MICROBIAL PRODUCTION AND APPLICATION OF ALKALITOLERANT XYLANASES BY *PENICILLIUM OXALICUM*

A THESIS

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

DOCTOR OF PHILOSOPHY

in

BIOTECHNOLOGY

by

PALLAVI DWIVEDI

DEPARTMENT OF BIOTECHNOLOGY
INDIAN INSTITUTE OF TECHNOLOGY ROORKEE
ROORKEE - 247 667 (INDIA)

JUNE, 2009

INDIAN INSTITUTE OF TECHNOLOGY ROORKEE
ROORKEE

CANDIDATE'S DECLARATION

I hereby certify that the work which is being presented in the thesis entitled **MICROBIAL PRODUCTION AND APPLICATION OF ALKALITOLERANT XYLANASES BY *PENICILLIUM OXALICUM*** in partial fulfilment of the requirements 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 a period from July 2004 to June 2009 under the supervision of Dr. R. P. Singh, Professor, Department of Biotechnology, Indian Institute of Technology Roorkee, Roorkee, India.

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

Pallavi Dwivedi
1.06.09
(PALLAVI DWIVEDI)

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

(R P Singh)
Supervisor

Date: 01 June, 2009

The Ph.D. Viva-Voce examination of **Ms. Pallavi Dwivedi**, Research Scholar, has been held on 27.8.09

Signature of Supervisor

Signature of External Examiner

ABSTRACT

The present investigation was undertaken to isolate a potent microorganism for production of xylanase stable at alkaline pH and deficient in undesirable cellulase activity. The process was economized by employing agro-horticultural residual resources.

The potent fungal strain secreting xylanase was isolated by selecting samples from soda ash and paper industry waste sites. The isolated strain SA-8 had remarkable levels of xylanase production and identified as *Penicillium oxalicum* by Indian Agricultural Research Institute, New Delhi. To achieve an improvement in enzyme production ability, the selected strain was subjected to mutagenesis. Mutagenesis by UV-irradiation and ethidium bromide led to the development of strain SAU_E-3.510 having notably higher levels of xylanase production. A detectable level of β-xylosidase production was also observed. Mutagenized strain *P. oxalicum* SAU_E-3.510 had discrete morphological features. The mycelia of mutant SAU_E-3.510 were coiled, rounded and had rough surface as compared to wild type SA-8 strain which were flattened, shiny, smooth surfaced and straight. Similarly, spores from the wild type were rounded with rougher surface while those from mutant strain were larger, smooth, flattened and crescent shaped.

Xylanase activity from mutant strain SAU_E-3.510 was evaluated by employing non food, weedy plant biomass (*Parthenium* sp. and *Eichhornia crassipes*) and other agroresidual materials, which are abundantly available and grow wildly to reduce the cost of production under submerged fermentation condition. An almost similar level of xylanase production was achieved by using congress grass as to that obtained with commercial oat spelt xylan. To develop an enzyme preparation possibly with a better ability for biobleaching applications, attempts were made to develop a xylanase-laccase mixture by co-cultivation of mutant *P. oxalicum* SAU_E-3.510 and *P. ostreatus* MTCC 1804. Production of mixed enzyme was evaluated by co-cultivation of *P. oxalicum* SAU_E-3.510 and *P. ostreatus* MTCC 1804 under surface, submerged and solid-state fermentation systems. Solid-state fermentation led to

maximum level of production. Among various solid supports used, combination of bagasse and black gram husk, in a ratio of 3:1, was found to be the most ideal for fungal colonization and enzyme production during co-cultivation. In order to further enhance the enzyme production levels, amount of substrate, moisture level and incubation period were evaluated.

Mixed enzyme preparation with both xylanase and laccase activity was evaluated for its bleach enhancing ability of mixed wood pulp in XCEPHHP and XODED sequences. Process of enzymatic bleaching was further ascertained by analysing the changes occurring in polysaccharide and lignin by HPLC and FTIR. The UV absorption spectrum of the compounds released during enzymatic treatment had denoted a characteristic peak at 280 nm, indicating the presence of lignin in released colouring matter. Variations in morphology and crystallinity of pulp were evaluated by scanning electron microscopy and X-ray diffraction analysis. The enzyme pretreatment led to decrease in kappa number, yellowness, AOX and COD. Additionally, it had improved mechanical and optical properties of paper along with reduction in chlorine consumption which in turn diminished the pollution load denoting it therefore, to be an ecofriendly and benign bleaching technology.

An attempt to scale up the production of mixed enzyme preparation was made by designing an intermittent rotating drum bioreactor using the fungal co-culture under derived conditions. A comparable level of scaled up SSF process was accomplished and significantly higher levels of xylanase and laccase were produced.

ACKNOWLEDGEMENT

"God understands our prayers even when we can't find the words to say them."

This piece of work would have been never accomplished without Almighty God's blessings and His power that work within me. I thank god for everything with which I am blessed with.

Today when I am intended to attain another milestone of my life in form of this thesis, it will be simple to name all those people that helped me to get this done, but it will be tough to thank them appropriately. I would like to thank God for blessing me the company of such nice people around me.

First and foremost, I would like to record my sincere gratitude to my supervisor, Prof. R. P. Singh for his inspiring guidance, valuable suggestions, constant motivation and timely discussions from the very early stage of this research. This thesis grew out of a series of dialogue only because of him, who shared with me a lot of his expertise and research insight. Above all and the most needed, he provided me unflinching encouragement and support in various ways. His truly scientist intuition has made him as a constant oasis of ideas and passions in science, which exceptionally inspire and enrich my growth as a student and a researcher. I am very much gratified to him for developing the scientific temperament in me and providing me a healthy, professional and free working atmosphere as a result, research life became smooth and rewarding for me.

My sincere thanks are to Prof. Ritu Barthwal, Head, Department of Biotechnology, for providing the necessary infrastructure and excellent working environment in the department during the course of my work. I would be failing in my duties without expressing my gratitude and thanks to the hon'ble faculty members, Prof. G. S. Randhawa, Prof. H. S. Dhaliwal, Dr. R. Prasad, Dr. V. Pruthi, Dr. A. K. Sharma, Dr. P. Roy, Dr. B. Chaudhary, Dr. S. Ghosh, Dr. P. Kumar, Dr. S. Tomar, Dr. N. K. Navani and Dr. R. Pathania for their valuable suggestions and encouragement.

I am delighted to express my gratefulness and respect to Dr. Dharm Dutt, Associate Professor, Department of Paper Technology, Saharanpur Campus, who persistently inspired and guided me all along the application work in spite his busy schedule. I am extremely thankful for his keen interest and perpetual guidance offered for this work. I've deep and sincere sense of gratitude towards Prof. G. S. Uppadhaya, Head, Department of Paper Technology, for providing me all in-house research facilities and excellent working environment in the department.

In recognition of all the help and support I would like to mention all the technical staff of the Biotechnology Department in particular, Mr. Mani, Mr. Lokesh, Mrs. Surita, Mrs. Shashi, Mr. Saini, Riayaz ji, Giri ji, Pankaj and Pradeep. I also extend my sincere thanks to Mr. Sinha, Mr Arora, Mrs Bhatjiwale, Mr. Rakesh Kumar and Harpal ji for their constant help at DPT, Saharanpur Campus.

My thesis would have been a distant dream without inspirative guidance and moral support from my respected seniors Dr. Ruma Ganguly, Dr. Amit Sharma, Dr. Nidhi Sabharwal, Dr. Soma Sen, Dr. Arshi Rastogi, Dr. N K Singh, Dr. Sujeet kumar (JNU new delhi). I would always be thankful to them for their generous help and efforts for teaching and improving my experimental skills along with boosting my analytical abilities.

I find a scarcity of words while conveying my heartfelt thanks to Dr. Vivekanand and Ms. Nidhi Pareek for their precious love, care, support and encouragement. They always provided a friendly, humorous and encouraging environment during the entire course of study which is a major driving force behind me to achieve my dreamed destination. I am especially grateful to Dr. Swarnima Agnihotri for always being helping, affectionate and paved my way through the struggles while writing this thesis.

"Wherever you are its your friends who make your world" its true because of the direct and indirect support and help from my friends Ms. Hina Ahmad, Ms. Lata Chauhan, Ms. Anjali Sardana, Dr. Priya Kapoor, Mrs. Kushuma Negi, Mrs. Shreela Dey, Mrs. Deepti Thakur, Mrs. Dhanpati Rana, Mrs. Prerana Gupta, Mrs. Shilpa Pal, Mrs. Pooja Mittal, Ms. Rajni Salunke, Ms. Sushmita Bhatacharya, Mrs. Divya Srivastava, Dr. Shalini Singh, Mrs. Harjeet Kaur, Dr. Mohanlal and Mr. Alok Kumar Tyagi. They provide me the joyful, affectionate and healthy environment during my stay at Roorkee for accomplishing my goal.

I would like to extend my sincere thanks to my friends Ms. Niti Paliwal, Ms. Neeta Purbia and Mrs. Saima Ruhela for their indirect support to make this journey so congenial and easy. I am thanking them for their memorable concern, affection and constant care.

I would like to avail the opportunity to express my deep respect and gratitude to Ma'am, Mrs. Pratima Singh for her kind affection and encouraging thoughts to achieve this destination. I never felt homesick since beginning only because of her incessant loving and caring attitude. I am unable to express my feelings in words for her constant help and inspiration in every possible way.

I am forever grateful to my grandparents, Amma and Dada, whose foresight and values paved the way for a privileged education since my childhood. No words and no language is ever adequate to express my heart felt admiration for my respected parents Mr. Mohan Krishna Dwivedi and Mrs. Rekha Dwivedi, who have always supported, encouraged and believed in me, in my all endeavors. They gently offered unconditional love, support and strength at each turn of the road. Particular thanks to my loving sisters Puja, Divya and Chhavi for their affection and moral boost-up whenever needed. They made me felt cheerful and joyful with their smile and humor during hectic and difficult hours. I also convey a special credit to my uncles, aunts and cousins in accomplishment of this tedious assignment with their constant supportive, helping and optimistic attitude.

A word of special thanks to a person sitting miles apart from me but always had been on my side during hard times to keep my moral up. Thanks Heerak, for your encouragement, inspiration, cooperation and affection that made me able to complete this assignment.

My heartfelt thanks and appreciation are due to my in-laws, Mr. Yogendra Kumar Vyas, Mrs. Kusum Vyas, Bhaiya- Bhabhi and Didi-Jijaji for their continuous encouragement, moral support and promptness that brought this investigation to completion. I would also like to thank my little nice & nephews Cherry and Julu for their refreshing smiles and timely allowance to complete my work.

And to the rest, I extremely thankful to those people, whose names have been unknowingly left, thank you very much for your prayers. It really helped a lot. I apologize and believe, they will be always with me as they were there in the time of need.

Last but not the least; I would like to thank Council of Scientific & Industrial Research (CSIR) for providing financial support for my research work.

Pallavi Dwivedi
1.06.09
(Pallavi Dwivedi)

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

ANOVA	Analysis of Variance
AOX	Adsorbable Organic Halide
bp	Base Pair
b-θ	Number of Block
COD	Chemical Oxygen Demand
°C	Degree Celsius
Cl_2	Chlorine
cm	Centimeter
df	Degree of Freedom
DNA	Deoxyribonucleic acid
DNS	Dinitrosalicylic Acid
DTT	Dithiothreitol
ECF	Elemental Chlorine Free
FTIR	Fourior Tranform Infrared Spectroscopy
g	Gram
HBT	N-hydroxybenzotriazole
HPLC	High Performance Liquid Chromatography
IU	International Unit
ISO	International Organization for Standardization
K-θ	Number of Treatment
kb	Kilobase
kD	Kilodalton
l	Litre

MALDI ToF/ToF	Matrix Assisted Laser Desoption/Ionisation Time of Flight
µg	Microgram
min	Minute
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
TPD	Tons per day
TCF	Total Chlorine Free
TLC	Thin Layer Chromatography
TOX	Total Organic Halide
UV	Ultra Violet
v/v	Volume per Volume
w/v	Weight per Volume
w/w	weight per weight
XRD	X-ray Diffraction

CHAPTER I

INTRODUCTION

Chapter - 1

INTRODUCTION

Xylan is a major, highly branched, polymeric component of hemicellulosic biomass, which is present in the plant cell wall along with other minor polysaccharides like mannans, glucans, arabinans and galactans. Due to its heterogeneity and complexity, the complete hydrolysis of xylan requires a cocktail of enzymes (Subramaniyan and Prema, 2002). Endo-1, 4-β-D-xylanase (EC 3.2.1.8) randomly cleaves the xylan back bone at endo-β-1,4-xylosidic linkage and releases xylooligosaccharides, while β-D-xylosidase (EC 3.2.1.37) cleaves the terminal β-1,4-xylosidic linkage and releases xylose monomers from the reducing end of xylooligosaccharides and xylobiose. Besides the above mentioned enzymes, removal of side group is catalyzed by α-L-arabinofuranosidase (EC 3.2.1.55), α-L-glucuronidase (EC 3.2.1.139), acetyl xylan esterase (EC 3.2.1.72) and ferulic acid esterase (EC 3.1.1.73).

Xylooligosaccharides, the end product of xylan degradation have considerable industrial applications like their bioconversion to biofuel (ethanol), artificial low calorie sweetener (xylitol and furfural) etc. Xylanases hold tremendous potential for biotechnological applications and together with cellulases and pectinases, account for 20% of the world's enzyme market (Polizeli et al. 2005). In juice, wine and beer-making, xylanases are used as clarification agent and in the feed industry both cellulases and xylanases have been shown to reduce the viscosity of the seed extracts, thereby ameliorating the uptake of nutrients. Besides these, xylanases play major role as a biobleaching agent for pulp and paper industry (Viikari et al. 1994).

The makeover of wood into a fully processed, bright and white paper is a result of a long, tedious, multistage process covering chipping, pulping, bleaching and sheet formation. Final brightness of paper mainly depends upon the efficiency of bleaching stage where lignin is removed from pulp which is responsible for the colour of pulp. Up to now paper industries rely

mainly upon chlorine and chlorine based chemicals for pulp bleaching and release huge amounts of toxic effluents. This effluent contains adsorbable organic halides (AOX) as well as total organic halides (TOX) and other chlorine based compounds which cause air and water pollution. They are toxic, mutagenic, persistent, bioaccumulating in nature and cause numerous harmful disturbances in biological systems. Therefore, environmentalists and government denounce the use of chlorine for bleaching of pulp and it therefore becomes essential to look for the alternatives. Some existing alternatives of chemical bleaching are the use of O_2, O_3, H_2O_2 and extended delignification but these are less preferable due to their non-selective nature. They also degrade cellulose fibres, which are responsible for the strength of paper. Removal of lignin with the help of microbes or microbial enzymes is referred as biobleaching. Biobleaching appears to be a suitable and promising alternative of chemical bleaching due to its selective and environment friendly nature. Two possible routes for biological bleaching are the use of xylanolytic or ligninolytic enzyme systems. Among these, ligninases confer a straight approach and directly break lignin while xylanases make use of an indirect approach. These attack on reprecipitated xylan as well as on lignin carbohydrate complex. Thus it facilitates the exposure of tightly bound lignin to other bleaching chemicals which is applied during subsequent bleaching stages, hence facilitating the bleaching of pulp during paper manufacturing.

Alkalophilic and also cellulase free enzymes are desirable for their compatibility to mill operating conditions for the economic benefits to industry. During enzymatic treatment, enzyme is added as an aqueous solution in the brown stock. The pH of the brown stock is around 9-11 as well as temperature is high. Therefore, alkalophilic enzyme preparation that is stable at high temperature is desirable as it will save the cost of cooling and that of the acid treatment. In addition, the other desirable feature is that, enzyme preparation should be free of any cellulase activity. Any cellulase activity will have serious economic implications in terms of cellulose loss, degraded pulp quality and increased effluent treatment cost. Cellulose is the component, which is mainly responsible for the strength of the paper. Xylanase preparation with cellulase

activity may degrade cellulose fibres and will affect the paper quality and efficacy of biobleaching. Many of the xylanases analysed appear to be of mesophilic or neutrophilic in origin hence alkalophilic and cellulase free enzyme could prove to be of great benefit for pulp and paper industry.

A wide variety of microorganisms including fungi, bacteria, actinomycetes, algae, protozoa and yeast are known for their xylanase producing ability. Although xylanase producing ability is detected in bacteria and yeast but fungi are superior producers because of higher titre, multifunctional and extracellular nature of enzyme. In addition to this, fungi typically produce several accessory xylanolytic enzymes, which are necessary for the debranching of substrate.

Xylanase production is found to be inducible in nature and smaller molecules, which are the degradation products of complex polysaccharide xylan, can act as inducers. A significant factor for higher xylanase production is the choice of an appropriate substrate. The substrate not only serves as carbon and energy source, but may also result into the production of inducing molecules following its utilisation. A major share of overall production cost corresponds to substrate, therefore, rather expensive, commercially available purified xylan is not appropriate for large scale production of enzyme. Although these can be excellent substrates and are frequently used for laboratory scale preparations and result in increased yields of xylanases. Replacement or supplementation of expensive, highly purified, commercially available xylan with some weedy and abundantly available plant biomass as a source of inducer for the xylanase biosynthesis offers a promising option to achieve a substantial decrease in the production cost.

Co-cultivation of two fungi which produce xylanase and laccase in their respective monoculture system is a budding approach for development of a mixed enzyme preparation possessing both xylanase and laccase activity. Both xylanase and laccase act synergistically and simultaneously on defined components of plant cell wall, resulting into appreciably effective biopulping and biobleaching.

Superior productivity, improved product recovery, reduced risk of contamination, low capital investment, simple technique, reduced energy requirement, closeness to the natural way of life for many microorganisms, low waste water output are some advantages of solid-state fermentation (SSF) over submerged fermentation. SSF may be defined as growth of microbes in absence or near absence of free flowing water on the surface of a natural substrate or inert solid support. The solid substrate not only supplies the nutrients to the microbial cultures, but also serves as an anchorage for the cells. A large number of agroresidues (sugar cane bagasse, wheat bran, wheat straw, corn cobs, rice husk, maize bran and many more) which are generated in huge quantities during agricultural practices are considered as the best substrates for SSF. Utilisation of these agro-residual materials in bioprocesses can be part of environmental pollution control on one hand and their consumption for production of cost effective products of commercial significance on the other hand, thus changing its status from waste to the potential provider. SSF holds a tremendous potential for the production of enzymes both under mono or dual culture conditions. It can be of special interest in those processes where the crude fermentation product may directly be used as the source of enzyme for example in food industry, animal feeds and paper-pulp industry (Tengerdy and Szakacs, 2003).

In order to save the environment from hazards of the chemical bleaching, an approach involving use of biological agent has been the major aim of the present investigation. Further, so as to develop a system for over production of the high quality xylanases and to analyze the preparation for its application in biobleaching process, the proposed work has the following as the major outlines of the objective:

a. Exploration of microbes from different sources mainly from soda ash and paper industry effluents. Screening of isolated strains for selecting strain producing alkalitolerant and cellulase free xylanases. Genetic manipulation of the selected fungal strain and derivation of cultural and nutritional conditions for achieving enhanced production of enzyme.

b. Analysis of enzyme inducibility by employing various substrates, inducer molecules and molecular characterisation of the enzyme.

c. Evaluation of co-cultivation conditions employing xylanase and ligninase producing fungal strains and analysis of its feasibility to the different fermentation systems, mainly submerged, surface and solid-state systems for evaluating production of the enzymes.

d. Evaluation of the mixed enzyme preparation for its biobleaching applications to the pulp and paper industry.

e. To design a bioprocess for scaling up the production of the enzymes through co-cultivation system.

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

CHAPTER II

LITERATURE REVIEW

Chapter - 2

LITERATURE REVIEW

Xylanases are hydrolytic enzymes that depolymerize the plant cell wall component xylan that is the second most abundant polysaccharide. This xylan degradative ability of xylanases had been explored for production of a variety of products with distinct commercial applications. Growing concern of people and environmentalists promote the implementation of enzyme alternative in place of existing chemical technologies. One such area is the pulp and paper industry where huge quantities of raw materials are processed which also undergo large scale chemical treatments. Xylanases-assisted removal of lignin from pulp is the basis of biobleaching which had began initially as an idea and now has reached to realistic proportions whereby these can be considered as worthwhile promising technologies of the future. Intense research and developmental activities so as to have a technology that would eventually be independent from using chemicals for bleaching would be a cherished dream come true.

2.1 XYLAN: STRUCTURE AND OCCURRENCE

The plant cell wall represents half of the organic carbon in the biosphere and consists of three major components i.e. cellulose, hemicellulose and lignin (Hazlewood and Gilbert, 1998). Out of these three polymers, hemicellulose is the second most abundant polysaccharide in nature accounting for 40% of the total carbohydrate content (Thomson, 1993). Xyloglucan, glucomannan, galactomannan and arabinogalactan constitute minor proportion of hemicellulose (Shallom and Shoham, 2003). Xylan as major hemicellulosic component, acts like a glue and binds with cellulose through non-covalent linkages and has covalent linkages with lignin. It is responsible for maintaining the structural integrity of plant cell wall (Kulkarni et al., 1999; Beg et al., 2001; Collins et al., 2005; Polizeli et al., 2005). Xylan is a complex, highly branched heteropolysaccharide. The homopolymeric back-bone of xylan consists of 1,4-linked-

β-D-xylopyranosyl units. It has varying degree of side group substitution with glucuronopyranosyl, α-L-arabinofuranosyl, acetyl, feruloyl or ρ-coumaroyl groups (Li et al., 2000). Linear unsubstituted xylan has also been reported in esparto grass, tobacco and certain marine algae. Xylopyranosyl residues occasionally are also linked with β-1, 3-linkage. Besides this, hardwood and softwood xylan are quite distinct. Table-1 shows some major differences between hard and soft wood xylan.

Table. 1 Major differences between hardwood and softwood xylan

Sl. No.	Properties	Hardwood xylan	Softwood xylan
1.	Degree of polymerisation	Higher degree of polymerisation (150-200 β-D-xylopyranosyl residues)	Lower degree of polymerisation (70-130 β-D-xylopyranosyl residues)
2.	Xylan content	15-30% of cell wall components	7-10% of cell wall components
3.	Side groups	Hardwood xylans are generally acetyl xylans	Softwood xylans are arabinoxylans
4.	Examples	Eucalyptus, Birch wood	Coniferous trees: Pine, Spruce etc.

2.2 ENZYMATIC DEGRADATION OF XYLAN

Owing to its heterogenous, complex highly branched polymeric structure, xylan degradation needs not just one enzyme but a synergistic action of a complex of several hydrolytic enzymes with diverse specificity and modes of action. Therefore, production of an arsenal of polymer degrading proteins by xylan degrading cells is not surprising. The xylanolytic enzyme system carrying out the xylan hydrolysis is usually composed of a repertoire of hydrolytic enzymes i.e. β- 1,4- endoxylanase, β-xylosidase, α-L-arabinofuranosidase , α-D-glucuronidase, acetyl xylan esterase, ferulic and ρ-coumaric acid esterase (Fig.1).

2.2.1 Endo-1, 4- β-D-xylanase (β-1, 4-D-xylanxylanohydrolase; E.C. 3.2.1.8)

Endo-1, 4- β-D-xylanase (EC 3.2.1.8) randomly cleaves the xylan back bone at endo-β-1, 4-xylosidic linkage, resulting in a decreased degree of polymerisation of the substrate

and release of xylooligosaccharides. The site of enzymatic hydrolysis depends on the nature of the substrate molecule i.e. the chain length, degree of branching and the presence of substituents (Li et al., 2000). During the early course of hydrolysis, the pre-dominant products include xylooligosaccharides, which are further hydrolysed to xylotriose, xylobiose and xylose (Dekker and Richards, 1976; Wong et al., 1988). The endo-acting xylanases have been differentiated according to the end products released from the xylan hydrolysis (e.g. xylose, xylobiose and xylotriose, and/or arabinose) as non-debranching (arabinose non-liberating) or debranching (arabinose liberating) enzymes (Dekker and Richards, 1976).

Endoxylanases have been identified in *Trichoderma reesei* (Tenkanen et al., 1992; Lappalainen et al., 2000), *Aspergillus* sp. (Gawand and Kamat, 1999), *A. fischeri* (Senthilkumar et al., 2005), *A. foetidus* (Shah and Madamwar, 2005), *A. niger* FAS 128 (Tapingkae et al., 2007), *A. carneus* M34 (Fang et al., 2007), *Cellulomonas* NCIM 2353 (Chaudhary and Deobagker, 1997), *Streptomyces* sp. (Beg et al., 2000), *Staphylococcus* sp. SG-13 (Gupta et al., 2001), *Bacillus* sp. (Mamo et al., 2006 ; Battan et al., 2007), *Streptomyces olivaceoviridis* A1 (Wang et al., 2007) and many other microorganisms (Kolenova et al., 2005; Lin et al., 2005).

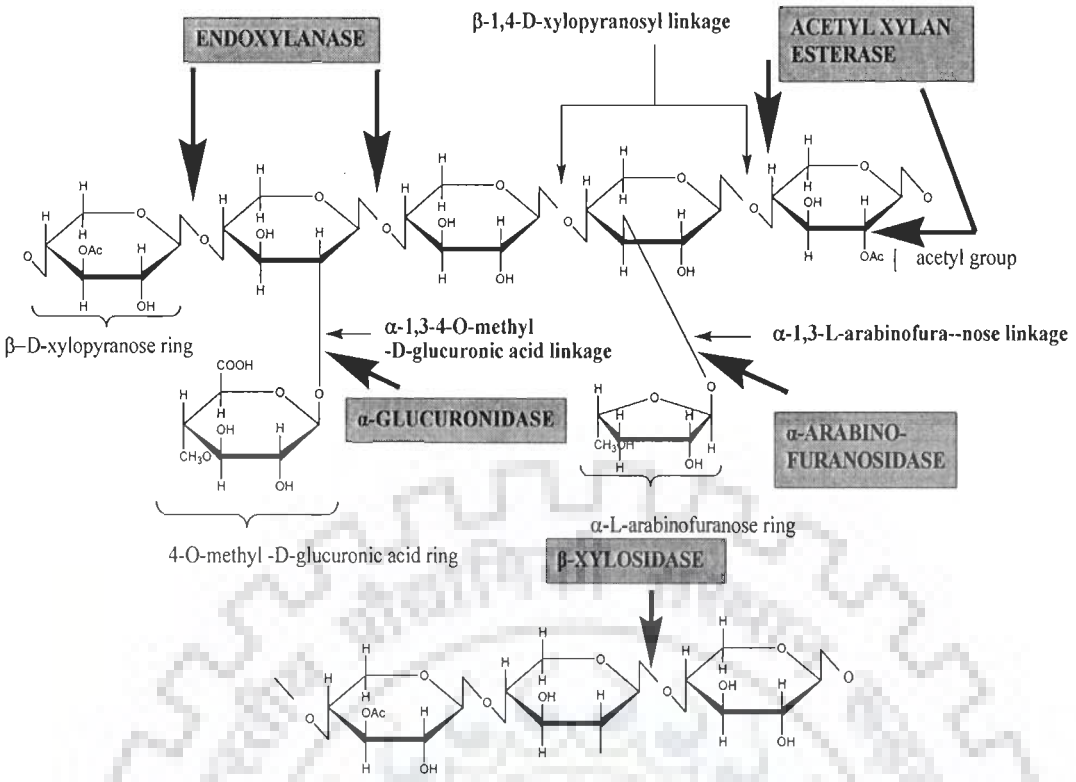


Fig. 1 Structure of xylan and various enzymes of xylanolytic system.

2.2.2 β-D-xylosidase (xylobiase, β-1, 4-D-xyloside xylanohydrolase, EC 3.2.1.37)

β-D-xylosidases hydrolyse smaller xylooligosaccharides and xylobiose from the non-reducing ends and liberate monomeric xylose (Wong et al., 1988). Purified β-xylosidases usually do not hydrolyse xylan; their preferred substrate is xylobiose and their affinity for xylooligosaccharides is inversely proportional to their degree of polymerisation. Role of β-xylosidases is attributed following successive cleavages of xylan by endoxylanase. This leads into the accumulation of short xylooligomers, which may cause feed back inhibition of endoxylanase. The elimination of these xylooligomers, due to the activity of β-xylosidase, causes removal of inhibition and increases the level of xylan hydrolysis (Andrade et al., 2004). Fungal β-xylosidase may be retained within the mycelium, detected in cell extracts, or liberated in the growth medium (extracellular) while those produced from bacteria and yeast are mainly intracellular. These enzymes can be monomeric or dimeric having the molecular weight ranging from 60 to 360 kD. Bacteria and fungi containing β-xylosidases include *Trichoderma harzianum*

(Ximenes et al., 1996), *Cellulomonas flavigena* (Perez-Avalos et al., 1996), *Melanocarpus albomyces* IIS 68 (Saraswat and Bisaria, 1997), *Thermoascus* sp. (Matsuo et al., 1998), *T. koningii* G-39 (Li et al., 2000), *Aspergillus fumigatus* (Lenartovicz et al., 2003) and *Bacillus thermantarcticus* (Lama et al., 2004). The thermostability of enzyme may vary. A thermophilic β-xylosidase was reported from fungus *Sporotrichum thermophile* strain ATCC 34628 was stable up to a temperature of 50°C and retained over 58% of its activity after 1h at 60°C (Katapodis et al., 2006). Enhanced production of β-xylosidase was reported from mutant *P. stipitis*, obtained through chemical mutagenesis (NTG) of wild type strain (Basaran and Ozcan, 2008).

2.2.3 α-L-arabinofuranosidase (EC 3.2.1.55)

Arabinosidases (EC 3.2.1.55) are exo-acting glycoside hydrolases catalyzing hydrolysis of α-L-arabinofuranosidic moieties or α(1-2) or/and α(1-3) bonds linking an L-arabinofuranose to xylose moiety in polymers such as arabinoxylan. Various groups have described the production and purification of α- arabinofuranosidase from *Melanocarpus albomyces* IIS 68 (Saraswat and Bisaria, 1997), *Trichoderma reesei* (Nogawa et al., 1999) and *Penicillium purpurogenum* (Ioannes et al., 2000). An α -arabinofuranosidase 54 (*AkAbf54*) gene from *Aspergillus kawachii* was cloned and expressed in *Pichia pastoris*. The effect of site directed mutational analysis of N-glycosylation site on the biochemical properties of the recombinant arabinofuranosidase was analysed. Two N-linked glycosylation motifs (Asn^{83}–Thr–Thr and Asn^{202}–Ser–Thr) were found in the AkAbf54 sequence. Asn^{83} and Asn^{202}, the two residues together were replaced with glutamine by site-directed mutagenesis. The N83Q mutant had the enzyme with similar catalytic activity and thermostability as the wild-type enzyme. On the other hand, the N202Q and N83Q/N202Q mutants exhibited enzymes with a considerable decrease in thermostability as compared to the glycosylated wild-type enzyme. These observations suggest that the glycosylation at Asn^{202} may contribute to thermostability and catalysis (Koseki et al., 2006a). A gene encoding an α-arabinofuranosidase (*deAFc*) was isolated

from a culture seeded with a compost starter mixed bacterial population and cloned into *Escherichia coli* with a C-terminal His-tag. The recombinant enzyme appeared to be monomeric and showed a broad pH profile, with the activity being essentially invariant between pH 5 and 8.5. The enzyme had a temperature maximum of 47°C and its stability decreased rapidly at 55°C (Wagschal et al., 2007).

2.2.4 α-D-glucuronidase (EC 3.2.1.131)

The xylan backbone is substituted with 4-O-methyl-α-D-glucopyranosyl, or α-D-glucopyranosyl acid groups. α-Glucuronidase (EC 3.2.1.131) hydrolyses the α-1,2-linkage between 4-O-methylglucuronic acid and xylose. Purified α-glucuronidases appeared to be large protein with molecular mass of around 100 kD. Purification of α-glucuronidase was initially reported from *Thermoascus aurantiacus* (Khandke et al., 1989). The analysis of α-glucuronidase structure from *Pseudomonas cellulosa* revealed three domains, the centre of which is a $(\beta/\alpha)_8$ barrel housing the catalytic apparatus (Nurizzo et al., 2002). The α-glucuronidase gene (*aguA*) of *Aureobasidium pullulans* NRRL Y-2311-1 was cloned and expressed in *Saccharomyces cerevisiae* Y294. Maximal activity was obtained at 65 °C and at pH 5.0- 6.0 (de Wat et al., 2006). Cloning, sequence analysis and expression profile of the α-glucuronidase from *Talaromyces emersonii* was also described (Heneghan et al., 2007).

2.2.5 Acetyl xylan esterase (EC 3.2.1.72)

Acetyl xylan esterases are responsible for deacetylation of xylan by removing acetate residues from the backbone of xylan. Deacetylation improves accessibility of xylan for xylanases. The enzyme has been isolated from many microorganisms including *Thermobifida* (Liu and Yang, 2002) and *Penicillium* (Chavez et al., 2006). Yang and Liu, (2008) had purified and analysed biochemical properties of acetyl xylan esterase from *Thermobifida fusca*. Though most of the acetyl xylan esterases are found in mesophilic microorganisms, enzyme was also detected in a thermophilic actinomycetes, *Thermomonospora fusca* (Bachmann and McCarthy, 1991). An acetyl xylan esterase gene from *Aspergillus oryzae* was cloned and expressed in *Pichia pastoris* (Koseki et al., 2006b).

2.2.6 Ferulic acid esterase (EC 3.1.1.73) and *p*-coumaric acid esterase (EC 3.1.1-)

Ferulic acid esterases and *p*-coumaric acid esterases are responsible for cleaving the ester-linkage on the main chain of xylan; the first one breaks the bond between arabinose and ferulic acid side groups, while second cleaves the bond between arabinose and *p*-coumaric acid (Crepin et al., 2004). Carbohydrate esters of ferulic acid can also be involved in ether linkages with lignin components providing the linkage between lignin and hemicellulose. Notable levels of feruloyl esterase along with other accessory enzymes of xylanolytic enzyme system had been reported in *Fusarium proliferatum* (Shin and Chen, 2006), *Penicillium brasilianum* (Panagiotou et al., 2007), *Talaromyces stipitatus* and *Humicola grisea* var *thermoidea* (Mandalari et al., 2008).

2.2.7 Synergism

Different enzymes of xylanolytic enzyme system degrade xylan in different manner. All of these enzymes act synergistically and action of one enzyme cooperatively increases the activity of other. It was observed that fungal esterases and xylanases act synergistically during the hydrolysis of acetyl xylan. The principle behind this phenomenon is that acetyl xylan esterase releases side chains of acetyl esters from acetyl xylan and creates more new sites on the xylan for the subsequent attack by xylanases. Bachmann and MaCarthy, (1991) investigated the interaction between endoxylanases, α-L-arabinofuranosidases, β-xylosidases and acetyl esterases from the thermophile *Thermomonospora fusca.* Similarly, complex synergistic action was observed among different enzymes of xylanolytic system of *Aspergillus* during degradation of water insoluble pentosan from wheat flour. Degradation of the xylan backbone by endoxylanase and β-xylosidase was affected most strongly by the action of α-arabinofuranosidase (de Vries et al., 2000).

2.2.8 Catalytic mechanism

Hydrolysis reaction, catalysed by xylanases proceeds through an acid base mechanism and involves two amino acid residues (Fig. 2). The first residue acts as a general

catalyst and protonates the oxygen of the glycosidic bond. The second residue acts as a nucleophile, which interacts with the oxycarbonium intermediate or promote the formation of an OH^- ion from a water molecule. Xylanases mainly exhibit a double displacement mechanism involving a glycosyl enzyme intermediate which is formed and hydrolysed via oxycarbonium ion (Kulkarni et al., 1999). Further, crystallographic studies had indicated that GLU ^{78} and GLU ^{172} act as nucleophile and acid base catalyst respectively. Xylanase of family-11 generally follows double displacement mechanism while family-10 xylanases catalyse by single displacement reaction.

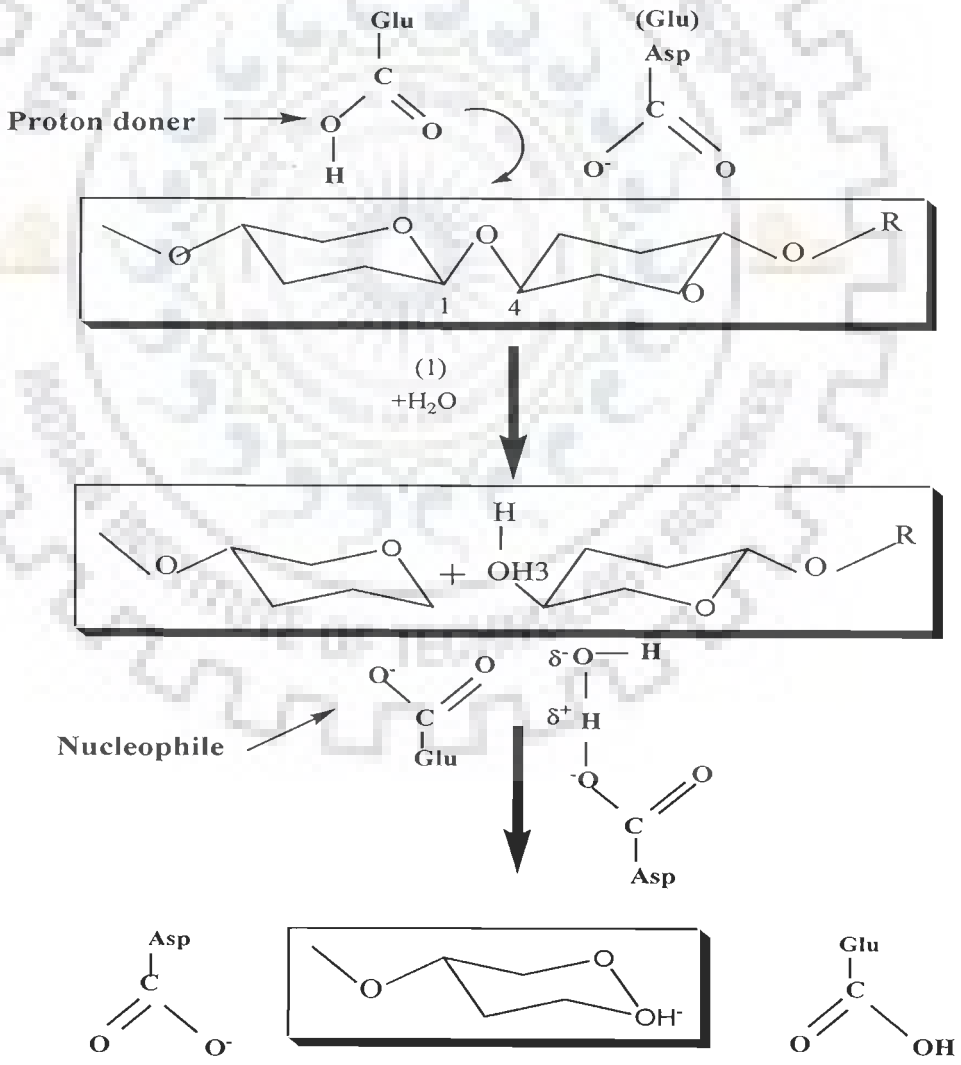


Fig. 2 Xylanase mediated catalysis by single displacement mechanism.

2.3 SOURCE OF XYLANASES

The presence of xylanase in groups of organisms including bacteria, algae, fungi, plants, protozoans, gastropods and arthropods confirm their widespread occurrence among living world (Dekker and Richards, 1976; Prade, 1995). The physiological role of xylanase in microbes concerns with the production of xylose, a primary carbon source for cell metabolism during growth and plant cell infection by plant pathogens. Intracellular and extracellular xylanases from various bacterial and fungal sources have been studied.

2.3.1 Xylanases of bacterial origin

Xylanases occur commonly among bacteria and production of xylanases from both aerobic and anaerobic bacteria has been observed. Among aerobic or facultative anaerobic species, xylanolytic activity has been detected from *Bacillus* sp. JB-99 (Virupakshi et al., 2005), *B. subtilis* (Yuan et al., 2005a), *B. circulans* (Heck et al., 2006) and *B. pumilus* (Asha poorna and Prema, 2007; Kapoor et al., 2008). The various *Streptomyces* species having activity includes *S. halstedii* JM8 (Ruiz-Arribas, 1995; Diaz et al., 2004), *S. lividans* (Faury et al., 2004), *S. olivaceoviridis* (Jiang et al., 2005; 2006), *Thermobifida fusca* (Kim et al., 2004; Chen and Wilson, 2007), *S. cyaneus* (Ninawe et al., 2008) and *S. thermonitrificans* (Cheng et al., 2008). Anaerobic microbes, which grow under mesophilic conditions, include *Clostridium absonum* CFR-702 (Swaroopa Rani and Nand, 2000), *C. thermocellum* (Kimura et al., 2003), *C. stercorarium* (Araki et al., 2004) and *C. cellulovorans* (Xu and Foong, 2008).

In addition, gram negative, aerobic, non-spore forming soil microbe, *Pseudomonas fluorescens* subsp. *cellulosa*, has also been shown to degrade xylan (Gilkes et al., 1991; Uffen, 1997). Other *Pseudomonas* type xylanolytic gram-negative bacteria seem to reside primarily in ruminant animals. These cells represent a large, fastidious anaerobic group of cells that include *Bacteriodes ovatus* (Whitehead and Hespell, 1990), *Butyrivibrio fibrisolvens* (Utt et al., 1991; Kobayashi et al., 1998) and *Ruminococcus flavefaciens* (Aurilia et al., 2000).

2.3.2 Xylanases of fungal origin

Fungal xylanases offer several benefits over bacterial in terms of higher titre and being extracellular hence having their relatively easier purification. Fungal xylanase show synergism and accessory enzymes of xylanolytic system are also secreted along with endoxylanases. However, disadvantage associated with fungal xylanases is due to the accompanying associated cellulase activity. Various studies have been taken up for xylanase production from fungi mainly *Penicillium capsulatum* (Filho et al., 1996), *Fusarium oxysporum* (Christakopoulos et al., 1996), *Aspergillus foetidus* (Shah and Madamwar, 2005), *Ceriporiopsis subvermispora* (Milagres et al., 2005), *Trichoderma viride* (Liu et al., 2006), *A. carneus* (Fang et al., 2007), *A. niger* (Tapingkae et al., 2007), *Trichoderma longibrachiatum* (Kadi and Crouzet, 2008) and *Fusarium graminearum* (Carapito et al., 2009).

2.3.3 Xylanases from yeasts

Around 95 strains of yeasts and yeast like organisms have been observed to have xylanase production when grown on xylose, xylan from deciduous trees and hemicellulose from conifers. *Cryptococcus albidus* was observed to produce xylanase and β-xylosidase simultaneously (Biely, 1981). *Aureobasidium pullulans* (Leathers et al., 1984) and *Trichosporon cutaneum* (Liu et al., 1999) had also been observed to produce extracellular endoxylanase.

2.3.4 Xylanases from extremophiles

The enzymes from extremophilic organisms could prove to be of profound industrial significance. The study of extremophiles and their enzymes can expand the understanding of protein chemistry in addition to its applications as biocatalyst. Their compatibility with industrial operating conditions consisting of pH and temperature variations delineates these to be economically viable. Attempt to disseminate the unexplored biodiversity of extreme environment is a step towards isolation and identification of newer enzymes which should have stability under unfavorable conditions of pH and temperature. Xylanase producing thermophilic

fungi include *Melanocarpus albomyces* (Jain et al., 1998), *Paecilomyces thermophila* (Li et al., 2006; Yang et al., 2008), *Marasmius* sp. (Ratanachomsri et al., 2006), *Termitomyces* sp. (Faulet et al., 2006) and *Thermoascus aurantiacus* (Battan et al., 2007). Among bacteria, mainly *Thermomonospora fusca* (Irwin et al., 1994), *Clostridium absonum* CFR-702 (Swaroopa Rani and Nand, 2000), *C. acetobutylicum* (Marichamy and Mattiason, 2005), *Sreptomyces cyaneus* (Ninawe and Kuhad, 2006), *Bacillus halodurans* (Mamo et al., 2006) and *B. pumilus* (Battan et al., 2007) have been observed to produce thermophilic xylanase. Xylanase from these organisms possess optimum temperature ranging between 60 -80° C. Attempts were made to define the thermostability of these enzymes and has denoted the presence of extra disulphide bridges, an N-terminal proline residue affecting into a reduced conformational variation, salt bridges and presence of hydrophobic side-chains characteristically lead to thermal stability (Turunen et al., 2001) Further, other minor modifications include, higher Thr/Ser ratio, increased charged residues especially Arg which result into enhanced polar interactions and improved stability due to compacting protein structure having higher number of ion pairs or aromatic residues on the protein surface (Hakulinen et al., 2003).

2.3.5 Xylanases from plants

Occurrence of xylanases is not only restricted to microbial population but also detected in higher plants. These hydrolases are involved in the degradation and reorganisation of cell wall polysaccharides during plant development and growth. Endoxylanases were detected in several fruits and cereal plants (Caspers et al., 2001; Wu et al., 2002 and Suzuki et al., 2002). Two xylan endohydrolases had been purified, characterised and their corresponding cDNAs and genes had been identified from barley (Caspers et al., 2001). Two β-D-xylosidases were purified, biochemically characterised and sequenced from young barley seedlings and *Arabidopsis thaliana* stems (Lee et al., 2001; Minic et al., 2004).

2.4 CLASSIFICATION OF XYLANASES

Taking into account the molecular weight and isoelectric point, Wong, (1988) had classified xylanases into two groups. All high molecular weight xylanases (>30 kD) with lower isoelectric point were subjected to one group while other group corresponded to xylanases of lower molecular weight (<30 kD) and high isoelectric point. Later on Henrissat et al. (1989) had classified cellulases and xylanases into six families (A-F) on the basis of amino acid sequence similarity and hydrophobic cluster analysis. In 1993, Henrissat and Bairoch upgraded these families and subdivided xylanase into two families that are family F/10 and family G/11, comprising high and low molecular weight xylanases respectively. Based on primary structure comparison of the catalytic domains, a precise classification system was proposed for glycosidases in general including xylanases. Currently 96 glycoside hydrolase families have been reported to exist (Coutinho and Henrissat, 1999). Enzymes within a particular family have a similar three dimensional structure and due to divergent evolution some families have the related three dimensional structures. Thus these families are grouped into higher hierarchical levels denoted as clans (Bourne and Henrissat, 2001). Presently, 14 different clans have been proposed (GH-A to GH-N), with most clans encompassing two to three families, apart from clan GH-A which currently contains 17 families. Within this classification system xylanases mainly are confined to family-F/10 and family-G/11. But enzymes with xylanase activity are also found in families 5, 7,8,16,26,43,52 and 62. Xylanase in families 5,7,8,10,11 and 43 contains a distinct catalytic domain with a demonstrated endo-1, 4-β-xylanase activity while xylanases of families 16, 52 and 62 are bifunctional with two catalytic domains.

2.5 XYLANASE MULTIPLICITY

Multiplicity corresponds to existence of multiple forms of the similar enzymes, which perform the `same function but differ in their physicochemical properties, such as molecular weight, isoelectric point etc. Such multiple forms of xylanases had been observed in

Penicillium purpurogenum (Belancic et al., 1995), *Aspergillus fischeri* (Chandra and Chandra, 1996), *Melanocarpus albomyces* IIS 68 (Saraswat and Bisaria, 1997), *Streptomyces* sp. strain AMT-3 (Nascimento et al., 2002), *Bacillus firmus* (Tseng et al., 2002), *Aeromonas caviae* (Liu et al., 2003), *Myceliophthora* sp. (Badhan et al., 2004), *Aspergillus foetidus* (Shah and Madamwar, 2005) and *Schizophyllum commune* (Kolenova et al., 2005). A higher extent of multiplicity was observed in *Fusarium graminearum*. A set comprising of 30 xylan related genes had been transcribed when the same was cultured in presence of different carbon sources like hop cell wall, xylan, xylose and carboxymethyl cellulose (Hatsch et al., 2006). Possible reasons for this multiplicity may be due to differential m-RNA processing, post translational modifications such as glycosylation and auto aggregation (Biely, 1985). Multiple xylanases can also be the product from different alleles of the same gene (Wong et al, 1988). However, some of the multiple xylanases may be due to the existence of independent genes (Hazlewood and Gilbert, 1993).

2.6 XYLANASE BIOSYNTHESIS

Xylanase synthesis in bacteria and fungi has been observed to be inducible in nature. Minimal level of xylanase is constantly present in the cell and due to its activity, substrate is degraded and small signaling molecules are generated. These molecules enter the cell and affect the induction of xylanase gene. However, in some organisms constitutive expression of xylanase had also been reported e.g. in *Schizophyllum commune* (Haltrich and Steiner, 1994) and in some other microbes (Srivastava and Srivastava, 1993). In general, xylanase induction is a complex phenomenon and level of induction varies within the organisms. An inducer amplifying xylanase activity in one species, may be the inhibitor of activity in other species (Hrmova et al., 1989). The potent inducer molecules are xylose, xylobiose, xylooligosccharides, heterosaccharides of xylose and glucose and their positional isomers. Purified xylan is reported to be a potent inducer for achieving maximum xylanase production in several organisms like *Bacillus polymyxa* (Pham et al., 1998), *Aspergillus awamori* (Sidenberg et al., 1998), *Fusarium oxysporum* (Kuhad et al., 1998; Jorge et al., 2005), *Trichosporon cutaneum* SL409 (Liu et al., 1999), *Streptomyces* sp.

strain AMT-3 (Nascimento et al., 2002), *Aspergillus foetidus* (Shah and Madamwar, 2005) and *Trichoderma harzianum* 1073D3 (Seyis and Aksoz, 2005). Xylobiose is also found to be an effective inducer in a range of microorganisms together with other xylooligosaccharides (Pinaga et al., 1994; Samain et al., 1997; Rajoka, 2005). There is evidence that positional isomers of xylobiose such as 1, 2- β-xylobiose and 1, 3- β-xylobiose are comparatively effective inducers than to simple cleavage product from xylan. As reviewed by Bajpai (1997), xylose appeared to act as repressor for xylanase biosynthesis in several organisms and considered to be under derepression control (Samain et al., 1997; Rajoka, 2005). Another possible reason for inhibitory effect of monosaccharides (xylose and glucose) is catabolic repression modulated by a catabolite repressor protein Cre 1. But in few organisms like *Aureobasidium pullulans* (Leathers et al., 1984), *Bacillus pumilus* (Paul and Verma, 1990) and *Streptomyces lividens* (Kluepfel et al., 1990) higher enzyme yields were obtained in presence of xylose. For β-xylosidase production, xylose always acts as an inducer. Enhanced levels of β-xylosidase were obtained in presence of xylose from *Cellulomonas flavigena* (Rajoka, 2005).

From economic and environmental point of view, agricultural and forestry residual materials have great potential for the production of xylanase due to the presence of xylan in plant cell wall. Levels of production depend upon various key factors such as substrate type, structure and accessibility etc. The use of sugarcane bagasse and grass as low cost raw material for xylanase production by *Bacillus circulans* D1 in submerged fermentation was investigated (Bocchini et al., 2005). 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 prepared at low cost. Fig. 3 shows the mechanism of xylanase biosynthesis. Xylan degrading organisms are often cellulolytic and secrete complex mixture of xylanases and cellulases concurrently (Nascimento et al., 2002). In a number of organisms xylanase synthesis not only occurs in the presence of xylan, but also in presence of cellulose especially in *Trichoderma reesei.* Selective production of xylanase is possible in presence of xylan or xylobiose but in presence of cellulose or cellobiose both xylanase and cellulase activities were reported in *T. reesei* (Hrmova et al., 1986) and *Aspergillus terreus* (Harmova et al., 1989).

The regulation and biosynthesis of xylanase at molecular level is comparatively less investigated. An analysis of DNA fragments containing β- xylanase genes from *Bacillus pumilus*, indicated that xylanase and xylosidase genes are closely associated and linked in a 14.4-Kb DNA fragment (Kulkarni et al., 1999). Coordinate control of xylanase and β-xylosidase gene was also reported in *Butyrivibrio fibriosolvens* GS-113 (Utt et al., 1991; Rajoka, 2005).

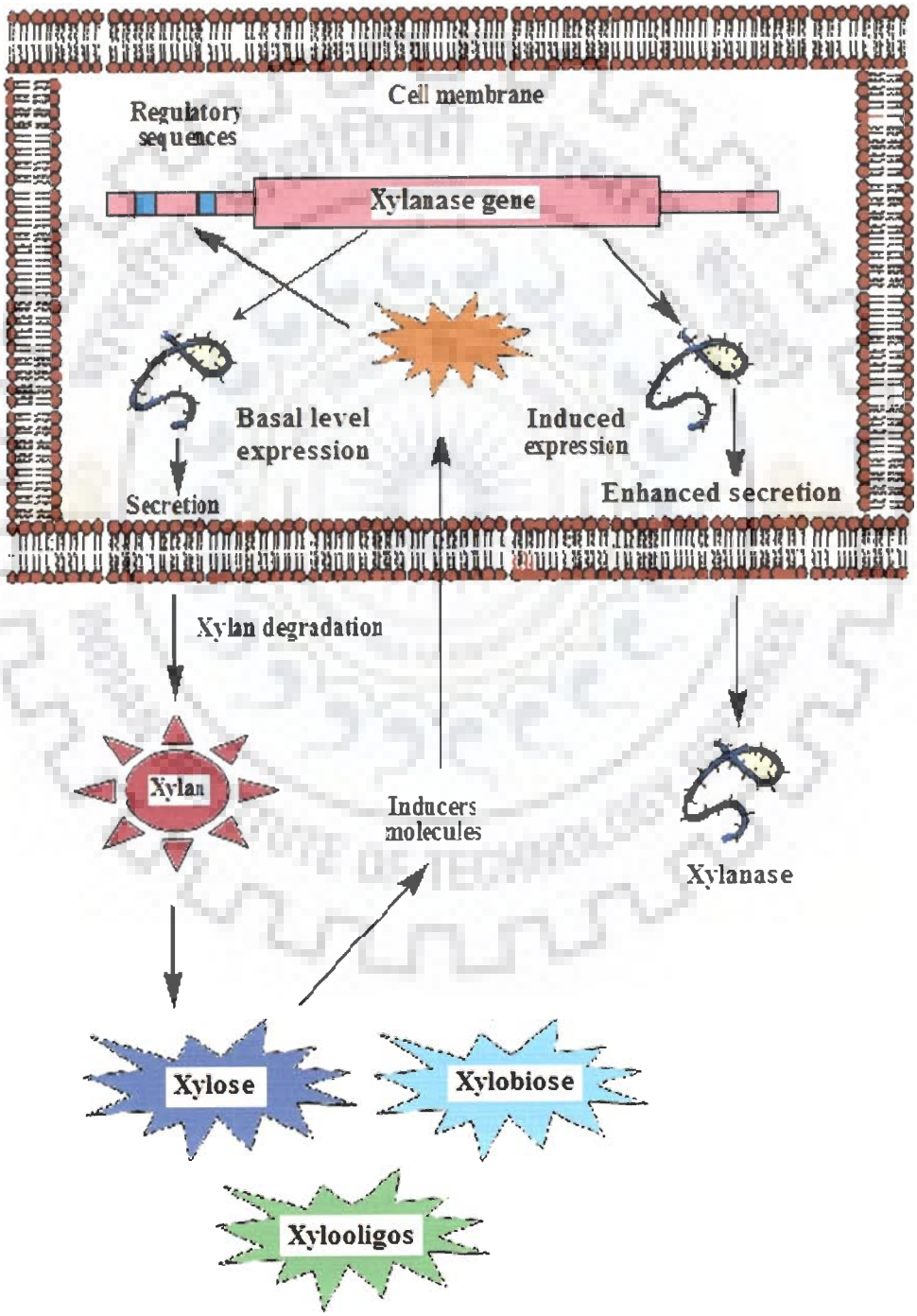


Fig. 3 Mechanism of xylanase biosynthesis.

