium dihydrogen phosphate (Rizzatti *et al.*, 2001) for xylanase production.

2.10.4 pH

An important environmental factor that significantly affects the production of xylanases is the pH of the medium during cultivation. Several fungi exhibit the increased yields of xylanase at unfavorable pH along with limited growth rate. Maximum enzyme production by *Trichoderma lanuginosus* was obtained at pH 7.5, however the suitable pH for growth observed was 6.5 (Purkarthofer *et al.*, 1993). Royer & Nakas (1989) while working with *Trichoderma longibrachiatum*, observed higher levels of xylanase production at a pH of 7.0 while the growth pattern remained normal. Bailey & Viikari (1993) found that xylanase production by *Aspergillus fumigatus* at a pH below 3.0 was essential for efficient production. The lower levels of enzyme production at higher pH values were probably due to proteolytic inactivation of the xylanase. High levels of protease was observed by this organism during growth at a high pH, but not when cultivated at a pH below 3.0. The significance of the pH for xylanase production by *Aspergillus awamori* has similarly been shown by another

group (Smith & Wood, 1991). Optimal levels of production was obtained at pH of 3.5- 4.0. Contrary to the results for *A. fumigatus*, significantly higher protease activity was observed by *A. awamori* at acidic pH values.

2.10.5 Temperature

Xylanase production is observed to be influenced significantly due to the temperature variations. Temperature will not only affect the growth rate of an organism, but also the levels of xylanase production (Suh *et al.*, 1988; Merivuori *et al.*, 1990). Different fungal species represent the optimum temperature of cultivation from a range of 27-37°C (Espinar *et al.*, 1994; Haltrich *et al.*, 1996). The thermophilic fungus *Allescheria terrestris* produced slightly higher levels of xylanase when cultivated at 40°C compared to 48°C. However, the preparation at 48 °C was shown to be thermostable, retaining 50% of its activity when incubated at 65°C for 6h, whereas the enzyme preparation obtained after cultivation at 40 °C lost more than half of its activity after 1 h at this temperature (Kvesitadze *et al.*, 1994).

2.10.6 Agitation

Mechanical agitation is a very crucial parameter for the xylanase production. The effects of shear stresses and mechanical forces on filamentous fungi, i.e. change in morphology and breakage of hyphae, together with leakage of intracellular material due to agitation, have been described (Thomas, 1990). Increasing the impeller speed resulted in a drastic increase in xylanase productivity. This is presumably caused due to the damage to the mycelium in the region of high shear stress. Rizzatti *et al.* (2001) has reported a low agitation of 100 rpm for xylanase production from the thermo tolerant *Aspergilluis phoenicis*. The optimal levels of xylanase production by

microorganism subjected to a constant agitation of 200 rpm have been reported by many workers (Copa-Patino *et al.*, 1993; Silva *et al.*, 1994). Haltrich *et al.* (1996) summarized different fungal strains, which maximally produce xylanase in shake flask conditions. However, xylanase production in static liquid cultivation condition has also been reported in *Aspergillus sydowii* MG 49 (Ghosh *et al.*, 1993), *Phanerochaete chrysosporium* ME446 (Copa-Patino *et al.*, 1993), and *Pyromyces* sp. strain E2 (Teunissen *et al.*, 1992), etc.

2.11 FERMENTATION CONDITION

2.11.1 Submerged Fermentation

The submerged fermentation process is well characterized where homogenous conditions can be maintained and mainly pH, temperature and dissolved oxygen can be easily controlled and scaling up of the process is feasible under defined conditions. It is the well-defined system for the production of xylanolytic enzymes. Different workers have summarized the production of xylanase in submerged fermentation processes ranging from shake flasks to fermenters (Gomes *et al.*, 1994; Haltrich *et al.*, 1996). It is however a energy intensive process that involve high capital investment and also generates significant volumes of liquid wastes. These factors make this mode of cultivation commercially unattractive.

2.11.2 Solid-State Fermentation

Solid-state fermentation stimulates the growth of microorganism in nature on moist solids and has been credited to be responsible for the beginning of fermentation technique in ancient times (Mitchell & Lonsane, 1990). It was, therefore not surprising that almost all the fermentations process used in ancient time were based

on the principles of solid-state fermentation. A glance at the history shows that after 1940, solid-state fermentation processes were nearly ignored in western countries due to adaptation of submerged fermentation technology. Perhaps there was no logical reasoning for this at that time. Since the development of penicillin took place in submerged fermentation and due to enormous development of this system of fermentation, researchers at that time had their major attention on submerged fermentation. Solid-state fermentation unknowingly, was neglected; still, in some isolated pockets research continued although slowly and during 1950-1960, solid-state fermentation for steroid transformation was reported using fungal cultures. The trend continued although slowly and solid-state fermentation attained another millstone during 1960-1970 when reports appeared on production of mycotoxins by solid-state fermentation. Production of protein enriched cattle feed was the next major activity reported, which involved utilization of agro-industrial residues thus offering a unique process development for value addition of these otherwise low cost residues. The solid-state process has comparatively lower energy requirements, generates lesser waste-water and resolves the problem of solid waste disposal. In fact this was one of the areas, which generated interest of researchers globally on solid-state fermentation. Since then there has been continuous increase in the extension of solid-state fermentation area, which, picked up strongly during the last one decade.

Solid-state fermentation (SSF) involves the growth of microorganisms on moist substrates in the absence of free flowing water. The necessary moisture in solid state fermentation exists in absorbed or complex form within the solid matrix, which is likely to be more advantageous for growth because of the required level availability of the air, unlike in the case of submerged fermentation (SmF). In case of submerged

fermentation (SmF), the amount of the solid substrates rarely reaches more than 50g/l where as in SSF the solid content typically varies between 20-70 % of the total weight. In SSF, free water is absent or nearly absent in the spaces between the solid particles. Microbes obtain water from moisture held within the substrates (Mitchell *et al.*, 2002; Raghavarao *et al.*, 2003).

The solid-state fermentation system which during the previous two decades was termed as a low-technology system appears to be promising one for the production of value added, low volume-high cost products. Filamentous fungi are the most important group of microorganisms for solid-state fermentation. They are ideally suited to solid-state fermentation due to their hyphal mode of growth and also due to their physiological capabilities. Further, many fungi grow on solid material such as wood and leaves in nature and typically produce high enzyme yields in this mode of growth. Filamentous fungi are well adapted to spread over and penetrate into solid substrate. In solid-state fermentation, the colonies arising from the adjacent spores soon merge and then the hyphal density within the mycelium increases. Hyphal growth also gives the fungus a much higher penetration than unicellular microorganism. Fungi also have enzymatic machinery to aid their penetration in the solid matrix.

2.11.2.1 Solid support

Most substrates used in solid-state fermentation are of agricultural origin. They are often relatively unprocessed, may be having only been ground or chopped from larger agricultural residues. In some cases the macromolecule is utilized as a carbon and energy source by the microorganism, while in others the structural macromolecule may simply provide an inert matrix within which smaller soluble

nutrients or other macromolecules are located. This macromolecular matrix has some important consequences. Firstly, if the structural macromolecule is the major source for carbon and energy, it is then hydrolyzed during the fermentation. Under these circumstances, the solid substrate will collapse or the particular size and properties of the solid matrix may change during the fermentation. This may not occur if the carbon source is simply a soluble molecule embedded within an inert macromolecular matrix. Secondly, such macromolecular structures may also involve several different molecular components that may affect the growth and to some extent the production by the microorganism. If the microorganism is capable of utilizing more than one, this can lead to complex patterns of substrate utilization. The most important physical property is the particle size, which affects the surface area to volume ratio of the substrate. Particle shape, along with particle size also affects the surface area to volume ratio of the substrate.

Various different processes have been investigated for the utilization of lignocellulosic substrates such as wheat straw, corn and rice straw, wheat bran, sugar beet pulps and wood. Some processes have the aim of degrading cellulose, lignin or hemicelluloses and the microorganism involved must be able to produce respectively the cellulolytic, lignolytic or xylanolytic enzymes. Effective hydrolysis requires the synergistic action of several enzymes. Fungal strains in some reports have been successfully shown for the production of xylanase by using the SSF (Gessesse & Mamo, 1999; Yuan *et al.*, 2005). A variety of different solid supports like wheat bran, barley straw, wheat straw, bagasse have been reported for xylanase production by *Humicola* sp., *Talaromyces emersonii, Chaetomium globosum*, *Melanocarpus albomyces* (Pandey *et al.*, 1999).

The addition of small amounts of purified xylan to the complex lignocellulosic

substrate has been found to be advantageous in some cases and resulted in considerable enhancement of the xylanase activities produced (Biswas *et al.*, 1988; Anthony *et al.*, 2003). Different effects of pre-treatment of the lignocellulosic substrates have been also reported.

2.11.2.2 Moisture content

Moisture content is very essential and is a very critical parameter for solid-state fermentation. The availability of moisture in solid-state fermentation can be expressed as either the water content or the water activity, that are inter-related and play a key role in solid-state bioprocessing since hyphal extension, spore germination and production of nucleotides are very sensitive to water stress (Gervais & Molin, 2003; Lenz *et al.*, 2004). It is water activity that mainly affects the microbial growth but on the other hand water content is easier to measure.

Different substrates have different water holding capacities, so the amount of water that they can hold before free water in visible can vary markedly. Therefore different SSF processes are carried out at varying percentage of water content (Moo-Young *et al.*, 1983). The effects of moisture are not limited to direct effects on the physiology of the fungus, only, the most important indirect effect is the alternations of the substrate characteristic. High moisture levels may displace gases from the interparticle spaces (Cannel & Moo Young, 1980) and may cause agglomeration of the substrate particles into lumps, which will restrict interparticle gas diffusion (Kim *et al.*, 1985).

Further, the high moisture level may lead to the swelling of the substrate, which increases porosity allowing better enzyme diffusion and cation and better mycelial penetration (Zardazil & Brunert, 1981; Narahara *et al.*, 1982). Low moisture

contents may allow inhibitory metabolite by-products to reach inhibitory concentration sooner, thereby limiting growth.

Additionally, the influence of water activity has been described either on enzymatic stability itself or fungal growth and enzyme expression. Fluctuations in the water activity of the medium for *Aspergillus parasiticus*, *Aspergillus niger*, *Coniothyrium minitans* affects the sporulation and growth rates of the microorganism.

Biophysical theory and computational methods have also been applied to study the impact and the dynamics of water in solid media (Gervais & Molin, 2003). Based on the theory of water transport, Gervais *et al.* (1999) had supported the hypothesis that the rate of hyphal growth is controlled by water influx at the apical tip. Sautour *et al.* (2001) developed an empirical model to describe the relationship between fungal growth and water activity. Nagel *et al.* (2001) and Penay-Lillo *et al.* (2001) incorporated water balance into their complex models of bioreactor. This allowed estimation of overall water content and hence the development of efficient strategies for moisture control in solid-state fermentation.

2.11.2.3 Temperature

Microbial growth in SSF generates significant amount of metabolic heat. However, heat removal from the substrate tends to be insufficient, leading to the establishment of temperature gradients and localized overheating of the substrate. The temperature often reaches higher values, which severely limit growth or even kill the microorganism. The heat generated must be dissipated immediately.

Temperature rise due to exothermic characteristic of fermentation processes is one of the main difficulties faced in SSF. Temperature control is more difficult in SSF processes as compared to the submerged processes. In case of SSF, the solid

characteristics of the substrate lack the homogeneity in the reactor and therefore results in the existence of temperature gradients. Such factors make the heat exchange difficult in the system. In an industrial context, monitoring and controlling this variable is critical for scaling up the process (Bellon-Maurel *et al.*, 2003). Conventionally aeration is the major approach (Raimbault, 1998; Raghavaro *et al.*, 2003) to control the temperature of the substrate. Because high aeration rates can reduce the water activity of the substrate by evaporation, water saturated air is usually used (Raghavaro *et al.*, 2003). The agitation of the fermentation mass can also help to control the temperature (Pérez-Guerra *et al.*, 2003).

2.11.2.4 pH

The measurement and control of this variable in SSF seems difficult. Nevertheless, the substrate employed in SSF usually has buffering effect due to their complex chemical composition. In these cases, the control of pH is not necessary. Whenever this variable needs to be controlled, buffering solutions are added as liquid phase, but this strategy can be inadequate when the process is subjected to a scale-up. Another possibility to control the evolution of the pH consists of adding a mixture of sources of nitrogen in such a way that it counteracts the effect of the pH variations. In this sense, ammonium salts have been used in SSF in combination with urea or nitrate salts due to the respective effects of acidification and alkalization of the former and the latter (Raimbault, 1998; Torrado *et al.*, 1998).

2.12 BIOPROCESS STRATEGIES

Solid-state fermentation processes can be conveniently operated in batch, fed-batch or continuous modes, although batch processes are most common (Raghavaro *et al.*, 2003). Reports dealing with bioreactor design for SSF are scarce. However, due to

some problems associated with solid beds like poor mixing, heat transfer, characteristics and material handling, SSF bioreactor systems are yet to reach a high degree of development. (Raghavaro *et al.*, 2003; Durand, 2003).

The wide variety of solid substrates can be employed in SSF, which may have important differences with respect to composition, size, mechanical resistance, porosity and water holding capacity. The SSF bioreactors must be constructed with a material, which must be anticorrosive and non-toxic to the process and organism. It should also be of lower cost. The entry of contaminants into the process as well as the uncontrolled release of the process organism into the environment must be avoided by using filters on outlet air stream and by a careful design of seals and filtration of the inlet air stream. Other important aspect to be considered during the construction of a bioreactor is the effective regulation of aeration, mixing and heat removal. This could avoid the problems related to an ineffective heat removal, evaporative loss of water from the substrate bed and thermal gradients, which affect the yield and quality of the desired product. In the same way, the control of operational parameters (temperature, water activity and oxygen concentration) and the maintenance of uniformity within the substrate bed could be as effective as possible. Despite centuries of use and renewed interest over the last 20 years in solid-state fermentation (SSF) technology, and despite its good potential for a range of products, there are currently relatively few large-scale commercial applications (Mitchell *et al.*, 2000).

2.13 STATUS OF PAPER INDUSTRY IN INDIA

The pulp and paper industry is one of the India's key industry sectors. Presently, about 380 paper mills are in existence in India with an installed capacity of about 4 million tons per annum as registered with Government of India. The raw

material wise classification of 380 mills (Table 2) enumerate that, 21 are large mills, each one having a capacity to produce about 33,000 TPA; small mills have a capacity below 10,000 TPA (Rao *et al.*, 1998; Rao, 1998). However, presently as many as 135 mills of large, medium and small capacity were lying closed due to limited supply of raw materials.

Table 2. Raw material wise classification of paper mills in India

Types of raw material	Number of mills	Installed capacity (million tons)	% of capacity
Forest based (Wood & Bamboo)	28	1.449	37
Agro-based (Straws, bagasse, etc)	111	1.240	31
Other wastes, paper, etc	241	1.265	32
Total	**380**	**3.954**	**100**

Source: Development council for pulp, paper and allied products

The basic cellulosic fibrous material of the pulp and paper industry is pulpwood. The Indian pulp and paper industries rely on forest-based raw materials, which are in short supply. The consumption of paper is an index of a country's development. But it is rather disappointing that the per capita consumption of paper in India is 5.0 kg per annum, which is one of the lowest in comparison to other developed nations of the world (Papermaker, Asia Pacific July, 2004). The Indian paper industries are facing multifarious problems like shortage of wood fibers, depreciation of money, less selling price with minimum profit level and also lack of advance technology. Hence, Indian manufacturers are unable to match their products both in quality and cost-wise in global competitive market. The stringent rules and

regulation has compelled the pulp and paper industries to minimize their pollution load, hence, there is a need to develop environmentally benign bleaching technology for pulp and paper industries. The other potentially available non-woody fibrous plants are both agro-based residues and other non-conventional raw materials. In present scenario, pulp from non-woody plants represent only 7% of the total world pulp production. About 70% of this non-wood pulp production occurs in India and China where domestic wood fibers were used by the pulp and paper industry. The estimated total availability of non-woody fibrous plants is 2300 metric tons of which about 50% are straws. Besides agro-based residues, plenty of other non-woody fibrous plants are available in India, whose morphological characteristics have tempted the pulp and paper technologists for their augment use for manufacturing of low-cost and high quality value added specialty papers where strength is the main criteria. The non-woody fibrous plants are able to meet most of the requirement, which are those from hard wood. Surprisingly, the chemical and morphological characteristics of selected non-woody fibrous plants indicate that they may be better substitute for expensive long fibred plants except having some strength deficiency. The selected non-woody fibrous plants, due to their open and loose anatomical features and low lignin content can be pulped easily with milder cooking condition to produce easy bleachable grade pulp. The fiber dimensions data and unbeaten pulp freeness values indicate that they do not require extensive refining to develop fibrillation hydration and inter-fiber bonding properties (Dutt *et al.*, 2004).

India has a landmass of 3.29 million sq km and 0.69 million sq. km of forest cover i.e. nearly 2.5% of world's geographical area and 1% of the world's forest area. The recorded forest area in the country is 7,65,210 sq. km i.e. 23.42% of the

geographical area of the country while the satellite data analysis indicates a forest cover of 6, 39,600 sq. km (19.47%). However, only 3,85,756 (11.73%) has good forest cover of over 40% crown density and up and rest is open forest with 10-40% density (2,49,311 sq. km) and there are nearly 60,000 sq km of blank area without tree cover (Akhtar, 2000).

In future the Indian pulp and paper industry will require more forest based raw materials and has to strive for providing the increasing demand (Table 3) for pulp and paper products. Some of the ways to meet the increased demand are more intensive forests, more efficient utilization and shorter rotation with increased use of juvenile wood and more intensive tree improvement such as selection, genetic engineering, breeding, short rotation and intensive culture systems, incorporate agronomic systems. Agro residues such as bagasse and straws could also be better option for the pulp and paper industry.

Table 3. Demand and supply scenario of cellulosic fiber in India (Rao *et al.*, 1998)

Year	Projected Demand (lakh tones)	Compounded growth rate (%)	Total possible production from indigenous fiber (lakh tones)	Short fall of indigenous fiber in paper equivalent (lakh tons)	Per capital consumption (kg /yr)
2000-01	**49.50**	**7.8**	**32.58**	**16.92**	**5.0**
2005-06	**67.0**	**6.5**	**41.61**	**25.39**	**6.5**
2010-11	**85.50**	**5.5**	**49.10**	**39.40**	**-**

2.14 PULP BLEACHING PROCESS

The pulp and paper industry is a large and growing sector of the world economy. A number of pulping and bleaching process have been developed to meet industrial and consumer needs. The traditional, effective approach of using chlorine-

containing chemicals as bleaching agents has been challenged, first by environmental groups and then by consumers. In response to these pressures, alternative bleaching technologies have been developed to partially or completely replace these chemicals, particularly elemental chlorine (Cl_2). The option open to pulp mills considering a change to chlorine-free bleaching includes oxygen delignification, extended cooking, and substitution of chlorine dioxide for chlorine, hydrogen peroxide and ozone. Most of these involve process modification and/or capital investment. Enzymatic processes have been developed as one of alternative bleaching technologies. Enzymes provide a very simple and cost-effective way to reduce the use of chlorine, chlorine compounds and other bleaching chemicals. Enzymes also offer a simple approach that allows for a higher brightness ceiling to be reached. This can all be achieved without resorting to major capital investment.

The primary goal of chemical pulp bleaching is to remove lignin from the pulp while retaining the carbohydrates in the pulp. Two different approaches using enzymes have been followed since the early 1980s to accomplish this goal, but only one has led to a successful commercial mill-scale process. The process involving use of hemicellulases (specifically xylanases) for enhancing the chemical removal of lignin in multi-stage bleaching sequences is termed as "Bleach Boosting", has been successfully used in a number of pulp and paper mills throughout the world. An alternative approach, i.e., direct delignification, using enzymes called ligninases or laccases, which act directly on lignin, is still in the exploratory stage and awaits possible future commercialization.

2.14.1 Biobleaching Approach: A Better Option

Environmental, customer and regulatory concerns have favored the investigation of enzymes in bleaching because they are readily biodegradable and don't contribute to organochlorine formation. So far, following three enzyme-based approaches have been investigated:

1. Hemicellulases; 2. Lignolytic enzymes; 3. Biomimetic agents.

2.14.1.1 Hemicellulases in bleaching

The basic idea of pre-bleaching with hemicellulase enzyme was first published in 1986 and originally developed at the VTT Biotechnical laboratory in cooperation with the Finnish Pulp and Paper Research Institute. The original idea was to hydrolyze a relatively lower amount of hemicelluloses in pulps. It was realized that enzymatic treatment could not enable the production of fully bleached kraft pulp when combined with chlorine free chemicals. However, it was clear that brightness was substantially increased by enzymatic treatment and that this improved brightness could be gained either in the prebleaching stage, as chlorine gas or in the first bleaching stage, as chlorine dioxide (Viikari *et al.*, 1986; Viikari *et al.*, 1987). For environmental reasons, the possibilities in minimizing the consumption of chlorine gas in bleaching were of particular interest. The enzymatic treatment was therefore undertaken for developing a more effective and eco-friendly approach for pulping and bleaching stages. Thus, utilization of enzyme may significantly reduce the release of chemical based effluents in the environment.

Combination of the enzymatic treatments with different bleaching sequences yields to the most reliable and practical results. Crude culture filtrates of hemicellulases generated by different micro-organisms were used in the initial

delignification experiments (Viikari *et al.*, 1986, 1987), and the most of the reports published are still based on results with unpurified enzymes. The culture filtrates used, however contained xylanases as the major constituents and the enzyme preparations are generally dosed as per the xylanase activity. Thus, when small lots were used, the amounts of other activities, mainly other hemicellulolytic and some cellulolytic enzymes, were very low. Even with the unpurified enzymes, identification of the sugars released in the enzymatic treatments denoted that xylanase was the major activity (Viikari *et al.*, 1987; 1990). Preliminary optimization of the amount of enzyme and the hydrolysis time had revealed that reduction in the kappa number of pure kraft pulp could be achieved using lower amounts of enzymes and a relatively shorter incubation time. Minimization of the overall hydrolysis of hemicelluloses is necessary in order to maintain a high pulp yield and the advantageous properties of hemicelluloses in the pulp (Viikari *et al.*, 1986, 1987).

In xylanase pre-bleaching, the side group cleaving enzymes can be expected to act in two possible ways. These may enhance the action of xylanases in a synergistic way by removing the side groups. If xylan is attached to lignin molecules through the side groups, hydrolysis of the linkages between the side groups and xylan may enhance delignification. Kulkarni *et al* (1999) studied the role of side group splitting enzymes with partially purified enzymes or in combination of xylanases with other debranching enzymes. The presence of cellulases in hemicellulase preparations has generally been considered to be detrimental in pulp treatments. This is especially true when both endoglucanase and cellobiohydrolase activities are present in crude enzyme preparations. Due to synergistic action of cellulolytic enzymes, a rapid depolymerisation of cellulose occurs. However, significant differences in the action of

individual cellulases in enzyme bleaching were observed. The endoglucanase was found to be most harmful to pulp quality while on the other hand, a non-specific endoglucanase of *T. reesei* was found to increase the bleaching ability of pulps (Buchert *et al.*, 1994).

Dunlop *et al.* (1995) have developed a method for selecting xylanases useful for boosting of the bleaching of the kraft pulp. The color difference of the pulp supernatant after xylanase treatment, compared with a suitable control, was found to have a higher co-relation with the brightness of the chemically bleached pulp. Various attempts have been made for using the hemicellulases (especially xylanases) in pre-bleaching of pulp (Onysko, 1993; Gessesse & Mamo, 1999; Medeiros *et al.*, 2003; Roncero *et al.*, 2000, 2003 a,b)

Results from the laboratory studies have shown about 35-41% reduction in active chlorine at the chlorination stage for hard wood and 10-26% for softwoods, whereas saving in total active chlorine was accounted to be 20-25% for hard woods and 10-15% for soft woods, if the pulp is pretreated with the xylanase enzyme. Xylanase pretreatment lead to reduced effluent concentrations of adsorbable organic halide (AOX), and dioxin, as less chlorine is needed to achieve a given brightness (Scott *et al.*, 1993). The enzyme treated pulps show unchanged or improved strength properties. Also these pulps are easier to refine than the reference pulps. Improved viscosity of the pulp had been noted as a result of xylanase treatment This was probably caused by the selective removal of xylan as indicated by the pentosan values.

A notable number of European, North American and Japanese mills are using enzyme preparations for bleaching purposes. Crestbrook Forest Industries, British

Columbia, Canada has been using the commercially available enzyme to remove the bottleneck in chlorine dioxide generation and to increase the production of Elemental Chlorine Free (ECF) pulp. As the 'totally chlorine free' (TCF) pulp market grows, Canadian mills, which are the World's largest exporters of market pulp, have started investigating chlorine-free bleaching with xylanase enzymes. European paper makers are now requesting TCF pulps or bleached pulps with extremely low AOX and/or TOX level in their effluents. Many Finnish companies mainly - Enso Gutseit OY, Kimi Kymmene, Metsa-Sellu OY, United Paper mills are making efforts to develop chlorine free bleaching which involves the use of enzymes, oxygen, peroxide and ozone.

2.14.1.2 Lignolytic enzymes

These enzymes, unlike hemicellulases attack lignin directly, hence appear to be more effective for bleaching. White-rot fungi are the main producers of lignolytic enzymes. These fungi secrete a number of oxidative enzymes and some unknown substances (mediators) into their surroundings, which together bring about the slow but continuous degradation of the lignin. The most important degrading enzymes are Lignin peroxidase, Manganese peroxidase and Laccase. Attempts of using these enzymes have suggested that these could prove useful in bleaching of the kraft pulp (Tavares *et al.*, 2004; Sigoillot *et al.*, 2005).

The exact mechanism by which lignolytic enzyme promotes bleaching is not yet defined. Researchers had attempted to develop a model of the 3-D structure of lignin to provide a framework for interpretation and prediction of interactions between the enzymes and lignin on molecular level. Inspection of the model of lignified secondary wall showed the accessibility restrictions upon lignin degrading

enzymes and points towards a concept of an “enzyme factory” outside the cell wall, producing chemicals, which have access to, and breakdown the lignin and allow its fragments to leave the network. Once out of the wall, these fragments may interact with enzymes directly, resulting in further reduction of the fragment size and finally mineralization.

Commercialization of the bleaching processes with lignolytic enzymes-manganese peroxidase and laccase-faces a number of challenges; major among those are, availability of enzymes, cost of mediators and enzyme stability. Currently, neither enzyme is available in sufficient quantity for mill trails, and scaling up of the enzyme production from fungal cultures may be costly. Cloning of genes for both enzymes have been reported and may act as an alternative production routes. The laccase mediators ABTS and HBT are expensive and alternatives are less effective. Chelating agents for Mn (III) in the manganese peroxidase reaction appeared to be a costly item.

2.14.1.3 Biomimetic agents

Some of the reactions catalyzed by lignin peroxidase and manganese dependent peroxidase can be catalyzed by non-enzymic compounds. As they could be more economical and easier to use than lignolytic enzymes, these biomimetic systems have received considerable attention as alternatives in biobleaching process. Porphyrins have been studied as mimics or models of lignin peroxidase enzymes by a number of groups. They have studied their interaction with lignin like model compounds to understand the role of the enzyme system in lignin degradation. These researchers have found that the porphyrins catalyse many of the same reactions as lignin peroxidase. However, there are problems that are associated with the use of porphyrins which are mainly their unstability and insolubility in aqueous

environment. Similarly, bleaching with hemoglobin in 90% dioxane resulted in considerable reduction in kappa number, a moderate decrease in viscosity, and increase of approximately 10 (ISO) brightness points.

