

**STUDIES ON THE MICROBIAL PRODUCTION 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

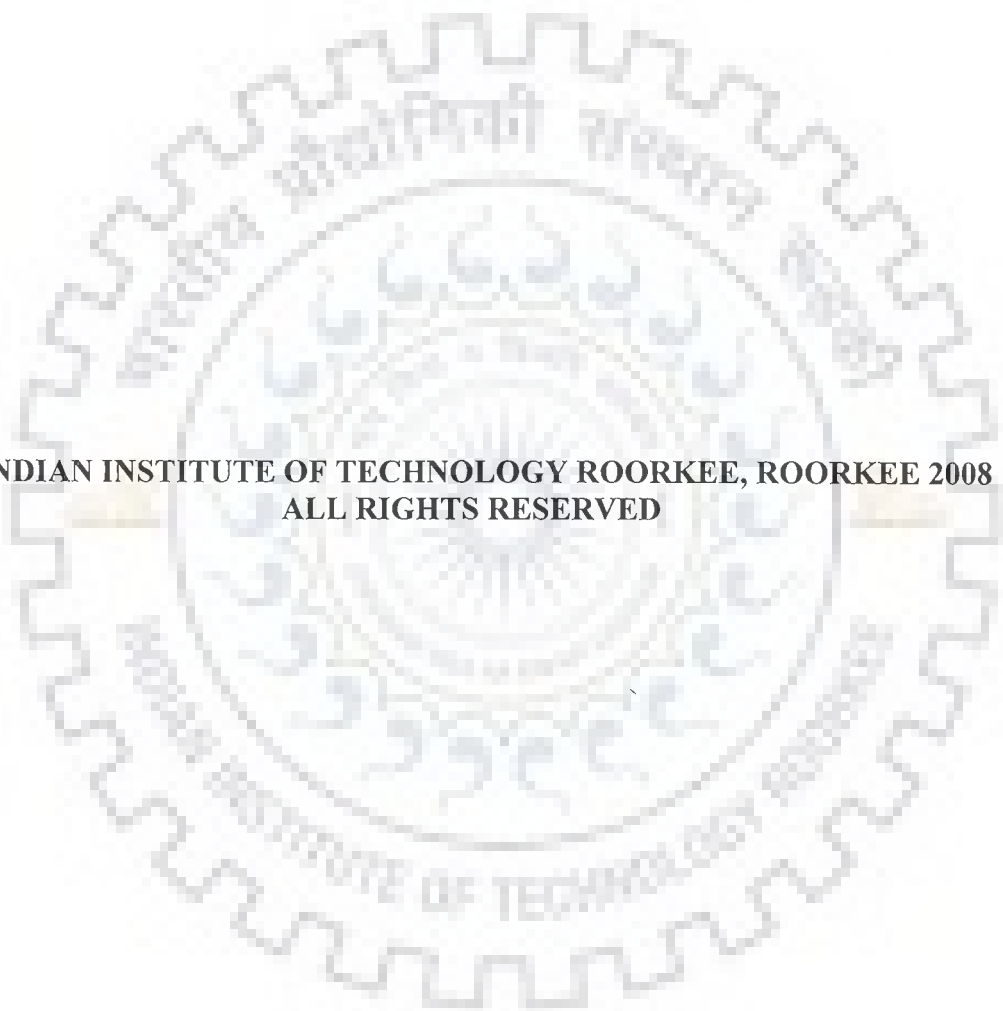
by

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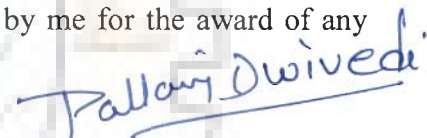


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CANDIDATE'S DECLARATION

I hereby certify that the work which is being presented in the thesis entitled **STUDIES ON THE MICROBIAL PRODUCTION OF 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 November 2008 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)

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: 26th November, 2008

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

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* from 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 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 with possibly having a better ability for biobleaching applications, attempts were made to develop a xylanase-laccase concoction by using the strategy of 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 investigated, 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, 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 coloring 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. Comparable levels of scaled-up SSF process was accomplished and significantly higher levels of xylanase and laccase were produced.

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

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Pallavi Dwivedi

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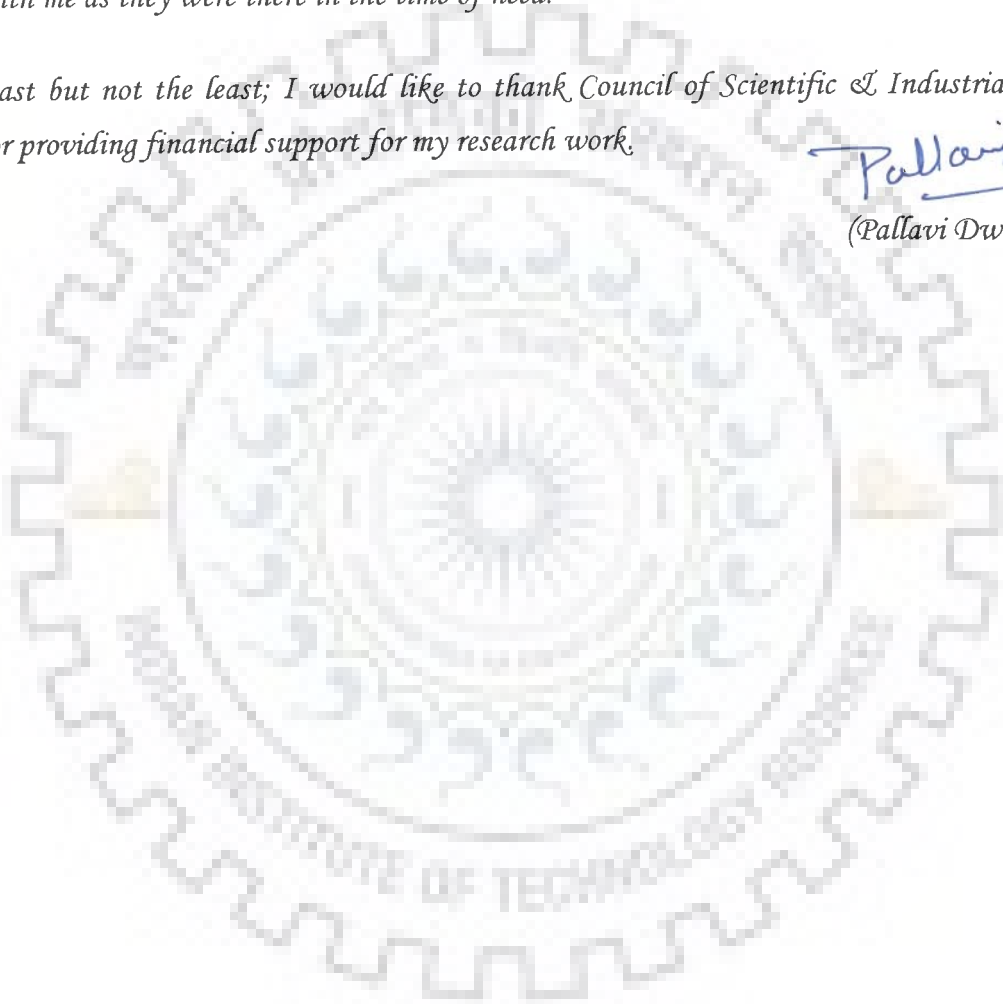


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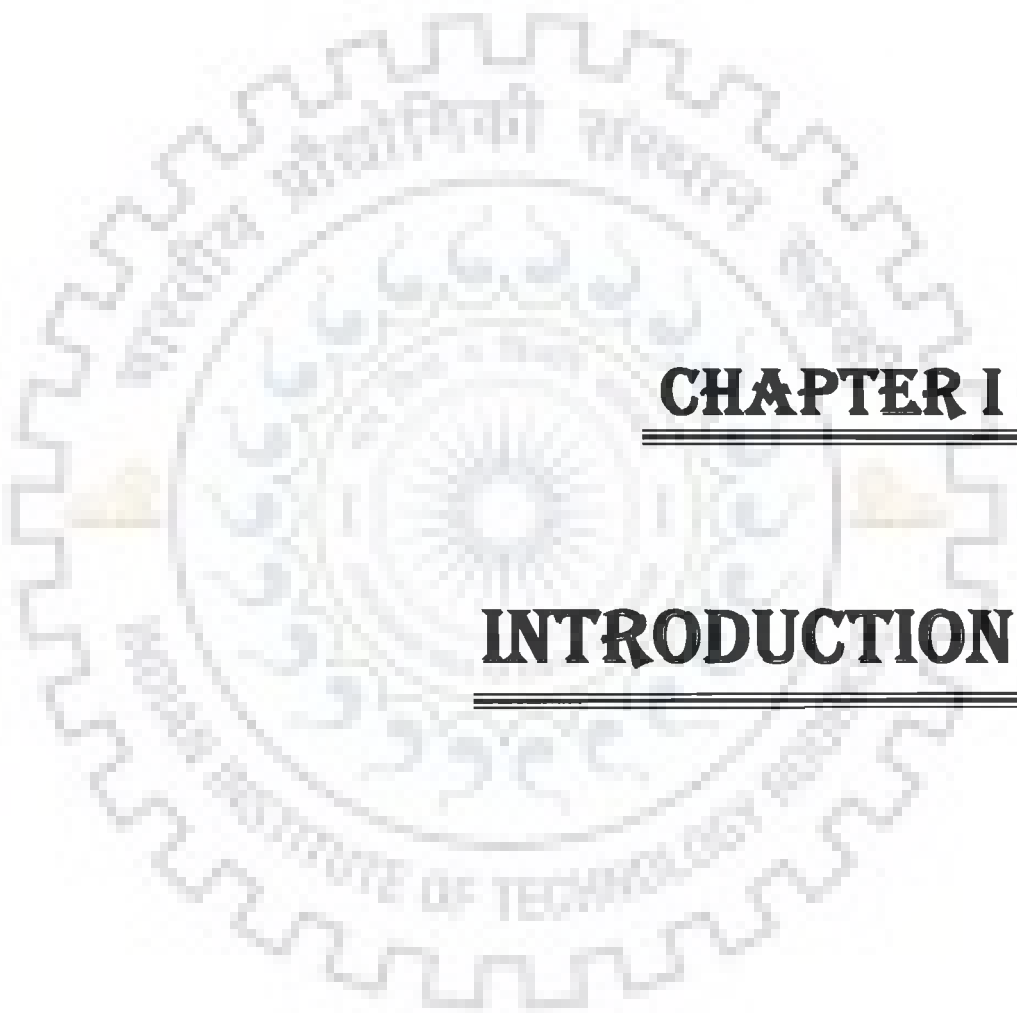
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ABBREVIATIONS



| | |
|--------------------|--|
| ANOVA | Analysis of Variance |
| AOX | Adsorbable Organic Halide |
| bp | Base Pair |
| b- θ | Number of Block |
| COD | Chemical Oxygen Demand |
| $^{\circ}\text{C}$ | Degree Celsius |
| Cl_2 | Chlorine |
| cm | Centimeter |
| df | Degree of Freedom |
| DNA | Deoxyribonucleic acid |
| DNS | Dinitrosalicylic Acid |
| DTT | Dithiothreitol |
| ECF | Elemental Chlorine Free |
| FTIR | Fourier Transform Infrared Spectroscopy |
| g | Gram |
| hrs | Hour |
| HBT | N-hydroxybenzotriazole |
| HPLC | High Performance Liquid Chromatography |
| IAA | Iodoacetamide |
| 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 Desorption/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

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 account together with cellulases and pectinases, 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 in which lignin is removed from pulp which is responsible for the colour of pulp. Up to now paper industries

rely mainly upon chlorine and other chlorine based chemicals for pulp bleaching and release huge amount 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 therefore it 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 fibers, 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 system. 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 and 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 fibers 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 titer, multifunctional and extracellular nature of enzyme. In addition to this, fungi typically produce several accessory xylanolytic enzymes, which are necessary for 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 utilization. A major share of overall production cost corresponds to substrate, therefore, rather expensive, commercially available purified xylan are not the appropriate inducers for large scale production of enzyme. Although, these can be excellent substrates and are frequently used for laboratory scale preparations and result into 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.