2.6.1 Structure of xylanase gene

In *Caldicellulosiruptor* Rt8B.4 a thermophilic bacterium, xylanase gene (*xyn-A*) was detected in operon consisting of genes associated with xylose metabolism. Screening of genomic library of *Aspergillus oryzae* KBN616 showed that the structural part of the gene is 725 bp long and was interrupted by a single intron of 62 bp. The intron / exon junction followed GT-AG rule and the size of the introns resembled with introns of other filamentous fungi. The open reading frame consisted of 221 amino acid residues. Comparison of amino acid sequence showed the presence of a 32 amino acid long signal sequence at the amino terminal of precursor xyn-G_1. The 5' non-coding region of the gene contained demarcated consensus sequences. A consensus sequence (TATAAA) which is similar to eukaryotic promoter sequence i.e. TATA box (TATAAAT) exists 86 bp upstream from the translation initiation site. Furthermore, a CT rich sequence, that is considered as a fungal promoter element, was located just downstream of the TATA box like sequence. A similar promoter sequence (TATAAT) was also detected in *Bacillus* sp. and was located 125 bases upstream of the ribosome binding site. Promoter like sequence and typical Shine-Dalgarno (SD) sequence were observed upstream from the possible start codon (TTG instead of ATG). A 14-bp inverted repeat, corresponding to a transcriptional terminator, occurred downstream from the TAG stop codon (Nakamura, 2003). The nucleotide sequence from *Streptomyces thermoviolaceus* OPC-520 showed that genes *stx-II* and *stx-III* were clustered on the genome. GGAG, the potential ribosome binding sites were located upstream of each initiation codon ATG. A characteristic A, T rich promoter sequence was found upstream of the ribosome binding site (Kobayashi et al., 1998). Xylanase gene expression was down regulated in the presence of easily metabolizable sugars such as glucose. Catabolic repression is the main reason behind this which is a common phenomenon observed during xylanase biosynthesis. The catabolic repression consensus sequence identified was TGT/AAANC/GNTNA/TGA, where underlined letters represented the most critical bases (Kulkarni et al., 1999).

2.7 APPROACHES TO ENHANCE THE PRODUCTION LEVEL

A significantly higher level of enzyme activity is a prerequisite for production of a particular product at commercial scale through microbes. The production level of the product of interest in naturally occurring strains is, in most cases, too low for commercial exploitation. Multiple approaches may be recruited for achieving improvement in the levels of production.

2.7.1 Genetic manipulation

2.7.1.1 *Mutagenesis*

Mutagenesis of a wild type strain is widely used for achieving improved levels of production. Mutagens, either chemical or physical, confer genetic changes and resultant mutants are then screened to select the one with improved productivity. Among physical mutagens, UV irradiation was employed for obtaining improved levels of xylanase in *Fusarium oxysporum* (Singh et al., 1995), *Aspergillus niger* GCBMX-45 (Butt et al., 2002) and in *Thermomyces lanuginosus* (Bakalova et al., 2002). Chemical mutagenesis was performed in the presence of various alkylating agents like N-methyl-N'-nitro-N-nitrosoguanidine for achieving increased levels of xylanase (Singh et al., 1995; Mayorga-Reyes and Ponce-Noyola, 1998; Bakalova et al., 2002), glucose-6-phosphate dehydrogenease (Liu et al., 2003), citric acid (Ikram-ul et al., 2004) and ethyl methane sulphonate for achieving improved levels of polysaccharide (West, 2002), citric acid (Lotfy et al., 2007) and cellulase (Adsul et al., 2007) production. In addition, ethidium bromide and acridine orange were also used. Ethidium bromide was used for achieving enhanced levels of laccase and cellulase (Dhawan et al., 2003) and (Chand et al., 2005) respectively.

2.7.1.2 *Gene cloning*

Genetic engineering has been a good alternative of traditional approaches for strain improvement. Till date xylanase genes from various sources have been isolated, cloned and

expressed into suitable hosts. Both homologous as well as heterologous hosts had been used to clone xylanase gene. The choice of a suitable host vector system is a major factor in order to achieve higher degree of success. Active recombinant xylanase HXYN2 was produced from *Trichoderma reesei* transformants harboring the xylanase gene (*xyn2*) of *Humicola grisea* var *thermoidea* under the control of cellobiohydrolase I (*cbhI*) promoter. The heterologous HXYN2 enzyme is processed in *T. reesei* in a similar way to its endogenous xylanases (de Faria et al., 2002). The mature peptide of *Aspergillus niger* xylanase A (AnxA) was successfully expressed in *Pichia pastoris* and 1.9 times higher level of production was obtained under the control of *AOX1* promoter (Liu et al., 2006). *Pichia pastoris* was also employed to express the recombinant xylanase from *A. niger* (Berrin et al., 2000; Korona et al., 2006), *Thermomyces lanuginosus* (Damaso et al., 2003) and *A. terreus* (Chantasingh et al., 2006) and an increment was achieved in the production level. The foreign xylanase gene, *xynA* from *Eubacterium ruminantium* was successfully introduced, replicated and expressed in *Butyrivibrio fibrisolvens* with 5-6 times higher levels of expression (Kobayashi et al., 1998). Xylanase genes from various microorganisms like *Cellulomonas pachnodae* (Cazemier et al., 1999); *Bacillus lyticus* (Srivastava and Mukherjee, 2001), *Bacillus subtilis* strain B10 (Huang et al., 2006), *Aspergillus usamii* (Zhou et al., 2008) and *Streptomyces thermonitrificans* NTU-88 (Cheng et al., 2008) were isolated, cloned and expressed in *E. coli*. The xylanase gene from a thermophilic bacterium *Thermotoga maritima* (*xynB*) was cloned and over expressed in *Escherichia coli* (Wu et al., 2008). Heterologous cloning of xylanase gene from thermophilic microorganism resulted into a recombinant strain producing xylanases with improved thermostability. Xylanase gene *xynB* from the thermophilic bacterium *Dictyoglomus thermophilum* was cloned and expressed in the filamentous fungus *T. reesei* (Te'O et al., 2000). The xylanase gene of *Bacillus circulans* Teri-42 was cloned in both *B. subtilis* and *E. coli*. The production of recombinant xylanase in *B. subtilis* was six times higher and was found to be stable at higher temperature (60°C) (Qureshy et al., 2000). A recombinant gene *XylB* encoding endo-1,4-β-xylanase, obtained from *A. niger*

BCC14405, was successfully cloned and secreted as a 21 kD protein in *Pichia pastoris* under the control of *AOX1* promoter. Maximum level of activity of the recombinant xylanase was observed at 55°C which was 5°C higher than native xylanase (Ruanglek et al., 2007)

2.7.2 Co-cultivation

Emphasis lately has been given towards using mixed culture for obtaining higher biosynthetic activity as well as for achieving desired goals in an effective manner. During co-cultivation, cultures interact in a complex manner which may have distinct effects on their morphology and physiology. The interactions between two organisms in a mixed culture could be competitive or may show neutralism, mutualism, commensalism or amensalism behaviour. Mixed fungal associations resulted into higher biosynthetic activity that had led into improved levels of degradation of nitrocellulose (Sharma et al., 1995), cellulose (Duenas et al., 1995), herbicide metachlor (Sanyal and Kulshrestha, 2003) and lignin (Ferreira Gregorio et al., 2006; Chi et al., 2007). The mixed culture fermentation has also been observed to be advantageous as compared to single culture in terms of increased levels of hydrolytic enzyme production (Garcia-Kirchner et al., 2002; Banerjee et al., 2005; Huang et al., 2008). Holmgren and Sellstedt (2008) identified the compatible fungi that are capable of increasing ethanol production from lignocellulose in spent sulfite liquor in co-culture with *Saccharomyces cerevisiae*.

2.7.3 Medium design and process optimisation

Besides manipulating organism, alterations in the medium constituents and culturing conditions is also a major tool for achieving improved level of production. The yield of the derived product from a microorganism to a greater extent depends upon its cultural and nutritional requirements.

2.7.3.1 *Medium constituents*

Medium components are significant prerequisite for obtaining optimal growth and high rates of product yield. Classical approaches to media design are frequently employed where

individual components such as carbon and nitrogen source are alternatively analysed as single variables. However, other strategies mainly statistical experimental design, evolutionary computational methods and artificial neural network are also employed to achieve significant improvements in product yields. Derivation of medium composition for alkalitolerant xylanase production by *Aspergillus fischeri* Fxn 1 in solid-state fermentation was accomplished by response surface methodology and central composite rotary design (Senthilkumar et al., 2005).

2.7.3.1.1 *Carbon and nitrogen sources*

The carbon source plays a pivotal role in determining the growth and enzyme production from microbes. During microbial production of xylanases that is inducible, substrate not only serves as energy source, but also generates the inducer components. Among carbon sources including mono, di, oligo and polysaccharides, purified xylan supports maximum xylanase production. The addition of xylose, glucose and other monosaccharides are not favorable due to quicker consumption by microbes. However, addition of glucose along with xylan appeared productive due to the shorter incubation time required for achieving maximum levels of enzyme production by *T. harzianum* 1073 D3 (Seyis and Aksoz, 2005). Uses of commercially available purified xylans are probably not technically feasible for enzyme production at industrial scale. Presence of xylan in plant cell wall makes use of agricultural and forest residual materials as an alternative and promising carbon source (Deschamps and Huet, 1985; Gomes et al., 1992). Besides, low cost or abundantly available substrates such as grasses like *Leptochloa fusca*, *Sesbania aculeate* and *Paspalum notatum* were utilised for xylanase production (Rajoka, 2005; Bocchini et al., 2005).

Different organic (peptone, beef extract, yeast extract, tryptone etc.) as well as inorganic nitrogen sources (NH_4Cl, $(NH_4)_2SO_4$, $NaNO_3$, NH_4NO_3 etc.) have been analysed for microbial production of xylanases. Higher titre of xylanase was achieved by using organic nitrogen source because amino acids are the constituents of such complex compounds which can be absorbed directly from media. Higher xylanase activity was observed by organic nitrogen sources, mainly,

peptone for *Fusarium oxysporum* (Kuhad et al., 1998), *Pleurotus ostreatus* (Qinnghe et al., 2003) and among inorganic compounds, $(NH_4)_2SO_4$ was most effective (Seyis and Aksoz, 2005). A combination of peptone, yeast extract and potassium nitrate appeared to stimulate xylanase synthesis in *Bacillus pumilus* ASH (Battan et al., 2007).

2.7.3.1.2 *Other medium constituents*

Production medium also contains inorganic salts like KH_2PO_4, $MgSO_4$, $CaCl_2$, NH_4^+ salts or NO_3^-, as a source of macro and micro nutrients for the microbial growth. Further, supplementation of medium with surfactants or fatty acids appeared suitable for increasing the production levels. It is assumed that these compounds increase permeability of cell membrane and, therefore, affect the secretion of certain proteins. Increased (40%) production of xylanase from *Fusarium oxysporum* was obtained by addition of oleic acid and olive oil (Kuhad et al., 1998). Stimulatory effect of rhamnolipids and tween-80 on xylanase production from *Trichoderma viride* under solid state fermentation condition was analysed (Liu et al., 2006). Addition of rhamnolipids was found to be more effective as compared to tween-80. Enhancement in xylanase production was observed by the addition of amino acids (phenyl alanine), vitamins (niacin) and surfactant (polyethyleneglycol) in *Bacillus pumilus* strain MK001 (Kapoor et al., 2007).

2.7.3.2 *Critical environmental parameters*

Microbial production of xylanase is affected by a wide range of environmental parameters, in addition to the effects due to the constituents of the culture medium as described in the previous sections.

2.7.3.2.1 *pH and temperature*

The pH of the enzyme production media markedly affects the growth and enzyme production in various bacteria and fungi. Acidic pH (4.0-6.0) generally favours fungal xylanases

(Kuhad et al., 1998; Qinnghe et al., 2003; Shah and Madamwar, 2005) while higher pH (7.0-9.0) favours bacterial xylanases (Battan et al., 2007). Although, xylanase production from various fungi and bacteria was observed at acidic and neutral pH, only few observations had pointed out the production of microbial xylanases at alkaline pH. *Micrococcus* sp AR-135, isolated from alkaline soda lake grows in a pH range of 8-11 but not at pH 7.0 (Gessesse and Mamo, 1998). Similarly, the optimal pH for xylanase production from *Bacillus pumilus* was in the range of 8.0-9.0 and at pH 7.0 and 10.0 activity was decreased (Asha Poorna and Prema, 2007). In several fungi, higher xylanase yield was obtained at a pH where the growth was limited. Maximum enzyme production by *Trichoderma lanuginosus* was obtained at pH 7.5, however the suitable pH for the growth of organism was found to be 6.5 (Purkarthofer et al., 1993). An interesting case of pH regulated production of xylanase isozymes was observed in *T. reesei* Rut C-30. The filamentous fungi respond to the changes in the environmental pH by modifying its enzyme production patterns. Four xylanase isoforms were observed in this fungus. Among these, xylanase I was produced at pH 4.0, xylanase III at pH 6.0 while xylanase II was produced at both the pH (Xiong et al., 2004).

Derivation of incubation temperature is also a fundamental part of bioprocess designing. The effect of incubation temperature on xylanase production by *Aspergillus niger* was investigated by Yuan et al., (2005b). The temperature shift from 33°C which is suitable for the growth of microbe to 27°C for xylanase production, significantly reduced the production time without any adverse effects on xylanase activity. Similar results were also reported by Stollnberger et al., (1996) for xylanase production by *T. reesei* Rut C-30 at 37 °C followed by a shift to 28 °C at the beginning of enzyme production phase. The effect of the temperature on growth, production and secretion of β-xylosidase by the thermotolerant fungi *A. fumigatus* was studied in submerged cultures. The β-xylosidase activity was predominantly in cell-bound form from the cultures that required an optimal temperature of 30°C, whereas the cultures that are

maintained at higher temperature i.e. 42°C had the activity in the cell free supernatants (Lenartovicz et al., 2003).

2.7.3.2.2 *Agitation*

Agitation is a significant parameter which affects the xylanase production mainly under submerged fermentation condition. Agitation provides adequate mixing for proper oxygen transfer, generates shear stress and mechanical force on filamentous fungi which ultimately affects the fungal morphology and enzyme secretion (Thomas, 1990). Mycelial damage and leakage of intracellular materials at high agitation speed, limits the volumetric biomass and enzyme productivity of the culture. Effects of agitation rate in combination with the aeration rate and dissolved oxygen tension on xylanase production have been studied by several groups (Hoq et al., 1994; Palma et al., 1996; Singh et al., 2000; Techapun et al., 2003). The effects of agitation rate on growth and extracellular xylanase production by *Aspergillus oryzae* NRRL 3485 was investigated in bioreactor cultures using spent sulphite liquor (SSL) and oat spelt xylan as the respective carbon substrates. It was observed that impeller tip velocities within the range of 1.56–3.12 m s^{-1}, equivalent to stirrer speeds of 400–800 rpm, did not have a marked effect on the xylanase activity in the *A. oryzae* culture. Even though the xylanase activity was not affected by agitation intensity but lower biomass generation was observed at higher impeller speeds (Chipeta et al., 2008).

2.8 FERMENTATION SYSTEMS

2.8.1 Surface fermentation

Surface fermentation is a conventional process, carried out in shallow pan and fungus is allowed to develop a mycelial mat over the fermentation medium. Purification and characterisation of a novel alkalophilic xylanase under static condition using corn stover as inducer was reported from mixed microbial community isolated from compost soil sample (Lv et al., 2008). The effect of culture condition on laccase production from *Phanerochaete chrysosporium* NCIM 1197 was evaluated and lower levels of laccase was observed under static

condition as compared to solid-sate fermentation condition (Gnanamani et al., 2006). Similarly, the effect of culture conditions on cellulase production from *Chaetomium erraticum* was studied and higher levels of exoglucanase and β-glucosidase was observed under static culture condition while submerged fermentation condition suited for the production of endoglucanase (Soni et al., 1999).

2.8.2 Submerged fermentation

The submerged fermentation process is well characterised where homogenous conditions can be maintained and mainly pH, temperature and dissolved oxygen can be suitably controlled and scaling up of the process is feasible under defined conditions. It is a well-defined system for the production of xylanolytic enzymes. Different workers have summarised the production of xylanase in submerged fermentation processes ranging from shake flasks to fermenters (Gomes et al., 1994; Singh et al., 1995; Nascimento et al., 2002; 2003; Shah and Madamwar, 2005). It is however an energy intensive process that involves high capital investment and also generates higher volumes of liquid wastes. These factors may therefore add on to the cost of production for commercial application of the process.

2.8.3 Solid-state fermentation

Growth of microorganisms on the surface of moist solid material in the absence or near absence of free flowing water is defined as solid-state fermentation (SSF). Microbes obtain water from moisture held within the substrates (Mitchell et al., 2002; Raghavarao et al., 2003). SSF offers several advantages over submerged fermentation like superior volumetric productivity, higher concentration of products, improved product recovery, less effluent generation, reduced risk of contamination, low capital investment, simple technique, reduced energy requirement, closeness to the natural habitat for many microorganisms and low waste water output (Lonsane et al., 1985). For the success of any SSF process, selection of a suitable strain, appropriate substrate and derivation of critical physico-chemical parameters are crucial (Pandey et al., 2001). Based on the microorganisms involved, SSF process can be grouped as,

natural (indigenous) SSF and pure culture SSF using individual strains or a mixed culture. Composting and ensiling are two main examples of SSF processes, which involves natural microflora. Bioprocess involving SSF for the production of a particular product of interest either primary or secondary metabolite, uses the pure culture or mixed culture of compatible microorganism. Filamentous fungi are the preferred group of microorganisms for solid-state fermentation. These are ideally suited to solid-state fermentation due to their hyphal mode of growth and physiological capabilities. The enzymatic system of filamentous fungi makes these well adapted to spread over and penetrate into solid substrate. The solid-state fermentation system which was termed as a low-technology system during the previous two decades, appears to be promising one for the production of value added, low volume-high cost products.

2.8.3.1 *Major factors affecting solid-state fermentation*

2.8.3.1.1 *Solid support*

The choice of an appropriate solid support is a major factor for the success of any SSF process. Cost, availability and chemical composition of solid supports are some major factors which should be considered during selection of support material for SSF. Availability of substrate in huge amounts at lower prices is desirable in order to make process economical. Agro-forestry residues which fulfill these requirements are suitable for SSF. These include crop residues such as wheat straw, rice bran, soy hull, cassava and sugarcane bagasse, corn cobs and coffee husk etc. Besides, residual materials from fruit processing industries such as pine-apple, banana and carrot processing wastes are also the promising substrates. Notably, higher yields of xylanase was obtained by using different agro-residues such as corn cobs (Ferreira et al., 1999; Shah and Madamwar, 2005), corn stover (Panagiotou et al., 2003), rice bran (Virupakshi et al., 2005), wheat straw (Yang et al., 2006), wheat bran (Khandeparkar and Bhosle, 2006), orange peels (Mamma et al., 2008) and distillery spent wash (Mohana et al., 2008). A mixture of wheat bran along with fish meal and soybean meal was also evaluated for xylanase production (Lu et al., 2003). In SSF these solid-supports not only provide support for microbial anchorage but also supply essential nutrients for microbial growth. Therefore, chemical composition of solid

supports is of prime significance during selection of support material.

2.8.3.1.2 *Moisture content*

Moisture content is an important parameter of SSF which determines the success of the process (Ramesh and Lonsane, 1990). The availability of moisture in solid-state fermentation can be expressed as either the water content or 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 and Molin, 2003; Lenz et al., 2004). Adjustment of the desired moisture level can be accomplished by various moistening agents like fermentation medium, distilled water and nutrient salt solution. A higher than optimum moisture level causes decreased porosity, alteration in particle structure, gummy texture, lower oxygen transfer and enhancement of the aerial mycelia (Narahara et al., 1982). Moreover, it leads to conglomeration of the substrate or sticking of the particles to the wall of the reactor and the substrate becomes more vulnerable to bacterial contamination (Lonsane et al., 1985).

Water activity is an indicator of water availability and is a critical factor in the solid-state fermentation. The water activity ($aw = Pm/Ps$) of the medium depends upon its moisture content and composition. It can be obtained by measuring the vapor pressure (Pm) in equilibrium with SSF medium of different moisture content and saturated vapor pressure (Ps) in equilibrium with pure water at 30 °C. The effect of water activity on xylanase production from *Aspergillus sulphureus* was studied by Lu et al. (2003). The optimal water activity for xylanase biosynthesis was in the range of 93–96%.

2.8.3.1.3 *Temperature*

Microbial growth under SSF condition generates metabolic heat. It is directly related to the metabolic activities of the microorganism and the depth of the substrate layer. Temperature regulation is an essential requirement for the success of SSF since temperature affects spore germination, growth, product formation and sporulation. However, heat removal

from the substrate tends to be insufficient, leading to the establishment of temperature gradient and localized overheating of the substrate. Temperature control is more difficult in SSF processes as compared to the submerged processes. Conventionally aeration is the major approach to control the temperature (Raimbault, 1998; Raghavaro et al., 2003). Because higher aeration rates can reduce the water activity of the substrate by evaporation, so water saturated air is usually used (Raghavaro et al., 2003). The agitation of the fermentation mass can also help to control the temperature. Scaled up production of xylanase in wooden trays by *Aspergillus sulphurous* under solid-state fermentation was analysed by Lu et al. (2003). Mild mixing of the fermentation medium and ventilating the culture room were two effective ways to release the metabolic heat to maintain the medium temperature.

2.8.3.1.4 pH

Although pH is one of the critical factors, monitoring and control of pH during fermentation is not usually attempted in SSF. Buffering capacity of the moistening medium used for SSF help in eliminating the need for pH control during fermentation. Further, some of the substrates used for SSF may aid in pH control during the process.

2.8.3.1.5 Aeration and oxygen transfer

Aeration is usually achieved by forcing in sterile air under pressure. The aeration rate desired for a process mainly depends upon the oxygen requirement for a process, amount of metabolic heat to be dissipated from the mass and the thickness of the substrate layer employed for the process.

The oxygen transfer may occur from the oxygen dissolved in the moistening medium. There is a linear relationship between oxygen uptake and growth rate. There are several ways in which free movement of air and hence better O_2 transfer can be achieved like use of a porous, coarsely granulated or fibrous substrate; lower thickness of substrate layer and extent of air spaces present in the substrate.

2.8.3.1.6 *Agitation*

Agitation of the fermenting mass has beneficial effects mainly by providing homogeneity throughout the fermentation period, promoting growth of mycelium on individual particles of the substrate, preventing aggregate formation and promotion of gas transfer and facilitation of heat exchange. The level of agitation may be decided by the similar factors as those governing the rate of aeration.

2.8.3.1.7 *Particle size*

Generally, smaller substrate particles provide larger surface area for microbial attack but if they are too small, may result in substrate agglomeration as well as poor growth. In contrast, larger particles provide better aeration but a limited surface for microbial attack. Therefore, a compromised particle may be derived for the particular process (Pandey et al., 1999).

2.9 BIOPROCESS DESIGN

Inspite of several advantages of solid-state fermentation over submerged fermentation for the production of metabolites, designing of the bioprocess is in its infancy. Many types of reactors are available to run at laboratory scale with small amounts of medium but scale up still requires intricate designing mainly due to intense heat generation and heterogeneity in the system. Wide varieties of matrices are used in SSF which vary in composition, porosity and water holding capacity. These factors affect reactor design (Durand, 2003). Solid media used for SSF contain less water which makes oxygen transfer inadequate because of the poor conductivity of the air as compared to water. The lack of free water and generation of metabolic heat may adversely affect the process and hence proper design for adequate aeration, cooling and agitation can be engineered for achieving higher yields in the process. Over the past 10 years, significant progress has been made in the design of solid-state reactors as enumerated below.

2.9.1 Tray bioreactor

It is the simplest kind of reactor consisting of wooden, metallic or plastic trays, possibly have perforations. A thin layer (maximum 15 cm) of solid substrate is placed in thermostated chambers. Although employed at commercial scale, this system requires larger areas (incubation chambers) and is labour intensive. No choice is available for aeration and temperature control. A significant level of laccase production was achieved from *Trametes hirsuta* which was grown in laboratory scale tray bioreactor using grape seeds (Couto et al., 2006) and orange peels as solid support (Rosales et al., 2007).

2.9.2 Packed bed reactors

These reactors are made up of a column that contains solid medium in the form of a bed and situated in a clean sterilised chamber. The characteristic design feature of packed-bed reactors is the introduction of sterile, moist air through a sieve located at the bottom of the column. Dissipation of metabolic heat is the major problem with such kind of reactors as temperature gradient is established due to microbial activities and thickness of medium. Packed bed bioreactors were employed for dihydroxy phenylalanine (Ates et al., 2007), enzymatic hydrolysis of lactose (Haider and Husain, 2008) and for lipase production (Ciftci et al., 2009).

2.9.3 Drum bioreactors

A cylindrical drum with or without perforations with continuous or intermittent rotation is a characteristic feature of these reactors. Rotation of drum at constant speed provides adequate mixing of the substrate and aeration. Supply of sterilised and moist air further facilitates the maintenance of temperature and moisture during fermentation. Damage of fungal mycelia presumably because of shear forces which are generated due to the continuous rotation of drum may have serious impact on productivity. Intermittent rotation of drum at defined speed may result into decreased mycelial damage thus attaining the desired productivity. A laboratory scale rotating drum bioreactor was designed for the production of cellulases, hemicellulases

from thermophilic fungus *Thermoascus aurantiacus* under solid-state fermentation (Kalogeris et al., 2003) and ligninases from *Phanerochaete chrysosporium* (Dominguez et al., 2001).

2.10 APPLICATION OF XYLANASES

Hydrolases constitute approximately 75% of the total market for industrial enzymes, with the glycosidases, including cellulases, amylases and hemicellulases, constituting the second largest group after protease (Bhat, 2000). Xylanase constitutes the major proportion of hemicellulases. The use of xylanase has grown remarkably in recent years (Aristidou and Penttila, 2000; Subramaniyan and Prema, 2000, 2002; Beg et al., 2000, 2001; Techapun et al., 2003; Moure et al., 2006). The biotechnological potential of xylanases rely in its applicability to various industrial sectors including pulp and paper, fruit juice clarification, textile, animal feed and fodder, baking and coffee extraction. Besides this, xylooligosaccharides which are degradation product of xylan, possess tremendous uses for the development of biofuels, artificial sweetener, food additive, neutraceutical and pharmaceutics. A large number of bacteria, fungi and yeast are known like *Pichia stipitis* (Nigam, 2002), *Pachysolen tannophilus* (Yablochkova et al., 2003), *Candida shehatae* (Yablochkova et al., 2003), *Zymomonas mobilis* (Aristidou and Penttila, 2000), *Piromyces* sp. (Kuyper et al., 2003), *Thermus thermophilus* (Lonn et al., 2003) and *Fusarium oxysporum* (Panagiotou et al., 2006) for their pentose fermenting ability into ethanol. The bioconversion of xylose to xylitol, which is better sweetener compared to that of sucrose (Parajo et al., 1998), was observed by bacteria, yeast and fungi including *Candida* sp. (Rao et al., 2006; Granstrom et al., 2007; Santos et al., 2008) and *Debaryomyces* (Converti et al., 2002 ; Sampaio et al., 2008).

Considered as food ingredients, xylooligosaccharides show favourable technological features, including stability in acidic media, heat resistance, save insulin secretion from pancreas and stimulate intestinal mineral absorption (Oku and Sadako, 2002). These cause probiotic effects when ingested as part of the diet through the modulation of colonic microbiota. Xylooligosaccharides alone or as active components of pharmaceutical preparations exhibit a range of biological activities including antioxidant activity, blood and skin related effects,

antiallergic, antimicrobial, anti-infection and anti-inflammatory properties, selective cytotoxic activity, immunomodulatory action, cosmetic and a variety of other properties (Moure et al, 2006).

During bread making, xylanases are employed along with α-amylase, glucose oxidase and protease. Like other hemicellulases, xylanase degrades hemicellulose in wheat flour, leaving the dough softer and easier to knead (Si and Lustenberger, 2002; Baillet et al., 2003). The use of xylanases increases the overall content of arabinoxylooligosaccharides in bread, thus increasing the nutritional value of the bread.

Xylanases are also employed as an active ingredient of poultry diet and non ruminant animals along with glucanases, pectinases, cellulases, amylases and phytases. Arabinoxylans, which is a major component of animal feed, may increase the viscosity of the ingested feed, interfere with the mobility and absorption of other components and therefore decrease the nutrient value of the animal feed. Addition of arabinofuranosidases in animal diet facilitates the degradation of this arabinoxylan by removing the arabinofuranose side group. This leads to decreased viscosity and improved mineral absorption in the digestive tract. Moreover, an enzyme fortified diet is found to reduce unwanted residues in the excreta (phosporus, nitrogen, copper and zinc), an effect that could have a role in reducing environmental contamination. The influence of crude xylanase produced from *Aspergillus niger* FAS128 or FAS128 on the *in vitro* digestibility of pig diets and production performance of piglets was investigated in comparison with two commercial crude enzyme products, IE1 and IE2 (Tapingkae et al., 2007).

Along with cellulase and pectinase, xylanases are employed for desizing, scouring and bleaching of plant fibres during textile manufacturing. Conventional scouring is chemical intensive that is unquestionably expensive in terms of energy and water. Concentrated alkaline solutions of scouring not only threaten the environment but also attack nonspecifically on cellulose fibres leading to weight and strength loss. Replacement of these harsh chemicals with

enzyme accelerates the degradation of non cellulolytic impurities and thus facilitates its subsequent bleaching. A number of reports are available on the use of cellulases and pectinases for bioprocessing of fabric but very few reports are on xylanases for the purpose of desizing and scouring. An alkalothermophilic xylanase from *Bacillus stearothermophilus* SDX was evaluated for its application in fibre processing (Dhiman et al., 2008). In combination with cellulases, amylases and pectinases, xylanases find extensive application in fruit juice clarification and wine making. By hydrolysing arabinoxylan and starch content from raw material, it facilitates liquefaction of fruit and vegetable, stabilisation of the fruit pulp and increased recovery of aromas, essential oils, vitamins, mineral salts, edible dyes, pigments etc. The main desirable properties of xylanases for use in the food industry are their high stability and optimum activity at acidic pH. During beer manufacturing, the cell wall of barley is hydrolysed releasing long chain arabinoxylans, which increases the viscosity of the beer rendering it "muddy" in appearance. Thus, xylanases are used to hydrolyse the released arabinoxylans to decrease the beer's viscosity and consequently eliminating its muddy appearance.

2.10.1 Xylanases for pulp and paper industry

2.10.1.1 *Current Scenario*

Demand for print quality paper is expected to go up in times to come, besides the demand for upstream market of paper products, like tissue paper, tea bags, filter paper, light weight online coated paper, medical grade coated paper, etc., is growing up as well. The pulp and paper industry comprises a large and growing portion of the world's economy. Today the paper industry accounts for almost 2.5% of the world's production and 2% of world trade (Mittal, 2005) and this industry sector is mainly dominated by North American (United States, Canada), Northern European (Finland, Sweden) and East Asian countries (such as Japan). The paper industry has an important role to play in the Indian economy. In the recent past, the Indian paper sector has maintained a growth rate of around 6%, which is a way above the world

average of about 2.8% (Jain, 2005). With such a growth turnover, the Indian paper industry has a vital role to play in the socio-economic development of the country. To date there are more than 600 pulp and paper mills producing nearly 5.5 million tons of paper and paper board and 0.68 million tons of newsprint against the present installed capacity of 7.5 million tons of paper and paper board and about 1.30 million tons of newsprint. The projected demand for paper board and newsprint is expected to touch 8.3 million tons by the year 2010. The per capita consumption of paper, which is the benchmark of modernization of any country and is directly related to the literacy rate, stands at 5.5 kg for India, which is poorer compared to the global average of about 50 kg. (Jain, 2005). Table-2 comprises the per capita consumption of paper in various countries.

Table. 2 Paper consumption in different countries (Report CPPRI, 2005)

Country	Paper consumption (Kg/capita)	Country	Paper consumption (Kg/capita)
USA	312	China	20
Singapore	228	Indonesia	14
Western Europe	160	Philippines	11
Malaysia	89	India	5.5

Pulp and paper industry is the major source of chlorine and chlorine based compounds in the environment. The effluents generated contain high concentration of organohalides, adsorbable organic halides (2.0-8.0 Kg of AOX/tonne of paper) as well as total organic halides (TOX). Besides this, the black liquor generated contains highly polluting, toxic and mutagenic compounds in form of lignin and lignin degradation products.

Pulping and bleaching are two major stages during paper manufacturing where most of the lignin is removed from pulp due to chemical treatments. Although, bleaching is an essential

step for the production of fully bleached white pulp, it causes the release of organohalides in the effluents.

2.10.1.2 *Alternatives to conventional bleaching*

To minimise the chlorine or chlorine compounds from bleaching sequences, alternative bleaching strategies have been developed. Replacement of chlorine with chlorine dioxide is characterised as elemental chlorine free (ECF) bleaching while the use of hydrogen peroxide, oxygen or ozone is described as totally chlorine free (TCF) bleaching processes. But these add on to the cost of production since it requires multiple alterations in the set up and hence are generally viable to large paper mills (Mathur et al., 2001). These set ups may not be technically feasible for agro residue based pulp mills smaller than 100 TPD in developing countries like India (Tendulkar et al., 1994). Besides this, nonselective nature of oxygen and ozone is also one of the limitations of this process which has a direct effect on the paper strength due to degradation of cellulose fibres. A simple, environmentally friendly and economical alternative of chemical bleaching is "biobleaching", namely the process of lignin removal with the help of microbes or microbial enzymes.

2.10.2 BIOBLEACHING: a promising alternative

During paper manufacturing, the bleaching stage is mainly responsible for the aesthetic qualities of paper. Among existing alternatives, biobleaching appears to be specific, relatively simpler and an eco-friendly process (Bajpai and Bajpai, 1992; Ragauskus et al., 1994; Tolan and Guenitte, 1997). Among enzymes of potential uses xylanases, ligninases and biomimetic agents look to be more promising.

2.10.2.1 *Xylanases*

Xylanases hydrolyse the xylan layer in the plant cell wall thus affecting the structural integrity and facilitating the lignin removal in the subsequent bleaching stages. At the same time it also attacks the xylan which gets reprecipitated on the surface of pulp fibres during

chemical pulping, thereby enhancing bleaching by exposing the lignin for the action of bleaching chemicals (Paice et al., 1992; Pham et al., 1995). The other proposed mechanism involves the cleavage of carbohydrate portion of lignin-carbohydrate complex to generate lignin molecules that are easier to be removed (Viikari et al., 1996; Allison et al., 1997; Roncero et al., 2000; 2005).

Combination of the enzymatic treatment with bleaching sequences may yield effective and reliable results. Crude culture filtrates of hemicellulases produced by different microorganisms were used in the initial delignification experiments (Viikari et al., 1987). In xylanase pre-bleaching, the side group cleaving enzymes act in a synergistic way by removing the side groups (Polizeli et al., 2005).

The colour of the pulp supernatant obtained after xylanase pretreatment, could be correlated and is normally proportional to the brightness of the chemically bleached pulp. Attempts were made for the use of hemicellulases (especially xylanases) in pre-bleaching of pulp (Gessesse & Mamo, 1999; Roncero *et al.*, 2000; Medeiros et al., 2002; 2003; Raghukumar et al., 2004; Sandrim et al., 2005; Atik et al., 2006; Khandeparkar and Bhosle, 2007; Shatalov and Pereira, 2007). It was observed that a saving about 20-25% of the total active chlorine for hardwoods and 10-15% for softwoods could be accomplished if the pulp is pretreated with xylanase. The pulp thus obtained had an improved viscosity and paper sheets made from enzyme treated pulps had unchanged or improved strength properties.

2.10.2.2 *Ligninases*

Ligninases are oxidative enzymes which not only are responsible for the degradation of lignin in their natural lignocellulosic substrates (Becker and Sinitsyn, 1993; Hatakka, 1994) but may also enable the degradation of various xenobiotic compounds (Scheibner et al., 1997; Pointing, 2001). White-rot fungi are the main producers of ligninolytic 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 lignin degrading enzymes are lignin peroxidase (LiP), manganese peroxidase (MnP) and laccase. Due to their ability to degrade lignin, these proved to be useful in bleaching of the kraft pulp (Sigoillot et al., 2005). A somewhat direct approach is followed by ligninases to achieve biobleaching. Unlike xylanases, the enzyme directly and specifically attacks lignin, oxidises it and makes it water soluble. Laccase, which is a major enzyme of the ligninolytic enzyme system, specifically attacks the phenolic groups through intermediate formation of phenoxy radicals with concomitant reduction of oxygen to water (Niku-Paavola et al., 1994). With respect to the more powerful oxidants LiP and MnP, laccase has a lower redox potential, hence it cannot target the non-phenolic subunits of lignin. In fact, the substrate range of laccase may be extended to non-phenolic structures by the use of easily oxidisable mediators (Bourbonnais and Paice, 1990). Laccase preparations have been used for biobleaching by various groups (Balakshin et al., 2001; Gronqvist et al., 2003; Lund et al., 2003; Ibarra et al., 2006; Camarero et al., 2007).

2.10.2.3 *Xylanase-Laccase mixture*

Xylanase and laccase both act in a different manner for achieving the similar goal of removal of lignin from pulp. Although the substrate is different for both enzymes, these can be utilised to act simultaneously and synergistically. Due to action of xylanase, lignin gets exposed which then can be degraded and removed by laccases, leading improved levels of delignification. Application of a cocktail of a hydrolytic and an oxidative enzyme during pulp bleaching could be an innovative approach and very few attempts have been made in this direction. Bleaching was attempted by using both the enzymes separately as well as using the mixed enzyme system. *Bacillus pumilus* strain MK001 was used for xylanase production and laccase was produced by *Cyathus stercoreus* and used in combination for biobleaching (Kapoor et al., 2007). Co-cultivation of two fungi producing a xylanase and laccase, respectively, in monoculture, may prove to be a fruitful approach for obtaining a mixed enzyme preparation for biobleaching applications.

2.10.2.4 *Biomimetic agents*

Biomimetic agents are compounds which mimic the catalytic mechanism of microbial enzymes for degradation of substrate. These agents have considerable potential in the development of new high-performance materials with low environmental impact (Teeri et al., 2007). The degradation of cellulosic and hemicellulosic substrates involving catechol and hydroxamate chelators in presence of hydrogen peroxide and Fe^{3+} was examined to understand the non-enzymatic mechanisms involved in wood degradation (Arantes and Milagres, 2006). Some of the reactions catalysed by lignin peroxidase and manganese dependent peroxidase can be catalysed by non-enzymatic compounds. Manganese and iron porphyrins had been evaluated for degradation of lignin and lignin model compounds and it was observed that manganese porphyrins are more effective compared to iron porphyrins (Crestini et al., 1999). However, there are limitations that are associated with the use of porphyrins that correspond to their unstability and insolubility in aqueous environment. These porphyrins are unstable in the presence of excess oxidants.

2.11 COMMERCIAL XYLANASES

Various groups of microorganisms, mainly fungi and bacteria, produce xylanases. These microorganisms differ with respect to the specificity and activities of the xylanases obtained. However, some of these strains have been found to be suitable for commercial production of xylanases for the paper industry; for improving the digestibility of animal and poultry feed in the food industry, for enhancing recovery of starch for wheat flour in the baking industry and in the horticulture industry for clarification of fruit juices. As given in Table-3, many of the preparations are active at neutral or acidic pH with moderate thermostability. An enzyme based bleaching technology viable for the pulp and paper industries could be suitably implemented by employing alkalophilic and thermostable enzyme preparations. Moreover,

enzyme preparation can be made more cost effective by choosing a suitable productive strain and an economically promising fermentation strategy. A list of commercially available xylanases are summarised in Table- 3.

Table. 3 Commercially available xylanases (Beg et al., 2001; Polizeli et al., 2005)

Sl. No.	Product name	Distributor	Microorganism	Optimum pH	Optimum temperature (° C)	Application
1.	Bel feed	Agrimex NV, Belgium	*Bacillus subtilis*	6.0-7.0	65	Improving the digestibility of grains
2.	Habio	Sichuan Habio Bioengineering Co., Ltd, China	na	na	na	Antinutrilite in feed products & rising the feed availability
3.	Grindazyme	Danisco Ingredients, Denmark	*Aspergillus niger*	na	na	Supplementa--tion of poultry & piglet feed
4.	Allzym PT	Alltech, USA	*A. niger*	5.3	65	Upgrading of animal feed
5.	Amano 90	Amano pharmaceutical, Japan	*A. niger*	4.5	50	Pharmaceutic al & food industry
6.	Bio-Feed Plus	Novo-Nordisk, Denmark	*Humicola insolens*	na	na	Animal feed
7.	Bleachzyme	Biocon, India	na	na	na	Paper industry
8.	Cartazyme	Clerient, UK	na	5.0	45-55	Pulp bleaching
9.	EcopulpX-200	Primalco, Finland	*Trichoderma reesei*	5.0-6.0	50-55	Improving the bleachability of soft wood & hard wood kraft pulp
10.	Irgazyme 40	Nalco-Genencor, Ciba-Geigy	*Trichoderma longibrachiatum*	na	na	Pulp & paper industry, animal feed
11.	Multifect XL	Genencor	*Trichoderma longibrachiatum*	5.0-5.5	55-60	Food industry
12.	Pulpzyme	Novozymes, Denmark	*Bacillus* sp	9.5	50	Paper industry

13.	Solvay pentonase	Solvay Enzymes, Germany	*T. reesei*	5.3-5.5	55	Starch processing & baking
14.	Sternzym HC 46	Stern-Enzym, Germany	*T. reesei*	5.0	30	Hydrolysis of plant raw materials, bakery, animal feed,
15.	Sumizyme X	Shin Nihon, Japan	*T. koningii*	5.0	55	Peeling of cereals, Bread making,
16.	Xylanase GS35	Iogen, Canada	*T. reesei*	4.5	40	Pulp cleaning & processing
17.	Xylanase 250	Hankyu bioindustry, Japan	*T. viridae*	4.5	40	Macerating fruits & vegetables
18.	Hemi cellulase 100	Hankyu bioindustry, Japan	*A. niger*	3.5	40	Improving the filtration speed of saccharified cereal solution & fruit juices
19.	Texzyme J	Texan chemicals, India	na	na	na	Bleaching of paper pulp

na, not available

2.12 PATENTS ON XYLANASES

Various patents had also been filed for the production and application of the xylanase as listed in Table-4

Table. 4 Patents for xylanase production and application during last five years

Sl No.	Patent No	Date	Title
1.	WO2007/115407	18.10.2007	Modification of xylanases to increase thermophilicity, thermostability and alkalophilicity
2.	US2007 7226772	05.06.2007	Recombinant xylanases derived from anaerobic fungi, and the relevant sequences, expression vectors and hosts
3.	WO 2007/025328	08.03.2007	Extracting and purifying β 1,4-xylanase
4.	WO 2006/104448	05.10.2006	Thermostable alkaline xylanase
5.	WO 2006/083240	10.08.2006	Microbially expressed xylanases and their use as feed additives and other uses
6.	WO 2006/078256	27.07.2006	Polypeptides having xylanase activity and polynucleotide encoding same
7.	WO 2005/093072	06.10.2005	Modified xylanases exhibiting improved expression
8.	WO 2005/052176	09.06.2005	Method of separating glycosaminoglycan from proteoglycan core protein with the use of xylanase
9.	US20046833259	21.12.2004	*Pseudomonas stutzeri* strain and process for preparation of xylanase
10.	EP1479765	24.11.2004	Enzymes with xylanase activity from *A. aculeatus*
11.	WO 2004/092479	28.10.2004	Enzymatic treatment of paper making pulps
12.	US2004 6768001	27.07.2004	Xylanase from *Trichoderma reesei*, method for production thereof, and methods employing this enzyme
13.	WO2004/023879	25.03.2004	Use of family 8 enzymes with xylanolytic activity in baking
14.	WO 2004/013322	12.02.2004	Degrading lignocellulosic materials
15.	US20036667170	23.12.2003	Sequences of xylanase and xylanase expression vectors
16.	US20036635464	21.10.2003	Xylanases, genes encoding them, and uses there of
17.	WO2003/048449	12.06.2003	Bleaching stage using xylanase with hydrogen peroxide, per acids, or a combination there of
18.	WO2003/020923	13.03.2003	Xylanase variants
19.	WO2003/012094	13.02.2003	Process for xylanase production

CHAPTER III

MATERIALS AND METHODS

Chapter 3

MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Chemicals and other materials

Oat spelt xylan, Birch wood xylan, xylose, ρ- nitrophenyl - β - D - xylopyranoside, 2,2'-azino - bis (3-ethylbenzothiazoline-6-sulfonic acid) ammonium salt (ABTS) were purchased from Sigma Chemicals Company, USA. Standard protein molecular weight markers were procured from Bangalore Genei. All other chemicals were of analytical grade and purchased from standard commercial manufacturers and were of the highest grade available.

3.1.2 Plant and agroresidual materials

Plant biomass i.e. congress grass (*Parthenium* sp.) and water hyacinth (*Eichhornia crassipes*) were collected from local sites. Sugarcane bagasse was obtained from a local sugar mill, Rai Bahadur Narayan Singh Sugar Mills Ltd., Laksar, Hardwar. Cotton hull, black gram husk, red gram husk, sawdust and wheat bran were purchased from the local market of Roorkee.

3.1.3 Microorganisms

Penicillium oxalicum SAU_E-3.510 was developed through mixed mutagenesis of wild type strain *Penicillium oxalicum* SA-8 (ITCC 6024) which had been isolated from the waste of a soda ash industry ('TATA Chemicals', Meethapur Gujrat, India) and identified by the Indian Type Culture Collection Bank, Indian Agricultural Research Institute, New Delhi. The alkaline condition (pH 8.5) was maintained during isolation and screening, in order to get an alkalitolerant strain. Three white rot fungal cultures, namely *Pleurotus ostreatus* MTCC 1804, *Trametes hirsuta* MTCC 136 and *Pycnoporus* sp. MTCC 137, were procured from the Microbial Type Culture Collection Bank, Institute of Microbial Technology, Chandigarh. The strains were maintained on PDA slants, stored at 4°C and subcultured periodically.

3.1.4 Pulp

The mixed wood pulp (Eucalyptus: Poplar; 60:40), corresponding to kappa number 14.4 (with 35.5% ISO brightness and viscosity 10.1 cp) obtained through kraft pulping, was procured from STAR Paper Mill, Saharanpur, India.

3.2 METHODS

3.2.1 Isolation of strains

A total of 18 different fungal strains were isolated from the waste of a soda ash industry and a paper industry effluent. Briefly, samples from the mentioned sites were collected and mixed with two parts of double distilled water. The suspension was centrifuged (5000 x g) at room temperature for 10 min. The clear supernatant following serial dilution was inoculated onto potato dextrose agar (pH 8.5) plates. Growth was observed after 48 hours of incubation at 30°C. On the basis of distinct morphological features, 18 fungal cultures were isolated. Isolated pure cultures were transferred to PDA slants, incubated at 30°C for 5 days and further stored at 4°C for future usage.

3.2.2 Screening of xylanase producing strains

Primary screening of the isolated strains for xylanase activity was carried out on xylan-agar plates as described by Mathrani and Ahring (1992). The xylan agar medium contained 1% xylan, 2% agar and autoclaved at 15 psi for 15 minutes. Cell free culture supernatant (100 µl) was placed in 0.8 cm diameter wells that were cut into the solidified medium and the plates were incubated at 30°C for 20-24 hours. Enzymatic hydrolysis of the surrounding xylan resulted in a clearing zone which was followed by staining with 0.1% Congo red then destaining with 1M NaCl (Teather and Wood, 1982). Controls with heat killed supernatant (100°C, 20 minutes) did not produce any clearing zones.

3.3 MUTAGENESIS AND SCREENING

P. oxalicum SA-8 was subjected to mutagenesis in an attempt to obtain improved levels of enzyme production. The schematic representation of the step-wise mutagenesis and screening is shown in Fig. 4.

3.3.1 Single-step mutagenesis

3.3.1.1 *Physical mutagenesis*

Physical mutagenesis was performed using UV radiation according to Petruccioli et al. (1995). A three ml aliquot of spore suspension (3.5 x 10^6 spores ml^{-1}) of a five day old culture of *P. oxalicum* SA-8 was exposed to UV light (2.5 $Jm^{-2}s^{-1}$, distance 0.70 m) in sterile Petri dishes (9 cm diameter) for different time periods ranging from 30 s to 10 min and stored in the dark at 4°C for 24 h to avoid photorepair. The lid of the Petri dish was removed while performing the UV irradiation. Following serial dilutions, treated spore suspensions were inoculated on potato dextrose agar and xylan agar media to detect their survivability and xylanase producing ability, respectively. Colonies showing a larger clearing zone diameter on xylan agar plates as compared to that obtained with the wild type strain were picked and subjected to quantitative estimation of xylanase activity spectrophotometrically. A parallel control was also maintained except 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 *P. oxalicum* SA-8 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 *P. oxalicum* SA-8 was treated with 150 µg ml^{-1} of NTG in sterile saline solution for different time periods (10-120 min) followed by washing with distilled water. Survivability and frequency of mutants were determined on PDA and XA-plates (stained with 0.1% Congo-red), respectively. Colonies showing a larger clearing zone diameter on xylan agar plates as compared to that of wild type strain were picked up and quantitatively analysed for their xylanase activity. A control conidial suspension was also identically treated, excluding the use of N-methyl-n'-nitro-N-nitrosoguanidine.

3.3.1.2.2 *Ethidium bromide treatment*

Mutagenesis of *P. oxalicum* SA-8 was also carried out using ethidium bromide as

described by Chand et al. (2005). A spore suspension of *P. oxalicum* SA-8 (35 x 10^5 spores ml^{-1}) was treated with 200 μg ml^{-1} of EtBr in sterile saline solution for different time periods (10-60 min) followed by washing with distilled water. Survivability and frequency of mutants were determined on PDA and XA-plates stained with 0.1% Congo-red, respectively. Colonies showing a greater clearing zone diameter on xylan agar plates as compared to the parental strain were selected and quantitatively analysed for their xylanase activity. A parallel control was also maintained where conidia were not exposed to EtBr treatment.

3.3.2 Mixed mutagenesis

Mutants SAU-3.5, SAN-60 and SAE-10 that were obtained after physical and chemical mutagenesis and had a comparatively greater enzyme production were subjected to mutagenesis to find out if further improvement in enzyme production level could be achieved. Mutant, SAU-3.5 obtained following UV mutagenesis, had shown a maximum increase in xylanase activity. Therefore, it was selected for second stage mutagenesis to determine if a further increase in activity could be observed. SAU-3.5 was further treated separately with NTG and EtBr. Colonies having higher zone diameter on xylan agar plates with respect to strain SAU-3.5 were selected and further quantitatively analysed for their xylanase activity. Mutant SAU_E-3.510, obtained as a result of mixed mutagenesis (UV + EtBr), had shown a maximum increase in xylanase activity and hence was selected for further studies.

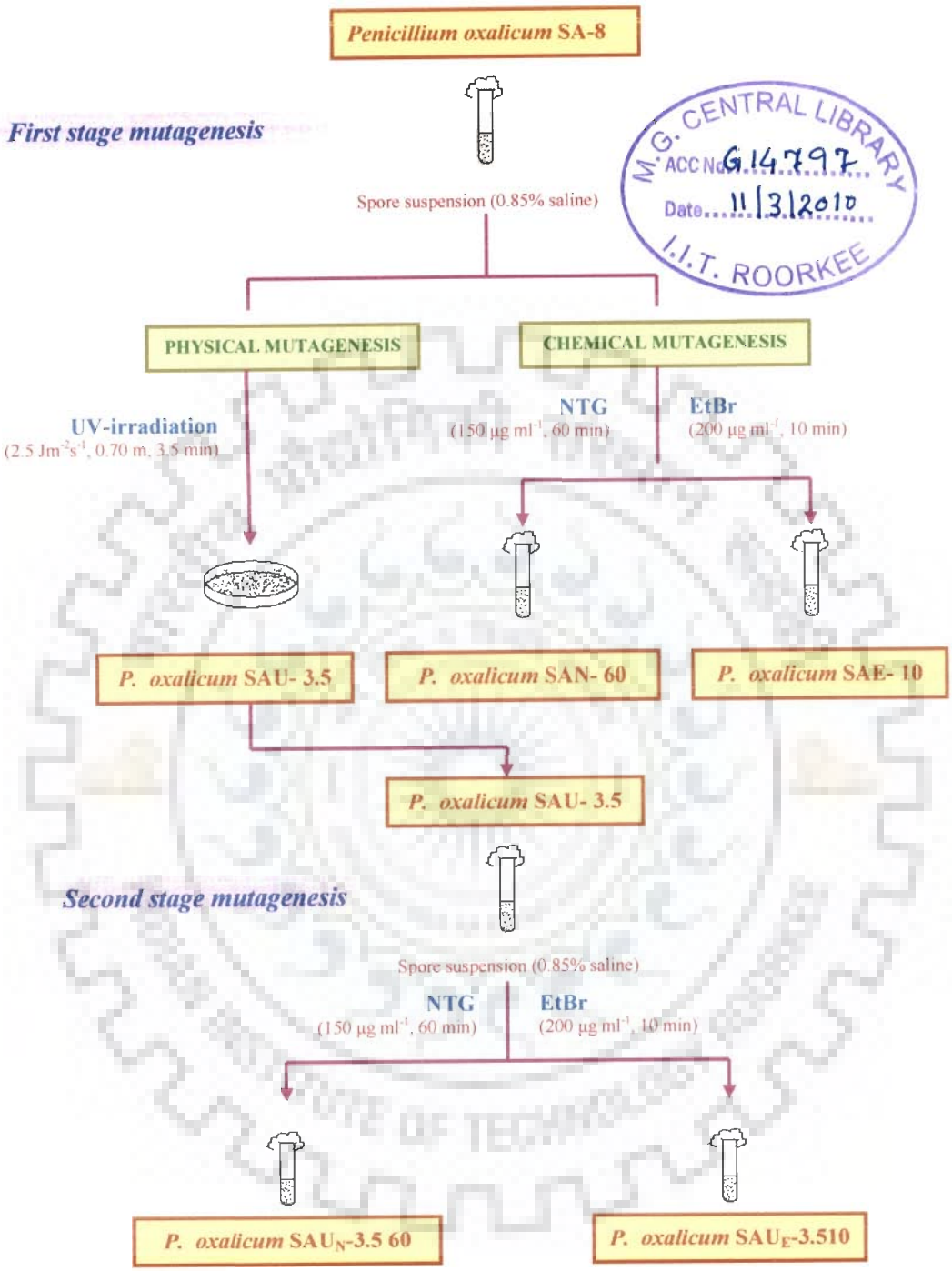


Fig. 4 Mutagenesis of *P. oxalicum* SA-8 for xylanase production.

3.4 SHAKE FLASK CULTIVATION

3.4.1 Xylanase production

Preliminary studies on xylanase production by mutant *P. oxalicum* SAU_E-3.510 as well as by different white rot fungi (*P. ostreatus*, *T. hirsuta*, *Pycnoporous* sp) were carried out as described by (Medeiros et al., 2003). The fermentation medium contained (w/v): oat spelt xylan, 0.5%; KH_2PO_4, 0.7%; K_2HPO_4, 0.2%; $MgSO_4.7H_2O$, 0.05%; $(NH_4)_2SO_4$, 0.1% and yeast extract, 0.06% (Medeiros et al., 2003). The pH of the fermentation medium was adjusted to 9.0 with 2N NaOH followed by autoclaving the medium at 121°C for 15 min. The medium was then inoculated with fungi and then incubated (30 °C, 150 rpm) for six days for xylanase production. This experiment was performed by using 50 ml of medium in 250 ml of Erlenmeyer flasks using the cotton plugs.