2.15 XYLANASE IN PULP AND PAPER INDUSTRY

For industrial processes of large volume, such as wood pulp bleaching, xylanase must be produced in a several grams per liter range to provide a competitive alternative/ additive to eliminate or decrease the amount of chlorine dioxide used for pulp bleaching. Over a decade ago, the pre-treatment of kraft pulp with xylanase was found to reduce the amount of chlorine required to bleach pulp to target brightness. This can significantly help in reduction in the generation of organochlorines in the bleach plant and thus can notably reduce the environment deterioration and degeneration by the effluent generation by the kraft mills. A number of kraft mills in Canada and Scandinavia now use xylanase to reduce the amount of organochlorines in their effluents and to maximize the production of bleached pulp using a limited amount of chlorine dioxide as bleaching agent.Various findings have shown that xylanase enzymes are consistently more effective than mannase enzymes, particularly for the pre-treatment of oxygen delignified pulps; Chlorine-based bleaching of pulp responds effectively to the xylanase pre-treatment than to the peroxide based bleaching of pulp. However, for peroxide bleaching of pulp, xylanase treatment is equally effective

2.16 COMMERCIAL XYLANASES

Xylanases are produced by a large number of different fungi. These organisms differ greatly with respect to xylanase activities and productivities attained. A number of xylanase-producing strains have been described but, commercial production is still

in its initial stage and the various companies producing xylanase have it's utilization in paper industry, cellulase containing xylanase has potential in food industry, where they can be used for baking products, enhancing recovery of starch from wheat flour, or aiding the extraction and clarification of fruit juices. In the feed industry, it is used to improve the digestibility of animal feed, thus increasing the feed efficiency. Commercial xylanases available worldwide are summarized in Table 4.

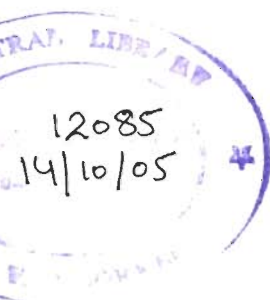
Table 4. List of commercially available xylanases

Supplier	Commercial Xylanase	Application
Alltech Inc, USA	Allzym PT	Upgrading animal feed
Amano Pharama ceutical Ltd. Japan	Amano 90	Pharmaceutical, food and feed industry
Danisco Ingredients, Denmark	Grindazyme GP 5000 Grindazyme PF	For supplementation of poultry and piglet feed
Gamma Chemie,	Gammazym X400OL	Production of wheat Starch, baking and brewing
GmbH, Germany	Gammafeed X	Feed and brewing industry
Genencor, Finland	Multifect XL Irgazyme 40	Food industry Pulp and paper industry and animal feed
Hankyu Bioindustry Co. Ltd., Japan	Xylanase 250	Baking industry; macerating vegetables and fruits
	Hemicellulase 100	Improving the filteration speed of saccharified cereal solutionand fruit juices
Nova Nordisk, Denmark	Bio-feed Plus Novozym 431	Animal feed Animal feed
Primalco Biotech., Finland	Ecopulp X-200	Bleaching of softwood and hardwood pulp
	Ecopulp XM	Bleaching of softwood kraft pulp
	Econase	Animal feed
Stern-Enzym GmbH &Co. KG,Germany	Sternzym HC46 Sternzym HC 40 & 42	Bakery industry Bakery, animal feed and hydrolysis of plant raw materials

Various patents have also been filed for the production and application of the xylanase as listed in Table 5.

Table 5. Patents for xylanase production and application

Patent No.	Date	Title
EP1479765	2004-11-24	Enzymes with xylanase activity from *Aspergillus aculeatus*
US2004077071	2004-04-22	Methods of xylanase treatment in bleaching
WO03074780	2003-09-12	Xylanase treatment of chemical pulp
US2003180895	2003-09-25	Xylanse variants having altered sensitivity to xylanse inhibitors
WO03012094	2003-02-13	Process for xylanase production
WO02057541	2002-07-25	Methods of xylanase treatment in bleaching
RU2183670	2002-06-20	Nutrient medium for culturing fungus culture *Penicillium canescens* as producer of endo(1→4)-beta-xylanase and beta-galactosidase
US2002037342	2002-03-28	Multi-enzyme product with gluco amylase, proteolytic and xylanase activities and method for producing same by solid state fermentation of wheat bran with *Aspergillus niger*
WO09320208	2001-07-12	*Trichoderma reesei* xylanase
US6200797	2001-03-13	Bacterial protein with xylanase activity
WO0068396	2000-11-16	Novel thermostable alkaliphilic xylanase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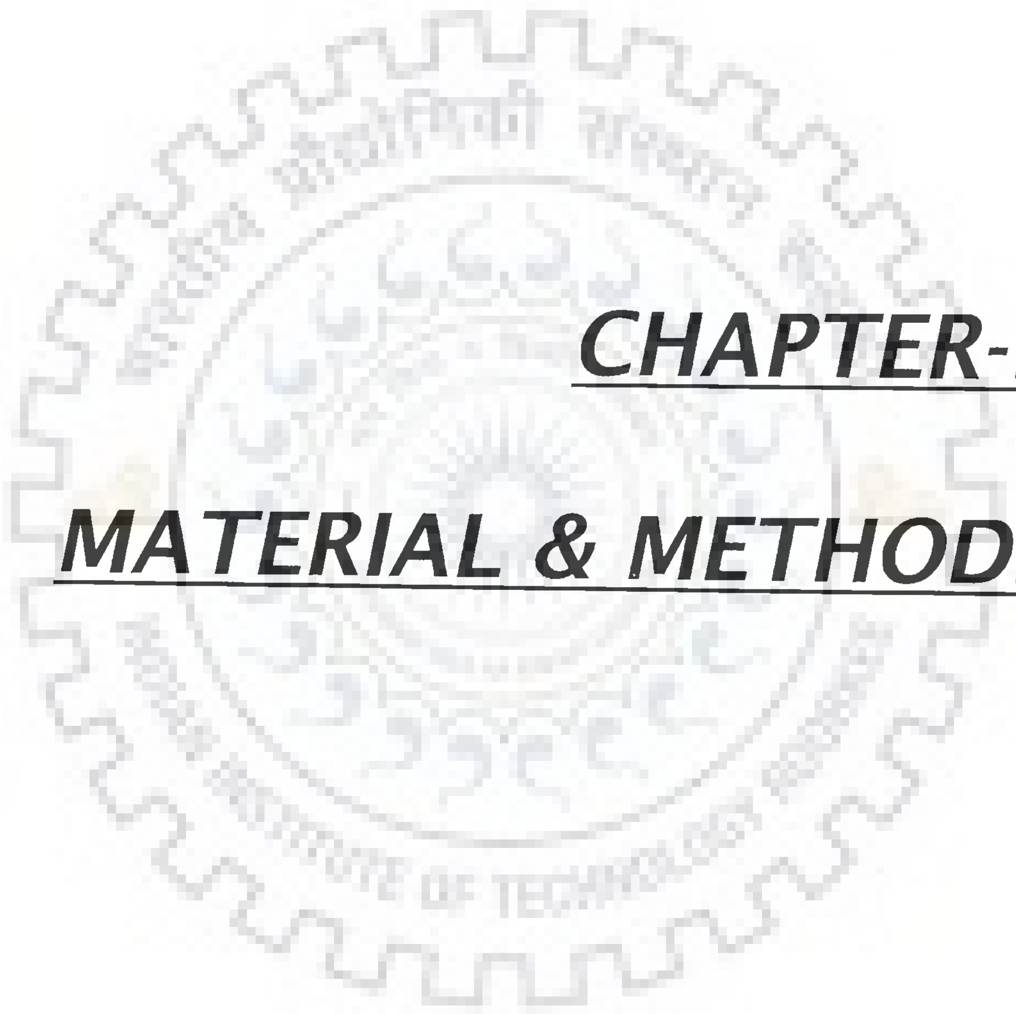


CHAPTER-3

MATERIAL & METHODS

Chapter - 3

MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Chemicals

Xylan, xylose, p-nitro phenyl-β-D glucopyranoside, β-xyloside, standard protein markers were purchased from Sigma Chemical Company, USA. Whatman No 1 filter paper was purchased from Whatman Asia Pacific, Singapore. All other chemicals were of analytical grade and purchased from standard commercial manufacturers and were of the highest grade available. Sugarcane bagasse was obtained from local sugarcane industry, Rai Bahadur Narayan Singh Sugar Mills Ltd., Laksar, Hardwar (UA), India. Cotton hull, oil cake, polyurethane sponge, coconut coir were purchased from the local market of Roorkee (UA). Wheat straw, wheat residue, rice straw, wheat bran, rice bran, luffa were purchased from the regional market.

3.1.2 Microorganisms

Aspergillus niger mutant NKUC$_N$-3.40 was generated by mixed mutagenesis of the wild type strain *Aspergillus niger* NK-23 ITCC 5454 (isolated from sugarcane dumping site), identified by Indian Type Culture Collection Bank, Indian Agricultural Research Institute, New Delhi. The strain was maintained on PDA (2% dextrose, 2% agar, 20% potato extract) slants, stored at 4°C and the culture was renewed monthly.

3.2 METHODS

3.2.1 Strain Selection

42 different fungal strains were isolated from the soil samples of sugarcane dumping site, forest soil, decomposing manure and paper industry waste site. Briefly, soil samples from the site was collected and mixed with two parts of double distilled water. This suspension was centrifuged (600 g) at room temperature for 10 minutes. The clear supernatant was inoculated onto xylan agar (XA; 1% xylan and 2% agar) plates. Growth was observed after 48 hours of incubation at 30^0C. Morphological observation of the colonies indicated that 42 different strains, which were then further purified by single spore isolation. Purified cultures were transferred to PDA slants, incubated at 30^0C for 5 days and further stored at 4°C for future usage. Of these, ten distinct colonies were selected for further investigation on the basis of clear zone appearance by staining of XA-plate with Congo red.

3.2.2 Screening of Xylanase Producing Strains

Primary screening of the selected strains for the xylanase was carried out on xylan-agar plates as described (Mathrani & Ahring, 1992). The xylan agar medium contained 10 g of xylan, 20 g of agar dissolved in 1 liter of double distilled water and autoclaved at 15 p.s.i. for 15 minutes. Cell free culture supernatant (50μl) was placed on 2-3 mm diameter wells cut into the solidified medium and the plates were incubated at 30^0C for approximately 24 hours. Enzymatic hydrolysis of the surrounding xylan resulted into clear zones in the medium. Controls with heat killed (140^0C, 20 minutes) supernatant did not produce any clear zones.

3.3 MUTAGENESIS AND SCREENING

The schematic representation of the step-wise approach used for mutagenesis and screening is shown in figure 4.

3.3.1 Single-Step Mutagenesis

3.3.1.1 Physical mutagenesis

Physical mutagenesis by UV irradiation was performed according to Petruccioli *et al.* (1995). Conidiospores from 5-day-old culture, grown on PDA medium at 30°C, were suspended in sterile 0.05M phosphate buffer (pH 7.0) containing 0.1% Tween-80. Three ml aliquots of this suspension (approximately $1x10^9$ conidia ml^{-1}), were transferred to sterile petridishes (9 cm diameter), and irradiated with UV light (2.5 $Jm^{-2}s^{-1}$, distance 0.70 m) for different time periods ranging from 60 sec to 10 minutes and stored in dark at 4°C for 24 h to avoid photorepair. These were then subjected by serial dilutions on XA medium. Control conidiospore was also treated identically expect that UV exposure was not performed.

3.3.1.2 Chemical mutagenesis

3.3.1.2.1 N-methyl-n'-nitro-N-nitrosoguanidine treatment

Mutagenesis of *A.niger* was also carried out using N-methyl-n'-nitro-N-nitrosoguanidine (NTG or MNNG) as described by Kuhad *et al.* (1994). Briefly, a spore suspension of *A.niger* NK-23 was prepared in saline solution. The spores ($2x10^6$ spores ml^{-1}) were treated with 100 μg ml^{-1} of NTG in saline solution for different time periods (10-60 min). Survivability and frequency of mutants were determined on PDA and XA-plate (stained with 0.1% Congo-red) respectively. Control conidiospore suspension was also treated identically excluding N-methyl-n'-nitro-N-nitrosoguanidine.

3.3.1.2.2 Colchicine treatment

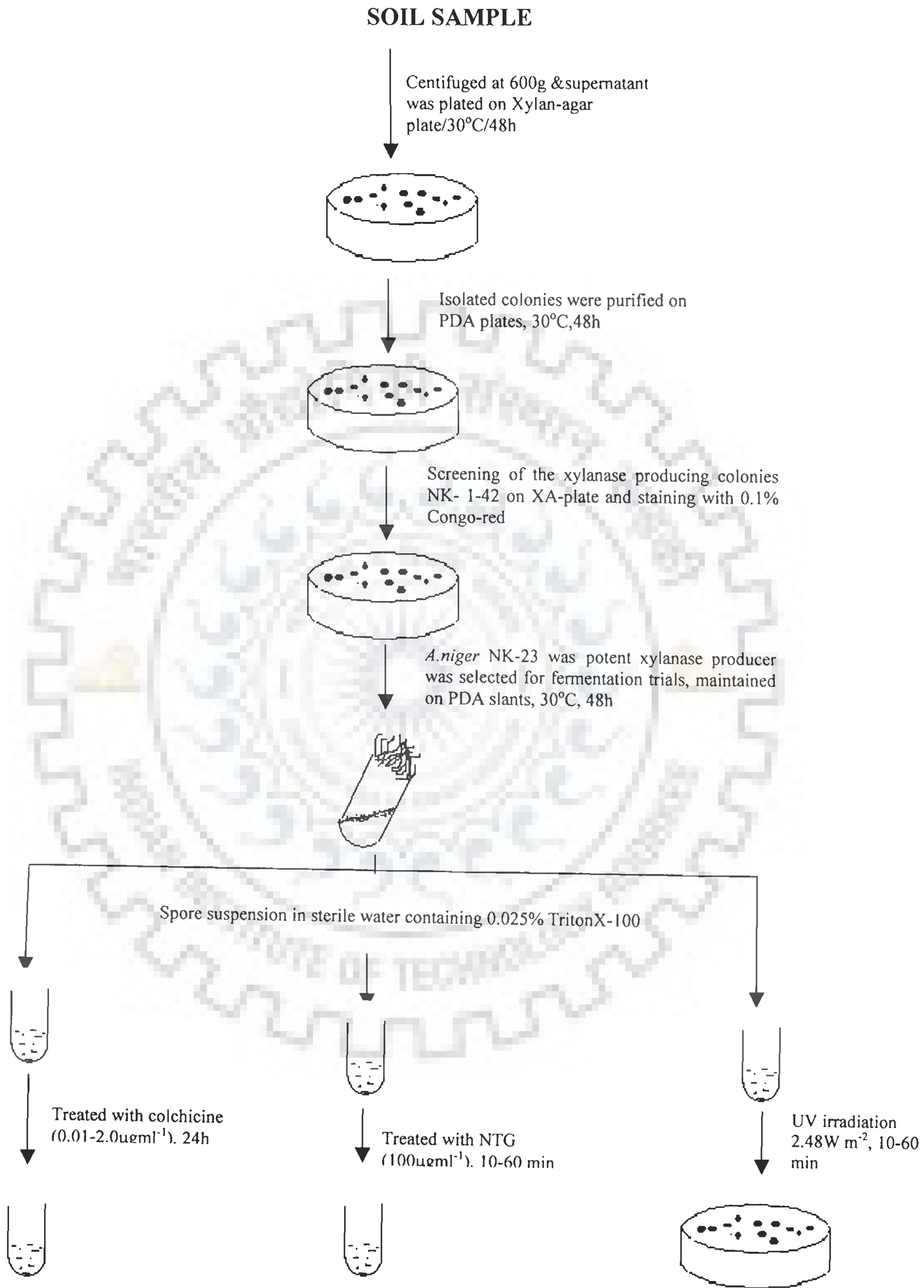
Mutagenesis using colchicine treatment was performed as described by Claudimara *et al.*, (1996). Briefly, fungal spores ($2x10^6$ spores ml^{-1}) obtained after 10 days of incubation, were suspended in distilled water and treated with different concentrations of colchicine 0.01, 0.02, 0.1, 0.2, 0.5, 1 and 2 $\mu g\ ml^{-1}$ for 24 h. The colchicine treated spores were then plated on PDA and xylan agar media. The surviving colonies were recultured and then denoted according to colchicine concentrations used for treatment.

3.3.1.3 Mixed mutagenesis

Analysis of the mutants after single treatments using ultra violet (UV) or chemical mutagenesis resulted into mutants NKU-2 and NKU-3 with maximum zone diameters as obtained after UV mutagenesis hence the same were chosen for mixed mutagenesis. The mutants NKU-2 and NKU-3 (selected after UV mutagenesis) were further treated with 100 $\mu g\ ml^{-1}$ of NTG (Kuhad *et al.*, 1994) for 30 minutes. The strains showing higher zone diameter on xylan agar plates as compared to UV treated mutant strains were selected. The mutants thus obtained after UV+NTG mixed treatment were finally selected for further studies.

3.4 MUTANT ISOLATION

After every treatment, the conidial survival was determined on PDA plate at 30 °C and the mutant selection of the survivor was performed on xylan agar medium. Rapid screening of higher xylanase producing strains was done on xylan agar medium stained with 0.1% Congo red. The different colonies selected on the basis of zone diameter were further streaked on PDA media.



SOIL SAMPLE
Centifuged at 600g &supernatant was plated on Xylan-agar plate/30°C/48h
Isolated colonies were purified on PDA plates, 30°C,48h
Screening of the xylanase producing colonies NK- 1-42 on XA-plate and staining with 0.1% Congo-red
A.niger NK-23 was potent xylanase producer was selected for fermentation trials, maintained on PDA slants, 30°C, 48h
Spore suspension in sterile water containing 0.025% TritonX-100
Treated with colchicine (0.01-2.0ugml^{-1}), 24h
Treated with NTG (100ugml^{-1}), 10-60 min
UV irradiation 2.48W m^{-2}, 10-60 min

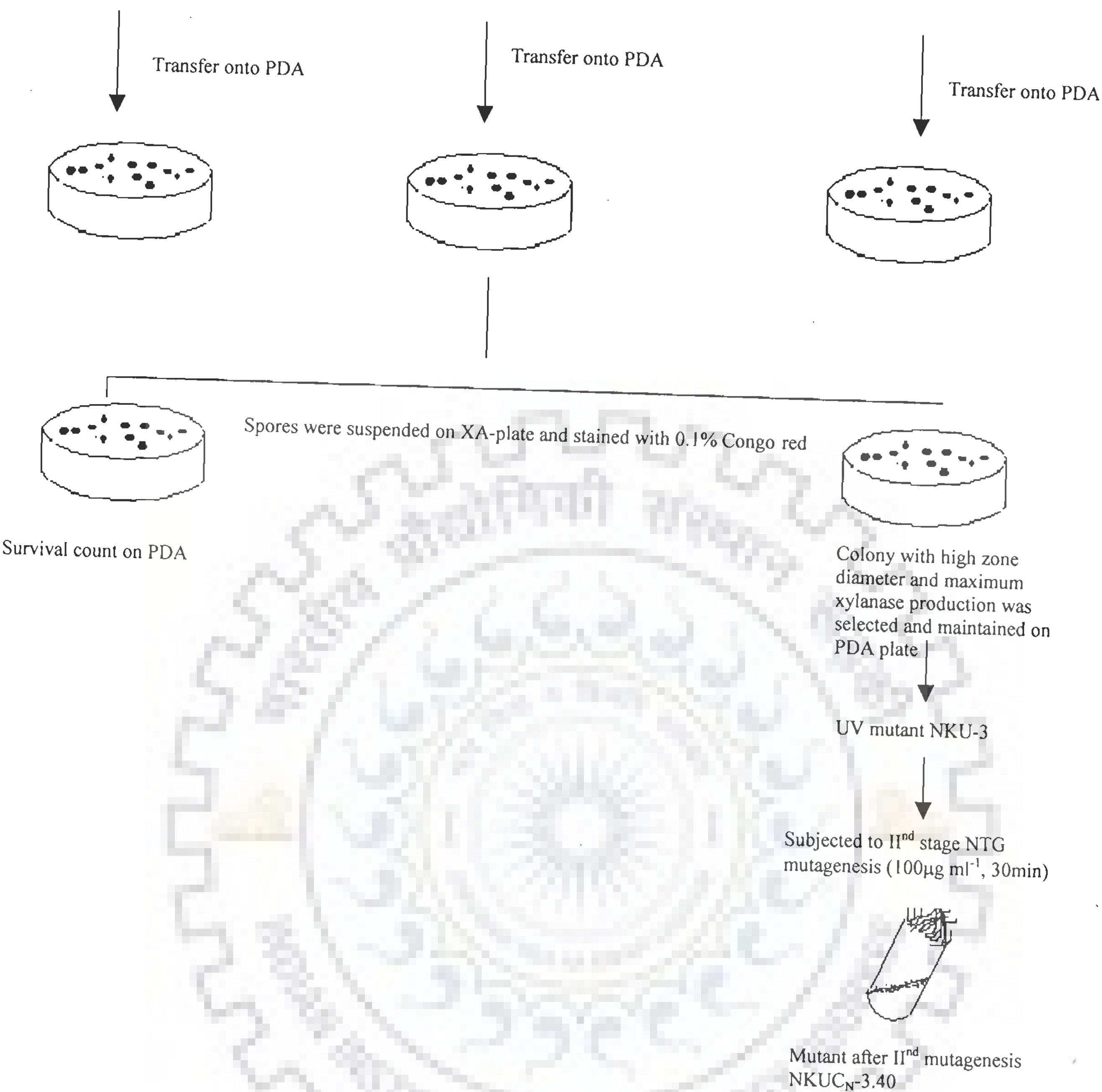


Fig. 4 Mutagenesis and screening of *Aspergillus niger* NK-23 for xylanase production

3.5 FERMENTATION MEDIUM

Preliminary studies of fermentation for xylanase production were carried out as previously described (Medeirosa *et al.*, 2003). The fermentation medium contained (w/v): oat spelt xylan, 0.5%; KH_2PO_4, 0.7%; K_2HPO_4, 0.2%; $MgSO_4.7H2O$, 0.05%; $(NH_4)_2SO_4$, 0.1% and yeast extract, 0.06%. The pH of the medium was adjusted to 7.0 and medium was then autoclaved at 121^0C for 15 minutes at 15 p.s.i.

3.5.1 Pretreatment of Substrate

Lignocellulosic substrates were milled and washed thoroughly in the double distilled water to remove the residual dust. For delignification these were soaked in NaOH (2N) for 24 hours and steamed (60 min) thereafter. The material was repeatedly washed with double distilled water until neutralized and then oven dried (Rajaram and Varma, 1990).

3.5.2 Inoculum

A.niger $NKUC_N$-3.40 and NK-23 from slants were transferred to petri-plates and incubated at 30°C for 5 days. The spores were aseptically harvested in 5 ml sterile 0.05 M phosphate buffer (pH 7.0) containing 0.1% Tween-80. A 2% (v/v) inoculum, containing nearly 10^7 spores ml^{-1} was used as inoculum for biotransformation process. In order to analyze the effect of inoculum on xylanase production, different inoculum sizes were taken.

3.5.3 Xylanase Production by Free Cells of *Aspergillus niger*

3.5.3.1 Submerged fermentation process

Fermentation was carried out in 50ml of fermentation medium in Erlenmeyer flasks (500ml). Medium was inoculated with *A. niger* $NKUC_N$-3.40 and NK-23 following incubation at 30°C in an orbital incubator shaker (Sanyo, Orbi-safe, UK) with constant shaking (120 rpm). The fermentation was carried out for 7 days. Samples for the enzyme activity were retrieved after particular intervals. Following completion of fermentation, biomass was filtered and autoclaved while the filtered supernatant obtained was assayed for xylanase, β-xylosidase and cellulase activities.

3.5.3.2 Surface fermentation

Medium (50ml) following inoculation were kept in an incubator at 30°C. The fungal mycelia were allowed to grow on the surface of the medium in the form of a mycelial mat with occasional shaking to maintain the pH of the media. When needed, the waste fermentation gases were removed by the water suction pump from the fermentation flask and at the same time circulating the fresh air (1.5-2.0 l min^{-1}) over the mycelia.

3.5.3.3 Solid-state fermentation

Solid-state fermentation was performed as described (Gawande & Kamat, 1999) until and otherwise indicated. Briefly, substrates were milled, washed and treated with 2N NaOH at room temperature, Substrates were then subjected to thorough washing with distilled water to remove the traces of alkali and autoclaved before use. A slurry of the fermentation medium containing solid support was prepared in Erlenmeyer flask (250 ml) and inoculated with *A niger* spore suspension. The culture flasks were incubated at 30°C for desired periods. Moisture content was maintained at 70% until and otherwise indicated (de Souza *et al.*, 2001).

3.5.3.3.1 Extraction of the enzyme

Enzyme was extracted from the fermentation system with 100 ml distilled water. Each time the whole content was squeezed, the extract was centrifuged (9000g, 10 min) and the clear supernatant was used as the enzyme source (Gessesse & Mamo, 1999). The remaining support was autoclaved and biomass was determined.

3.6 SCALING UP OF THE PROCESS

Solid-state fermentation using cotton hulls that had resulted into maximum yields of xylanase was subjected for a scaled up process. A tray bioreactor was

designed for semi-continuous production of xylanase as shown in fig 5. Flattened rectangular trays of steel of the size 0.6m x 0.38m x 0.025m (length x breadth x height) were used for the process. Cotton hulls were processed as described earlier. 450 gm of cotton hulls were mixed with 1 l of fermentation media and spread evenly in the fermentation tray. Each of the fermentation trays was connected with an outlet at corner to regulate the removal of fermentation media, which was connected to the product recovery unit. Application of the suction pressure to the trays via outlet, which were occasionally blocked during the fermentation period, enhanced the product recovery. These fermentation trays of above defined specifications were interconnected for the process. Fermentation medium was supplied through medium tank whenever required. The inlet and outlet ports for air were provided on each terminus from the inside bottom surface of the bioreactor. This had enabled the circulation of air over the solid surface. After 4 days of fermentation cycle, the broth was recovered aseptically and the same set up of fungal mat and the solid-support was used for the next batch of fermentation. Following initial batch of fermentation desired amount of nutrients was supplied from the inlet of the medium tank. Following every batch of fermentation, the recovered medium was analyzed for the enzyme production. At the completion of final batch, the cotton hulls were squeezed for the enzyme extraction and sterilized to analyze the total biomass production during complete fermentation process.