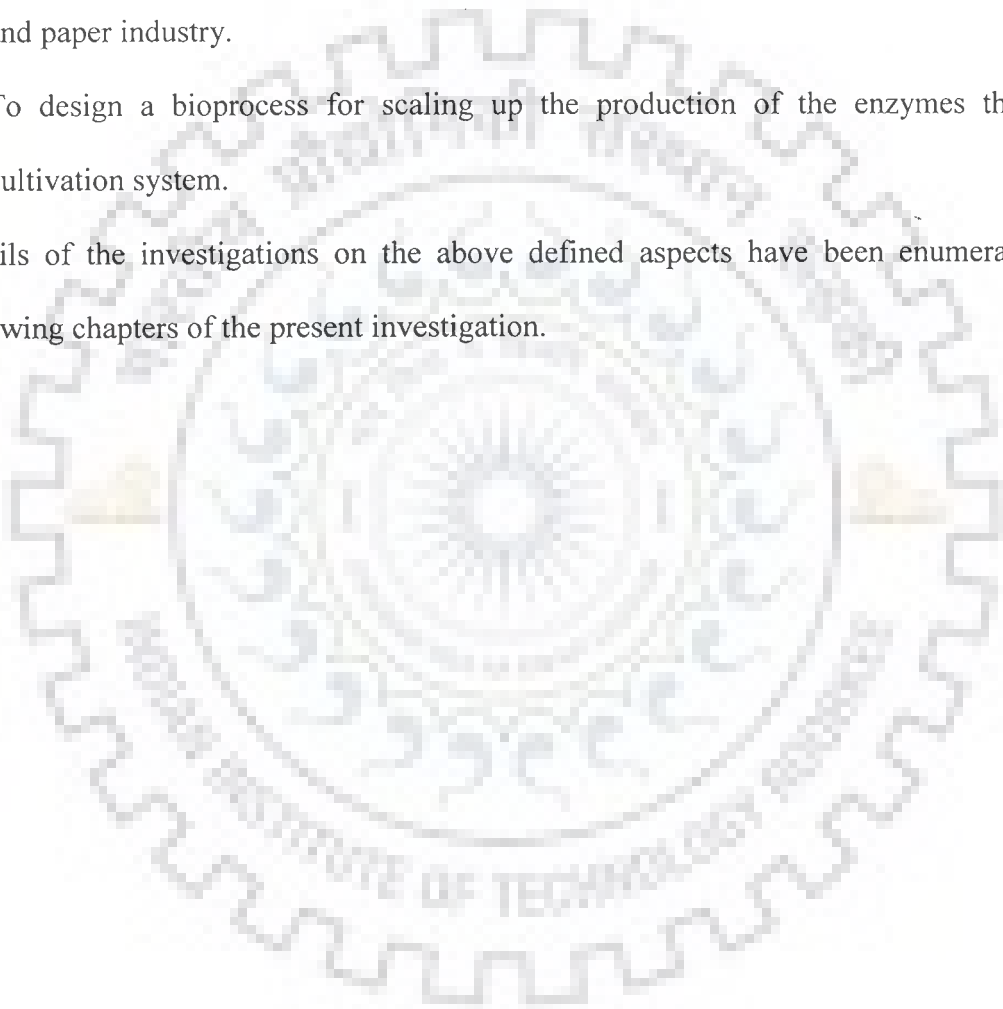
Superior productivity, improved product recovery, reduced risk of contamination, low capital investment, simple technique, reduced energy requirement, closeness to the natural way of life for many microorganisms, low waste water output are some advantages of solid-state fermentation (SSF) over submerged 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. Utilization 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 grade 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).

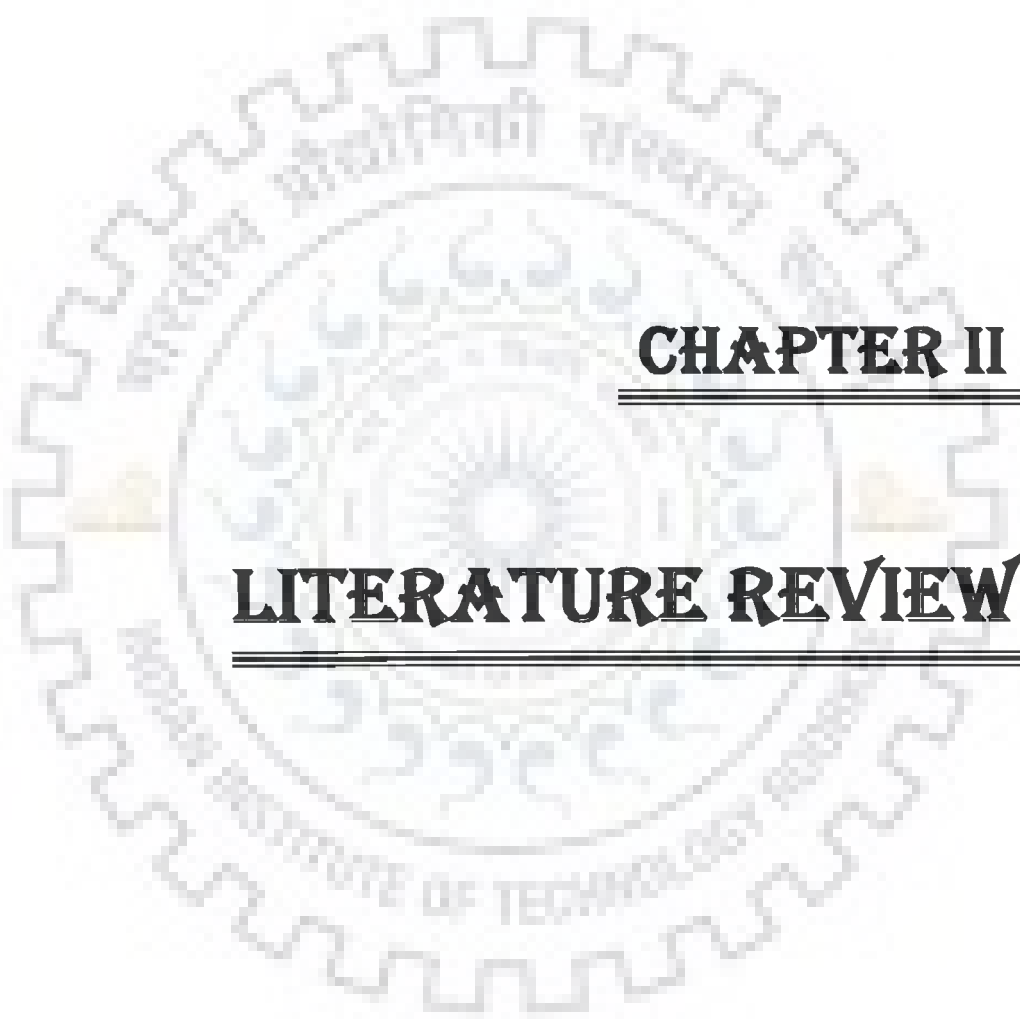
In order to save the environment from hazards of the chemical bleaching, an approach involving use of biological agent has been the major aim and further so as to develop a system for over production of the high quality xylanases, and to analyze the preparation for its application in biobleaching process.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

- a. Exploration of micro flora 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 characterization 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

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 begun 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 linkage and has covalent linkage 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 p-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 difference between hardwood and softwood xylan

| S. No. | Properties | Hardwood Xylan | Softwood Xylan |
|--------|--------------------------|---|--|
| 1. | Degree of polymerization | Higher degree of polymerization (150-200 β -D-xylopyranosyl residues) | Lower degree of polymerization (D70-130 β -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 is required. 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 p-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 polymerization of the substrate and release of xylooligosaccharides. The site of enzymatic hydrolysis depends on the nature of the substrate molecule i.e. on the chain length, the 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 hydrolyzed to xylotriase, 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 xylotriase, 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), *Bacillus* sp. (Mamo et al., 2006 ; Battan et al., 2007), *Streptomyces* sp. (Beg et al., 2000), *Streptomyces olivaceoviridis* A1 (Wang et al., 2007), *Staphylococcus* sp. SG-13 (Gupta et al., 2001), and many other microorganisms (Kolenova et al., 2005; Lin et al., 2005).

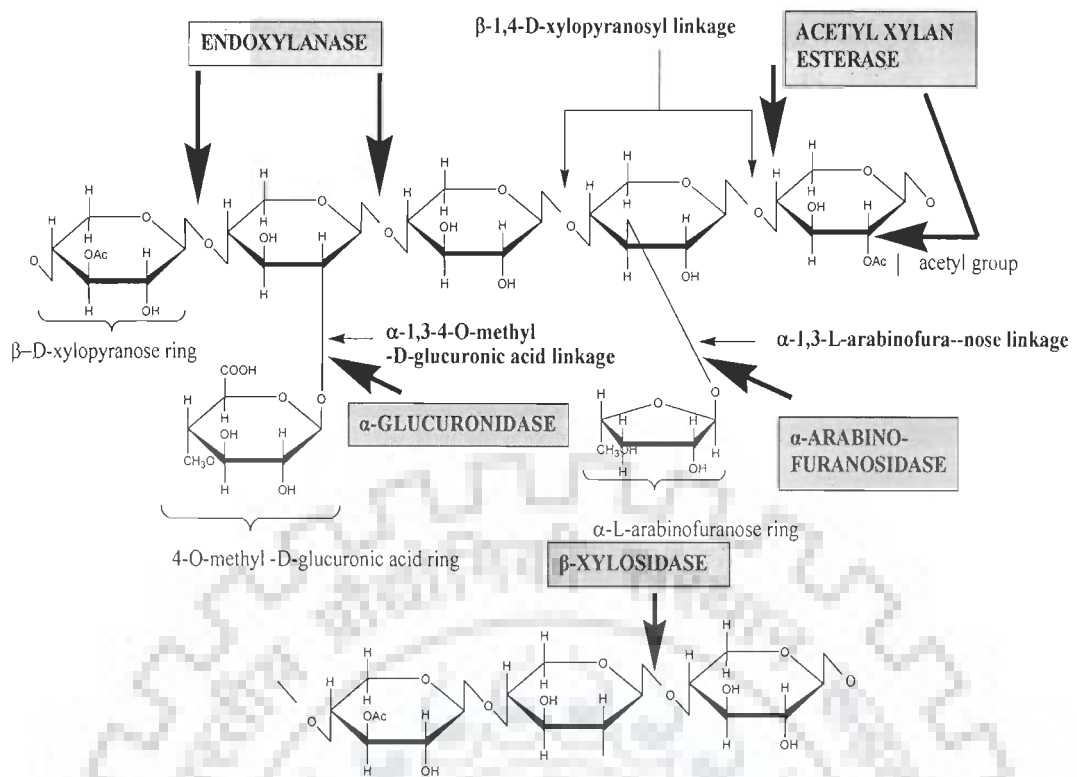


Fig. 1 Structure of xylan and various enzymes of xylanolytic system

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

β-D-xylosidases hydrolyze smaller xylooligosaccharides and xylobiose from the non-reducing ends and liberate monomeric xylose (Wong et al., 1988). Purified β-xylosidases usually do not hydrolyze xylan; their preferred substrate is xylobiose and their affinity for xylooligosaccharides is inversely proportional to their degree of polymerization. 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 into 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), *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 a 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 (Asn⁸³-Thr-Thr and Asn²⁰²-Ser-Thr) were found in the *AkAbf54* sequence. Asn⁸³ and Asn²⁰², 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 considerable decrease in thermostability as compared to the glycosylated wild-type enzyme. These observations suggest that the glycosylation at Asn²⁰² 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) hydrolyzes 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). 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, catalyzed 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 interact 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 oxocarbonium 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.

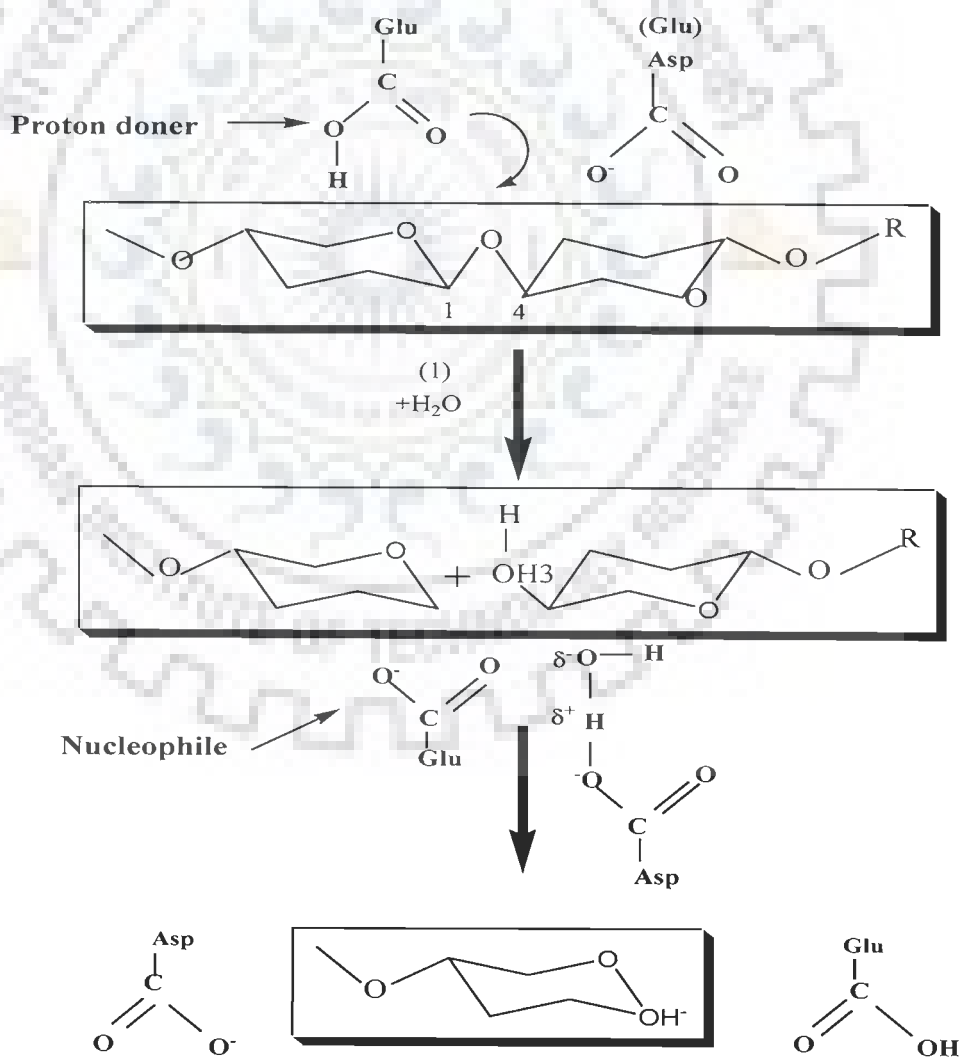


Fig. 2 Xylanase mediated catalysis by single displacement mechanism

2.3 SOURCE OF XYLANASES

Presence of xylanase in groups of organisms including bacteria, algae, fungi, plants, protozoans, gastropods and anthropods confirm their widespread occurrence among living world (Dekker and Richards, 1976; Prade, 1995). 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 xylanase production 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, includes, *Clostridium absonum* CFR-702 (Swaroop 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 titer and mainly being extracellular hence having its 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 the xylanase production from fungi mainly *Penicillium capsulatum* (Filho et al., 1996), *Fusarium oxysporum* (Christakopoulos et al., 1996), *Aspergillus foetidus* (Shah and Madamwar, 2005), *Ceriporiopsis subvermispota* (Milagres et al., 2005), *Trichoderma viride* (Liu et al., 2006), *A. carneus* (Fang et al., 2007), *A. niger* (Tapingkae et al., 2007), *Fusarium graminearum* (Carapito et al., 2009) and *Trichoderma longibraghiatum* (Kadi and Crouzet, 2008).