3.4.2 Laccase production

The preliminary studies for laccase production from mutant *P. oxalicum* SAU_E-3.510 and selected white rot fungi (*P. ostreatus*, *T. hirsuta*, *Pycnoporous* sp) were carried out by using glucose peptone medium as described (Koroleva et al., 2002) that contained: glucose, 1.0%; peptone, 0.3%; KH_2PO_4, 0.06%; K_2HPO_4, 0.04%; $MgSO_4$, 0.05%; $ZnSO_4$, 0.0001%; $FeSO_4$, 0.00005%. and $MnSO_4$, 0.005%. After sterilisation, the fermentation medium was inoculated with fungi and incubated (30 °C, 150 rpm) for twenty days for laccase production. This experiment was performed by using 50 ml of medium in 250 ml of Erlenmeyer flasks using the cotton plugs. Samples were taken on every second day and used for measuring the enzyme activity.

3.4.3 Xylanase-Laccase mixture through mixed culture

The medium used for growth and enzyme production by co-culturing of fungi included: glucose, 1.0%; KH_2PO_4, 0.7%; K_2HPO_4, 0.2%; $MgSO_4.7H_2O$, 0.05%; $(NH_4)_2SO_4$, 0.1%; yeast extract, 0.06%; $ZnSO_4$, 0.0001%; $FeSO_4$, 0.00005%; $MnSO_4$, 0.005% and xylan, 0.25% which was added on the 6^{th} day of incubation under aseptic conditions. The pH of fermentation

medium was adjusted to 9.0. Medium was inoculated with *P. ostreatus* MTCC 1804 and after 6 days of incubation, it was further inoculated with mutant *P. oxalicum* SAU_E-3.510 and incubation continued to a total of 12 days (30°C, 150 rpm). This experiment was performed by using 50 ml of medium in 250 ml of Erlenmeyer flasks using the cotton plugs.

3.4.4 Pretreatment of substrate

Congress grass (*Parthenium* sp.) was collected from local areas during the rainy season (July-September) prior to the flowering stage; leaves and nascent buds were removed. Fresh water hyacinth plants (*Eichhornia crassipes*) with long stems were collected from ponds and bagasse was collected from a local sugar mill. These were washed to remove adhering dirt and chopped into small pieces, dried in an oven at 85-90°C to a constant weight and powdered to a particle size of 0.1-0.5 cm (Nigam, 2002). Wheat bran and saw dust were procured from the local market, milled and washed thoroughly in the double distilled water to remove the residual dust and dried. For delignification, all substrates were soaked in NaOH (2N) for 24 h and subsequently steamed for 60 min. After treatment, substrates were thoroughly washed with water to neutral pH, dried and reground (Rajaram and Verma, 1990). A 40-45 % yield of the substrates was obtained following pretreatment process.

3.5 PLATE CULTIVATION AND FUNGAL COMPATIBILITY ASSAY

For testing compatibility among the selected fungi, the white rot fungi and mutant *P. oxalicum* SAU_E-3.510 were inoculated aseptically on the same potato dextrose agar plate and allowed to grow at 30°C. Four discs of 0.8 cm each were obtained from actively growing cultures of white rot fungi (*P. ostreatus* MTCC 1804, *T. hirsuta* MTCC 136 and *Pycnoporus* sp. MTCC 137) and placed diagonally on the PDA plate. After that, the similar plate was point inoculated with mutant *P. oxalicum* SAU_E-3.510. The parallel controls of all four fungi were also established for comparative study of growth pattern under dual and mono cultures (Stepnova et al., 2003).

3.6 CO-CULTIVATION OF MUTANT *P. OXALICUM* SAU_E-3.510 AND *P. OSTREATUS* MTCC 1804

3.6.1 Shake flask cultivation

Fermentation medium (50 ml) in a 250 ml Erlenmeyer flask was inoculated with *P. ostreatus* and incubated at 30 °C, 150 rpm. Mutant *P. oxalicum* SAU_E-3.510 was inoculated on the 6th day due to its faster growth rate. Cultures were harvested following a total of 12 days of incubation for estimation of xylanase and laccase activities.

3.6.2 Surface cultivation

Medium (50 ml) following inoculation with mutant *P. oxalicum* SAU_E-3.510 and *P. ostreatus* MTCC 1804, as mentioned above, were kept in an incubator at 30°C under static conditions. 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 medium.

3.6.3 Solid-state fermentation (SSF)

Co-cultivation of mutant *P. oxalicum* SAU_E-3.510 and *P. ostreatus* under solid-state fermentation (SSF) initially was performed at 70% initial moisture by using 5 g of *Parthenium* biomass as substrate in a 250 ml Erlenmeyer flask as described (Ferreira et al., 1999). The flasks were incubated statically at 30°C. *P. ostreatus* MTCC 1804 was inoculated by using three fungal discs (0.8 cm) which were obtained from an actively growing culture and this was followed by inoculation with mutant *P. oxalicum* SAU_E-3.510 after 48 h of incubation. At the end of fermentation, 25 ml of distilled water was added to each flask. The enzymes produced were extracted on a rotary shaker (150 rpm) at 4°C for 1 h. The slurry was squeezed through cheese cloth. The extract was clarified by centrifugation (10 000 x g, 15 min) and the clear supernatant obtained was used for measuring the enzyme activity (Kalogeris et al., 1998).

3.7 SCALING UP OF THE SOLID-STATE CULTIVATION PROCESS

The solid-state fermentation that had resulted in maximum yields of xylanase using a mixture of bagasse and black gram husk in a ratio of 3:1 was scaled up. An intermittent rotating

drum bioreactor was designed for simultaneous production of xylanase and laccase through co-culturing of mutant *P. oxalicum* SAU_E-3.510 and *P. ostreatus* MTCC 1804 as shown in Fig 5. The bioreactor was operated in a batch mode. Bagasse and black gram husk were processed as described earlier. The cultivation was performed in a cylindrical drum (diameter, 28cm; length, 38 cm; capacity, 23 l) made up of acrylic sheets (polymethyl methacrylate; PMMA). The bioreactor drum contained with 368 g of solid support (276 g bagasse and 92 g black gram husk) which was mixed with fermentation medium to have an initial moisture content of 80%. The prepared slurry was sterilised by autoclaving at 121°C, 15 psi. Pre-cultures of mutant *P. oxalicum* SAU_E-3.510 and *P. ostreatus* MTCC 1804 were made. Inoculum and presterilised substrate were added to the bioreactor through an opening in its left and right walls under aseptic conditions. The cylinder was connected to a geared motor to provide gentle rotation at definite time intervals (10 rpm, within an interval of 3 hrs). The intermittent rotation facilitated the proper mixing of substrate as well as fungal inoculum along with appropriate aeration. A cooling tray filled with sterile distilled water was used to provide humidity and to dissipate metabolic heat generated during cultivation. The complete unit was placed in an incubator to maintain the desired temperature (30 °C) for growth and enzyme production. At the end of the cultivation, the required amount of water was added and enzyme was extracted by rotating the cylinder at 10 rpm for 60 min. The sample thus collected was subjected to filtration and centrifugation and then used for estimation of the enzyme activity.

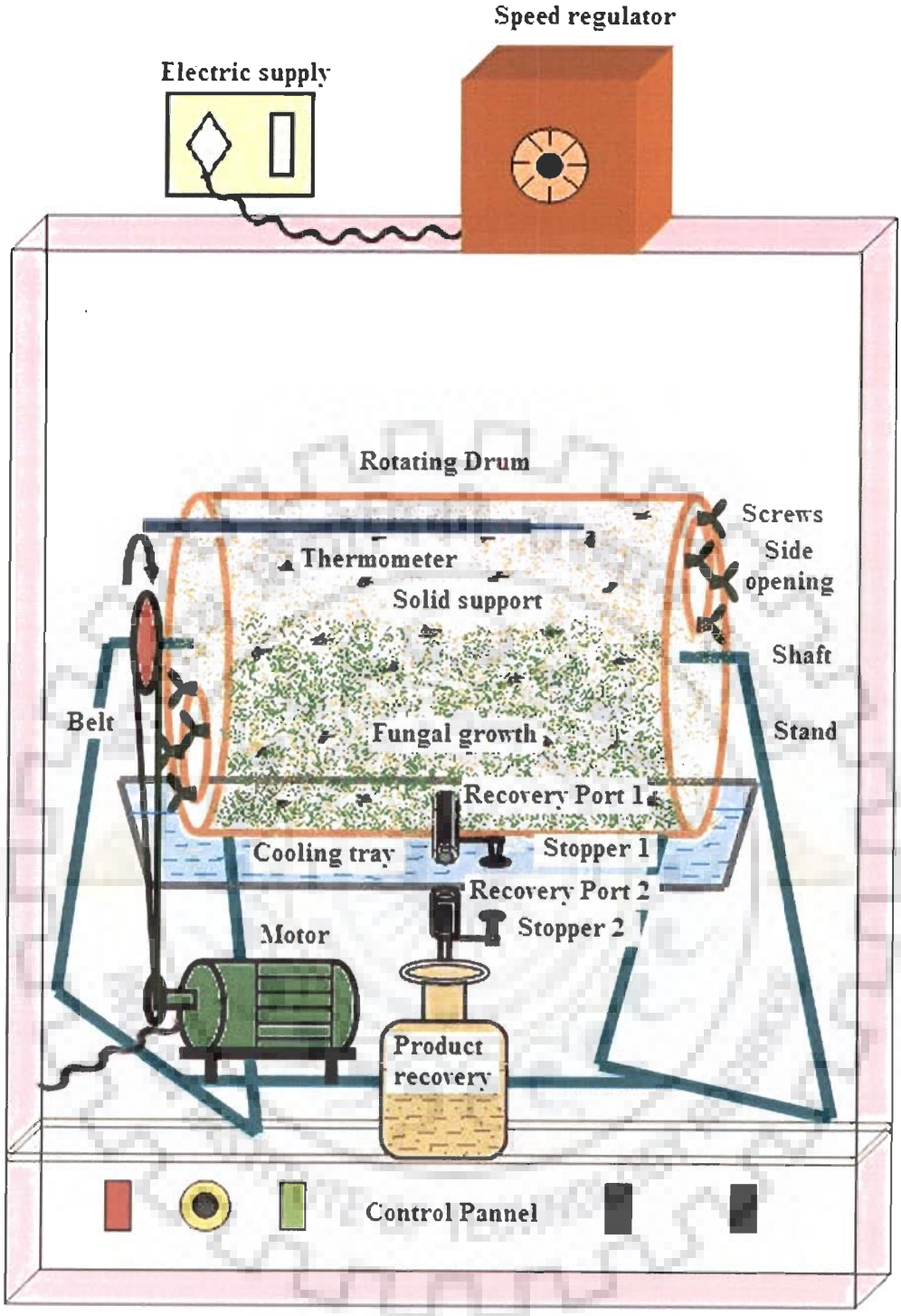


Fig. 5 Schematic representation of the intermittent rotating drum bioreactor for simultaneous biosynthesis of xylanase-laccase by co-culture of *P. oxalicum* SAU_E-3.510 and *P. ostreatus* MTCC 1804 under solid-state fermentation.

3.8 BIOBLEACHING EMPLOYING MIXED ENZYME PREPARATION

3.8.1 Production of enzymes

The mutant *P. oxalicum* SAU_E-3.510 was used for xylanase production under solid-state fermentation using bagasse and black gram husk (3:1) as solid substrate. Mixed enzyme having both xylanase and laccase activities was produced by co-cultivation of *P. oxalicum* SAU_E-3.510 and *P. ostreatus* MTCC 1804 using the same solid support (bagasse and black gram husk, 3:1).

3.8.2 Enzymatic treatment of pulp

3.8.2.1 *Selection of enzyme*

The effect of enzyme treatment on bleaching of mixed wood pulp was evaluated by treating the pulp with two different enzyme preparations viz. xylanase and xylanase-laccase mixture (in a ratio of 22:1) respectively (10 IU $g^{-1}$of pulp, 55°C, pH 9.0 and 2 hrs). Enzymatic treatment was followed by measurement of kappa number and the formation of paper pads for brightness measurement.

3.8.2.2 *Determination of enzyme dose*

To find out the optimum dose of mixed enzyme (xylanase and laccase, in a ratio of 22:1) for achieving maximum delignification, pulp was treated with varying doses of enzyme ranging from 0-20 IU g^{-1} of pulp.

3.8.2.3 *Determination of treatment time*

The pulp samples were treated with enzyme mixture containing both xylanase and laccase activity in a ratio of 22:1 for varying time durations ranging from 0-5 h to determine the suitable time for achieving maximum bleaching effects.

3.8.2.4 *Effect of mediator concentration*

To have maximum levels of lignin removal from pulp, enzyme treatment was performed in presence of 1-hydroxybenzotriazol (HBT) at different concentrations ranging from 0-3% w/w of pulp. The compound acts as an artificial mediator for laccase.

All the experiments were performed in triplicate using 10 g of pulp in polythene bags at 55° C, pH 9.0 and at a 10% pulp consistency. After enzymatic treatment, the pulp was washed with distilled water and the filtrate collected for further analysis. The release of reducing sugars was measured by the dinitrosalicylic acid (DNS) method (Miller, 1959) and phenolic compounds were estimated by measuring the absorbance at a wavelength of 237 nm. Hydrophobic compounds were determined by measuring the absorbance of the effluent at a wavelength of 465 nm (Patel et al., 1993; Gupta et al., 2000).

3.8.2.5 ***Colour removal from pulp***

After enzyme treatment and washing, the absorbance of collected filtrate was measured spectrophotometrically from 200 nm to 400 nm for detecting the lignin removal. The colour of effluent is due to presence of lignin (Khandeparkar and Bhosle, 2007).

3.8.2.6 ***Application of mixed enzyme preparation in multi-step bleaching sequences***

Fresh pulp samples were collected and pre-bleached with enzyme under derived conditions followed by conventional (CEPHHP) and elemental chlorine free (ODED) bleaching sequences. Samples without enzyme treatment were also processed for chemical bleaching under the same conditions. These were taken as control to analyze the effect of enzymatic pre-treatment in the bleaching process on paper properties. Conditions applied during the bleaching are given in Table-5. The treated and untreated pulp samples were filtered and respective effluents were evaluated for the AOX, COD etc. as per standard test procedures given in Table-6. 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 analysed according to the standard methods of TAPPI 1996 (Table-6).

Table. 5 Conditions applied during different bleaching sequences

Sl. No.	Bleaching sequences
1.	**CEPHHP/XCEPHHP**
X	Dose, 8 IU g^{-1} ODP; Consistency, 10%; pH, 9.0; Temperature, 50°C; Time, 180 min
C	Dose, 50% of TCD; Consistency, 3%; pH, ≥ 2.0; Temperature, ambient; Time, 45 min
E	Dose, $Cl_2/2$ + 0.03; Consistency, 10%; pH, 10.8; Temperature, 90°C; Time, 90 min
P_1	Dose, 0.5% H_2O_2 + 0.1% EDTA + 0.1% $MgSO_4$; Consistency, 10%; pH, 11.8; Temperature, 90°C; Time, 60 min
H_1	Dose, 70% of remaining 50% TCD; Consistency, 10%; pH, 11.5; Temperature, 45°C; Time, 60 min
H_2	Dose, 30% of remaining 50% TCD; Consistency, 10%; pH, 11.8; Temperature, 90°C; Time, 60 min
P_2	Dose, 0.5% H_2O_2 + 0.1% EDTA + 0.1% $MgSO_4$; Consistency, 10%; pH, 11.8; Temperature, 90°C; Time, 60 min
2.	**ODED/XODED**
X	Dose, 8 IU g^{-1} ODP; Consistency, 10%; pH, 9.0; Temperature, 50°C; Time, 180 min
O	Dose, 5 Kg cm^{-3} + 0.1% $MgSO_4$; Consistency, 10%; pH, 11.5; Temperature, 90°C; Time, 90 min
D_1	Dose, 1%; Consistency, 10-12%; pH, 3.5-4.5; Temperature, 70°C; Time, 180 min
E	Dose, 2.5%; Consistency, 10%; pH, 11.7; Temperature, 60°C; Time, 60 min
D_2	Dose, 0.6%; Consistency, 10-12%; pH, 3.5-4.5; Temperature, 70°C; Time, 180 min

ODP, Oven dried pulp; TCD, Total chlorine demand; $Cl_2/2$, Half of the added chlorine in C Stage
X, enzyme treatment; C, chlorination; E, alkali extraction; H_1, hypochlorite stage 1; H_2, hypochlorite stage 2; P_1, peroxide stage 1; P_2, peroxide stage 2; O, oxygen; D_1, chlorine dioxide stage 1; D_2, chlorine dioxide stage 2.

Table. 6 Standard test procedures followed for analysis of pulp and paper

Sl. No.	Test No.	Description
1.	T 610 om-87	Preparation of indicators and standard solutions
2.	SCAN-C 18:65	Disintegration of pulp for testing
3.	SCAN-C 24:67	Beating of pulp in PFI mill
4.	T227 cm-99	Determination of ° SR
5.	SCAN-C 26:76	Forming handsheets for physical testing of pulp
6.	T236 cm-85	Kappa number of pulp
7.	T403	Bursting strength of paper
8.	T404 cm-92	Tensile breaking length and elongation of paper
9.	T414 cm-98	Internal tearing resistance of paper
10.	SCAN-C 11:75	Pulp ISO brightness
11.	T410 cm-98	Grammage of paper
12.	SCAN-P 17:77	Folding endurance of paper
13.	SCAN-C 29:72	Chlorine Consumption of pulp
14.	SCAN-C 16:62	Preparation of cupriethylenediamine (CED) solution
15.	T230 cm-99	Viscosity of pulp (capillary viscometer method)
16.	COD	Closed reflux titrimetric method – Thermoreactor CR

3.9 ANALYTICAL METHODS

3.9.1 Estimation of xylanase activity

Xylanase was assayed by mixing 500 µl of suitably diluted enzyme in 50 mM glycine-NaOH buffer (pH 9.0) with 0.5 ml of xylan solution (1%) in a final volume of 1 ml and incubating at 55°C for 30 min. The reducing sugars released were measured by the dinitrosalicyclic acid method (Miller, 1959). One unit of enzyme corresponded to one µmol of xylose released per minute under the assay conditions.

3.9.2 Estimation of β-xylosidase activity

β-Xylosidase activity was measured according to Kuhad et al. (1998). The reaction mixture contained 0.5 ml of suitably diluted enzyme and 0.5 ml of 1 mM ρ-nitrophenyl- β-D-xylopyranoside prepared in glycine-NaOH buffer (50 mM, pH 9.0) and incubated for 15 min at 50°C. The reaction was terminated by adding 2 ml of $Na_2CO_3/NaHCO_3$ buffer (1M, pH 10.0) and the absorbance read at 410 nm. A unit of activity is the amount of enzyme releasing 1 µ mol of ρ-nitrophenol per minute under the assay conditions.

3.9.3 Estimation of cellulase activity

Carboxymethyl cellulase (CMCase) activity was determined as described by Shah et al. (1999). Briefly, the assay mixture in a total volume of 1 ml contained 0.5 ml of 1% carboxymethyl cellulose (CMC) in 50 mM potassium phosphate buffer (pH 8.0) and 0.5 ml of suitably diluted fermentation broth as the source of enzyme. The mixture was incubated at 50 °C for 30 min. The reducing sugars released were measured using DNS reagent as described by Miller (1959). One unit of enzyme activity corresponded to one µmol of glucose liberated per minute of the reaction under the above defined conditions.

3.9.4 Estimation of laccase activity

Laccase assay was performed using ABTS (2, 2'-azino-bis-(3-ethylbenz-thiazoline-6-sulphonic acid) as substrate. Reaction was carried out by taking 50 mM of glycine-NaOH buffer at pH 9.0, enzyme extract and 1.0 mM of ABTS in a total volume of 1 ml. The reaction was

monitored at 420 nm using UV-Vis spectrophotometer Cary 100 Bio (Varian Australia) at 25^{0}C (de Souza-Cruz et al., 2004).

3.9.5 Biomass estimation

Growth was measured by determining the dry cell weight. Dry biomass was obtained by filtering the samples and drying the mycelia to constant weight at 75 °C (Oliveira et al., 2006).

3.10 BIOCHEMICAL AND MOLECULAR CHARACTERISATION

3.10.1 Optimum temperature and thermostability

The optimum temperature was determined by assaying the xylanase activity at various temperatures (25°C - 85°C) for 30 minutes in 50 mM glycine-NaOH buffer (pH 9.0). The thermostability of enzyme was determined by measuring the residual activity at 55°C by incubating the enzyme at various temperatures ranging from 50° -100°C for 2 h.

3.10.2 Optimum pH and stability

The optimum pH was determined by measuring the activity at 55°C over a pH range of 4.0-11.0 using the following buffers: 100 mM citrate buffer (4.0-5.0), 100 mM phosphate buffer (6.0-8.0), 50 mM glycine-NaOH buffer (9.0-10.0) and 100 mM carbonate-bicarbonate buffer (11.0). The stability of enzyme at higher pH values was determined after incubating the enzyme in the buffers described above for 24 h at room temperature and measuring the residual activity after every 4 h.

3.10.3 Sodium-dodecylsulphate polyacrylamide gel electrophoresis

SDS-PAGE of the samples was performed according to Laemmli (1970). Polyacrylamide gels (10%) of 1.5 mm thickness were prepared for electrophoretic analysis.

3.10.3.1 *Reagents*

Solution A: Acrylamide solution 29.0 % (w/v) containing 1.0 % (w/v) bisacrylamide (N, N'-methylene-bisacrylamide)

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

Solution C: Stacking buffer, 1.0 M Tris-buffer, 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, 0.02 M Tris, 0.2 M glycine, pH 8.3

Preparation of resolving and stacking gels

Reagents	Resolving gel (10 %) (ml)	Stacking gel (4%) (ml)
Solution A	3.3	0.67
Solution B	2.5	-
Solution C	-	0.5
Solution D	0.1	0.04
Solution E	0.1	0.04
Solution F	0.004	0.004
Water	4.0	2.7
Total volume (ml)	10.0	4.0

3.10.3.2 *Casting of gel*

A sandwich of glass plates (8 x 7 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. The mixture was degassed for 15 min and ammonium persulphate and TEMED were added to it. The mixture was gently mixed and poured between the plates, leaving the required space at the top for pouring the stacking gel. After polymerisation of the resolving gel, the assembly was tilted to pour off the top 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 polymerisation of stacking gel, comb was carefully removed, wells formed were rinsed with reservoir buffer and the polymerised gel was used for electrophoresis.

3.10.3.3 ***Sample preparation***

Fractions containing proteins were concentrated by micro-concentrators (Centricon C-10, Amicon, USA) and added with SDS-PAGE sample buffer containing Tris-HCl (0.125 M, pH 6.8), 4% (w/v) SDS, 20% (v/v) glycerol, 10% (v/v) β-mercaptoethanol, 0.004% (w/v) bromophenol blue. Samples were heated in a boiling water bath for 3-5 min and loaded onto gel for electrophoresis

3.10.3.4 ***Electrophoresis***

Samples (100 µg of the protein) were gently loaded on the gel using a micropipette. Electrophoresis was carried out at constant voltage of 100 V until the tracking dye reached about 1 cm above the bottom of the gel. After electrophoresis, the gel was removed and stained overnight with gentle shaking in Coomassie brilliant blue R-250 0.1% in methanol, glacial acetic acid and water in a ratio of 5:2:5 (v/v/v) at room temperature. The gel was destained by using the methanol: acetic acid: water (30:10:60) solution.

3.10.3.5 ***Zymogram analysis***

Native polyacrylamide gel electrophoresis (PAGE) using 10 % gel was performed for visualization of enzyme activities in situ as described (Chadha et al., 1999). Oat spelt xylan (1%, 500 µl) was added to 3.50 ml of water during the preparation of resolving gel. The enzyme samples were mixed with sample buffer lacking SDS and β-merceptoethanol. The gel for activity staining was incubated in 50 mM Glycine - NaOH buffer, pH 9.0 for 2 min at 55°C followed by staining the gel in Congo-Red solution (0.5% w/v Congo-Red and 5% v/v ethanol in distilled water) for 15 min. The gel was de-stained with 1 M NaCl to visualize the clearing zone following hydrolysis. The gel was further exposed to 5% acetic acid to increase colour contrast between the hydrolysis zone and the remaining portion of gel.

For visualization of laccase activity, after electrophoresis the gel was washed with 50 mM of glycine-NaOH buffer, pH 9.0 for 5 min and then placed in the substrate solution (0.25% guaiacol; v/v) until the bands appeared (Vasdev et al., 2005).

3.10.4 Scanning electron microscopy

The detailed morphological studies of fungal strains were carried out using scanning electron microscopy (SEM, Leo 435 VP, England). The samples were taken and subjected for fixation using 3% (v/v) glutaraldehyde and 2% (v/v) formaldehyde (4:1) for 24 h. Following primary fixation, these were washed thrice with double distilled water and 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. For scanning electron microscopy of unbleached and enzymatically bleached pulp, fibres were washed three times in deionized water and treated with 2.0% (v/v) glutaraldehyde in 100 mM phosphate buffer (pH 7.2) for 1 h. Further, fibres were separated from glutaraldehyde, washed with the same buffer three times and dehydrated with an ethanol gradient (30-100%) (Kapoor *et al.*, 2007). After treating with 100% alcohol, samples were air dried and examined under SEM using a gold shadowing technique (Gabriel, 1982). Electron photomicrographs were taken at desired magnifications.

3.10.5 Matrix-assisted laser desorption/ionization time-of-flight (MALDI-ToF/ToF) mass spectrometry

For MALDI-ToF/ToF analysis of the protein, initially the broth was concentrated using Centricon C-10 microconcentrators and then electrophoresed on native gel (10 %). Half of the gel was stained with Coomassie brilliant blue R-250 and the other half was subjected to staining with Congo red for zymogram analysis. Protein bands in the Coomassie stained gel with the one corresponding to the active band in zymogram were carefully excised and repeatedly washed with 100 mM ammonium bicarbonate-acetonitrile buffer (pH 8.0). The gel slice was dissolved in acetonitrile and vacuum dried. Reduction was carried out with 1% dithiothreitol (DTT)

followed by modification using iodoacetamide (2%). Samples were then subjected to tryptic digestion overnight and acidified by adding 5 µl of 10% tri-fluoroacetic acid (TFA). The digested product was mixed with α-cyano-4-hydroxycinnapic acid matrix at a 1:1 ratio. The matrix was made by mixing 0.1% TFA to mixture of acetic acid and water (1:1). Spectra were recorded (Bruker Daltronix Autoflex ToF/ToF, Germany) in a positive mode and the N_2 laser intensity was set at a wave length of 337 nm. The peptide fragments obtained were analysed with the Flex Analysis Software and database homology search for protein identification was carried out manually using short sequence BLAST (Basic Local Alignment Search Tool) at NCBI (National Center for Biotechnology Information) (Schwend and Gustafsson, 2006).

3.10.6 High performance liquid chromatography

Hydrolysis of congress grass xylan (1%,) was evaluated after its incubation with suitably diluted enzyme preparations (5 IU ml^{-1}) in a final volume of 1 ml, for 24 h at 55 °C and pH 9.0, by examination of released reducing sugars through high performance liquid chromatography (Waters 1500 series, Ireland). The samples were centrifuged (10,000 x g) at 4 °C for 45 min. Following centrifugation, samples were membrane filtered (0.2 µm) to remove any particulate impurities and suitably diluted using the mobile phase (Barreca et al., 2003). Reducing sugars released were analysed by using a hydrocarbide column (4.6 x 250 mm, Waters, Ireland), with acetonitrile: water (70:30) as mobile phase (0.5 ml min^{-1}), using an injection volume of 20 µl and a run time of 15 min. A refractive index detector was used (Sa-Pereira et al., 2002). The peaks were analysed by using xylose purchased from Sigma chemical company as standard. The lignin degradation products (monophenols) were analysed using a Novo Pak C18 column at 40°C and using a mixture of acetonitrile-water-tetramethyl ammonium hydroxide-phosphoric acid (20:80:1:1) as eluent and using a UV-detector at 240 nm (Niku-Paavola et al., 1988).

3.10.7 X-ray diffraction (XRD)

Laboratory sheets were prepared according to the standard test methods (SCAN C 26:76) and the crystallinity of these sheets was measured through X-ray diffraction using a

diffractometer (Bruker AXS D8) operated at 40 kV and 30 mA. The samples were scanned at 1° min^{-1} from 2θ= 5° to 50° (Roncero et al., 2003).

3.10.8 Fourier-transformed infrared spectroscopy (FTIR)

Residual lignin from treated and untreated pulp was extracted with methanol-tetrahydrofuran (1:1, v/v) solution in a soxhlet extractor for 3 h and analyzed by FTIR spectroscopy for comparison (Geng and Li, 2002). Lignin samples were embedded in KBr disc and the fourier-transformed infrared spectra were recorded using a Perkin-Elmer 1600 series spectrometer at room temperature. The assignment of absorbance peaks were based on the database for lignin (Buta *et al.*, 1989; Faix, 1992).

3.10.9 Analysis of bleach liquor

3.10.9.1 *Calcium hypochlorite*

In 10 ml of diluted bleach liquor, 10 ml potassium iodide (10%) and 10 ml acetic acid (10%) were added. This solution was titrated with standard sodium thiosulphate solution (0.1N) with starch (0.5%) as indicator (Laboratory manual, 1993). The end point was blue to colourless. The active chlorine (gpl) was calculated as below:

Active chlorine (gpl) = Normality of bleach liquor X 35.5

3.10.9.2 *Chlorine dioxide*

Sodium chlorite solution (20 gl^{-1}) was titrated using the same procedure as for analysis of the calcium hypochlorite solution (Laboratory manual, 1993).

3.10.9.3 *Analysis of H_2O_2*

A 10 ml aliquot of H_2O_2 solution was diluted and made up to 250 ml in a volumetric flask. To 5 ml of this diluted solution, 10 ml of KI (10%), 10 ml H_2SO_4 (4N) and 1 ml of ammonium molybdate (1%) were added. This solution was titrated with standard sodium thiosulphate solution (0.1N) with starch (0.5%) as an indicator (Vogel, 2002). The end point was blue to colourless. The concentration of H_2O_2 solution was calculated as below:

Concentration of H_2O_2 solution = Normality of H_2O_2 solution X 34

3.10.9.4 *Analysis of residual chlorine*

The same procedure as described above was used except that the volume of spent bleach liquor was increased to 100 ml and titrated with standard sodium thiosulphate solution (0.1N) (Laboratory manual, 1993).

3.11 STATISTICAL ANALYSIS

All fermentation reactions were carried out in triplicate and experimental results represent the mean of three identical fermentations. The statistical analysis of variance (ANOVA) was performed as described (Somasegaran and Hoben, 1994). The 1% and 5% levels for the ANOVA test was used for comparing the calculated F ratio by using standard tables (Table-7).

Table. 7 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.

CHAPTER IV

RESULTS

Chapter 4

RESULTS

4.1 ISOLATION, PURIFICATION AND SCREENING OF STRAINS

A total of 18 xylanase producing fungal cultures were isolated initially on the basis of colour and colony appearance at 30°C. Waste from a soda ash industry resulted in 11 isolates, whereas 7 isolates were obtained from paper mill industry effluent. A primary screening of the isolated fungal cultures for their xylanase producing ability was performed on the basis of clearing zone formation on xylan agar plates. Morphological features and clearing zone diameter of the isolated strains are given in Table-8. Out of 18 isolates, SA-2, SA-3, SA-4, SA-5, SA-7, SA-8, SA-11 SB-2 and SB-3 resulted in zone formation with a considerable diameter (≥3.1 cm). Among these, two isolates each from the soda ash industry waste and paper mill effluents were further subjected to quantitative estimation of xylanase, β-xylosidase and cellulase activity in submerged condition. Maximum xylanase production (242.05 ± 3.91 IU ml^{-1}) was observed from SA-8 (Table-9) along with significant amounts of β-xylosidase (16.20 ± 2.00 IU $ml^{-1)}$ and negligible levels of cellulase activity (0.18 ± 0.03 IU ml^{-1}). The strain SA-8 was, therefore, selected for further studies and identified as *Penicillium oxalicum* SA-8 ITCC 6024 by the Indian Type Culture Collection Bank, Indian Agricultural Research Institute, New Delhi, India.

Table. 8 Morphological features and clearing zone diameter of fungal isolates

Isolated strains	Site of isolation	Morphology	Spore colour	Zone diameter (cm)
SA-1	Soda ash industry waste	White hyphae, forming flat mat-like colony, horizontally growing mycelia	White	2.2 ± 0.43
SA-2	Soda ash industry waste	Pink hyphae which turned green in later stage	Green	3.6 ± 0.11
SA-3	Soda ash industry waste	Light green and orange in colour	Green	3.2 ± 0.43
SA-4	Soda ash industry waste	White erect hyphae which form dome shaped colony; it appears green at margin	-	3.9 ± 0.08
SA-5	Soda ash industry waste	Yellowish white hyphae	Black	4.1 ± 0.05
SA-6	Soda ash industry waste	White mycelium	Green	2.2 ± 0.14
SA-7	Soda ash industry waste	Erect white hyphae	Pinkish green	3.2 ± 0.14
SA-8	Soda ash industry waste	White hyphae	Light green	4.2 ± 0.14
SA-9	Soda ash industry waste	Off-white mycelium	-	2.9 ± 0.10
SA-10	Soda ash industry waste	Transparent off-white mycelium	-	2.5 ± 0.06
SA-11	Soda ash industry waste	Light pink hyphae	-	3.1 ± 0.17
SB-1	Paper mill effluent	Yellowish green	Pale yellow	2.6 ± 0.08
SB-2	Paper mill effluent	White	Light brown	3.2 ± 0.14
SB-3	Paper mill effluent	Light green with white spots	light green	3.1 ± 0.08
SB-4	Paper mill effluent	Erect white cottony hyphae	Black	2.6 ± 0.08
SB-5	Paper mill effluent	White	Green	2.6 ± 0.08
SB-6	Paper mill effluent	White	Green	2.0 ± 0.05
SB-7	Paper mill	White	Black	2.3 ± 0.06

(Values are mean of three replicates ± SE).

Table. 9 Enzyme production from the selected fungal strains

Potent strains	Xylanase activity (IU ml^{-1})	β-xylosidase activity (IU ml^{-1})	Cellulase activity (IU ml^{-1})
SA-5	147.90 ± 6.94	2.55 ± 0.06	0.34 ± 0.02
SA-8	242.05 ± 3.91	16.20 ± 2.00	0.18 ± 0.03
SB-2	152.08 ± 5.29	3.96 ± 0.80	0.21 ± 0.02
SB-3	135.56 ± 3.93	13.13 ± 1.20	0.18 ± 0.02

(Values are mean of three replicates ± SE).

4.2 MUTAGENESIS OF *P. OXALICUM* SA-8 FOR IMPROVED XYLANASE PRODUCTION

The spores of strain *P. oxalicum* SA-8, isolated from the waste of soda ash industry was subjected to physical (UV-irradiation), chemical (N-methyl-N'-nitro-N-nitrosoguanidine, ethidium bromide) and mixed mutagenesis to determine if improved levels of xylanase can be obtained. Following mutagenic treatment, survivability and percent distribution of positive, negative and corresponding or unaltered mutants were determined. Among the mutants generated as a result of initial stage of mutagenesis, mutant SAU-3.5 obtained following UV mutagenesis showed the maximum clearing zone formation (4.7 cm diameter) on a xylan agar plate. Out of the 88.9% spores that survived following 3.5 min of UV exposure, 13% had a greater zone diameter compared to the parent strain and were termed as positive mutants, whereas 8% of the colonies had a lesser diameter and were denoted as negative mutants; 79% of the mutants had the zone diameter (~3.8-4.0 cm) that corresponded to the parental strain, hence termed the corresponding mutants (Fig. 6a). SAU-3.5 produced 430.81 ± 8.33 IU ml^{-1} of xylanase in submerged fermentation. The parental strain SA-8 was also subjected to NTG (150 μg ml^{-1}) and to EtBr (200 μg ml^{-1}) treatments. Among positive mutants, SAN-60 obtained

following 60 minutes of exposure with NTG, had a zone-diameter of 4.5 cm with a 70% spore survival. Out of the total mutants obtained, 27%, 7% and 66%, respectively, were positive, negative and unaltered mutants (Fig. 6b). Further, mutant SAE-10 generated after 10 min of EtBr treatment (200 μg ml^{-1}) had a zone diameter of 4.7 cm with an 85% spore survivability. Among these, 5% of the total mutants obtained were negative, 10% positive and 84% were the corresponding mutants (Fig. 6c).

Among the mutants obtained following UV, NTG and EtBr mutagenesis, i.e. SAU-3.5, SAN-60 and SAE-10, mutant SAU-3.5, obtained after UV irradiation resulted in a comparatively higher zone diameter, had a maximum increase (78%) in xylanase production ability. Mutant SAU-3.5 was further subjected to a second round of mutagenesis. Mutant SAU_E-3.510, obtained following EtBr exposure, exhibited a further increase in the enzyme production level (453.02 ± 5.70 IU ml^{-1}) (Table-10). Of the total mutants thus obtained, 9%, 3% and 88% were the positive, negative and corresponding mutants, respectively (Fig. 6e).

Table. 10 Xylanase production by the selected mutants obtained following successive stages of mutagenesis

Mutagen	Strain	Xylanase activity (IU ml^{-1})	Percent increase
None	SA-8	242.05 ± 3.91	-
UV	SAU-3.5	430.81 ± 8.33	78
NTG	SAN-60	368.84 ± 4.53	52
EtBr	SAE-10	390.86 ± 5.21	61
UV+EtBr	SAU_E-3.510	453.02 ± 5.70	87
UV+NTG	SAU_N-3.560	421.94 ± 6.81	74

(Values are mean of three replicates ± SE).

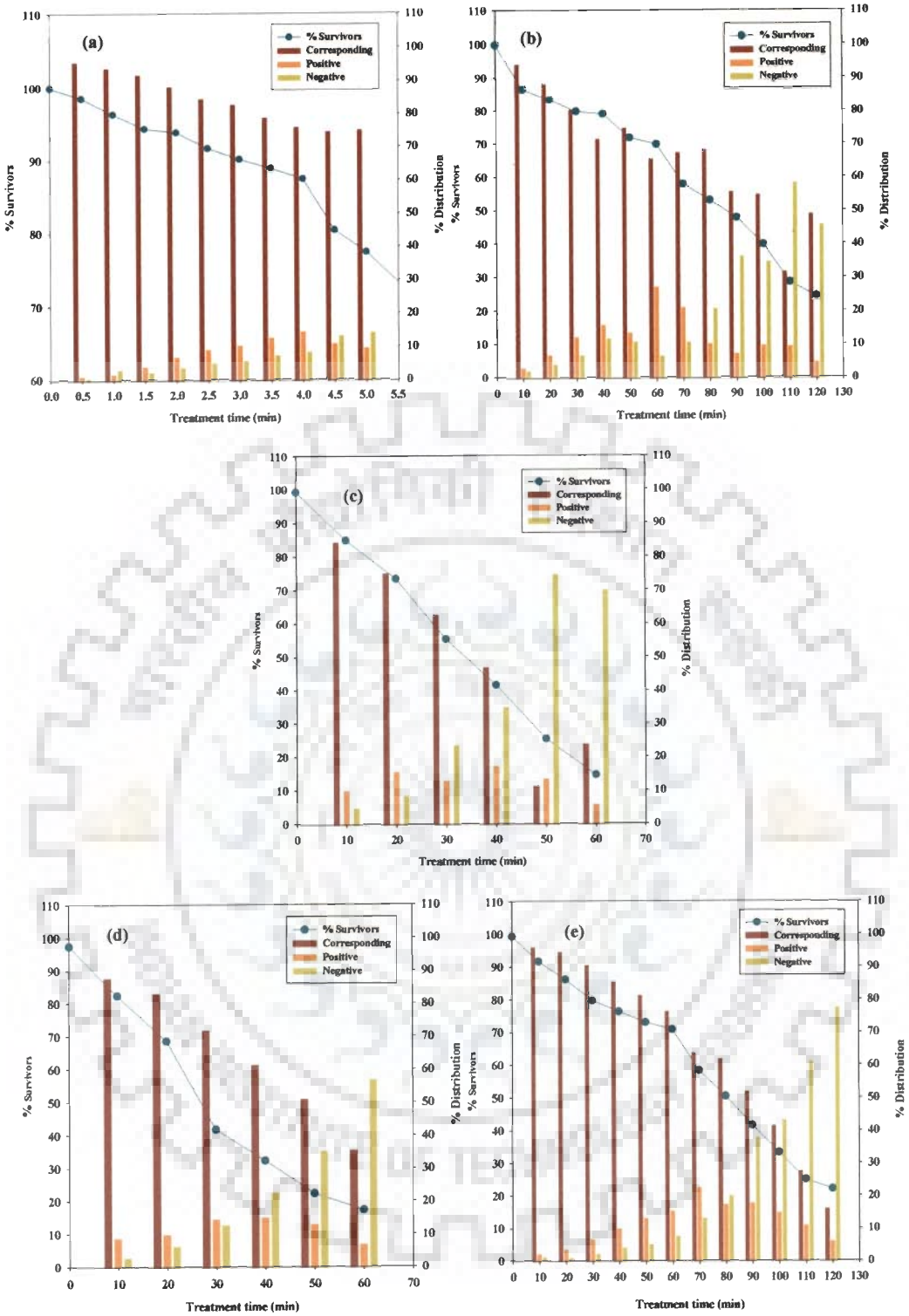


Fig. 6 Analysis of mutagenesis of *P. oxalicum* SA-8 by (a) UV-irradiation; (b) N-methyl-N'-nitro-N-nitrosoguanidine; (c) Ethidium bromide; (d) Mixed (UV+ EtBr); (e) Mixed (UV + NTG).

4.2.1 Morphological features

The morphological features of wild type *P. oxalicum* SA-8 and mutant *P. oxalicum* SAU_E-3.510 are shown in Fig. 7a, b. The wild type and mutant of *P. oxalicum*, both had white hyphae with dark green spores. However, the colony obtained following growth of mutant SAU_E-3.510 had irregular margins and was flattened, compared to the smooth margins of the wild type strain (Fig. 7a, b). However, substantially greater xylanase production ability was found with mutant SAU_E-3.510 compared to the wild type strain (Fig. 7c, d).

Scanning electron microscopy of fungal mycelia and spores showed that the hyphae of wild type *P. oxalicum* SA-8 were flattened, shiny, smooth surfaced and straight, whereas the hyphae of mutant *P. oxalicum* SAU_E-3.510 appeared coiled, rounded and had a rough surface (Fig. 8a, b). Similarly, spores of the wild type and mutant had characteristically distinct features. Spores from the wild type were rounded with a rougher surface, whereas the spores from the mutant strain were smooth, flattened, crescent shaped and they also appear to be considerably larger than those of the parental strain (Fig. 9a, b).

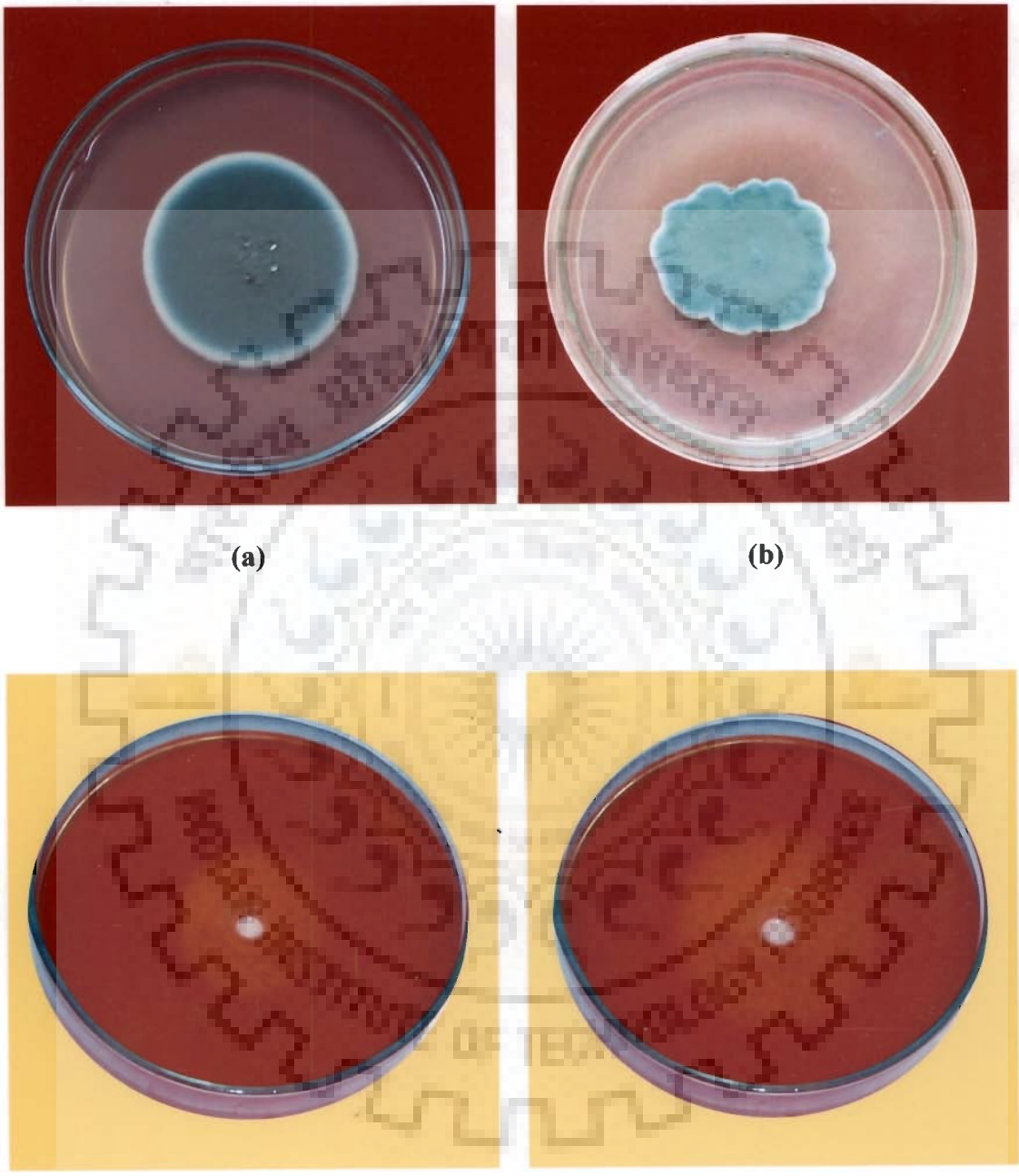
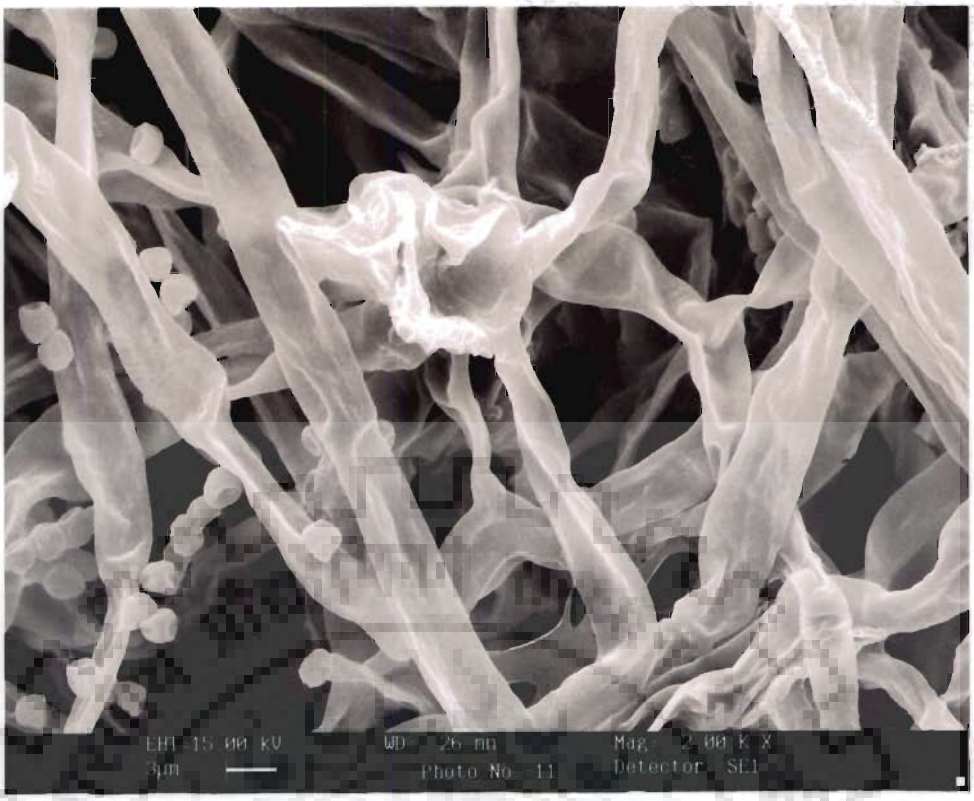
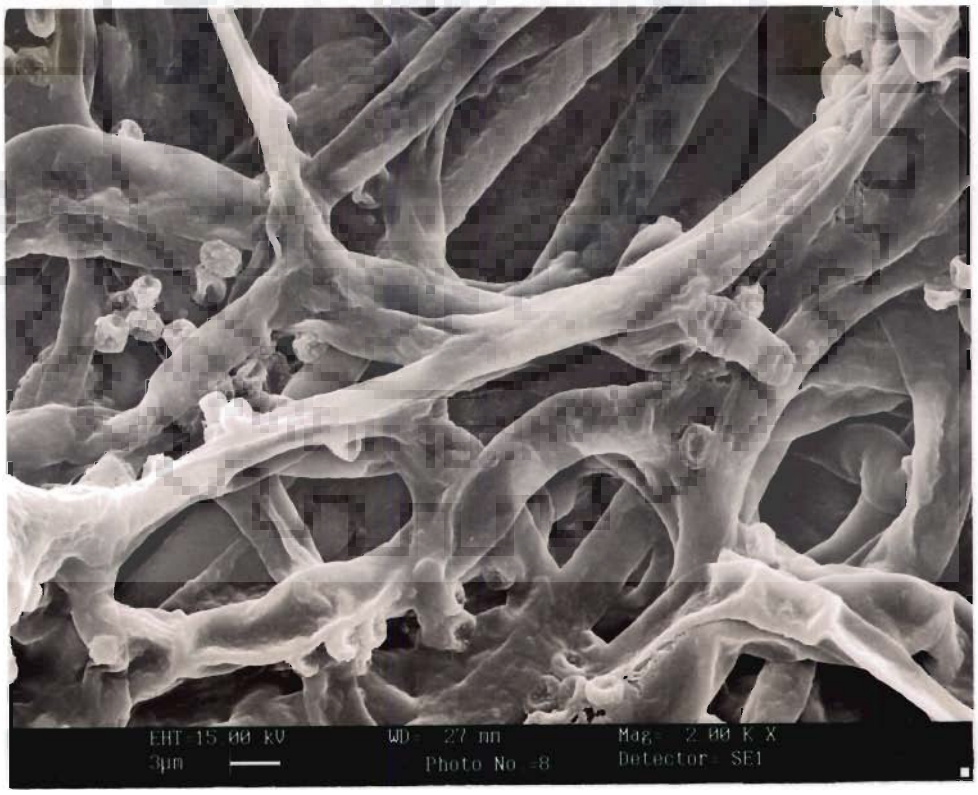


Fig. 7 Colonial morphology (a,b) and xylanase assay (c,d) of wild type *P. oxalicum* SA-8 (a, c) and mutant *P. oxalicum* SAU_E- 3.510 (b, d) strains.

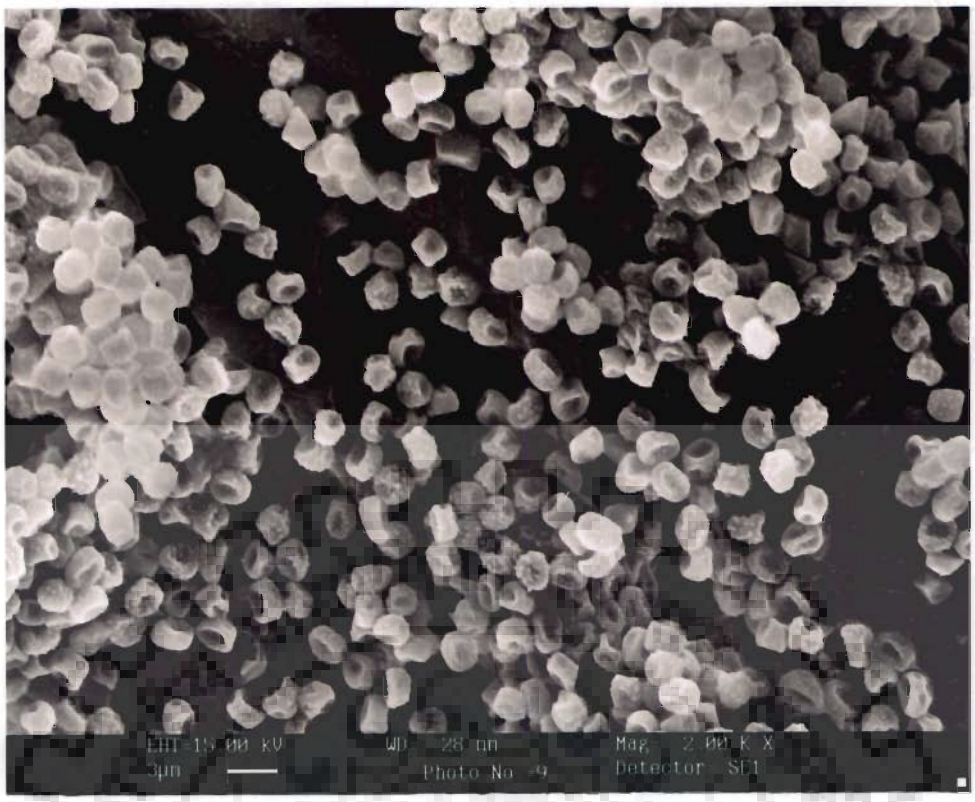


(a)

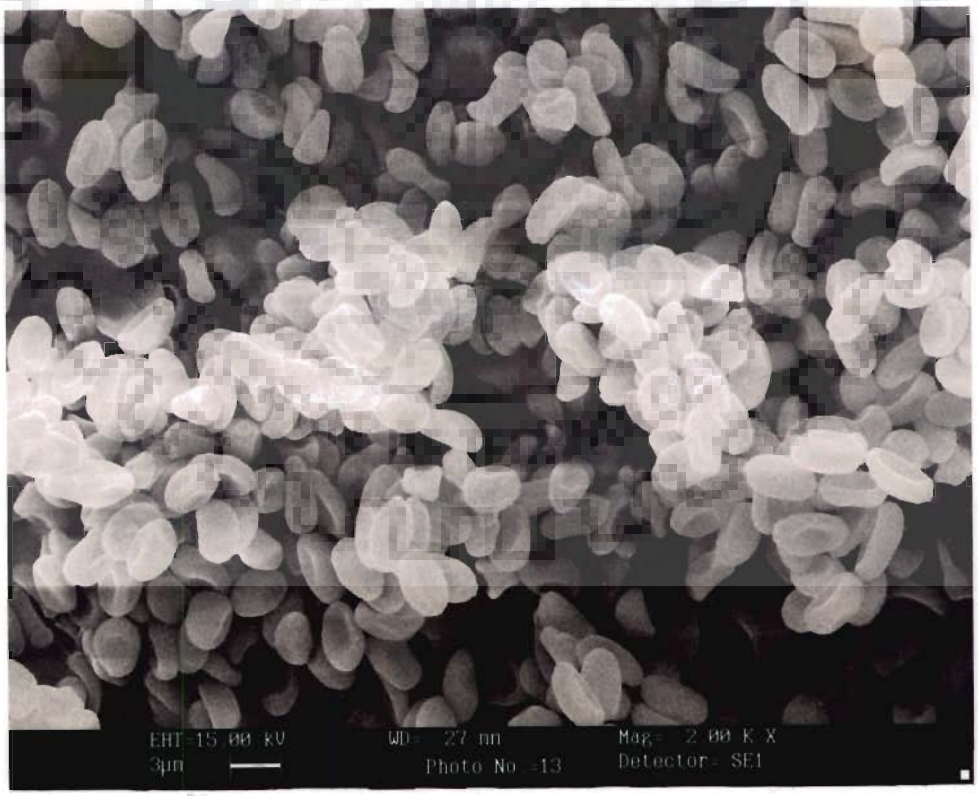


(b)

Fig. 8 Scanning electron microscopy of wild type SA-8 (a) and mutant SAU_E-3.510 (b) mycelia.