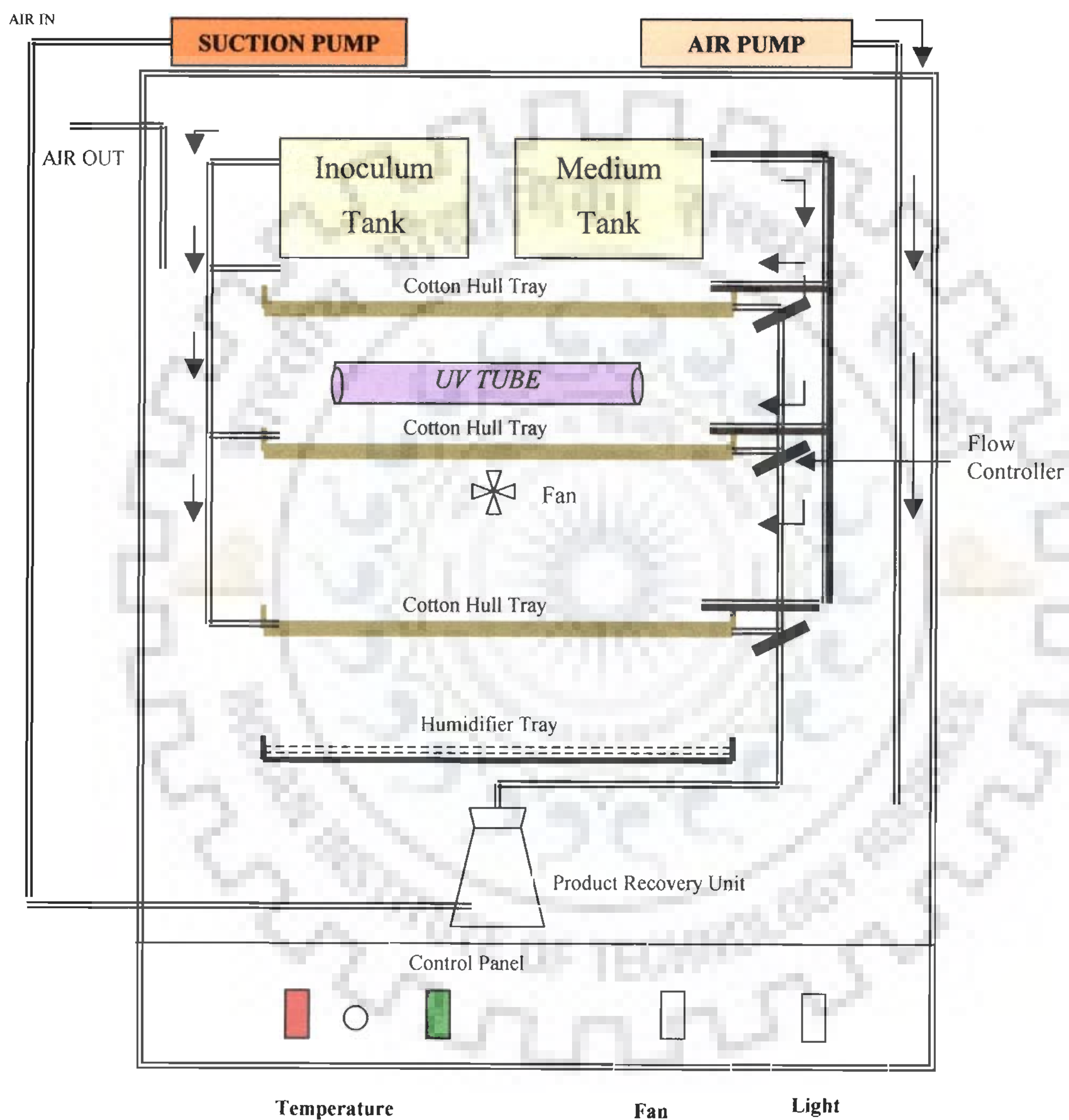


Fig. 5 Schematic representation of the tray bioreactor for semi-continuous production of xylanase under solid-state fermentation

3.7 ANALYTICAL METHODS

3.7.1 Estimation of Xylanase Activity

Xylanase activity was estimated by analysis of the xylose released by DNS method as described (Miller, 1959). 0.5ml of 1% xylan solution was mixed with 0.5 ml of suitably diluted culture filtrate in 50mM phosphate buffer (pH 7.0) and incubated at room temperature for 30 min. 3 ml of 1% dinitrosalicylic acid (DNS) reagent was then added, heated in boiling water bath for 5 min and the xylose released was estimated calorimetrically at 540 nm (Meister, 1961). One unit of enzyme corresponded to one μmol of xylose released per minute per ml under the assay conditions.

DNS reagent

i. Dinitrosalicylic acid, 1 g

ii. Phenol, 0.2 g

iii. Sodium sulphite, 0.05 g

iv. Sodium potassium tartarate, 18.2 g

Dissolved the above components sequentially in 100 ml of 1% sodium hydroxide solution. Mixture was shaken for 5 min and filtered through Whatman filter paper (No.1). The regent was stored in dark at 4°C for future use.

3.7.2 Estimation of β-xylosidase Activity

The β-xylosidase activity was assayed as described (John *et al.*, 1997). Briefly, 1 mM of *p*-nitrophenyl-β-D-xylopyranoside (PNPX) as substrate was incubated with diluted enzyme solution in a total volume of 1 ml reaction mixture for 5 minutes at 60°C. The *p*-nitrophenol released was measured by detecting the increase in absorbance at 405nm. One unit of activity was defined as the amount of enzyme that led to the release of 1μmol of *p*-nitrophenol per minute per ml at 60°C.

3.7.3 Estimation of Cellulase (CMCase) Activity

Carboxymethyl cellulase (CMCase) activity was determined as described by Mandel (1975). Briefly, the assay mixture, in a total volume of 2 ml, contained 0.5ml of 1mM of carboxymethyl cellulose (CMC) in 50 mM citrate buffer (pH 4.8) and 0.5 ml of the supernatant obtained from fermentation broth as the source of enzyme. The mixture was incubated at 50 °C for 30 min. The reducing sugar released was measured using DNS reagent as described (Miller, 1959). One unit of enzyme activity was expressed as one µmol of glucose liberated per minute per ml of the reaction under above defined conditions.

3.8 CHARACTERIZATION OF THE ENZYME

3.8.1 Thermostability

The thermostability of the enzyme was determined at temperatures ranging between 30-75 °C for upto 2 hrs. Samples were periodically withdrawn and analysed for xylanase activity.

3.8.2 Optimum pH and pH Stability

The stability was determined in the pH range of 3.0-11.0 by incubating the enzyme in buffers of different pH. Citrate-phosphate, pH 3.0-6.0; Phosphate, pH 6.0-8.0; Tris- HCl, pH 8.0-9.0; Glycine- NaOH, pH 9.0-10.5;Carbonate- Bicarbonate, pH 10.5-11.0. After 30 min and 1 hr incubation, the relative enzyme activities were determined, under standard assay conditions described earlier.

3.9 PARTIAL PURIFICATION OF ENZYME

Solid-state fermentation using cotton hulls as solid support was used for maximum enzymatic production. Broth (100 ml) after 4 days of bioconversion was

collected and centrifuged (9000 g, 10 min). Clear supernatant was added with ammonium sulphate (80% saturation) (Yeoh *et al.*, 1986; Singh *et al.*, 1990) and incubated at 4°C with continous shaking. Precipitate obtained after centrifugation (4500 g, 30 min) at 4°C was dissolved in 50mM phosphate buffer (pH 7.0) (Singh *et al.*, 1990). Preparation was then dialyzed for 14-16 h using 50mM phosphate buffer (pH 7.0) with buffer changes at appropriate intervals.

3.9.1 Sodium-dodecylsulphate Polyacrylamide Gel Electrophoresis

SDS-PAGE of the samples was performed according to Laemmli (1970). Polyacrylamide gels (12%) of 1.5 mm thickness were prepared for electrophoretic analysis.

3.9.1.1 Reagents

Solution A: Acrylamide solution 29.2% (w/v) containing 0.8% (w/v) bisacrylamide (N, N'-methylene-bisacrylamide),

Solution B: Resolving buffer, 1.5 M Tris-HCl, pH 8.8,

Solution C: Stacking buffer, 0.125 M Tris-HCl, pH 6.8,

Solution D: SDS (10% (w/v)

Solution E: Ammonium persulphate 10% (w/v) (freshly prepared)

Solution F: TEMED (N, N, N', N'-tetramethylethylenediamine),

Electrophoresis buffer, 0.25 M Tris, 0.192 M glycine, pH 8.3,

3.9.1.2 Preparation of resolving and stacking gels

Reagents	Resolving gel (12%) (ml)	Stacking gel (4%) (ml)
Solution A	12.0	2.66
Solution B	7.50	-
Solution C	-	5.00
Solution D	0.30	0.20
Solution E	0.15	0.10
Solution F	0.015	0.02
Water	10.035	12.2
Total volume (ml)	30.0	20.0

3.9.1.3 Casting of gel

A sandwich of glass plates (16 x 18 x 0.3 cm) was assembled by plastic spacers (1.5 mm) and the plates were held together by plastic clamps. The base of the gel mould was sealed. Resolving gel mixture was prepared by mixing all the components except ammonium persulphate and TEMED. Mixture was degassed for 15 min, ammonium persulphate and TEMED were added to it. The mixture was gently mixed and poured between the plates, and leaving the required space at the top for pouring the stacking gel. After polymerization of the resolving gel, the assembly was tilted to pour off the over layer, washed with sterile distilled water and wiped gently to dry the empty space. Stacking gel mixture was then similarly degassed and layered on the resolving gel; comb was immediately inserted into the mixture to form the wells. After polymerization of stacking gel, comb was carefully removed, wells formed were rinsed with reservoir buffer and the polymerized gel was used for electrophoresis.

3.9.1.4 Sample preparation

Fractions containing proteins were concentrated using Centricon (C-10) concentrators (Amicon, USA) and added with SDS-PAGE sample buffer containing Tris-HCl (pH 6.8), 2% (w/v) SDS, 5% (v/v) glycerol, 2% (v/v) β-mercaptoethanol, 0.01% (w/v) bromophenol blue. Samples were heated in a boiling water bath for 3-5 min and loaded onto the gel for electrophoresis.

3.9.1.5 Electrophoresis

Samples (100 μg of the protein) were gently loaded on the gel using a micropipette. Electrophoresis was carried out at constant voltage (stacking at 60 V, resolving at 90 V) until the tracking dye reached to about 1 cm above from the bottom of the gel. Gel after electrophoresis was removed and stained overnight with gentle shaking in coomassie brilliant blue R-250 0.1% in methanol: glacial acetic acid: water (5:2:5 v/v/v) at room temperature. Gel was then destained by washing in 10% isopropanol-7% acetic acid solution (Walker, 1996). Destaining was done so as to get the stained bands over the clear background. Gel was stored in 7% acetic acid until photographed.

3.9.2 Zymogram Analysis of Xylanase Components

Native polyacrylamide gel electrophoresis (PAGE) using 12% gel was performed for visualization of enzyme activities in situ as described (Schwarz *et al.*, 1989; Reyes and Noyola, 1998). Substrate i.e. xylan to the final concentrations of 0.1% was incorporated into separating gel before adding the ammonium persulphate and TEMED for polymerization. The enzyme samples were mixed with sample buffer lacking SDS and β-merceptoethanol. After electrophoresis, the gel was soaked with

0.1% (w/v) Congo red for 5 min with mild shaking. Excess dye was decanted and gel was washed with 5% (w/v) NaCl until excess stain was totally removed and the background was clear.

3.10 SCANNING ELECTRON MICROSCOPY

The detailed morphological studies of lignocellulosic wastes (before and after treatment) and fungal strains were carried out using scanning electron microscopy (SEM, Leo 435 VP, England). Lignocellulosic substrates or the fungal mat were taken and subjected for fixation using 3% (v/v) glutaraldehyde-2% (v/v) formaldehyde (4:1) for 24 h. Following the primary fixation, samples were washed thrice with double distilled water. The samples were then treated with the alcohol gradients of 30%, 50%, 70%, 80%, 90% and 100% for dehydration. Samples were kept for 15 min each up to 70% alcohol gradient, thereafter treated for 30 min each for subsequent alcohol gradients. After treating with 100% alcohol, samples were air dried and examined under SEM using gold shadowing technique (Gabriel, 1982). Electron photomicrographs were taken at desired magnifications.

3.11 STATISTICAL ANALYSIS

All fermentations were carried out in triplicate and experimental results represent the mean of three identical fermentations. The statistical analysis (ANOVA) was performed as described by Somasegaran and Hoben (1994). 1% and 5% levels for ANOVA test was used for comparing the calculated F ratio by using standard tables.

Table for Analysis of Variance

Source of Variation	Sum of square	Degree of freedom (df)	Mean squares (Ms)	F ratio
Between the table (T)	SST	k-1	SST/ (k-1)	$\frac{SST}{SSE} \times \frac{bkn-k-b+1}{(K-1)}$
With in block (B)	SSB	b-1	SSB/ (b-1)	$\frac{SSB}{SSE} \times \frac{bkn-k-b+1}{(b-1)}$
Error (E)	SSE	Bkn-k-b+1	SSE/ (bkn-k-b+1)	
Total	SS	Bkn-1		

d.f., Degree of freedom; SST, Treatment sum of squares; SSB, Block sum of squares; SSE, Error sum of squares; SS, Total sum of squares; K, Number of treatment; b, Number of blocks (number of replicates per treatment); n, Number of replicates per treatment per block.

3.12 BIOBLEACHING OF PULP WITH XYLANASE FROM *A. NIGER* NKUC$_N$-3.40

During application studies of xylanase from *A.niger* NKUC$_N$-3.40 for bio bleaching of pulp, the physical and chemical characteristics of pulp and paper were determined according to the standard methods of Technical Association of Pulp and Paper Industry (TAAPI, 2002).

3.12.1 Pulping Process

Screened wood chips of *Populas deltoidea, Eucalyptus tetrecornis, Bambusa aurandacea and Pinus roxumberghii* in the ratio 17:13: 10:2 were cooked in Weverk made laboratory digester of capacity 0.02 m^3 having four bombs of 200ml capacity at different cooking doses i.e. 14%, 15%, 16% and 17% as Na_2O, sulphidity 20%, maximum temperature 165±2°C for 75 minutes, liquor to wood ratio =3.0:1 and digester pressure 6.512 g cm^{-2}. At the end of cooking the pulp was squeezed, pressed, washed and screened in Weverk rotary screen. The unbleached pulp was evaluated for permanganate number, residual alkali, pulp yield and screened rejects.

In the similar manner, de-dusted wheat straw was cooked in Weverk made laboratory digester of capacity 0.02 m^3 having four bombs of 200ml capacity by soda pulping process. The cooking conditions involves active alkali dose as 11%,12%,13% and 14% (as Na_2O), maximum cooking temperature, 160°C for 120 minutes, liquor to wood ratio =5.0:1 and digester pressure 6.512 g cm^{-2}.

3.12.2 Biobleaching

Both hard wood and wheat straw pulps were treated with XCEHH and conventional CEHH bleaching sequences (X, xylanase; C, Chlorination; E, alkali extraction; H, Hypochlorite).

3.12.2.1 Optimization of Enzyme Dose

The unbleached pulp composed of wheat straw and mixed wood pulp containing *Populas deltoidea* (71%), *Populus tetrecornis* (13%), *B. aurandacea* (10%) and *P. roxumberghii* (2%) was obtained from Star Paper Mills, Saharanpur (U.P.), India. Oven dried pulp samples (1g each) were soaked in 9 ml of distilled water for overnight at room temperature. These samples were added with enzyme dosage ranging between 0-30 IU/g of oven dried pulp and incubated in a water bath at 65°C for 2 hrs. The treated pulp samples were filtered through muslin cloth and the respective filtrates were collected. The filtrates were studied for the BOD, COD and release of chromophores and hydrophobic compounds (Gupta et al., 2000). The amount of reducing sugars released in the filtrates were estimated as described earlier (Miller, 1959). The treated pulp samples were dried at 80°C for 24 hrs and used for estimation of kappa number (residual lignin content) following the standard method of TAPPI (Anonymous. 1991) as described below.

2g of pulp sample was dispensed in 500 ml of distilled water in a reaction beaker (2L) and volume was made up to 800 ml with distilled water. The reaction was incubated in a shaking water bath for 30 min at 25°C to make the pulp free of fiber clots. This was followed by addition of 100 ml of potassium permanganate solution (0.1N) and 100 ml of sulphuric acid solution (4N). After 10 min, the reaction was stopped by addition of 20 ml of 1 N potassium iodide solution. The mixture was titrated with sodium thiosulphate solution (0.2 N) with the addition of few drops of starch solution as an indicator to detect the end point of the reaction. Volume of sodium thiosulphate solution required for complete titration of each sample was noted.

Kappa number is the volume (in ml) of 0.1 N potassium per manganate solution consumed by 1 g of moisture free pulp under the assay conditions. Kappa number is used as an indicator of lignin content (kappa number divided by 7 gives the approximate percentage of lignin content in the pulp). NaOH extraction of pulp is directed by the kappa number of the pulp (chemical required =0.11x kappa number).

Kappa number is determined as follows

$$K= (PXF)/W$$

and

$$P= [(B-A) N]/0.1$$

Where, K, kappa number; F, factor for correction to 50% permanganate consumption, dependent on the value of P; W, weight (g) of moisture free pulp in the specimen P, volume (ml) of 0.1 N permanganate consumed by the test specimen (g); B, volume (ml) of thiosulphate consumed by the test specimen; A, amount of thiosulphate consumed (in ml) by the test specimen; N, normality of thiosulphate

3.12.4 Optimization of Reaction Time

The optimum time for maximum enzymatic bleaching of pulp was determined by treating the pulp samples (1g each) in different polythene packages added with enzyme (10 IU g^{-1} of oven dried pulp) at 65°C for 3h. The samples were withdrawn at regular intervals of 30 min each (1 package at a time) and the pulp treated with xylanase was examined for release of chromophores, hydrophobic compounds, release of reducing sugars and reduction in kappa number as described above.

3.12.5 Application of Xylanase in Multi-step Bleaching Process

Fresh pulp samples were collected and pre-bleached with optimum dose of xylanase (10 IUg^{-1} of odp) for 2h at 65°C followed by CEHH sequences. Samples without enzyme application were also processed for chemical treatment under the same conditions. These were taken as control to analyze the effect of xylanase pre-treatment in the bleaching process on paper properties. The treated and untreated pulp samples were filtered and respective filtrates were studied for release of chromophores, hydrophobic compounds, release of reducing sugars and reduction in kappa number as described earlier. Pulp samples (both treated and untreated) were used for making hand made paper sheets. Physical parameters like brightness, tear index, tensile index and burst index were analyzed according to the standard methods of TAPPI.

3.1253.1 Brightness

Absolute brightness is defined as the reflectance of blue light with a specified spectral distribution peaking at 457nm (because it coincides with lignin absorption) compared to that of a perfectly reflecting, perfectly diffusing surface. The ISO method

uses diffused illumination. Lignin is the "glue" that holds fibres together and gives a yellow tint to the paper. The brightness value shows the bleaching ability of the pulp and paper. Brightness of the handmade sheets was determined in terms of colour number using brightness and colour meter and expressed as % ISO brightness.

3.12.5.2 Tear Index

Tear strength is a major of the energy required to create new area by tearing. The two flaps of pre-cut paper were clamped in separate jaws of an Elmendorf Pendulum and with a swing of a pendulum, the sample was torn. The energy removed from the pendulum by a process of tearing the sample was estimated from the final magnitude of the swing and the result was directly read off the pendulum.

Tearing Strength ($g\ f^{1}$)= Scale reading x pendulum factor,

Tear Factor = Tearing Strength x 100/ Basis weight,

Tear Index ($mN.m^{2}\ g^{-1}$) = Tear Factor x 0.098

3.12.5.3 Tensile Index

It is the measure of the maximum tensile stress developed in a test specimen before rupture on a tensile tester under specified conditions. Short length (5-8 cm) of paper strips were made from hand sheets and placed in the center in-between the open jaws of Clarke's attachment so that the grain direction of the strip (it's strongest direction) was parallel to the length of the attachment. The jaws were closed and pressure was applied over the clamping screws till the paper strip broke and the values were recorded. Tensile strength is estimated as follows:

Tensile Index ($N.m\ g^{-1}$)= Breaking Length x 0.0098

and Breaking Length (m)= Tensile Strength x $10^{6}/15$ x Basis Weight

3.12.5.4 Burst Index

It is widely used as a measure of resistance to bursting on application of hydrostatic pressures. Burst factor is defined as the hydrostatic pressure in kilopascals or pounds per square inch or *psi*, required to rupture the hand sheet when the pressure was increased at a controlled constant weight through a rubber diaphragm to a circular area of 30.5 mm diameter. The area of the material under test was initially flat and held rigidly at the circumference but was free to bulge during the test. This was followed by the application of hydrostatic pressure until the specimen ruptures and the maximum pressure registered was recorded.

Burst index is calculated as follows:

Burst Index (kPa. m^2g^{-1})= Burst Factor x 0.098

Burst Factor= Burst Strength (kPa) x 1000/ Basis Weight

3.12.5.5 Copper Number

The copper number may be regarded as an index of those impurities in paper, such as oxycellulose, hydrocellulose, lignin and sugars, which possesses reducing properties. It is useful for determining the changes accompanying deterioration and may therefore be considered as a factor having an indirect bearing on the performance of paper. Copper number is defined as the number of grams of metallic copper (as Cu_2O) resulting from the reduction of Cu SO_4 by 100 g of the pulp or paper fibers.

5 ml of copper sulphite (10%) solution was mixed with carbonate-bicarbonate solution and boiled for 2 min, mixture was poured on 1.5 g of odp and incubated for 3 h in water bath. At the end of 3 h, the slurry was filtered on ash less filter paper in buchner funnel with suction. It was then washed with 100 ml of the Na_2CO_3 (5%) solution at 20°C and then by flooding it with 250 ml of hot water at about 95°C.

Filtrate was discarded and the pulp fiber and filter paper was transferred in a beaker (100 ml), followed by the addition of the 25 ml of the molybdophosphoric acid. The mixture was transferd to buchner funnel with ash less filter paper and washed thoroughly with cold water collecting the filtrate, until the blue colour was removed. Diluted the filtrate to approximately 700 ml followed by titrating with 0.05 N $KMnO_4$. Blank contained all reagents except pulp.

Copper number can be calculated as follows:

$$\text{Copper number} = 6.36\,(V-B)\,N/\,W$$

Where,

V, Volume (ml) of $KMnO_4$ solution to titrate the filtrate from the specimen;

B, Volume (ml) of $KMnO_4$ solution to titrate the blank filtrate;

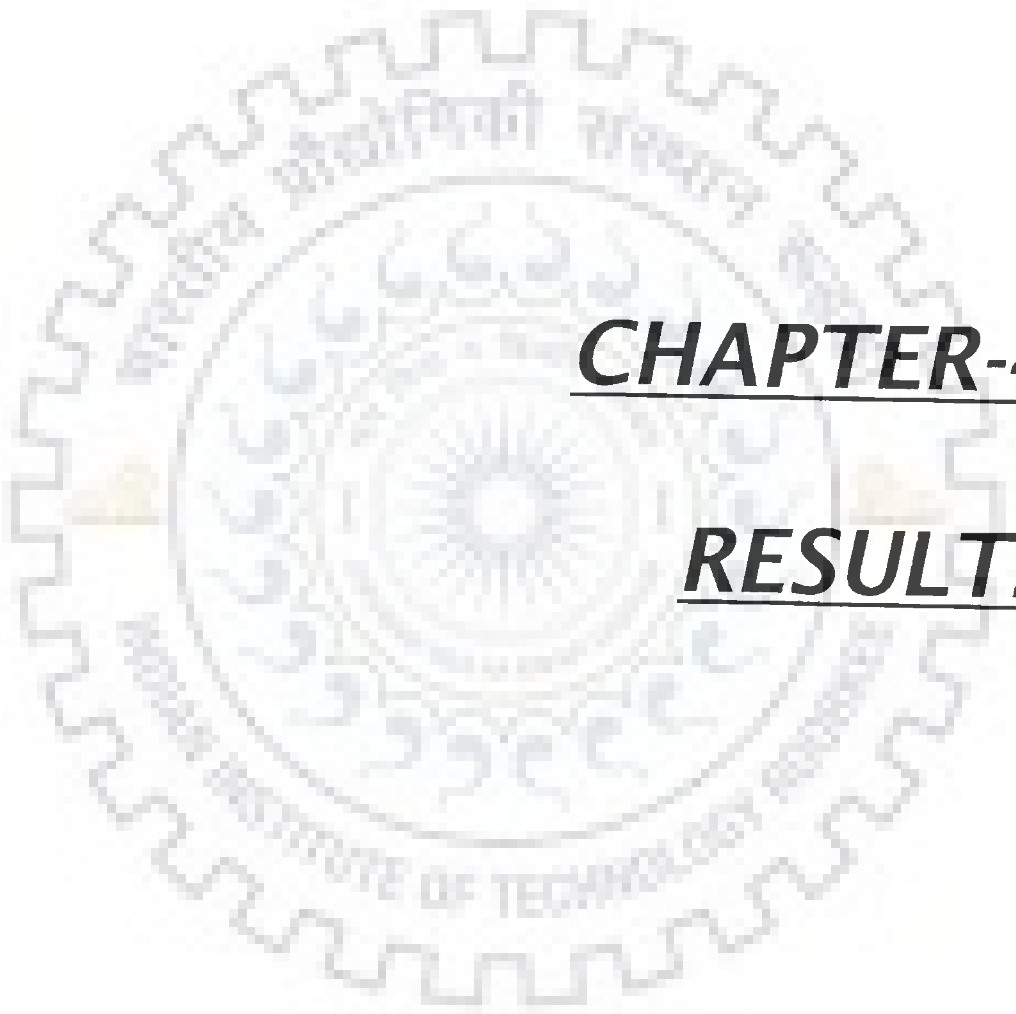
N, Normality of $KMnO_4$ solution

3.12.5.6 Double fold

It is an indicative of the ability of paper to get folded without tearing. The ends of pre-cut paper strip were clamped in separate jaws of double fold analyzer. The number of revolution is the indicator of the double fold of paper.

3.12.5.7 Smoothness

It measures the opposite to the extent to which the surface of the paper deviates from a plane and involves the depth, width and number of departures from the plane. Bendtsen smoothness and porosity tester is used to measure smoothness of the hand sheet. The test piece is pressed under a definite pressure by one or two annular metal lands and against a smooth, plane, and hard surface. Air is supplied at a constant pressure to the center of the annular land or between the lands, and the airflow passing between the annular land and the surface of the paper is measured. The instrument reading is greater with rougher papers.



CHAPTER-4

RESULTS

Chapter - 4
RESULTS

4.1 ISOLATION, PURIFICATION AND SCREENING OF STRAINS

A total of 42 fungal isolates were screened in the present study. These distinct colonies were obtained from different sites viz., forest soil, sugarcane dumping site, paper industry waste site, decomposing manure and were isolated initially on the basis of the colour and colony appearance at 32°C following 48 hrs of incubation. These strains were primarily screened for the xylanase production ability on the basis of clear zone diameter obtained after using the xylan-agar (XA) plate (Table 6). Of the 42 strains, 22 isolates resulted into zone formation onto xylan-agar (XA) plates. Five among these twenty-two isolated, that had shown maximum zone formation were further subjected to secondary screening for xylanase production ability in submerged condition. This had resulted into NK-23 and NK-35 having relatively higher production of xylanase (Table 7) and a maximal of 335.4 IU ml^{-1} was obtained from NK-23. This strain, therefore, was selected for further studies. The strain was identified as *Aspergillus niger* from Indian Agricultural Research Institute, New Delhi, India and denoted as *Aspergillus niger* NK-23. The culture was maintained by periodical transfers on potato dextrose agar slants and was stored at 4°C for further use.