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). *Trichosporon cutaneum* (Liu et al., 1999) and *Aurobasidium pullulans* had also been observed to produce extracellular endoxylanase (Leathers et al., 1984).

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 (Swaroop Rani and Nand, 2000), *C. acetobutylicum* (Marichamy and Mattiason, 2005), *Sreptomycetes 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 reorganization 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, characterized and their corresponding cDNAs and genes had been identified from barley (Caspers et al., 2001). Two β -D-xylosidases were purified, biochemically characterized 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 had xylanases having 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) using 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 known 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 autoaggregation (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 resulting into 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 amplified 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, xylooligosaccharides, heterosaccharides of xylose and glucose and their positional isomers. Purified xylan is reported to be a best inducer for achieving maximum xylanase production in several organisms like *Bacillus polymyxa* (Pham et al., 1998), *Fusarium oxysporum* (Kuhad et al., 1998; Jorge et al., 2005), *Aspergillus awamori* (Sidenberg et al., 1998), *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 fibriosolvans* GS-113 (Utt et al., 1991; Rajoka, 2005).

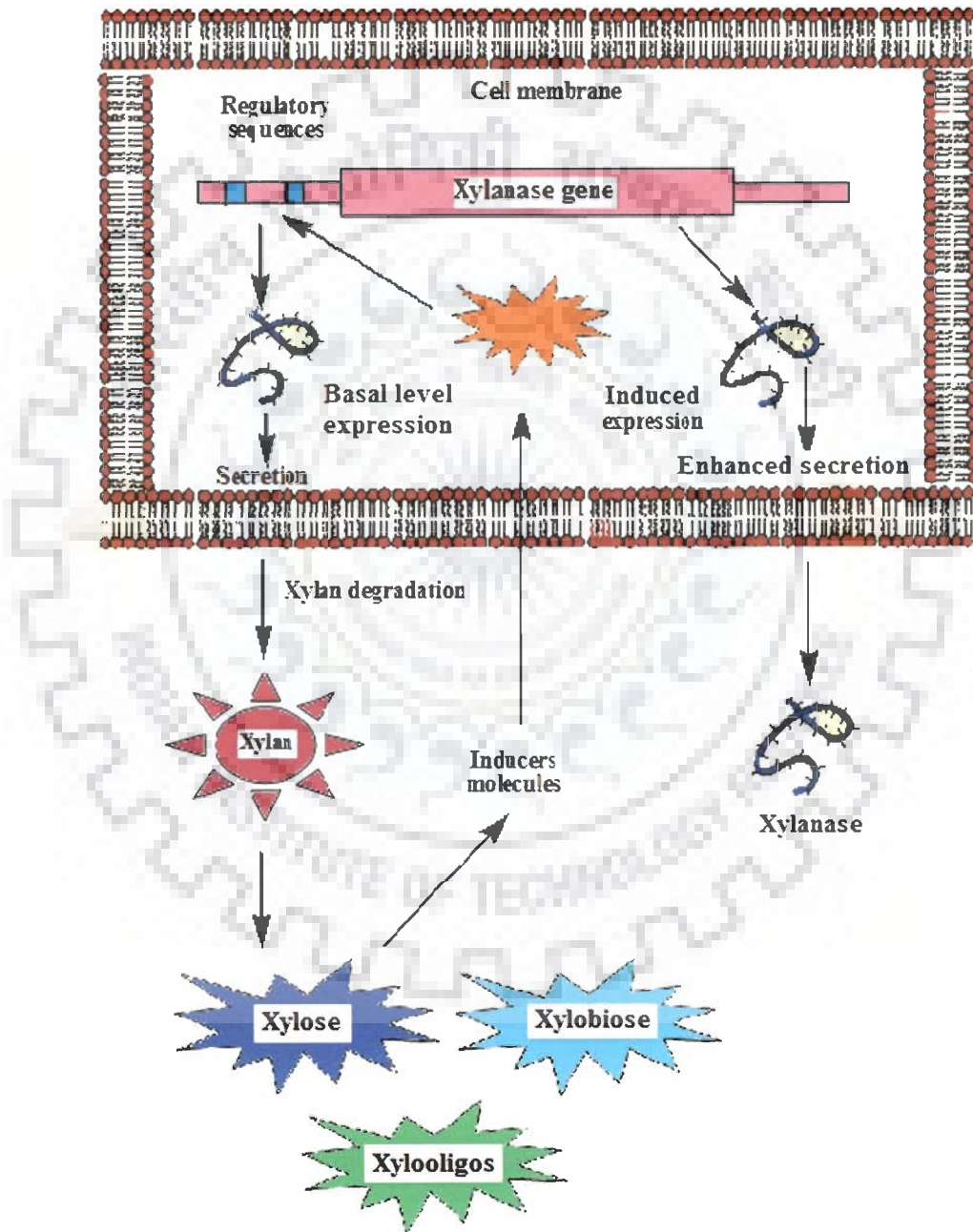


Fig. 3 Mechanism of xylanase biosynthesis

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 consists 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-G₁. 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 (TATAAAT) 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/AANC/GNTNA/TGA, where underlined letters represented the most critical bases (Kulkarni et al., 1999).

2.7 APPROCHES 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 obtaining improved level of production of xylanase (Singh et al., 1995; Mayorga-Reyes and Ponce-Noyola, 1998; Bakalova et al., 2002), glucose-6-phosphate dehydrogenase (Liu et al., 2003), citric acid (Ikram-ul et al., 2004) and ethyl methane sulphonate for achieving improved level 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 enhancement in laccase and cellulase levels by Dhawan et al., (2003) and Chand et al., (2005) respectively. Efforts were made with yeast (*Saccharomyces cerevisiae*) for the development of petite mutants (Pinto et al., 1975) and in *Kluyveromyces marxianus* for inulin assimilation along with β -fructosidase activity (Guiraud et al., 1987).

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 host 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 level of expression (Kobayashi et al., 1998). Xylanase gene 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) had been isolated, cloned and expressed in *E. coli*. The xylanase gene from a thermophilic bacterium *Thermatoga maritima* (*xynB*) was cloned and overexpressed 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 *AOXI* 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 behavior. Mixed fungal associations had resulted into higher biosynthetic activity that had led into improved levels of degradation of nitrocellulose (Sharma et al., 1995), herbicide metachlor (Sanyal and Kulshrestha, 2003), lignin (Ferreira Gregorio et al., 2006; Chi et al., 2007) and cellulose (Duenas et al., 1995). 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 optimization

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 source, 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 alkali-tolerant 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 inducing components. Among synthetic 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 level of enzyme production by *T. harzianum* 1073 D3 (Seyis and Aksoz, 2005). Use of commercially available purified xylans is 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 utilized 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 , $(\text{NH}_4)_2\text{SO}_4$, NaNO_3 , NH_4NO_3 etc) had been analysed for microbial

production of xylanases. Higher titer 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, $(\text{NH}_4)_2\text{SO}_4$ 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 media components*

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 the 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 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 favors fungal xylanases (Kuhad et al., 1998; Qinnqhe et al., 2003; Shah and Madamwar, 2005) while higher pH (7.0-9.0) favors 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 the, 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 are developed at an optimal temperature of 30°C whereas from the cultures that are maintained at higher temperature i.e. 42°C predominantly it is detected in the cell free supernatants (Lenartovicz et al., 2003). Microbial growth under SSF condition generates significant amount of metabolic heat. However, heat removal from the substrate tends to be insufficient, leading to the establishment of temperature gradients and localized overheating of the substrate. Temperature control is more difficult in SSF processes as compared to the submerged processes. Conventionally aeration is the major approach (Raimbault, 1998; Raghavaro et al., 2003) to control the temperature. Because high 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). Mildly mixing the fermentation medium and ventilating the culture room were two effective ways to release the metabolic heat and maintain the medium temperature.

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 effect 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 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 a lower biomass concentration was observed at high 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 characterization 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 level of laccase was observed under static condition as compared to solid-state fermentation condition (Gnanamani et al., 2006). Similarly, the effect of culture conditions on cellulose production from *Chaetomium erraticum* was studied and higher level 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 characterized where homogenous conditions can be maintained and mainly pH, temperature and dissolved oxygen can be easily controlled and scaling up of the process is feasible under defined conditions. It is the well-defined system for the production of xylanolytic enzymes. Different workers have summarized the production of xylanase in submerged fermentation processes ranging from shake flasks to fermenters (Gomes et al., 1994; 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 significant volumes of liquid wastes. These factors may therefore add on to the cost of production for commercial scale 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 way of life 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 specific 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 amount at lower prices is desirable in order to make process economical. Agro-

forestry residues which fulfill these two requirements are suitable for SSF. These include crop residues such as bran and straw of wheat and rice, hull of soy, corn and rice, bagasse of sugarcane and cassava, residues of coffee processing industry such as coffee husk, residues of fruit processing industries such as wastes of pine-apple and carrot processing, banana waste, and others such as saw dust, corn pods, tea waste, chicory roots etc. A significantly higher yield of xylanase had been 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 (water activity a_w) 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 desired moisture level can be accomplished by various moistening agents like fermentation media, 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).

2.8.3.1.3 Particle size

Generally, smaller substrate particles provide a 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 each particular process (Pandey et al., 1999).

2.9 BIOPROCESS DESIGNING

In spite 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. 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 sterilized chamber. The characteristic design feature of packed-bed reactors is the introduction of sterilized, 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 sterilized 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 affect 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 to 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, nutraceutical and pharmaceuticals. A large number of bacteria, fungi and yeast are known like *Pichia stipitis* (Nigam, 2002) *Pachysolen tannophilus* (Yablochkova et al., 2003) and *Candida shehate* (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 favorable 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 microflora. 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, antiinfection and antiinflammatory 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 degrade hemicellulose in wheat flour, leaving the dough softer and easier to knead (Si and Lustenberger, 2002; Baillet et al., 2003). The use of xylanases, increase 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 viscosity of the ingested feed, interfere with the mobility and absorption of other components and therefore decreasing 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 lead to decreased viscosity and improved mineral absorption in digestive tract. Moreover enzyme fortified diet is found to reduce unwanted residues in the excreta (phosphorus, 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 fibers 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 cause threat to the environment but also attack nonspecifically on cellulose of the fiber leading to weight and strength loss. Replacement of these harsh chemicals with enzyme accelerates the degradation of noncellulolytic 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 there are very few reports on xylanases for the purpose of desizing and scouring. An alkalothermophilic xylanase from *Bacillus stearothermophilus* SDX was evaluated for its application in fiber processing (Dhiman et al., 2008). In combination with cellulases, amylases and pectinases, xylanases find extensive application in fruit juice clarification and wine making. By hydrolyzing arabinoxylan and starch portion from raw material, it facilitates liquefaction of fruit and vegetable, stabilization 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 hydrolyzed releasing long chain arabinoxylans, which increases the viscosity of the beer rendering it “muddy” in appearance. Thus, xylanases are used to hydrolyze the releasing 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, 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. As on 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 | Phillipines | 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 of conventional bleaching

To minimize the chlorine or chlorine compounds from bleaching sequences, alternative bleaching strategies have been developed. Replacement of chlorine with chlorine dioxide is characterized 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 fibers. So a, simple, environment friendly and economical alternative of chemical bleaching is the “biobleaching”. The process of removal of lignin with the help of microbes or microbial enzymes is referred as Biobleaching.