(a)



(b)

Fig. 9 Scanning electron microscopy of wild type SA-8 (a) and mutant SAU_E-3.510 (b) spores.

4.3 GROWTH AND XYLANASE ACTIVITY AT DIFFERENT pH VALUES

The effect of pH in the range of pH 3.0 to12.0 on xylanase production was studied in shake flasks. The pH of the production medium was adjusted using acid/alkali solutions and the enzyme assay was done after adjusting the pH of the crude enzyme preparation to pH 9.0. The xylanase activity was dependent on the pH of the growth medium. The strain was able to grow and produced significant levels of xylanase over a wide range of pH values (5.0-10.0) and the maximum level of xylanase production was detected at pH 9.0 with both the mutant and wild type strains and a decrease in the xylanase production level was observed at the higher pH values. The ability of both the mutant and wild type strains to produce a high level of xylanase activity under alkaline conditions (pH 9.0) indicates the alkalitolerant nature of these fungi (Fig. 10a).

The activity profile of xylanase was evaluated at different pH values ranging from pH 4.0-11.0. The maximum activity of the xylanase of mutant *P. oxalicum* SAU_E-3.510 (487.44 ± 3.57 IU ml^{-1}) as well as of the wild type *P. oxalicum* SA-8 (252.50 ± 6.27 IU ml^{-1}) was found at pH 9.0. A notable level of enzyme activity was also observed at the higher pH values. Interestingly, a high enzyme activity was also observed at pH 5.0 with the xylanase of both mutant *P. oxalicum* SAU_E-3.510 (444.89 ± 7.60 IU ml^{-1}) as well as of the wild type strain (197.81 ± 6.94 IU ml^{-1}) (Fig. 10b).

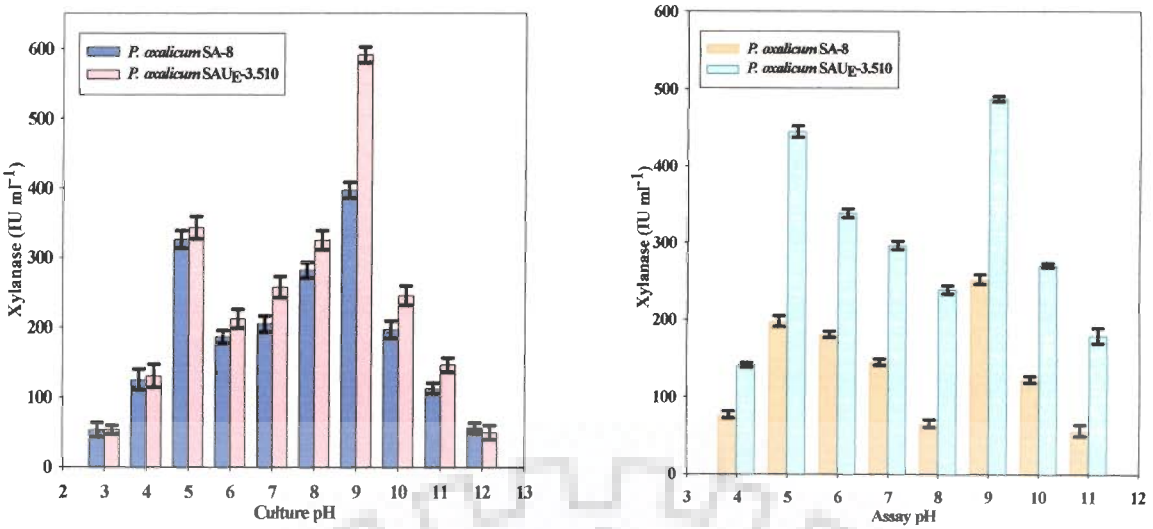


Fig. 10 Effect of pH on the production (a) and xylanase activity (b) of wild type *P. oxalicum* SA-8 and mutant *P. oxalicum* SAU_E-3.510 (values are mean of three replicates ± SE).

4.4 CRITICAL FACTORS AFFECTING ENZYME PRODUCTION UNDER SHAKE FLASK CULTIVATION

4.4.1 Enzyme production as a function of incubation period

Xylanase production by *P. oxalicum* SAU_E-3.510 at 30°C was monitored for up to 8 days in the medium containing 0.5% oat spelt xylan as substrate. Xylanase production was detected after 24 to 48 h of incubation and reached to its maximum level following six days of incubation (Fig. 11). Production declined sharply thereafter. Maximum β-xylosidase activity was also observed following a similar period of incubation. Interestingly, the significant levels of cellulase activity (0.58 ± 0.06 IU ml^{-1}) that were detected after 24 h of incubation declined to nearly undetectable levels (0.05 ± 0.02 IU ml^{-1}) at the time when maximum levels of xylanase activity was observed. As indicated in Fig. 11, exponential growth had been observed. Biomass concentrations when plotted on an exponential scale had resulted into a straight line (Fig. 12). The slope of this curve was used for calculation of specific growth rate i.e. μ_{max}=0.3854 day^{-1}.

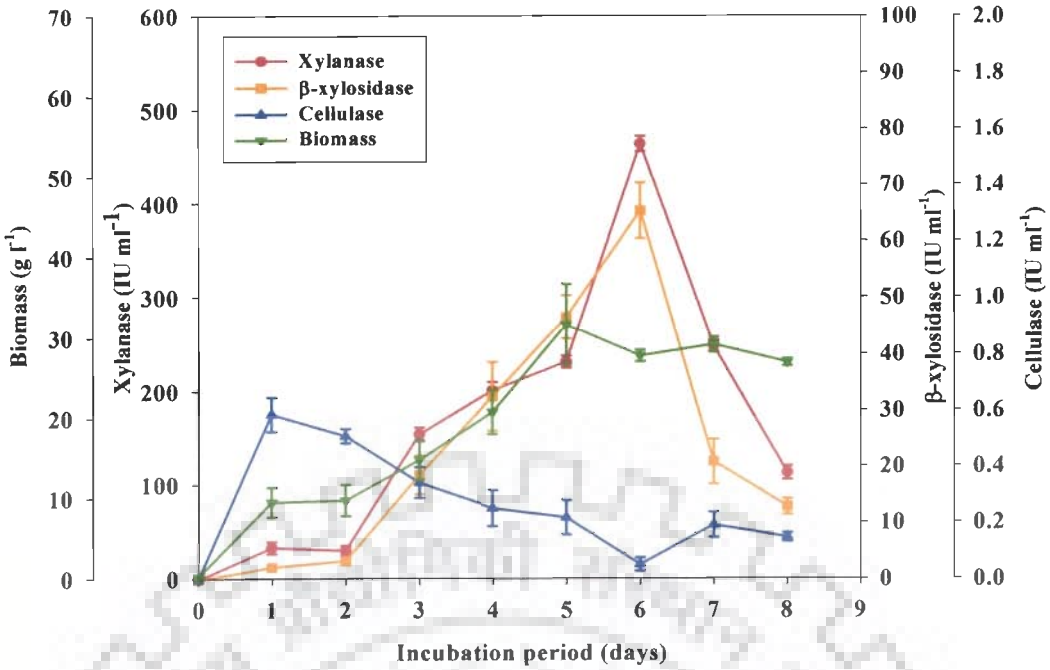


Fig. 11 Enzyme production at varying periods of incubation.

(Cultivation was performed at 30° C at a pH value of 9.0 and at 150 rpm under shake flask condition; values are mean of three replicates ± SE).

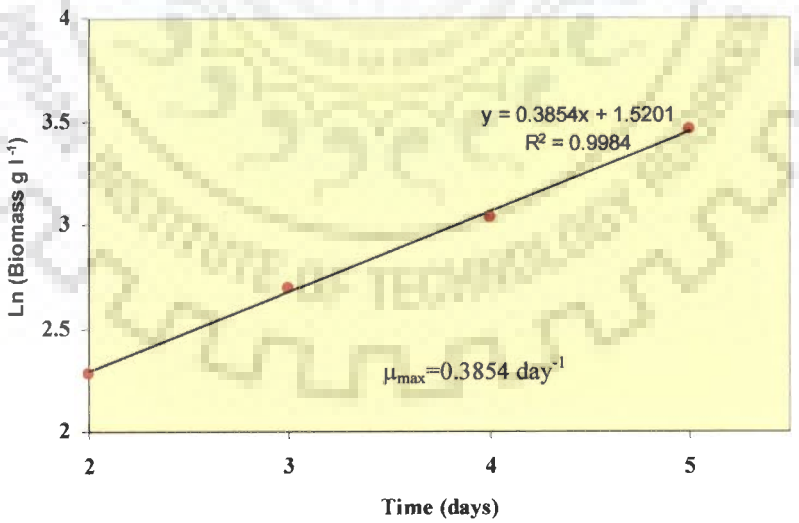


Fig. 12 Exponential growth during shake flask cultivation.

(Cultivation was performed at 30° C at a pH value of 9.0 and at 150 rpm under shake flask condition; values are mean of three replicates).

4.4.2 Effect of carbon source on enzyme production

Oat spelt xylan in the production medium was replaced with different mono, di and polysaccharides, viz. fructose, galactose, glucose, xylose, CMC, avicel, lactose, oat spelt xylan and birch wood xylan. Maximum xylanase activity was observed with oat spelt xylan (488.56 ± 6.53 IU ml^{-1}), followed by birch wood xylan (354.75 ± 8.01 IU ml^{-1}) (Fig. 13a). Besides the above, xylose also appeared to induce the production levels. Various hemicellulose-containing natural substrates, including non-food plant biomass and agricultural residues, were evaluated for xylanase production. Of the various natural substrates used, alkali treated congress grass as well as treated water hyacinth resulted in remarkably high levels of activities (475.23 ± 6.05 IU ml^{-1}, 397.05 ± 7.53 IU ml^{-1}) (Fig 13b). Xylanase activity obtained using the congress grass was about at similar levels to that obtained with commercial oat spelt xylan.

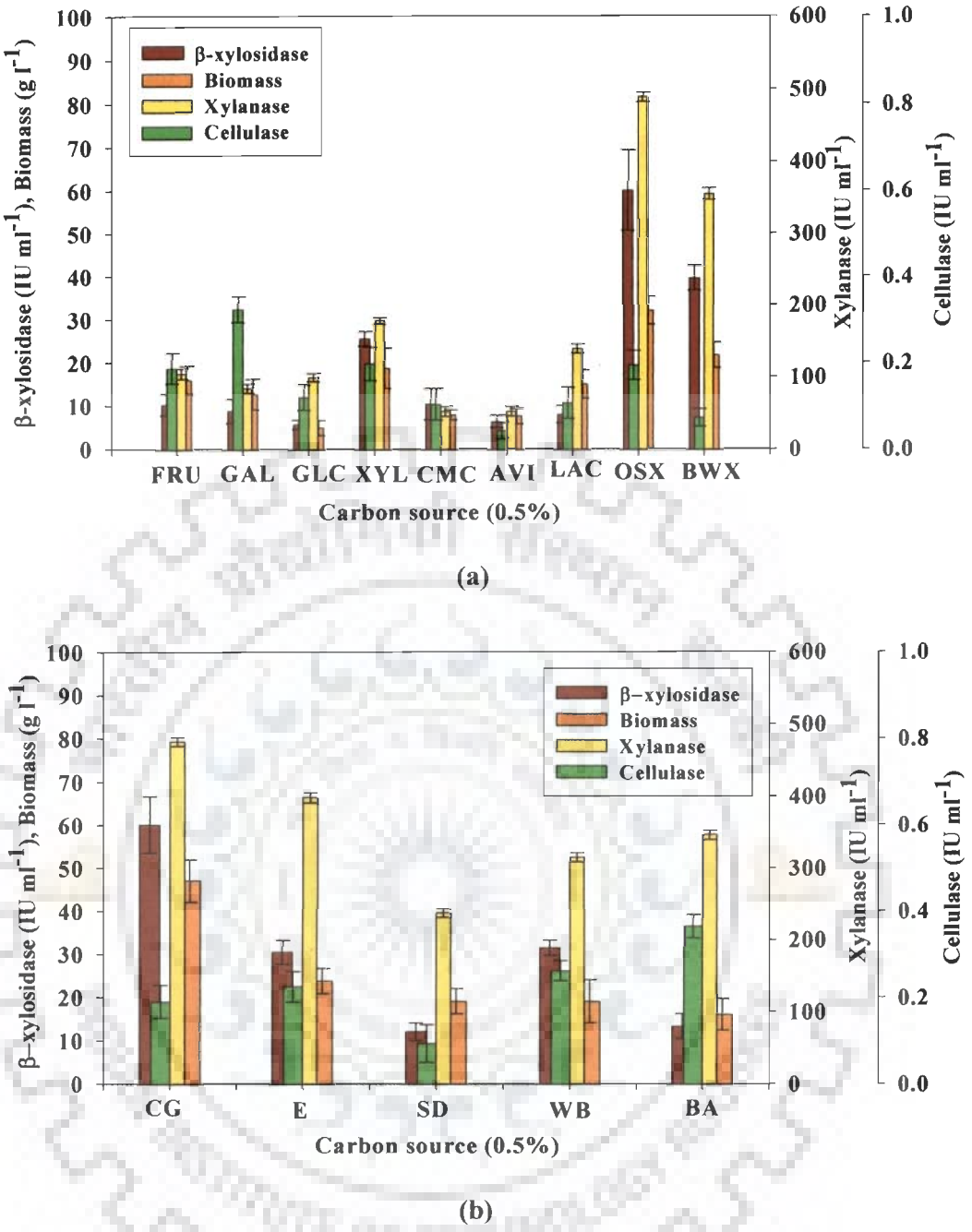


Fig. 13 Production of enzymes on different mono, di and polysaccharides (a) and complex carbon substrates (b) by mutant *P. oxalicum* SAU_E-3.510.

(**a**, Mono, di and polysaccharides: FRU, Fructose; GAL, Galactose; GLC, Glucose; XYL, Xylose; CMC, Carboxymethyl cellulose; AVI, Avicel; LAC, Lactose; OSX, Oat spelt xylan and BWX, Birch wood xylan;

b, Complex carbon substrates: CG, Congress grass; EI, *Eichhornia*; SD, Saw dust; WB, Wheat bran and BA, Bagasse).

(Cultivation was performed at 30° C at a pH value of 9.0 and at 150 rpm under shake flask condition; values are mean of three replicates ± SE).

Table. 11 Analysis of variance for critical factors for xylanase production by *P. oxalicum* SAU_E-3.510 in shake flask cultivation

Parameters	Source of variation	Degree of freedom	Sum of square	Mean square	F ratio observed
Incubation period	Between the Samples	8	501587.6	62698.4	454.5
	With in the Samples	2	160.3	80.1	0.5
	Error	16	2207.1	137.4	
Carbon source (Mono, di and Polysaccharides)	Between the Samples	8	542589.8	67823.7	2000.4
	With in the Samples	2	1713.9	856.9	25.2
	Error	16	542.4	33.9	
Carbon source (Complex carbon Substrates)	Between the Samples	9	204827.3	22758.5	214.5
	With in the Samples	2	397.1	198.5	1.8
	Error	18	1908.9	106.0	
Carbon source concentration	Between the Samples	4	296041.9	74010.4	821.6
	With in the Samples	2	591.9	295.9	3.2
	Error	8	720.5	90.0	
pH for Growth	Between the Samples	9	701320.7	77924.5	227.3
	With in the Samples	2	4106.1	2053.0	5.9
	Error	18	6168.9	342.7	
Inducers	Between the Samples	5	159757.2	31951.4	63.4
	With in the Samples	2	1546.9	773.4	1.5
	Error	10	5033.9	503.3	

Significant at 5% level.

4.5 CO-CULTIVATION STRATEGY FOR SIMULTANEOUS BIOSYNTHESIS OF XYLANASE AND LACCASE

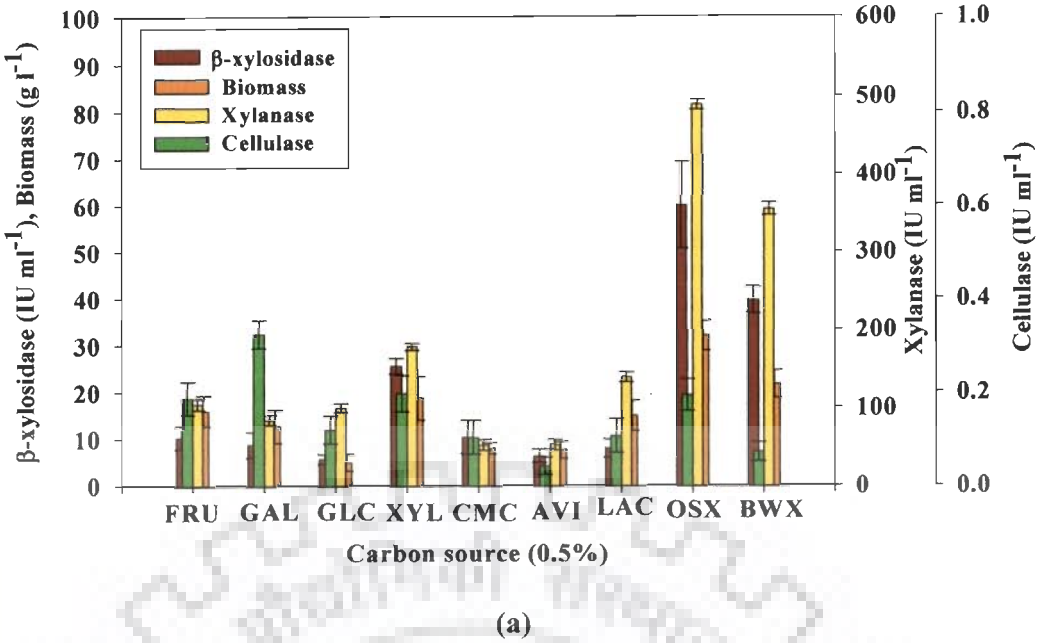
Co-cultivation of fungi which produce xylanase and laccase in their respective monoculture system may be used for developing a mixed enzyme preparation possessing both xylanase and laccase activity. Such an enzyme preparation could be a promising approach for its use in biobleaching. The co-culturing of fungi belonging to two different taxonomic groups for xylanase and laccase production may be a novel approach for developing an enzyme concoction having both xylanase and laccase activities and no attempts for the same has been made so far.

4.5.1 Fungal compatibility assay

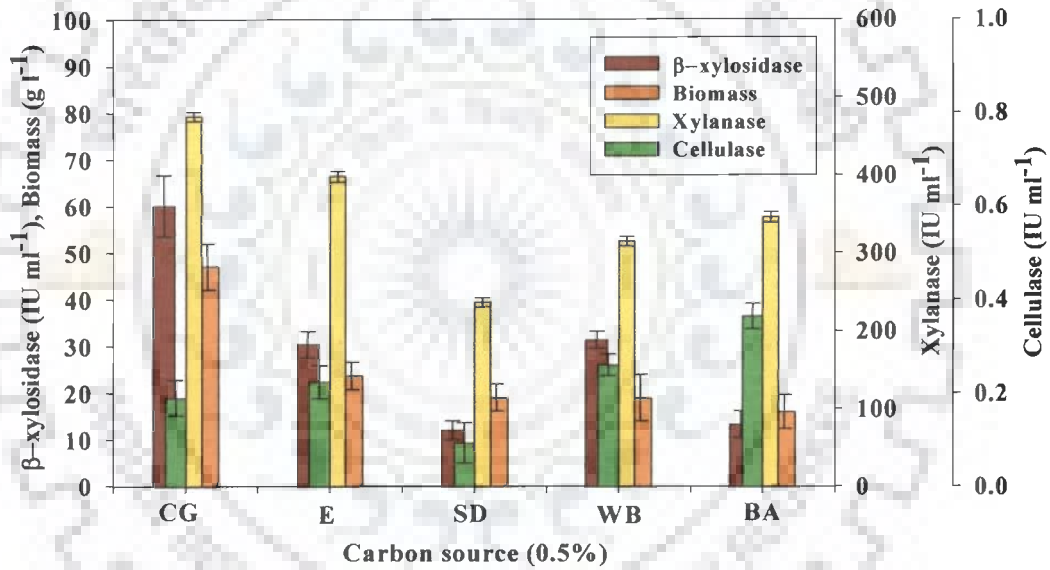
The compatibility of fungi belonging to two different groups, viz ascomycetes and basidiomycetes, was evaluated by growing fungi on potato dextrose agar medium. To analyse if fungal co-cultivation could lead to an enhanced productivity of enzymes, the ability of cultures to coexist in a similar culture medium is desirable. All selected white rot fungi, viz *P. ostreatus* MTCC 1804, *T. hirsuta* MTCC 136 and *Pycnoporus* MTCC 137, were grown in association with mutant *P. oxalicum* SAU_E-3.510 on PDA plates and their growth was compared with their respective monoculture (Fig. 16a, b). The diameter of the fungal colony was measured every day after inoculation up to five days and was used as a criterion to check their compatibility during co-cultivation (Table-12). *P. ostreatus* MTCC 1804 showed the maximum compatibility with mutant *P. oxalicum* SAU_E-3.510 as its growth remained unaffected when it came into contact with mutant hyphae and the growth pattern of both fungi was also similar in dual culture to their respective monocultures (Fig. 16a, b). Some thickening and browning of *P. ostreatus* MTCC 1804 hyphae were observed in the contact zone. This could possibly have been due to a higher activity of oxidative enzymes. A diminished growth pattern of *T. hirsuta* MTCC 136 and *Pycnoporus* MTCC 137 during their co-culturing with mutant *P. oxalicum* SAU_E-3.510 was noted compared to their growth in monocultures (Figure 16a, b). Initially the growth appeared

normal but as soon as their colony diameter increased, their hyphae appeared scanty. This was not observed in their axenic cultures.

Interaction between *P. ostreatus* MTCC 1804 and mutant *P. oxalicum* SAU_E-3.510 during their co-culture on PDA plates was also enumerated by scanning electron microscopy. Hyphae of mutant *P. oxalicum* SAU_E-3.510 were thick, flattened with a rougher and peeled surface (Fig. 17a), while that of *P. ostreatus* MTCC 1804 were thinner, smooth surfaced, shiny and whitish in colour (Fig. 17b). Interaction of both fungal hyphae is shown in Fig. 17c, in which both can be easily demarcated. The contact regions for both fungi denote a close association and branching, deformities or injuries in the hyphae were not observed. Slight coiling and roughness was seen in the hyphae of *P. ostreatus* MTCC 1804 and the hyphal tip of mutant *P. oxalicum* SAU_E-3.510 became thick and globous, while no change was observed in its surface. This suggested that the interaction between two fungi was not antagonistic and they tend to support the growth of each other.



(a)



(b)

Fig. 13 Production of enzymes on different mono, di and polysaccharides (a) and complex carbon substrates (b) by mutant *P. oxalicum* SAU_E-3.510.

(**a**, Mono, di and polysaccharides: FRU, Fructose; GAL, Galactose; GLC, Glucose; XYL, Xylose; CMC, Carboxymethyl cellulose; AVI, Avicel; LAC, Lactose; OSX, Oat spelt xylan and BWX, Birch wood xylan;

b, Complex carbon substrates: CG, Congress grass; EI, *Eichhornia*; SD, Saw dust; WB, Wheat bran and BA, Bagasse).

(Cultivation was performed at 30° C at a pH value of 9.0 and at 150 rpm under shake flask condition; values are mean of three replicates ± SE).

4.4.3 Enzyme production as a function of substrate concentration

Congress grass at varying concentrations was used to define the suitable concentration for achieving maximum production of xylanase in shake flask fermentation. As shown (Fig. 14), the levels of xylanase, β-xylosidase, cellulase and biomass synthesis varied markedly with mutant strain *P. oxalicum* SAU_E-3.510. Maximum levels of xylanase (563.05 ± 6.49 IU ml^{-1}) and β-xylosidase (85.57 ± 5.30 IU ml^{-1}) were obtained at a 0.25% (w/v) concentration of congress grass.

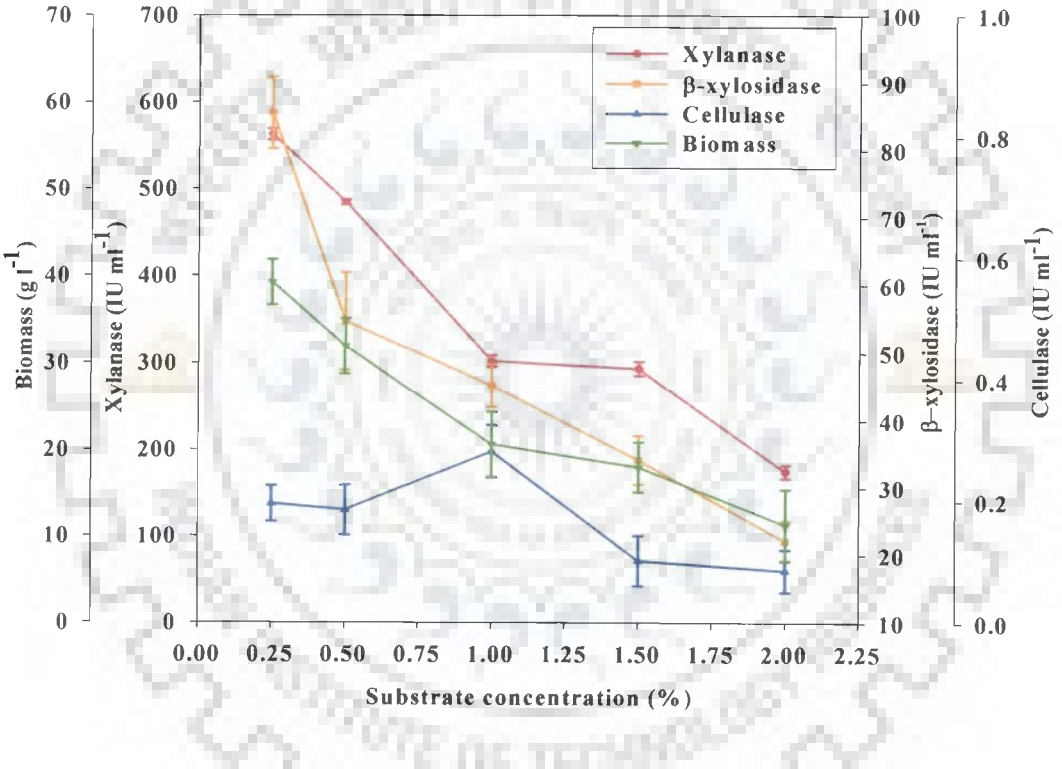


Fig. 14 Effect of concentration of congress grass on enzyme production.
(Cultivation was performed at 30° C at a pH value of 9.0 and at 150 rpm under shake flask condition; values are mean of three replicates ± SE).

4.4.4 Effect of lignin model compounds on xylanase production

In order to further enhance the level of xylanase production, various lignin model compounds viz; veratryl alcohol, guaiacol, syrinzaldazine, ABTS and vanilline following filter

sterilisation (0.2 μm membrane filter, Millipore, USA) were added in the production media along with inoculum. As shown (Fig. 15) veratryl alcohol and ABTS had mildly enhanced the xylanase biosynthesis while the production level decreased in presence of guaiacol, syrinzaldazine and vanilline. The maximum level of xylanase (714.13 ± 15.40 IU ml^{-1}) was achieved in the presence of veratryl alcohol. The statistical analysis of the factors derived is given in Table-11.

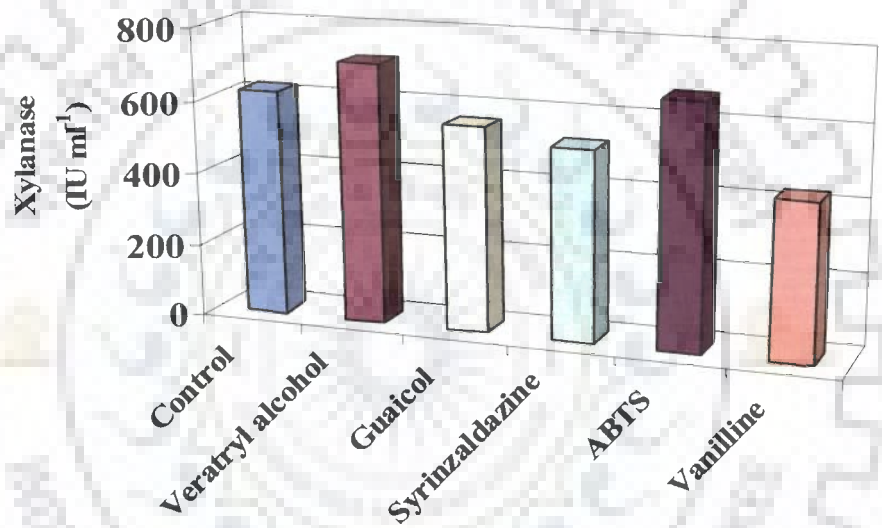


Fig. 15 Effect of lignin model compounds on xylanase biosynthesis in shake flask cultivation.

(Cultivation was performed at 30° C at a pH value of 9.0 and at 150 rpm under shake flask condition; values are mean of three replicates).

Table. 11 Analysis of variance for critical factors for xylanase production by *P. oxalicum* SAU_E-3.510 in shake flask cultivation

Parameters	Source of variation	Degree of freedom	Sum of square	Mean square	F ratio observed
Incubation period	Between the Samples	8	501587.6	62698.4	454.5
	With in the Samples	2	160.3	80.1	0.5
	Error	16	2207.1	137.4	
Carbon source (Mono, di and Polysaccharides)	Between the Samples	8	542589.8	67823.7	2000.4
	With in the Samples	2	1713.9	856.9	25.2
	Error	16	542.4	33.9	
Carbon source (Complex carbon Substrates)	Between the Samples	9	204827.3	22758.5	214.5
	With in the Samples	2	397.1	198.5	1.8
	Error	18	1908.9	106.0	
Carbon source concentration	Between the Samples	4	296041.9	74010.4	821.6
	With in the Samples	2	591.9	295.9	3.2
	Error	8	720.5	90.0	
pH for Growth	Between the Samples	9	701320.7	77924.5	227.3
	With in the Samples	2	4106.1	2053.0	5.9
	Error	18	6168.9	342.7	
Inducers	Between the Samples	5	159757.2	31951.4	63.4
	With in the Samples	2	1546.9	773.4	1.5
	Error	10	5033.9	503.3	

Significant at 5% level.

4.5 CO-CULTIVATION STRATEGY FOR SIMULTANEOUS BIOSYNTHESIS OF XYLANASE AND LACCASE

Co-cultivation of fungi which produce xylanase and laccase in their respective monoculture system may be used for developing a mixed enzyme preparation possessing both xylanase and laccase activity. Such an enzyme preparation could be a promising approach for its use in biobleaching. The co-culturing of fungi belonging to two different taxonomic groups for xylanase and laccase production may be a novel approach for developing an enzyme concoction having both xylanase and laccase activities and no attempts for the same has been made so far.

4.5.1 Fungal compatibility assay

The compatibility of fungi belonging to two different groups, viz ascomycetes and basidiomycetes, was evaluated by growing fungi on potato dextrose agar medium. To analyse if fungal co-cultivation could lead to an enhanced productivity of enzymes, the ability of cultures to coexist in a similar culture medium is desirable. All selected white rot fungi, viz *P. ostreatus* MTCC 1804, *T. hirsuta* MTCC 136 and *Pycnoporus* MTCC 137, were grown in association with mutant *P. oxalicum* SAU_E-3.510 on PDA plates and their growth was compared with their respective monoculture (Fig. 16a, b). The diameter of the fungal colony was measured every day after inoculation up to five days and was used as a criterion to check their compatibility during co-cultivation (Table-12). *P. ostreatus* MTCC 1804 showed the maximum compatibility with mutant *P. oxalicum* SAU_E-3.510 as its growth remained unaffected when it came into contact with mutant hyphae and the growth pattern of both fungi was also similar in dual culture to their respective monocultures (Fig. 16a, b). Some thickening and browning of *P. ostreatus* MTCC 1804 hyphae were observed in the contact zone. This could possibly have been due to a higher activity of oxidative enzymes. A diminished growth pattern of *T. hirsuta* MTCC 136 and *Pycnoporus* MTCC 137 during their co-culturing with mutant *P. oxalicum* SAU_E-3.510 was noted compared to their growth in monocultures (Figure 16a, b). Initially the growth appeared

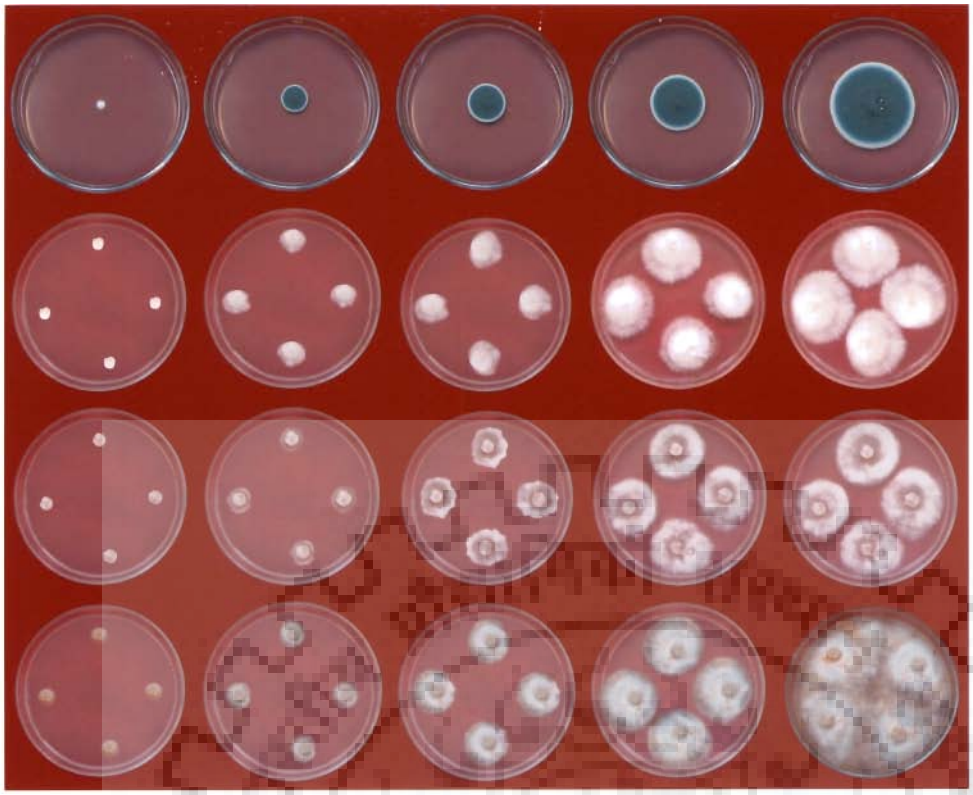
normal but as soon as their colony diameter increased, their hyphae appeared scanty. This was not observed in their axenic cultures.

Interaction between *P. ostreatus* MTCC 1804 and mutant *P. oxalicum* SAU_E-3.510 during their co-culture on PDA plates was also enumerated by scanning electron microscopy. Hyphae of mutant *P. oxalicum* SAU_E-3.510 were thick, flattened with a rougher and peeled surface (Fig. 17a), while that of *P. ostreatus* MTCC 1804 were thinner, smooth surfaced, shiny and whitish in colour (Fig. 17b). Interaction of both fungal hyphae is shown in Fig. 17c, in which both can be easily demarcated. The contact regions for both fungi denote a close association and branching, deformities or injuries in the hyphae were not observed. Slight coiling and roughness was seen in the hyphae of *P. ostreatus* MTCC 1804 and the hyphal tip of mutant *P. oxalicum* SAU_E-3.510 became thick and globous, while no change was observed in its surface. This suggested that the interaction between two fungi was not antagonistic and they tend to support the growth of each other.

Table. 12 - Growth compatibility of mutant *P. oxalicum* SAU_E-3.510 (M) with *P. ostreatus* MTCC 1804 (Plu), *Pycnoporus* sp. MTCC 137 (Pyc) and *T. hirsuta* MTCC 136 (Tra) during mono and dual culture conditions

Incubation period (days)	Colony diameter (cm)									
	Monoculture				Co-cultivation					
	M	Plu	Pyc	Tra	M	: Plu	M	: Pyc	M	: Tra
1	Point	0.8 ± 0.00	0.8 ± 0.00	0.8 ± 0.00	Point	0.8 ± 0.00	Point	0.8 ± 0.00	Point	0.8 ± 0.00
2	1.7 ± 0.11	1.9 ± 0.11	1.6 ± 0.11	1.1 ± 0.17	1.8 ± 0.05	1.9 ± 0.14	1.9 ± 0.06	1.1 ± 0.11	1.9 ± 0.11	1.2 ± 0.11
3	2.3 ± 0.88	2.5 ± 0.14	2.8 ± 0.20	1.9 ± 0.20	2.5 ± 0.11	2.8 ± 0.05	2.3 ± 0.14	2.3 ± 0.20	2.2 ± 0.23	1.6 ± 0.11
4	3.3 ± 0.26	3.5 ± 0.11	3.4 ± 0.20	2.2 ± 0.17	3.4 ± 0.23	4.0 ± 0.23	3.6 ± 0.11	3.2 ± 0.05	3.1 ± 0.11	1.9 ± 0.11
5	4.1 ± 0.17	4.4 ± 0.11	3.8± 0.05	3.3 ± 0.11	Mix	Mix	4.1 ± 0.14	3.6 ± 0.11	3.8 ± 0.20	2.2 ± 0.11

(Values are mean of three replicates ± SE)



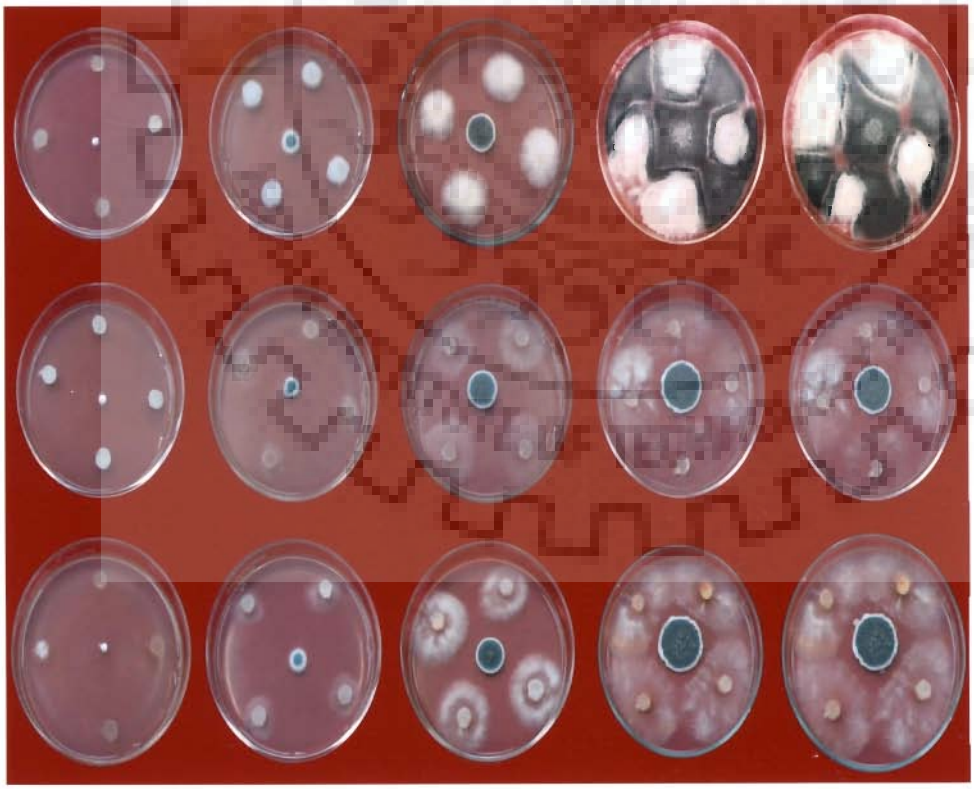
P. oxalicum SAU_E-3.510

P. ostreatus MTCC 1804

Pycnoporus sp. MTCC 137

T. hirsuta MTCC 136

(a)



P. oxalicum SAU_E-3.510 + *P. ostreatus* MTCC 1804

P. oxalicum SAU_E-3.510 + *Pycnoporus* sp. MTCC 137

P. oxalicum SAU_E-3.510 + *T. hirsuta* MTCC 136

1st D 2nd D 3rd D 4th D 5th D

(b)

Fig. 16 Growth compatibility of mutant *P. oxalicum* SAU_E-3.510 with *P. ostreatus* MTCC 1804, *Pycnoporus* sp. MTCC 137 and *T. hirsuta* MTCC 136 under single (a) and paired culture (b) conditions.

(a)

(b)

(c)

Fig. 17 Scanning electron micrographs showing fungal hyphae of mutant *P. oxalicum* SAU_E-3.510 (a), *P. ostreatus* MTCC 1804 (b) and interaction of mutant *P. oxalicum* SAU_E-3.510 and *P. ostreatus* MTCC 1804 during paired culture (c).

4.5.2 Evaluation of enzyme production at varying periods by white rot fungi

The mutant *P. oxalicum* SAU_E-3.510 is a potent xylanase producer with maximum (714.13 ± 15.40 IU ml^{-1}) xylanase activity after the 6th day of incubation under derived conditions but no laccase activity was observed in submerged cultivation. *P. ostreatus* MTCC 1804, *T. hirsuta* MTCC 136 and *Pycnoporus* sp. MTCC 137 were cultured in glucose peptone medium and laccase activity was measured for up to 20 days. Among the three selected fungi, *P. ostreatus* MTCC 1804 resulted in maximum laccase activity (13.02 ± 1.53 IU ml^{-1}) following 12 days of incubation (Fig. 18). A low level of xylanase activity (16.00 ± 2.84 IU ml^{-1}) was also produced by *P. ostreatus* MTCC 1804 after 6 days of incubation. High levels of enzyme biosynthesis by *P. ostreatus* MTCC 1804 along with its compatibility with mutant *P. oxalicum* SAU_E-3.510 indicated this strain to be superior to the other two strains (*T. hirsuta* MTCC 136, *Pycnoporus* sp. MTCC 137) hence *P. ostreatus* MTCC 1804 was selected for further studies.

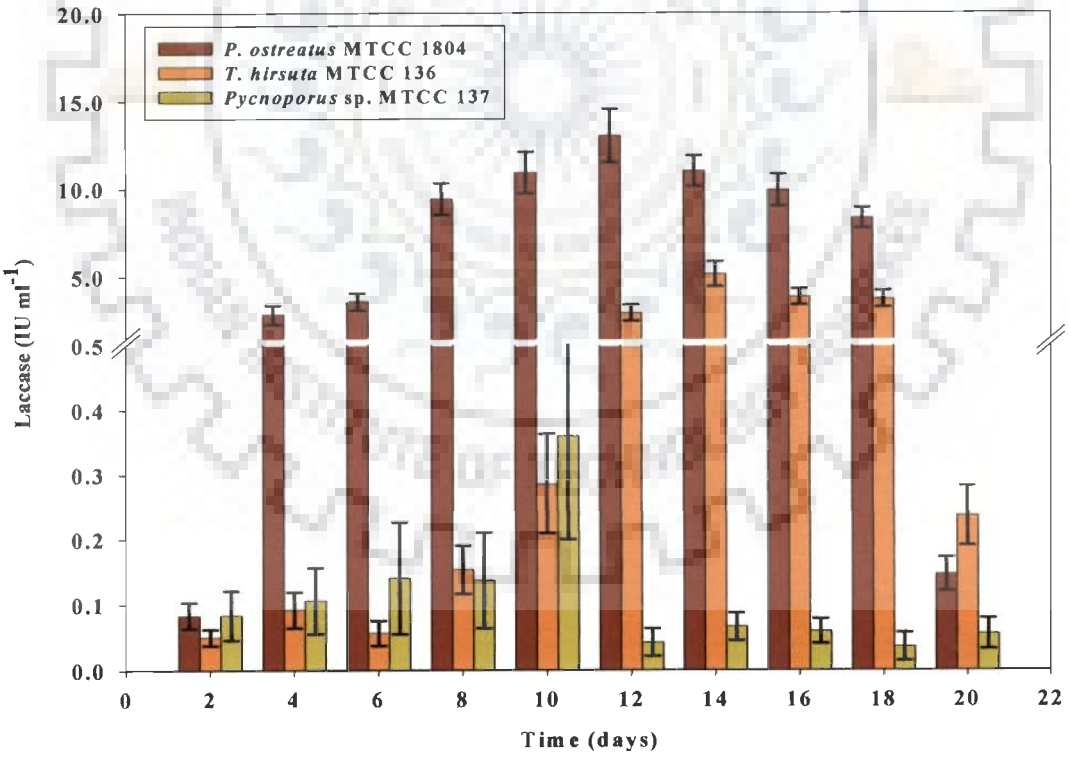


Fig. 18 Production of laccase by axenic cultures of white rot fungi at varying time intervals (Cultivation was performed at 30° C at a pH value of 9.0 and at 150 rpm under shake flask condition; values are mean of three replicates ± SE).

4.5.3 Enzyme production by a mixed culture of *P. oxalicum* SAU_E-3.510 and *P. ostreatus* MTCC 1804

A mixed enzyme preparation having both xylanase and laccase activity was developed through co-cultivation of mutant *P. oxalicum* SAU_E-3.510 and *P. ostreatus* MTCC 1804. A remarkable increase in xylanase and laccase biosynthesis was observed through co-culturing of mutant *P. oxalicum* SAU_E-3.510 and *P. ostreatus* MTCC 1804. Co-cultivation was performed in media containing glucose, xylan and combinations of the two to derive the most suitable carbon source for their growth and enzyme production. The medium containing glucose followed by subsequent addition of xylan on the 6th day, resulted in maximum levels of xylanase (1208.00 ± 15.01 IU ml^{-1}) and laccase activity (19.40 ± 2.40 IU ml^{-1}) (Table-13). An over all increase of 58% and 33% was achieved in xylanase and laccase activity respectively under derived conditions for co-culturing of mutant *P. oxalicum* SAU_E-3.510 and *P. ostreatus* MTCC 1804 (Fig. 19).

Table. 13 Enhanced production of xylanase through co-cultivation of mutant *P. oxalicum* SAU_E-3.510 (M) and *P. ostreatus* MTCC 1804 (Plu) as compared to their respective axenic cultures

Sl. No.	Carbon source	Xylanase activity (IU ml^{-1})*			
		M	Plu	M : Plu	% Change
1.	Glucose	99.10 ± 13.33	0.88 ± 0.18	99.13 ± 5.64	-
2.	Xylan	714.46 ± 15.58	17.53 ±1.94	934.36 ± 6.03	27.6
3.	Glucose and xylan	715.95 ± 10.95	18.35 ± 3.45	1087.00 ± 7.28	48.0
4.	Glucose and xylan added on 6th day of incubation	735.80 ± 17.37	27.63 ± 3.37	1208.00 ± 15.01	58.2

* The fermentation media were initially inoculated with *P. ostreatus* MTCC 1804. Mutant *P. oxalicum* SAU_E-3.510 was inoculated on 6th day of incubation.

* Total duration for incubation was 12 days, following which cultures were harvested and supernatant was used for estimating the enzyme activity.

* Values are mean of three replicates ± SE.

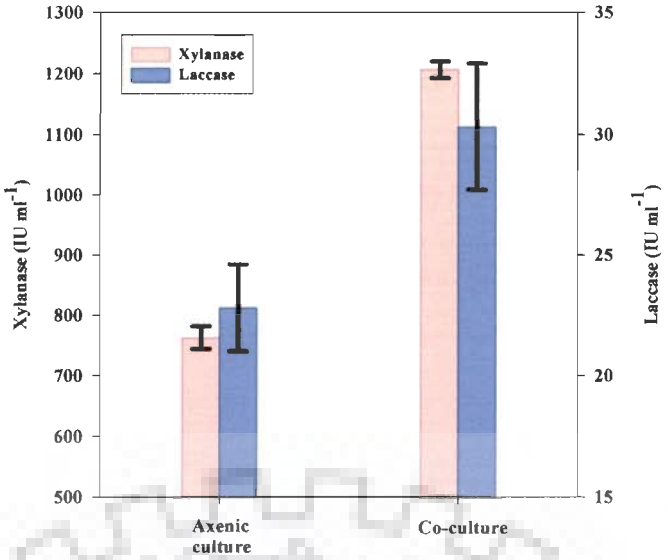


Fig. 19 Analysis of xylanase and laccase activity during co-cultivation of mutant *P. oxalicum* SAU_E-3.510 with *P. ostreatus* MTCC 1804.

(Co-cultivation was performed at 30° C at a pH value of 9.0 and at 150 rpm under shake flask condition; values are mean of three replicates ± SE).

4.5.4 Effect of cultivation conditions

Enzyme production using a mixed culture of the mutant *P. oxalicum* SAU_E-3.510 and *P. ostreatus* MTCC 1804 was evaluated in different fermentation set-ups, namely in surface, submerged and solid state cultivations. The highest level of xylanase (1301.15 ± 12.73 IU ml^{-1}) and laccase activity (43.70 ± 3.72 IU ml^{-1}) was obtained when the fungi were co-cultured under solid-state conditions (Table-14).

Table. 14 Production of enzymes under various fermentation conditions by co-cultivation of mutant *P. oxalicum* SAU_E-3.510 and *P. ostreatus* MTCC 1804

Fermentation conditions	Activity (IU ml^{-1}) *		Productivity (IU $l^{-1}h^{-1}$)*	
	Xylanase	Laccase	Xylanase	Laccase
Surface fermentation	976.55 ± 12.20	15.00 ± 1.90	6781.62 ± 76.06	52.08 ± 6.62
Submerged fermentation	1201.84 ± 11.72	20.83 ± 2.94	8346.15 ± 81.38	72.33 ± 10.22
Solid-state fermentation	1301.51 ± 12.73	43.70 ± 3.72	9038.26 ± 88.42	151.73 ± 12.9

* The fermentation media were initially inoculated with *P. ostreatus* MTCC 1804. Mutant *P. oxalicum* SAU_E-3.510 was inoculated on 6th day of incubation.
* Total duration for incubation was 12 days, following which cultures were harvested and supernatant was used for estimating the enzyme activity.
* Values are mean of three replicates ± SE.

4.6 CO-CULTIVATION IN SOLID-STATE FERMENTATION

Co-cultivation in solid-state fermentation was performed and various critical conditions for maximizing the level of production were evaluated.

4.6.1 Inoculation time

P. ostreatus MTCC 1804 was initially inoculated followed by inoculation with *P. oxalicum* SAU_E-3.510 at different time intervals. *P. oxalicum* SAU_E-3.510 when inoculated after 48 h had resulted in maximum levels of xylanase (6210.76 ± 56.79 IU g^{-1}) and laccase (247.16 ± 32.47 IU g^{-1}) production (Fig. 20). However, contrary to this, inoculation with the mutant after 144 h resulted in maximum levels of production in submerged cultivation. This discrepancy may be due to the fact that the growth of the white rot fungus was faster during solid-state cultivation as compared to submerged cultivation because conditions in SSF are closer to the natural environmental conditions.

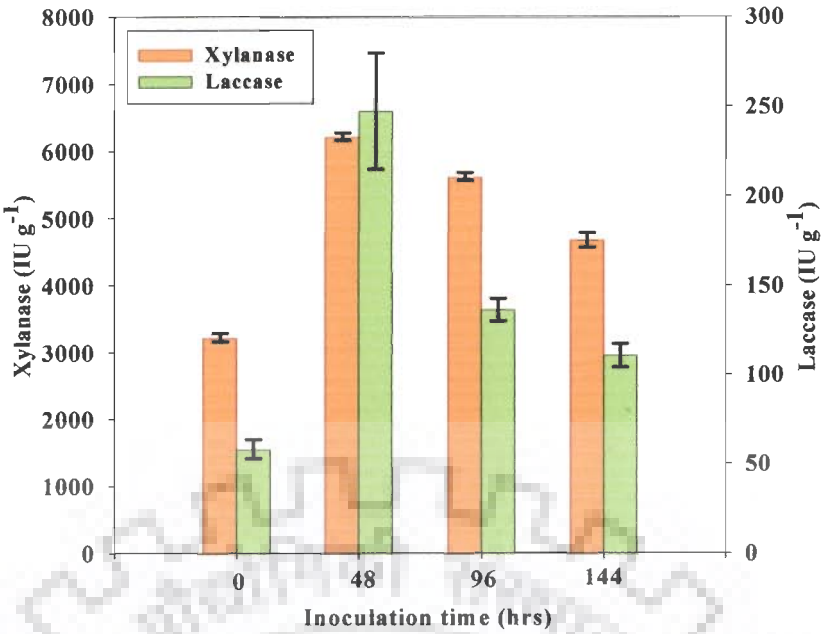


Fig. 20 Evaluation of inoculation time of mutant *P. oxalicum* SAU_E-3.510 with *P. ostreatus* 1804 for enzyme production in solid-state fermentation.

(Co-cultivation was performed at 30° C and at 70% initial moisture content; values are mean of three replicates ± SE).

4.6.2 Enzyme production on different substrates

Co-cultivation of fungi in solid-state fermentation led to higher levels of xylanase and laccase production. Thus, to obtain further improved levels of production and to improve the economy of the process, solid-state fermentation was performed using a variety of agro-horticultural residues that are easily and abundantly available either at considerably lower price or with no cost (Fig. 21). Fig. 22 denotes the activity of xylanase and laccase by using various agroresidual materials. Almost similar levels of xylanase were obtained with sugarcane bagasse (6837.33 ± 37.46 IU g^{-1}), black gram husk (6551.31 ± 81.61 IU g^{-1}), wheat bran (6542.94 ± 68.39 IU gl^{-1}) and congress grass (6305.64 ± 48.9 IU g^{-1}), whereas saw dust resulted in a lower xylanase activity. However, combination of bagasse with wheat bran and with black gram husk demonstrated maximum levels of both xylanase (7121.11 ± 67.74 IU g^{-1}) and laccase (316.02 ± 13.01 IU g^{-1}) production during co-culturing of mutant *P. oxalicum* SAU_E-3.510 and *P. ostreatus* MTCC 1804.