Table 6. Morphological analysis of the fungal isolates

S.No.	Isolated Strains	Site of Isolation	Mycelial Colour	Spore Colour	Zone Diameter (mm)
1.	NK-1	Forest soil	Brown	-	10.0
2.	NK-2	Forest soil	Brown	-	6.7
3.	NK-3	Forest soil	Whitish Grey	Black	-
4.	NK-4	Forest soil	Pinkish Brown	Brown	9.6
5.	NK-5	Forest soil	Off-white	Black	8.8
6.	NK-6	Forest soil	Light Yellow	-	10.1
7.	NK-7	Forest soil	White	Black	-
8.	NK-8	Forest soil	White	White	6.7
9.	NK-9	Forest soil	Dull Green	Green	6.8
10.	NK-10	Forest soil	Dirty White	Green	14.1
11.	NK-11	Forest soil	White	Black	-
12.	NK-12	Forest soil	White	White	-
13.	NK-13	Forest soil	White	-	11.2
14.	NK-14	Forest soil	Creamish	-	-
15.	NK-15	Forest soil	White	Black	-
16.	NK-16	Forest soil	Yellowish Green	Green	12.4
17.	NK-17	Forest soil	Bluish	-	-
18.	NK-18	Sugarcane dumping site	Dirty White	Greenish	7.2
19.	NK-19	Sugarcane dumping site	White	Black	16.5
20.	NK-20	Sugarcane dumping site	Pink	-	4.6
21.	NK-21	Sugarcane dumping site	Off-white	Black	-
22.	NK-22	Sugarcane dumping site	Brown	-	-
23.	NK-23	Sugarcane dumping site	Creamish	Black	18.5
24.	NK-24	Sugarcane dumping site	White	Black	7.1
25.	NK-25	Sugarcane dumping site	-	Dirty green	11.8
26.	NK-26	Sugarcane dumping site	Off-white	Green	-
27.	NK-27	Decomposing manure	-	Black	-
28.	NK-28	Decomposing manure	White	Black	16.4
29.	NK-29	Decomposing manure	Grey	-	16.4

30.	NK-30	Decomposing manure	Off-white	Black	-
31.	NK-31	Decomposing manure	Yellow	-	-
32.	NK-32	Decomposing manure	-	Black	-
33.	NK-33	Decomposing manure	White	Black	-
34.	NK-31	Decomposing manure	-	Black	-
35.	NK-35	Decomposing manure	Yellow	Black	17.1
36.	NK-36	Decomposing manure	Red	Red	-
37.	NK-37	Paper industry waste site	Pale yellow	Black	15.0
38.	NK-38	Paper industry waste site	Pink	Red	5.03
39.	NK-39	Paper industry waste site	-	Black	-
40.	NK-40	Paper industry waste site	Whitish yellow	Brown	-
41.	NK-41	Paper industry waste site	Dirty white	Black	11.9
42.	NK-42	Paper industry waste site	-	Black	-

Table 7. Xylanase production from the selected fungal isolates

S. No.	Fungal Strain	Zone diameter (mm)	Xylanase (IU ml^{-1})
1.	NK-23	18.5	335.4
2.	NK-35	17.1	270.3
3.	NK-19	16.5	197.3
4.	NK-37	15.01	189.4
5.	NK-29	16.4	165.2

4.2 MUTAGENESIS OF *Aspergillus niger* NK-23 FOR IMPROVED XYLANASE PRODUCTION

NK-23 strain isolated from the sugarcane-dumping site was subjected to UV irradiation, NTG, colchicine and mixed mutagenic treatments for achieving an enhancement in xylanase production. Spore suspensions of *Aspergillus niger* NK-23

were exposed to UV or chemical mutagens and the percentage distribution of positive, negative and the corresponding or unaltered colonies were determined. The mutant exhibiting the highest zone diameter (22.3 mm) on xylan agar plate was referred as NKU-3. Out of 78.4% spore survived following 3 minutes of UV irradiation, 26% had the higher zone diameter compared to the parent strain and were termed as positive mutants, whereas 7% of the colonies had the lesser zone diameter and denoted as negative mutants; 67% of the mutants had the zone diameter (~18-19 mm) that corresponded to the parental strain, hence termed as the corresponding mutants (Fig 6a). NKU-3 produced 448.7 $IUml^{-1}$ of xylanase under submerged fermentation. The parental strain NK-23 was also subjected to NTG (100 $\mu g\ ml^{-1}$) and to colchicine (0.01-1.0 $\mu g\ ml^{-1}$) treatments. NKC_N-30 mutant obtained after 30 minutes of exposure of NTG had a zone-diameter of 21.2 mm with 69% spore survival, of the total mutants obtained, 29%, 11% and 65% respectively were negative, positive and unaltered mutants (Fig. 6b) whereas mutant NKC_C-0.2 generated after colchicine (0.2 $\mu g\ ml^{-1}$) treatment had a zone diameter of 19.4 mm (Table 6). 10% of the total mutants obtained were negative, 9% positive and 81% were corresponding mutants (Fig. 6c). Prolonged treatment with NTG or colchicines leads to the reduction in the number of corresponding mutants.

Among the strains obtained following UV, NTG and colchicine mutagenesis, mutant NKU-3, obtained after UV irradiation had resulted into maximal zone diameter and was subjected to second round of NTG mediated mutagenesis for achieving a further improvement in the xylanase production ability of the strain. The mutant thus obtained after 40 minutes of treatment had the highest zone diameter (Fig. 9b) and was termed as $NKUC_N$-3.40 (Table 6). Out of the total number of mutants thus obtained 27%, 24% and 49% were positive, negative and corresponding mutants respectively.

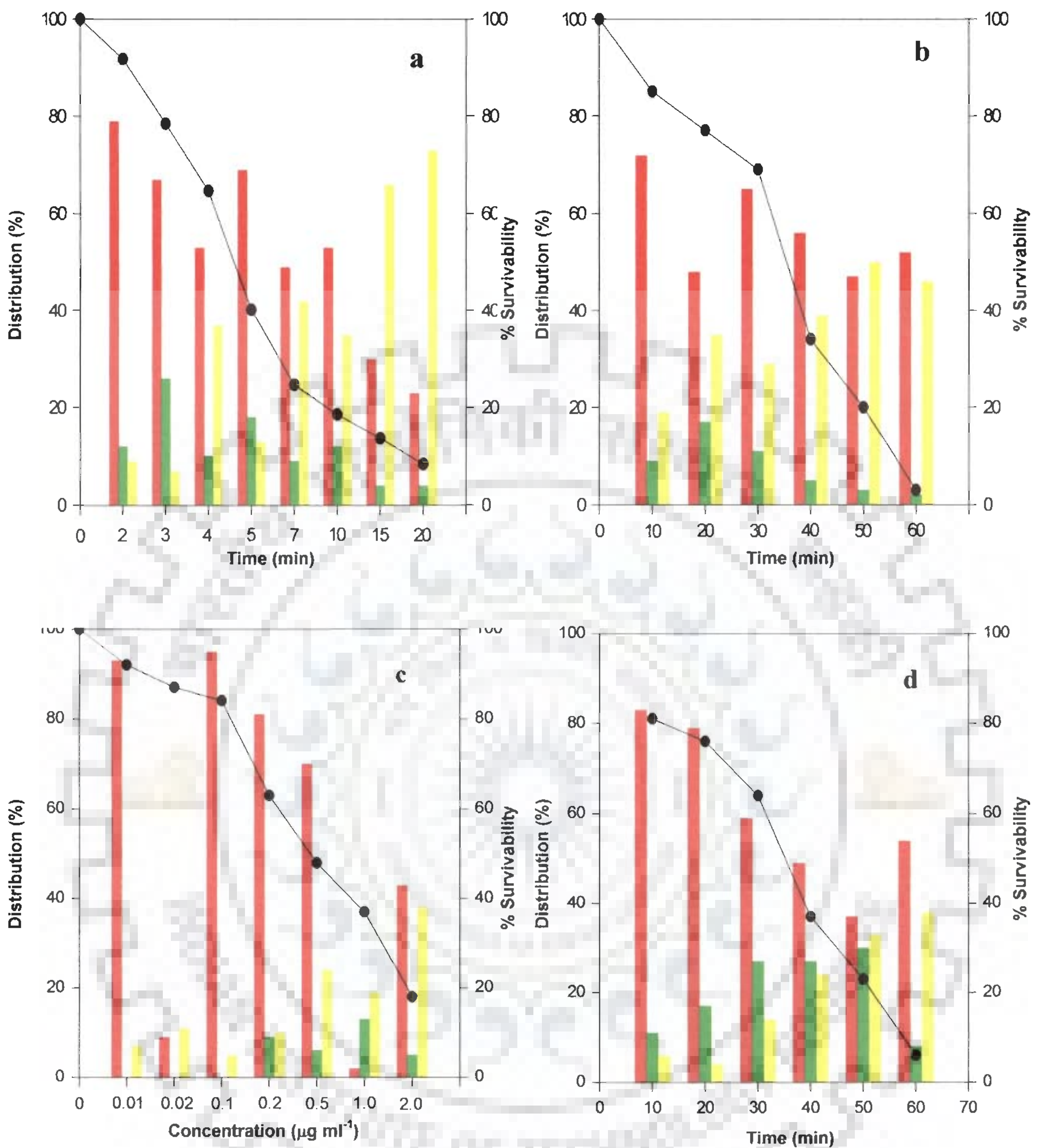


Fig. 6 ANALYSIS OF MUTAGENESIS OF *A. NIGER* NK-23

(a) UV irradiation; (b) N-methyl-N'-nitro-N-nitrosoguanidine treatment; (c) Colchicine treatment; (d) Mixed (UV+ NTG) treatment (■, unaltered; ■, positive; □, negative mutants); ● Survivability

The mutant strains thus selected on the basis of clear zone were analyzed for the xylanase, β-xylosidase and cellulase production under submerged fermentation condition.

Table 8. Xylanase production of the selected mutants obtained after mutagenesis

S.No.	Mutagenic agent	Strains of *A. niger*	Zone diameter (mm)	Xylanase (IU ml^{-1})	β-Xylosidase (IU ml^{-1})	Cellulase (IU ml^{-1})
1.	None	NK-23	18.5	335.4	97.3	2.6
2.	UV	NKU-3	22.3	448.7	113.4	1.7
3.	NTG	NKC_N-30	21.2	345.3	92.8	3.2
4.	Colchicine	NKC_C-0.2	19.4	339.3	105.2	3.7
5.	UV+NTG	$NKUC_N$-3.40	25.8	469.6	127.6	1.63

Among these, $NKUC_N$-3.40 obtained after mixed mutagenesis strategy had the maximum levels of xylanase, that represents 40% enhancement in xylanase production as compared to the wild type NK-23 strain. The $NKUC_N$-3.40 also had significant increase in β-xylosidase production (127.6 IU ml^{-1}) and had comparatively lower levels of cellulase production (1.63 IU ml^{-1}) during successive generations with minor variations (Fig.7).

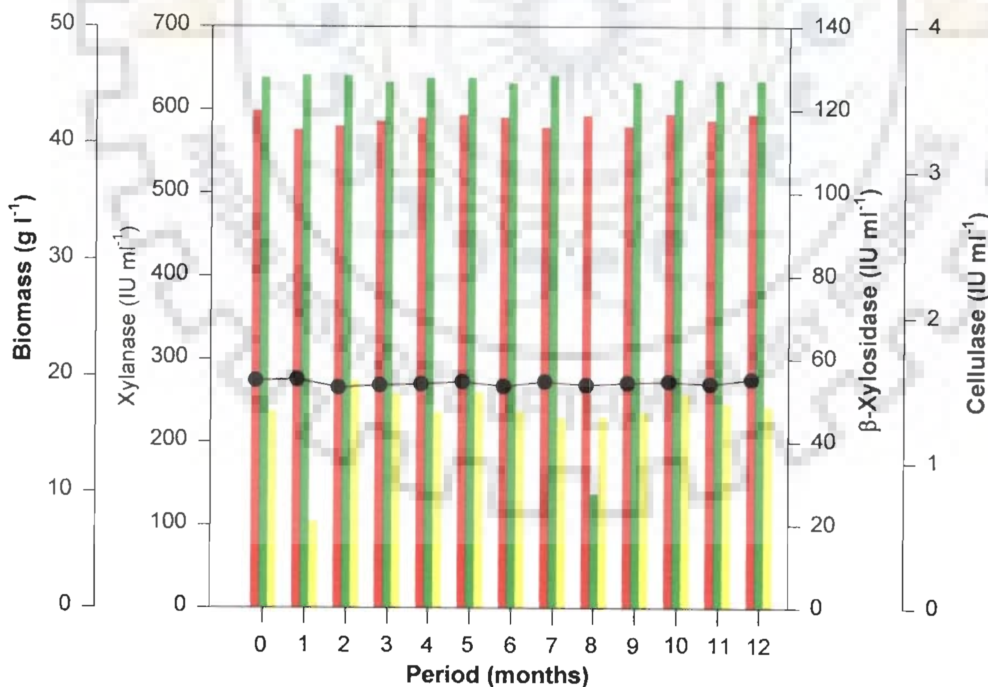


Fig. 7 Production of xylanase, β-xylosidase and cellulase following repeated sub-culturing of *A. niger* $NKUC_N$-3.40

■, ■, ■, ● , represent xylanase, β-xylosidase, cellulase and biomass produced respectively.

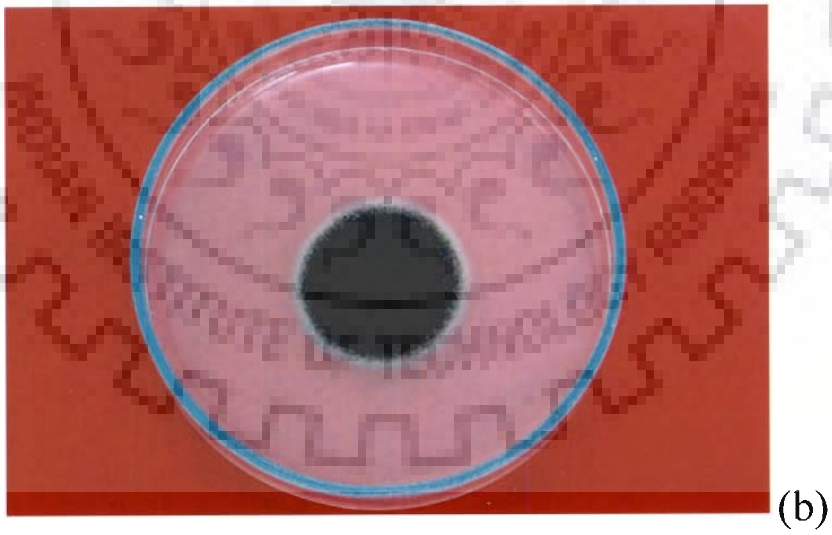
4.2.1 Morphological features

The characteristic features of the wild type *A. niger* NK-23 and mutant strain NKUC$_N$-3.40 are shown in fig.8. Distinct changes in colour are seen in the mycelia of the fungal strains. Mycelia of wild type strain NK-23 was loose and brownish yellow as compared to the compact and dirty white coloured mycelia of the mutant strain NKUC$_N$-3.40. However, the spores produced by the mutants obtained after mixed mutagenesis appeared to be black in colour like that of the parental strain NK-23.

The finer structural details of the mycelium as observed through the scanning electron microscopy (SEM) are shown in fig.10 and 11. The hyphae of wild type *A. niger* NK-23 are thin, elongated and straight (Fig 10a) whereas, the hyphae of the strain NKUC$_N$-3.40 are thick, compact and coiled (Fig 10b).

Extent of sporulation observed was lower for mutant NKUC$_N$-3.40 as compared to the wild type NK-23 strain. In addition, spores of mutant *A. niger* NKUC$_N$-3.40 had uneven or rough surface compared to the wild type strain *A. niger* NK-23 that had smoother surface (Fig. 11).

(a)



(b)

Fig. 8 (a-b) Morphological features of wild type NK-23 (a) and mutant NKUC$_N$-3.40 (b) strains

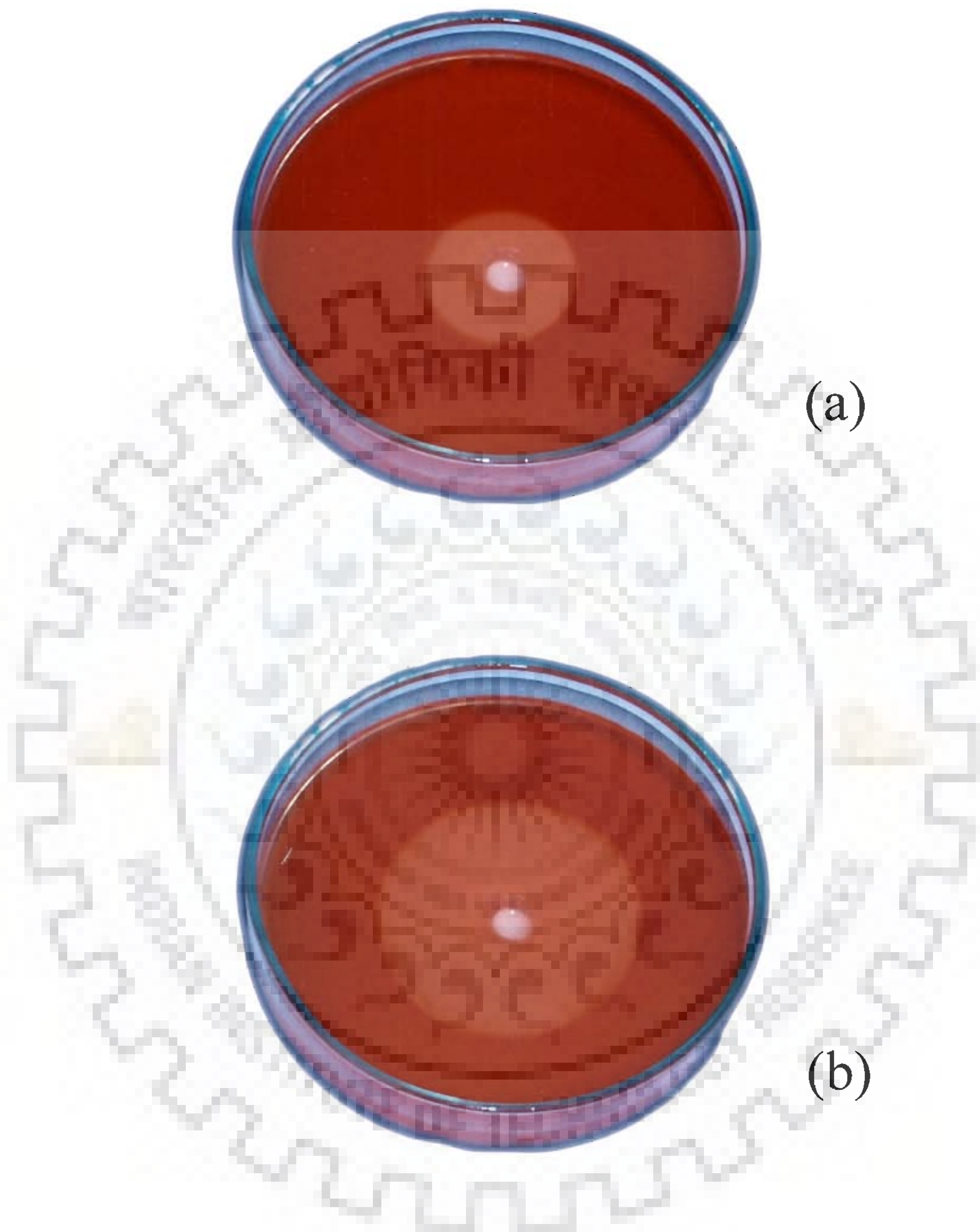
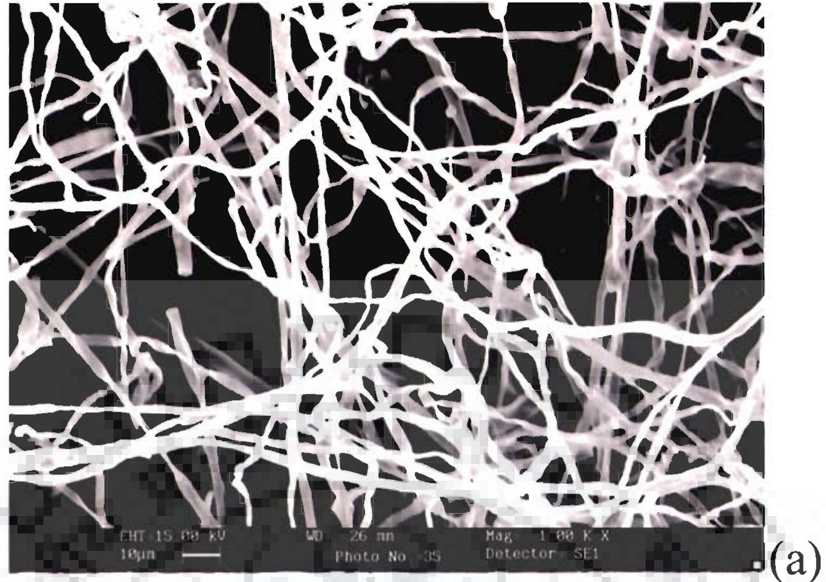


Fig 9 (a-b) Zone diameters obtained with wild type NK-23 (a) and mutant NKUC$_N$-3.40 (b) srains



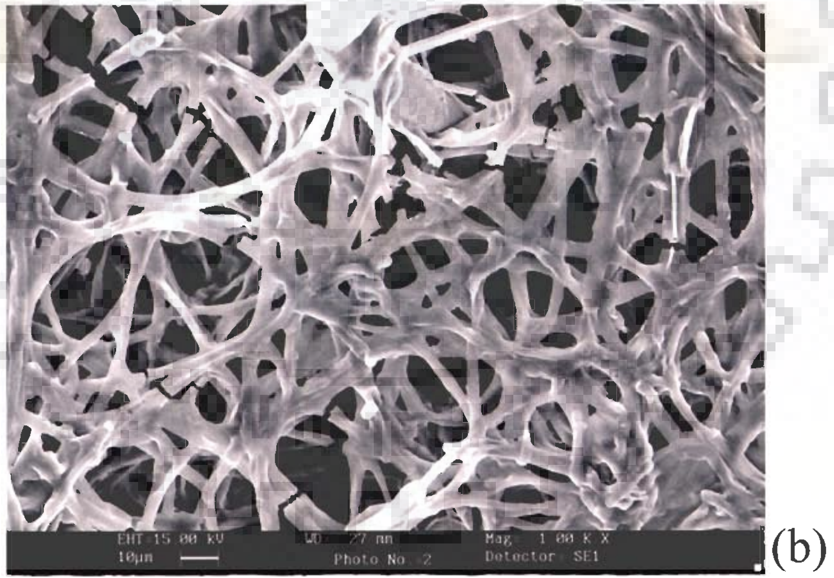
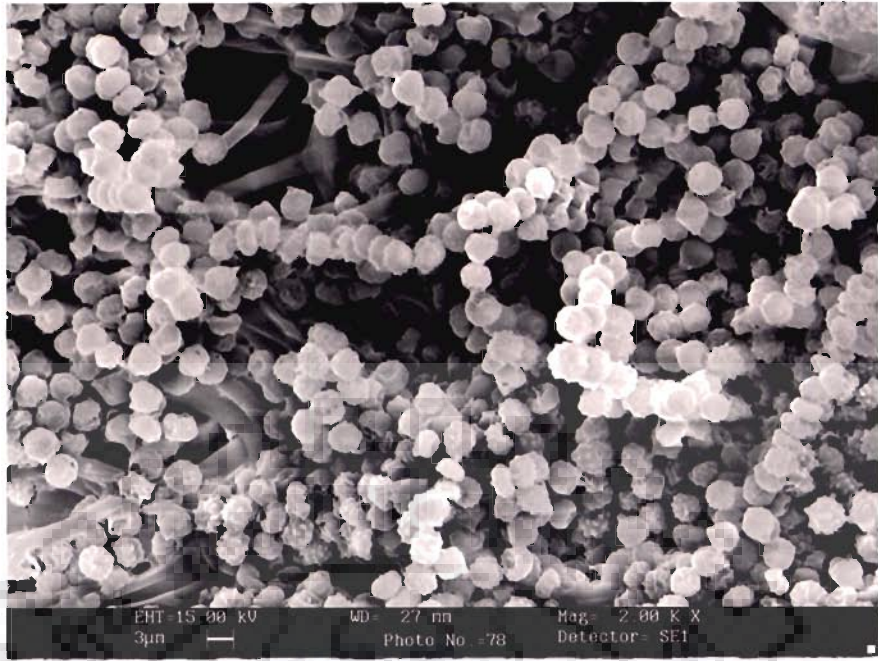


Fig.10 Scanning electron micrograph of the wild type NK-23 (a) and mutant $NKUC_N$-3.40 (b) mycelia



(a)

(b)

Fig.11 Scanning electron micrograph of wild type NK-23 (a) and mutant NKUC$_N$-3.40 (b) spores

4.3 ANALYSIS OF THE CRITICAL PARAMETERS FOR IMPROVING THE XYLANASE PRODUCTION

Submerged cultivation was carried out for optimizing various parameters using hyper-xylanase producing mutant strain *A. niger* NKUC$_N$-3.40. Oat spelt xylan was used as the substrate unless mentioned otherwise. Using these conditions, critical physicochemical and biological factors were analyzed for deciphering the most favorable conditions for achieving enhanced levels of enzymatic production.

4.3.1 Analysis of Enzyme Production as a Function of Different Carbon Sources

Among the various carbon substitutes analyzed, xylan followed by xylose were the most favorable substrates and had resulted into 584.2 and 480.6 IU ml^{-1} of xylanase respectively by mutant *A. niger* NKUC$_N$-3.40. Oat spelt xylan was preferred over birch wood xylan for the production of xylanase. These were followed by maltose and sucrose (Fig.12).

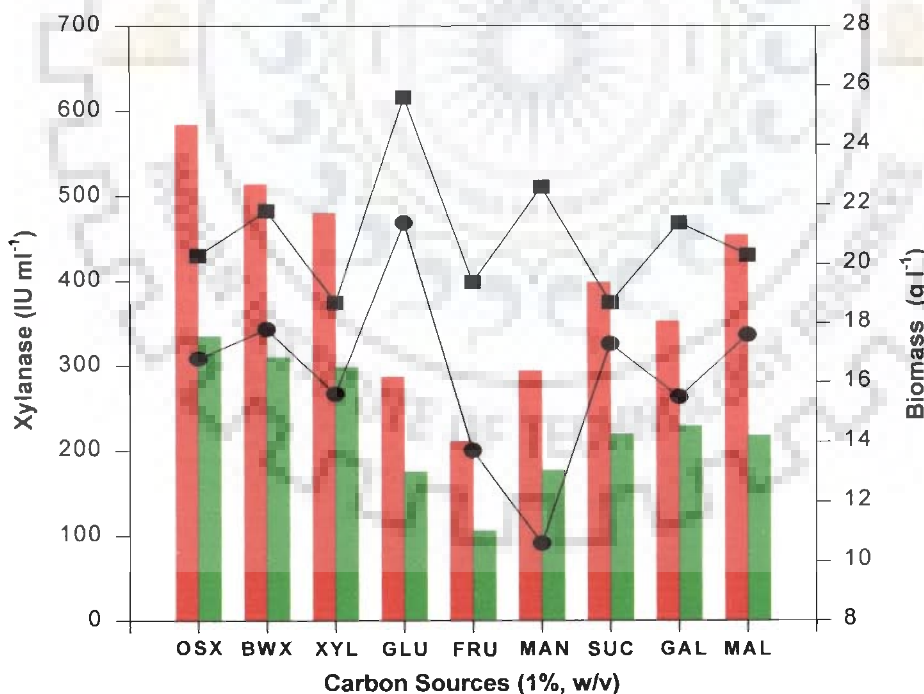


Fig. 12 Effect of different carbon sources on xylanase production

■, ■ ; represent xylanase production by mutant NKUC$_N$-3.40 and wild type NK-23 strains respectively

■, ● ; represent biomass generated by mutant NKUC$_N$-3.40 and wild type NK-23 strains respectively.