2.10.2 BIOBLEACHING: a promising alternative

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

2.10.2.1 *Xylanases*

Xylanase hydrolyze 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 on the xylan which gets reprecipitated on the surface of pulp fibers during chemical pulping therefore 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 most 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 color 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 hard woods and 10-15% for soft woods can be accomplished, if the pulp is pretreated with the xylanase enzyme. The pulp thus obtained had 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 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, manganese peroxidase 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, enzyme directly and specifically attacks on lignin, oxidizes it and makes it water soluble. Laccase which is a major enzyme of the ligninolytic enzyme system, specially molest the phenolic groups by concomitant reduction of oxygen to water through intermediate formation of phenoxy radicals (Niku-Paavola *et al.*, 1994). With respect to the more powerful oxidants LiP and MnP, laccase has a lower redox potential, hence it can not 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 oxidizable mediators (Bourbonnais and Paice, 1990).

Laccase preparations had 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 concoction*

Xylanase and laccase both act in different manner for achieving similar goal for removal of lignin from pulp. Although substrate is different for both enzymes, but moreover these can be utilized to act simultaneously and synergistically. Due to action of xylanase, lignin gets exposed which then could be degraded and removed simultaneously by laccases, leading therefore into improved levels of delignification. Application of a cocktail of a hydrolytic and a 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 from *Cyathus stercoreus*, these enzymes were used in combination for biobleaching purposes (Kapoor et al., 2007). Co-cultivation of two fungi, producing 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 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 catalyzed by lignin peroxidase and manganese dependent peroxidase can be catalyzed 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 that of iron porphyrins (Crestini et al., 1999).

However, there are limitations that are associated with the use of porphyrins that correspond to their instability and insolubility in aqueous environment. These porphyrins are unstable in 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 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 the 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 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 List of commercially available xylanases

| S. No. | Product name | Distributor | Microorganism | Optimum pH | Optimum temperature (° C) | Application |
|--------|---------------|--|------------------------------------|------------|---------------------------|---|
| 1. | Bel feed | Agrimex NV, Belgium | <i>Bacillus subtilis</i> | 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 | <i>Aspergillus niger</i> | na | na | Supplimentation of poultry & piglet feed |
| 4. | Allzym PT | Alltech, USA | <i>A. niger</i> | 5.3 | 65 | Upgrading of animal feed |
| 5. | Amano 90 | Amano pharmaceutical, Japan | <i>A. niger</i> | 4.5 | 50 | Pharmaceutical & food industry |
| 6. | Bio-Feed Plus | Novo-Nordisk, Denmark | <i>Humicola insolens</i> | 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 | <i>Trichoderma reesei</i> | 5.0-6.0 | 50-55 | Improving the bleachability of soft wood & hard wood kraft pulp |
| 10. | Irgazyme 40 | Nalco-Genencor, Ciba-Geigy | <i>Trichoderma longibrachiatum</i> | na | na | Pulp & paper industry, animal feed |
| 11. | Multifect XL | Genencor | <i>Trichoderma longibrachiatum</i> | 5.0-5.5 | 55-60 | Food industry |
| 12. | Pulpzyme | Novozymes, Denmark | <i>Bacillus sp</i> | 9.5 | 50 | Paper industry |

| | | | | | | |
|-----|--------------------|---------------------------|--------------------|---------|----|---|
| 13. | Solvay pentonase | Solvay Enzymes, Germany | <i>T. reesei</i> | 5.3-5.5 | 55 | Starch processing & baking |
| 14. | Sternzym HC 46 | Stern-Enzym, Germany | <i>T. reesei</i> | 5.0 | 30 | Hydrolysis of plant raw materials, bakery, animal feed, |
| 15. | Sumizyme X | Shin Nihon, Japan | <i>T. Koningii</i> | 5.0 | 55 | Peeling of cereals, Bread making, |
| 16. | Xylanase GS35 | Iogen, Canada | <i>T. reesei</i> | 4.5 | 40 | Pulp cleaning & processing |
| 17. | Xylanase 250 | Hankyu bioindustry, Japan | <i>T. viridae</i> | 4.5 | 40 | Macerating fruits & vegetables |
| 18. | Hemi cellulase 100 | Hankyu bioindustry, Japan | <i>A. niger</i> | 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 have 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

| S. 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 | <i>Pseudomonas stutzeri</i> ' strain and process for preparation of xylanase |
| 10. | EPI479765 | 24.11.2004 | Enzymes with xylanase activity from <i>A. aculeatus</i> |
| 11. | WO 2004/092479 | 28.10.2004 | Enzymatic treatment of paper making pulps |
| 12. | US2004 6768001 | 27.07.2004 | Xylanase from <i>Trichoderma reesei</i> , 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

MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Chemicals and other materials

Xylan, xylose, ρ -nitrophenyl - β - D - xylopyranoside, 2,2'-azino - bis (3-ethylbenzothiazoline-6-sulfonic acid) ammonium salt (ABTS) were purchased from Sigma Chemical 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, saw dust, 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 was isolated from the waste of soda ash industry ('TATA Chemicals', Meethapur Gujrat, India) and was identified from 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 Microbial Type Culture Collection Bank, Institute of Microbial Technology, Chandigarh. The strains were maintained on PDA slants, stored at 4°C and renewed 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 strain

A total of 18 different fungal strains were isolated from the waste of soda ash industry and 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 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. Purified 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 the xylanase was carried out on xylan-agar plate 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 had resulted into the clear zone which was followed by staining with 0.1% congo red then destaining with 1M NaCl (Teather and Wood, 1982). Controls with heat killed (100°C, 20 minutes) supernatant did not produce any clear 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). Three ml aliquot of spore suspension (3.5×10^6 spores ml^{-1}) of five day old culture of *P. oxalicum* SA-8 was exposed to UV light ($2.5 \text{ Jm}^{-2}\text{s}^{-1}$, distance 0.70 m) in sterile Petri dishes (9 cm diav) for different time periods ranging from 30 sec to 10 minutes and stored in dark at 4°C for 24 h to avoid photorepair. Following serial dilutions, treated spore suspension was inoculated on potato dextrose agar and xylan agar media to detect their survivability and xylanase producing ability respectively. Colonies showing higher zone diameter on xylan agar plate as compared to that obtained from the wild type strain were picked and subjected to quantitative estimation of xylanase spectrophotometrically. 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 $150 \mu\text{g ml}^{-1}$ 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 higher zone diameter on xylan agar plate as compared to that of wild type strain were picked up and analyzed for their xylanase activity quantitatively. Control conidial suspension was also treated identically excluding 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 (35×10^5 spores ml^{-1}

¹) was treated with 200 $\mu\text{g ml}^{-1}$ 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-plate (stained with 0.1% Congo-red) respectively. Colonies showing higher zone diameter on xylan agar plate as compared to parent were picked and analyzed for their xylanase activity quantitatively. A parallel control was also maintained where conidia were not exposed to EtBr treatment.

3.3.2 Mixed mutagenesis

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

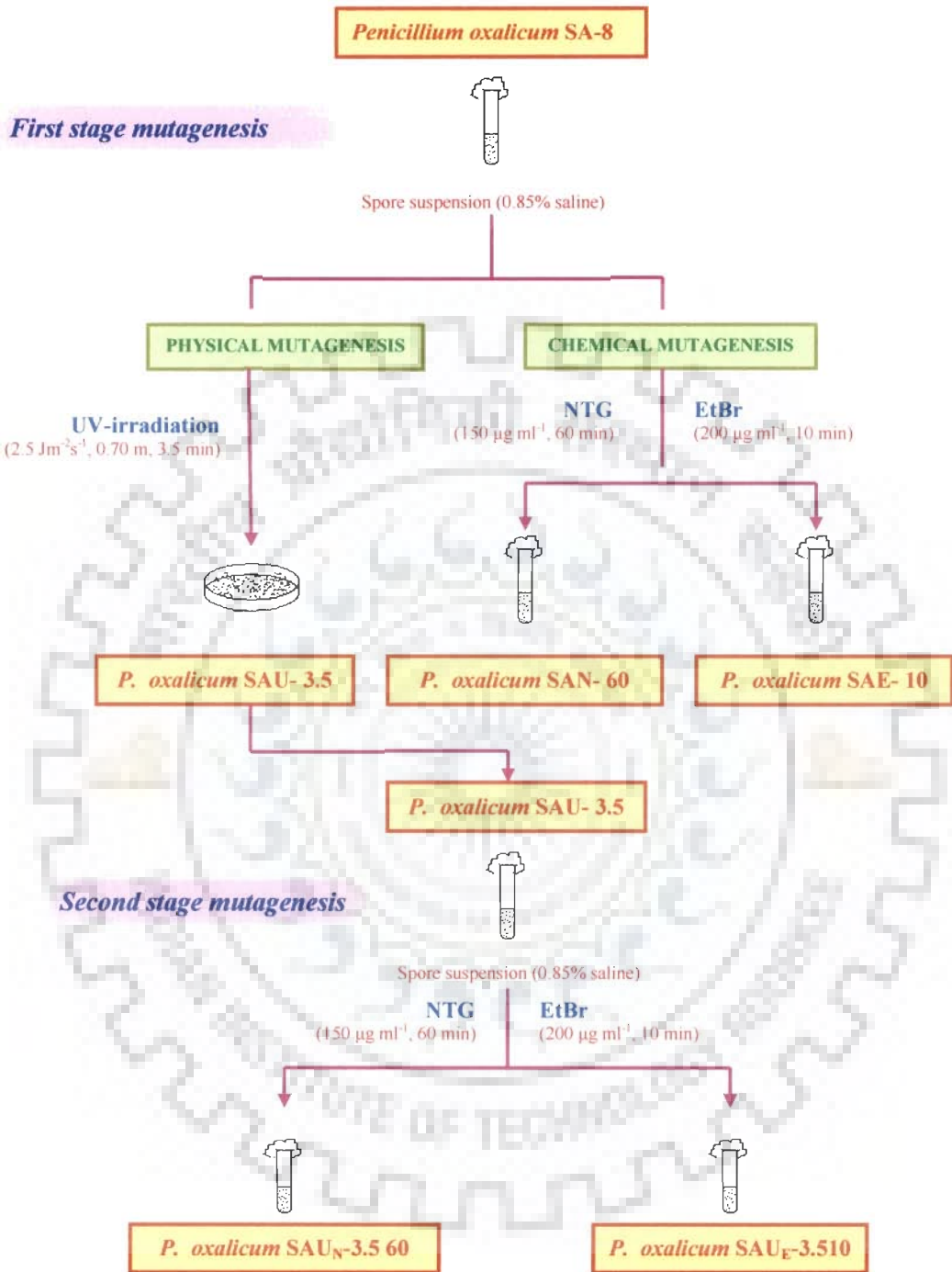


Fig. 4 Mutagenesis of *P. oxalicum* SA-8 for xylanase production

3.4 FERMENTATION MEDIUM

3.4.1 Xylanase production

Preliminary studies on xylanase production from mutant *P. oxalicum* SAU_E-3.510 as well as from 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): xylan, 0.5%; KH₂PO₄, 0.7%; K₂HPO₄, 0.2%; MgSO₄.7H₂O, 0.05%; (NH₄)₂SO₄, 0.1% and yeast extract, 0.06% (Medeiros *et al.*, 2003). The pH of fermentation medium was adjusted to 9.0 with 2N NaOH followed by autoclaving the medium (121°C, 15 minutes). The medium was then inoculated with fungi and the flasks were then incubated (30 °C, 150 rpm) for six days for xylanase production.

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 media as described (Koroleva *et al.*, 2002), that contained: glucose, 1.0%; peptone, 0.3%; KH₂PO₄, 0.06%; K₂HPO₄, 0.04%; MgSO₄, 0.05%; ZnSO₄, 0.0001%; FeSO₄, 0.00005%. and MnSO₄, 0.005%. After sterilization, the fermentation media were inoculated with fungi and incubated (30 °C, 150 rpm) for twenty days for laccase production. Samples were taken on every second day and used for measuring the enzyme activity.

3.4.3 Xylanase-Laccase mixture through dual culture

The media used for growth and enzyme production by co-culturing of fungi included: Glucose, 1.0%; KH₂PO₄, 0.7%; K₂HPO₄, 0.2%; MgSO₄.7H₂O, 0.05%; (NH₄)₂SO₄, 0.1%; yeast extract, 0.06%; ZnSO₄, 0.0001%; FeSO₄, 0.00005%; MnSO₄, 0.005% and xylan, 0.25% which was added on 6th day of incubation under aseptic conditions. The pH of fermentation media was adjusted to 9.0. Media was inoculated with *P. ostreatus* MTCC 1804, after 6 days of incubation, medium was further inoculated with mutant *P. oxalicum* SAU_E-3.510 and incubation continued to a total of 12 days (30°C, 150 rpm).