Fig. 21 Different solid supports used for solid-state fermentation (a) Bagasse; (b) Black gram husk; (c) Red gram husk; (d)Wheat bran; (e) Congress grass; (f) Rice bran; (g) Cotton hull; (h) Saw dust; (i) Bagasse + Wheat bran; (j) Bagasse + Black gram husk.

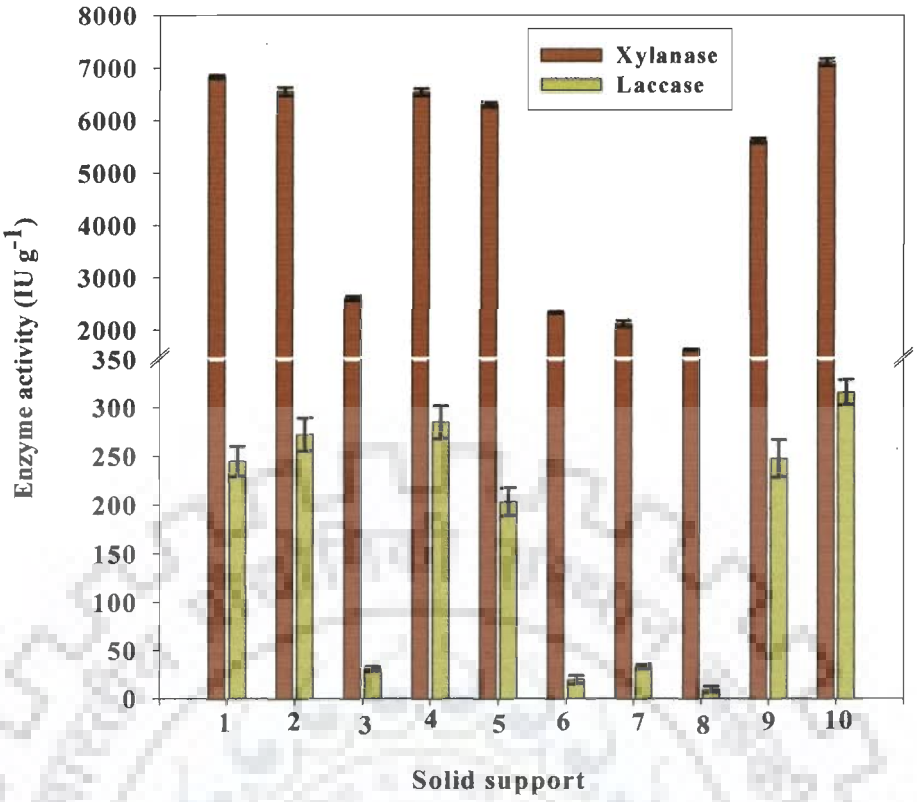


Fig. 22 Production of enzymes using different solid supports : 1, Bagasse; 2, Black gram husk; 3, Red gram husk; 4, Wheat bran; 5, Congress grass; 6, Rice bran; 7, Cotton hull; 8, Saw dust; 9, Bagasse + Wheat bran (1:1) and 10, Bagasse + Black gram husk (1:1).

(Co-cultivation was performed at 30° C and at 70% initial moisture content; values are mean of three replicates ± SE).

As observed with scanning electron microscopy, a mixture of bagasse and black gram husk served as an ideal solid support for fungal entrapment and anchorage. Uninoculated mixed substrate appeared porous and following inoculation with mixed fungal culture showed profound colonization by mutant *P. oxalicum* SAU_E-3.510 and *P. ostreatus* MTCC 1804 (Fig. 23).

(a)

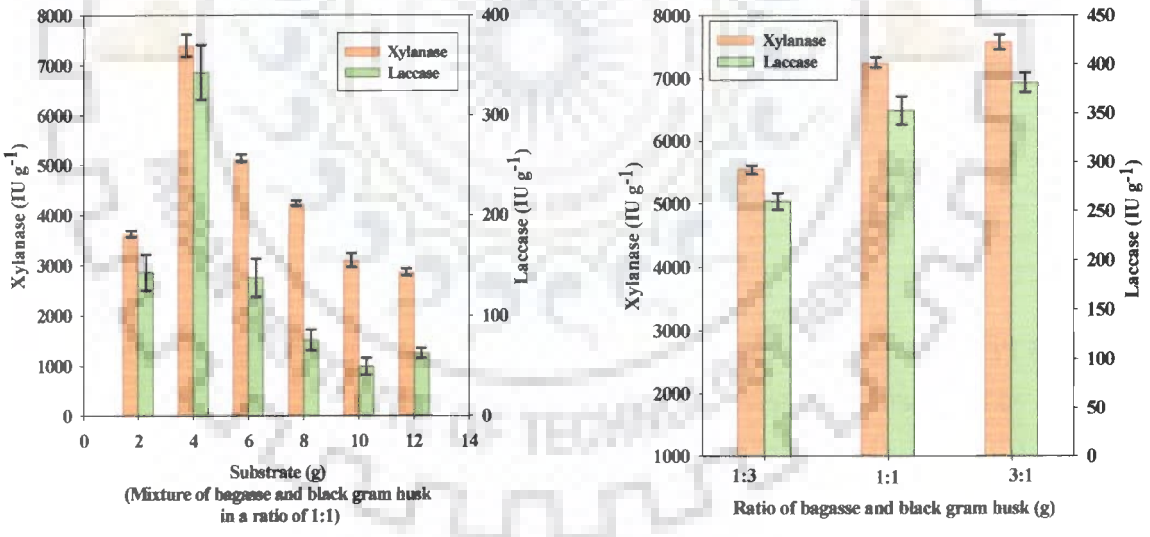
(b)

Fig. 23 Scanning electron micrograph of bagasse and black gram husk in mixture as solid substrate (a) uninoculated and (b) entrapped with *P. oxalicum* SAU_E-3.510 and *P. ostreatus* MTCC 1804.

4.6.3 Analysis of amount of substrate and ratio of two substrates

The amount of substrate is of prime importance and 4g of substrate in 250 ml Erlenmeyer flask (bagasse: black gram husk, 1:1) was found to be the most suitable for the production of xylanase (7396.64 ± 222.38 IUg^{-1}) and laccase (342.95 ± 27.44 IUg^{-1}) as shown in Fig. 24a. A further increase in the amount of substrate resulted into decreased enzyme production.

Two selected substrates (bagasse and black gram husk) were mixed in different proportions and used for the co-cultivation of *P. oxalicum* SAU_E-3.510 and *P. ostreatus* MTCC 1804. Estimation of xylanase and laccase activity demonstrated that bagasse and black gram husk used in the ratio of 3:1 resulted in a further increase in xylanase (7574.24 ± 118.99 IU g^{-1}) and laccase activity (381.33 ± 10.01 IU g^{-1}) (Fig. 24b).



(a) **(b)**

Fig. 24 Enzyme production at different amounts (a) and ratio (b) of bagasse and black gram husk under solid-state fermentation by co-cultivation of mutant *P. oxalicum* SAU_E-3.510 with *P. ostreatus* MTCC 1804.

(Co-cultivation was performed at 30° C and at 70% initial moisture content; values are mean of three replicates ± SE).

4.6.4 Enzyme production at different time periods

Production of xylanase and laccase by a mixed culture of *P. oxalicum* SAU_E-3.510 and *P. ostreatus* MTCC 1804 under SSF was evaluated at different time intervals. Maximum levels of xylanase (7904.27 ± 52.36 IUg^{-1}) and markedly higher levels of laccase activity (348.42 ± 32.27 IUg^{-1}) were observed after 8 days of incubation following inoculation with the mutant (Fig. 25). However, a marginally higher level of laccase was observed after 6 days of incubation. Decreasing levels of enzymes were observed on further incubation; hence the culture was harvested after the 8th day of incubation to get maximum levels of both enzymes.

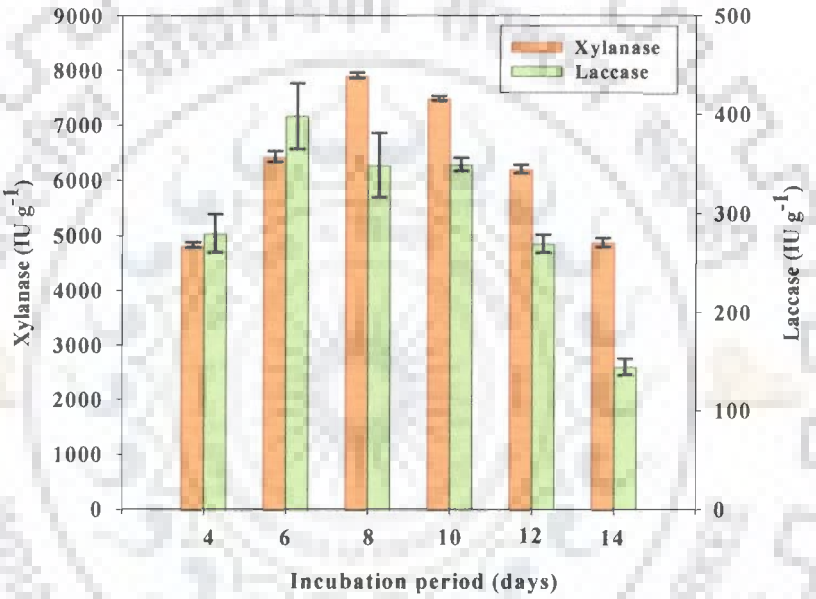


Fig. 25 Production of enzymes by mixed culture of *P. oxalicum* SAU_E-3.510 and *P.ostreatus* MTCC 1804 at different periods of incubation.

(Co-cultivation was performed at 30° C and at 70% initial moisture content; values are mean of three replicates ± SE).

4.6.5 Effect of moisture level

The initial moisture content is a critical factor for achieving microbial growth and enzyme production under solid-state fermentation condition. Co-cultivation was performed at varying moisture contents ranging from 20% to 120% and as shown in Fig. 26, a moisture level

of 80% was best suited for enzyme production and yielded 8205.31 ± 168.31 IU g^{-1} of xylanase and 375.53 ± 34.17 IU g^{-1} of laccase under these conditions. A higher moisture content was inhibitory to production. A high moisture level during SSF interferes with air circulation and heat transfer generated due to the biological activities of the microbial strains.

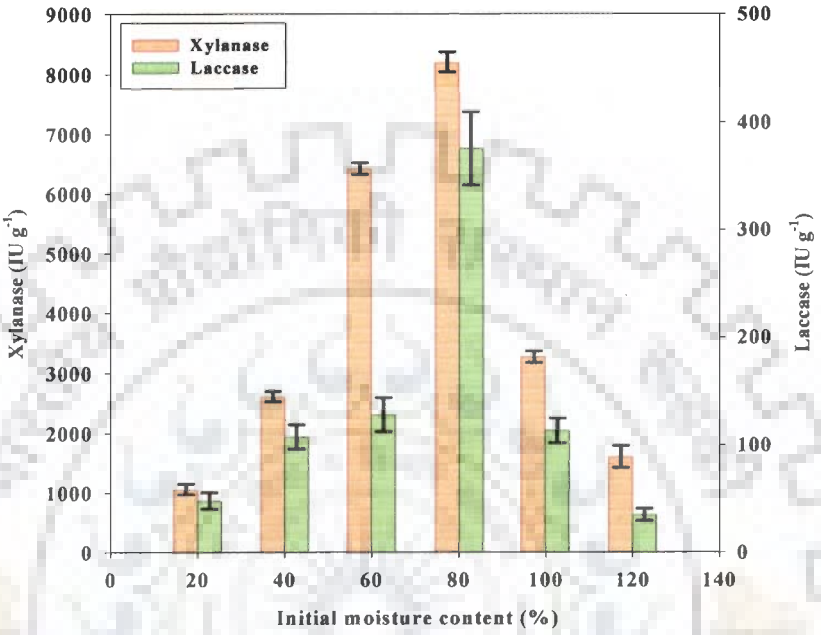


Fig. 26 Effect of moisture content on enzyme production during co-cultivation of mutant *P. oxalicum* SAU_E-3.510 with *P. ostreatus* MTCC 1804 under solid-state fermentation.
(Co-cultivation was performed for 8 days at 30° C; values are mean of three replicates ± SE).

ANOVA was performed to assess the statistical significance of differences of means between more than two groups. Variations in the enzyme production under solid-state fermentation process were studied by the statistical analysis and results of ANOVA are given in Table-15.

Table. 15 Analysis of variance for critical factors for production of a xylanase-laccase mixed preparation by co-culture of *P. oxalicum* SAU_E-3.510 and *P. ostreatus* in solid-state fermentation

Parameters	Source of variation	Degree of freedom	Sum of square	Mean square	F ratio observed
Inoculation sequence	Between the Samples	3	15283780.0	5094593.0	268.6
	Within the Samples	2	24226.0	12113.0	0.63
	Error	6	113799.0	18966.4	
Solid support	Between the Samples	9	139745510.7	15527279.0	1659.9
	Within the Samples	2	3842.1	1921.0	0.20
	Error	18	168374.7	9354.1	
Substrate concentration	Between the Samples	5	42617025.0	8523405.0	543.5
	Within the Samples	2	356391.9	178196.0	11.3
	Error	10	156810.2	15681.0	
Substrate ratio	Between the Samples	2	7156432.0	3578216.0	116.5
	Within the Samples	2	28588.0	14294.0	0.46
	Error	4	122794.9	30698.7	
Incubation period	Between the Samples	5	24541669.0	4908334.0	312.7
	Within the Samples	2	25646.9	12823.4	0.81
	Error	10	156961.5	15696.5	
Moisture content	Between the Samples	5	120856359.3	24171272.0	2007.3
	Within the Samples	2	461896.6	230948.3	19.1
	Error	10	120415.1	12041.5	

Significant at 5% level.

4.7 CHARACTERISATION OF XYLANASE FROM MUTANT *P. OXALICUM* SAU_E-3.510

4.7.1 Biochemical properties

The profiles obtained for pH stability of xylanase from mutant *P. oxalicum* SAU_E-3.510 is presented in Fig. 27a. The enzyme appeared stable and maintained 93% of the residual activity at pH 9.0 for 24 hrs, 93% of the residual activity remained after four h of incubation in the buffer at pH 11.0 and about a 50% decrease in the activity was detected after 20 h. The effect of incubation temperature on xylanase activity was determined by incubating the enzyme with substrate at different temperatures (25°C - 85°C) and 55°C was found to be most appropriate for achieving maximum activity (Fig. 27c).The influence of temperature on the stability of xylanase from mutant *P. oxalicum* SAU_E-3.510 was evaluated at different temperatures (50°C-100°C, 2 h). The enzyme also appeared to be thermostable. The half life of the enzyme was found to be two h at 80°C where 50% of the residual activity was estimated (Fig. 27b).

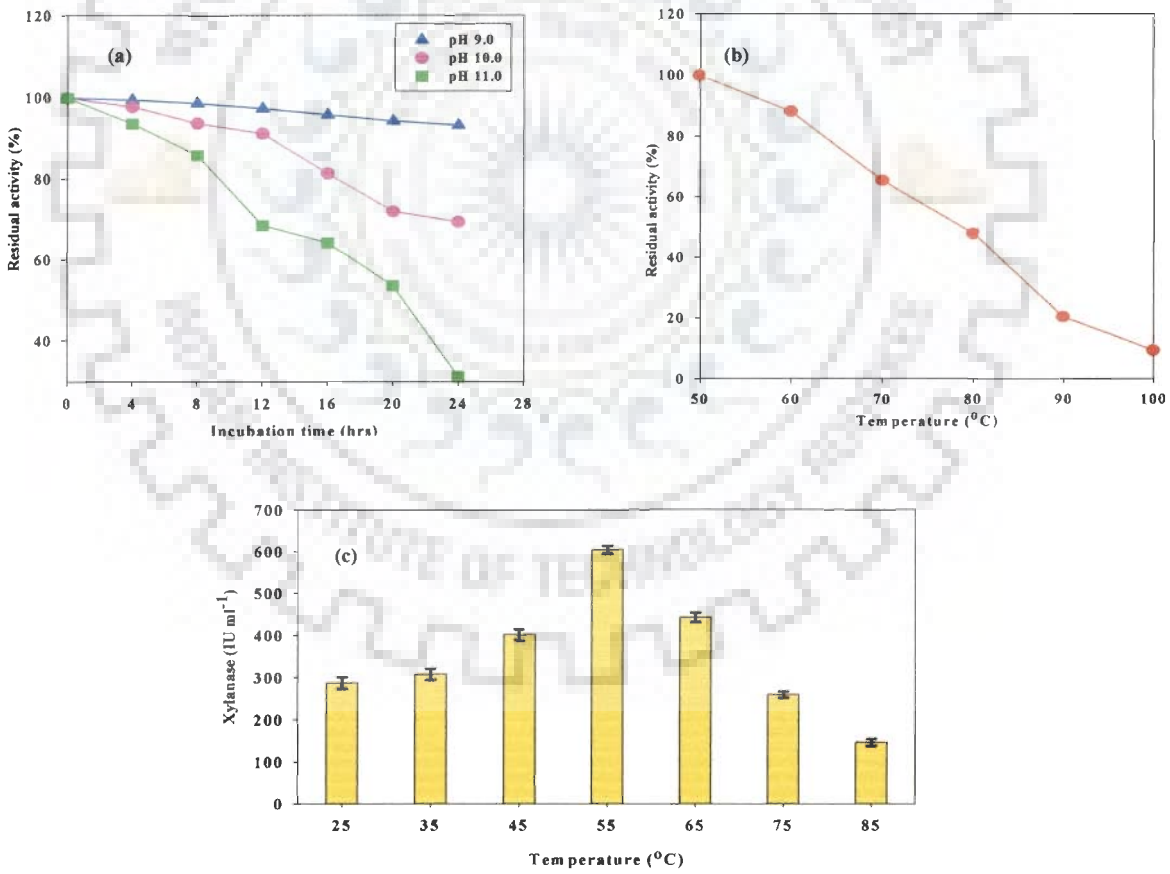


Fig. 27 pH (a) and temperature (b) stability profiles and optimum temperature (c) for xylanase activity from mutant *P. oxalicum* SAU_E-3.510.

4.7.2 Molecular characterisation

The protein profile of mutant *P. oxalicum* SAU_E-3.510 was analysed through SDS PAGE and compared with that of the wild type *P. oxalicum* SA-8 (Fig 28 a). SDS-PAGE and zymogram analysis had indicated the presence of two isoforms of xylanase in both the wild type and mutant strains and the molecular mass of these was deduced to be 121 kD and 77 kD respectively (Fig 28 a, b). The activity bands of the mutant strain were pronounced as compared to wild type strain, indicating a higher level of xylanase expression in mutant *P. oxalicum* SAU_E-3.510. Similarly, proteins produced during co-cultivation of mutant *P. oxalicum* SAU_E-3.510 and *P. ostreatus* MTCC 1804 during solid-state fermentation were analysed by polyacrylamide electrophoresis and zymogram analysis (Fig. 29 a-c). Two xylanase isozymes of 121 kD and 77 kD, which were specific to mutant *P. oxalicum* SAU_E-3.510, were present in the sample obtained by co-culture along with an extra xylanase of 40 kD that corresponded to *P. ostreatus* MTCC 1804. No laccase was detected in the axenic culture of mutant, whereas laccase corresponding to 29 kD was detected for *P. ostreatus* MTCC 1804 during co-cultivation.

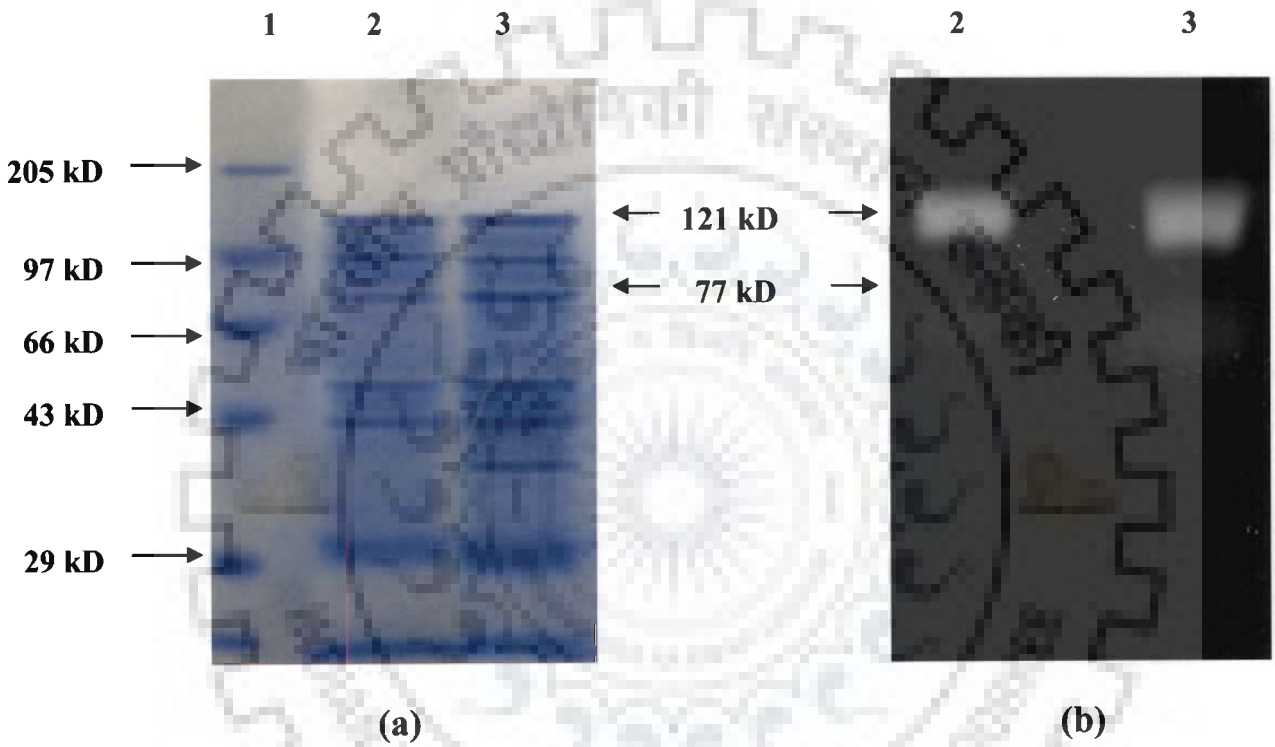


Fig 28 SDS-PAGE (a) and zymogram (b) analysis of proteins produced by wild type *P. oxalicum* SA-8 and mutant *P. oxalicum* $SAU_E3.510$ following 6 days of incubation. Lane 1, protein molecular weight markers; 2 and 3, culture supernatant of wild type and mutant strains respectively.

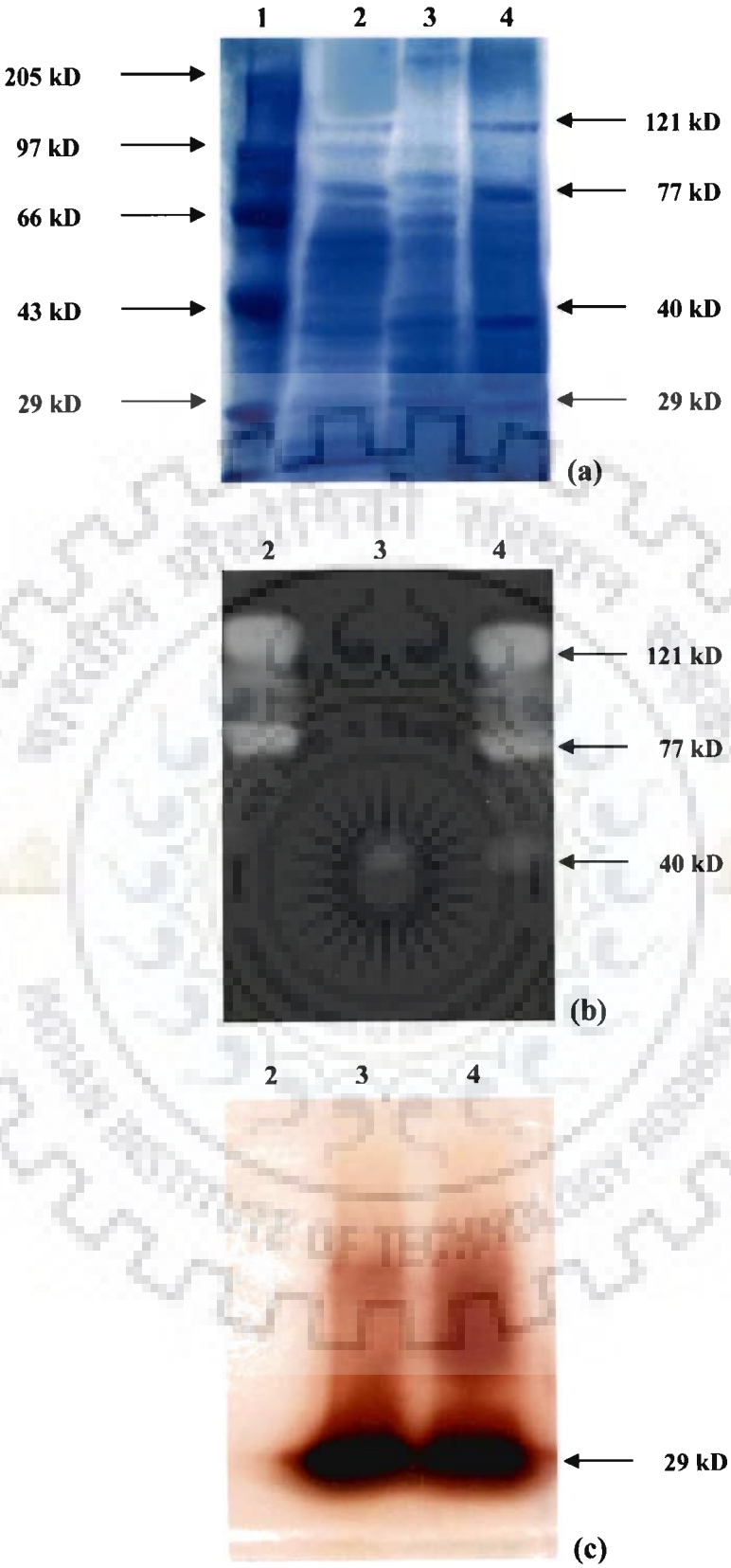


Fig. 29 SDS-PAGE (a), zymogram analysis for xylanase (b) and laccase (c) produced during mono and dual culture conditions. Lane 1, protein molecular weight markers; 2, culture supernatant of mutant *P. oxalicum* SAU_E-3.510; 3, *P. ostreatus* MTCC 1804 and 4, co-culture of mutant *P. oxalicum* SAU_E-3.510 with *P. ostreatus* MTCC 1804.

4.7.3 Determination of internal peptide sequence

The internal amino acid sequencing of xylanase was performed by MALDI-ToF/ToF analysis that provides significant insights into the nature of xylanase and also the sequence homology search observations. Interestingly, xylanase from *P. oxalicum* SAU_E-3.510 was found to have significant identity with xylanases of bacterial origin. A level of identity of 40-60% was found with the xylanases from *Bacillus stearothermophilus*, *Geobacillus stearothermophilus*, *Clostridium* sp. and *Paenibacillus* sp. On the other hand, xylanase from *P. oxalicum* SAU_E-3.510 exhibited less identity with xylanases of fungal origin viz *Aspergillus versicolor*, *A. fumigatus* and *Gibberella zeae* (Table-16).

4.7.4 Hydrolysis studies

Xylan extracted from *Parthenium* sp (congress grass) was used as substrate to analyse the mode of action of xylanase from mutant *P. oxalicum* SAU_E-3.510. The congress grass xylan (1%) was incubated with xylanase and xylanase-laccase mixed enzyme preparation for 24 h at 55°C and hydrolysis products were analyzed by high performance liquid chromatography (HPLC). The peaks were identified by running a parallel standard of xylose. Xylose was the major hydrolysis product released by the action of enzyme, while the presences of other oligosaccharides (xylobiose, xylotriose, etc.) were not detected. The high concentration of xylose indicated the improved degree of substrate hydrolysis with xylanase in the presence of laccase (Fig. 30c) as compared to xylanase alone (Fig. 30b), whereas no xylose was detected in the untreated samples (Fig. 30a). These results showed that the mutant secreted a set of enzymes (endo and exo acting) that synergistically promoted efficient substrate degradation. These results also suggested that congress grass xylan could be effectively hydrolysed by xylanase and could be utilised for the production of monosaccharides (xylose) which has wide range of industrial applications.

Table. 16 Sequence homology for internal peptides of xylanase by NCBI BLAST short sequence search

Accession no	Organism		Sequence		Identity %
Peptide 1	*Penicillium oxalicum*	248	-VLDDAARRLR-	257	-
1HIZ	*B. stearothermophilus*	289	FLDQAARYDR	298	60
BAA05669.1	*G. stearothermophilus*	530	FLDQAARYDR	539	60
XP_754103.1	*Aspergillus fumigatus*	27	NLDKLARRNG	36	50
YP_001560948.1	*Clostridium sp.*	2119	LTDDGTRPLR	1128	40
CA179477.1	*Paenibacillus sp.*	457	TQAWQARRLP	466	40
ABM55502.1	*Aspergillus versicolor*	203	NLDDANYAKT	212	40
AAT84259.1	*Gibberella zeae*	220	LGDDHDRRFF	229	40
Peptide 2	*Penicillium oxalicum*	193	-ISELNALKKAAK-	204	-
ABZ80916.1	*Paenibacillus sp.*	651	TDYKNALRKAPW	662	42
1HIZ	*B. Stearothermophilus*	12	ISALNAPQLDQR	23	42
Peptide 3	*Penicillium oxalicum*	151	-AGSQGPSNAETIR-	163	-
YP_001560106.1	*Clostridium sp.*	12	FLIGAAVNASTIR	24	38
BAA05669.1	*G. stearothermophilus*	478	IQIGWPSEAEIEK	490	31
1HIZ	*B. Stearothermophilus*	237	IQIGWPSEAEIEK	249	31
BAF49077.1	*Paenibacillus sp.*	120	RLSQGPAPDGEEF	132	31
Peptide 4	*Penicillium oxalicum*	117	-DPGVRCPDCNR-	127	-
AAV98257.1	*Gibberella zeae*	271	YPGGRCPVLAP	281	45
CAP53700.1	*Xanthomonas campestris*	324	HPGVRAP----	335	45
YP_001360303.1	*Kineococcus radiotolerans*	128	DAIVRFAEENR	138	36
Peptide 5	*Penicillium oxalicum*	243	-WKVGRVLDDAAR-	254	-
AAD32559.2	*Streptomyces sp.*	376	AAAGRVLDEPAG	387	50
BAA05669.1	*G. stearothermophilus*	325	IPKQKFLDQAAR	336	42
1HIZ	*B. Stearothermophilus*	284	IPKQKFLDQAAR	295	42
AAT37531.1	*Clostridium sp.*	233	LKPDYVLDKAAC	244	42
ABM55502.1	*Aspergillus versicolor*	197	YINDYNLDDANY	208	33
Peptide 6	*Penicillium oxalicum*	55	-GTFCLEHRTETAHR	68	-
YP_001360820.1	*Kineococcus radiotolerans*	446	DRALLEQRMETHIK	459	36
AAV98623.1	*Bacillus halodurans*	213	GTDYIKVAFETARK	226	36
AAT84254.1	*Gibberella zeae*	31	PSGLLEKRTSPTTG	44	28
Peptide 7	*Penicillium oxalicum*	295	-QGHTIVLLRGAGVILGK-	311	-
AAT84255.1	*Gibberella zeae*	22	QGHLIVGETPSRSELTT	39	35
YP_001560948.1	*Clostridium sp.*	1112	DAAKGDGWSGANIILGN	1128	29
BAC45001.1	*Paenibacillus sp.*	1112	QGKTIVVPNGRYDAKSG	1128	29
YP_366089.1	*Xanthomonas campestris*	204	YLHTIQLLQQEHLIDAI	220	29

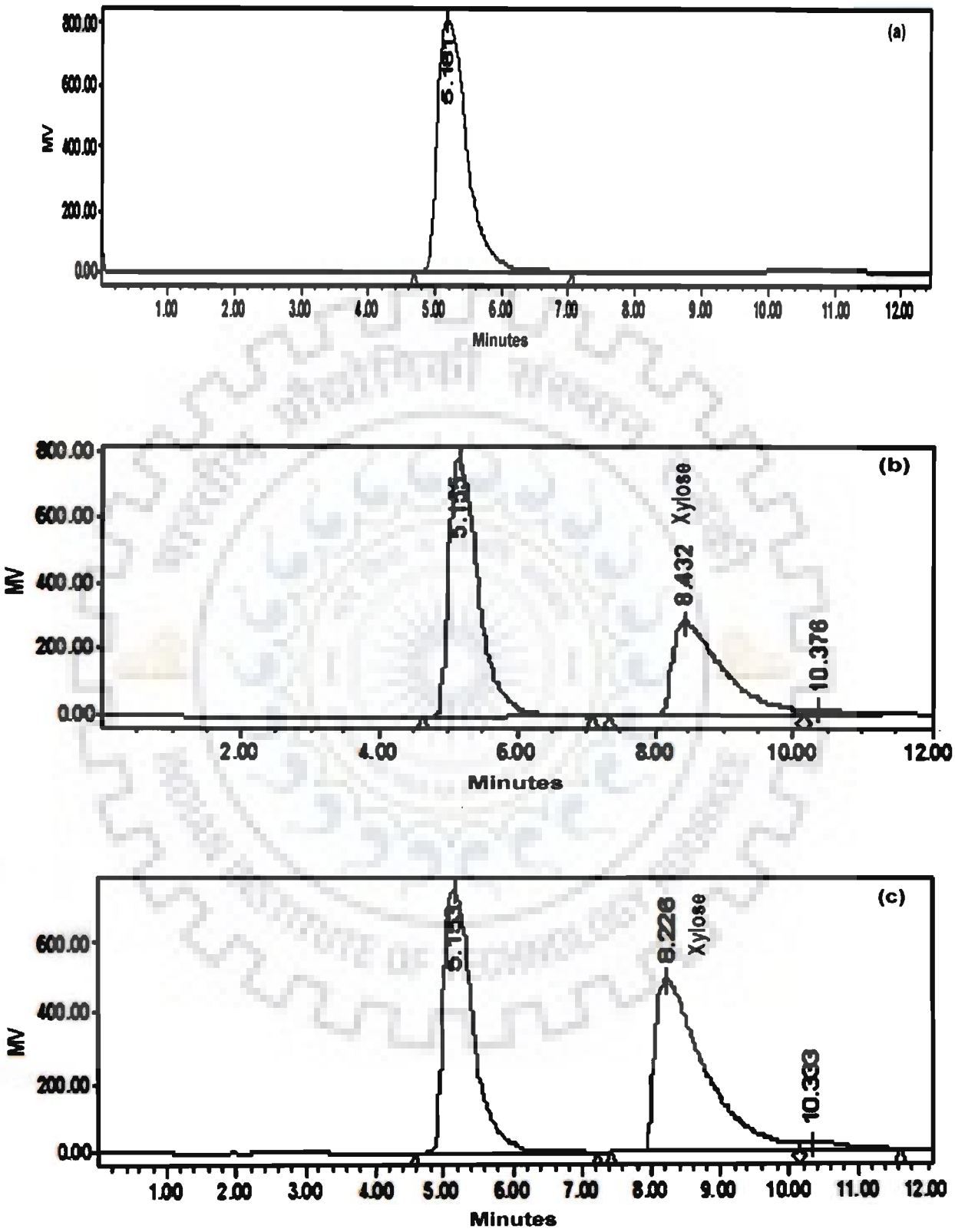


Fig. 30 Analysis of hydrolysis products released as a result of congress grass xylan hydrolysis (a) untreated (b) xylanase treated (c) xylanase-laccase mixture treated.

4.8 BIOBLEACHING OF MIXED WOOD KRAFT PULP

4.8.1 Enzymatic pretreatment

4.8.1.1 *Selection of enzyme system*

Unbleached mixed wood pulp was subjected to enzymatic treatment by using xylanase from mutant *P. oxalicum* SAU_E-3.510 as well as a xylanase- laccase mixture produced through co-cultivation of mutant *P. oxalicum* SAU_E-3.510 and *P. ostreatus* MTCC 1804 in solid-state fermentation. A significant decrease in kappa number and increase in brightness was achieved as result of enzymatic treatment due to the removal of lignin as compared to untreated pulp. Compared to xylanase alone, the mixed enzyme preparation yielded better results in terms of kappa number and brightness (Fig. 31). A significantly higher lignin removal (kappa number decrease was 5 % higher compared to using xylanase alone) was obtained by using a mixture of xylanase and laccase produced through co-cultivation in solid-state fermentation. Similarly, brightness of the sheets that had been treated with the mixed enzyme preparation was also higher (38.3 ISO) as compared to treatment with xylanase alone (37.0 ISO). These results suggested the mixed enzyme preparation was an effective and suitable alternative to xylanase for the bleaching of mixed wood pulp, hence the enzyme mixture was used in further experiments.

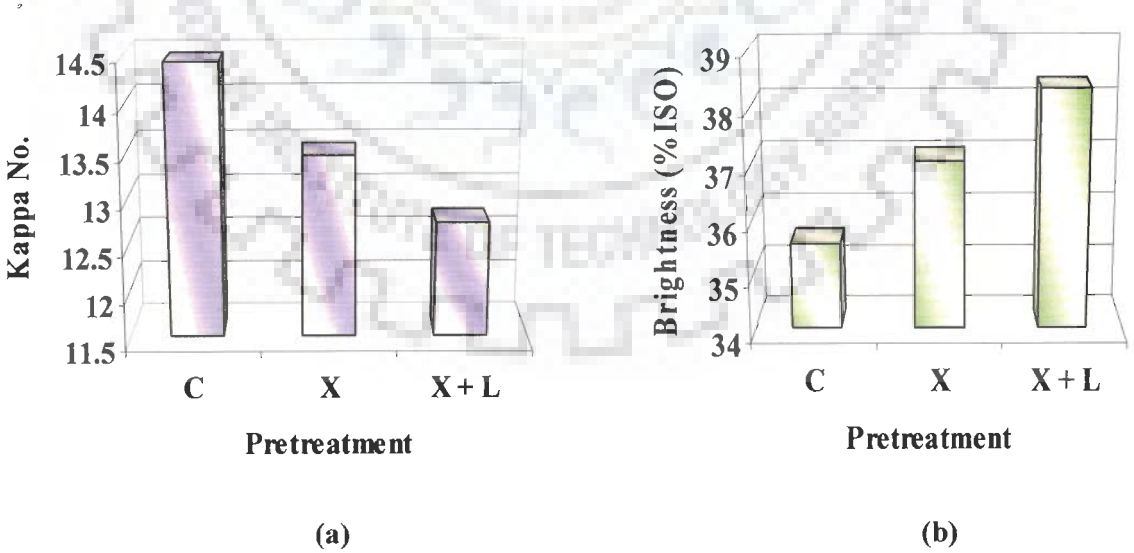


Fig. 31 Alteration in pulp kappa no. (a) and brightness (b) following enzymatic treatment (C, untreated; X, xylanase; X+ L, xylanase-laccase mixture).

4.8.1.2 *Optimisation of enzyme dose*

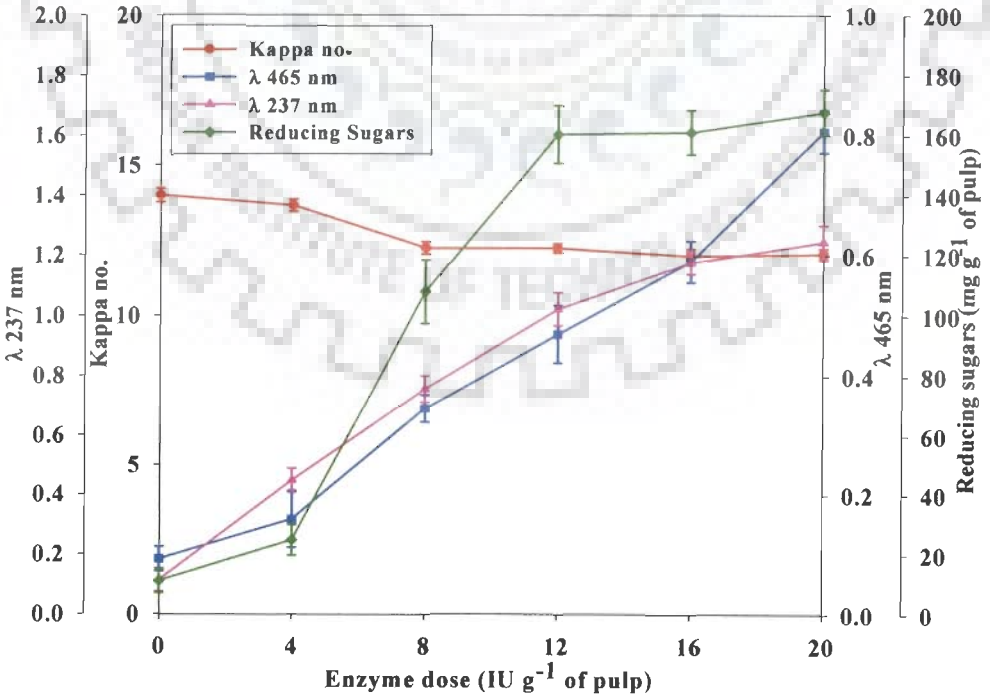
The biobleaching efficacy of the xylanase-laccase preparation was evaluated using different doses ranging from 0-20 IU g^{-1} of pulp. A maximum decrease in kappa no. (12.8%) was achieved by employing 8 IU g^{-1} of enzyme. The kappa no. of pulp remained constant even after further increase in the enzymatic dose, which suggested that a dosage of 8 IU g^{-1} was most effective for achieving the maximum degree of lignin removal. Release of reducing sugars, phenolic and hydrophobic compounds in the bleaching effluent also increased along with the increasing dosage of enzyme (Fig 32a).

4.8.1.3 *Optimisation of reaction time*

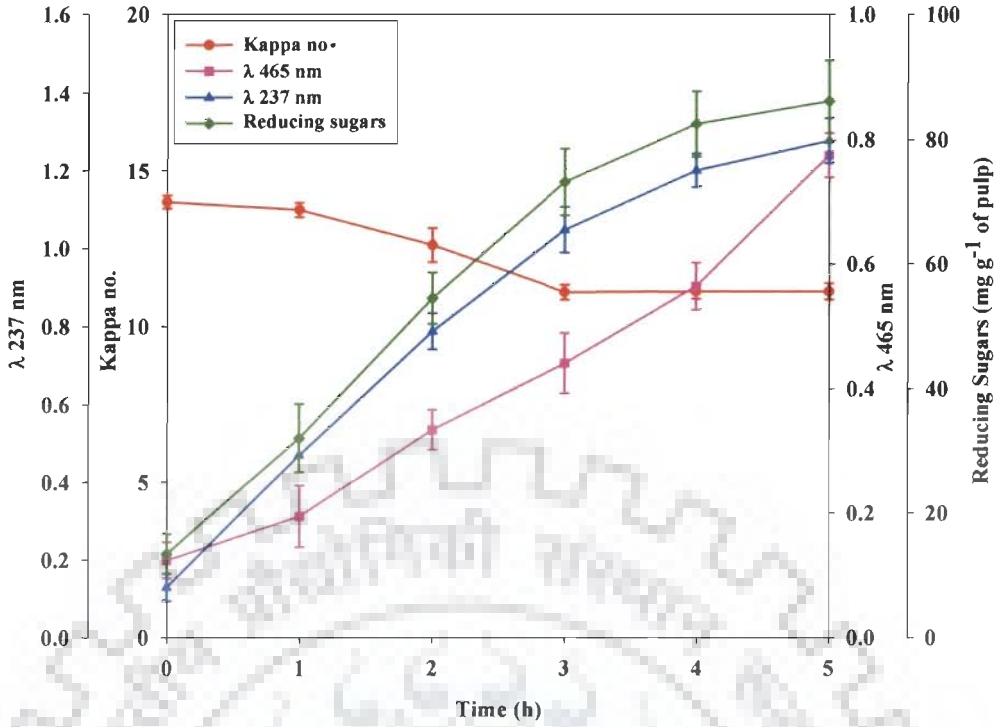
A treatment time of 3 h using a mixed enzyme preparation was found to be the most suitable leading to a maximum decrease in kappa no. (20.7 %) and the maximum release of reducing sugars, phenolic and hydrophobic compounds (Fig. 32b).

4.8.1.4 *Effect of mediator concentration*

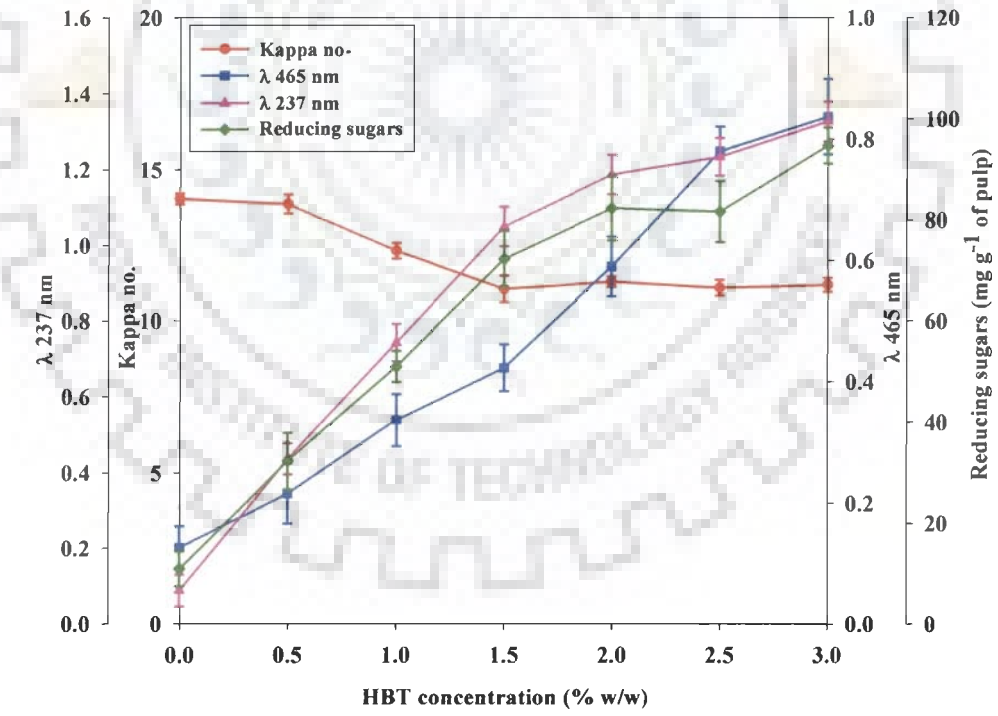
An increased level of lignin removal was achieved when a mediator, 1-hydroxybenzotriazole hydrate (HBT), was added to the mixed enzyme preparation. Maximum decrease in pulp kappa no. (21.4%) was observed by using 1.5 % of HBT and no further change in kappa no. was obtained by increasing the mediator concentration (Fig. 32c).



(a)



(b)



(c)

Fig. 32 Evaluation of (a), enzyme dose; (b), reaction time and (c), mediator concentration for enzyme mediated prebleaching.

(Values are mean of three replicates ± SE).

The maximum level of delignification was achieved following 3 h of treatment with the xylanase-laccase mixture (8 IU g^{-1} of pulp) in the presence of 1-hydroxybenzotriazol (HBT, 1.5% w/w) which acted as a mediator for laccase at a 10 % pulp consistency (Fig. 32 a-c). After enzymatic treatment of pulp under derived conditions, a 21% reduction of kappa number, an 8% increment in brightness and a 5% increase in pulp viscosity was obtained (Table-17). Fig 33 represents the colour of handsheets made from unbleached and enzymatically bleached mixed wood pulp. Increased release of phenolic compounds (λ 237 nm) and hydrophobic compounds (λ 465 nm), along with an increase in the release of reducing sugars, was observed in the derived bleaching conditions (Fig. 32 a-c). The correlation between chromophores release (λ 237 nm), hydrophobic compounds (λ 465 nm) and reduction in kappa number coupled to the release of reducing sugars suggested the dissociation of the lignin carbohydrate complex from pulp fibres.

Table. 17 Effect of enzymatic treatment with a xylanase-laccase mixture on the properties of mixed wood pulp

Properties	Initial	Final	% improvement
Kappa number	14.0 ± 0.17	11.0 ± 0.44	21.0
Brightness	35.5 ± 0.34	38.3 ± 0.26	8.0
Yellowness	30.3 ± 0.14	29.3 ± 0.14	3.0
Viscosity	10.1 ± 0.08	10.6 ± 0.08	5.0

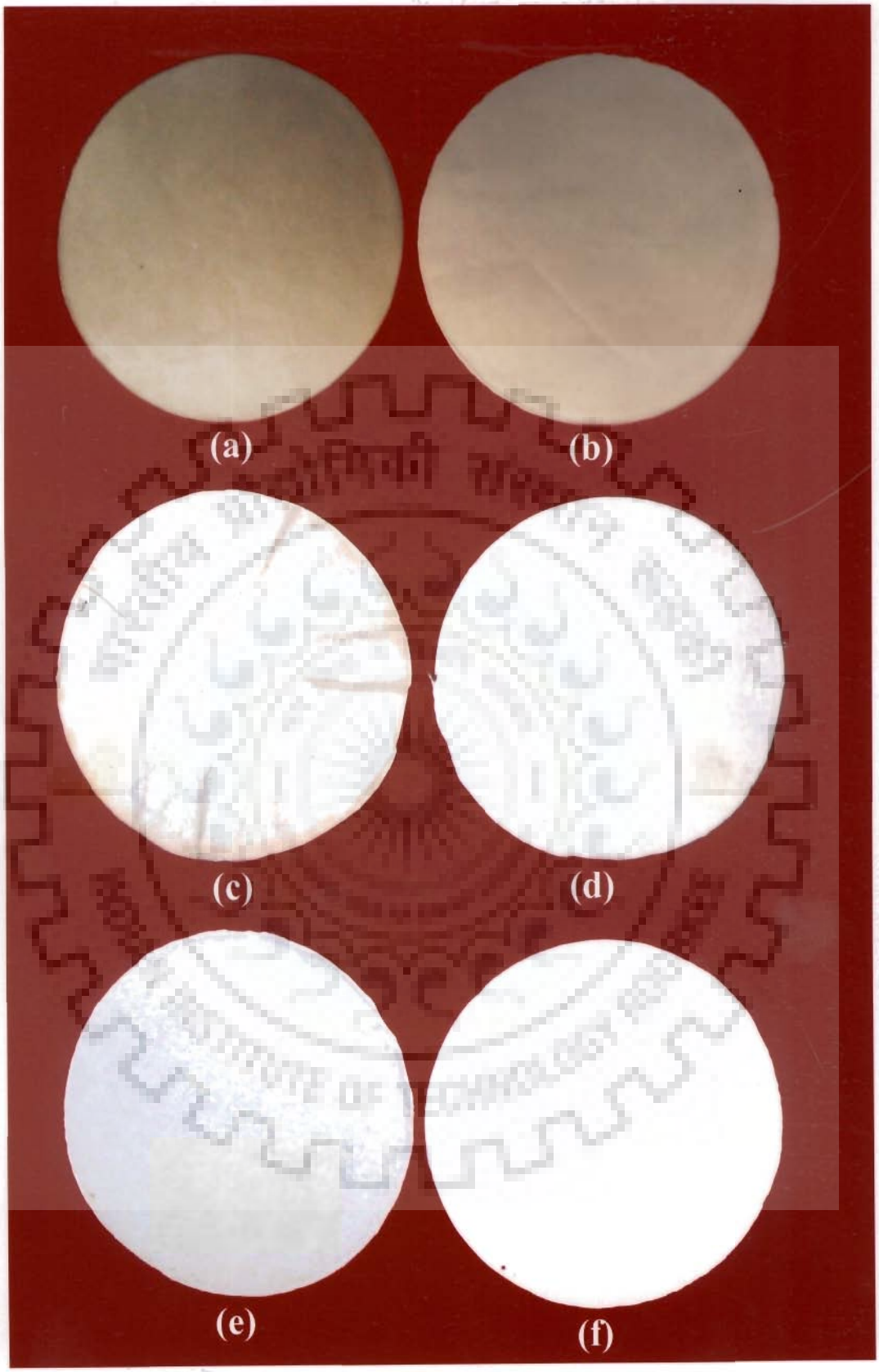


Fig. 33 Colour of the handsheets made from mixed wood pulp (a), untreated; (b), enzyme treated; (c), employing CEPHHP; (d), employing XCEPHHP; (e), employing ODED and (f), employing XODED bleaching sequences.

4.8.2 Effect of enzymatic treatment on the chemical requirement and physicochemical properties of pulp and effluent

Both enzyme treated as well as untreated (control) pulp samples were subjected to conventional (CEPHHP) and elemental chlorine free (ODED) bleaching. As compared to untreated samples, xylanase-laccase treated samples required less bleaching chemicals to achieve a particular brightness standard (≥70% ISO). Following enzymatic treatment, 22% less chlorine and 26% less chlorine dioxide were required during their subsequent bleaching by conventional and elemental chlorine free bleaching sequences. Also, an increase of 13.5% and 14.7% was observed in final brightness of paper sheets prepared from enzymatically bleached pulp (Table-18). Hence, enzymatic pre-treatment would lead to a reduced chemical load during bleaching of the pulp in paper manufacturing. The reduced level of chemical consumption may lead into improved economics of the bleaching process and may effectively curb the environmental pollution.

A reduced level of adsorbable organic halides (AOX) and chemical oxygen demand (COD) was detected in the bleaching effluents of treated pulp samples (Table-18). As a result of the decrease in requirement for chlorine compounds due to enzymatic bleaching, the discharge of chlorinated compounds had also decreased. Therefore, the application of biobleaching plays a major role in decreasing the pollution load of the pulp and paper industry by minimising the release of organic halides.

Table. 18 Effect of enzymatic treatment on pulp and effluent properties

Bleaching sequences	Brightness (%ISO)	Chemical consumption (%)	AOX (kg t^{-1})	COD (mg l^{-1})
CEPHHP	71.5 ± 0.53	95.0	5.8	921 ± 12.7
XCEPHHP	81.2 ± 0.53	74.0	4.0	849 ± 7.7
ODED	73.9 ± 0.20	92.0	4.1	673 ± 15.0
XODED	84.8 ± 0.20	68.0	3.4	528 ± 8.8

4.8.3 Effect of enzymatic treatment on the paper properties

Strength properties of handsheets made from enzymatically bleached and unbleached mixed wood pulp are represented in Table-19. Pretreatment of pulp with a mixed enzyme preparation and its subsequent bleaching using the ODED procedure enhanced various physical properties, viz tensile strength, tear strength and burst strength while less superior strength properties were found with the conventional bleaching sequence. These results indicated that the mixed enzyme preparation was more effective and preserved paper strength during the elemental chlorine free bleaching sequence.

Table. 19 Effect of enzyme treatment on paper properties

Bleaching sequence	Tensile strength ($N\ mg^{-1}$)	Tear strength ($m\ N\ m^2\ g^{-1}$)	Burst strength ($k\ pam^2 g^{-1}$)
CEPHHP	36.6 ± 0.17	10.3 ± 0.77	3.7 ± 0.05
XCEPHHP	33.4 ± 0.17	4.6 ± 0.02	2.5 ± 0.08
ODED	34.6 ± 0.13	5.1 ± 0.08	1.7 ± 0.05
XODED	46.5 ± 0.27	6.4 ± 0.05	2.4 ± 0.05

4.8.4 Analysis of effluent from enzymatically bleached pulp

4.8.4.1 *Colour removal from the pulp*

After bleaching of pulp with different doses of mixed enzyme preparation, effluents were collected and scanned (λ 200- 400 nm) for analysing the colour due to presence of lignin. The peak at 280 nm in the UV spectrum indicated the presence of lignin in the released colour compounds and colour removal increased with an increase in enzyme dose (Fig. 34).