(OSX, Oat spelt xylan; BWX, Birch wood xylan; XYL, Xylose; GLU, Glucose; FRU, Fructose; MAN, Mannose; SUC, Sucrose; GAL, Galactose; MAL, Maltose)

4.3.2 Analysis of Enzyme Production as a Function of Substrate Concentration

Xylan at varying concentrations was used to define the suitable concentration of xylan for maximal production of xylanase levels in submerged fermentation after 3 days of incubation. As shown (Fig. 13), the rate of enzyme production and biomass varied remarkably for both mutant NKUC$_N$-3.40 and wild type NK-23 strains of *A.niger*. The xylanase production along with the β-xylosidase prodution was notably higher for mutant strain than the wild type. Maximum levels of production were obtained at 1% (w/v) concentration of xylan.

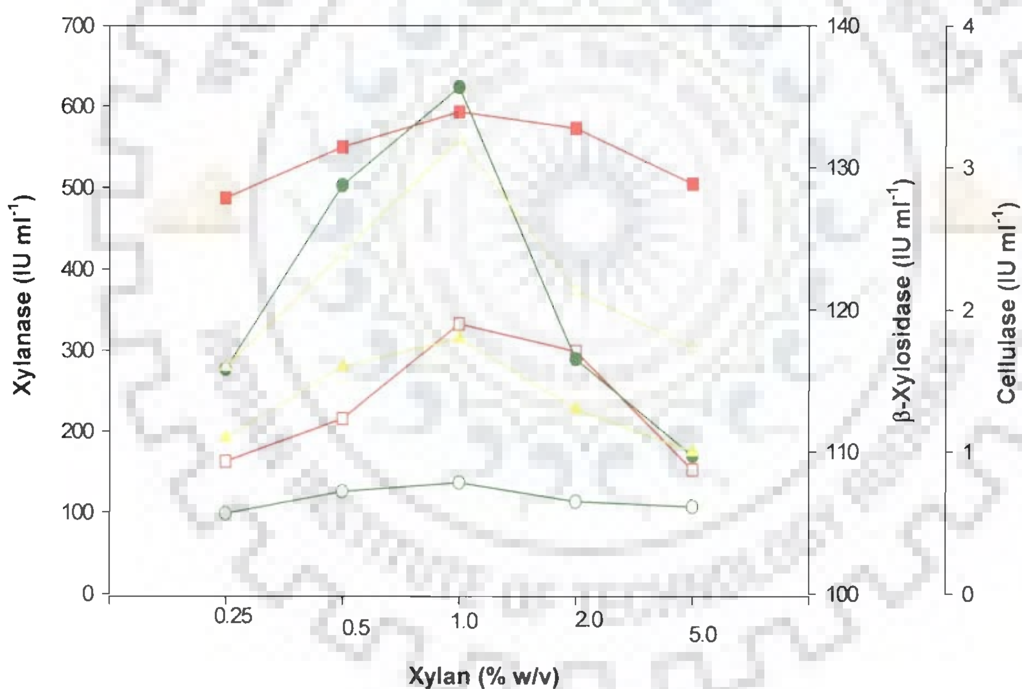


Fig. 13 Effect of different xylan concentrations on xylanase production
Xylanase (■, □), β-xylosidase (● , ○) and cellulase (▲ , △) production by mutant NKUC$_N$-3.40 and wild type NK-23 strains respectively

4.3.3 Analysis of enzyme production as a function of incubation period

The influence of fermentation time on enzymatic production in a batch process is depicted in fig. 14. Maximal xylanase production was observed on 3rd day of incubation, whereas β-xylosidase production increased to its maximum levels after 4

days of incubation. Interestingly higher levels of xylanase production sustained upto 7 days of incubation, whereas β-xylosidase levels dropped following 4 days of incubation.

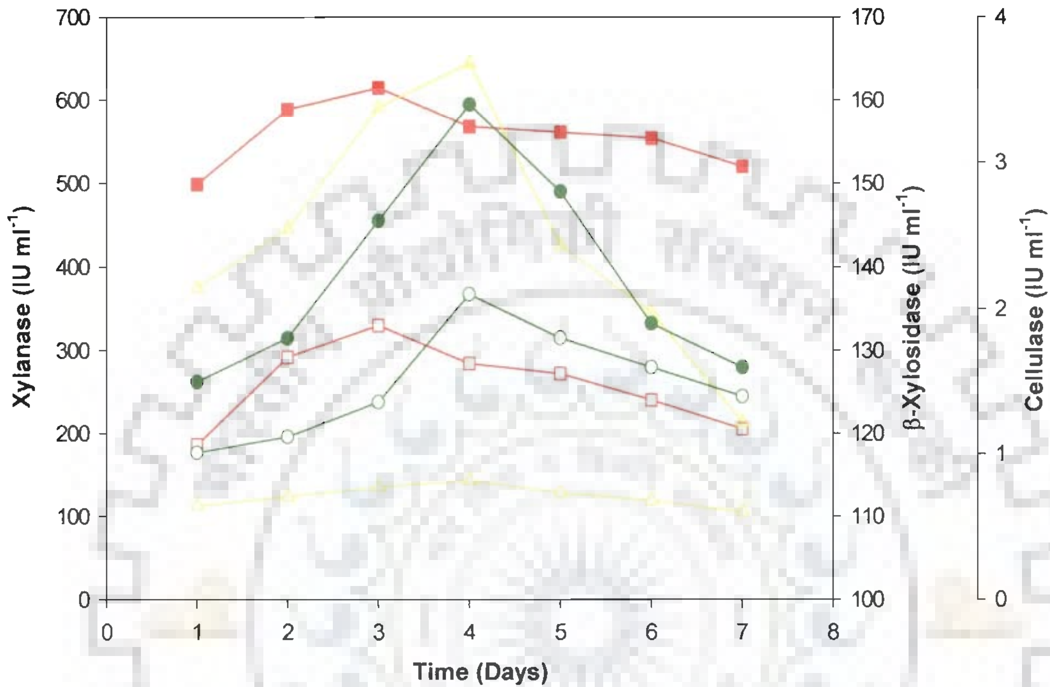


Fig. 14 Production of xylanase at different time intervals
Xylanase (■ , □), β-xylosidase (● , ○) and cellulase (▲ , △) production by mutant NKUC$_N$-3.40 and wild type NK-23 strains respectively

4.3.4 Analysis of enzyme production as a function of agitation, pH, temperature and inoculum level

Among the other major factors analyzed, an agitation at 120 rpm and a pH range of 6.5-7.5 was suitable for achieving the maximum xylanase and β-xylosidase production (Fig.15a, b). Among the factors analyzed, preferable temperature and inoculum level for maximal xylanase production were 30°C and 1-3% respectively for maximal enzyme production (Fig. 15c,d). Highest level of xylanase (584.6 IUml^{-1}) was obtained with 2% inoculum levels (10^7 spores ml^{-1}), higher levels of inoculum led to decreased level of production.

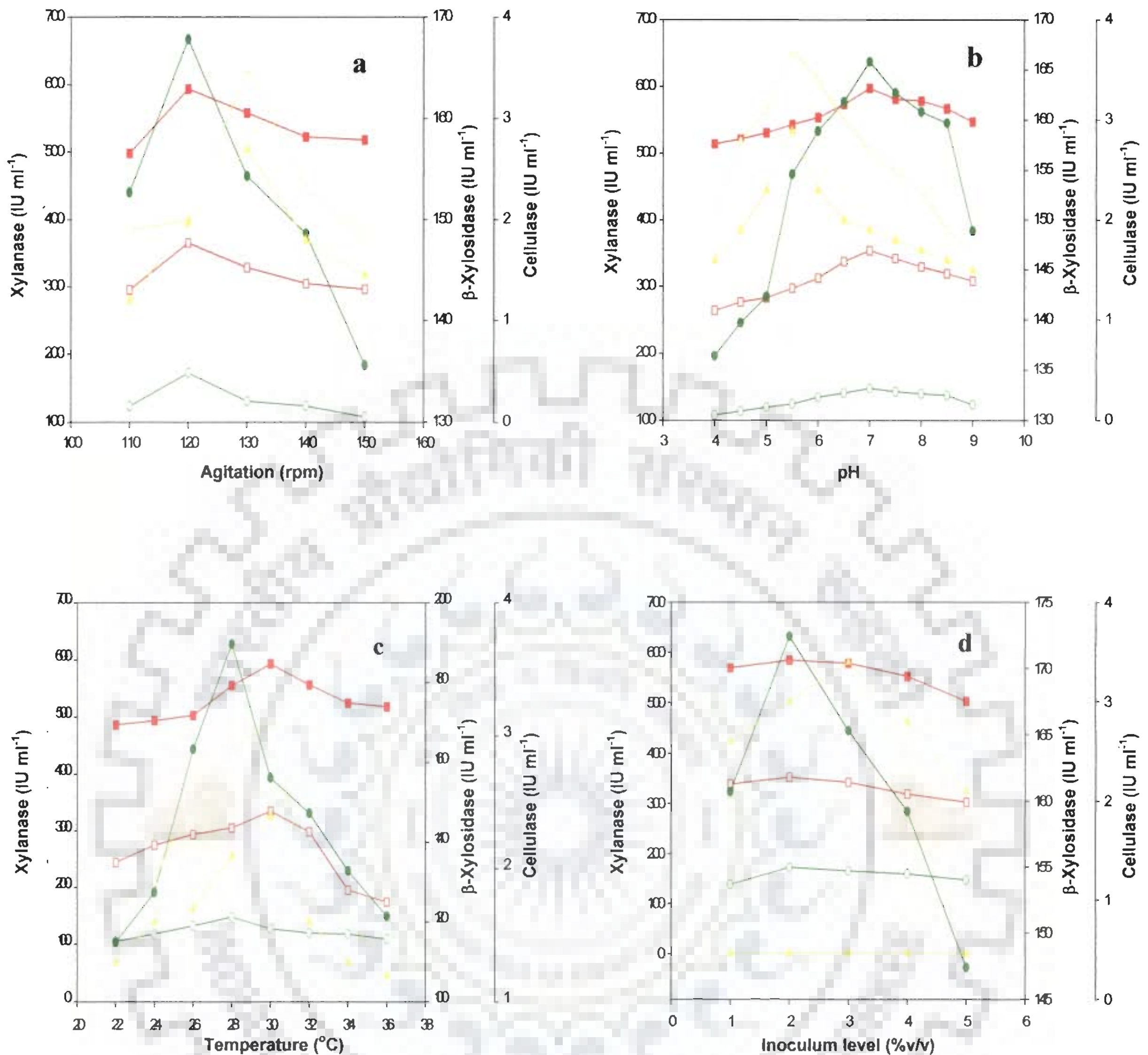


Fig. 15 Effect of agitation (a), pH (b), temperature (c) and inoculum level (d) on xylanase production
Xylanase (■ , □), β-xylosidase (● , ○) and cellulase (▲ , △)production by mutant NKUC$_N$-3.40 and wild type NK-23 strains respectively.

4.3.5 Analysis of Enzyme Production as a Function of Natural Substrate

On analyzing various natural substrates as the sources of carbon, the treated bagasse resulted into maximum production (719.6 IU ml^{-1}). The level of xylanase production increased further when oat spelt xylan was added along with bagasse in 1:1 ratio (935.2 IU ml^{-1}) (Fig.16).

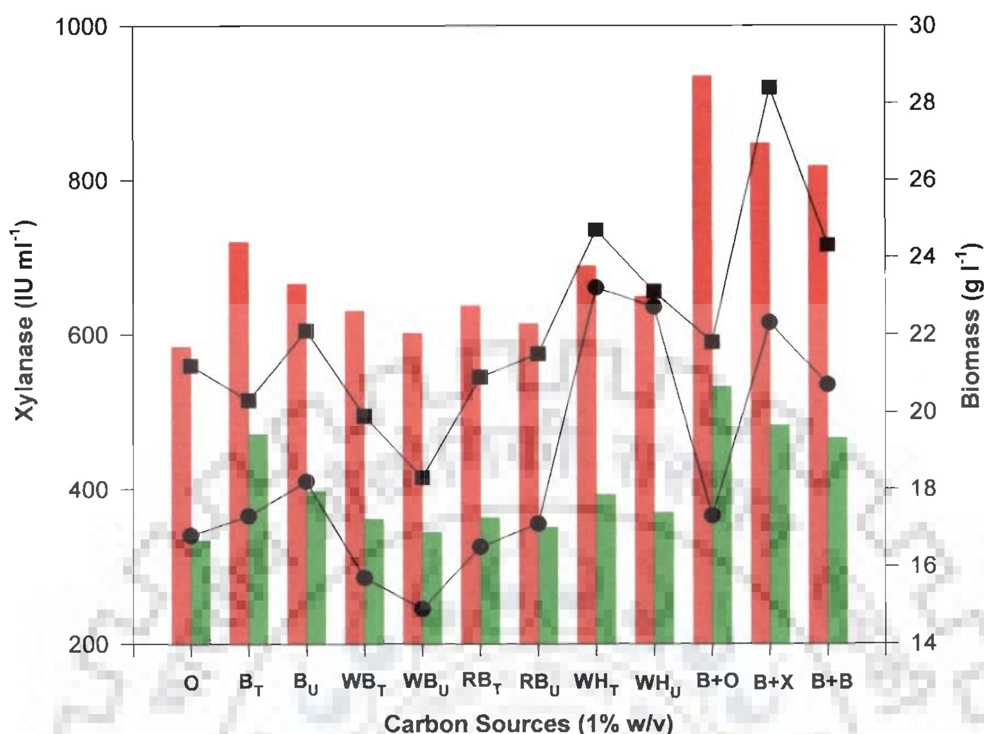


Fig. 16 Effect of different natural and synthetic carbon sources (1% w/v) on xylanase production
Xylanase (■ , ■) production and biomass generated (■, ●) by mutant $NKUC_N$-3.40 and wild type NK-23 strains.
(O, Oat spelt xylan; B_T, Treated bagasse; B_U, Untreated bagasse; WB_T, Treated wheat bran; WB_U, Untreated wheat bran; RB_T, Treated rice bran; RB_U, Untreated rice bran; WH_T, Treated wheat husk; WH_U, Untreated wheat husk; B+O, Bagasse +Oat spelt xylan; B+X, Bagasse + xylose; B+B, Bagasse +Birch wood xylan)

4.3.6 Analysis of Enzyme Production as a Function of Media Composition

On optimizing various media components maximum xylanase activity is obtained at 0.2% K_2HPO_4, 0.7% KH_2PO_4, 0.2% $MgSO_4$, 0.1% $(NH_4)_2SO_4$ and 0.06% Yeast extract (Fig. 17). Phosphate and magnesium are essential constituents regulating growth of the organism . Ammonium and yeast extract are sources of nitrogen and yeast extract in particular provides additional nutrients regulating growth and enzyme production. The various factors thus derived are defined in Table 9.

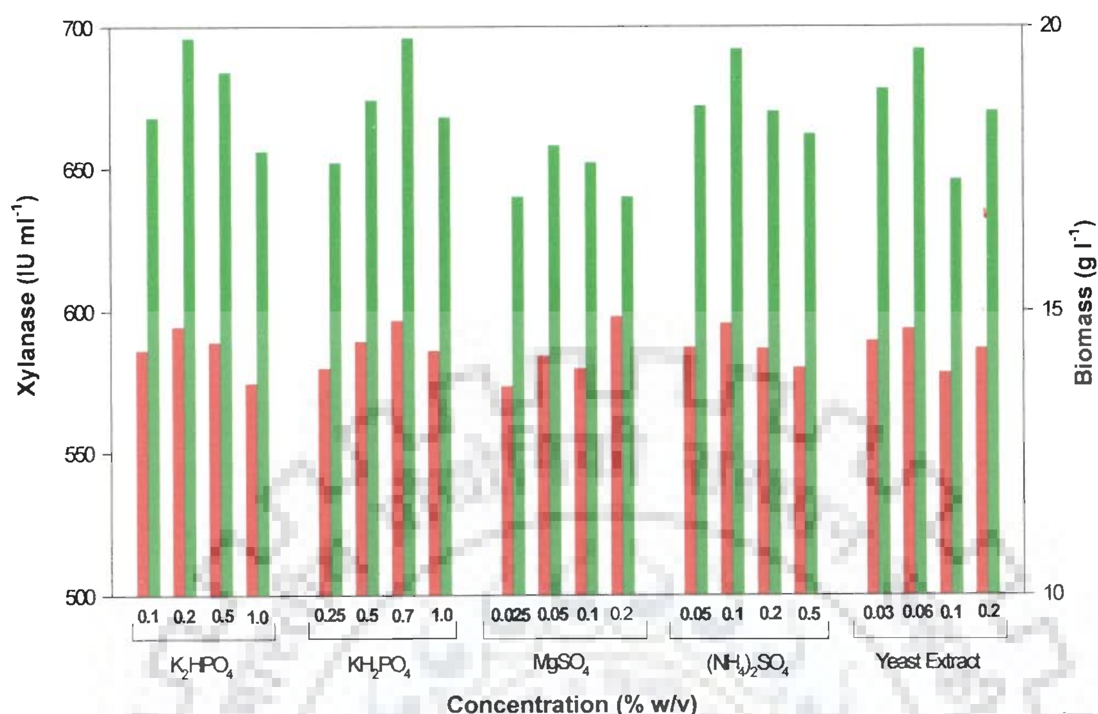


Fig. 17 Effect of varying concentrations of nutrients on xylanase production

Xylanase ( ) and biomass ( ) production by mutant *A.niger* $NKUC_N$-3.40

Table 9. Derivation of various factors for xylanase production by *Aspergillus niger* $NKUC_N$-3.40.

Parameters	Conditions/Factors
Physical Parameters	
(i) pH	7.0
(ii) Temperature	30°C
(iii) Agitation speed	120 rpm
Biological Parameters	
(i) Inoculum Level	2%
Chemical Parameters	
(i) Carbon source	Xylan (1% w/v)
(ii) Nitrogen source	$(NH_4)_2SO_4$ (0.1%) Yeast Extract (0.06%)
(iii) Phosphate source	KH_2PO_4 (0.7%) K_2HPO_4 (0.2%)
(iv) Magnesium source	$MgSO_4.7H_2O$ (0.2%)

Variations in the xylanase levels under submerged cultivation were

statistically analyzed and shown in Table 10.

Table 10. Analysis of variance for physico-chemical and biological factors for xylanase production by mutant *Aspergillus niger* NKUC$_N$-3.40.

Parameters	Source of Variation	Degree of Freedom	Sum of square	Mean square	F ratio observed
Carbon Substrate	Variation in xylanase production between sugar substrate	11	403913.9	36719.4	15.64**
	Variation in xylanase production within the sugar substrates	2	3152.3	1576.1	0.671
	Error	22	51634.60	2347.02	
Substrate Concentration	Variation in xylanase production between different sugar concentration	4	23936.67	5984.16	43.59*
	Variation in xylanase production within the sugar concentration	2	95.11	47.55	0.34
	Error	8	1098.20	137.27	
Temperature	Variation in xylanase production between different temperature	7	37189.51	5312.78	673.75*
	Variation in xylanase production within the temperature	3	388.30	129.43	16.41
	Error	21	165.59	7.88	
Time	Variation in xylanase production between different time	6	14135.6	2355.9	28.9**
	Variation in xylanase production within the time	2	501.6	250.8	3.08

	Error	12	976.57	81.38	
pH	Variation in xylanase production between different pH	10	21082.8	2108.2	21.24**
	Variation in xylanase production within the pH	2	886.85	443.42	4.46
	Error	20	1984.60	99.23	
Agitation	Variation in xylanase production between different agitation	4	5362.0	1340.5	33.33**
	Variation in xylanase production within the agitation	2	553.58	276.79	6.88
	Error	8	321.68	40.21	
Inoculum Level	Variation in xylanase production between different inoculum level	4	12847.7	3211.9	23.29*
	Variation in xylanase production within the inoculum level	2	99.85	49.92	0.36
	Error	8	1102.84	137.85	
K_2HPO_4	Variation in xylanase Concentration production Between different K_2HPO_4 concentration	3	594.33	198.11	5.75*
	Variation in xylanase production within the K_2HPO_4 concentration	2	197.58	98.79	2.86
	Error	6	206.53	34.42	
KH_2PO_4	Variation in xylanase concentration production between different KH_2PO_4 concentration	3	444.68	148.22	3.91

	Variation in xylanase production within the KH_2PO_4 concentration	2	60.62	30.31	0.80
	Error	6	227.24	37.87	
$MgSO_4$ Concentration	Variation in xylanase production between different $MgSO_4$ concentration	3	986.36	328.78	22.73*
	Variation in xylanase production within the $MgSO_4$ concentration	2	147.09	73.54	5.08
	Error	6	86.78	14.46	
$(NH_4)_2SO_4$ Concentration	Variation in xylanase production between different $(NH_4)_2SO_4$ concentration	3	377.0	125.66	1.96
	Variation in xylanase production within the $(NH_4)_2SO_4$ concentration	2	298.56	149.28	2.33
	Error	6	383.17	63.86	
Yeast Extract Concentration	Variation in xylanase production between different yeast extract concentration	3	391.22	130.40	2.45
	Variation in xylanase production within the yeast extract concentration	2	162.64	81.32	1.53
	Error	6	318.87	53.14	

* Significant at 1% ; ** Significant at 5%.

4.4 ANALYSIS OF FERMENTATION CONDITIONS FOR XYLANASE PRODUCTION

The fermentation medium as derived (Table 9) was used for defining the different fermentation conditions for xylanase production. Fermentation conditions were analyzed using mutant *A. niger* NKUC$_N$-3.40. Table 11 represents the comparative xylanase production under surface, submerged and solid state fermentation, the latter was found to result into maximum levels of production.

Table 11. Analysis of various fermentation conditions for xylanase production by mutant *A. niger* NKUC$_N$-3.40

Fermentation Condition	Xylanase
Surface fermentation	406 ± 8.24 (IU ml^{-1})
Submerged Fermentation	935.8 ± 7.33 (IU ml^{-1})
Solid state Fermentation (Bagasse as solid support)	1337.33 ± 18.18 (IU g^{-1})

4.5 ANALYSIS OF SOLID-STATE FERMENTATION CONDITIONS FOR XYLANASE PRODUCTION

Solid-state fermentation led to comparatively higher levels of the xylanase production. Thus in order to achieve further higher levels of production and to economize the process, solid-state fermentation was performed using a range of solid supports of agricultural origin as well as with other synthetic inert support materials (Fig.18).

Fig.18 Different solid supports used during solid-state fermentation

(a), Poly Urethane Sponge; (b), Glass Beads; (c), Loofa Sponge; (d), Wheat Re
(e), Oil Cake; (f), Coconut Coir; (g), Bagasse; (h), Cotton Hull; (i), Rice Bran;
(j), Wheat Bran

The majority of support materials chosen for solid-state fermentation are agro-residues that are abundantly and easily available either at considerably cheaper prices or for free. Various agro-residues used for xylanase productions are listed in Table 12. All the solid supports promoted the growth and enzyme production by the mutant strain *A. niger* NKUC$_N$-3.40, but cotton hull among all solid supports led to maximum levels of production. The order of the substrate suitability was Cotton Hull> Bagasse >Rice bran> Wheat Residue> Wheat Bran > Coconut Coir> Loofa Sponge> Oil Cake> Polyurethane Sponge> Glass Beads. Hence, Cotton hull was selected for the further studies.

Table 12. Effect of different solid supports on xylanase production in solid state fermentation

S. No.	Solid supports	Xylanase (IU g^{-1}) ±S.D.
1.	Rice Bran	1218.33 ± 13.65
2.	Wheat Bran	982.66 ± 10.06
3.	Wheat Residue	1042.66 ± 7.76
4.	Bagasse	1337.33 ± 18.18
5.	Coconut Coir	871.66 ± 15.94
6.	Loofa Sponge	843.00 ± 28.67
7.	Oil Cake	679.75 ± 18.24
8.	Cotton Hull	1705.67 ± 1.88
9.	Glass Beads	613.25 ± 10.14
10.	Poly Urethane Sponge	653.00 ± 31.14

4.5.1 Analysis of the Amount of Substrate on Xylanase Production Under Solid-state Fermentation

Amount of substrate is of prime importance and 4g of substrate was found to be the most suitable for the production of xylanase (1704 IUg^{-1}) and β-xylosidase (465 IUg^{-1}) as shown in fig. 19. Cellulase production observed was lower under these conditions.

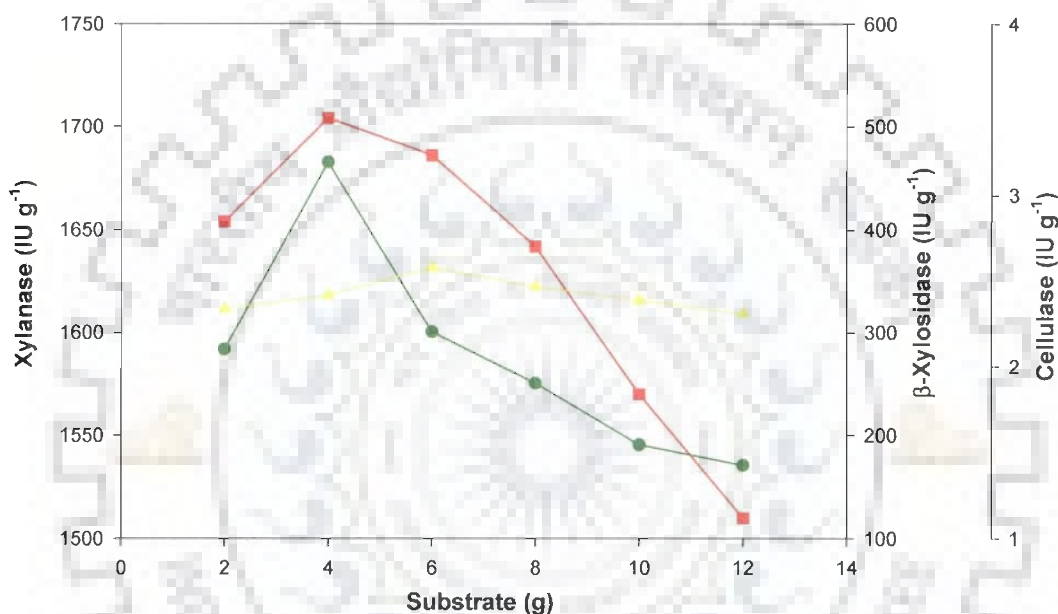


Fig. 19 Effcet of the amount of cotton hull on xylanase production under solid-state fermentation by *A. niger* NKUC$_N$-3.40

■, ●, ▲ represent xylanase, β-xylosidase and cellulase production respectively

4.5.2 Analysis of Temperature and Inoculum Level on Xylanase Production Under Solid-state Fermentation

Temperature regulation is one of the crucial factors for solid-state fermentation. Stoichiometric global equation and respiration is highly exothermic and heat generation was due to higher levels of fungal activity within the solids leads to thermal gradients due to the limited heat transfer in the solid substrates. The thermal gradation can be controlled by maintaining the desired level of humidity. The most suitable temperature was found to be 34°C for the xylanase production (1679 IU g^{-1})

as shown in fig. 20a. However the maximum level of β-xylosidase production (456.4 IU g^{-1}) was observed at 32°C.

Maximum xylanase production (1683 IU g^{-1}) was found when 4% inoculum was used (Fig. 20b). 3% and 5% inoculum was also favorable for the xylanase production. Higher inoculum density resulted into more biomass production and effected into nutrient scarcity and decreased enzyme production.