3.4.4 Pretreatment of substrate

Congress grass (*Parthenium* sp.) was collected from local areas during rainy season (July-September) prior to stage of flowering; leaves and nascent buds were removed. Fresh water hyacinth plants (*Eichhornia crassipes*) with long stem 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 (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 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 hours and steamed (60 min) thereafter. 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 thus obtained following pretreatment process.

3.5 PLATE CULTIVATION AND FUNGAL COMPATIBILITY ASSAY

For testing compatibility among fungi, both white rot fungi and mutant *P. oxalicum* SAU_E-3.510, were inoculated aseptically on 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 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 Submerged fermentation process

Fermentation medium (50 ml) in 250 ml erlenmeyer flask was inoculated with *P. ostreatus* and incubated (30 °C, 150 rpm). Mutant *P. oxalicum* SAU_E-3.510 was inoculated on

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 fermentation condition

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

3.6.3 Solid-state fermentation condition (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 actively growing culture and this was followed by inoculation with mutant *P. oxalicum* SAU_E-3.510 after 48 hrs 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 PROCESS

Solid-state fermentation that had resulted into maximum yields of xylanase using a mixture of bagasse and black gram husk in a ratio of 3:1 was subjected for scaling up the process. 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 fermentation 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 was added with 368 g of solid support (276 g bagasse and 92 g black gram husk) which was mixed with fermentation medium to have 80% of initial moisture content. The prepared slurry was sterilized 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 presterilized substrate were added to bioreactor through an opening at its left and right wall under aseptic conditions. The cylinder is connected to a geared motor to provide gentle rotation at definite time intervals (10 rpm, within an interval of 3 hrs). The intermittent rotation facilitates the proper mixing of substrate as well as fungal inoculum along with appropriate aeration. A cooling tray filled with sterile distilled water was also kept to provide humidity and to dissipate metabolic heat generated during cultivation of fungi. The complete unit was placed in an incubator to maintain the desired temperature (30 °C) for growth and enzyme production. At the end of fermentation, required amount of water was added and enzyme was extracted by rotating the cylinder (10 rpm, 60 min). The sample thus collected was subjected to filtration and centrifugation and then used for enzyme activity estimation.

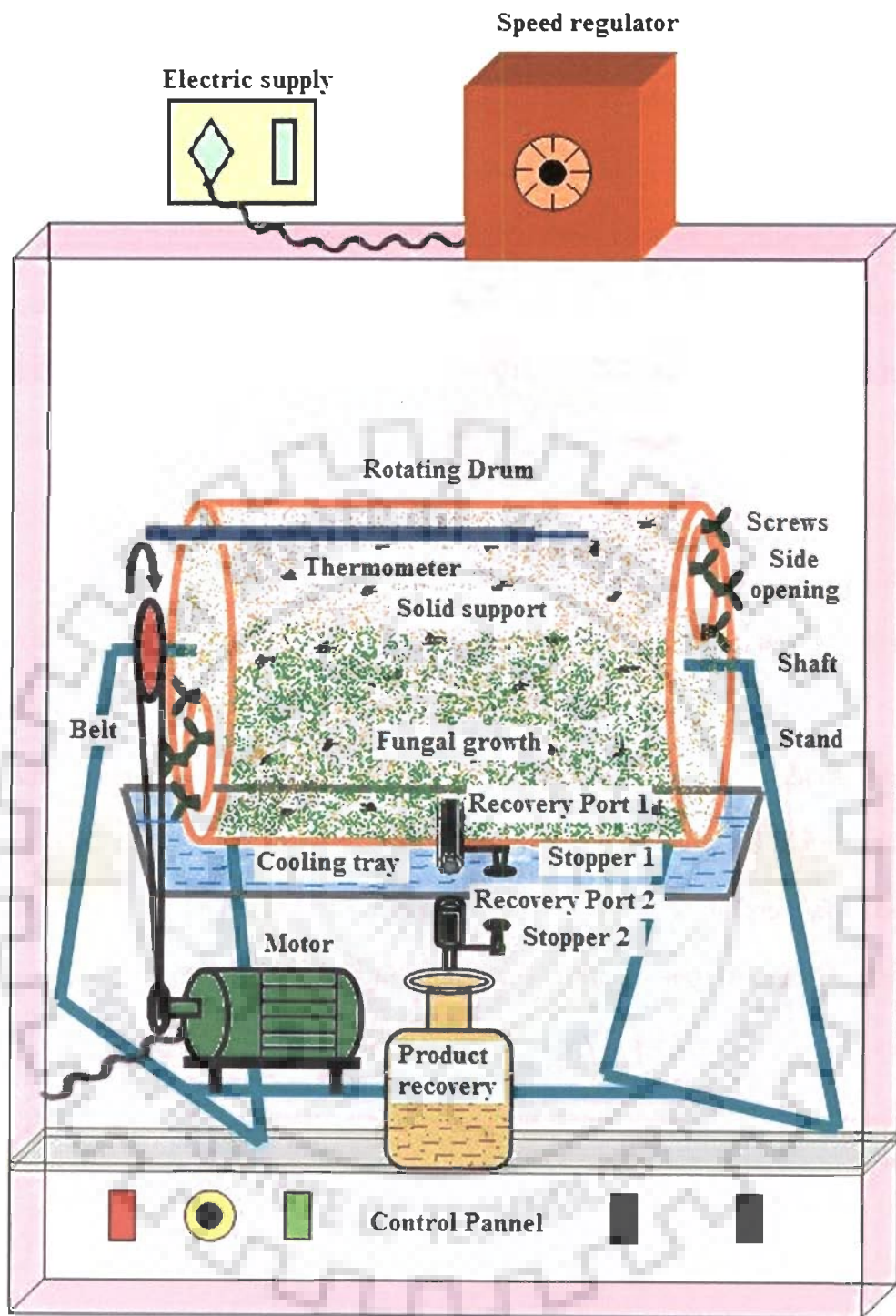


Fig. 5 Schematic representation of the intermittent rotating drum bioreactor for simultaneous biosynthesis of xylanase-laccase by co-culture of *P. oxalicum* SAU_E-3.510 and *P. ostreatus* MTCC 1804 under solid-state fermentation

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 (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 formation of paper pads for brightness measurement.

3.8.2.2 Derivation of enzyme dose

To find out the optimum dose of mixed enzyme (xylanase and laccase, 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 Derivation 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 hrs 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 a synthetic mediator for laccase enzyme.

All the experiments were performed in triplicate using 10 g of pulp, in polythene bags at 55° C, pH 9.0 and at 10% pulp consistency. After enzymatic treatment, pulp was washed with distilled water and the filtrate was 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 λ 237 nm. Hydrophobic compounds were determined by measuring the absorbance of the effluent at λ 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 λ 200 nm to λ 400 nm to determine removal of lignin. 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 analyzed according to the standard methods of TAPPI 1996 (Table 6).

Table. 5 Conditions applied during different bleaching sequences

| S. 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 ₂ O 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 ₂ O 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; Cl₂/2, Half of the added chlorine in C Stage
X, enzyme treatment; C, chlorination; E, alkali extraction; H₁, hypochlorite stage 1; H₂, hypochlorite stage 2; P₁, peroxide stage 1; P₂, peroxide stage 2; O, oxygen; D₁, chlorine dioxide stage 1; D₂, chlorine dioxide stage 2

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

| S. 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

Xylanase was assayed by mixing the 500 μ l 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 dinitrosalicylic method (Miller, 1959). One unit of enzyme corresponds to one μ mol of xylose released per minute under the assay conditions.

3.9.2 Estimation of β -xylosidase

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 *p*-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 Na₂CO₃/NaHCO₃ 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 *p*-nitrophenol per minute under the assay conditions.

3.9.3 Estimation of cellulase

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 was expressed as one μ mol of glucose liberated per minute of the reaction under above defined conditions.

3.9.4 Estimation of laccase

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 Glycine NaOH buffer at pH 9.0, enzyme extract and 1.0 mM of ABTS in a total volume of 1 ml. Reaction was monitored

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

3.9.5 Biomass estimation

Growth was evaluated 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 CHARACTERIZATION

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 hrs.

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 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 was determined after incubating the enzyme in the buffers described above for 24 hrs at room temperature and measuring the residual activity after every four hrs.

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. Mixture was degassed for 15 min, ammonium persulphate and TEMED were added to it. The mixture was gently mixed and poured between the plates, and leaving the required space at the top for pouring the stacking gel. After polymerization of the resolving gel, the assembly was tilted to pour off the over layer, washed with sterile distilled water and wiped gently to dry the empty space. Stacking gel mixture was then similarly

degassed and layered on the resolving gel; comb was immediately inserted into the mixture to form the wells. After polymerization of stacking gel, comb was carefully removed, wells formed were rinsed with reservoir buffer and the polymerized gel was used for electrophoresis.

3.10.3.3 *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 μ g of the protein) were gently loaded on the gel using a micropipette. Electrophoresis was carried out at constant voltage (100 V) until the tracking dye reached to about 1 cm above from the bottom of the gel. Gel after electrophoresis was removed and stained overnight with gentle shaking in coomassie brilliant blue R-250 0.1% in methanol: glacial acetic acid: water (5:2:5 v/v/v) at room temperature. 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 μ l) 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 -mercaptoethanol. 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 clear zone following hydrolysis. The gel was further exposed to 5% acetic acid to increase colour contrast between the hydrolysis zone and to remaining portion of gel.

For visualization of laccase activity, after electrophoresis, gel was washed with 50 mM Glycine-NaOH buffer, pH 9.0 for 5 min and then placed in 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-2% (v/v) formaldehyde (4:1) for 24 h. Following primary fixation, these were washed thrice with double distilled water. Samples were then treated with the alcohol gradients of 30%, 50%, 70%, 80%, 90% and 100% for dehydration. Samples were kept for 15 min each up to 70% alcohol gradient, thereafter treated for 30 min each for subsequent alcohol gradients. For scanning electron microscopy of unbleached and enzymatically bleached pulp, fibres were washed thrice 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 thrice and dehydrated with ethanol gradient (30-100%) (Kapoor *et al.*, 2007). After treating with 100% alcohol, samples were air dried and examined under SEM using gold shadowing technique (Gabriel, 1982). Electron photomicrographs were taken at desired magnifications.

3.10.5 Matrix-Assisted Laser Desorption/Ionization Time-of-Flight (MALDI-ToF/ToF)

Mass Spectrometry

For MALDI-ToF/ToF analysis of the protein, initially broth was concentrated using Centricon C-10 microconcentrators and then electrophoresed on native gel (10 %). The 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). Gel slice was dissolved in acetonitrile and vacuum dried. Reduction was carried out with 1% dithiothreitol

(DTT) followed by 2% iodoacetamide (IAA) modification and tryptic digestion overnight and acidified by adding 5 μ l of 10% tri-fluoroacetic acid (TFA). The digested product was mixed with α -cyano-4-hydroxycinnamic acid matrix in 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 Daltonix Autoflex ToF/ToF, Germany) in a positive mode and the N₂ laser intensity was set at λ 337 nm. The peptide fragments obtained were analyzed 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 for 45 min, at 10,000g at 4 °C. Following centrifugation samples were membrane filtrated (0.2 μ m) to remove any particulate impurities and suitably diluted using the mobile phase (Barreca et al., 2003). Reducing sugars released were analysed by using hydrocarbide column (4.6 x 250 mm), with acetonitrile: water (70:30) as mobile phase (0.5 ml min⁻¹), using an injection volume of 20 μ l and runtime of 15 minutes. A refractive index detector was used (Sa-Pereira et al., 2002). The peaks were analysed by using xylooligomers as standard. The lignin degradation products (monophenols) were analysed using Novo Pak C18 column at 40°C using a mixture of acetonitrile-water-tetramethyl ammonium hydroxide-phosphoric acid (20:80:1:1) as eluent, and 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 $2\theta = 5^\circ$ 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 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 colorless. 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 g l^{-1}) was titrated by same procedure as used for analysis for calcium hypochlorite solution (Laboratory manual, 1993).

3.10.9.3 *Analysis of H₂O₂*

10 ml of H₂O₂ 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 H₂SO₄ (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's; 2002). The end point was blue to colorless. The concentration of H₂O₂ solution was calculated as below:

Concentration of H₂O₂ solution = Normality of H₂O₂ solution X 34

3.10.9.4 Analysis of residual chlorine

Same procedure as described above was used except that 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). 1% and 5% levels for ANOVA test was used for comparing the calculated F ratio by using standard tables 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) | $\frac{SST \times bkn-k-b+1}{SSE (K-1)}$ |
| With in block (B) | SSB | b-1 | SSB/ (b-1) | $\frac{SSB \times bkn-k-b+1}{SSE (b-1)}$ |
| 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

4.1 ISOLATION, PURIFICATION AND SCREENING OF STRAINS

Total 18 xylanase producing fungal cultures were isolated initially on the basis of colour and colony appearance at 30°C. Waste from soda ash industry had resulted into 11 isolates whereas remaining 7 isolates were obtained from paper mill industry effluent. Primary screening of the isolated fungal cultures for their xylanase producing ability was performed on the basis of clear zone formation on xylan agar plates. Morphological features and 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 had resulted into zone formation with 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 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 Indian Type Culture Collection Bank, Indian Agricultural Research Institute, New Delhi, India.