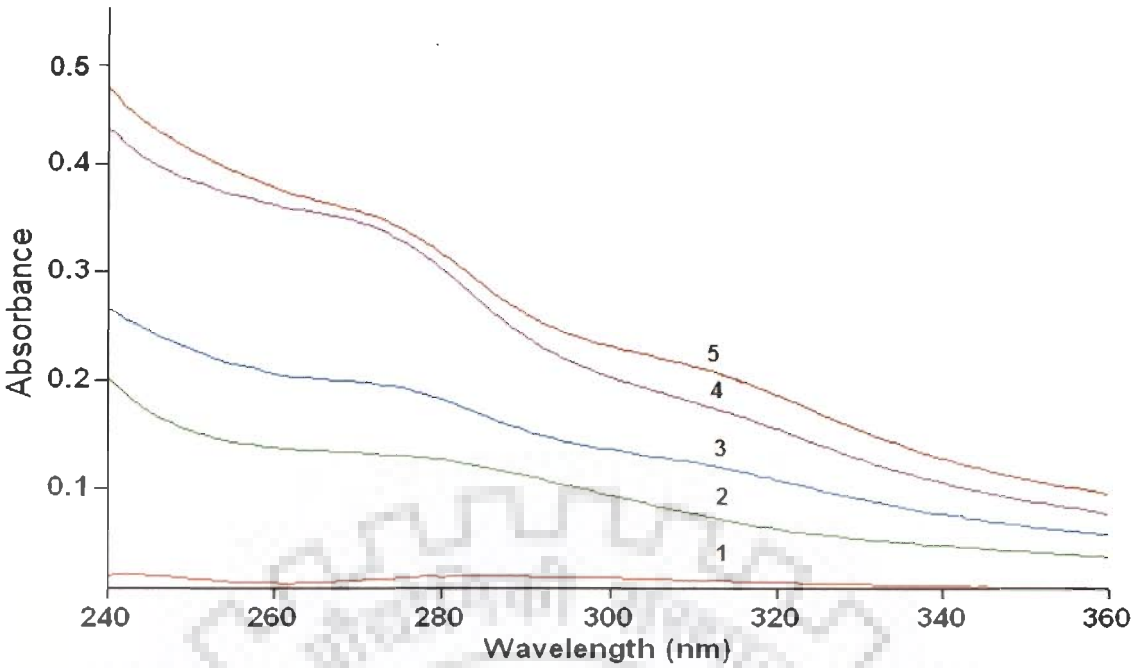


Fig. 34 UV spectra of coloured compounds released during enzymatic treatment at different enzyme doses (1, untreated; 2, 4 IU g^{-1}; 3, 8 IU g^{-1}; 4, 12 IU g^{-1}; 5, 16 IU g^{-1} of oven dried pulp).

4.8.4.2 *Analysis of solubilized products*

The release of xylooligomers and monophenols due to degradation of xylan and lignin, respectively, during enzymatic bleaching was analysed by HPLC. Xylose was found to be the major product in the effluent of pulp treated with the mixed enzyme preparation, while marginally lower levels of the same was found in the effluents of pulp treated with xylanase (Fig. 35a-c). These results indicated that the presence of laccase may have synergistically enabled the xylanase attack on the xylan layer, yielding xylooligomers (xylopentose, xyloteraose, xylotriose and xylobiose) which were further degraded into the monomeric sugar xylose. Following enzymatic treatment, effluents were also analysed for the presence of lignin degradation products (phenols). A similar pattern for released phenols was observed. The mixed enzyme preparation consisting of xylanase and laccase led to an increased release of catechol, syringaldazine and some extra phenols (Fig. 36a-c). Lower levels of catechol and syringaldazine was observed in the laccase treated samples.

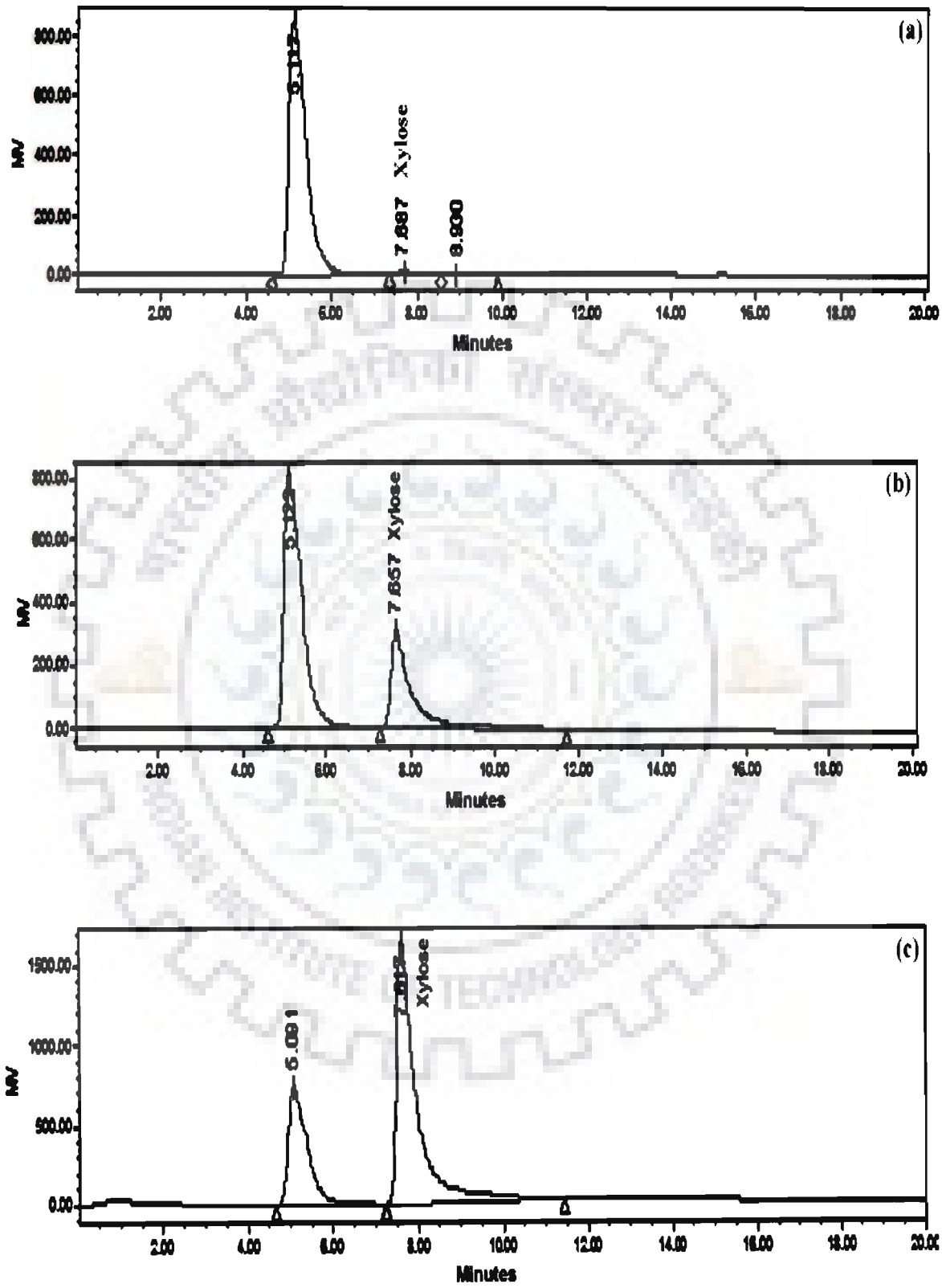


Fig. 35 High performance liquid chromatography of xylooligomers released (a, untreated; b, xylanase treated and c, xylanase-laccase treated).

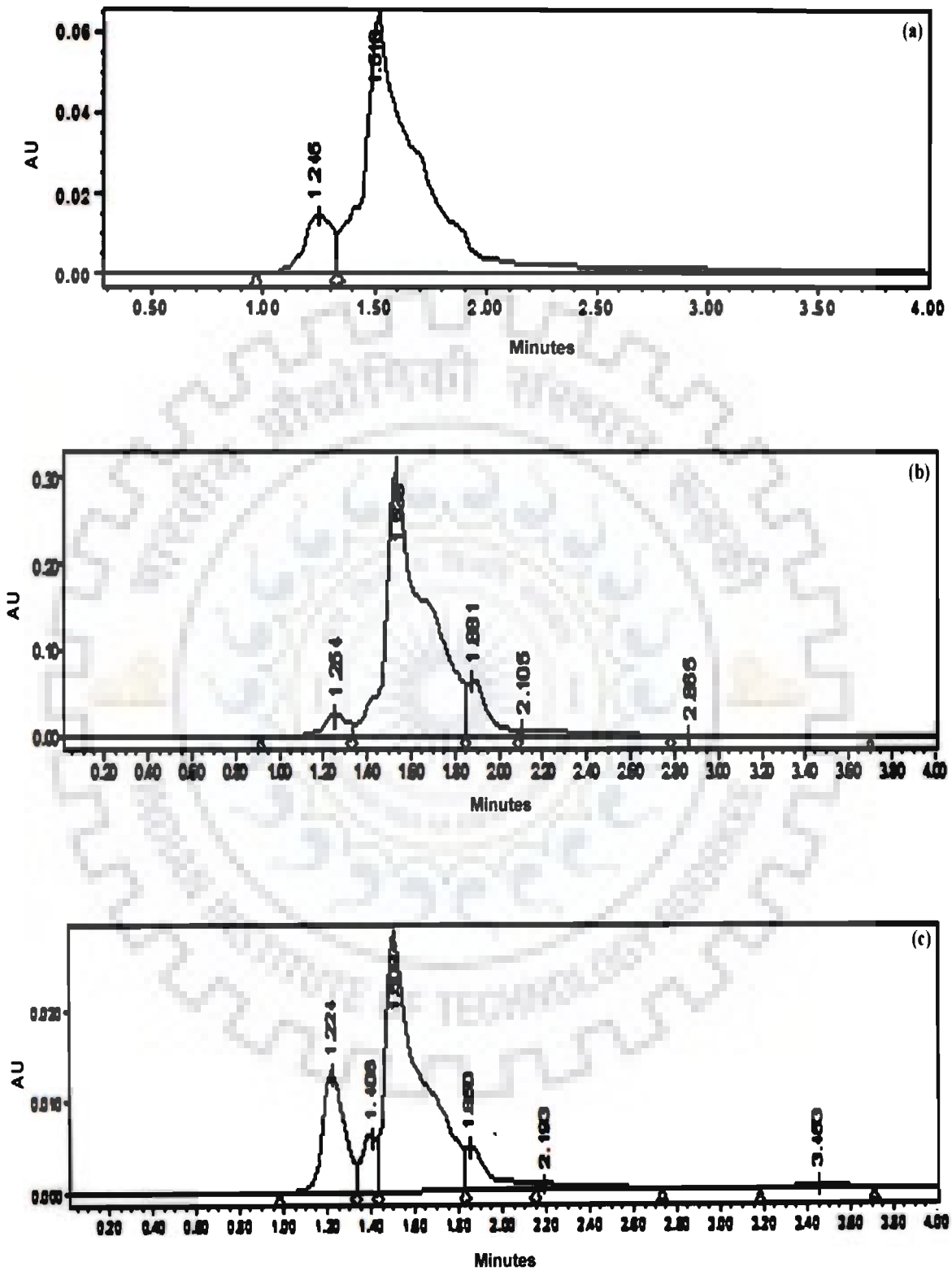


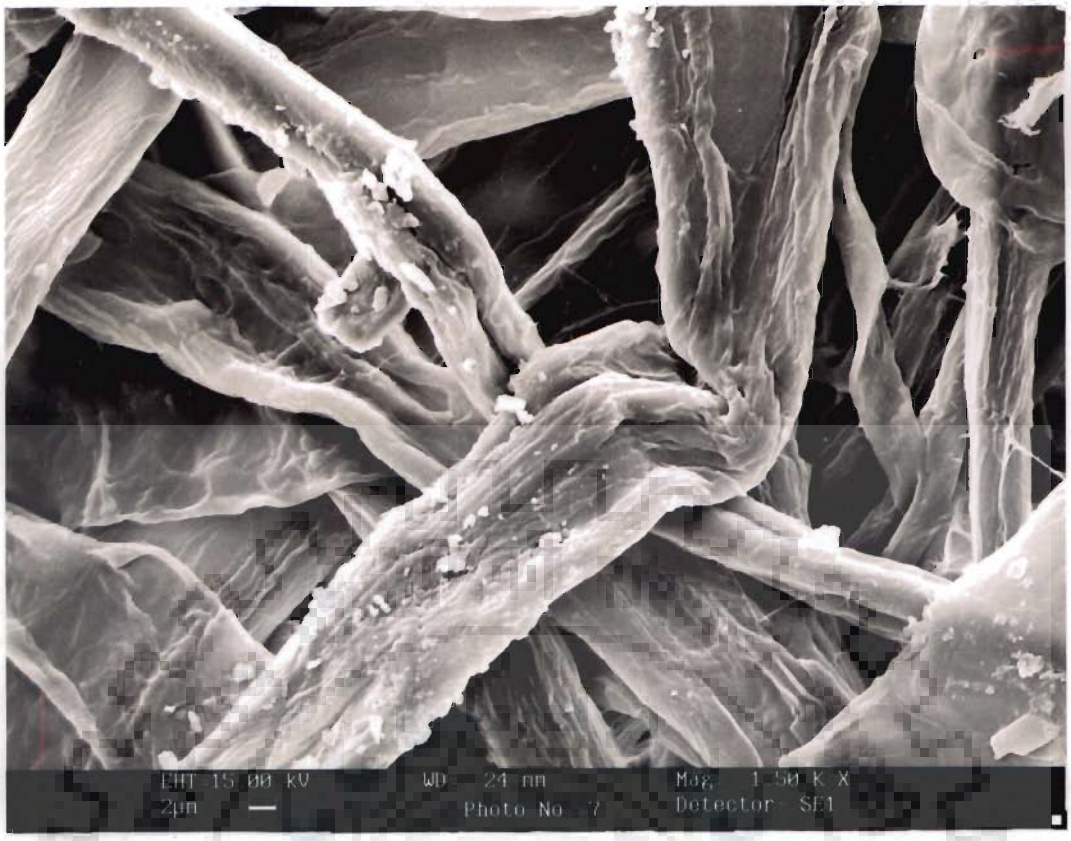
Fig. 36 High performance liquid chromatography of lignin degradation products released (a, untreated; b, xylanase treated and c, xylanase-laccase treated).

4.8.5 Analysis of fibre morphology

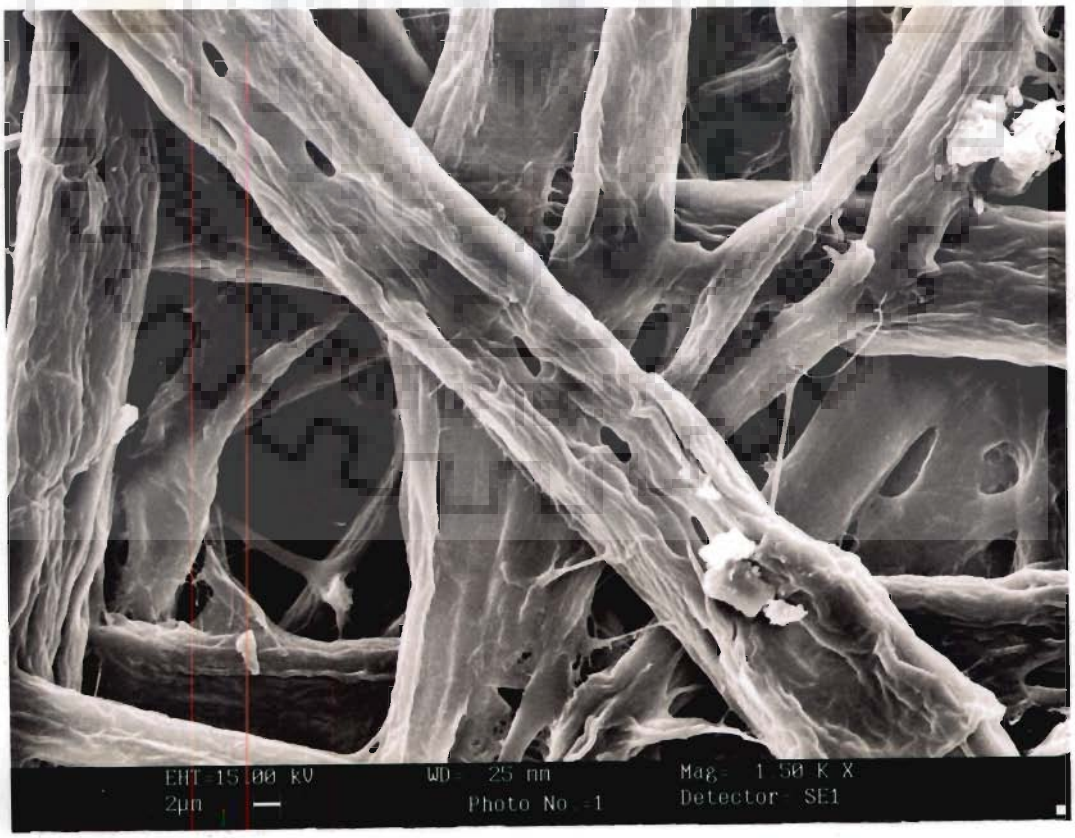
Morphological features of pulp following enzymatic treatment were analysed by scanning electron microscopy. The enzymatic treatment introduced greater porosity, swelling, separation and peeling of pulp fibres (Fig. 37 b) compared to the smoother surface of untreated pulp (Fig. 37a). The swelling, separation and loss in compactness in the pulp fibres rendered them more susceptible to chemical bleaching as it facilitated the entry of various chemicals during subsequent bleaching stages. Grooves and cracks appeared to have formed in distinct regions of the fibre, although they were not evenly distributed. It is well known that during the pulping process, short xylan chains precipitated on the surface of pulp fibres and acted as physical barrier for the entry of bleaching chemicals in subsequent bleaching stages. The separation of this xylan leads to formation of grooves or cracks enabling the bleaching agents to penetrate more readily.

4.8.6 Analysis of pulp crystallinity by X-ray diffraction technique

Crystallinity of pulp is a major criterion which is used to achieve better understanding of pulp delignification during biobleaching. As a result of enzymatic action (xylanase-laccase), hemicellulose and lignin materials were removed, leaving therefore high proportions of amorphous cellulose which led to a decrease in the pulp crystallinity. The degree of pulp crystallinity was determined on the basis of peak width at half maximum (PWHM). As detected following X-ray diffraction analysis (Fig. 38), it is highest for xylanase-laccase treated (XL) pulp, hence it is least crystalline. Thus the bleaching ability of the mixed enzyme preparation was superior compared to xylanase alone, as it had enabled a greater extent of lignin removal from the lignocellulosic materials.



(a)



(b)

Fig. 37 Scanning electron microscopy of the pulp (a) before and (b) after treatment with mixed enzyme preparation.

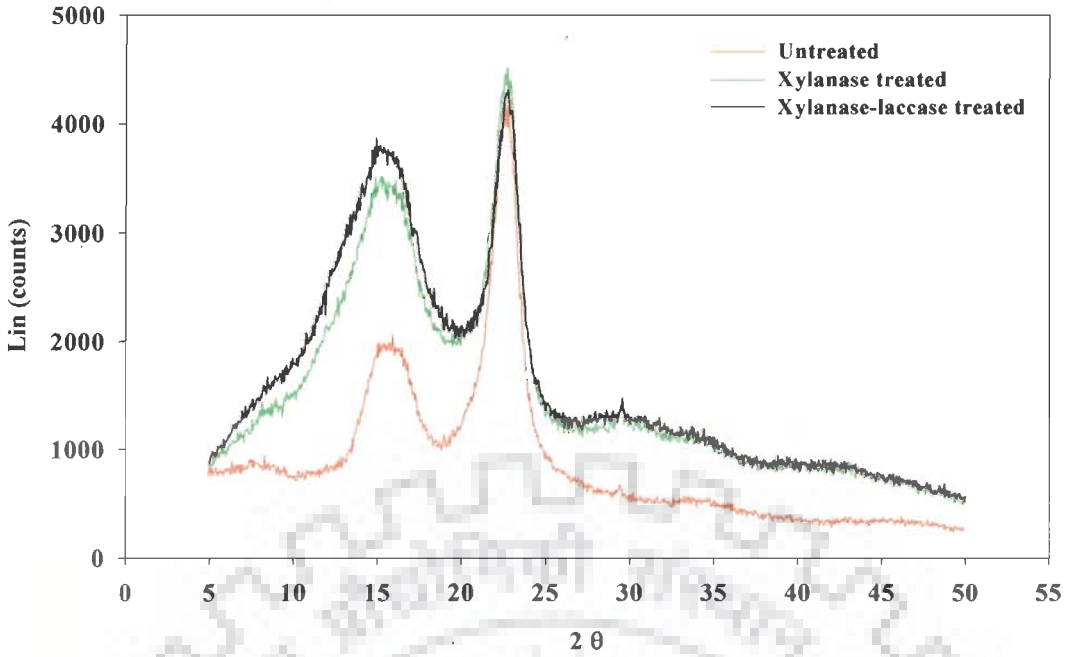


Fig. 38 X-ray diffraction analysis for crystallinity of pulp samples

4.8.7 Analysis of residual lignin by fourier transform infrared spectroscopy

FTIR spectra of untreated, xylanase treated (X) and xylanase-laccase (XL) treated pulp samples indicated structural changes in the lignin. Due to enzymatic treatment there was removal and degradation of lignin, hence there were some characteristic changes in the FTIR spectra before and following treatment, possibly due to alterations in lignin's functional groups. The peak at 3400 cm^{-1} denoted the presence of -OH groups in lignin and the intensity and number of these peaks were higher in xylanase-laccase treated samples as compared to xylanase and untreated samples (Fig 39 a-c). The peak intensity at 2900 cm^{-1} in FTIR spectra of pulp treated with mixed enzyme was higher as compared to the remaining two (xylanase treated and untreated) and a few more peaks appeared in this region (Fig. 39c). The peak intensity at this wave number was attributed due to presence of methyl/methylene (-CH) groups. A new, strong absorption peak at 1720 cm^{-1} in XL and X treated pulp appeared. However, its intensity was lower in X treated pulp and it was totally absent in untreated pulp. This peak was attributed to carboxylic acid and unconjugated carbonyl groups (C=O) due to action of the enzyme on lignin's aromatic ring. In untreated samples these carbonyl groups remain associated with aromatic rings, hence affected into no absorption. Besides this, more peaks appeared at wave number 1600-1400 cm^{-1} and 1300-1200 cm^{-1} due to the changes in the ratio of syringyl and guaiacol groups.

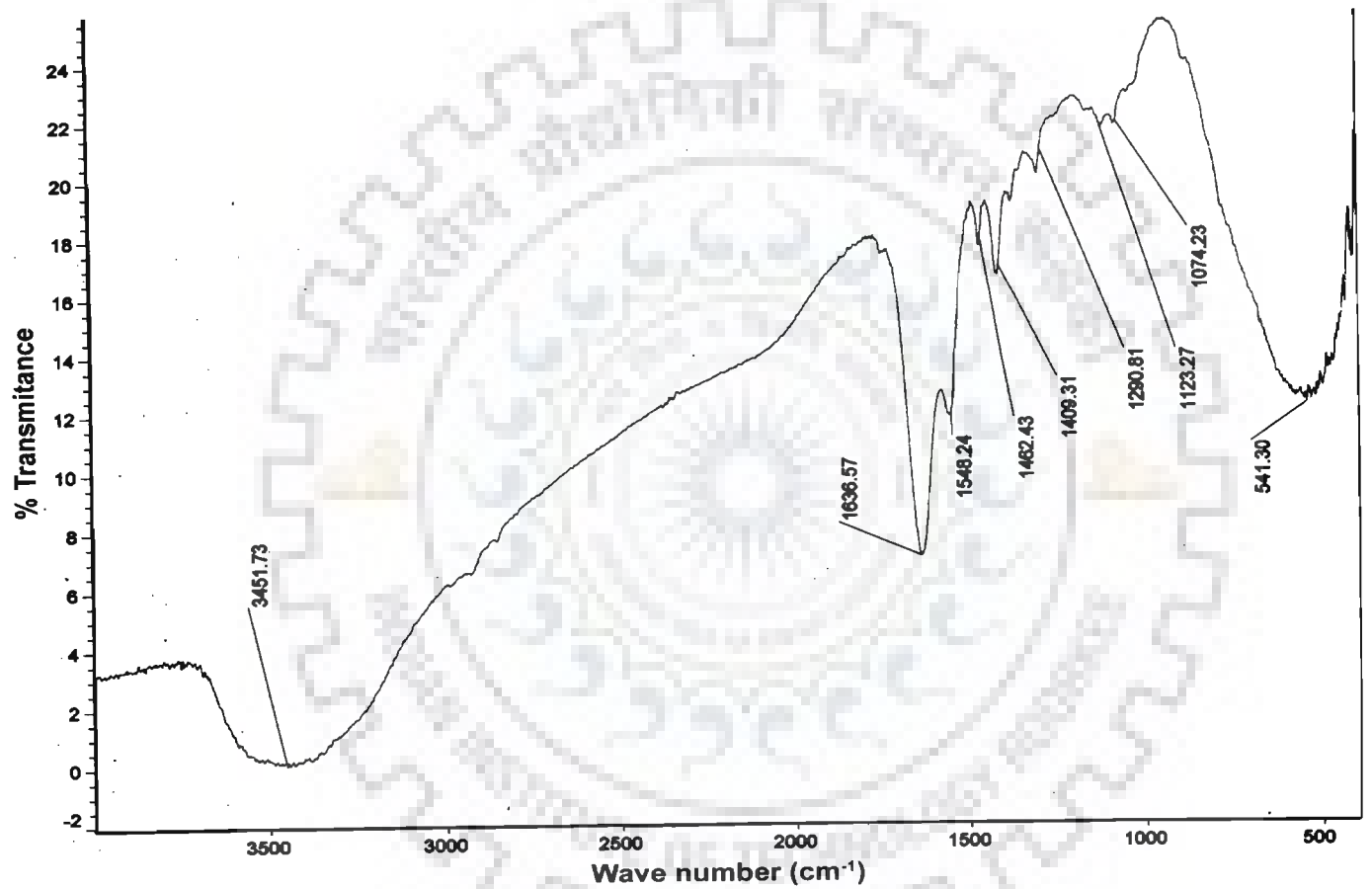


Fig. 39a FTIR spectra of lignin extracted from untreated pulp.

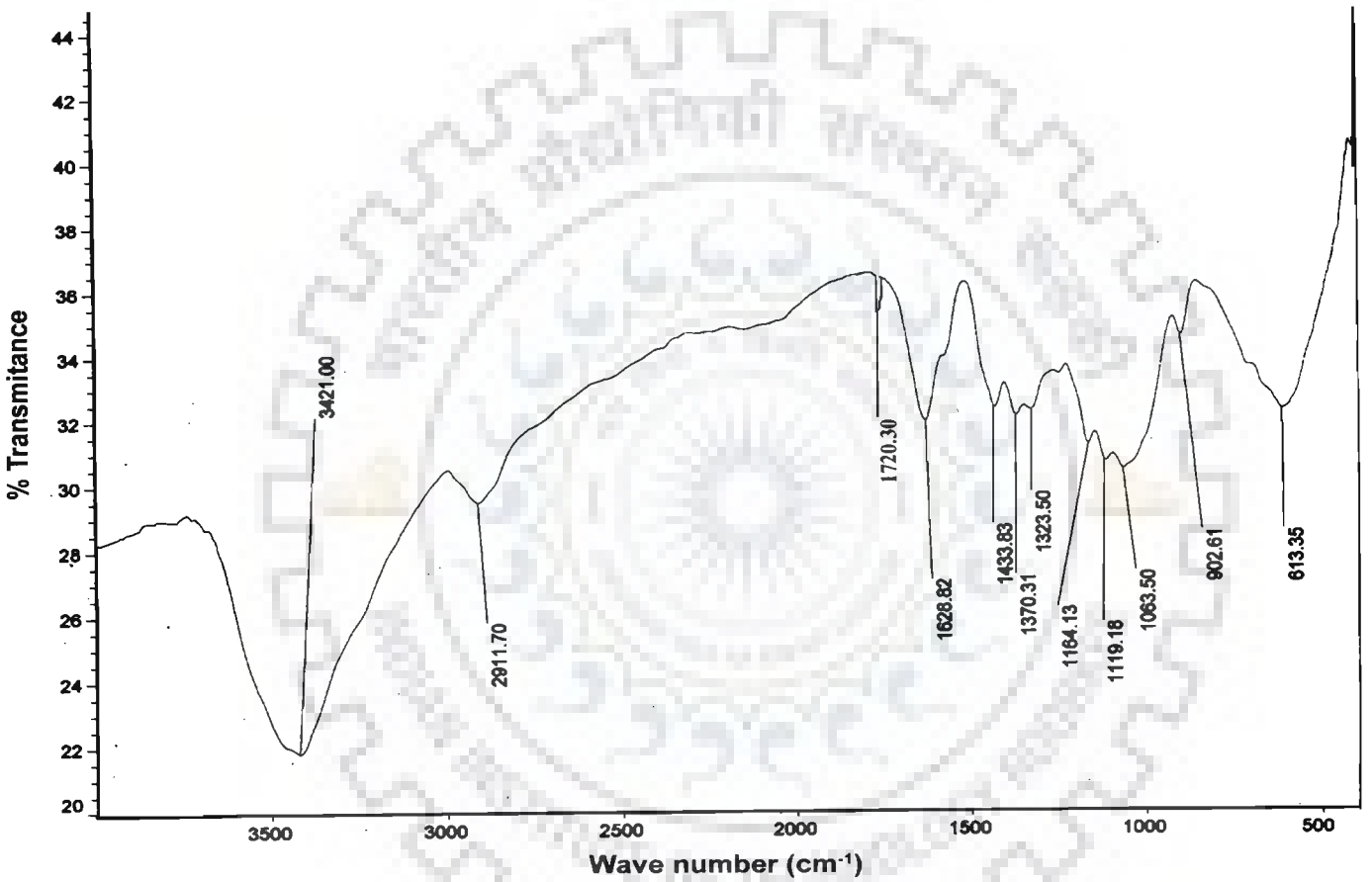


Fig. 39b FTIR spectra of lignin extracted from xylanase treated pulp.

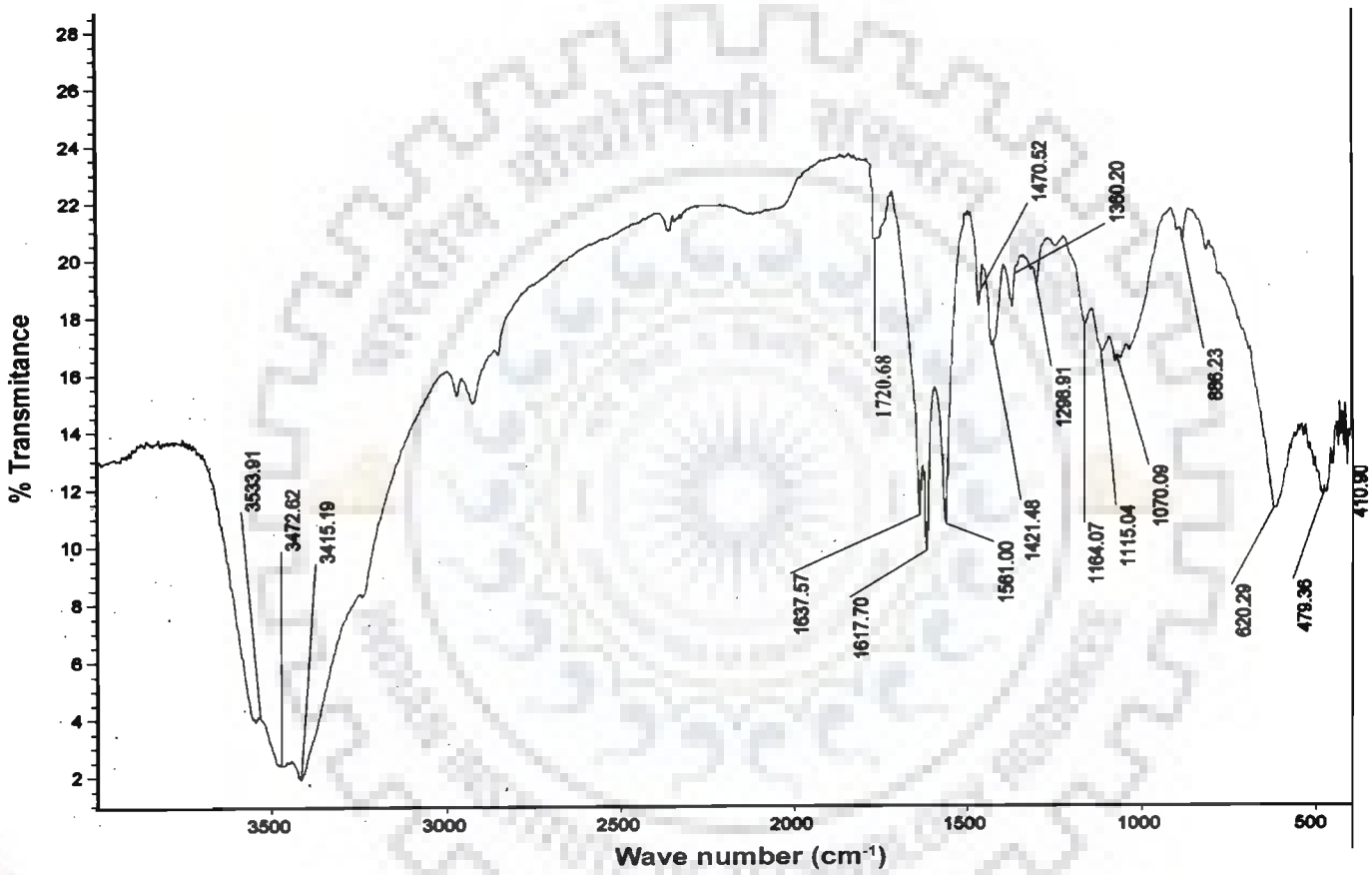


Fig. 39c FTIR spectra of lignin extracted from xylanase-laccase mixture treated pulp.

4.9 BIOPROCESS DESIGN

Scaling up of the solid-state fermentation process was attempted by designing an intermittent rotating drum bioreactor (Fig 40). Simultaneous production of xylanolytic and ligninolytic enzymes in a batch mode was evaluated through co-cultivation of mutant *P. oxalicum* SAU_E-3.510 and white rot fungus *P. ostreatus* MTCC 1804. The fermentation was carried out in a cylindrical drum having the dimensions of diameter, 28 cm; length, 38 cm with the total capacity of 23 l and was made up of acrylic sheet (polymethyl methacrylate, PMMA). The bioreactor drum contained 368 g of solid support (276 g bagasse and 92 g black gram husk) which was mixed with 1.5 l of fermentation medium to have an 80% initial moisture content. A typical growth associated production of enzymes was observed by using the optimised culture medium and the growth conditions consisting of 80% initial moisture and at 30°C (Fig. 41). Production of xylanase and laccase began after the 4^{th} day of incubation, which increased progressively thereafter and maximum level of xylanase (7832.46 ± 353.71 IU g^{-1}) and laccase activity (280.76 ± 19.05 IU g^{-1}) was obtained after 8 days of incubation (Fig. 41). Enzyme activity declined thereafter. A similar pattern of enzyme production was also observed for β-xylosidase, but maximal cellulase activity (1.33 ± 0.02 IU g^{-1}) was achieved following 6 days of incubation that decreased after further incubation.

(a)

(b)

Fig. 40 Intermittent rotating drum bioreactor for simultaneous production of xylanase and laccase through co-cultivation of mutant *P. oxalicum* SAU_E-3.510 and *P. ostreatus* MTCC 1804 under solid-state fermentation (a) Exterior view; (b) Enlarged view.

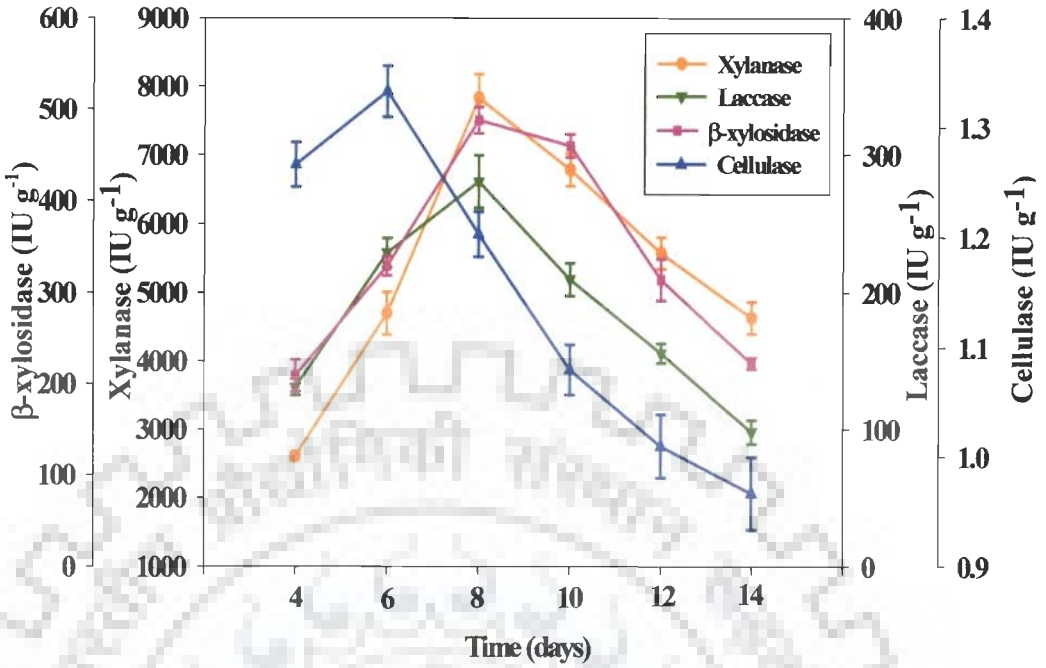


Fig. 41 Simultaneous production of xylanase and laccase in a laboratory scale intermittent rotating drum bioreactor by co-cultivation of mutant *P. oxalicum* SAU_E-3.510 and *P. ostreatus* MTCC 1804.

(Values are mean of three replicates ± SE).

To assess the efficacy of bioreactor, the levels of production of enzymes obtained in the bioreactor were compared with the levels obtained in the smaller scale laboratory experiments. Comparable levels of enzyme activities were obtained in the bioreactor (Table-20). The xylanase yield obtained in the bioreactor was around 12% lower than that obtained in laboratory scale flasks, however.

Table. 20 Effect of scaling up on enzyme production by co-culture of *P. oxalicum* SAU_E-3.510 and *P. ostreatus* MTCC 1804 under SSF using bagasse and black gram husk mixture as solid support

Enzymes	IRDB		Laboratory flask scale	
	Production level (IU g^{-1})	Productivity (IU g^{-1} d^{-1})	Production level (IU g^{-1})	Productivity (IU g^{-1} d^{-1})
Xylanase	7832.46 ±353.71	1304.60 ± 44.61	8965.66 ±194.54	1494.27 ±32.42
Laccase	280.76 ±19.05	46.00 ±2.08	390.00 ±15.01	65.00 ± 2.50
β-xylosidase	488.28 ±14.42	81.44 ±5.44	591.33 ± 4.91	98.55 ±0.81
Cellulase	1.20 ±0.02	0.18 ±0.009	1.21 ±0.06	0.20 ±0.01

(Values are mean of three replicates ± SE).

Thus it is apparent that the levels of production as achieved with intermittent rotating drum bioreactor are almost comparatively similar as that obtained under shake flask conditions. Minor modifications in the rotating drum bioreactor system may further enable in improving the levels of production.

CHAPTER V

DISCUSSION

Chapter 5

DISCUSSION

5.1 IDENTIFICATION OF ALKALITOLERANT XYLANASE PRODUCING MICROORGANISMS

Microorganisms are considered as "miniature cell factories" and are exploited for the production of enzymes of commercial significance. Enzymes are recognised as potential alternatives to harsh chemical technologies. This has led to intensive efforts to explore natural microbial diversity to discover enzymes that could be used for environmentally friendly "Dream technologies" in the immediate future. Microorganisms surviving in the extreme environmental set-ups present a system interesting enough to explore and also to utilise these for industrial applications.

Many workers have employed the stepwise screening approaches for selecting xylanase producing microorganisms (Breccia et al., 1998; Kohli et al., 2001; Ratanachomsri et al., 2006). In an attempt to derive an alkalitolerant, stable and cellulase free xylanase, two potential sources namely, waste of a soda ash industry (pH 11.0) and effluents from a pulp and paper industry (pH 11.0) were explored for their natural microbial biodiversity. A total of 18 fungal isolates were obtained and screened for their xylanase production ability as evident through screening on xylan agar plates. Out of these strains, SA-8 was selected due to its ability to produce the maximum xylanase activity (242.05 ± 3.91 IU ml^{-1}) with a noteworthy β-xylosidase activity (16.20 ± 2.00 IU ml^{-1}) under alkaline conditions of growth (pH 9.0) and also due to its exhibiting negligible levels of cellulase activity (0.18 ± 0.03 IU ml^{-1}). The strain was, therefore, selected for further studies and identified as *Penicillium oxalicum* SA-8 ITCC 6024 by the Indian Type Culture Collection Bank, Indian Agricultural Research Institute, New Delhi, India. Attempts also have been made to isolate alkalophilic strains from an alkaline lake (Mamo et al., 2006,

Gessesse and Mamo, 1999), the effluent of a pulp and paper industry (Anthony et al., 2003) and decomposed plant samples (Swaroopa Rani and Nand, 2000) for obtaining a xylanase stable at high pH.

The development of an industrially viable fermentation process largely depends on the production efficiency of the microbial strain involved in the process. The strain selected in the investigation had shown significant levels of xylanase production. Thus, to explore the possibilities for commercial exploitation of the enzyme, attempts were made for achieving a further increase in the enzyme production levels. The strain was subjected to mutagenesis for achieving an improvement in production level and the process economy was further improved by evaluating low cost agro-industrial residual materials as substrate in the production medium.

5.2 MUTAGENESIS OF *P. OXALICUM* SA-8

Isolate *P. oxalicum* SA-8 was subjected to UV and ethidium bromide mutagenesis. Several mutants thus obtained were evaluated for their xylanase production ability. Mutant *P. oxalicum* SAU_E-3.510 produced 87% higher xylanase levels as compared to the wild type strain. Induced mutagenesis and strain selection had been followed by various groups for achieving increased levels of xylanase (Singh et al., 1995; Bakalova et al., 2002), laccase (Dhawan et al., 2003), tannase (Purohit et al., 2006) and cellulase (Chand et al., 2005; Adsul et al., 2007). The mutant strains not only were able to produce enhanced levels of enzymes (1.2 to 2.5 fold higher) but also had an improved bioconversion efficiency.

Mutagenesis not only resulted in varying levels of enzyme production but had also led to characteristic variations in morphological features. The hyphae of wild type *P. oxalicum* SA-8 were flattened, shiny, smooth surfaced and straight, whereas those of mutant *P. oxalicum* SAU_E-3.510 were coiled, rounded with a rough peeled surface. Similarly, distinct features were also observed for their spores, viz spores of the wild type were rounded with a rougher surface whereas spores of mutant strain were larger, smooth, flattened and crescent shaped.

5.3 ANALYSIS OF XYLANASE PRODUCTION AT DIFFERENT pH VALUES

Production of xylanase in media having a wide range of pH values indicated the alkalitolerant nature of the *P. oxalicum*, since a higher level of xylanase production was found under alkaline conditions (pH 9.0). A specific pattern of xylanase activity was observed for both the mutant and wild type strains, when activity was measured at different pH values from 4 to 11. The maximum xylanase activity, i.e. 487.44 ± 3.57 IU ml^{-1} and 252.50 ± 6.27 IU ml^{-1}, was obtained by mutant and wild type strain of *P. oxalicum* respectively at pH 9.0 followed by a subsequent decrease in activity with increasing pH. However, at pH 5.0 again a markedly higher xylanase activity was observed with the mutant *P. oxalicum* SAU_E-3.510 (444.89 ± 7.60 IU ml^{-1}) and wild type *P. oxalicum* SA-8 (197.81 ± 6.94 IU ml^{-1}). The similar profile of xylanase activity was also reported with *A. niger* NIOCC, which was isolated from a marine habitat. The culture filtrate showed peaks of xylanase activity at pH 3.5 and pH 8.5 (Raghukumar et al., 2004). The varying levels of growth and xylanase production at different pH values as observed with *Trichoderma reesei* C-30 also resulted in the expression of distinct isoforms of xylanases with defined pH optima (Xiong et al., 2004). Multiplicity is a commonly observed phenomenon among fungal xylanases and might be a possible reason for xylanase activity at two different pH values. Xylanase activity at two different pH values was also observed for an alkalophilic *Bacillus* sp. which was isolated from kraft pulp (Yang et al., 1995) and *Bacillus halodurans* S7 (Mamo et al., 2006). Most of the xylanases of the genus *Penicillium* are optimally active in the acidic range (pH 5.0). Recently, a cellulase free, alkalophilic xylanase was discovered from alkalitolerant *P. citrinum* which was isolated from soil (Dutta et al., 2007).

An alkalistable and possibly thermostable xylanase would be of immense value to the pulp and paper industry due to its environmental and technological benefits. During paper manufacturing, the fibres are broken apart and most of the lignin is removed at the time of pulping. The kraft pulping, which is achieved through chemical means, is commonly used around the world and requires cooking of wood chips at a high temperature and pH. This process

removes around 95% of total lignin but the remaining 5% lignin, which is responsible for the brown colour of pulp, is removed during subsequent bleaching stages either by using chemicals or enzymes (Damiano et al., 2003). Therefore, the pulp for enzymatic bleaching remains hot and alkaline. Thus the use of a thermostable and alkalophilic xylanase would be more suitable. Very few reports are available regarding alkalophilic xylanases from fungi like *Aspergillus fischeri* (Chandra Raj and Chandra, 1996), *Cepahalosporium* sp. RYM-202 (Kang et al., 1996), *A. fumigatus* (Anthony et al., 2003) and *P. citrinum* (Dutta et al., 2007).

5.4 CRITICAL FACTORS AFFECTING ENZYME PRODUCTION IN SHAKE FLASK FERMENTATION

The carbon source used for the production medium is one of the major factors affecting enzyme production. As reviewed by Bajpai (1997), fungal xylanases appear to be inducible in nature and remain under derepression control. Among various mono, di and polysaccharides analysed for synthesis of xylanase by mutant *P. oxalicum* SAU_E-3.510, oat spelt xylan resulted in the maximum level of xylanase (488.56 ± 6.53 IU ml^{-1}), followed by xylose. It is therefore apparent that xylan acted as inducer for xylanase biosynthesis because it released mono, di and largely oligosaccharides on enzymatic degradation. Xylose appeared to act as marginal inducer, apparently due to its quick utilisation by microbes which resulted in lower levels of xylanase induction.

The use of commercial xylan as carbon source is uneconomical for large scale production of endo-xylanase and β-xylosidase due to its high cost. Various hemicellulose rich natural substrates, including non-food plant biomass and agricultural residues, were evaluated for xylanase production. Of the various natural substrates, alkali treated congress grass (*Parthenium* sp.), led to a remarkably higher xylanase activity (475.23 ± 6.05 IU ml^{-1}) followed by the activity (397.05 ± 7.53 IU ml^{-1}) obtained with treated water hyacinth (*Eichhornia crassipes*). Xylanase activity obtained with congress grass was about similar to that obtained with commercial oat spelt xylan. The replacement of oat spelt xylan in the production medium

with congress grass (*Parthenium* sp.) for xylanase production, provides environmental and economic advantages. These herbaceous plants grow wildly and abundantly in tropical countries like India and their eradication in proper manner is an arduous task for the Government. Utilisation of these plant biomass materials will not only address the hazards due to their wide spread occurrence but would also offer an economic alternative for the production of value added products.

Enzyme production to a greater extent depends upon the concentration of the carbon source. An increase in the enzyme production (563.05 ± 6.49 IU ml^{-1}) was observed when congress grass was added at a concentration of 0.25% and decreased upon a further increase in the substrate concentration. The decreased enzyme activity at the higher substrate concentration possibly may have been due to catabolic repression (Ikram-ul Haq et al., 2002) or improper mixing due to the high viscosity of the medium (Kuhad et al., 1998). Furthermore, parthenin present in congress grass, which is a sesquiterpene lactone of the pseudoguaianolide, may result in decreased levels of xylanase at increasing concentrations of congress grass (Shah et al., 2007).

An increased production of xylanase was observed in presence of veratryl alcohol and ABTS. An improved rate of xylan hydrolysis was also reported in the presence of lignin or lignin model compounds at lower concentrations. The affinity of xylanase for lignin or lignin model compounds has been observed earlier (Kaya et al., 2000). This interaction may possibly regulate the molecular conformation of xylanase resulting in an increase in xylanase activity, as observed. Soluble lignin-xylanase complex apparently accelerates hydrolysis of soluble xylan, possibly by introducing some change in the structure of xylanase.

5.5 CO-CULTIVATION OF MUTANT *P. OXALICUM* SAU_E-3.510 WITH *P. OSTREATUS* MTCC 1804

Fungal co-culture often is associated with improved abilities as compared to the activities obtained during respective monoculture cultivation conditions. During co-cultivation, improved activities, mainly related to biodegradation of cellulose (Bernalier et al., 1991),

nitrocellulose (Sharma et al., 1995), lignin (Watanabe et al., 2003; Chi et al., 2007), pollutants in potato chips industry waste water (Mishra et al., 2004) and cyanide (Ezzi and Lynch, 2005) had been observed. Mixed fungal cultivation appears to have a number of advantages over a mono culture. As compared to monoculture, a paired fungal culture effects better substrate utilisation, increased productivity, improved adaptability to changing conditions and has a superior resistance to contamination by unwanted microbes. It results in a higher level of production of enzymes, antibiotics, several types of fermented food, composting, dairy fermentation and domestic waste water sludge in an economic manner (Duenas et al., 1995; Vinogradova and Kushnir, 2003; Alam et al., 2003; Mukherjee and Banerjee, 2006). Co-culturing of two fungi having growth compatibility under the same cultural and nutritional conditions will provide economic advantages due to a reduction in the overall cost of production. Nutritional limitations between compatible partners may be overcome due to synergistic interaction (Gutierrez-Correa et al., 1999). During co-cultivation, fungi behave in a complex manner and the interaction may be antagonistic, parasitic or synergistic. Mixed fungal cultures could lead to higher enzyme production through synergistic interaction, but the final outcome seems to depend on the particular combination of species, mode of interaction between them and on the microenvironment or nutritional conditions provided by the substrate under colonisation (Gutierrez-Correa and Tengerdy, 1998). Interaction between *Laccaria laccata* and *Trichoderma virens* in co-culture is of the antagonistic type and the growth of *T. virens* appeared to be inhibited in co-culture. Shortened, more branched and deformed or injured hyphae of *T. virens* were observed in the scanning electron micrograph of the hyphae from the contact zone (Werner et al., 2002). A similar kind of antagonistic interaction was also observed by Stepanova et al. (2003) during co-cultivation of *T. reesei* with three basidiomycetes. The mould *T. reesei* fully dominated and had occupied the complete plate surface. Thickening and brown pigmentation in the hyphal contact zone is a commonly occurring phenomenon and was also observed by other groups who had studied the interaction between *Lentinula edodes* and *Trichoderma*. Browning

and higher levels of polyphenol oxidase in the area of interaction have been reported by Tokimoto (1982). The probability behind this mutual growth stimulation between two fungi may be due to the fact that both can colonise the same microenvironment in nature or due to the formation of complementary enzymes xylanase and laccase which may act synergistically during wood decay in nature. The possibility of co-cultivation of two different fungi on oat straw was demonstrated by Stepanova et al. (2003). *Coriolus hirsutus* and *Cerrena maxima* co-cultivation intensely consumed both lignin and cellulose under solid-state fermentation employing oat straw as solid-support.

The mutant *P. oxalicum* SAU_E-3.510 was characterized as a hyper xylanase (714.13 ± 15.40 IU ml^{-1}) producing strain under derived cultural and nutritional conditions and its co-cultivation was performed with *P. ostreatus* MTCC 1804 due to its ability to produce significant levels of laccase in monoculture (13.00 ± 1.53 IU ml^{-1}). Both fungal strains nurtured well in the same cultural media showing growth compatibility, which indicated their synergistic mode of interaction that also resulted in stimulation of enzyme production. The mixed association of the macromycete *Schizophyllum commune* and the micromycete *Mucor* sp. was highly suitable for the production of hydrolytic enzymes. Levels of endoglucanase and protease, increased to 4 and 1.5 times, respectively, during co-cultivation condition (Vinogradova and Kushnir, 2003). Koroleva et al. (2002) studied the production of lignin modifying enzymes by co-cultivation of white rot fungi *Cerrena maxima* and *Coriolus hirsutus* and reported a moderate increase in production of both lignin peroxidase and manganese peroxidase. Changes in activities of some extracellular enzymes were reported in dual cultures of *L. edodes* and *Trichoderma* strains (Hatvani et al., 2002) and results had indicated that these enzymes play important role in the antagonistic interaction between the two species. Chi et al. (2007) analysed the production of lignin degrading enzymes under co-cultivation and their role in lignin degradation. Further, chemical analysis of decayed aspen wood blocks showed that *Ceriporiopsis subvermispora* with *P. ostreatus* during co-cultivation could significantly stimulate wood decay, compared to

monocultures. A mixed submerged fermentation system was developed employing *A. niger* and *T. reesei* strains for enhanced cellulose production with lactose and lactobionic acid as inducers. Significant enhancement was observed in the level of cellulase activity (1.5 to 2.1 fold), volumetric productivity (2.1 fold) and total protein content (1.5 fold) in mixed cultures as compared to their mono cultures (Ahamed and Vermette, 2008). An increased level of ethanol production from biomass was reported by co-culturing of *Chalara parvispora* and *Trametes hirsuta* as compared to the fermentation using *Saccharomyces cerevisiae* (Holmgren and Sellstedt 2008).

5.6 CO-CULTIVATION OF MUTANT *P. OXALICUM* SAU_E-3.510 AND *P. OSTREATUS* MTCC 1804 FOR SIMULTANEOUS BIOSYNTHESIS OF XYLANASE AND LACCASE IN SOLID-STATE FERMENTATION

The advantages of mixed culturing may be even more evident in solid-state fermentation because the colonisation of the substrate may be accomplished in a better manner in symbiotic associations. Superior productivity, improved product recovery, reduced risk of contamination, low capital investment, simpler set up, reduced energy requirement, almost closely similar to natural surroundings for microorganisms and low waste water output are some of the advantages of solid-state fermentation (SSF) over submerged fermentation (Lonsane et al., 1985; Pandey et al., 2001). A large number of agroresidues (sugar cane bagasse, wheat bran, wheat straw, corn cobs, rice husk, maize bran and many more) which are generated in enormous amounts during agricultural practices are considered as the promising substrates for SSF (Pandey et al., 1999). Utilisation of these residual materials for bioprocesses also eases the pollution menace, which otherwise is a major concern (Pandey et al., 2000). A maximal level of xylanase (1301.51 ± 12.73 IU ml^{-1}) and laccase (43.70 ± 3.72 IU ml^{1}) was obtained when mutant *P. oxalicum* SAU_E-3.510 and *P. ostreatus* MTCC 1804 were co-cultivated in solid-state fermentation.