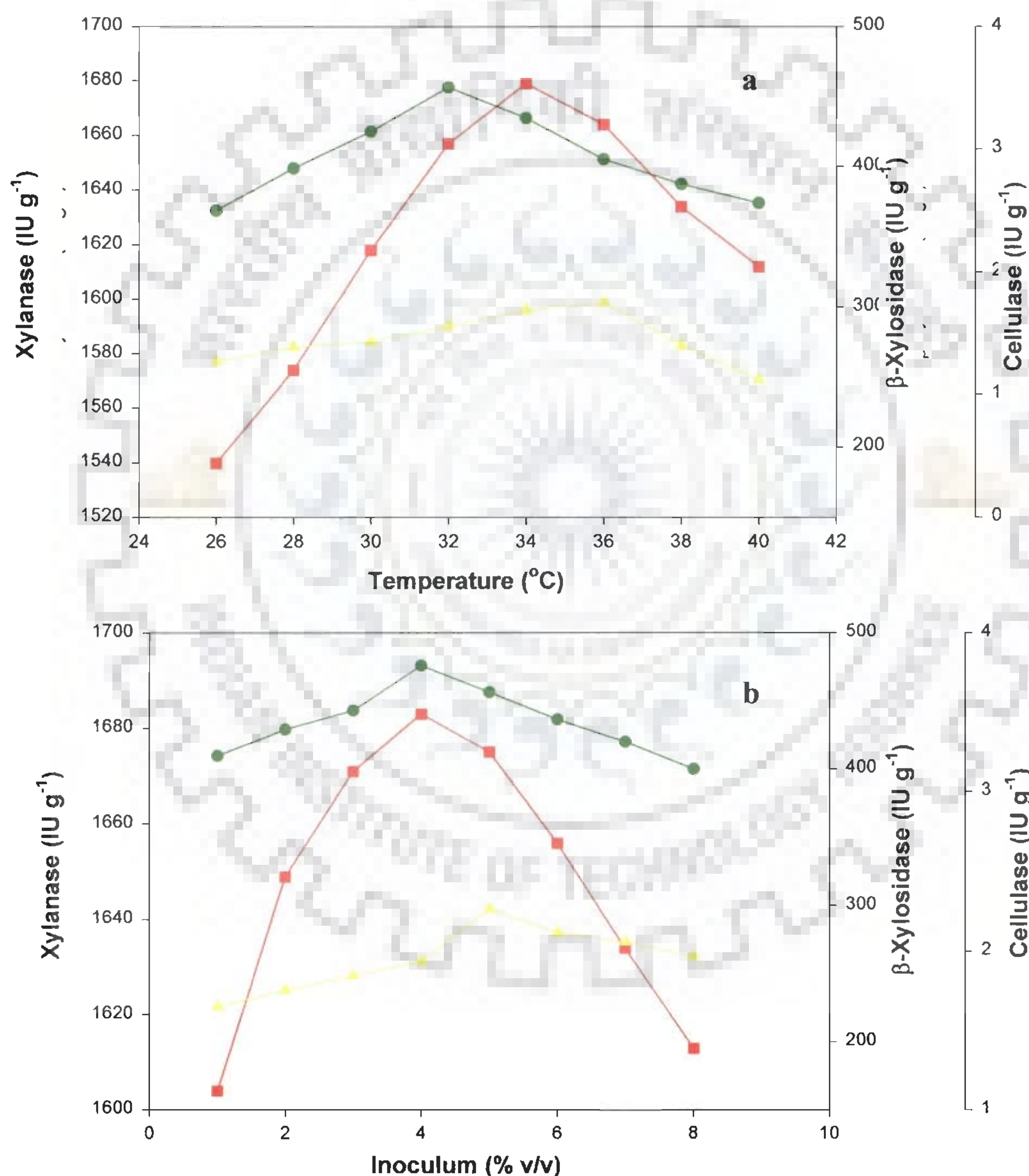


Fig. 20 Effect of (a), temperature and (b), inoculum level on xylanase production under solid-state fermentation by *A. niger* NKUC$_N$-3.40

■, ●, ▲ represent xylanase, β-xylosidase and cellulase production respectively

4.5.3 Analysis of xylanase production at different time interval under solid-state fermentation

A notable level of xylanase production by NKUC$_N$-3.40 during solid-state fermentation using cotton hull was achieved during 3-5 days of incubation and maximum levels of production occurred on 4th day of incubation. Levels of xylanase dropped thereafter (Fig. 21).

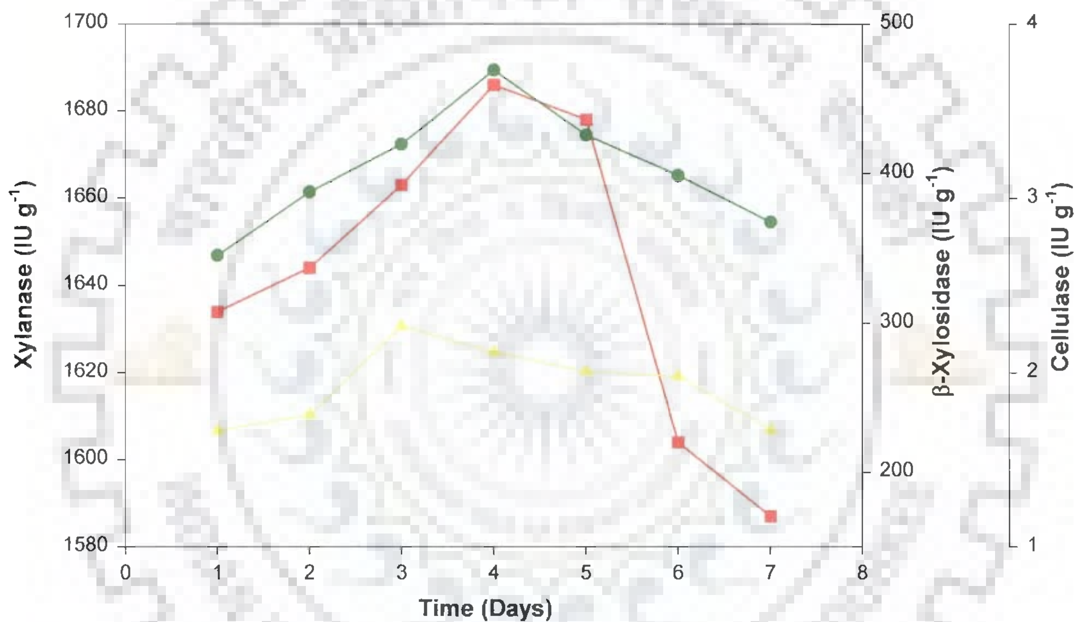


Fig. 21 Effect of the incubation time on xylanase production under solid-state fermentation by *A. niger* NKUC$_N$-3.40

■, ●, ▲ represent xylanase, β-xylosidase and cellulase production respectively

4.5.4 Analysis of the Moisture Level on Xylanase Production Under Solid-state Fermentation

Solid-state fermentation is distinguished from the submerged fermentation by the fact that microbial growth and product formation occurs at low moisture content. Fig. 22 represents xylanase production at different moisture levels. Low moisture content causes reduction in solubility of nutrients and hence decrease the xylanase

production as represented in fig. 22; while, high moisture content can cause reduction in the enzyme yield due to steric hindrance of the growth of the microorganism by reducing the porosity of solid support and interfering with the oxygen transfer for the fungus entrapped in the hull. A moisture content of 70% was observed suitable for maximal enzyme production.

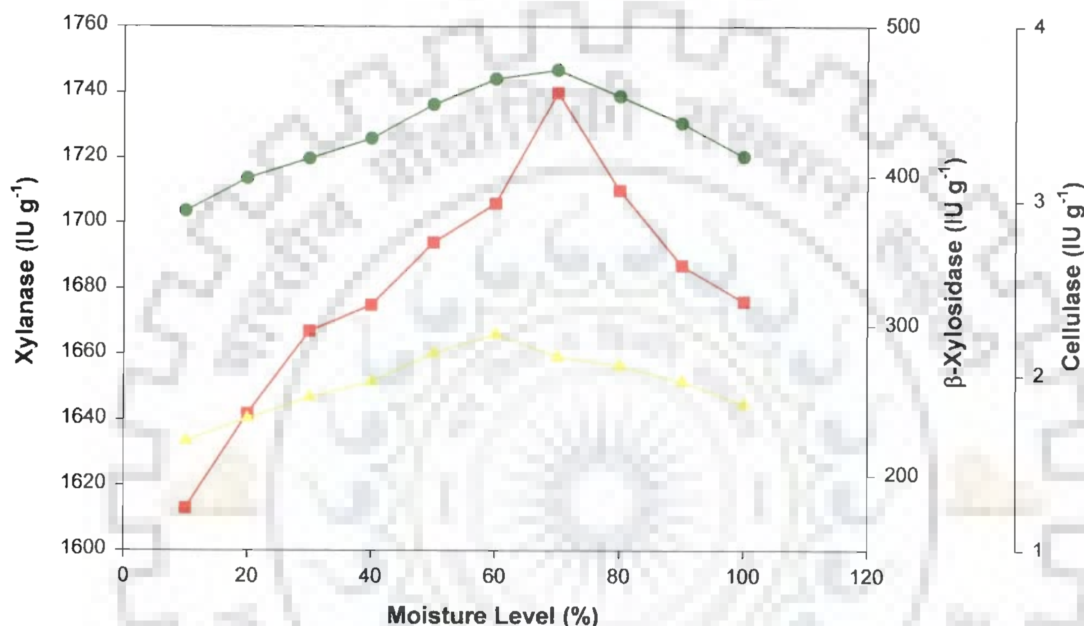


Fig. 22 Effect of the moisture level (%) on xylanase production under solid-state fermentation by *A. niger* NKUC$_N$-3.40

■, ●, ▲ represent xylanase, β-xylosidase and cellulase production respectively

4.5.5 Analysis of Additives on Xylanase Production Under Solid-state Fermentation

The various additives were analyzed to detect their effect on xylanase production. Among the various additives used mustard oil cake was found to be the best inducer for xylanase production (1783 IU g^{-1}) (Fig. 23). Corn steep liquor and yeast extract also led to an increase in xylanase production.

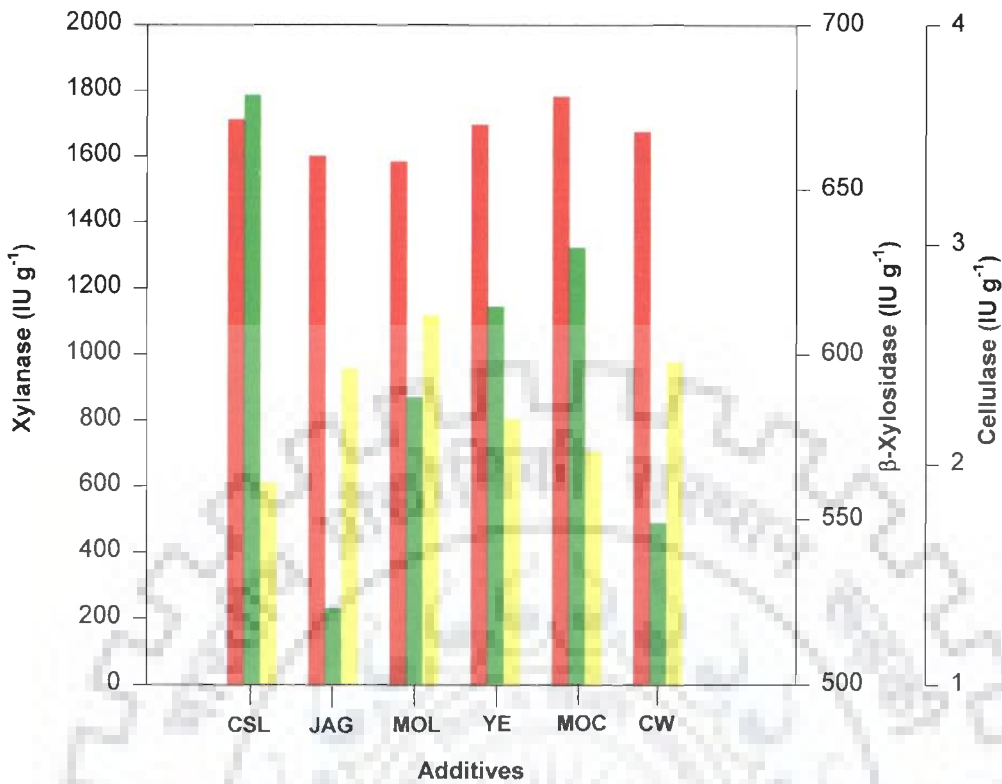


Fig. 23 Effect of the additives (5% w/w) on enzyme production under solid-state fermentation by *A. niger* NKUC$_N$-3.40

■, ■, ■ represent xylanase, β-xylosidase and cellulase produced respectively (CSL: Corn Steep Liquor; JAG: Jaggery; MOL: Molasses; YE: Yeast Extract; MOC: Mustard Oil Cake; CW: Cheese Whey)

The various factors thus derived under solid-state fermentation are defined in Table 13.

Table 13. Derivation of various factors for xylanase production by *Aspergillus niger* NKUC$_N$-3.40 under solid-state fermentation

Parameters		Conditions/Factors
Physical/Biological Parameters		
(i)	Temperature	34°C
(ii)	Incubation Duration	4 days
(iii)	Inoculum Level	4%
(iv)	Moisture content	70%
Chemical Parameters		
(v)	Solid support	Cotton hull
(vi)	Additive	Mustard oil cake, 5% w/w
(vii)	Medium components	As derived earlier*

*other components of fermentation medium were as described in Table 9

Variations in the xylanse levels under solid-state fermentation process were studied by the statistical analysis and results of ANOVA are given (Table 14).

Table 14. Analysis of variance for different physico-chemical and biological factors for xylanase production by mutant *Aspergillus niger* NKUC$_N$-3.40 under solid-state fermentation using cotton hull as solid support.

Parameters	Source of Variation	Degree of Freedom	Sum of square	Mean square	F ratio observed
Substrate Concentration	Variation in xylanase production between solid substrate	5	81898	16379.6	1038.87*
	Variation in xylanase production within the sugar substrates	2	770.3	365.16	23.16
	Error	10	157.66	15.76	
Time	Variation in xylanase production between different time	6	24628.28	4104.71	114.55**
	Variation in xylanase production within the time	2	342	171	4.77
	Error	12	430	35.83	
Temperature	Variation in xylanase production between different temperature	7	46576.5	6653.78	212.5**
	Variation in xylanase production within the temperature	2	379.75	189.87	6.06
	Error	14	438.25	31.30	

Inoculum Level	Variation in xylanase production between different inoculum level	7	17714.6	2530.6	12.52**
	Variation in xylanase production within the inoculum level	2	180.7	90.37	0.44
	Error	14	2829.25	202.08	
Moisture Level	Variation in xylanase production between different moisture level	9	34662	3851.3	38.90**
	Variation in xylanase production within the moisture level	2	456.20	228.10	2.30
	Error	18	1781.8	98.98	
Additives	Variation in xylanase production between different additives	5	80956.5	6191.3	352.75**
	Variation in xylanase production within the additives	2	181	90.5	1.97
	Error	10	459	45.9	

*Significant at 1%; ** Significant at 5%

4.6 BIOCHEMICAL CHARACTERIZATION OF XYLANASE PRODUCED BY *ASPERGILLUS NIGER* NKUC$_N$-3.40 UNDER SOLID STATE FERMENTATION

4.6.1 Temperature and pH stability of the xylanase

The broth containing enzyme was kept for 2 hours at various temperatures and enzyme activity thereafter was determined. Xylanase appeared to be quite stable upto

a temperature of 50°C (Fig. 24a). Increase in temperature up to 65°C led to a 50 % loss in activity.

Crude xylanase was found to be stable between the pH of 7.0-9.0 and retained upto 85% of the relative activity of xylanase (Fig. 24b) Higher and lower pH decreased the enzyme levels.

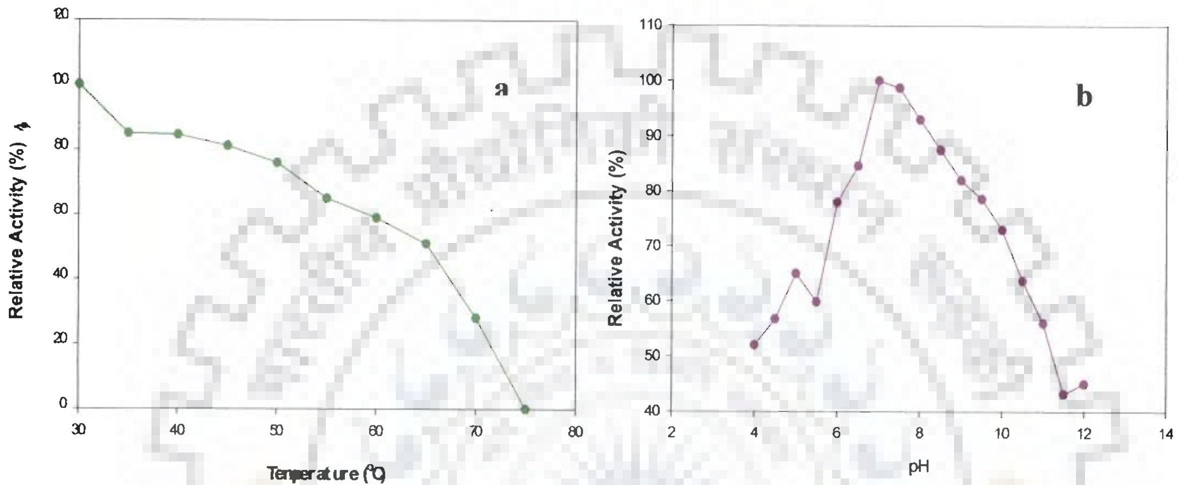
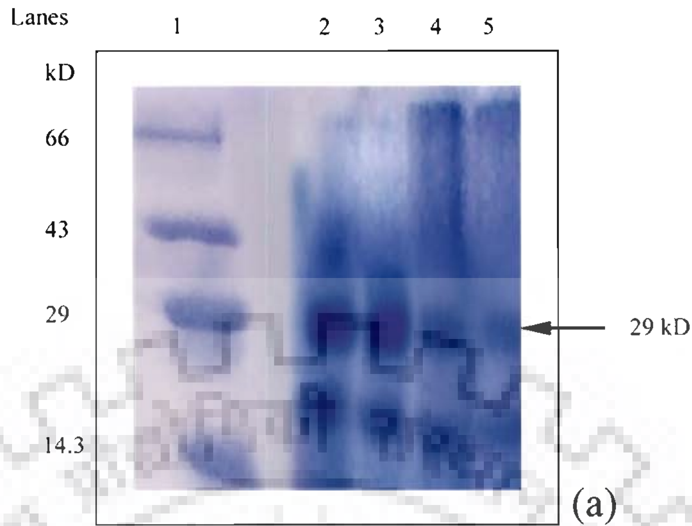


Fig. 24 Temperature (a) and pH (b) stability of the crude xylanase obtained from *A. niger* NKUC$_N$-3.40

4.6.2 SDS-PAGE and Zymogram Analysis

The protein profile of the fermentation broth was analyzed using mutant NKUC$_N$-3.40 and compared with that of wild type NK-23. The SDS-polyacrylamide gel analysis had shown increased levels of 29 kD protein (Fig. 25a) from mutant NKUC$_N$-3.40 as compared to the similar bands from wild type NK-23.

Fermentation broth was subjected for ammonium sulphate precipitation followed by dialysis as described in methods. The zymogram analysis of the partially purified xylanase had shown high level of enzyme activity from mutant NKUC$_N$-3.40 compared to the NK-23 strains. Molecular weight analysis of the respective bands had indicated the xylanase having a molecular weight of 29 kD (Fig.25b).



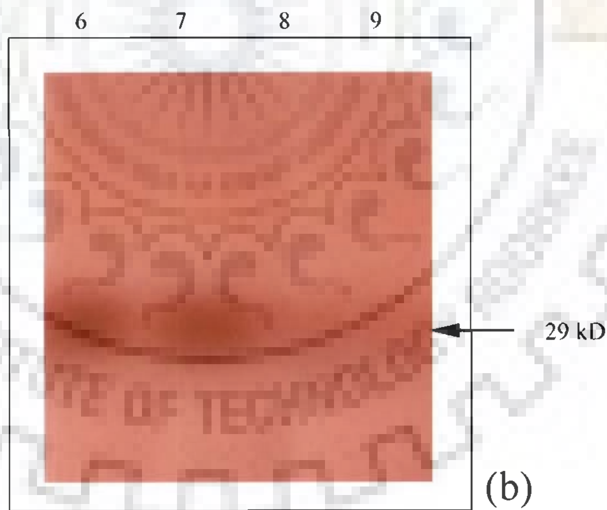


Fig. 25 SDS-PAGE (a) and Zymogram (b) analysis of proteins produced by mutant *Aspergillus niger* NKUCN-3.40 and wild type NK-23 after 4 days of incubation

Lane 1, Marker proteins; Lane 2-3, Protein profile of mutant *Aspergillus niger* NKUC
Lane 4-5,Proteins profile of wild type NK-23; Lane 6-7, Zymogram analysis of mutar
Aspergillus niger NKUCN-3.40;Lane 8-9, Zymogram analysis of wild type NK-23

4.7 TRAY BIOREACTOR FOR XYLANASE PRODUCTION

Comparison of the enzyme production in submerged and solid state fermentation had indicated later system yielding higher levels of xylanases. Thus, a tray bioreactor was designed as shown in fig. 27 for the semi-continuous production of the enzyme. In this bioreactor, inoculum was added along with the fermentation medium from the inoculum and media tanks respectively. After Ist cycle the fungal biomass generated was utilized for enzyme production in the subsequent cycles. At the end of each fermentation cycle the broth was collected in recovery unit and recovery was facilitated by applying vacuum as shown in fig. 27. Humidifier tray contained autoclaved water for maintaing the desired humidity. Sterilized air was passed through air pump for proper aeration.

Enzymatic activities from the culture filtrates of the various fermentation cycle were detected. Significant levels of xylanase were produced during I–III fermentation cycles. Enzyme production in the IVth and subsequent cycles decreased due to the overgrowth and thus higher biomass generated during the cycles (Fig. 26).

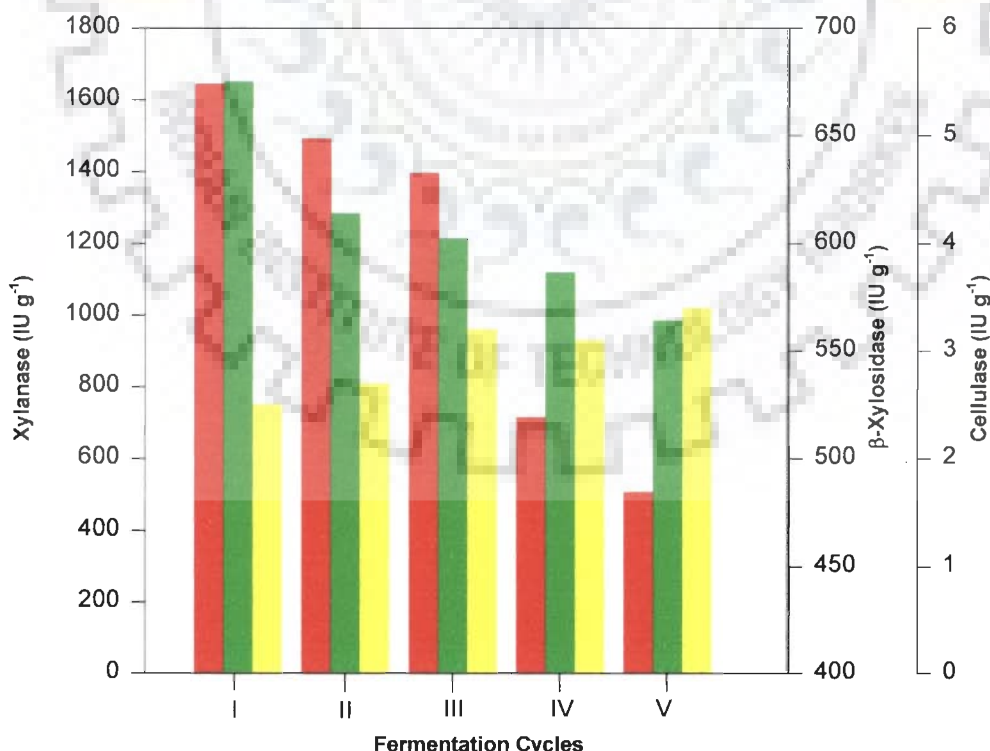


Fig. 26 Enzyme production during various cycles of fermentation in tray bioreactor

■, ■, ■ represent xylanase, β-xylosidase, cellulase produced respectively.

Fig. 27 Tray bioreactor for semi-continuous production of xylanase using cotton hull as solid support

(a), Exterior view; (b), Interior view

Morphological features of cotton hull through scanning electron microscopy is shown in fig.28. Uninoculated cotton hull (fig. 28a) was found to be porous and hence appeared as the better substratum for fungus to adhere and penetrate. Cotton hull after inoculation (fig. 28b) heavy colonization by *A. niger* NKUC$_N$-3.40. Thus, cotton hull appeared to be the suitable matrix for high metabolic activity of fungus and thus lead to increase in the enzyme secretion as shown in fig. 26.

4.8 PULPING OF WOOD FIBRES

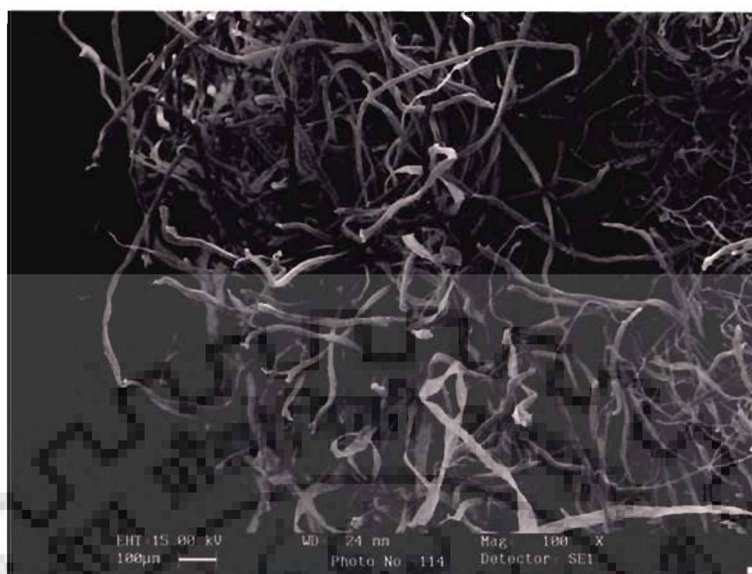
4.8.1 Pulping of Mixed Wood

Mixed wood was subjected to pulping by cooking the wood chips using different concentrations of active alkali (Na_2O). Table 15 shows the results of kraft pulping of mixed wood chips of *Populas deltoidea, Eucalyptus tetrecornis, Bambusa aurandacea and Pinus roxumberghii* (71:13:10:2). An optimum screened pulp yield of 47.90% of kappa number 19.6 (Fig 29) was obtained at active alkali 16%, sulphidity 20%, maximum pulping at 165°C for 75 minutes, digester pressure 6.5kg cm^{-2} and at a bath ratio of 1:3.