Table. 8 Morphological features and zone diameter of fungal isolates

| S. No. | Isolated Strains | Site of Isolation | Mycelial Colour | Spore Colour | Zone Diameter (cm) |
|--------|------------------|-------------------------|---|---------------|--------------------|
| 1. | SA-1 | Soda ash industry waste | White hyphae, forming flat mat like colony, horizontally growing mycelia | White | 2.2 ± 0.43 |
| 2. | SA-2 | Soda ash industry waste | Pink hyphae which turned into green in later stage | Green | 3.6 ± 0.11 |
| 3. | SA-3 | Soda ash industry waste | Light green and orange in colour | Green | 3.2 ± 0.43 |
| 4. | SA-4 | Soda ash industry waste | White erect hyphae which forms dome shaped colony, it appears green at margin | - | 3.9 ± 0.08 |
| 5. | SA-5 | Soda ash industry waste | Yellowish white hyphae | Black | 4.1 ± 0.05 |
| 6. | SA-6 | Soda ash industry waste | White mycelium | Green | 2.2 ± 0.14 |
| 7. | SA-7 | Soda ash industry waste | Erect white hyphae | Pinkish green | 3.2 ± 0.14 |
| 8. | SA-8 | Soda ash industry waste | White hyphae | Light green | 4.2 ± 0.14 |
| 9. | SA-9 | Soda ash industry waste | Off white mycelium | - | 2.9 ± 0.10 |
| 10. | SA-10 | Soda ash industry waste | Transparent off white mycelium | - | 2.5 ± 0.06 |
| 11. | SA-11 | Soda ash industry waste | Light pink hyphae | - | 3.1 ± 0.17 |
| 12. | SB-1 | Paper mill effluent | Yellowish green | Pale yellow | 2.6 ± 0.08 |
| 13. | SB-2 | Paper mill effluent | White | Light brown | 3.2 ± 0.14 |
| 14. | SB-3 | Paper mill effluent | Light green with white spots | light green | 3.1 ± 0.08 |
| 15. | SB-4 | Paper mill effluent | Erect white cottony hyphae | Black | 2.6 ± 0.08 |
| 16. | SB-5 | Paper mill effluent | White | Green | 2.6 ± 0.08 |
| 17. | SB-6 | Paper mill effluent | White | Green | 2.0 ± 0.05 |
| 18. | SB-7 | Paper mill | White | Black | 2.3 ± 0.06 |

Table. 9 Enzyme production from the selected fungal strains

| S. No. | Potent Strains | Xylanase activity (IU ml ⁻¹) | β -xylosidase activity (IU ml ⁻¹) | Cellulase activity (IU ml ⁻¹) |
|--------|----------------|--|---|---|
| 1. | SA-5 | 147.90 \pm 6.94 | 2.55 \pm 0.06 | 0.34 \pm 0.02 |
| 2. | SA-8 | 242.05 \pm 3.91 | 16.20 \pm 2.00 | 0.18 \pm 0.03 |
| 3. | SB-2 | 152.08 \pm 5.29 | 3.96 \pm 0.80 | 0.21 \pm 0.02 |
| 4. | SB-3 | 135.56 \pm 3.93 | 13.13 \pm 1.20 | 0.18 \pm 0.02 |

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

P. oxalicum SA-8 strain, 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 attempt 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 had shown the maximum zone formation (4.7 cm diameter) on xylan agar plate. Out of 88.9% spore survived following 3.5 min of UV exposure, 13% had the higher zone diameter as compared to the parent strain and were termed as positive mutants, whereas 8% of the colonies had the lesser diameter and 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 as the corresponding mutants (Fig. 6a). SAU-3.5 had produced 430.81 ± 8.33 IUml⁻¹ of xylanase under submerged fermentation. The parental strain SA-8 was also subjected to NTG (150 μ g ml⁻¹) and to EtBr (200 μ g 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 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 ($200 \mu\text{g ml}^{-1}$) had a zone diameter of 4.7 cm with 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 had resulted into comparatively higher zone diameter and had the maximum increase (78%) in xylanase production ability. Mutant SAU-3.5 was further subjected to second round of mutagenesis. Mutant SAU_E-3.510 obtained following EtBr exposure had resulted into a further increase in enzyme production level ($453.02 \pm 5.70 \text{ IUml}^{-1}$) (Table-10). Of total mutants thus obtained, 9%, 3% and 88% were the positive, negative and corresponding mutants respectively (Fig. 6e).

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

| S. No. | Mutagen | Strain | Xylanase activity (IU ml^{-1}) | Percent increase |
|--------|---------|-------------------------|---|------------------|
| 1. | None | SA-8 | 242.05 ± 3.91 | - |
| 2. | UV | SAU-3.5 | 430.81 ± 8.33 | 78 |
| 3. | NTG | SAN-60 | 368.84 ± 4.53 | 52 |
| 4. | EtBr | SAE-10 | 390.86 ± 5.21 | 61 |
| 5. | UV+EtBr | SAU _E -3.510 | 453.02 ± 5.70 | 87 |
| 6. | UV+NTG | SAU _N -3.560 | 421.94 ± 6.81 | 74 |

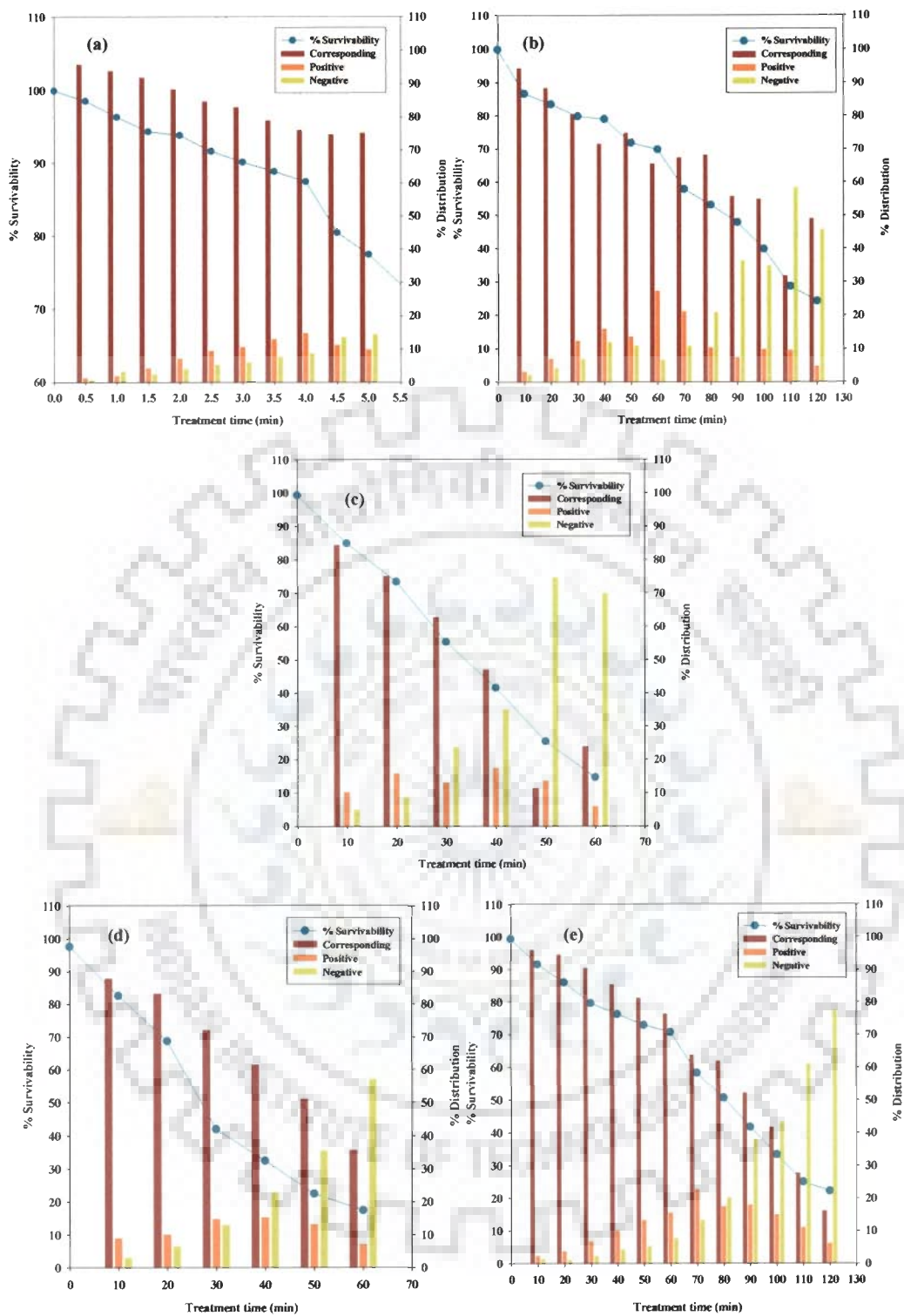


Fig. 6 Analysis of mutagenesis of *P. oxalicum* SA-8 (a) UV-irradiation; (b) N-methyl-N'-nitro-N-nitrosoguanidine; (c) Ethidium bromide; (d) Mixed (UV+ EtBr); (e) Mixed (UV + NTG)

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. Wild type and mutant *P. oxalicum*, both had white hyphae with dark green spores. However, colony obtained following growth of mutant SAU_E-3.510 had irregular margins and was flattened, compared to the smooth margins obtained following the growth of wild type strain (Fig. 7a, b). However, a remarkably higher xylanase production ability was observed from the mutant SAU_E-3.510 as compared to the wild type strain (Fig. 7c, d).

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

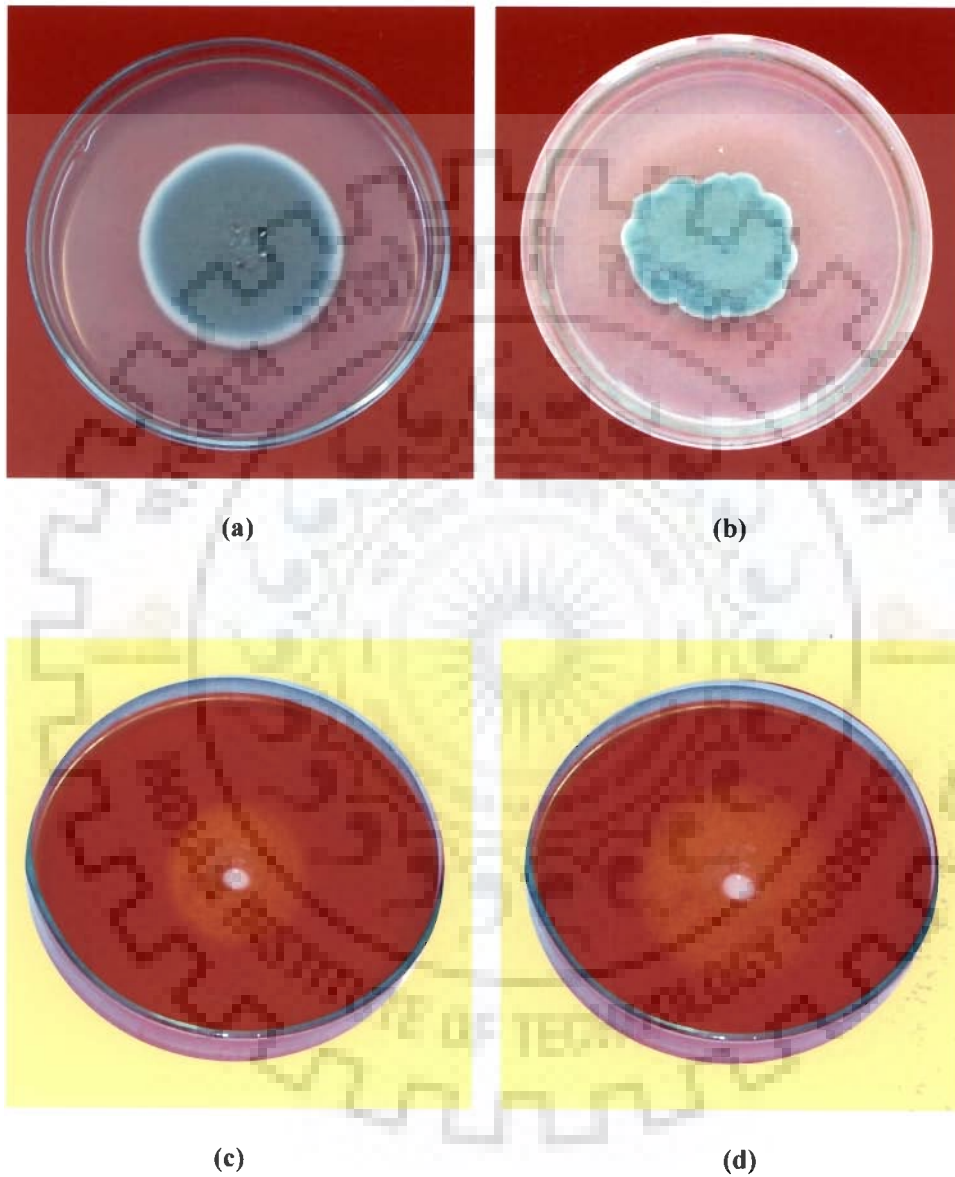
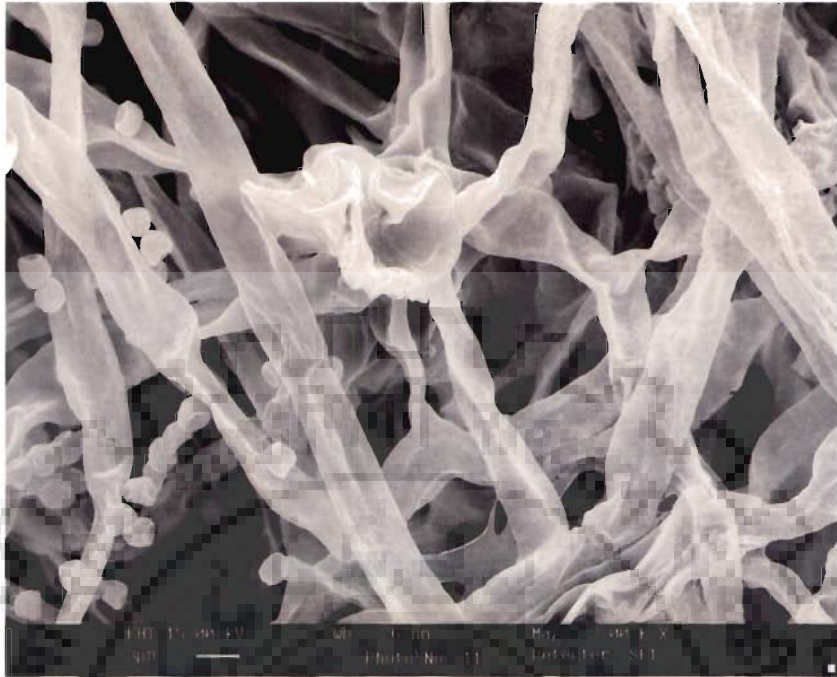