To obtain a further improvement in the production level, SSF was performed using a variety of solid supports of agricultural origin. Medium constituents, particularly the substrate,

affects the cost of production. This can be further economised by using low cost and easily available agro-industrial residual materials (Sandhya et al., 2005). A mixture of sugarcane bagasse and black gram husk in a ratio of 3:1 was most productive for both xylanase (7574.24 ± 118.99 IU g^{-1}) and laccase (381.33 ± 10.01 IU g^{-1}). Sugar cane bagasse and black gram husk both are abundantly available in India due to agricultural practices and served as a solid substrate during SSF in this investigation. The husks of pulses are known to be rich not only in nitrogen but also in mineral contents; hence, when used in combination with bagasse, supported the growth and production of enzymes. Supplementation of green gram husk was found to be beneficial for attaining higher levels of pectinases under solid-state fermentation by *A. niger* (Patil and Dayanand, 2006).

Moisture content during SSF is a critical factor for achieving microbial growth and production of enzyme. Co-cultivation was performed at different moisture contents ranging from 20% to 120% and a moisture level of 80% was best suited for enzyme production. Due to their particulate nature both bagasse and black gram husk did not show agglomeration at higher moisture levels up to 85% and resulted in proper mixing of substrates, better heat and mass transfer and thus improved productivity of enzymes (Kumar et al., 2003). Higher moisture levels during SSF interfere with air circulation and heat transfer that is generated due to biological activities of the inoculated strains (Gawande and Kamat, 1999; Gautam et al., 2002).

5.7 CHARACTERISTIC FEATURES OF XYLANASE FROM MUTANT *P. OXALICUM* SAU_E-3.510

The optimal pH and temperature for xylanase activity was found to be 9.0 and 55°C, respectively. The enzyme was quite stable and maintained 93% residual activity at pH 9.0 for 24 h. A 93% residual activity was observed after 4 h of incubation in the buffer at pH 11.0, while about a 50% decrease in the activity was detected after 20 h. Similarly, enzyme was also detected to be appreciably thermostable. Maximal activity of the enzyme was obtained at 55°C and the half life of enzyme was found to be 2 h at 80°C with 50% of the activity remaining.

SDS-PAGE and zymogram analysis indicated the presence of two isoforms of xylanase in both the wild type and mutant strains. The molecular mass of these two isoforms were deduced to be 121 kD and 77 kD respectively. These two xylanase isoforms were also synthesised by mutant during its paired culture with *P. ostreatus* MTCC 1804 under solid-state fermentation along with an extra xylanase of 40 kD that corresponded to *P. ostreatus* MTCC 1804. Similarly, no laccase was detected in the axenic culture of the mutant, whereas laccase corresponding to 29 kD was detected for *P. ostreatus* MTCC 1804 during co-culturing. Multiple xylanases with such a high molecular weight have not been reported from any other species of *Penicillium* to date, while higher molecular mass isoforms of xylanases have been reported from *Aspergilllus fumigatus* AR1 (Anthony et al., 2003), *Aeromonas caviae* ME-1 (Liu et al., 2003) and *Streptomyces olivaceoviridis* E-86 (Jiang et al., 2005). Multiplicity is a commonly occurring phenomenon among fungal xylanases. Such multiple forms of xylanases have also been reported in different species, namely *Cephalosporium* (Wong et al., 1988), *Aeromonas* (Liu et al., 2003), *Myceliophthora* (Badhan et al., 2004), *Aspergillus* (Anthony et al., 2003; Shah and Madamwar, 2005) and *Streptomyces* (Jiang et al., 2005). Several factors could be responsible for multiplicity among xylanases, viz differential m-RNA processing, post translational modifications, etc. (Biely, 1985). Further, multiple xylanases can also be the product of different alleles of the same gene (Wong et al., 1988) or the product of independent gene (Hazlewood and Gilbert, 1993).

Internal peptide sequence elucidation of xylanase from mutant *P. oxalicum* SAU_E-3.510 was performed by MALDI-ToF/ToF analysis, the sequences derived were subjected for homology search with the sequence of xylanases reported in the database. Maximum degree of identity was revealed mainly with xylanase from bacterial species i.e. *Bacillus stearothermophilus*, *Geobacillus stearothermophilus*, *Penibacillus* sp. and *Clostridium* sp. A comparatively lower extent of identity was observed with xylanases from fungal strains, i.e. 35-45% identity was observed with xylanase from *Gibberella zeae* and *Aspergillus versicolor*.

To derive the mode of enzymatic action, the hydrolysis products of congress grass xylan due to the action of xylanase from mutant *P. oxalicum* SAU_E-3.510 and a paired culture of mutant *P. oxalicum* SAU_E-3.510 and *P. ostreatus* MTCC 1804 were analysed by performing high performance liquid chromatography. The release of xylose as the major hydrolysis product suggested the synergistic action of endoxylanase and β-xylosidase. Endoxylanase attacks the internal xylosidic bonds and releases xylooligosaccharides. These xylooligosaccharides, on prolonged incubation are further degraded into xylobiose which serves as a substrate for β-xylosidase. On further incubation maximum hydrolysis was achieved and xylose was released as the major end product. The release of xylose as the main hydrolysis product suggests the unique mechanism of xylanase action of mutant *P. oxalicum* SAU_E-3.510, possessing both endo and exo degrading activities. These observations suggest that xylanase from the mutant strain could be used for the production of monomeric xylose from congress grass which grows wildly and is abundantly available. The release of xylose and xylobiose as the main hydrolysis products was also reported using xylanases from other microorganisms during degradation of oat spelt xylan (Dhillon et al., 2000), birch wood xylan (Bataillon et al., 2000; Zeng et al., 2007). Xylotriose was reported to be the major hydrolysis product of birch wood xylan hydrolysis by the recombinant xylanase of *Aspergillus niger*, which suggest its endoxylanase nature (Liu et al., 2006; Liu and Liu, 2008).

5.8 BIOBLEACHING OF MIXED WOOD KRAFT PULP BY MIXED ENZYME PREPARATION

To date, biobleaching of pulp was approached mainly by using xylanolytic (Gupta et al., 2000; Sandrim et al., 2005; Li et al., 2005; Ninawe et al., 2006; Battan et al., 2007) or ligninolytic enzymes (Balakshin et al., 2001; Sigoillot et al., 2005; Ibarra et al., 2006) alone. Biobleaching and bioprocessing of pulp using a mixture of xylanase and laccase further broadens the horizon of enzymes in the pulp and paper industry. The mixture of xylanase-laccase which was developed by co-culturing of fungi in solid-state fermentation was evaluated

for its biobleaching efficacy of mixed wood kraft pulp. An improved level of delignification was achieved by using the xylanase laccase mixture as it resulted in a 5% greater decrease in kappa number as compared to xylanase alone. Similarly, brightness of the sheets which had been treated with the mixed enzyme preparation was also greater (38.3 ISO) as compared to the brightness achieved by using the xylanase alone (37.0 ISO). This possibly could be due to the simultaneous separation and degradation of lignin into smaller phenolic compounds due to the synergistic action of xylanase and laccase. The size of the laccase enzyme is considered to be too large for penetration into the fibre wall (Goodell et al., 1998), but action of xylanase may increase the porosity of the cell wall and lignin is exposed on the surface for the action of laccase. Xylanase attacks the xylan layer and the reprecipitated xylan on the fibre surface and breaks the lignin carbohydrate complex. The reaction products that are released by the xylanase (xylooligomers and xylose) may quench the radicals created in the lignin structure by lignin modifying enzymes (Niku-paavola et al., 1994). Therefore, a xylanase-laccase mixture appears to be more effective for pulp delignification. The synergistic action of xylanase and laccase mediator system in combination (one after other) on the bleaching of soda and waste pulp was evaluated by Kapoor et al. (2007). Both enzymes were produced separately by using different microbial cultures (*Bacillus pumilus* strain MK001 and *Cyathus stercoreus* for xylanase and laccase respectively).

After treatment of pulp with mixed enzyme preparation under derived conditions, a 21% reduction of kappa number, an 8% increment in brightness and a 5% increase in pulp viscosity was obtained. The application of xylanases for the improvement of pulp bleaching was reported by several workers by using xylanase from *Aspergillus caespitosus* (Sandrim et al., 2005), *Thermomyces lanuginosus* (Li et al., 2005), *Streptomyces cyaneus*-SN32 (Ninawe and Kuhad, et al., 2006), *Bacillus pumilus* ASH (Battan et al., 2007) and *Arthrobacter* sp. MTCC 5214 (Khandeparkar and Bhosle, 2007). They reported an 8-20% reduction in pulp kappa number, a 3.5-9.6% improvement in brightness and a 10-29% lesser chemical load. The pulp viscosity

remained unchanged or slightly increased, which indicated the selective nature of enzyme that left cellulose fibres intact. The synergistic effect of xylanase and laccase for the bleaching of soda pulp was studied by the sequential addition of xylanase and laccase. A 17.6 % reduction in kappa number and a 20.4 % gain in the brightness were observed when the soda pulp was pretreated with either xylanase (40 IU g^{-1}) or laccase (200 IU g^{-1}) in combination (one after the other). The findings reported here on the optimisation of enzyme dose and treatment period revealed the correlation between the release of chromophores, hydrophobic compounds and the reduction in kappa number, which suggested the dissociation of the lignin carbohydrate complex from the fibres. Release of chromophores as well as hydrophobic compounds was greater under optimised bleaching conditions (8 IU g^{-1} of pulp, 3h, and 55°C). Release of reducing sugars continued at a reduced rate due to xylanase mediated hydrolysis of soluble xylooligosaccharides which were released by the initial depolymerization of the xylan. Thus, the release of hydrophobic and phenolic compounds could be a better indicator of the kinetics of enzyme attack on the pulp (Garg et al., 1998; Beg et al., 2000; Khandeparkar and Bhosle, 2007).

After bleaching of pulp with different doses of mixed enzyme preparation, effluents thus collected were scanned from λ 200 nm to λ 400 nm to evaluate the colour due to presence of lignin. The peak at 280 nm in the UV spectrum indicated the presence of lignin in the released colour compounds and the colour removal increased with the increase in enzyme dose (Khandeparkar and Bhosle, 2007).

As a result of enzymatic action, there is loosening and swelling of pulp fibers which facilitates the penetration of bleaching chemicals in subsequent bleaching stages, thereby, enabling the decreased requirement of bleaching chemicals. Thus, requirement of chemicals for bleaching were decreased by 22-26% for enzymatically treated pulp as compared to untreated pulp. In addition to this, enzymatic bleaching with a xylanase-laccase mixture reduced the discharge of adsorbable organic halides (17-30%) and the chemical oxygen demand (7-21%),

respectively. These findings suggested the superiority of the xylanase-laccase mixture for bleaching of mixed wood pulp in an economic and environmentally friendly manner.

Improvement in the mechanical strength properties of pulp during elemental chlorine free bleaching (XODED) indicated the selective removal of xylan and lignin, leaving cellulose fibres intact which are responsible for maintaining the desirable strength of paper. The superior physical strength properties after treatment of pulp with xylanase have been observed by other groups (Battan et al., 2007; Kapoor et al., 2007). Contrary to this, a loss in mechanical strength properties was observed when pulp was bleached using the conventional sequence (XCEPHHP). Excessive removal of hemicellulosic components that contribute to inter-fibrillar bonding during paper sheet formation during the multistage bleaching sequence could lead to decreased strength properties. A similar loss in physical properties was also observed for fully bleached pulp of *Eucalyptus globulus* and wheat straw following their treatment with commercial xylanases (Li et al., 2005; Shatalov and Pereira, 2008).

5.8.1 Solubilized products after enzymatic treatment

The release of xylooligomers and monophenols due to degradation of xylan and lignin, respectively, during enzymatic bleaching was analysed by high performance liquid chromatography. The release of xylose as the major hydrolysis product suggested the synergistic mode of action of endoxylanase and β-xylosidase. Endoxylanase attacks on the internal xylosidic bonds and releases xylooligosaccharides. These xylooligosaccharides, on prolonged incubation are further degraded into xylobiose which serves as a substrate for β-xylosidase. In untreated pulp the xylan remained intact. Therefore, there was no release of degradation products. Detection of increasing levels of phenolic compounds in a xylanase-laccase treated sample indicated the delignification efficacy of a mixed enzyme preparation. Niku-Paavola et al. (1994) also investigated the enzymatic delignification on pine kraft pulp and obtained similar results.

5.8.2 Fibre morphology

Pulp fibres before and following enzymatic treatment revealed distinct changes in the surface architecture. Morphological changes such as "cracks" and "peeling" of fibre surface were evident after enzymatic treatment, as has also been observed by other groups (Pham et al., 1995; Torres et al., 2000 and Roncero et al., 2000, 2005). An increase in pulp fibrillation, water retention and increased freeness in fibers are the major indicators of pronounced enzymatic action on pulp during bleaching.

5.8.3 Pulp crystallinity

Crystallinity of pulp is a significant criterion that enables the understanding of pulp delignification during biobleaching. As a result of enzymatic action (xylanase-laccase), hemicellulose and lignin components are removed, leaving high proportions of amorphous cellulose, thus resulting in a decrease in pulp crystallinity. A mixed enzyme preparation as indicated had a better bleaching ability as compared to xylanase alone by X-ray diffraction analysis. Decreased pulp crystallinity due to an improvement in fibre fragility was also observed following treatment of ramie fibres with xylanase from *Bacillus* sp. (Zheng et al., 2000). Ammonia pretreated soybean straw also showed a decline in crystallinity following enzymatic treatment due to the removal of lignin and hemicellulose (Xu et al., 2007).

5.8.4 Residual lignin

Due to enzymatic treatment there was a removal and degradation of lignin, hence accordingly, characteristic variations in FTIR spectra before and following treatment due to alterations in lignin's functional groups were observed. The appearance of a strong absorption peak at 1720 cm^{-1} in FTIR spectra of pulp samples treated with a xylanase-laccase mixture suggested lignin degradation, as the same peak was not detected in the spectra of untreated pulp samples. This peak was attributed to carboxylic acid and unconjugated carbonyl groups (C=O) due to the action of the enzyme on lignin's aromatic ring (Buta et al., 1989). The peak, therefore, specifically denoted lignin degradation due to enzymatic action. In untreated samples these

carbonyl groups remained associated with aromatic rings, hence resulting in no absorption. Oxidation of phenolic components and enrichment in carboxylic acid groups (absorpation peak at 1719 cm^{-1}) were also detected during laccase mediated bleaching of kraft pulp by evaluating the structure of residual lignin by FTIR analysis (Sealey and Ragauskas, 1998). FTIR analysis of residual lignin from the Eucalyptus pulp by laccase-HBT treatment showed typical lignin patterns having characteristic absorption band corresponding to carbonyl groups conjugated with the aromatic ring (Ibarra et al., 2006). El Mansouri and Salvado (2007) determined various functional groups in lignins of five different origins, i.e. kraft, sulfite, soda, organosolve and thanol process by using FTIR. The main functional groups reported in lignin are hydroxyl, methoxyl, carbonyl and the carboxylic groups.

5.9 INTERMITTENT ROTATING DRUM BIOREACTOR FOR SCALED-UP PRODUCTION OF MIXED ENZYME PREPARATION IN BATCH CULTIVATION

In contrast to submerged fermentation, scaling-up of a solid-state fermentation is cumbersome due to the difficulty in controlling the major process parameters such as heat transfer, aeration, mixing, temperature and moisture content. A large amount of metabolic heat is generated during SSF and is directly proportional to the metabolic activity in the system (Banks, 1984; Trilli, 1986). Transmission of generated heat in SSF is not as efficient as in a submerged process due to the poorer heat transfer ability of the solid-support used. The problem of heat generation is more pronounced in tray bioreactors, whereas in drum bioreactors the rotation facilitates heat transfer and a more uniform distribution of nutrients and fungal inoculum (Lonsane et al., 1992).

A rotating drum bioreactor for simultaneous biosynthesis of xylanase and laccase through co-cultivation of mutant *P. oxalicum* SAU_E-3.510 and *P. ostreatus* MTCC 1804 was designed. Intermittent rotation (10 rpm, at an interval of 3 h) of the fermentation vessel facilitated the proper mixing of substrate and fungal inoculum during the production period,

which led to high levels of xylanase, β-xylosidase and laccase production. Growth of the fungi in the drum bioreactor was more pronounced and uniform as compared to that in tray fermenters (Pandey et al., 2001). The yield of xylanase and laccase obtained was significantly higher than those observed using other SSF bioreactors. Solid-state fermentation in a laboratory scale horizontal bioreactor using the derived medium produced 320 U g^{-1} of xylanase activity by *Sporotrichum thermophile* (Topakas et al., 2003). Similarly, high levels of xylanase was observed from *Fusarium oxysporum* (1840 U g^{-1}) and *Thermoascus aurantiacus* (4490 U g^{-1}) during solid-state fermentation at bioreactor scale (Panagiotou et al., 2003; Kalogeris et al., 2003). The enzyme yield obtained in the designed bioreactor was marginally lower (12 %) than those obtained in the laboratory scale fermentation process. These results are in agreement with those reported for the scaling up the processes for xylanase production from *Thermoascus aurantiacus* in solid state culture (Kalogeris et al., 1998, 2003; Panagiotou et al., 2003). Although mixing promotes proper heat transfer, aeration, distribution of inoculum and nutrients during cultivation, it may to some extent damage mycelia due to shear forces, causing a decrease in the product yield. The system may also have some heat build-up due to growth and metabolic activities of the fungal mycelia. Therefore, the designed system had a cooling tray at the base of fermenter vessel to minimize the effect of heat build-up. These findings therefore denote that solid-state process can be effectively scaled up and the intermittent rotating drum bioreactor system can be successfully utilised for scaling up the production level. Minor modifications in this system may further improve the levels of production.

CHAPTER VI

SUMMARY AND CONCLUSIONS

Chapter 6

SUMMARY AND CONCLUSIONS

An attempt was made towards an effective, ecofriendly bleaching technology by using an industrially compatible enzyme preparation to minimise the pollution load generated by paper industries. With the aim of developing the production of an alkalitolerant, cellulase free xylanase, waste from a soda ash industry (pH- 11.0) was explored for its microbial biodiversity. A potent xylanase producing fungal strain (SA-8) having a notable xylanase activity at an alkaline pH (242.05 ± 3.91 IU ml^{-1}) was isolated and identified as *Penicillium oxalicum* SA-8 (ITCC 6204) by Indian Agricultural Research Institute, New Delhi, India. Due to its alkalitolerant nature and negligible cellulase activity (0.18 ± 0.03 IU ml^{-1}), the strain was selected for further studies and subjected to mutagenesis in an attempt to enhance the level of xylanase secretion.

A mutant strain *P. oxalicum* SAU_E-3.510 was developed by mutagenesis using successive treatments of UV irradiation and ethidium bromide. A higher level of xylanase (453.02 ± 5.70 IU ml-1) was found with from *P. oxalicum* SAU_E-3.510. Mutant and wild type strains of *P. oxalicum* could be differentiated on the basis of growth patterns as well as morphological features of hyphae and spores. The hyphae of mutant *P. oxalicum* SAU_E-3.510 were coiled, rounded with a rough peeled surface, whereas those of wild type *P. oxalicum* SA-8 were flattened, shiny, smooth surfaced and straight. Similarly, spores of mutant were large, smooth and crescent shaped, whereas the wild type spores were rounded with rough surface.

A specific pattern of xylanase activity was observed with the mutant as well as with the wild type strains. The maximum xylanase activity was observed at pH 9.0 from both mutant (487.44 ± 3.57 IU ml^{-1}) and wild type (252:50 ± 6.27 IU ml^{-1}) strains respectively. Decrease in xylanase activity was observed on further increase in pH. Interestingly, a high xylanase activity

was also observed at pH 5.0 from mutant as well as the wild type strain i.e. 444.89 ± 7.60 IU ml^{-1} and 197.81 ± 6.94 IU ml^{-1} respectively.

Production of xylanase was evaluated under shake flask condition by using wildly growing and abundantly available plant biomass namely *Parthenium* sp. (congress grass) and *Eichhornia crassipes* (water hyacinth). A markedly higher level of xylanase activity (475.23 ± 6.05 IU ml^{-1}) was observed when treated congress grass was used as the carbon source in the production medium. The activity thus observed was comparable with that obtained with commercial oat spelt xylan (488.56 ± 6.53 IU ml^{-1}) in the production medium. Thus the use of congress grass biomass will not only help in addressing the challenges relating to environmental hazards due to its widespread and wild occurrence, but would substantially contribute towards decreasing the cost of xylanase production. The mutant *P. oxalicum* SAU_E-3.510 produced 714.13 ± 15.40 IU ml^{-1} of xylanase at the 6^{th} day of incubation under derived conditions along with significant levels of β-xylosidase activity.

A mixed culture fermentation, consisting of inoculums from two or more organisms, is a promising system for the biosynthesis of industrially significant value added products like antibiotics, enzymes, several types of fermented food, etc. During co-cultivation, cultures interact in a complex way and the interaction may be synergistic or antagonistic which may lead to changes in enzymatic activities. As compared to monocultures, co-cultivation of fungi may lead to better substrate utilisation, increased productivity and increased resistance to contamination. In addition to this, co-culturing of compatible microbial partners may result in an enhanced enzyme productivity and, therefore, may prove to be economically viable. The efficacy of biobleaching of pulp during paper manufacturing is based upon the synergistic action of xylanolytic and ligninolytic enzyme systems. Bioprocessing of pulp utilising a mixture of xylanase and laccase may prove to be superior for biobleaching in pulp and paper industry. Hydrolysis of xylan due to action of xylanases leads to exposure of the lignin layer. The exposed lignin layer is effectively attacked by laccase, resulting in improved delignification and therefore

requires less bleaching chemicals in the subsequent bleaching stages. Therefore, bleaching of pulp with xylanase-laccase mixture may be economically beneficial to the paper industry in an ecofriendly manner.

A novel strategy of fungal co-cultivation was evaluated for developing a xylanase-laccase mixture. Analysis of growth compatibility revealed the suitability of mutant *P. oxalicum* SAU_E-3.510 to be grown with *P. ostreatus* MTCC 1804, which is known as hyper laccase producing strain. *P. oxalicum* SAU_E-3.510 was found to be a potent xylanase producer while laccase activity was not detected. Scanning electron microscopy indicated the closely associated contact regions for both the fungi and any type of branching, deformities or injuries were not observed. Co-cultivation of mutant *P. oxalicum* SAU_E-3.510 and *P. ostreatus* MTCC 1804 yielded an overall increase of 58% and 33% in the xylanase and laccase activities, respectively.

Enzyme production during co-cultivation was also evaluated by employing different fermentation systems. The highest level of xylanase (1301.51 ± 12.73 IU ml^{-1}) and laccase activity (43.70 ± 3.72 IU ml^{-1}) were obtained when the fungi were co-cultured under solid-state fermentation as compared to production using surface and submerged fermentation. The advantages of mixed cultures are more evident in solid-state fermentation due to the proper colonisation of the substrate by the fungus. The SSF system led to maximum levels of enzyme production and an attempt to further improve the level and economy of production was made by evaluating low cost or freely available agro-horticultural residual materials as solid support for fermentation. Among various lignocellulosic residues that were evaluated for enzyme production under SSF, a mixture of bagasse and black gram husk in a ratio of 3:1 was the most promising for xylanase (7574.24 ± 118.99 IU g^{-1}) and laccase (381.33 ± 10.01 IU g^{-1}) production.

The xylanase of mutant *P. oxalicum* SAU_E-3.510 was quite stable at a high pH and temperature. The enzyme maintained 93% of its residual activity at pH 9.0 for 24 h. The enzyme was also thermostable. The half life of the enzyme was found to be two hours at 80°C.

Polyacrylamide gel electrophoresis and zymogram analysis revealed the presence of two isoforms (121 kD and 77 kD respectively) from mutant *P. oxalicum* SAU_E-3.510, whereas co-cultivation of *P. oxalicum* SAU_E-3.510 with *P. ostreatus* MTCC 1804 yielded an additional xylanase (40 kD) corresponding to that of *P. ostreatus* MTCC 1804 along with the laccase of 29 kD. The amino acid sequence of the xylanase from *P. oxalicum* SAU_E-3.510 shared significant identity with xylanase sequences from *Bacillus stearothermophilus*, *Geobacillus stearothermophilus*, *Paenibacillus* sp. and *Clostridium* sp. The amino acid sequences of xylanases are diverse in composition and the degree of identity could be found in various groups of organisms. Analysis of the hydrolysis product of congress grass xylan with xylanase by high performance liquid chromatography indicated the synergistic action of endoxylanase and β-xylosidase, as xylose was the major product obtained following hydrolysis.

Biobleaching of mixed wood pulp was attempted using a xylanase-laccase preparation as well as xylanase alone. An improved level of bleaching was obtained with the mixed enzyme preparation. An overall 21% reduction in kappa number, an 8% increase in brightness and a 5% increase in viscosity was obtained following 3 h of treatment with the xylanase-laccase mixture (8 IU g^{-1} of pulp) in the presence of mediator (1.5% HBT) at a 10% pulp consistency. Thus an additional 5% decrease in pulp kappa number and a higher level of brightness (3.6%) was obtained with the mixed enzyme preparation as compared to xylanase alone. Following enzymatic treatment, further bleaching of pulp was achieved by performing conventional and elemental chlorine free bleaching sequences. Enzymatic treatment resulted in a 22-26% decrease in the requirement for bleaching chemicals as compared to untreated pulp samples. The paper generated from treated pulp showed superior mechanical and optical properties as compared to paper made from untreated pulp. A greater bleaching ability of the xylanase-laccase mixture was also observed due to the increased release of xylose and monophenols in the effluent as compared to the treatment using xylanase alone or without any treatment. During scanning electron microscopy, cracks and peelings were observed in

enzymatically treated pulp which had enabled swift action of bleaching chemicals, thus leading to enhanced delignification and resulting in a lower chemical consumption. As a result of enzyme prebleaching, crystallinity of the pulp decreased due to the removal and degradation of hemicellulose and lignin, thereby resulting in amorphous cellulose. Degradation of lignin was further evident from FTIR analysis of the treated mixed wood pulp. An extra peak appeared at the 1720 cm^{-1} wave number, which indicated the oxidative action of laccase on lignin. These findings revealed the efficacy of a mixed enzyme preparation for modification of lignin during biobleaching involving demethylation and oxidation reactions.

An attempt to scale up the production by co-cultivation of *P. oxalicum* SAU_E-3.510 and white rot fungus *P. ostreatus* MTCC 1804 was made by designing an intermittent rotating drum bioreactor and using a mixture of bagasse and black gram husk as solid support. Cultivation was performed in batch mode and levels of enzymes were achieved at almost a similar yield as obtained in small scale laboratory experiments. The maximum level of xylanase (7832.46 ± 353.71 IU g^{-1}) and laccase (280.76 ± 19.05 IU g^{-1}) was attained on the 8^{th} day of incubation. The observed levels of xylanase and laccase production were significantly higher than those obtained earlier for other SSF bioreactors. Mixing by rotating the reactor vessel promoted proper heat transfer, aeration, distribution of inoculum and nutrients during cultivation, but may lead into minor damage to fungal mycelia which may cause marginal decrease in overall product production level.

Therefore, pulp bleaching using a xylanase-laccase mixture appears to be effective, eco-friendly and a safer process. The following are the major conclusions derived from the present study,

a. Among the eighteen fungal isolates, *P. oxalicum* SA-8, isolated from the waste of a soda ash industry, was found to be a potential strain producing higher levels of xylanase, β-xylosidase with negligible amounts of cellulase at an alkaline pH of 9.0.
b. Strain SA-8 was subjected to mutagenesis by successive treatments employing UV-

irradiation and ethidium bromide. Mutant *P. oxalicum* SAU_E-3.510 thus obtained exhibited an 87% higher level of xylanase production.

c. *Parthenium* sp. when added in production media as carbon source led to comparable levels of xylanase as obtained with oat spelt xylan. Thus this low cost and abundantly available plant biomass could be a potential substitute for commercial oat spelt xylan.

d. Co-cultivation of mutant *P. oxalicum* SAU_E-3.510 with *P. ostreatus* MTCC 1804 resulted in an improved biosynthesis of xylanase and laccase under solid-state fermentation and a mixture of bagasse and black gram husk in a ratio of 3:1 was found to be most suitable for production of a xylanase-laccase mixture through co-cultivation.

e. Two xylanase isoforms (121 kD and 77 kD) were produced by *P. oxalicum* SAU_E-3.510, while one extra xylanase (40 kD) was detected during co-culturing using *P. ostreatus* MTCC 1804 along with laccase production (29 kD). The amino acid sequence of the xylanase of *P. oxalicum* SAU_E-3.510 had significant identity with the xylanase from *B. stearothermophilus, G. stearothermophilus, Paenibacillus* sp. and *Clostridium* sp.

f. Pre-treatment of mixed wood pulp with xylanase-laccase mixture resulted in a significant increase in ISO brightness and improved paper quality. Enzymatic pre-bleaching reduced the chlorine requirement by 26 %, thus the process can be considered as effective and ecofriendly process.

g. The solid-state process was scaled up by designing an intermittent rotating drum bioreactor using the earlier derived parameters. A high level of xylanase (7832.46 ± 353.71 IU g^{-1}) and laccase (280.76 ± 19.05 IU g^{-1}) was obtained under these conditions.

REFERENCES

REFERENCES

1. Adsul MG, Bastawde KB, Varma AJ and Gokhale DV (2007). Strain improvement of *Penicillium janthinellum* NCIM1171 for increased cellulase production. *Bioresource Technol.* **98**, 1467-1473.
2. Ahamed A and Vermette P (2008). Enhanced enzyme production from mixed cultures of *Trichoderma reesei* RUT-C30 and *Aspergillus niger* LMA grown as fed batch in a stirred tank bioreactor. *Biochem. Eng. J.* **42**, 41-46.
3. Alam MZ, Razi F, Aziz SA and Molla AH (2003). Optimisation of compatible mixed cultures for liquid state bioconversion of municipal waste water sludge. *Water Air Soil Pollut.* **149**, 113-126.
4. Allison RW, Mc Grouther KG and Ellis MJ (1997). Optimizing the effects of interstage peroxymonosulphate treatment on two-stage oxygen delignification. *J. Pulp Paper Sci.* **23**, 433-438.
5. Andrade SV, Teixeira ML, Polizeli M, Terenzi HF and Jorge JA (2004). Effect of carbon source on the biochemical properties of β-xylosidases produced by *Aspergillus versicolor*. *Process Biochem.* **39**, 1931-1938.
6. Anthony T, Chandra RK, Rajendran A and Gunasekaran P (2003). High molecular weight cellulose free xylanase from alkali tolerant *Aspergillus fumigatus* AR1. *Enzyme Microb. Technol.* **32**, 647-654.
7. Araki R, Ali MK, Sakka M, Kimura T, Sakka K and Ohmiya K (2004). Essential role of the family-22 carbohydrate-binding modules for β-1, 3-1,4-glucanase activity of *Clostridium stercorarium* Xyn10B. *FEBS Lett.* **561**, 155-158.
8. Arantes V and Milagres AMF (2006). Degradation of cellulosic and hemicellulosic substrates using a chelator-mediated fenton reaction. *J. Chem. Technol. Biotechnol.* **81**, 413-419.
9. Aristidou A and Penttila M (2000). Metabolic engineering applications to renewable resource utilisation. *Curr. Opin. Biotechnol.* **11**, 187-98.
10. Asha Poorna C and Prema P (2007). Production of cellulase-free endoxylanase from novel alkalophilic thermotolerent *Bacillus pumilus* by solid-state fermentation and its application in wastepaper recycling. *Bioresource Technol.* **98**, 485–490.
11. Ates S, Cortenlioglu E, Bayraktar E and Mehmetoglu U (2007). Production of l-DOPA using Cu-alginate gel immobilized tyrosinase in a batch and packed bed reactor. *Enzyme Microb. Technol.* **40**, 683–687.

12. Atik C, Imamoglu S and Bermek H (2006). Impact of xylanase pre-treatment on peroxide bleaching stage of biokraft pulp. *Int. Biodeter. Biodegr.* **58**, 22-26.

13. Aurilia V, Martin JC, Scott KP, Mercer DK, Johnston MEA and Flint HJ (2000). Organisation and variable incidence of genes concerned with the utilisation of xylans in the rumen cellulolytic bacterium R*uminococcus flavefaciens*. Anaerobe **6**, 333-340.

14. Bachmann SL and Mc Carthy AJ (1991). Purification and cooperative activity of enzymes constituting the xylan-degrading system of *Thermomonospora fusca*. *Appl. Environ. Microbiol.* **57**, 2121-2130.

15. Badhan AK, Chadha BS, Sonia KG, Saini HS and Bhat MK (2004). Functionally diverse multiple xylanases of thermophilic fungus *Myceliophthora* sp. IMI 387099. *Enzyme Microb. Technol.* **35**, 460-466.

16. Baillet E, Downey G and Tuohy M (2003). Improvement of texture and volume in white bread rolls by incorporation of microbial hemicellulase preparations, In: *Recent Advances in Enzymes in Grain Processing*. Courtin CM, Veraverbeke WS, Delcour JA (Eds), Laboratory of Food Chemistry, Katholieke Universiteit Leuven, Leuven, 255–259.

17. Bajpai P (1997). Microbial xylanolytic enzyme system: properties and applications. *Adv. Appl. Microbiol.* **43**, 141-194.

18. Bajpai P and Bajpai PK (1992). Biobleaching of kraft pulp. *Process Biochem.* **27**, 319-325.

19. Bakalova NG, Petrova SD, Atev AP, Bhat MK and Koleve DN (2002). Biochemical and catalytic properties of endo-1,4-β-xylanases from *Thermomyces lanuginosus* (wild and mutant strains). *Biotechnol. Lett.* **24**, 1167-1172.

20. Balakshin M, Capanemaa E, Chen CL, Gratzl J, Kirkman A and Gracz H (2001). Biobleaching of pulp with dioxygen in the laccase-mediator system-reaction mechanisms for degradation of residual lignin. *J. Mol. Catal. B- Enzym.* **13**, 1–16.

21. Banerjee R, Mukherjee G and Patra KC (2005). Microbial transformation of tannin-rich substrate to gallic acid through co-culture method. *Bioresource Technol.* **96**, 949-953.

22. Banks GT (1984). Scale up of fermentation processes. In: *Topics in Enzyme and Fermentation Biotechnology*. Wiseman A (Eds), Ellis Horwood, Chichester, UK, PP. 170-266.

23. Barreca AM, Fabbrini M, Galli C, Gentili P and Ljunggren S (2003). Laccase mediated oxidation of lignin model for improved delignification procedures. *J. Mol.*

Catal. B- Enzym. **26**, 105-110.

24. Basaran P and Ozcan M (2008). Characterisation of β -xylosidase enzyme from a *Pichia stipitis* mutant. *Bioresource Technol.* **99**, 38–43.

25. Bataillon M, Cardinali APN, Castillon N and Duchiron F (2000). Purification and characterisation of a moderately thermostable xylanase from *Bacillus* sp. strain SPS-0. *Enzyme Microb. Technol.* **26**, 187-192.

26. Battan B, Sharma J, Dhiman SS and Kuhad RC (2007). Enhanced production of cellulase-free thermostable xylanase by *Bacillus pumilus* ASH and its potential application in paper industry. *Enzyme Microb. Technol.* **41**, 733–739.

27. Becker HG and Sinitsyn AP (1993). Mn-peroxidase from *Pleurotus ostreatus*: the action on the lignin. *Biotechnol. Lett.* **15**, 289– 94.

28. Beg QK, Bhushan B, Kapoor M and Hoondal GS (2000). Production and characterisation of thermostable xylanase and pectinase from a *Streptomyces* sp. QG-11-3. *J. Ind. Microbiol. Biotechnol.* **16**, 211-213.

29. Beg QK, Kapoor M, Mahajan L and Hoondal GS (2001). Microbiol xylanases and their industrial application: a review. *Appl. Microbiol. Biotechnol.* **56**, 326-338.

30. Belancic A, Scrapa J, Peirano A, Diaz R, Steiner J and Eyzaguirre J (1995). *Penicillium purpurogenum* produces several xylanases: purification and properties of two of the enzymes. *J. Biotechnol.* **41**, 71-79.

31. Bernalier A, Fonty G and Gouet P (1991). Cellulose degradation by two rumen anaerobic fungi in monoculture or in co-culture with rumen bacteria. *Animal Feed Sci. Technol.* **32**, 131-136.

32. Berrin JG, Williamson G, Puigserver A, Chaix JC, McLauchlan WR and Juge N (2000). High-level production of recombinant fungal endo-β -1,4-xylanase in the methylotrophic yeast *Pichia pastoris*. *Protein Expr. Purif.* **19**, 179–87.

33. Bhat MK (2000). Cellulases and related enzymes in biotechnology. *Biotechnol. Adv.* **18**, 355-383.

34. Biely P (1981). Substrate binding site of endo-1,4-β xylanase of the yeast *Cryptococcus albidus*. *Eur. J. Biochem.* **119**, 559-571.

35. Biely P (1985). Microbiol xylanolytic systems. *Trends Biotechnol.* **11**, 286-290.

36. Bocchini DA, Oliveira OMMF, Gomes E, Silva RD (2005). Use of sugarcane bagasse and grass hydrolysate as carbon sources for xylanase production by *Bacillus circulans* D1 in submerged fermentation. *Process Biochem.* **40**, 3653-3659.

37. Bourbonnais R and Paice MG (1990). Oxidation of non-phenolic substrates: an

expanded role for laccase in lignin biodegradation. *FEBS Lett.* **267**, 99-102.

38. Bourne Y and Henrissat B (2001). Glycoside hydrolases and glycosyltransferases: families and functional modules. *Curr. Opin. Struct. Biol.* **11**, 593-600.

39. Breccia JD, Sineriz F, Baigori MD, Castro GR and Hatti-Kaul R (1998). Purification and characterisation of a thermostable xylanase from *Bacillus amyloliquefaciens*. *Enzyme Microb. Technol.* **22**, 42–49.

40. Buta JG, Zardrazil F and Gallettti GC (1989). FTIR determination of lignin degradation in wheat straw by fungus *S. rugosoannulata* with different oxygen concentrations. *J. Agr. Food Chem.* **37**, 1382-1384.

41. Butt WA, Haq IU and Iqbal J (2002). Biosynthesis of xylanase by UV- treated mutant strain of *Aspergillus niger* GCBMX-45. *Biotechnology* **1**, 10-14.

42. Camarero S, Ibarra D, Martinez AT and Romero J (2007). Paper pulp delignification using laccase and natural mediators. *Enzyme Microb. Technol.* **40**, 1264-1271.

43. Carapito R, Carapito C, Jeltsch J and Phalip V (2009). Efficient hydrolysis of hemicellulose by a *Fusarium graminearum* xylanase blend produced at high levels in *Escherichia coli*. *Bioresource Technol.* **100**, 845-850.

44. Caspers MP, Lok F, Sinjorgo KM, van Zeijl MJ, Nielsen KA and V Cameron-Mills (2001). Synthesis, processing and export of cytoplasmic endo- β-1,4-xylanase from barley aleurone during germination. *Plant J.* **26**, 191–204.

45. Cazemier AE, Verdoes JC, Van Ooyen AJJ, and Op Den Camp HJM (1999). Molecular and biochemical characterisation of two xylanase-encoding genes from *Cellulomonas pachnodae*. *Appl. Environ. Microbiol.* **65**, 4099–4107.

46. Chadha BS, Jaswinder K, Rubinder K, Saini HS and Singh S (1999). Xylanase production by *Thermomyces lanuginosus* wild and mutant strains. *World J. Microbiol. Biotechnol.* **15**,195–198.

47. Chand P, Aruna A, Maqsood AM and Rao LV (2005). Novel mutation method for increased cellulase production. *J. Appl. Microbiol.* **98**, 318-323.

48. Chandra RK and Chandra TS (1996). Production of cellulose-free xylanase from an alkalitolerant *Aspergillus fischeri* Fxn 1. *Biotechnol. Lett.* **17**, 309-314.

49. Chantasingh D, Pootanakit K, Champreda V, Kanokratana P and Eurwilaichitr L (2006). Cloning, expression, and characterisation of a xylanase 10 from *Aspergillus terreus* (BCC129) in *Pichia pastoris*. *Protein Expr. Purif.* **46**, 143–149.

50. Chaudhary P and Deobagker D (1997). Purification and characterisation of xylanase from *Cellulomonas sp.* N. C. I. M. 2353. *Appl. Biochem. Biotechnol.* **25**, 127-33.

51. Chavez R, Bull P and Eyzaguirre J (2006). The xylanolytic system from the genus *Pinicillium*. *J. Biotechnol.* **123**, 413-433.

52. Chen S and Wilson DB (2007). Proteomic and transcriptomic analysis of extracellular proteins and mRNA levels in *Thermobifida fusca* grown on cellobiose and glucose. *J. Bacteriol.* **189**, 6260-6265.

53. Cheng HL, Wang PM, Chen YC, Yang SS and Chen YC (2008). Cloning, characterisation and phylogenetic relationships of *stx*I, a endoxylanase-encoding gene from *Streptomyces thermonitrificans* NTU-88. *Bioresource Technol.* **99**, 227–231.

54. Chi Y, Hatakka A and Maijala P (2007). Can co-culturing of two white-rot fungi increase lignin degradation and the production of lignin-degrading enzymes? *Int. Biodeter. Biodegr.* **59**, 32–39.

55. Chipeta ZA, du Preez JC and Christopher L (2008). Effect of cultivation pH and agitation rate on growth and xylanase production by *Aspergillus oryzae* in spent sulphite liquor. *J. Ind. Microbiol. Biotechnol.* **35**, 587-594.

56. Christakopoulos P, Nerinckx W, Kekos D, Macris B and Claeyssens M (1996). Purification and characterisation of two low molecular mass alkaline xylanases from *Fusarium oxysporum* F3. *J. Biotechnol.* **51**, 181-189.

57. Ciftci ON, Fadıloglu S and Gogus F (2009). Conversion of olive pomace oil to cocoa butter-like fat in a packed-bed enzyme reactor. *Bioresource Technol.* **100**, 324–329.

58. Collins T, Gerday, C and Feller G. (2005). Xylanases, xylanase families and extremophilic xylanases. *FEMS Microbiol. Rev.* **29**, 3-23.

59. Converti A, Perego P, Sordi A and Torre P. (2002). Effect of starting xylose concentration on the microaerobic metabolism of *Debaryomyces hansenii*: the use of carbon material balances. *Appl. Biochem. Biotechnol.* **101**, 15-29.

60. Coutinho PM and Henrissat B (1999). Carbohydrate- active enzyme server (CAZY) at URL: http:// afmb.cnrs-mrs.fr/~cazy/CAZY/.

61. Couto SR, Lopez E and Sanroman MA (2006). Utilisation of grape seeds for laccase production in solid-state fermentors. *J. Food Eng.* **74**, 263-267.

62. Crepin VF, Faulds CB and Connerton IF (2004). Functional classification of the microbial feruloyl esterase. *Appl. Microbiol. Biotechnol.* **63**, 647-652.

63. Crestini C, Saladino R, Tagliatesta P and Boschi T (1999). Biomimetic degradation of lignin and lignin model compounds by synthetic anionic and cationic water soluble managanese and iron porphyrins. *Bioorg. Med. Chem.* **7**, 1897-1905.

64. Damaso MCT, Almeida MS, Kurtenbach E, Martins OB, Pereira Jr, Andrade CMMA and Albano RM (2003). Optimised expression of a thermostable xylanase from *Thermomyces lanuginosus* in *Pichia pastoris*. *Appl. Microbiol. Biotechnol.* **69**, 6064-6072.

65. Damiano VB, Bocchini DA, Gomes E and Da Silva R (2003). Application of crude xylanase from *Bacillus licheniformis* 77-2 to the bleaching of eucalyptus kraft pulp. *World J. Microbiol. Biotechnol.* **19**, 139-144.

66. de Faria FP, Te'O VSJ, Berggquist PL, Azevedo MO and Nevalainen KMH (2002). Expression and processing of a major xylanase (xyn 2) from the thermophlic fungus *Humicola grisea* var. thermoidea in *Trichoderma reesei*. *Lett. Appl. Microbiol.* **34**, 119-123.

67. de Vries RP, Kester HCM, Poulsen CH, Bene JAE, and Vissar J (2000). Synergy between enzymes from *Aspergillus* involved in the degradation of plant cell wall polysaccharide. *Carbohydr. Res.* **327**, 401-410.

68. de Wet BJM, van Zyl WH and Prior BA (2006). Characterisation of the *Aureobasidium pullulans* α- glucuronidase expressed in *Saccharomyces cerevisiae*. *Enzyme Microb. Technol.* **38**, 649–656.

69. Dekker RFH and Richards GN (1976). Hemicellulases: their occurrence, purification, properties and mode of action. *Adv. Carbohydr. Chem. Biochem.* **32**, 277-352.

70. Deschamps F and Huet MC (1985). Xylanase production in solid state fermentation: a study of its properties. *Appl. Microbiol. Biotechnol.* **22**, 177-180.

71. de-Souza Cruz PB, Freer J, Siika-aho M and Ferraz A (2004). Extraction and determination of enzyme produced by *Cerriporiopsis subvermispora* during biopulping of *Pinus taeda* wood chips. *Enzyme Microb. Technol.* **34**, 228-234.

72. Dhawan S, Lal R and Kuhad RC (2003). Ethidium bromide stimulated hyper laccase production from bird's nest fungus *Cyathus butleri*. *Lett. Appl. Microbiol.* **36**, 64-67.

73. Dhillon A, Gupta JK and Khanna S (2000). Enhanced production, purification and characterisation of a novel cellulose-poor thermostable, alkalitolerant xylanase from *Bacillus circulans* AB 16. *Process Biochem.* **35**, 849-856.

74. Dhiman SS, Sharma J and Battan B (2008). Pretreatment processing of fabrics by alkalothermophilic xylanase from *Bacillus stearothermophilus* SDX. *Enzyme Microb. Technol.* **43**, 262–269.

75. Diaz M, Rodriguez S, Fernández-Abalos JM, Rivas JDL, Ruiz-Arribas A, Shnyrov and Santamaría RI (2004). Single mutations of residues outside the active center of the xylanase from *Streptomyces halstedii* JM8 affect its activity. *FEMS Microbiol. Lett.* **240**, 237-243.

76. Dominguez A, Rivela I, Couto SR and Sanroman MA (2001). Design of a new rotating drum bioreactor for ligninolytic enzyme production by *Phanerochaete chrysosporium* grown on an inert support. *Process Biochem.* **37**, 549-554.

77. Duenas R, Tengerdy RP and Gutierrez-Correa M (1995). Cellulase producton by mixed fungi in solid-substrate fermentation of bagasse. *World J. Microbiol. Biotechnol.* **11**, 333-337.

78. Durand A (2003). Bioreactor designs for solid state fermentation. *Biochem. Eng. J.* **13**, 113-125.

79. Dutta T, Sengupta R, Sahoo R, Sinha SR, Bhattacharjee A and Ghosh S (2007). A novel cellulase free alkaliphilic xylanase from alkali tolerant *Penicillium citrinum*: production, purification and characterisation. *Lett. Appl. Microbiol.* **44**, 206-211.

80. El Mansouri NE and Salvado J (2007) Analytical methods for determining functional groups in various technical lignins. *Ind. Crop Prod.* **26**, 116-124.

81. Ezzi MI and Lynch JM (2005). Biodegradation of cyanide by *Trichoderma* sp. and *Fusarium* sp. *Enzyme Microb. Technol.* **36**, 849–854.

82. Faix O (1992). Fourier transform infrared spectroscopy. In: *Methods in Lignin Chemistry*. Lin SY and Dence CW (Eds), Springer, Berlin, Heidelberg, pp 83-109.

83. Fang HY, Chang SM, Hsieh MC and Fang TJ (2007). Production, optimisation growth conditions and properties of the xylanase from *Aspergillus carneus* M34. *J. Mol. Catal. B-Enzym.* **49**, 36–42.

84. Faulet BM, Niamke S, Gonnety JT and Kouame LP (2006). Purification and biochemical properties of a new thermostable xylanase from symbiotic fungus *Termitomyces* sp. *Afr. J. Biotechnol.* **5**, 273-282.

85. Faury D, Saidane S, Li H and Morosoli R (2004). Secretion of active xylanase C from *Streptomyces lividans* is exclusively mediated by the Tat protein export system. *Biochim. Biophys. Acta.* **1699**, 155– 162.

86. Ferreira G, Boer CG and Peralta RM (1999). Production of xylanolytic enzymes by *Aspergillus tamarii* in solid state fermentation. *FEMS Microbiol. Lett.* **173**, 335-339.

87. Ferreira-Gregorio AP, Da Silva IR, Sedarati MR and Hedger JN (2006). Changes in production of lignin degrading enzymes during interactions between mycelia of the

tropical decomposer basidiomycetes *Marasmiellus troyanus* and *Marasmius pallescens*. *Mycol. Res.* **110**, 161-168.

88. Filho EXF, Puls J and Coughlan MP (1996). Purification and characterisation of two arabinofuranosidases from solid-state cultures of the fungus *Penicillium capsulatum*. *Appl. Environ. Microbiol.* **62**, 160-173.

89. Gabriel BL (1982). In: *Biological Scanning Electron Microscopy*. Von Nostrand Reinhold Company, New York, Toronto, London, Melbourne, 186.

90. Garcia-Kirchner O, Muoz-Aguilar M, Perez-Villalva R and Huitron-Vargas C (2002). Mixed submerged fermentation with two filamentous fungi for cellulolytic and xylanolytic enzyme production. *Appl. Biochem. Biotechnol.* **98**, 1105-1114.

91. Garg AP, Roberts JC and Mc Carthy AJ (1998). Bleach boosting effect of cellulase free xylanase of *Streptomyces thermoviolaceus* and its comparison with two commercial enzyme prepartions on birchwood kraft pulp. *Enzyme Microb. Technol.* **22**, 594-598.

92. Gautam P, Sabu A, Pandey A, Szakacs G and Soccol CR (2002). Microbial production of extracellular phytase using polystyrene as inert solid support. *Bioresource Technol.* **83**, 229-233.

93. Gawande PV and Kamat MY (1999). Poduction of *Aspergillus* xylanase by lignocellulosic waste fermentation and its application. *J. Appl. Microbiol.* **87**, 511-519.

94. Geng X and Li K (2002). Degradation of nonphenolic lignin by the white-rot fungus *Pycnoporus cinnabarinus*. *Appl. Microbiol. Biotechnol.* **60**, 342-346.

95. Gervais P and Molin P (2003). The role of water in solid-state fermentation. *Biochem. Eng. J.* **13**, 85-101.

96. Gessesse A and Mamo G (1998) Purification and characterisation of an alkaline xylanase from alkaliphilic *Micrococcus* sp. AR-135, *J. Ind. Microbiol. Biotechnol.* **20**, 210-214.

97. Gessesse A and Mamo G (1999). High-level xylanase production by an alkalophilic *Bacillus* sp. by using solid-state fermentation. *Enzyme Microb. Technol.* **25**, 68-72.

98. Gilkes NR, Henrissat B, Kilburn DG, Miller Jr RC and Warren RAJ (1991). Domains in Microbiol β-1, 4-glycanases: sequence conservation, function and enzyme families. *Microbiol. Rev.* **55**, 303-315.

99. Gnanamani A, Jayaprakashvel M, Arulmani M and Sadulla S (2006). Effect of inducers and culturing processes on laccase synthesis in *Phanerochaete*

chrysosporium NCIM 1197 and the constitutive expression of laccase isozymes. *Enzyme Microb. Technol.* **38**, 1017-1021.

100. Gomes DJ, Gomes J and Steiner W (1994). Factors influencing the induction of endoxylanase by *Thermoascus aurentiacus*. *J. Biotechnol.* **33**, 87-94.

101. Gomes I, Gomes J, Steiner W and Esterbauer H (1992). Production of cellulase and xylanase by a wild strain of *Trichoderma viride*. *Appl. Microbiol. Biotechnol.* **36**, 701-707.

102. Goodell B, Yamamoto K, Jellison J, Nakamura M, Fujii T, Takabe K and Hayashi N (1998). Laccase immunolabelling and microanalytical analysis of wood degradation by *Lentinus edodes*. *Holzforschung* **52**, 345-350.

103. Granstrom TB, Izumori K and Leisola M (2007). A rare sugar xylitol. Part II: biotechnological production and future applications of xylitol. *Appl. Microbiol. Biotechnol.* **74**, 273-276.

104. Gronqvist S, Buchert J, Rantanen K, Viikari L, Suurnakki A (2003). Activity of laccase on unbleached and bleached thermomechanical pulp. *Enzyme Microb. Technol.* **32**, 439-445.

105. Gupta S, Bhushan B, Hoondal GS (2000). Isolation, purification and characterisation of xylanase from *Staphylococcus* sp. SG-13 and its application in biobleaching of kraft pulp. *J. Appl. Microbiol.* **88**, 325-334.

106. Gupta S, Kuhad RC, Bhushan B and Hoondal GS (2001). Improved xylanase production from a haloalkalophilic *Staphylococcus* sp. SG-13 using inexpensive agricultural residues. *World J. Microbiol. Biotechnol.* **17**, 5-8.

107. Gutierrez-Correa M and Tengerdy RP (1998). Cellulolytic enzyme production by fungal mixed culture soild substrate fermentation. *Biotechnol. Lett.* **20**, 45-47.

108. Gutierrez-Correa M, Portal L, Moreno P and Tengerdy RP (1999). Mixed culture solid-substrate fermentation of *Trichoderma reesei* with *Aspergillus niger* on sugar cane bagasse. *Bioresource Technol.* **68**, 173-178.

109. Haider T and Husain Q (2009). Hydrolysis of milk/whey lactose by β-galactosidase: A comparative study of stirred batch process and packed bed reactor prepared with calcium alginate entrapped enzyme. *Chem. Eng. Process: Process Intens.* **48**, 576-580.

110. Hakulinen N, Turunen O, Jänis J, Leisola M and Rouvinen J (2003). Three dimensional structures of thermophilic β-1,4-xylanases from *Chaetomium thermophilum* and *Nonomuraea flexuosa*. Comparison of twelve xylanases in relation to their thermal stability. *Eur. J. Biochem.* **270**, 1399-1412.

111. Haltrich D and Steiner W (1994). Formation of xylanase by *Schizophyllum commune*: effect of medium components. *Enzyme Microb. Technol.* **16**, 229-235.

112. Hatakka A (1994). Lignin-modifying enzymes from selected white-rot fungi: production and role in lignin degradation. *FEMS Microbiol. Rev.* **13**, 125– 35.

113. Hatsch D, Phalip V, Petkovski E and Jeltsch JM (2006). *Fusarium graminearum* on plant cell wall: No fewer than 30 xylanase genes transcribed. *Biochem. Biophys. Res. Commun.* **345**, 959–966.

114. Hatvani N, Kredics L, Antal Z and Mecs I (2002). Changes in activity of extracellular enzymes in dual cultures of *Lentinula edodes* and mycoparasitic *Trichoderma* strains. *J. Appl. Microbiol.* **92**, 415-423.

115. Hazlewood GP and Gilbert HJ (1993). Molecular biology of hemicelluloses. In: *Hemicelluloses and Hemicellulases*. Coughlan MP and Hazlewood GP (Eds), London: Portland Press. 103-26.

116. Hazlewood GP and Gilbert HJ (1998). Structure and function analysis of *Pseudomonas* plant cell wall hydrolyses. *Prog. Nucleic Acid Res. Mol. Biol.* **61**, 211-241.