Table 15. Cooking conditions and results of kraft pulping of mixed chips of *Populas deltoidea*, *Eucalyptus tetrecornis*, *Bambusa aurandacea and Pinus roxumberghii* in ratio of 71:13:10:2

Particulars	Active alkali, % (as Na_2O)			
	14	15	16	17
Screened yield (%)	41.50	44.65	47.90	46.50
Screen rejects (%)	9.10	5.50	1.85	0.65
Unscreened yield (%)	50.60	50.15	49.75	47.15
Kappa number	28.4	24.5	19.6	18.6
Residual alkali	1.57	1.24	0.23	0.11

Cooking parameters: Time from room temperature to 105°C = 60 min, time from 105 to 165°C = 60 min, time at 165°C = 75 min, sulphidity, % = 20.4, bath ratio =1:3, digester pressure = 6.5 kgcm^{-2}



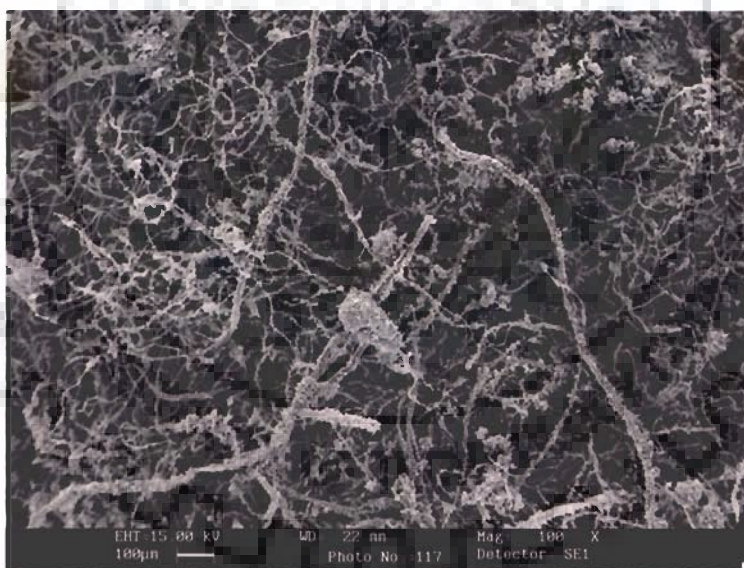


Fig. 28 Scanning electron micrograph of the *A. niger* NKUC$_N$-3.40 under solid state fermentation

(a), Uninoculated cotton hull (100x); (b), Inoculated cotton hull (100x)

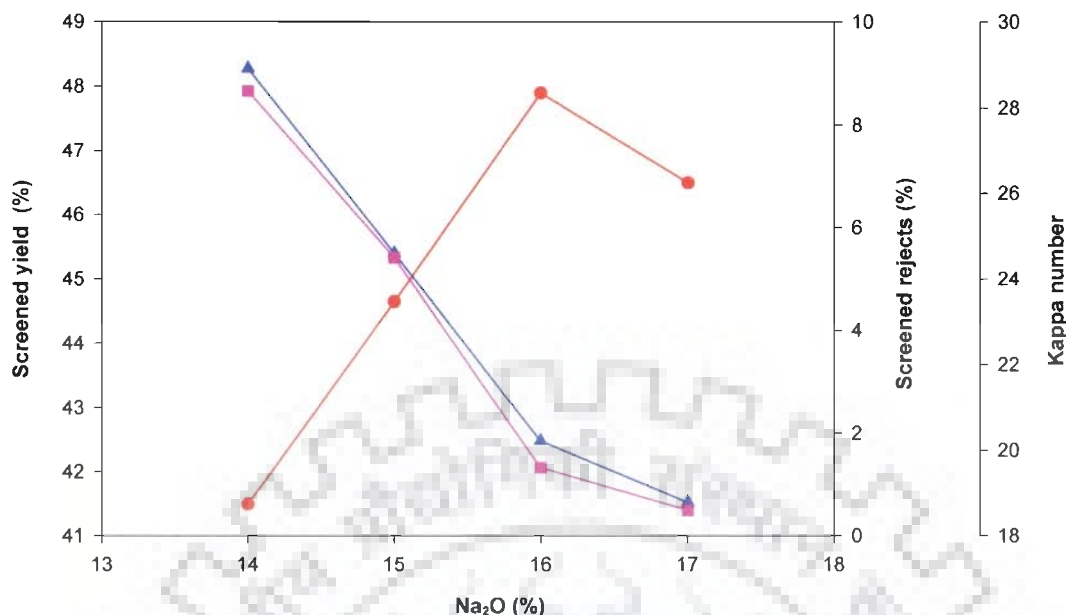


Fig. 29 Kraft pulping of mixed wood chips (*Populas deltoidea, Eucalyptus tetrecornis, Bambusa aurandacea and Pinus roxumberghii*)

●, ■, ▲ represent screened yield, screen rejects (%) and kappa number respectively.

4.8.2 Pulping of Wheat Straw Pulp

Pulping of wheat straw was done by cooking wheat straw with different concentration of active alkali (Na_2O). For cooking the wheat straw, 12% active alkali was found to be better and led to a notable reduction of the kappa number (Fig. 30) and had resulted into achieving maximum screened yield (Table 16).

Table 16. Cooking conditions and results of soda pulping of wheat straw

Particulars	Active alkali, % (as Na_2O)			
	11	**12**	**13**	**14**
Screened yield (%)	44.41	46.23	45.86	44.35
Screen rejects (%)	5.51	2.3	6.85	7.65
Unscreened yield (%)	49.92	48.53	52.71	52.0
Kappa number	38.8	34.3	33.1	32.8
Residual alkali	0.45	0.23	0.12	0.10

Cooking parameters: Time from room temperature to 105°C = 45 min, time from 105 to 165°C = 45 min, time at 165°C = 120 min, bath ratio =1:5, digester pressure = 6.5 kg cm^{-2}

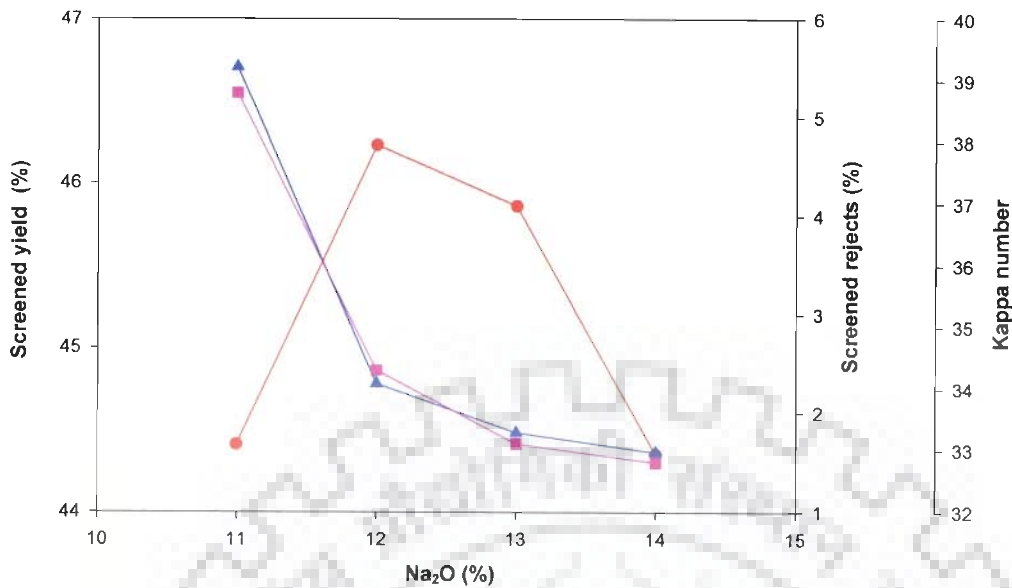


Fig. 30 Soda pulping of wheat straw

●, ■, ▲ represent screened yield, screen rejects (%) and kappa number respectively.

4.9 DERIVATION OF REACTION CONDITIONS FOR ENZYMATIC PRE-TREATMENT

4.9.1 Enzyme dosage

Wheat straw and mixed wood pulp was treated with different dosage of enzyme as shown in Fig. 31. 20 IU of xylanase per gm of oven-dried pulp was found to be optimal dosage and resulted into decreased kappa number of wheat straw pulp (30.8) and mixed wood pulp (16.4).

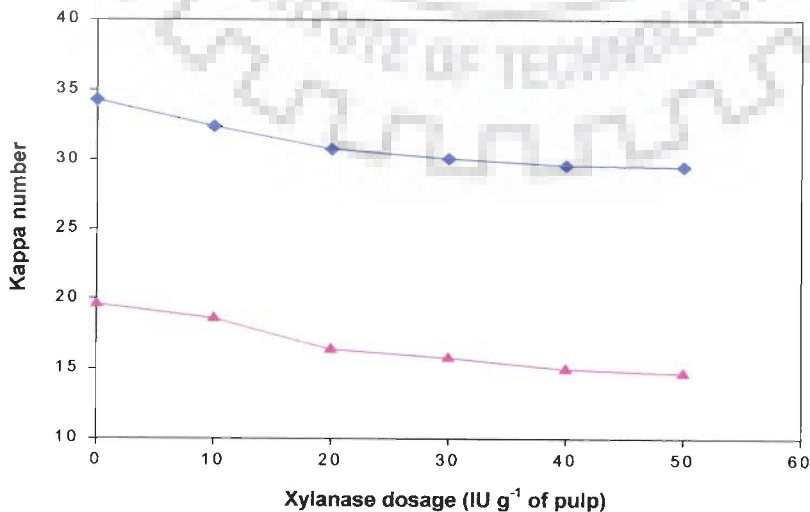


Fig. 31 Derivation of dosage for enzyme treatment of the pulp

◆ , Wheat straw pulp; ▲ , Mixed wood pulp

4.9.2 Reaction time

120 minutes of treatment time was found to be effective when 20 IU of xylanase per gm of oven dried pulp was used for both wheat straw and mixed wood pulp. Further increase in treatment period caused almost no reduction of kappa number (Fig.32).

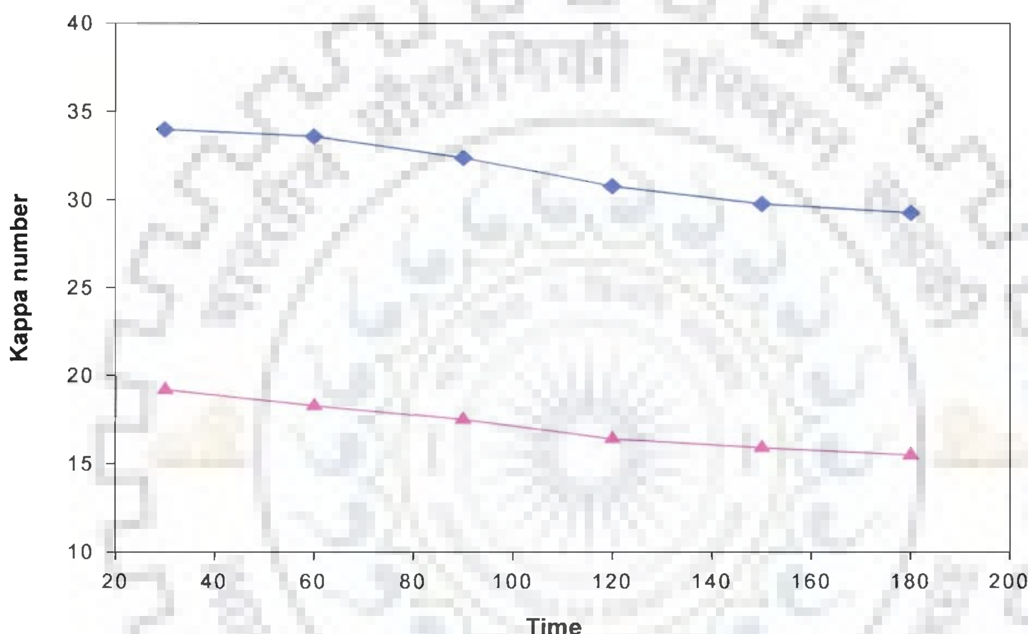


FIG. 32 Derivation of reaction time for the enzyme treatment of the pulp

◆ ,Wheat straw pulp; ▲, Mixed wood pulp

4.9.3 pH

pH is a critical parameter for the enzymatic pre-bleaching. Maximum release of lignin was accomplished at pH 8.0 that was reflected by the reduction in the kappa number (Fig. 33).

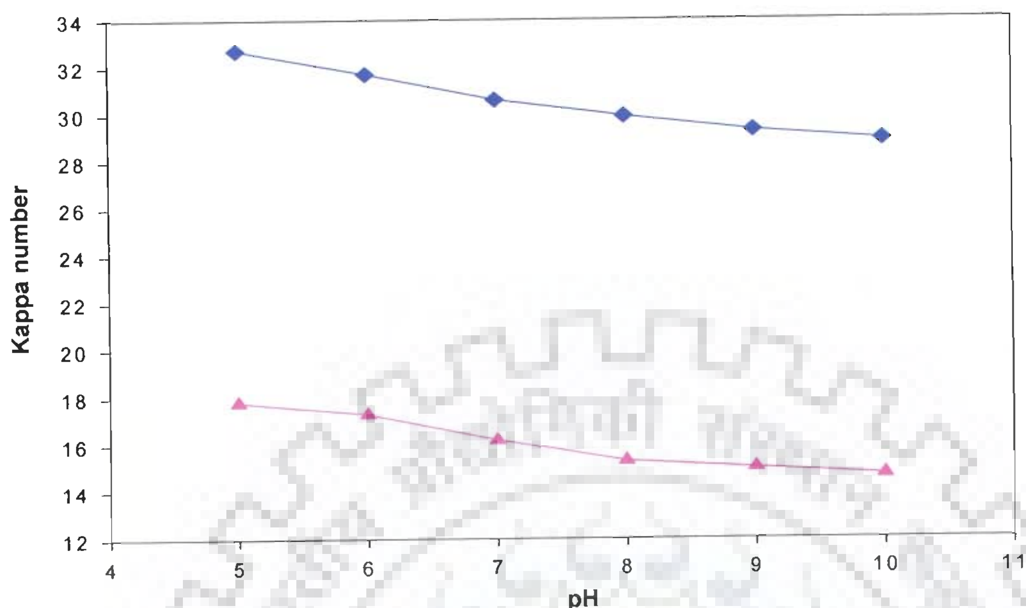


Fig. 33 Optimization of pH of the reaction mixture for the enzyme treatment of the pulp

◆ , Wheat straw pulp; ▲ , Mixed wood pulp

4.10 ANALYSIS OF PULP PROPERTIES

Decrease in the kappa number by enzymatic pre-treatment of pulp results into decrease in the Cl_2 consumption. The bleaching sequence CEHH required more chemical (Cl_2) consumption as compared to the XCEHH sequence as shown in Table 17. Hence, enzymatic pre-treatment would lead into reduced chemical load in pulp and paper manufacturing. Enzymatic pre-treatment also enhanced the pulp brightness to 79.6% (ISO) in case of wheat straw pulp and 80.8% (ISO) in case of mixed wood pulp in comparison to untreated wheat straw pulp (78.6%) and untreated mixed wood pulp (79.6%).. Total pulp yield was also observed to be higher with XCEHH bleaching in comparison to the CEHH based bleaching sequence.

Table 17. CEHH and XCEHH bleaching of wheat straw and mixed wood pulps

S.No.	Particulars	Wheat straw pulp		Mixed wood pulp	
1.	Initial kappa number	34.3	34.3	19.6	19.6
2.	**Xylanase (X) stage**				
	Dosage (IU g^{-1})	0	20	0	20
	Initial pH	7.0	7.0	7.0	7.0
	Kappa number	34.3	30.6	19.6	16.2
3.	**Chlorination (C) stage**				
	Cl_2 applied as active chlorine on pulp basis(%)	4.3	3.8	2.5	2.0
	Final pH	2.5	2.4	2.4	2.4
4.	**Extraction (E) stage**				
	NaOH applied on pulp basis (%)	2.2	2.0	1.3	1.1
	Initial pH	11.6	11.2	11.1	11.1
	Final pH	9.6	9.7	9.4	9.4
5.	**H_1 stage**				
	$Ca(OCl)_2$ added as active chlorine on pulp basis(%)	3.0	2.7	1.7	1.5
	Initial pH	11.6	11.5	11.5	11.6
	Final pH	10.0	9.6	9.6	9.6
6.	H_2 stage				
	$Ca(OCl)_2$ added as active chlorine on pulp basis(%)	1.3	1.1	0.9	0.5
	Initial pH	11.7	11.4	11.4	11.5
	Final pH	10.2	9.8	9.6	9.6
7.	Bleaching losses (%)	10.2	9.4	9.2	8.5
8.	Total pulp yield (%)	41.53	41.89	44.0	44.4
9.	Pulp brightness (%, ISO)	78.8	79.6	79.6	80.8
10.	Post colour number	2.7	1.9	1.6	1.2
11.	Pulp viscosity, cps (cupramonium)	7.02	7.56	10.8	13.5
12.	BOD_{5days} (mg l^{-1})	225	195	145	72
13.	COD (mg l^{-1})	625	510	385	255
14.	AOX (kg t^{-1}) of pulp	5.4	4.2	3.8	2.6

Bleaching conditions	**X**	**C**	**E**	**H_1**	**H_2**
Reaction time (min)	120	30	120	60	120
Reaction temperature (°C)	60±2	Ambient	60±2	45±2	45±2
Consistency(%)	10	3.0	10.0	9.0	9.0

4.11 ANALYSIS OF PAPER PROPERTIES

Evaluation of the paper properties of XCEHH and CEHH bleached pulp, had indicated increase in tensile index, burst index and double fold and reduction in tear index of paper due to the enzymatic pre-treatment of the pulp. ^{O}SR also increased for treated pulp in comparison to the untreated pulp (Table 18).

Table 18. Paper properties of the hand sheets made enzymatic treated various pulps

Samples	^{o}SR	Tear index ($mN\ m^2\ g^{-1}$)	Tensile index (Nmg^{-1})	Burst index ($kPam^2g^{-1}$)	Double Fold (no)
Treated wheat Straw pulp	62°	4.75	38.2	1.76	6
Untreated wheat Straw pulp	52°	5.33	35.95	1.53	3.8
Treated mixed hard wood pulp	34°	2.39	28.12	1.09	2.5
Untreated mixed hard wood pulp	22°	3.17	26.70	0.75	2.25

4.12 ANALYSIS OF COLOUR OF PAPER SHEETS

Fig. 34 represents the colour of hand sheets before any treatment (a), after enzyme treatment (b) and after final chemical leaching (c). Paper directly made by untreated wheat straw pulp is darker in colour as compared to the mixed wood pulp due to high kappa number. Enzyme pretreatment followed by chemical bleaching caused decrease in kappa number and resulted into desired brightness of the paper.

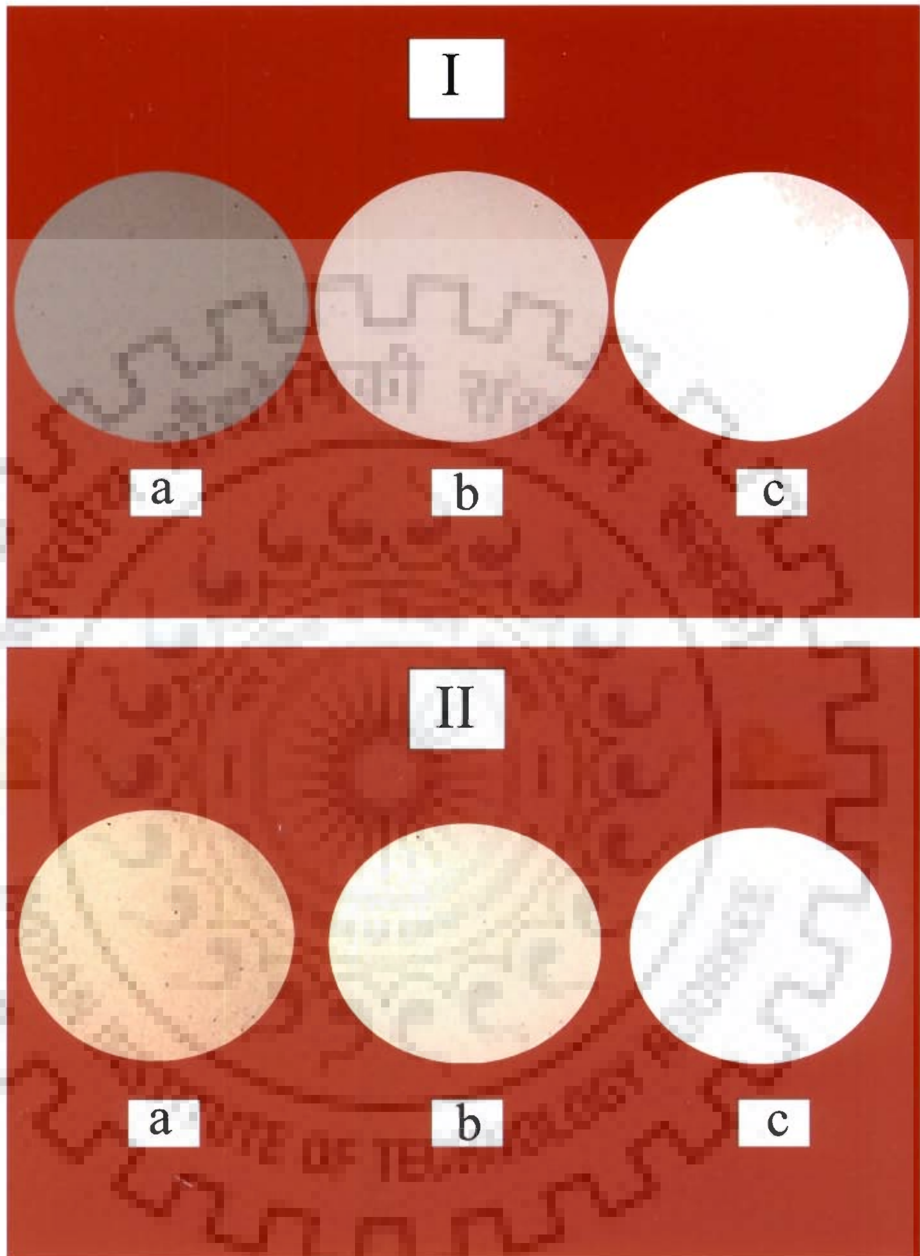
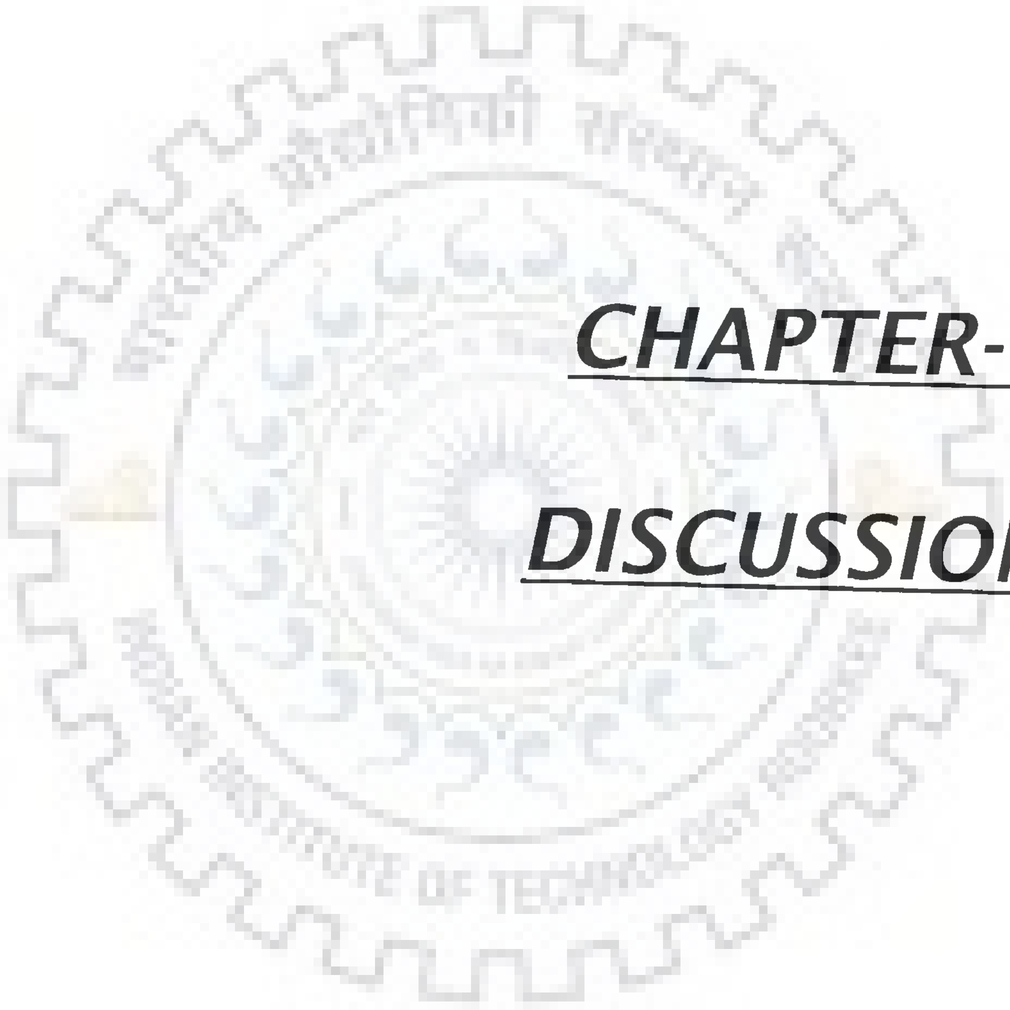


Fig. 34 Colour of the wheat straw (I) and mixed wood paper sheets (II)
(a), Unbleached; (b), Enzyme pretreated (X); (c), XCEHH treated



CHAPTER-5

DISCUSSION

Chapter - 5
DISCUSSION

5.1 IDENTIFICATION OF XYLANASE PRODUCING MICROORGANISM

Nature has plentiful variety of micro-flora and fauna with various vital functions. A stable microbe having distinctive production ability of the compound of interest is a precondition for any triumphant fermentation process. Screening and selection of potent microbial strain producing the compound of interest is a critical and exhaustive step for accomplishing the production of a particular compound. Most of the information regarding the isolation, development and improvement of microorganisms of industrial significance wasn't normally disclosed by R & D centers. Nevertheless, the general information regarding the production strategy was disclosed. A common practice among the researchers is to look for and select the microorganism, which secrete the required enzymatic component for a specific process. Many workers have employed the stepwise screening approaches for selecting the xylanase producing microorganism. The xylanase production was thought to be carried out by various bacterial and fungal organisms but fungal system was found to be effective due to multiple enzyme system, which enhance the degradation of the hemicellulose.

Among 42 fungal strains isolated from a variety of natural sites out of which 17 were from forest soil sample, 9 from sugarcane dumping site and 6 from paper industry waste site. Of the 42 isolates, 22 isolates with higher zone diameter were selected for further study. Following the quantitative analysis for xylanase; NK-23,

NK-35, NK-19, NK-37 and NK-29 were found to result into higher levels of xylanase; the maximal level was achieved by NK-23 (335.4 IU ml^{-1}). The strain was identified as *Aspergillus niger* from Indian Agricultural Research Institute, New Delhi and denoted as *A. niger* NK-23 strain. The strain although had shown significant levels of xylanase production but in order to explore the possibilities for commercial exploitation of the enzyme, attempt was made for further increase in the production levels. The development of an industrially viable fermentation process largely depends on the efficiency of the production strain involved for the process. Strain improvement was therefore attempted by mutagenesis and an attempt was made to further economize the fermentation process by incorporating the cheaper agroindustrial residues as the carbon source for xylanase production.

5.2 STRAIN IMPROVEMENT BY MUTAGENESIS

The development of an economical fermentation process largely depends on the efficiency of the production strain involved in the process. The use of a high yielding strain is a crucial factor for the success of the process. The strains isolated from natural sources, usually are not very high yielding. The major conquest for increasing the production of enzyme can be due to the strain improvement by extensive application of mutation and selection. Hence, this approach was applied to NK-23 for making the process more economical.