Fig. 7 Colonial morphology (a,b) and xylanase assay (c,d) of wild type *P. oxalicum* SA-8 (a, c) and mutant *P. oxalicum* SAU_E-3.510 (b, d) strains

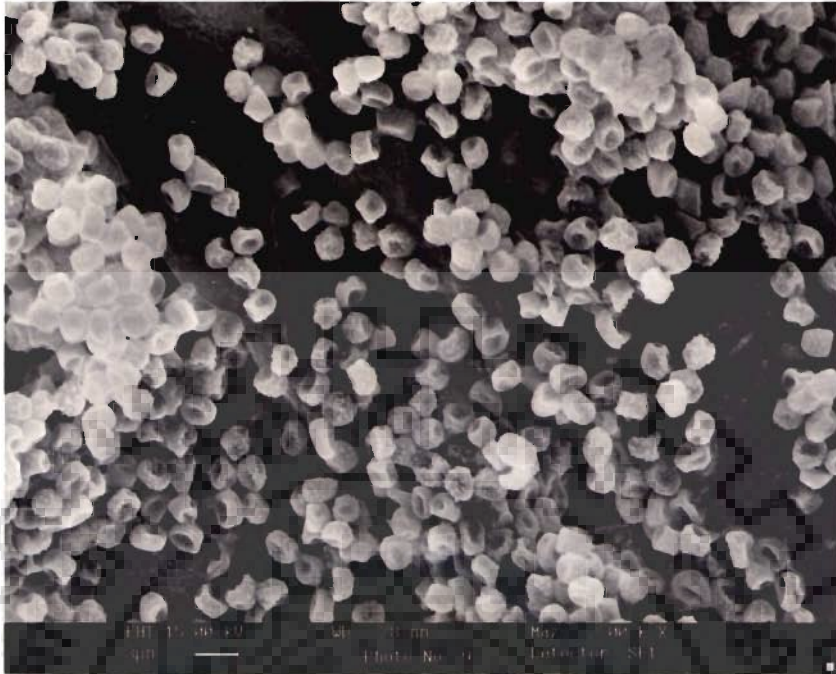


(a)

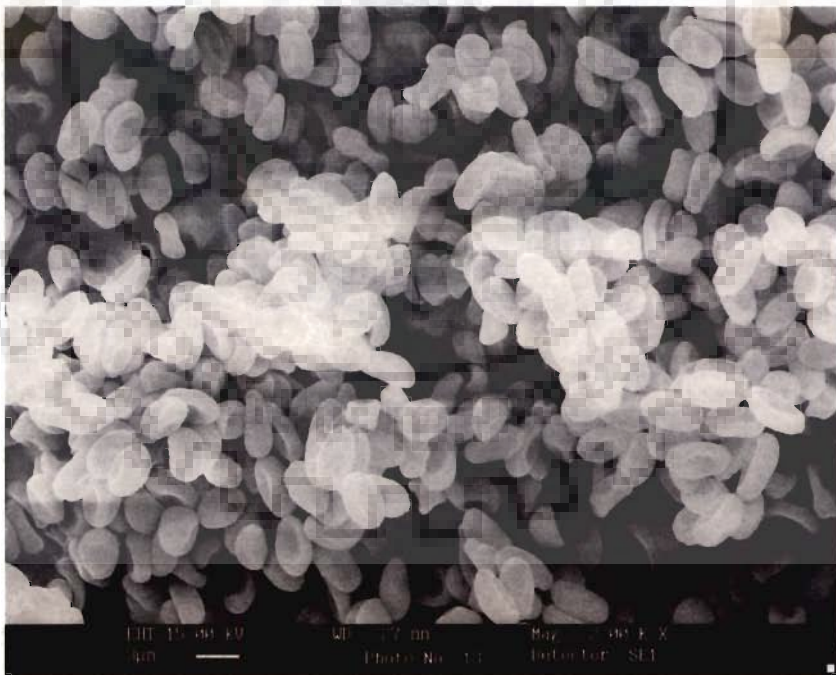


(b)

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 ANALYSIS OF GROWTH AND XYLANASE ESTIMATION AT VARYING pH

The effect of varying range of pH (3.0-12.0) on xylanase production was studied. The pH of the production media were maintained during the fermentation 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 at a wide range of pH (5.0-10.0) and maximum level of xylanase production was detected at pH 9.0 from both mutant and wild type strains and decrease in the xylanase production level was observed at higher pH. The ability of both mutant and wild type strains to produce remarkably higher level of xylanase under alkaline conditions (pH 9.0) indicates the alkali-tolerant nature of fungi (Fig. 10a).

The activity profile of xylanase was evaluated at varying pH ranging from 4.0-11.0. It was observed that maximum activity of xylanase was observed from mutant *P. oxalicum* SAU_E-3.510 (487.44 ± 3.57 IU ml⁻¹) as well as from wild type *P. oxalicum* SA-8 (252.50 ± 6.27 IU ml⁻¹) at pH 9.0. Notable levels of enzyme activity was also observed at higher pH. Interestingly, significantly higher level of enzyme activity was also observed at pH 5.0 from both mutant *P. oxalicum* SAU_E-3.510 (444.89 ± 7.60 IU ml⁻¹) as well as from the wild type strain (197.81 ± 6.94 IU ml⁻¹) (Fig. 10b).

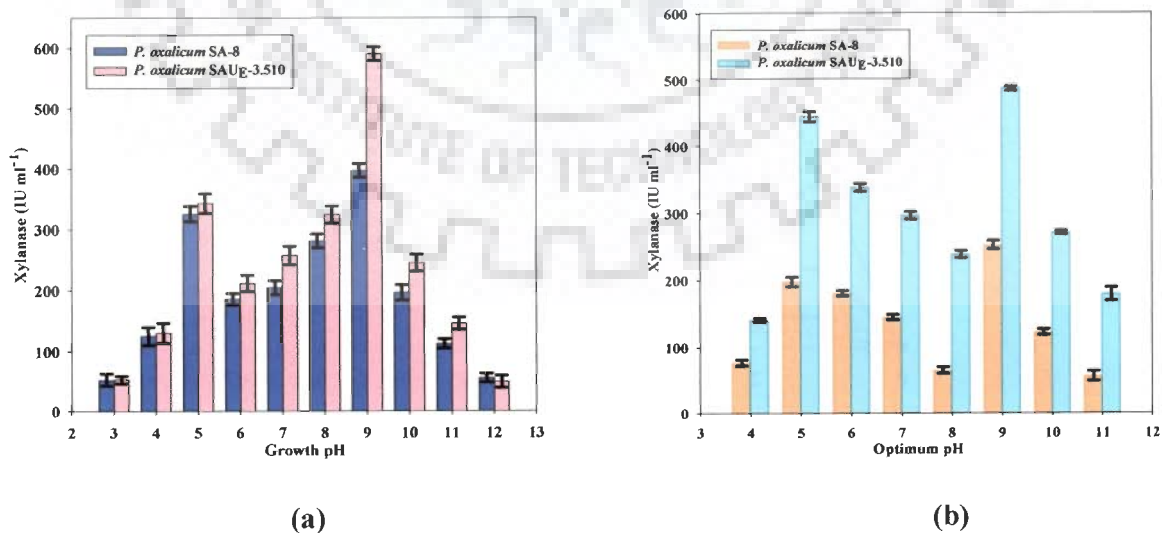


Fig. 10 Effect of pH on production (a) and xylanase activity (b) from wild type *P. oxalicum* SA-8 and mutant *P. oxalicum* SAU_E-3.510

4.4 CRITICAL FACTORS AFFECTING ENZYME PRODUCTION UNDER SUBMERGED FERMENTATION CONDITION

4.4.1 Analysis of enzyme production as a function of incubation period

Xylanase production by *P. oxalicum* SAU_E-3.510 was periodically analyzed at 30°C for up to 8 days in the medium containing 0.5% oat spelt xylan as substrate. Xylanase production was detected after 24 to 48 hrs 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 similar period of incubation. Interestingly, notable levels of cellulase activity (0.58 ± 0.06 IU ml⁻¹) that was detected after 24 hrs of incubation declined to nearly undetectable levels (0.05 ± 0.02 IU ml⁻¹) at the period when maximum levels of xylanase activity was observed.

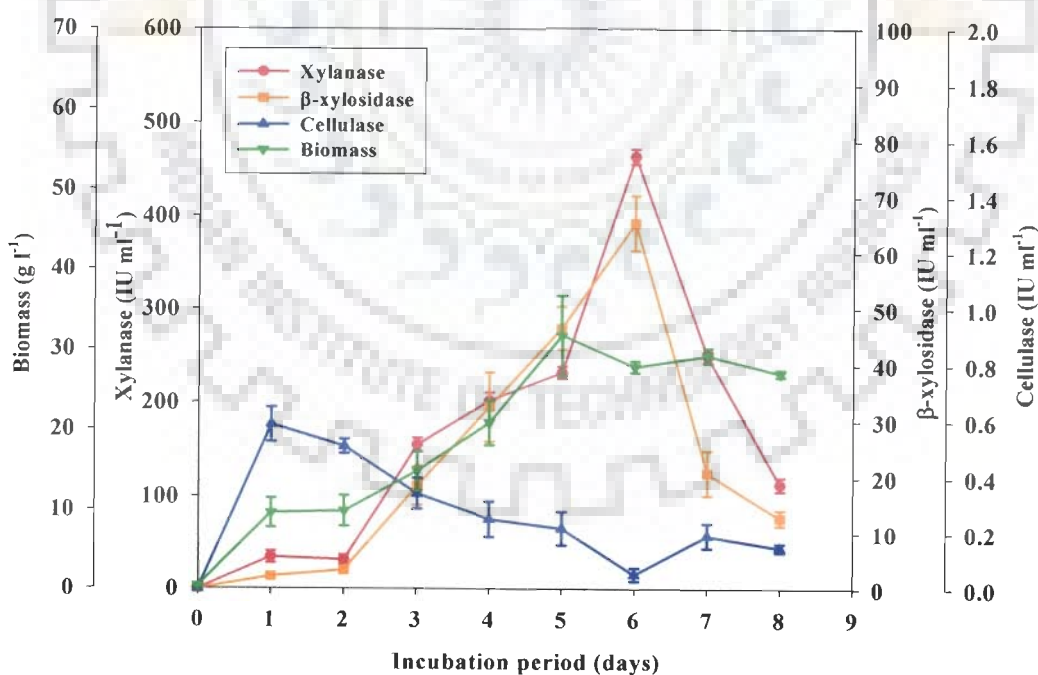
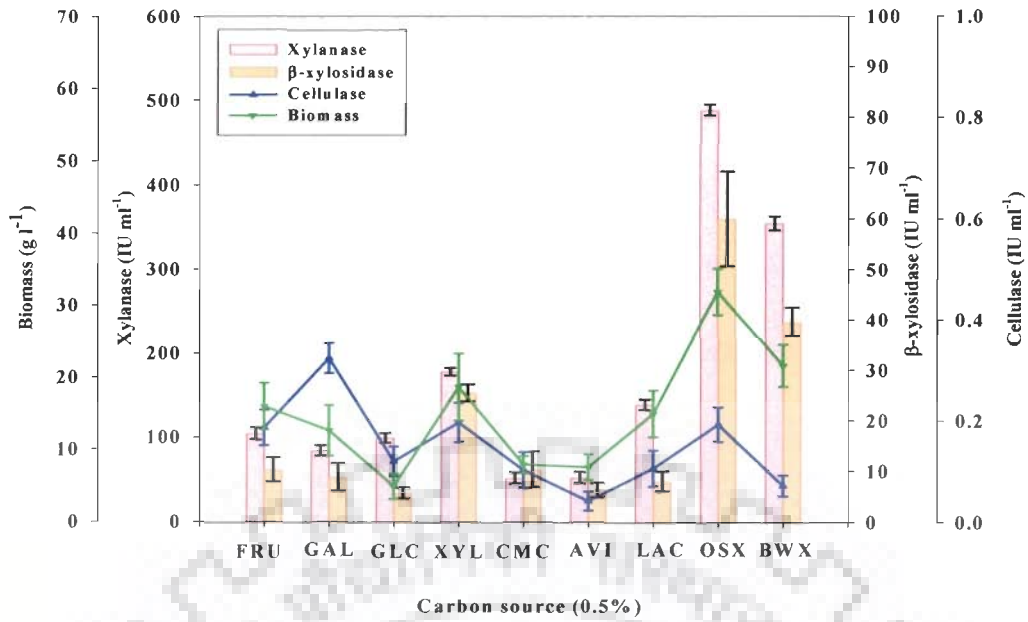