117. Heck JX, de Barros Soares LH, Hertz PF, Ayub MAZ (2006). Purification and properties of a xylanase produced by *Bacillus circulans* BL53 on solid-state cultivation. *Biochem. Eng. J.* **32**, 179–184.

118. Heneghan MN, McLoughlin L, Murray PG and Tuohy MG (2007). Cloning, characterisation and expression analysis of α-glucuronidase from the thermophilic fungus *Talaromyces emersonii* . *Enzyme Microb. Technol.* **41**, 677–682.

119. Henrissat B and Bairoch A (1993). New families in the classification of glycosyl hydrolases based on amino acid sequence similarities. *Biochem. J.* **293**, 781-788.

120. Henrissat B, Claeyssens M, Tomme P, Lemesle L and Mornon JP (1989). Cellulase families revealed by hydrophobic cluster analysis. *Gene* **81**, 83-95.

121. Holmgren M and Sellstedt A (2008). Identification of white rot and soft rot fungi increasing ethanol production from spent sulphite liquor in co-culture with *Saccharomyces cerevisiae*. *J. Appl. Microbiol.* **105**, 134-140.

122. Hoq MM, Hempel C and Deckwer WD (1994). Cellulase-free xylanase by *Thermomyces lanuginosus* RT9: Effect of agitation, aeration, and medium components on production. *J. Biotechnol.* **37**, 49-58.

123. Hrmova M, Biely P and Vrsanska M (1986). Specificity of cellulase and β-xylanase induction on *Trichoderma reesei* QM 9414. *Arch. Microbiol.* **144**, 307-311.

124. Hrmova M, Biely P and Vrsanska M (1989). Cellulose and xylan degrading enzymes of *Aspergillus terreus*. *Enzyme Microb. Technol.* **11**, 610-616.

125. Huang J, Wang G and Xiao L (2006). Cloning, sequencing and expression of the xylanase gene from a *Bacillus subtilis* strain B10 in *Escherichia coli*. *Bioresource Technol.* **97**, 802–808.

126. Huang W, Niu H, Li Z, He Y, Gong W and Gong G (2008). Optimisation of ellagic acid production from ellagitannins by co-culture and correlation between its yield and activities of relevant enzymes. *Bioresource Technol.* **99**, 769–775.

127. Ibarra D, Romero J, Martinez MJ, Martinez AT and Camarero S (2006). Exploring the enzymatic parameters for optimal delignification of eucalyptus pulp by laccase mediator. *Enzyme Microb. Technol.* **39**, 1319-1327.

128. Ikram-Ul H, Ali S, Qadeer MA and Iqbal J (2004). Citric acid production by selected mutants of *Aspergillus niger* from cane molasses. *Bioresource Technol.* **93**, 125-130.

129. Ioannes PD, Peirano A, Steiner J and Eyzaguirre J (2000). An α-L-arabinofuranosidase from *Penicillium purpurogenum*: production, purification and properties. *J. Biotechnol.* **76**, 253–258.

130. Irwin D, Jung ED and Wilson DB (1994). Characterisation and sequence of a *Thermomonospora fusca* xylanase. *Appl. Environ. Microbiol.* **60**, 763-770.

131. Jain A, Garg SK and Johri BN (1998). Properties of a thermostable xylanase produced by *Melanocarpus albomyces* IIS-68 in solid-state fermentation. *Bioresource Technol.* **64**, 225-228.

132. Jain RK (2005). About Cess Commitae of Development Council for Pulp, Paper and Allied Industries. Second workshop on Frontier Technologies addressed under Cess Project and Energy Norms for Pulp and Paper Industry, 1-5.

133. Jiang ZQ, Deng W, Li XT, Ai ZL, Li LT and Kusakabe I (2005). Characterisation of a novel, ultra-large xylanolytic complex (xylanosome) from *Streptomyces olivaceoviridis* E-86. *Enzyme Microb. Technol.* **36**, 923–929.

134. Jiang Z, Deng W, Yan Q, Zhai Q, Li L and Kusakabe I (2006). Subunit composition of a large xylanolytic complex (xylanosome) from *Streptomyces olivaceoviridis* E-86. *J. Biotechnol.* **126**, 304–312.

135. Jorge I, de la Rosa O, Navas-Cortes JA, Jimenez-Diaz RM and Tena M (2005). Extracellular xylanases from two pathogenic races of *Fusarium oxysporum* f.sp. ciceris: enzyme production in culture and purification and characterisation of a

major isoform as an alkaline endo-β-(1,4)-xylanase of low molecular weight. *Antonic van Leeuwenhoek* **88**, 49-59.

136. Kadi N and Crouzet J (2008). Transglycosylation reaction of endoxylanase from *Trichoderma longibrachiatum*. *Food Chem.* **106**, 466–474.

137. Kalogeris E, Christakopoulos P, Kekos D and Macris BJ (1998). Studies on the solid state production of thermostable endoxylanases from *Thermoascus aurantiacus*. Characterisation of two isozymes. *J. Biotechnol.* **60**, 155-163.

138. Kalogeris E, Iniotaki F, Topakas E, Christakopoulos P, Kekos D and Macris BJ (2003). Performance of an intermittent agitation rotating drum type bioreactor for solid-state fermentation of wheat straw. *Bioresource Technol.* **86**, 207–213.

139. Kang MK, Maeng PJ and Ha Rhee Y (1996). Purification and Characterisation of two xylanases from alkalophilic *Cephalosporium* sp. Strain RYM-202. *Appl. Environ. Microbiol.* **62**, 3480-3482.

140. Kapoor M, Kapoor RK and Kuhad RC (2007). Differential and synergistic effects of xylanase and laccase mediater system (LMS) in bleaching of soda and waste pulps. *J. Appl. Microbiol.* **103**, 305-317.

141. Kapoor M, Nair LM and Kuhad RC (2008). Cost-effective xylanase production from free and immobilized *Bacillus pumilus* strain MK001 and its application in saccharification of *Prosopis juliflora*. *Biochem. Eng. J.* **38**, 88–97.

142. Katapodis P, Nerinckx W, Claeyssens M and Christakopoulos P (2006). Purification and characterisation of a thermostable intracellular β-xylosidase from the thermophilic fungus *Sporotrichum thermophile*. *Process Biochem.* **41**, 2402–2409.

143. Kaya F, Heitmann JA and Joyce TW (2000). Influence of lignin and its degradation products on enzymatic hydrolysis of xylan. *J. Biotechnol.* **80**, 241-247.

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

145. Khandeparkar RDS and Bhosle NB (2006). Isolation, purification and characterisation of the xylanase produced by *Arthrobacter* sp. MTCC 5214 when grown in solid-state fermentation. *Enzyme Microb. Technol.* **39**, 732–742.

146. Khandke KM, Vithayathil PJ and Murthy SK (1989). Purification and characterisation of an α-D-glucuronidase from a thermophilic fungus *Thermoascus aurantiacus*. *Arch. Biochem. Biophys.* **274**, 511-517.

147. Kim JH, Irwin D and Wilson DB (2004). Purification and characterisation of *Thermobifida fusca* xylanase 10B. *Can. J. Microbiol.* **50**, 835-843.

148. Kimura T, Mizutani T, Sakka K and Ohmiya K (2003). Stable expression of a thermostable xylanase of *Clostridium thermocellum* in cultured tobacco cells. *J. Biosci. Bioeng.* **95**, 397-400.

149. Kluepfel D, Mehta S, Aumount F, Morosoli R and Shareck F (1990) Purification and characterisation of a new xylanase (xylanase B) produced by *Streptomyces liridans* 66. *Biochem. J.* **267**, 45-50.

150. Kobayashi Y, Okuda N, Matsumoto M, Inoue K, Wakita M and Hoshino S (1998). Constitutive expression of a heterologous *Eubacterium ruminantium* xylanase gene (xyn A) in *Butyrivibrio fibriosolvens*. *FEMS Microbiol. Lett.* **163**, 11-17.

151. Kohli U, Nigam P, Singh D and Chaudhary K (2001). Thermostable, alkalophilic and cellulase free xylanase production by *Thermoactinomyces thalophilus* subgroup C. *Enzyme Microb. Technol.* **28**, 606-610.

152. Kolenova K, Vrsanska M and Biely P (2005). Purification and characterisation of two minor endo-β-1,4-xylanases of *Schizophyllum commune*. *Enzyme Microb. Technol.* **36**, 903-910.

153. Koroleva OV, Stepanova EV, Gavrilova VP, Yakovleva S, Landesman EO, Yavmetdinov IS and Yaropolov A (2002). Laccase and Mn-peroxidase production by *Coriolus hirsutus* strain 075 in a jar fermentor. *J. Biosci. Bioeng.* **93**, 449-455.

154. Korona B, Korona D and Bielecki S (2006). Efficient expression and secretion of two co-produced xylanases from *Aspergillus niger* in *Pichia pastoris* directed by their native signal peptides and the *Saccharomyces cerevisiae* α-mating factor. *Enzyme Microb. Technol.* **39**, 683–689.

155. Koseki T, Miwa Y, Mese Y, Miyanaga A, Fushinobu S, Wakagi T, Shoun H, Matsuzawa H and Hashizume K (2006a). Mutational analysis of N-glycosylation recognition sites on the biochemical properties of *Aspergillus kawachii* α-L-arabinofuranosidase 54. *Biochim. Biophys. Acta.* **1760**, 1458–1464.

156. Koseki T, Miwa Y, Akao T, Akita O and Hashizume K (2006b). An *Aspergillus oryzae* acetyl xylan esterase: Molecular cloning and characteristics of recombinant enzyme expressed in *Pichia pastoris*. *J. Biotechnol.* **121**, 381–389.

157. Kuhad RC, Kumar M and Singh A (1994). A hyperxylanolytic mutant of *Fusarium oxysporum*. *Lett. Appl. Microbiol.* **19**, 397-400.

158. Kuhad RC, Manchanda M and Singh A (1998). Optimisation of xylanase production

by a hyperxylanolytic mutant strain of *Fusarium oxysporum*. *Process Biochem.* **33**, 641-647.

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

160. Kumar D, Jain VK, Shanker G and Srivastava A (2003). Citric acid production by solid-state fermentation using sugarcane bagasse. *Process Biochem.* **38**, 1731-1738.

161. Kuyper M, Harhangi HR, Stave AK, Winkler AA , Jetten MSM, de Laat WTAM, den Ridder JJJ, den Camp HJMO, van Dijken JP and Pronk JT (2003). High-level functional expression of a fungal xylose isomerase: the key to ancient ethanolic fermentation of xylose by *Saccharomyces cerevisiae*? *FEMS Yeast Res.* **4**, 69-78.

162. Laboratory manual for pulp and paper laboratory (1993), NORAD-COURSE, University of Trondheim, The Norwegian Institute of Technology, Department of Chemical Engineering.

163. Laemmli UK. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* (London) **277**, 680-685.

164. Lama L, Calandrelli V, Gambacorta A and Nicolaus B (2004) Purification and characterisation of thermostable xylanase and β-xylosidase by the thermophilic bacterium *Bacillus thermantarcticus*. *Res. Microbiol.* **155**, 283-289.

165. Lappalainen A, Sikka-Aho M, Kalkleinen N, Fagerstorm R and Tenkenen M (2000). Endoxylanase II from *Trichoderma reesei* has several isoforms with different isoelectric points. *Appl. Biochem. Biotechnol.* **31**, 61-68.

166. Leathers TD (1986). Colour variants of *Aureobasidium pullulans* over produce xylanase with extremely high specific activity. *Appl. Environ. Microbiol.* **52**, 1026-1030.

167. Leathers TD, Kurtzman CP and Detroy RW (1984). Overproduction and regulation of xylanase in *Aureobasidium pullulans* and *Cryptococcus albidus*. *Biotechnol. Bioeng.* **14**, 225-250.

168. Lee RC, Burton RA, Hrmova M and Fincher GB (2001). Barley arabinoxylan arabinofuranosidases: purification, characterisation and determination of primary structures from cDNA clones. *Biochem. J.* **356**, 181–189.

169. Lenartovicz V, de Souza CGM, Moreira FG and Peralta RM (2003). Temperature and carbon source affect the production and secretion of a thermostable β-xylosidase by *Aspergillus fumigatus*. *Process Biochem.* **38**, 1775-1780.

170. Lenz J, Hofer M, Krasenbrink JB and Holker U (2004). A survey of computational

and physical methods applied to solid state fermentation. *Appl. Microbiol. Biotechnol.* **65**, 9-17.

171. Li K, Azadi P, Collins R, Tolan J, Kim J and Eriksson K (2000). Relationships between activities of xylanases and xylan structures. *Enzyme Microb. Technol.* **27**, 89-94.

172. Li XT, Jiang ZQ, Li LT, Yang SQ, Feng WY, Fan JY and Kusakabe I (2005). Characterisation of a cellulase free, neutral xylanase from *Thermomyces lanuginosus* CBS 288.54 and its biobleaching effect on wheat straw pulp. *Bioresource Technol.* **96**, 1370-1379.

173. Li L, Tian H, Cheng Y, Jiang Z and Yang S (2006). Purification and characterisation of a thermostable cellulase-free xylanase from the newly isolated *Paecilomyces themophila*. *Enzyme Microb. Technol.* **38**, 780–787.

174. Lin CC, Hsieh PC, Mau JL and Teng DF (2005). Construction of an intergeneric fusion from *Schizosaccharomyces pombe* and *Lentinula edodes* for xylan degradation and polyol production. *Enzyme Microb. Technol.* **36**, 107-117.

175. Liu CJ, Suzuki T, Hirata S and Kawai K (2003). The processing of high- molecular weight xylanase (Xyn E, 110 kDa) from *Aeromonas caviae* ME-1 to 60 -kDa xylanase ($XynE_{60}$) in *E. coli* and purification and characterisation of $XynE_{60}$. *J. Biosci. Bioeng.* **95**, 95-101.

176. Liu J, Yuan X, Zeng G, Shi J and Chen S (2006). Effect of biosurfactant on cellulase and xylanase production by *Trichoderma viride* in solid substrate fermentation. *Process Biochem.* **41**, 2347–2351.

177. Liu MQ and Liu GF (2008). Expression of recombinant *Bacillus lichiniformis* xylanase A in *Pichia pastoris* and xylooligosaccharides released from xylans by it. *Protein Expr. Purif.* **57**, 101-107.

178. Liu W, Lu Y and Ma G (1999). Induction and glucose repression of endo-β-xylanase in the yeast *Trichosporon cutaneum* SL409. *Process Biochem.* **34**, 67-72.

179. Liu WH and Yang CH (2002). The isolation and identification of a lignocellulollyltic and thermophilic actinomycete. *Food Sci. Agric. Chem.* **4**, 89–94.

180. Lonn A, Traff-Bjerre KL, Otero RRC, van Zyl WH and Hahn-Hagerdal B (2003). Xylose isomerase activity influences xylose fermentation with recombinant *Saccharomyces cerevisiae* strains expressing mutated *xylA* from *Thermus thermophilus*. *Enzyme Microb. Technol.* **32**, 567–573.

181. Lonsane BK, Ghildyal NP, Budiatman S and Ramakrishna SV (1985). Engineering aspects of solid-state fermentation. *Enzyme Microb. Technol.* **7**, 258-265.

182. Lonsane BK, Saucedo-Castaneda G, Raimbault M, Roussos S, Viniegra-Gonzalez G, Ghildyal NP, Ramakrishna M and Krishnaiah MM (1992). Scale up strategies for solid state fermentation systems. *Process Biochem.* **27**, 259-273.

183. Lotfy WA, Ghanem KM and El-Helow ER (2007). Citric acid production by a novel *Aspergillus niger* isolate: I. mutagenesis and cost reduction studies. *Bioresource Technol.* **98**, 3464-3469.

184. Lu W, Li D and Wu Y (2003). Influence of water activity and temperature on xylanase biosynthesis in pilot-scale solid-state fermentation by *Aspergillus sulphureus*. *Enzyme Microb. Technol.* **32**, 305-311.

185. Lund M, Eriksson M and Felby C (2003). Reactivity of fungal laccase towards lignin in softwood kraft pulp. *Holzforschung* **57**, 21-26.

186. Lv Z, Yang J and Yuan H (2008). Production, purification and characterisation of an alkaliphilic endo-β-1,4-xylanase from a microbial community EMSD5. *Enzyme Microb. Technol.* **43**, 343-348.

187. Mamma D, Kourtoglou E and Christakopoulos P (2008). Fungal multienzyme production on industrial by-products of the citrus-processing industry. *Bioresource Technol.* **99**, 2373-2383.

188. Mamo G, Hatti-Kaul R and Mattiasson B (2006). A thermostable alkaline active endo- β-1-4-xylanase from *Bacillus halodurans* S7: Purification and characterisation. *Enzyme Microb. Technol.* **39**, 1492–1498.

189. Mandalari G, Bisignano G, Lo Curto RB, KW Waldron and Faulds CB (2008). Production of feruloyl esterases and xylanases by *Talaromyces stipitatus* and *Humicola grisea* var. thermoidea on industrial food processing by-products. *Bioresource Technol.* **99**, 5130-5133.

190. Marichamy S and Mattiasson B (2005). Rapid production of cellulase-free xylanases by solventogenic *Clostridia* from rumen. *Enzyme Microb. Technol.* **37**, 497–504.

191. Mathrani IM and Ahring BK (1992). Thermophillic and alkalophilic xylanases from *Dictyoglomus* isolates. *Appl. Microbiol. Biotechnol.* **38**, 23-27.

192. Mathur S, Kumar S and Rao NJ (2001). Application of commercial xylanases in bleaching – a review. *IPPTA* **13**, 13-23.

193. Matsuo M, Endon A, Okada T and Yamaoka T (1998). Purification and characterisation of β-xylosidase from *Thermoascus* sp. *J. Ferment. Bioeng.* **86**, 403-

405.

194. Mayorga-Reyes L and Ponce-Noyola T (1998). Isolation of a hyperxylanolytic *Cellulomonas flavigena* mutant growing on continuous culture on sugar cane bagasse. *Biotechnol. Lett.* **20**, 443-446.

195. Medeiros RG, Silva FG Jr, Salles BC, Estelles RS and Filho EXF (2002) The performance of fungal xylan-degrading enzyme preparations in elemental chlorine free bleaching for eucalyptus pulp. *J. Ind. Microbiol. Biotechnol.* **28**, 204-206.

196. Medeiros RG, Hanada R and Filho EXF (2003). Production of xylan-degrading enzymes from amazon forest fungal species. *Int. Biodeter. Biodegr.* **52**, 97-100.

197. Milagres AMF, Magalhaes PO and Ferraz A (2005). Purification and properties of a xylanase from *Ceriporiopsis subvermispora* cultivated on *Pinus taeda*. *FEMS Microbiol. Lett.* **253**, 267–272.

198. Miller GL (1959). Use of dinitrosalicylic acid reagent for determinatioin of reducing sugar. *Anal. Chem.* **31**, 426-428.

199. Minic Z, Rihouey C, Do CT, Lerouge P and Jouanin L (2004). Purification and characterisation of enzymes exhibiting β -D-xylosidase activities in stem tissues of *Arabidopsis*. *Plant Physiol.* **135**, 867–878.

200. Mishra BK, Arora A and Lata (2004). Optimisation of a biological process for treating potato chip industry waste water using a mixed culture of *Aspergillus foetidus* and *Aspergillus niger*. *Bioresource Technol.* **94**, 9-12.

201. Mitchell DA, Berovic M and Krieger N (2002). Overview of solid state bioprocessing. Biotechnol. *Ann. Rev.* **8**, 183-225.

202. Mittal SK (2005). Strategic moves of pulp & paper industry towards achieving sustainable development. Keynote address at IPPTA Annual General Meeting, ii-vii.

203. Mohana S, Shah A, Divecha J and Madamwar D (2008). Xylanase production by *Burkholderia* sp. DMAX strain under solid state fermentation using distillery spent wash. *Bioresource Technol.* **99**, 7553–7564.

204. Moure A, Gullon P, Dominguez H and Parajo JC (2006). Advances in the manufacture, purification and applications of xylo-oligosaccharides as food additives and nutraceuticals. *Process Biochem.* **41**, 1913-1923.

205. Mukherjee G and Banerjee R (2006). Effects of temperature, pH and additives on the activity of tannase produced by a co-culture of *Rhizopus oryzae* and *Aspergillus foetidus*. *World J. Microbiol. Biotechnol.* **22**, 207-212.

206. Nakamura S (2003). Structure and function of a multidomain alkaline xylanase from alkalophilic *Bacillus* sp. Strain 41 M-1. *Catalysis Surveys from Asia* **7**, 157-164.

207. Narahara H, Koyama Y, Yoshida T, Pichangkura S, Ueda R and Taguchi H (1982). Growth and enzyme production in a solid-state culture of *Aspergilles oryzae*. *J. Ferment. Technol.* **60**, 311-319.

208. Nascimento RP, Coelho RRR, Marques S, Alves L, Girio F, da Silva Bon EP and Amaral-Collaco MT (2002). Production and partial characterisation of xylanase from *Streptomyces* sp. Strain AMT-3 isolated from brazillian cerrado soil. *Enzyme Microb. Technol.* **31**, 549-555.

209. Nascimento RP, Marques S, Alves L, Girio F, Amaral-Collaco MT, Sacramento RR, da Silva Bon EP and Coelho RRR (2003). A novel strain of *Streptomyces malaysiensis* isolated from brazilian soil produces high endo-β-1,4-xylanase titres. *World J. Microbiol. Biotechnol.* **19**, 879-881.

210. Nigam JN (2002). Bioconversion of water hyacinth (*Eichhornia crassipes*) hemicellulose acid hydrolyasate to motor fuel ethanol by xylose-fermenting yeast. *J. Biotechnol.* **97**, 107-116.

211. Niku-Paavola M-J, Karhunen E, Salola P and Raunio V (1988). Ligninolytic enzymes of the white-rot fungus *Phlebia radiata*. *Biochem. J.* **254**, 877-884.

212. Niku-Paavola ML, Ranua M, Suurnakki A and Kantelinen A (1994). Effects of lignin modifying enzymes on pine kraft pulp. *Bioresource Technol.* **50**, 73-77.

213. Ninawe S and Kuhad RC (2006). Bleaching of wheat straw-rich soda pulp with xylanase from a thermoalkalophilic *Streptomyces cyaneus* SN32. *Bioresource Technol.* **97**, 2291–2295.

214. Ninawe S, Kapoor M and Kuhad RC (2008). Purification and characterisation of extracellular xylanase from *Streptomyces cyaneus* SN32. *Bioresource Technol.* **99**, 1252-1258.

215. Nogawa M, Yatsui K, Tomioka A, Okada H and Morikawa Y (1999). An α –L arabinofuranosidase from *Trichoderma reesei* containing a noncatalytic xylan-binding domain. *Appl. Environ. Microbiol.* **65**, 3964-3968.

216. Nurizzo D, Nagy T, HJ Gilbert and Daviesl GJ (2002). The structural basis for catalysis and specificity of the *Pseudomonas cellulosa* α-glucuronidase, GlcA67A. *Structure*, **10**, 547–556.

217. Oku T and Sadako N (2002). Digestion, absorption, fermentation and metabolism of functional sugar substitutes and their available energy. *Pure Appl. Chem.* **74**, 1253–61.

218. Oliveira LA, Porto Ana LF and Tambourgi EB (2006). Production of xylanase and protease by *Penicillium janthinellum* CRC 87M-115 from different agricultural wastes. *Bioresource Technol.* **97**, 862-867.

219. Paice MG, Gurnagul N, Page DH and Jurasek L (1992). Mechanism of hemicellulose directed prebleaching of kraft pulp. *Enzyme Microb. Technol.* **14**, 272-276.

220. Palma MB, Milagres AMF, Prata AMR and Mansilha IM (1996). Influence of aeration and agitation rate on the xylanase activity from *Penicillium janthinellum*. *Process Biochem.* **31**, 141-145.

221. Panagiotou G, Kekos D, Macris BJ and Christakopoulos P (2003). Production of cellulolytic and xylanolytic enzymes by *Fusarium oxysporum* grown on corn stover in solid state fermentation. *Ind. Crop. Prod.* **18**, 37-45.

222. Panagiotou G, Christakopoulosb P, Grotkjæra T and Olssona L (2006). Engineering of the redox imbalance of *Fusarium oxysporum* enables anaerobic growth on xylose. *Metab. Eng.* **8**, 474–482.

223. Panagiotou G, Olavarria R and Olsson L (2007). *Penicillium brasilianum* as an enzyme factory; the essential role of feruloyl esterases for the hydrolysis of the plant cell wall. *J. Biotechnol.* **130**, 219–228.

224. Pandey A, Selvakumar P, Soccol CR and Nigam P (1999). Solid state fermentation for the production of industrial enzymes. *Curr. Sci.* **77**, 149-162.

225. Pandey A, Soccol CR and Mitchell D (2000). New developments in solid-state fermentation. I. Bioprocesses and products. *Process Biochem.* **35**, 1153-1169.

226. Pandey A, Soccol CR, Rodriguez-leon JA and Nigam P (2001). *Solid state fermentation in biotechnology fundamentals and applications*, Asiatech Publishers Inc, New Delhi.

227. Parajo JC, Dominguez H and Domfnguez JM (1998). Biotechnological production of xylitol. Part 1" Interest of xylitol and fundamentals of its biosynthesis. *Bioresource Technol.* **65**, 191-2111.

228. Patel AN, Grabski AC and Jeffries TW (1993). Chromophore release from kraft pulp by purified *Streptomyces roseiscleroticus* xylanase. *Appl. Microbiol. Biotechnol.* **39**, 405-412.

229. Patil SR and Dayanand A (2006). Production of pectinase from deseeded sunflower head by *Aspergillus niger* in submerged and solid-state conditions. *Bioresource Technol.* **97**, 2054–2058.

230. Paul J and Verma K (1990). Influence of sugars on endoglucanase and p-xylanase activities of a *Bacillus* strain. *Biotechnol. Lett.* **12**, 1-64.

231. Perez-Avalos O, Noyela TP, Plaza IM and Torre M (1996). Induction of xylanase and β-xylosidase in *Cellulomonas flavigena* growing on different carbon sources. *Appl. Microbiol. Biotechnol.* **46**, 405-409.

232. Petruccioli M, Piccioni P, Federici F and Polsinelli M (1995). Glucose oxidase overproducing mutants of *Penicillium variabile* (P 16). *FEMS Microbiol. Lett.* **128**, 107-112.

233. Pham PL, Alric I and Delmas M (1995). Incorporation of xylanase in TCF bleaching sequences using ozone and hydrogen peroxide. *APPITA J.* **48**, 213–217.

234. Pham PL, Taillandier P, Delmas M and Strehaiano P (1998). Production of xylanases by *Bacillus polymyxa* using lignocellulosic wastes. *Ind. Crop. Prod.* **7**, 195-203.

235. Pinaga F, Fernandez-Espinar MT, Vallea S and Ramon D (1994). Xylanase production in *Aspergillus nidulans*: induction and carbon catabolite repression. *FEMS Microbiol. Lett.* **115**, 319-324.

236. Pointing SB (2001). Feasibility of bioremediation by white-rot fungi. *Appl. Microbiol. Biotechnol.* **57**, 20-33.

237. Polizeli MLTM, Rizzatti ACS, Monti R Terenzi HF, Jorge JA and Amorim DS (2005). Xylanases from fungi: properties and industrial applications. *Appl. Microbiol. Biotechnol.* **67**, 577-591.

238. Prade RA (1995). Xylanases: from biology to biotechnology. *Biotechnol. Genet. Eng. Rev.* **13**, 101-131.

239. Purkarthofer H, Sinner M and Steiner W (1993). Effect of shear rate and culture pH on the production of xylanase by *Thermomyceus lanuginosus*. *Biotechnol. Lett.* **15**, 405-410.

240. Purohit JS, Dutta JR, Nanda RK and Banerjee R (2006). Strain improvement for tannase production from co-culture of *Aspergillus foetidus* and *Rhizopus oryzae*. *Bioresource Technol.* **97**, 795-801.

241. Qinnghe C, Xiaoyu Y, Tiangui N, Cheng J and Qiugang M (2003). The screening of culture condition and properties of xylanase by white-rot fungus *Pleurotus ostreatus*. *Process Biochem.* **39**, 1561-1566.

242. Qureshy AF, Khan LA and Khanna S (2000). Expression of *Bacillus* Teri 42 xylanase gene in *Bacillus subtilis*. *Enzyme Microb. Technol.* **27**, 227-233.

243. Ragauskus AJ, Poll KN and Cesternino A (1994). Effects of xylanase pretreatment procedures on non-chlorine bleaching. *Enzyme Microb. Technol.* **16**, 492-495.

244. Raghavarao KSMS, Ranganathan and Karanth NG (2003). Some engineering aspects of solid-state fermentation. *J. Biochem. Eng.* **13**, 127-135.

245. Raghukumar C, Muraleedharan U, Gaud VR and Mishra R (2004). Xylanases of marine fungi of potential use for biobleaching of paper pulp. *J. Ind. Microbiol. Biotechnol.* **31**, 433-441.

246. Raimbault M (1998). General and microbiological aspects of solid state fermentation. *Electron. J. Biotechnol.* **1**, 174-188.

247. Rajaram S and Varma A (1990). Production and characterisation of xylanase from *Bacillus thermoalkalophillus* grown on agricultural wastes. *Appl. Microbiol. Biotechnol.* **34**, 141-144.

248. Rajoka MI (2005). Regulation of synthesis of endo-xylanase and β-xylosidase in *Cellulomonas flavigena*: a kinetic study. *World J. Microbiol. Biotechnol.* **21**, 463-469.

249. Ramesh MV and Lonsane BK (1990). Critical importance of moisture content of the medium in alpha-amylase production by *Bacillus licheniformis* M27 in a solid state fermentation system. *Appl. Microbiol. Biotechnol.* **33**, 501-505.

250. Rao RS, Jyothi CP, Prakasham RS, Sarma PN and Rao LV (2006). Xylitol production from corn fiber and sugarcane bagasse hydrolysates by *Candida tropicalis*. *Bioresource Technol.* **97**, 1974–1978.

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

252. Roncero MB, Torres AL, Colom JF and Vidal T (2000). Effects of xylanase treatment on fibre morphology in totally chlorine free bleaching (TCF) of Eucalyptus pulp. *Process Biochem.* **36**, 45–50.

253. Roncero MB, Torres AL, Colom JF and Vidal T (2003). TCF bleaching of wheat straw pulp using ozone and xylanase. Part A: paper quality assessment. *Bioresource Technol.* **87**, 305-314.

254. Roncero MB, Torres AL, Colom JF and Vidal T (2005). The effect of xylanase on lignocellulosic components during the bleaching of wood pulps. *Bioresource Technol.* **96**, 21-30.

255. Rosales E, Couto SR and Sanroman MA (2007). Increased laccase production by *Trametes hirsuta* grown on ground orange peelings. *Enzyme Microb. Technol.* **40**, 1286-1290.

256. Ruanglek V, Sriprang R, Ratanaphan N, Tirawongsaroj P, Chantasingh D, Tanapongpipat S, Pootanakit K and Eurwilaichitr L (2007). Cloning, expression, characterisation, and high cell-density production of recombinant endo-1, 4-β-xylanase from *Aspergillus niger* in *Pichia pastoris*. *Enzyme Microb. Technol.* **41**, 19-25.

257. Ruiz-Arribas A, Fernandez-Abalos JM, Sanchez P, Gorda A and Santamaria RI (1995). Overproduction, purification and biochemical characterisation of a xylanase (Xys1) from *Streptomyces halstedii* JM8. *Appl. Environ. Microbiol.* **61**, 2414-2419.

258. Samain E, Debeire PH and Touzel JP (1997). High level production of a cellulase free xylanase in glucose-limited fed batch cultures of a thermophilic *Bacillus* strain. *J. Biotechnol.* **58**, 71-78.

259. Sampaio FC, Chaves-Alves VM, Converti A, Lopes Passos FM and Cavalcante Coelho JL (2008). Influence of cultivation conditions on xylose-to-xylitol bioconversion by a new isolate of *Debaryomyces hansenii*. *Bioresource Technol.* **99**, 502–508.

260. Sandhya C, Binod P, Nampoothiri KM, Szakacs G and Pandey A (2005). Microbial synthesis of chitinase in solid cultures and its potential as biocontrol agent against phytopathogenic fungus *Colletotrichum gloeosporioides*. *Appl. Biochem. Biotechnol.* **127**, 1-16.

261. Sandrim VC, Rizzatti ACS, Terenzi HF, Jorge JA, Milagres AMF, Polizeli MLTM (2005). Purification and biochemical characterisation of two xylanases produced by *Aspergillus caespitosus* and their potential for kraft pulp bleaching. *Process Biochem.* **40**, 1823-1828.

262. Santos DT, Sarrouh BF, Rivaldia JD, Converti A and Silva SS (2008). Use of sugarcane bagasse as biomaterial for cell immobilization for xylitol production. *J. Food Eng.* **86**, 542–548.

263. Sanyal D and Kulshrestha G (2003). Degradation of metolachlor in soil inoculated with a mixed fungal culture. *Biol. Fertil. Soils* **38**, 253-256.

264. Sa-Pereira P, Mesquita A, Duarte JC, Barros MRA and Costa-Ferreira M (2002). Rapid production of thermostable cellulase free xylanase by a strain of *Bacillus subtilis* and its properties. *Enzyme Microb. Technol.* **30**, 924-933.

265. Saraswat V and Bisaria VS (1997). Biosynthesis of xylanolytic and xylan

debranching enzymes in *Melanocarpus albomyces* IIS 68. *J. Ferment. Bioeng.* **83**, 352-57.

266. Scheibner K, Hofrichter M and Fritsche W (1997). Mineralization of 2-amino-4,6 dinitrotoluene by manganese peroxidase of the white-rot fungus *Nematoloma frowardii*. *Biotechnol. Lett.* **19**, 835–839.

267. Schwend and Gustafsson (2006). False positives in MALDI-TOF detection of ERβ in mitochondria. *Biochem. Biophys. Res. Commun.* **343**, 707-711.

268. Sealey J and Ragauskas AJ (1998). Residual lignin studies of laccase-delignified kraft pulps. *Enzyme Microb. Technol.* **23**, 422-426.

269. Senthilkumar SR, Ashokkumar B, Raj BC and Gunasekaran P (2005). Optimisation of medium composition for alkali-stable xylanase production by *Aspergillus fischeri* Fxn 1 in solid-state fermentation using central composite rotary design. *Bioresource Technol.* **96**, 1380-1386.

270. Seyis I and Aksoz N (2005). Effect of carbon and nitrogen sources on xylanase production by *Trichoderma harzianum* 1073 D3. *Int. Biodeter. Biodegr.* **55**, 115-119.

271. Shah AK, Sidid SS, Ahmad A and Rele MV (1999). Treatment of bagasse pulp with cellulase-free xylanases from an alkalophilic *Bacillus* sp. *Sam-3*. *Bioresource Technol.* **68**, 133-140.

272. Shah BA, Taneja SC, Sethi VK, Gupta P, Andotra SS, Chimni SS and Qazi GN (2007). The formation of novel 1,3-dioxolanes: atypical Baylis–Hillman reaction of a sesquiterpene lactone parthenin, *Tetrahedron. Lett.* **48**, 955–960.

273. Shah AR and Madamwar D (2005). Xylanase production by a newly isolated *Aspergillus foetidus* strain and its characterisation. *Process Biochem.* **40**, 1763-1771.

274. Shallom D and Shoham Y (2003). Microbial hemicellulases. *Curr. Opin. Microbiol.* **6**, 219-228.

275. Sharma A, Sundaram ST, Zhang Y-Z and Brodman BW (1995). Nitrocellulose degradation by a coculture of *Sclerotium rolfsfi* and *Fusarium solani*. *J. Ind. Microbiol.* **15**, 1-4.

276. Shatalov AA and Pereira H (2007). Xylanase pre-treatment of giant reed organosolv pulps: direct bleaching effect and bleach boosting. *Ind. Crop. Prod.* **25**, 248–256.

277. Shin Hyun-Dong and Chen RR (2006). Production and characterisation of a type B feruloyl esterase from *Fusarium proliferatum* NRRL 26517. *Enzyme Microb. Technol.* **38**, 478–485.

278. Si JQ and Lustenberger CD (2002). Enzymes for bread, pasta and noodle products. In: *Enzymes in food technology*. Hitehurst RJ, Law BA (Eds), She Yeld Academic Press Ltd., She Yeld, UK, pp. 19–56.

279. Siedenberg D, Gerlach SR, Schugerl K, Giuseppin MLF and Hunik J (1998). Production of xylanase by *Aspergillus awamori* on synthetic medium in shake flask cultures. *Process Biochem.* **33**, 429-433.

280. Sigoillot C, Camarero S, Vidal T, Record E, Asther M, Perez-Boada M Martınez, MJ and Sigoillot JC (2005). Comparison of different fungal enzymes for bleaching high-quality paper pulps. *J. Biotechnol.* **115**, 333–343.

281. Singh A, Kuhad RC and Kumar M (1995). Xylanase production by a hyperxylanolytic mutant of *Fusarium oxysporum*. *Enzyme Microb. Technol.* **17**, 551-553.

282. Singh S, Pillay B, Dilsook V and Prior BA (2000). Production and properties of hemicellulases by a *Thermomyces lanuginosus* strain. *J. Appl. Microbiol.* **88**, 975-982.

283. Somasegaran P and Hoben HJ (1994). In: *Handbook of Rhizobia*: Methods in Legume-Rhizobium technology, Publ. Springer-verlag. New York, 402-408.

284. Soni R, Sandhu DK and Soni SK (1999). Localisation and optimisation of cellulase production in *Chaetomium erraticum*. *J. Biotechnol.* **73**, 43-51.

285. Srivastava P and Mukherjee KJ (2001). Cloning, characterisation, and expression of xylanase gene from *Bacillus lyticus* in *Escherichia coli* and *Bacillus subtilis*. *Prep. Biochem. Biotechnol.* **31**, 389-400.

286. Srivastava R and Srivastava AK (1993). Characterisation of a bacterial xylanase resistant to repression by glucose and xylose. *Biotechnol. Lett.* **15**, 847-852.

287. Statistics of the Indian Paper Industry (2005). Paper, Paperboard & Newsprint. A report by *CPPRI*, **1**, 3-23.

288. Stepanova SV, Koroleva OV, Vasilchenko LG, Karapetyan KN, Landesman EO, Yavmetdinov IS, Kozlov YP and Rabinovich ML (2003). Fungal decomposition of oat straw during liquid and solid-state fermentation. *Appl. Biochem. Microbiol.* **39**, 65-74.

289. Stollnberger W, Bichler H and Hampel WA (1996). Effects of temperature on the secretion of xylanolytic enzymes by *Trichoderma reesei* Rut C-30. In: *Biotechnology in the pulp and paper industry*. Proc. 6th Intern Conf Biotechnol. Pulp Paper Ind, ed. Srebotnik E and Messner K. Facultas-Universitatsverlag, Vienna, 517-520.

290. Subramaniyan S and Prema P (2002). Biotechnology of microbial xylanases, enzymology, molecular biology and application. *Crit. Rev. Biotechnol.* **22**, 33-64.

291. Subramaniyan S and Prema P (2000). Cellulase free xylanases from *Bacillus* and other microorganisms. *FEMS Micobiol. Lett.* **183**, 1-7.

292. Suzuki M, Kato A, Nagata N and Komeda Y (2002). A xylanase, AtXyn1, is predominantly expressed in vascular bundles, and four putative xylanase genes were identified in the *Arabidopsis* genome. *Plant Cell Physiol.* **43**, 759–767.

293. Swaroopa Rani D and Nand K (2000). Production of thermostable cellulase free xylanase by *Clostridium absonum* CFR-702. *Process Biochem.* **36**, 355-362.

294. Tapingkae W, Yachai M, Visessanguan W, Pongtanya P and Pongpiachan P (2008). Influence of crude xylanase from *Aspergillus niger* FAS128 on the *in vitro* digestibility and production performance of piglets. *Animal Feed Sci. Technol.* **140**, 125-138.

295. TAPPI test methods (1996). Technical association of the pulp and paper industry. Atlanta, GA, TAPPI press.

296. Te'O VSJ, Cziferszky AE, Vergquist PL and Nevalinen KMH (2000). Codon optimisation of xylanase gene *xyn*B from the thermophilic bacterium *Dictyoglomus thermophilum* for expression in the filamentous fungus *Trichoderma reeseii*. *FEMS Microbiol. Lett.* **190**, 13-19.

297. Teather RM and Wood PJ (1982). Use of congo red polysaccharide interactions in enumeration and characterisation of cellulolytic bacteria from the bovine rumen. *Appl. Environ. Microbiol.* **43**, 777-780.

298. Techapun C, Pooraran N, Watanabe M and Sasaki K (2003). Thermostable and alkaline-tolerant microbial cellulase free xylanases produced from agricultural wastes and the properties required for use in pulp bleaching bioprocesses: a review. *Process Biochem.* **38**, 1327-1340.

299. Teeri TL, Brumer H, Daniel G and Gatenholm P (2007). Biomimetic engineering of cellulose-based materials. *Trends Biotechnol.* **25**, 299-306.

300. Tendulkar SR, Shinde JK and Mokashi AG (1994). Elemental chlorine free

bleaching of bagasse chemical Pulp. *IPPTA*, **6**, 99-107.

301. Tengerdy RP and Szakacs G (2003). Bioconversion of lignocellulose in solid substrate fermentation. *Biochem. Eng. J.* **13**, 169-179.

302. Tenkanen M, Puls J and Poutanen K (1992). Two major xylanases of *Trichoderma reesei*. *Enzyme Microb. Technol.* **14**, 566-574.

303. Thomas CR (1990). Problems of shear in biotechnology. In: *Chemical Engineering Problems in Biotechnology*. Winkler MA (Eds), Elsevier Applied Sciences, London, 23-93.

304. Thomson JA (1993). Molecular biology of xylan degradation. *FEMS Microbiol. Rev.* **104**, 65-82.

305. Tokimoto K (1982). Lysis of the mycelium of *Lentinus edodes* caused by mycolytic enzymes of *Trichoderma harzianum* when the two fungi were in an antagonistic state. *Transactions of the Mycological Society of Japan* **23**, 13-20.

306. Tolan JS and Guenette M (1997). Using enzymes in pulp bleaching: mill applications. *Adv. Biochem. Eng. Biotechnol.* **157**, 290-309.

307. Topakas A, Katapodis P, Kekos D, Macris BJ and Christakopoulos P (2003). Production and partial characterisation of xylanase by *Sporotrichum thermophile* under solid-state fermentation. *World J. Microbiol. Biotechnol.* **19**, 195-198.

308. Torres AL, Roncero MB, Colom JF, Pastor FIJ, Blanco A and Vidal T (2000). Effect of a novel enzyme on fibre morphology during ECF bleaching of oxygen delignified eucalyptus kraft pulps. *Bioresource Technol.* **74**, 135-140.

309. Trilli A (1986). Scale up fermentation, In: *Industrial Microbiology and Biotechnology*. Demain AL, Solomon NA (Eds), American Society of Microbiology, Washington, DC, USA, 227-307.

310. Tseng MJ, Yap MN, Ratanakhanokchai K, Kyu KL and Chen ST (2002). Purification and characterisation of two cellulase free xylanases from an alkaliphilic *Bacillus firmus*. *Enzyme Microb. Technol.* **30**, 590-595.

311. Turunen O, Etuaho K, Fenel F, Vehmaanpera J, Wu X, Rouvinen J and Leisola M (2001). A combination of weakly stabilising mutations with a disulfide bridge in the α- helix region of *Trichoderma reesei* endo-1,4- β-xylanase II increases the thermal stability through synergism. *J. Biotechnol.* **88**, 37-46.

312. Uffen RL (1997). Xylan degradation: a glimpse at microbial diversity. *J. Ind. Microbiol. Biotechnol.* **19**, 1-6.

313. Utt EA, Eddy CK, Keshov KF and Ingram LO (1991). Sequencing and expression

of the *Butyrivibrio fibriosolvens xyl* B gene encoding a novel bifunctional protein with β-D-xylosidase and α-L-arabinofuranosidase activities. *Appl. Environ. Microbiol.* **57**, 1227-1234.

314. Vasdev K, Dhawan S, Kapoor RK and Kuhad RC (2005). Biochemical characterisation and molecular evidence of a laccase from the bird's nest fungus *Cythus bulleri*. *Fun. Gen. Biol.* **42**, 684-693.

315. Viikari L, Kantelinen A, Sundguist J and Linko M (1994). Xylanases in bleaching, from an idea to the industry. *FEMS Microbiol. Rev.* **13**, 335-350.

316. Viikari L, Ranua M, Kantelinen A, Linko M and Sundquist J (1987). Application of enzymes in biobleaching. In : Proc. *of 4th Int. Symposium on Wood & Pulping Chemistry* Paris, April 27-30, 1, 151-154.

317. Viikari L, Suurnakki A and Buchert J (1996). Enzymatic bleaching of kraft pulps: fundamental mechanisms and practical applications. *Abstracts of Paper of the American Chemical Society* 211 Meet, Pt 1, CELL 211.

318. Vinogradova SP and Kushnir SN (2003). Biosynthesis of hydrolytic enzymes during co-cultivation of macro- and micromycetes. *Appl. Biochem. Microbiol.* **39**, 573-575.

319. Virupakshi S, Babu KG, Gaikwad SR and Naik GR (2005). Production of xylanolytic enzyme by thermoalkaliphilic *Bacillus* sp. JB-99 in solid state fermentation. *Process Biochem.* **40**, 431-435.

320. Vogel AI (2002). A text book of quantitative inorganic analysis. 6th edition, 2nd Indian reprint, 422-436.

321. Wagschal K, Franqui-Espiet D, Lee CC, Kibblewhite-Accinelli RE, Robertson GH, and Wong DWS (2007). Genetic and biochemical characterisation of an α-l-arabinofuranosidase isolated from a compost starter mixture. *Enzyme Microb. Technol.* **40**, 747–753.

322. Wang Y, Zhang H, He Y, Luo H and Yao B (2007). Characterisation, gene cloning, and expression of a novel xylanase XYNB from *Streptomyces olivaceoviridis* A1. *Aquaculture* **267**, 328–334.

323. Watanabe T, Watanabe Y and Nakamura K (2003). Biodegradation of wood in dual cultures of selected two fungi detrmined by chopstick method. *J. Biosci. Bioeng.* **95**, 623-626.

324. Werner A, Zadworny M and Idzikowska K (2002). Interaction between *Laccaria laccata* and *Trichoderma virens* in co-culture and in the rhizosphere of *Pinus sylvestris* grown in vitro. *Mycorrhiza* **12**, 139–145.

325. West TP (2002). Isolation of a mutant strain of *Pseudomonas* sp. ATCC 31461 exhibiting elevated polysaccharide production. *J. Ind. Microbiol. Biotechnol.* **29**, 185-188.

326. Whitehead TR and Hespell RB (1990). The genes for three xylanase degrading activities from *Bacteroides ovatus* are clustered in a 3.8-kilobase region. *J. Bacteriol.* **172**, 2408-2412.

327. Wong KKY, Tan LUL and Saddler JN (1988). Multiplicity of β-1, 4-xylanases in microorganisms: functions and applications. *Microbiol. Rev.* **52**, 305-317.

328. Wu H, Pei J, Wu G and Shao W (2008). Overexpression of GH10 endoxylanase XynB from *Thermotoga maritima* in *Escherichia coli* by a novel vector with potential for industrial application. *Enzyme Microb. Technol.* **42**, 230-234.

329. Wu SSH, Suen DF, Chang HC and Huang AHC (2002). Maize tapetum xylanase is synthesised as a precursor, processed and activated by a serine protease, and deposited on the pollen. *J. Biol. Chem.* **277**, 49055–49064.

330. Ximenes FA, Silveira QP and Philho EXF (1996). Production of β-xylosidase activity by *Trichoderma harzianum* strains. *Curr. Microbiol.* **33**, 71-77.

331. Xiong H, von Weymarn N, Leisola M and Turunen O (2004). Influence of pH on the production of xylanases by *Trichoderma reesei* Rut C-30. *Process Biochem.* **39**, 731-736.

332. Xu Yin and Foong FC (2008). Characterisation of a cellulose binding domain from *Clostridium cellulovorans* endoglucanase-xylanase D and its use as a fusion partner for soluble protein expression in *Escherichia coli*. *J. Biotechnol.* **135**, 319–325.

333. Xu Z, Wang Q, Jiang Z, Yang X and Ji Y (2007). Enzymatic hydrolysis of pretreated soybean straw. *Biomass and Bioenergy* **31**, 162-167.

334. Yablochkova FN, Bolotnikova OI, Mikhailova NP, Nemova NN and Ginak AI (2003). Specific features of fermentation of D-xylose and D-glucose by xylose assimilating yeasts. *Appl. Biochem. Microbiol.* **39**, 265-269.

335. Yang CH and Liu WH (2008). Purification and properties of an acetylxylan esterase from *Thermobifida fusca*. *Enzyme Microb. Technol.* **42**, 181–186.

336. Yang S, Huang Z, Jiang Z and Li L (2008). Partition and purification of a thermostable xylanase produced by *Paecilomyces thermophila* in solid-state fermentation using aqueous two-phase systems. *Process Biochem.* **43**, 56–61.

337. Yang SQ, Yan QJ, Jiang ZQ, Li LT, Tian HM and Wang YZ (2006). High-level of xylanase production by the thermophilic *Paecilomyces themophila* J18 on wheat straw in solid-state fermentation. *Bioresource Technol.* **97**, 1794–1800.

338. Yang VW, Zhuang Z, Elegir G and Jeffries TW (1995). Alkaline-active xylanase produced by an alkaliphilic *Bacillus* sp. isolated from kraft pulp. *J. Ind. Microbiol.* **15**, 434-441.

339. Yuan X, Wang J, Yao H and Venant N (2005 a). Separation and identification of endoxylanases from *Bacillus subtilis* and their actions on wheat bran insoluble dietary fibre. *Process Biochem.* **40**, 2339-2343.

340. Yuan Q, Wang J, Zhang H and Qian Z (2005 b). Effect of temperature shift on production of xylanase by *Aspergillus niger*. *Process Biochem.* **40**, 3255–3257.

341. Zeng H, Xue Y, Peng T and Shao W (2007). Properties of xylanolytic enzyme system in *Bifidobacteria* and their effects on the utilisation of xylooligosaccharides. *Food chem.* **101**, 1172-1177.

342. Zheng L, Du Y and Zhang J (2000). Biobleaching effect of xylanase preparation from an alkalophilic *Bacillus* sp. on ramie fibers. *Biotechnol. Lett.* **22**, 1363-1367.

343. Zhou C, Bai J, Deng S, Wang J, Zhu J, Wu M and Wang Wu (2008). Cloning of a xylanase gene from *Aspergillus usamii* and its expression in *Escherichia coli*. *Bioresource Technol.* **99**, 831–838.

RESEARCH PUBLICATIONS

Research papers in refereed international journals

1. Ganguly R, **Dwivedi P** and Singh R P (2007). Production of lactic acid with loofa sponge immobilised *Rhizopus oryzae* RBU2-10. *Bioresource Technol.* **98**, 1246-1251.
2. Vivekanand V, **Dwivedi P,** Sharma A, Sabharwal N and Singh R P (2008). Enhanced delignification of mixed wood pulp by *Aspergillus fumigatus* laccase mediator system. *World J. Microbiol. Biotechnol. 24, 2799-2804.*
3. **Dwivedi P,** Vivekanand V, Ganguly R and Singh R P (2009). *Parthenium* sp. as a plant biomass for the production of alkalitolerant xylanase from mutant *Penicillium oxalicum* SAU_E-3.510 in submerged fermentation. *Biomass and Bioenergy*, **33**, 581-588.
4. **Dwivedi P**, Vivekanand V, Pareek N, Sharma A and Singh R P (2009). Bleach enhancement of mixed wood pulp by xylanase-laccase concoction derived through co-culture strategy. *Appl. Biochem. Biotechnol.* (DOI: 10.1007/s12010-009-8654-4.).
5. **Dwivedi P**, Vivekanand V, Pareek N, Sharma A and Singh R P (2009). Co-cultivation of mutant *Penicillium oxalicum* SAU_E-3.510 and *Pleurotus ostreatus* for simultaneous biosynthesis of xylanase and laccase under solid-state fermentation. *J. Appl. Microbiol.* (*Communicated*).
6. Vivekanand V, **Dwivedi P**, Pareek N and Singh R P (2009). Solid-state fermentation for laccase production by *Aspergillus fumigatus* VkJ2.4.5 using banana peel as the solid support. *World J. Microbiol. Biotechnol.* (*Communicated*).

Papers in International Conference

1. Singh R P, Kapur N and **Dwivedi P** (2005). Regulation and engineering of xylanases possibilities and implications. *International Conference on Microbial Diversity, University of Delhi, New Delhi, April 16th – 18th, p 30-31.*
2. Sharma A, Vivekanand, **Dwivedi P** and Singh R P (2007). Solid-state fermentation for gluconic acid production from sugarcane molasses by *Aspergillus niger* ARNU-4 strain employing tea waste as the novel solid support. *15th European Biomass Conference & Exhibition. Biomass for Energy, Industry and Climate Protection. , International Congress ICC Centre Berlin, Germany, May 07th -11th.*
3. **Dwivedi P**, Vivekanand, Sabharwal N and Singh R P (2007). "Fungal co-cultivation: an approach for simultaneous production of xylanase and laccase under submerged fermentation using *Parthenium* sp. as a novel plant biomass". *29th Symposium on Biotechnology for Fuels and Chemicals, Denver Adams Mark Hotel, Denver, Colorado, USA, 29th April – 2nd May, 2007.*
4. Vivekanand, **Dwivedi P**, Sabharwal N and Singh R P (2007). "SSF: a novel strategy for enhanced production of laccase by mutant *Aspergillus fumigatus* VkJ2-4.5 using banana peel as an ideal solid support". *29th Symposium on Biotechnology for Fuels and Chemicals, Denver Adams Mark Hotel, Denver, Colorado, USA, 29th April – 2nd May, 2007.*
5. Pareek N, **Dwivedi P**, Vivekanand V and Singh R P (2009). Chitin deacetylase from *Penicillium oxalicum* ITCC 6965: A novel enzyme for production of chitosan. *3rd Congress of European Microbiologists-FEMS 2009, Gothenburg, Sweden, June 28-July 02, 2009* (*Accepted*).
6. **Dwivedi P**, Vivekanand V, Pareek N and Singh R P (2009). An intermittent rotating drum bioreactor for the production of xylanase-laccase concoction through co-cultivation under solid-state fermentation. *3rd Congress of European*

Microbiologists-FEMS 2009, Gothenburg, Sweden, June 28- July 02, 2009. (*Accepted*).

Papers in National Conference

1. **Dwivedi P**, Vivekanand and Singh R P (2006). Alkalitolerant, cellulase free xylanase from *P. oxalicum* SAU_E-3.510 using cheaper lignocellulosic materials. *National Symposium Biohorizon-2006, IIT Delhi, New Delhi, India, March 10th -11th, p, 3.*

   (This poster got First Prize in Technical Poster Session 1 at National Symposium Biohorizon-2006, IIT Delhi, New Delhi, India.)

Chapters in Book

1. Singh R P, **Dwivedi P**, Vivekanand and Kapur N (2007). Xylanases: structure, molecular cloning and regulation of expression. *In: Lignocellulose Biotechnology: Future Prospects, (Kuhad R C & Singh A eds.) I K international New Delhi, India, pp: 149-161.*
2. Singh R P, Vivekanand and **Dwivedi P** (2009). "Laccase Regulation and Laccase Dependent Bioremediation"; *In: "Biotechnological Applications of Microorganisms - A Techno-Commercial Approach", (Maheshwari D K & Dubey R C eds.) I K International, New Delhi, India (In Press).*