Various research groups had attempted strain improvement for xylanase production earlier in different organisms (Kuhad *et al.*, 1990; Butt *et al.*, 2002; Trujillo *et al.*, 2003; Lu *et al.*, 2003; Suryanarayan, 2003). The technique of induced mutagenesis and strain selection is widely accepted as a routine procedure for strain improvement. Therefore, the wild type parental strain *A.niger* NK-23 was treated with

physical (UV), chemical (N-methyl-n'-nitro-N- nitrosoguanidine and colchicine) and mixed mutagenesis (UV + N-methyl-n'-nitro-N- nitrosoguanidine) for different time intervals to induce mutagenesis. The mutants were screened for xylanase production by zone analysis on XA-plate after staining with congo red. The screening procedure is reported to be accurate, rapid and expedient, ensuring vigorous screening of the mutants (Teather, 1982; Srivastava *et al.*, 1991). Thus, the zone analysis led to determination of the efficiency of each mutant without prolonged and laborious fermentation trials.

The strain *A.niger* NK-23 was subjected to various dosage of UV, N-methyl-n'-nitro-N- nitrosoguanidine and colchicine mutagens and number of positive and negative mutants were scored on the basis of zone diameter. The viability of the colonies obtained after 3 minutes UV exposure followed by 40 minutes of exposure with NTG, had yielded both positive and negative mutants. The proportion of different mutants obtained after simultaneous treatment of UV and NTG had yielded 27% positive, 24% negative and 49% unaltered mutants. Among different mutants obtained, *A. niger* NKUC$_N$-3.40 obtained after mixed mutagenesis was found to have maximum xylanase (469.6 IU ml^{-1}) secretion in the fermentation medium along with 127.6 IU ml^{-1} of β-xylosidase and 1.63 IU ml^{-1} of cellulase production. Subculturing for several generations had indicated that NKUC$_N$-3.40 was stable with minor variations in levels of production. Chemical mutagenesis by N-methyl-n'-nitro-N-nitrosoguanidine and colchicine treatment led into development of mutants with higher rates of survival but comparatively lesser levels of enzyme production.

Mutagenesis not only had resulted in strains having variations in enzyme production but also denoted characteristic morphological variations. The mycelia of

the mutant type $NKUC_N$-3.40 were dirty white in colour with black spores while wild type exhibited brownish yellow mycelia and black spores. The yellowish colour of the wild type might be due to over expression of the asperenone (Jefferson, 1967). In addition, hyphae of mutant strain $NKUC_N$-3.40 were thick, compact and coiled in comparison to the thin, straight and elongated mycelia of the parental wild type strain NK-23. Such distinguishing deviations were also observed earlier by Longacre *et al.*, 1997 and Bai *et al.*, 2004.

5.3 FACTORS AFFECTING XYLANASE PRODUCTION

Choice of various components and conditions for fermentation reaction is of vital significance. Attempts have earlier been made by several groups to identify and regulate the critical parameters for xylanase production using a variety of microorganisms mainly, *Trichoderma reesei* (Tenkanen *et al.*, 1992), *Thermomyces lanuginosus* (Purkarthofer *et al.*, 1993), *Aspergillus foetidus* (Shah & Madamwar, 2005), *Trichoderma harzianum* (Seyis & Aksoz, 2005). The improved mutant strain $NKUC_N$-3.40 developed in our laboratory was evaluated for most desirable sets of conditions for achieving maximum level of xylanase production. Initial trials with synthetic sugars had undoubtedly indicated that xylan was the most favorable substrate followed by xylose (Rajaram & Varma, 1990; Keskar, 1992; Karni *et al.*, 1993). Sucrose, galactose and maltose were not so effective. Glucose, fructose and mannose induced only marginal xylanase production. Xylan and xylose constitutively induced the expression of xylanase and β-xylosidase production when present in the fermentation medium (Beg *et al.*, 2001; Collins *et al.*, 2005). These enzymes appeared to be specifically induced depending upon the carbon source present in the

fermentation medium. The presence of glucose in the culture medium resulted in strong repression of xylanase (Loera & Cordova, 2003)

The choice of an appropriate substrate is of great importance for the successful enzyme production but the concentration is also equally important. Concentration of the substrate not only regulates the enzyme synthesis but is also used for the growth of the organism. Sugar substrates at lower concentration are mainly utilized for growth of the fungus, their presence in comparatively higher amounts leads to the synthesis of enzyme. Further increase in concentration leads to decrease in enzyme production due to osmotic problems faced by the microorganism in conjunction with the inhibition due to catabolite repression. These results are in agreement with the results obtained by the earlier workers (Haltrich *et al.*, 1996; Kulkarni, 1999). 1% (w/v) concentration of xylan in fermentation broth led to maximum production of xylanase and β-xylosidase. Both the enzymes show synergism for the hydrolysis of xylan (Poutanen and Puls, 1988).

The enzyme production level varies as a function of time in different organisms. *Aspergillus niger* NKUC$_N$-3.40 represents maximal production on 3rd day which declines with increase in time. Similar results with respect to time dependent production were also reported earlier (Biswas *et al.*, 1990; Gawande & Kamat, 1999; Loera and Cordova, 2003; Shah *et al.*, 2004). The H^+ concentration in the fermentation medium has profound effect on the xylanase production. It was observed that the pH range 6.5-7.5 supported the growth and enzyme production by *A. niger* NKUC$_N$-3.40. Fermentation at lower and higher pH proved to be detrimental perhaps because of the inactivation of the enzyme system. However, the higher pH did not

significantly affect the growth of the fungus. The reason for poor production at higher pH was probably due to proteolytic inactivation of the xylanase.

The effect of shear stress and mechanical forces as a result of agitation on filamentous fungi, i.e. change in morphology and breakage of hyphae, along with the leakage of intracellular material due to agitation has been described (Thomas, 1990). An agitation speed of 120 rpm favored the xylanase and β-xylosidase production. Higher agitation speed results in shear stress that presumably caused damage to the mycelium and decreased the enzyme production.

5.4 ALTERNATIVE CARBOHYDRATE SOURCES FOR XYLANASE PRODUCTION BY *ASPERGILLUS NIGER* NKUC$_N$-3.40

Since, cost of the substrate plays a crucial role in the economics of an enzyme production process, different lignocellulosic substrates that are easily available and are considerably cheaper were compared and assessed in relation to pure substrate, so that these lignocellulosic substrates can be exploited for the enzyme production (Toughy & Couglan, 1992; Haltrich *et al.*, 1993, Yuan *et al.*, 2005). In the present study several agro-residual wastes were evaluated alone or in combination with purified substrates for xylanase production. The agro-residues, primarily, in some case, are used as animal feed and otherwise have no major application and are burnt. Some of these agro-residual wastes performed notably better than the isolated xylans, thus these appear to be realistic alternatives as the carbon source.

In addition to the nature of inducing substrate, the pre-treatment of these complex compounds may lead into achieving the increased production levels. Pre-treatment methods were employed to make the substrate more amenable to the organism. Bagasse was found to be a better alternative for the xylan. Pre-treatment of

bagasse further induced the production levels. Similar observations have been noted by earlier groups (Rajaram & Varma, 1990). Untreated substrates resulted into lower yields of xylanase and that may be due to its inaccessibility to the enzymatic attack and alteration of the substrate by the pre-treatment process.

5.5 COMPARATIVE ANALYSIS OF DIFFERENT FERMENTATION CONDITIONS

It is often useful to try out fermentation under submerged, surface and other modified conditions for achieving maximal levels of production. In the present study, xylanase production was evaluated under submerged, surface and solid-state fermentation processes. Solid-state fermentation was found to be better than the submerged followed by the surface fermentation.

Xylanase production using *A. niger* has been largely carried out under submerged fermentation by various groups. However, some groups had mainly evaluated the solid-state fermentation (SSF) using *Trichoderma reesei* (Tenkanen *et al.*, 1992); *Melanocarpus albomyces* IIS68 (Narang *et al.*, 2001), *Trichoderma harzianum* (Rezende *et al.*, 2002); *Fusarium oxysporum* (Panagiotou *et al.*, 2003) for xylanase production. Solid-state fermentation (SSF) is advantageous over submerged fermentation (SmF) due to its simplicity and its compatibility to the natural surroundings that are suitable for growth and survival of many microorganisms (Virupakshi *et al.*, 2005). Enzymes including xylanase, β-xylosidase and cellulase production ability of mutant *A. niger* $NKUC_N$-3.40 strain was evaluated under solid state fermentation. Moreover, higher enzyme titre and productivity, as well as increased stability of secreted enzymes have been the other notable features of SSF (Montiel Gonzalez *et al.*, 2003; Holker *et al.*, 2004). Few species of *Aspergillus* mainly *Aspergillus ocharaceus* (Biswas *et al.*,1990), *Aspergillus niger* 44 and

Aspergillus terreus 5 (Gawande & Kamat,1999), *Aspergillus sulphureus* (Lu *et al.*, 2003), *Aspergillus* spp. (Shah *et al.*, 2004) and *Aspergillus fischeri* Fxn1(Senthilkumar *et al.*, 2005) have been reported by respective groups using different solid supports but the enzymatic levels were lower as compared to the xylanase secreted from *A. niger* $NKUC_N$-3.40.

5.6 ANALYSIS OF CRITICAL PARAMETERS FOR SOLID STATE FERMENTATION PROCESS

Solid-state fermentation for the production of enzymes and other products appear advantageous over submerged fermentation (Ramesh &Losane, 1990). Some of the advantages are, no need for a complex machinery and sophisticated control systems; less volume of liquid generated which could be advantageous for product recovery and could also reduce the cost of downstream processing and subsequent waste treatment; usability of simple and cheap media for fermentation; lower energy demand, and often a higher product yield; lower risk of contamination due to absence of free flowing water. Further, the utilization of various agro-residues makes the process more economical and industrially viable.

For solid-state fermentation in the present study, ten different solid supports viz. rice bran, wheat bran, wheat residue, bagasse, coconut coir, loofa sponge, oil cake, cotton hull, glass beads, poly urethane sponge impregnated with the fermentation medium as derived in this study, were used. Among the solid supports used, maximum xylanase production was obtained with cotton hull (1705.67 IU ml^{-1}) as the solid support followed by the sugarcane bagasse (1337.33 IU ml^{-1}). The difference in the production levels was probably due to the structural differences of the solid matrices used, that may cause difference in nutrient absorption and fungal

entrapment, therefore resulting into differential levels of growth of *A. niger* $NKUC_N$-3.40 and hence consecutively affecting the enzyme secretion. Oxygen appeared likely to limit the growth than the carbon substrate. This can be explained by the fact that oxygen always has to pass through an active mesh of biomass at the surface of the solid support interacting to the mycelial biomass in order to reach the interior regions (Lenz *et al.*, 2004). Aeration was maintained by periodically aerating the medium.

Cotton hull was found to be the best solid support for the xylanase production, it may be due to its high porosity that may help fungal biomass to penetrate the hull. The porosity of cotton hull also helps in efficient absorption of nutrient and air inside the hull, that, therefore can easily be made available to *A.niger* for growth and production of enzyme. Cotton hull have hard shell which are made up of the cell wall consisting of the hemicellulose that can also act as inducer for the microorganism. The solid-state fermentation normally led to higher levels of production with a number of the substrates analyzed because of close association of substrate and organism.

The duration for achieving the maximal enzyme production was slightly higher in solid-state fermentation than the submerged system. This may be because of initial lag due to time utilized by the mycelia to penetrate into cotton hull and hydrolyse xylan. Thus the time taken by the microorganism to achieve the exponential phase of growth was higher during SSF and hence maximum levels of enzyme production required longer durations (Ramesh & Lonsane, 1987; Ramachandran *et al.*, 2004)

The influence of moisture content on metabolic activity of filamentous fungi had been well documented. Sporulation and growth rates have been found to be affected as per the moisture content in the medium. Water content in SSF ranges from

30% to almost 80% depending on the solid support used. NKUC$_N$-3.40 had the optimum production level at 70% moisture. Moisture less than 60% or more than 80% didn't effectively endorse xylanase production. At higher moisture conditions, void spaces of the support is filled with moisture which may interfere with the oxygen transfer to the microorganism and hence reduce the ability of xylanase secretion in fermentation process (Gervais *et al.*, 2003; Gawande & Kamat, 1999).

Among the different additives used, mustard oil cake led to maximum enhancement in the xylanase production followed by the corn steep liquor. Yeast extract and cheese whey had also induced the production. Most of the additives used were the source for the nitrogen along with other nutrients and led to an enhancement in the enzyme production level.

5.7 CHARACTERISTIC FEATURES OF XYLANASE FROM MUTANT *ASPERGILLUS NIGER* NKUC$_N$-3.40

Thermal stability of the enzyme observed generally was higher when it was produced by the solid-state fermentation process. Similar results were obtained with the xylanase produced by mutant NKUC$_N$-3.40. Enzyme preparation from NKUC$_N$-3.40 was observed stable at higher temperature. 50% of the enzymatic activity remains after the same was kept at a temperature of 65°C for 2 hrs. The stability of the enzyme preparation was also evaluated at different pH. Up to 85% of the enzyme activity was observed at a pH range of 7.0-9.0. Thus the enzyme preparation had significant levels of activity at alkaline pH and thus appears suitable for its application to the pulp and paper industry.

Analysis of the xylanase secreted by the *A. niger* NKUC$_N$-3.40 indicated this to be a 29 kD protein. Xylanase of similar molecular mass, mainly, a 22 kD protein from *Rhizopus oryzae* (Bakir *et al.*, 2001), 22.4 kD from *Fusarium proliferatum*

(Saha, 2002), 22.4 kD from *Penicillium capsulatum* (Ryan *et al.*, 2003), 27.5 kD from *Acrophialophora nainiana* (Cardosa and Filho, 2004) and 32 kD from *Aspergillus versicolor* (Carmona *et al.*, 2004) have been identified by the respective groups.

5.8 EVALUATION OF TRAY BIOREACTOR FOR SEMI-CONTINUOUS PRODUCTION OF XYLANASE

The promising results obtained at flask level led us to analyze the feasibility of xylanase production at larger scale using cotton hull as the solid support. However, at larger scale the substrate bed in solid-state fermentation process often resulted into increased temperature due to inconsistent regulation of temperature (Mitchell *et al.*, 2000). This rise in temperature may also be due to higher metabolic activity of the microorganism and due to the lower thermal conductivity of the matrices used for solid-state fermentation; hence the heat removal process may be cumbersome and a time-consuming process. The increase in temperature may thus affect fungal growth and secretion of enzyme during fermentation. Initially the temperature and oxygen levels remain uniform throughout the substrate but as the fermentation continues, heat transfer into or out of solid-state fermentation system may be related to the aeration of the fermentation process (Pandey, 2003). Aeration commonly was thus performed by blowing the air over the surface of the sheets and cooling thus can be achieved by heat conduction. A humidifier tray containing water that was placed at the bottom of the bioreactor further helped in maintaining the temperature of the process. This practice of water evaporation to regulate the temperature was also followed by the various research groups (Raimbault, 1998; Smits *et al.*, 1999; Lu *et al.*, 2003; Suryanarayan, 2003; Lenz *et al.*, 2004).

Besides scaling up the process of production, process can be made more viable and economical by reusing the biomass for multiple cycles of production. Thus biomass was subjected to repeated cycles for enzyme production by replacing medium after every cycle of fermentation.

The mutant strain *A. niger* $NKUC_N$-3.40 had the significant levels of xylanase production for three cycles of fermentations. Thus higher and economical production of xylanase can be accomplished from this setup. The enzyme levels decrease thereafter. This may reflect the decreased metabolic activity of the fungus or may be due to the depletion of nutrients from the cotton hull resulting due to complete colonization of the hull by the fungus.

5.9 PULPING OF WHEAT STRAW AND MIXED WOOD CHIPS

Mixed wood pulping reveals that on increasing active alkali the screened pulp yield increased upto when 16% active alkali as Na_2O. Further increase in active alkali beyond 16% led to a decline in screened pulp yield due to carbohydrate degradation. The decrease in screening rejects and kappa number beyond active alkali at 16% level appeared almost constant. Therefore 16% active alkali dosage may be taken as an optimum cooking dose for kraft pulping of mixed wood chips of *Populas deltoidea, Eucalyptus tetrecornis, Bambusa aurandacea and Pinus roxumberghii.*

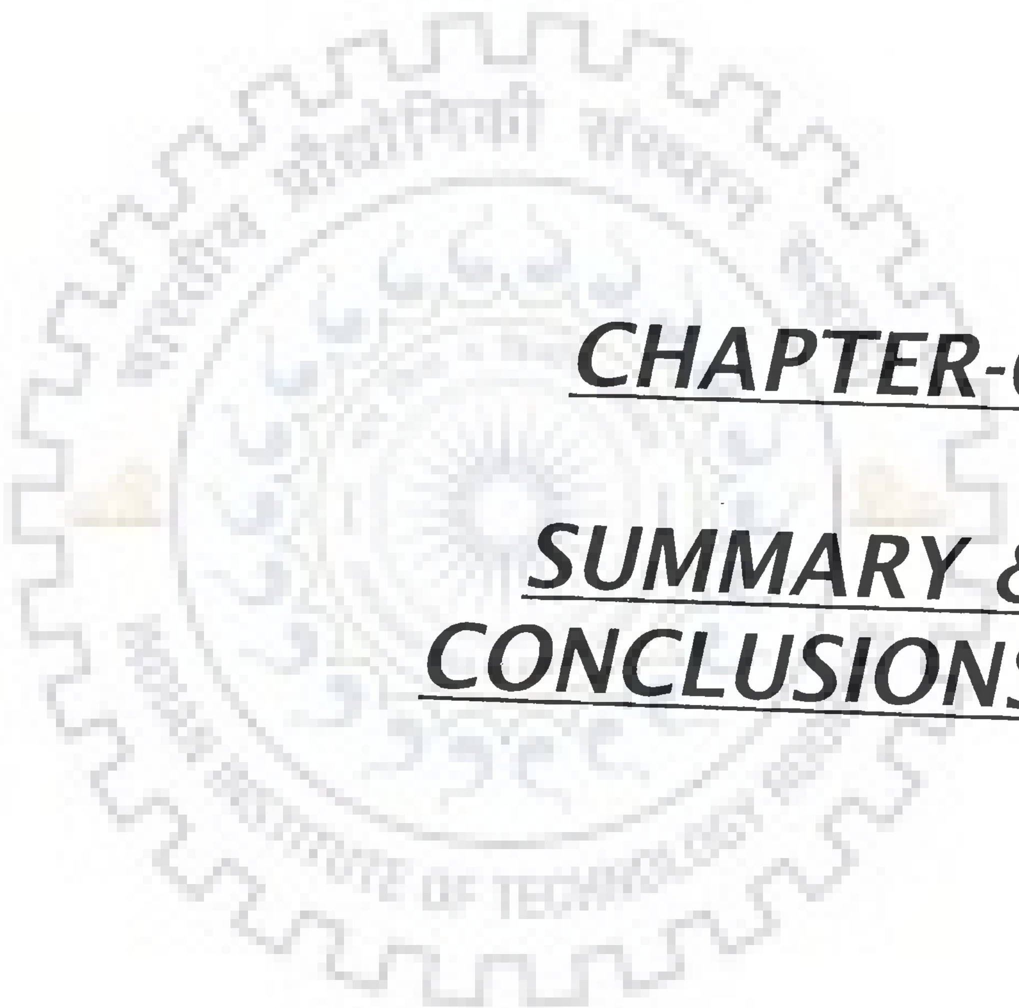
Similarly, pulping of wheat straw revealed that pulping of wheat straw also followed the similar pattern as that of kraft pulping of mixed pulp. 12% active alkali dosage was sufficient to produce bleachable grade pulp with minimum carbohydrate degradation.

5.10 BIOBLEACHING OF WHEAT STRAW AND MIXED WOOD CHIPS

Enzymatic pretreatment of mixed wood as well as wheat straw pulp resulted into 11.6% and 20% decrease in chlorine consumption respectively due to reduction

of kappa number by the action of xylanase from *A. niger* $NKUC_N$-3.40. This had also decreased the biological oxygen demand, chemical oxygen demand, adsorbable organic halide and post color number of both wheat straw and mixed wood pulp by 13.3%, 18.4%, 22.2%, 29.6% and 50%, 33.7%, 31.5% and 255 respectively. Increment in the pulp brightness and pulp viscosity was observed in both the pulp types. The enzymatic pre-bleaching reduced the total Cl_2 demand of the pulp. It resulted in the minimum degradation of carbohydrate fraction. In this way, enzymatic pre-bleaching pays interms of increased pulp yield with improved mechanical strength properties, high brightness with reduced pollution load.

Pulp properties were observed to further improve after the action of the enzyme. Tear index, tensile index and double fold of the paper made out of the treated mixed wood and wheat straw pulp observed was higher as compared to the untreated mixed wood and wheat straw pulp. Hence the preparation of xylanase enzyme from the mutant $NKUC_N$-3.40 represents a better option for the biobleaching in the pulp and paper industry.



CHAPTER-6

SUMMARY & CONCLUSIONS

Chapter - 6
SUMMARY AND CONCLUSIONS

Among forty-two fungal strains isolated from forest soil, sugarcane dumping site, paper loading site and decomposing manure, 22 isolates were selected on the basis of zone-formation on xylan-agar (XA) plates. These strains had distinct morphological features, colour appearance and growth patterns. The strain denoted as NK-23 and resulting into maximum zone diameter was identified to be *Aspergillus niger* from Indian Agricultural Research Institute, New Delhi and was selected for further study. The xylanase production level was found to be higher as compared to the other strains along with the notable β-xylosidase and lower cellulase levels. Attempts to improve the xylanase production ability of the selected strain were undertaken by physical, chemical and mixed mutagenesis of the strain. The survival rate and distribution profile i.e. positive, negative and corresponding mutants were determined after every stage of mutagenesis. The strain NKU-3 was the most productive for xylanase production obtained after primary mutagenic treatment. Chemical mutagenesis led to a marginal increment in the enzyme production ability in comparison to physical mutagenesis. NKU-3 was subjected to a second exposure of NTG treatment, which resulted in positive mutants (27%) of the total surviving colonies. These steps thus led to the isolation of *A. niger* $NKUC_N$-3.40 having the xylanase production ability that was 40% higher than the wild type *A. niger* NK-23. The mutant $NKUC_N$-3.40 after multiple periodical sub-culturing sustained the ability of enzyme production for a period of 12 months. Scanning electron microscopic studies revealed notable changes in the morphological features of the fungal strain

following mutagenic treatments. The wild type *A. niger* NK-23 had loose and brownish yellow mycelia, whereas mycelia of the mutant *A. niger* $NKUC_N$-3.40 was compact and dirty white in colour. Moreover in identical fermentation condition *A. niger* $NKUC_N$-3.40 produced notably higher levels of xylanase in comparison to the wild type strain *A. niger* NK-23. The various physico-chemical and biological parameters for achieving enhanced level of xylanase production by *A. niger* $NKUC_N$-3.40 were evaluated in submerged fermentation condition. Among various carbon sources, xylan at 1% with 2% inoculum (10^7 spores ml^{-1}) was observed to be the most promising for xylanase production. Using *A. niger* $NKUC_N$-3.40, maximum production of xylanase was obtained on 3rd day of fermentation (at 30°C, pH 7.0) under submerged condition (120 rpm). Besides these conditions, xylanase production increased after supplementing the fermentation medium with 0.7%, KH_2PO_4; 0.2%, K_2HPO_4; 0.2%, $MgSO_4$; 0.1%, $(NH_4)_2SO_4$ and 0.06% of Yeast extract. Among the various natural substrates in combination with the synthetic substrates, bagasse with oat spelt xylan in 1:1 ratio had shown further enhancement in enzyme production level.

An attempt was made to further economize the process of production by evaluating the solid state fermentation using agro-industrial residues as the solid support. Among ten different solid supports used, cotton hull was found to be the most effective for xylanase production. Under solid-state fermentation process maximum xylanase production was achieved on 4th day of incubation. A moisture level of 70% with an inoculum level of 4% (10^7 spores ml^{-1}) was suitable for production process. Of the various additives evaluated, mustard oil cake at 5% (w/v) enhanced the xylanase production ability of *A. niger* $NKUC_N$-3.40. The solid-state fermentation using cotton-hull had led to a maximum of 1783 IU g^{-1} of xylanase production.

The enzyme following characterization was found to be a 29 kD protein and the 50% of its activity was retained after incubating the same at 65°C for 2 hours. Further, 85% of the activity was retained at the pH range of 7.0-9.0.

The semi-continuous production of xylanase was attempted by designing a tray bioreactor and the production was evaluated under the conditions as derived earlier. In the bioreactor, biomass was recycled for different cycles of the fermentation and significant levels (1513 to 1645 IU g^{-1}) of enzyme were produced upto 3 cycles of fermentation. The level of production declined thereafter and that may be due to the depletion of nutrients and sporulation of fungi. Xylanase obtained was critically evaluated for the biobleaching of the mixed wood pulp as well as wheat straw pulp. Conditions optimized for the enzymatic pre-bleaching was found out to be 20 IU of xylanase g^{-1} of oven-dried pulp was used for 120 minutes at pH 8.0 which led to a significant reduction in the kappa number from 34.3 to 30.6 in case of wheat straw pulp and 19.6 to 16.2 in case of mixed wood pulp.

Enzymatic pre-treatment also led to a further decrease in the chemical demand as treated wheat straw pulp required 7.6 gpl Cl_2 in comparison to untreated wheat straw pulp that required 8.6 gpl Cl_2. The treated mixed wood pulp on the other hand required 4.0 gpl Cl_2 in comparison to untreated mixed wood pulp that required 5.1 gpl Cl_2. Pre-treatment could also effectively increase the viscosity of the pulp, which is desirable for the pulp and paper industry. Enzymatic pre-treatment also significantly decreased the BOD and COD of the effluent and also led to the decrease in the absorbable organic halide in the effluent. Hence, enzymatic treatment could lead into the development of a safer and eco-friendly process. The paper obtained after the enzymatic treatment by the xylanase produced by *A. niger* $NKUC_N$-3.40 had

shown better mechanical and optical properties in comparison to pulp that was not treated with the enzyme. Hence, treatment of pulp using xylanase appears a safer, effective and eco-friendly process. The following are the major conclusions from the present study,

a. Among the forty-two fungal isolates, *A. niger* NK-23 that was obtained from the sugarcane dumping site was found to be the potent xylanase producer.
b. Mixed mutagenic treatment i.e. ultraviolet followed by N-methyl-n'-nitro-N-nitrosoguanidine was found to be an effective strategy for strain improvement and mutant *A. niger* $NKUC_N$-3.40 thus obtained was remarkably a better xylanase producer as compared to the *A. niger* NK-23.
c. Solid-state fermentation resulted into higher levels of xylanase production than the submerged fermentation. Cotton hull was found to be the most preferred solid support for xylanase production under solid-state fermentation condition.
d. Xylanase produced by the *A.niger* $NKUC_N$-3.40 was stable in a pH range of 7.0-9.0 and upto a temperature of 65°C.
e. Process of production was scaled-up by designing a tray bioreactor to obtain the semi-continuous production of the xylanase.
f. Pre-treatment of mixed wood and wheat straw pulp with xylanase obtained by *A.niger* $NKUC_N$-3.40 had resulted into desired brightness and better quality of the paper.
g. Pre-treatment with xylanase obtained by *A.niger* $NKUC_N$-3.40 had also reduced the chemical as well eco-toxic effluent load.

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