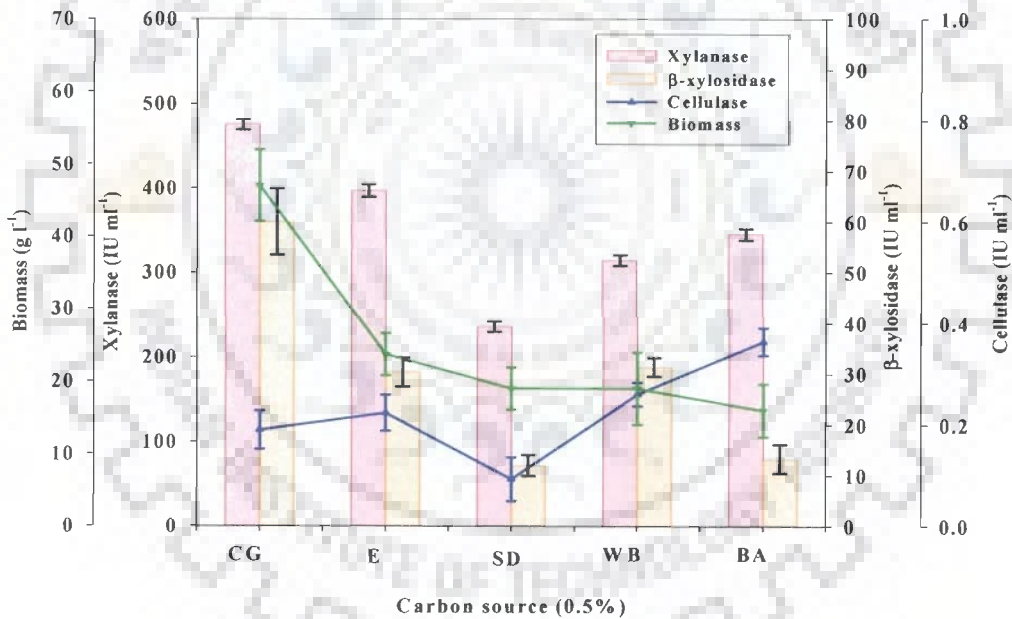
Fig. 11 Enzyme production at varying periods of incubation

4.4.2 Effect of carbon source on enzyme production

Xylan in the production medium when replaced with different synthetic mono, di and oligosaccharides, 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) which was followed by birch wood xylan (354.75 ± 8.01 IU ml⁻¹) (Fig. 12a). Besides the above, xylose also appear 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 had resulted into remarkably higher levels of activities (475.23 ± 6.05 IU ml⁻¹, 397.05 ± 7.53 IU ml⁻¹) (Fig 12b). Xylanase activity, obtained from the congress grass was about at similar levels to that obtained with commercial oat spelt xylan.



(a)



(b)

Fig. 12 Production of enzymes on different synthetic (a) and natural (b) carbon sources by mutant *P. oxalicum* SAU_E-3.510 in submerged fermentation condition

(a, Synthetic carbon sources: 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, Natural carbon sources: CG, Congress grass; EI, *Eichhornia*; SD, Saw dust; WB, Wheat bran and BA, Bagasse)

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 submerged fermentation. As shown (Fig. 13), the levels of xylanase, β -xylosidase, cellulase and biomass synthesis varied remarkably for mutant strain of *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 0.25% (w/v) concentration of congress grass.

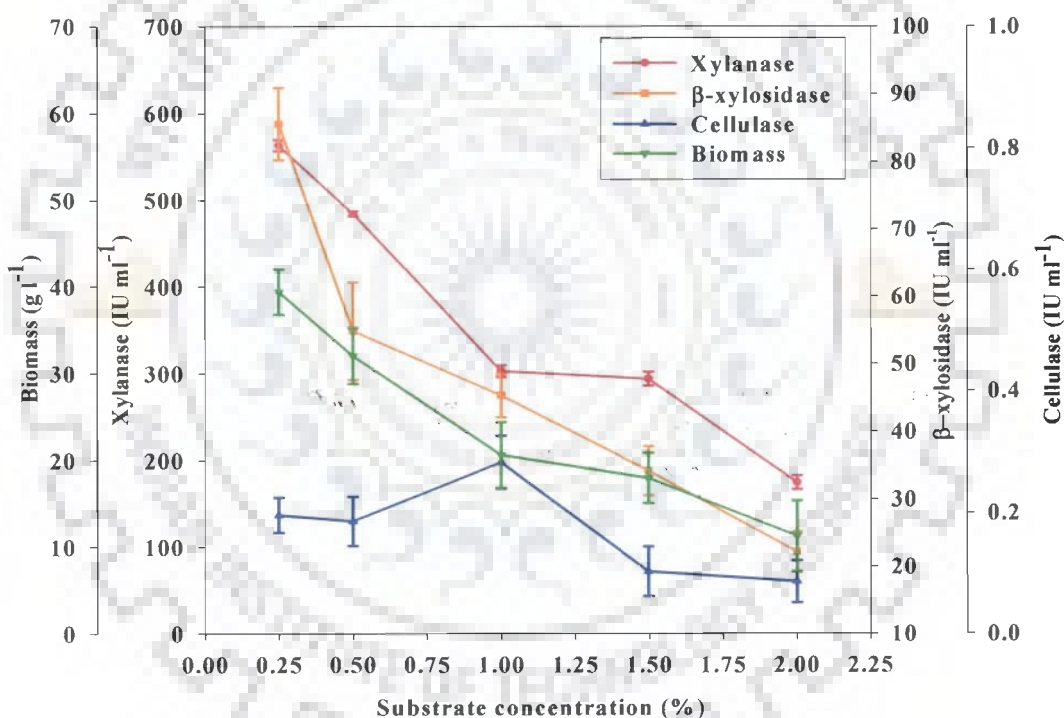


Fig. 13 Effect of concentration of congress grass on enzyme production

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, syringaldazine, ABTS and vanilline following filter sterilization (0.2 μ m membrane filter, Millipore, USA) were added in the production media

along with inoculum. As shown (Fig. 14) veratryl alcohol and ABTS had enhanced the xylanase biosynthesis while the production level decreased in presence of guaiacol, syrinzaldazine and vanilline. Maximum level of xylanase ($714.13 \pm 15.40 \text{ IU ml}^{-1}$) was achieved in presence of veratryl alcohol. The statistical analysis of the factors derived has been given in table 11.

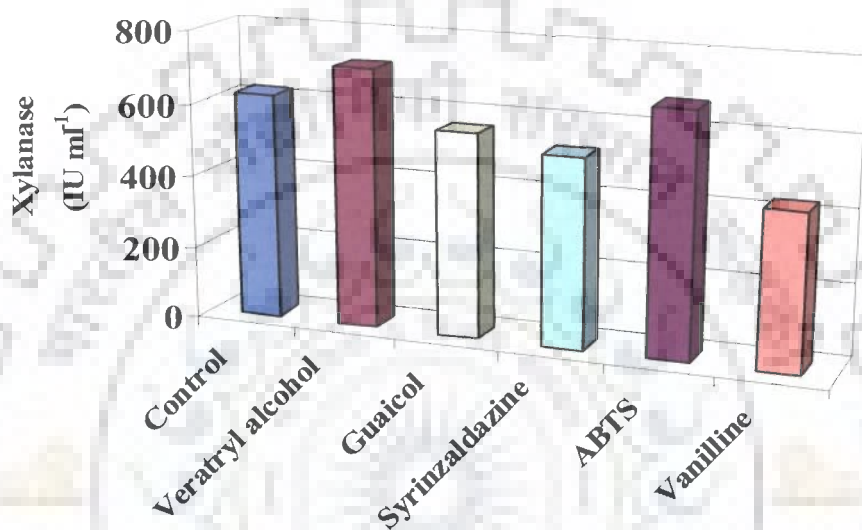


Fig. 14 Effect of lignin model compounds on xylanase biosynthesis

Table. 11 Analysis of variance for critical factors for xylanase production by *P. oxalicum* SAU_E-3.510 under submerged condition

| 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 synthetic | 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 natural | 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 enzyme preparation could be a promising approach for its use in the biobleaching. The co-culturing of fungi belonging to two different 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

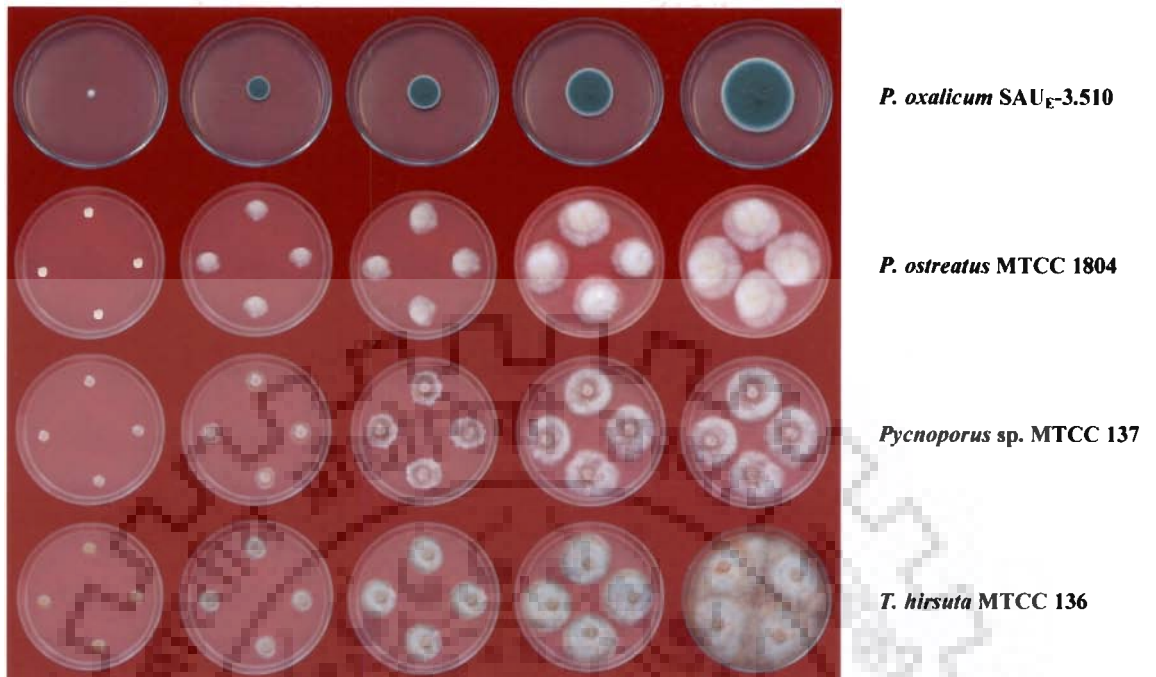
The compatibility of fungi belonging to two different groups viz; ascomycetes and basidiomycetes was evaluated by growing fungi on potato dextrose agar media. To analyse if fungal co-cultivation could lead into enhanced productivity of enzymes, ability of cultures to coexist in similar culture medium is a desirable feature. 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 behaviour was compared with their respective monoculture (Fig. 15a, b). Diameter of fungal colony was measured everyday 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 comes in contact with mutant hyphae and growth pattern of both fungi was also similar in dual culture as it appeared in their respective monoculture conditions (Fig. 15a, b). Although some thickening and browning of *P. ostreatus* MTCC 1804 hyphae were observed in the contact zone but it could be possible due to higher activity of oxidative enzymes. 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 as compared with their growth during monoculture cultivation

(Figure 15a, b). Initially the growth appeared normal but as soon as their colony diameter increased, their hyphae appeared scanty that was not observed in their axenic cultures.

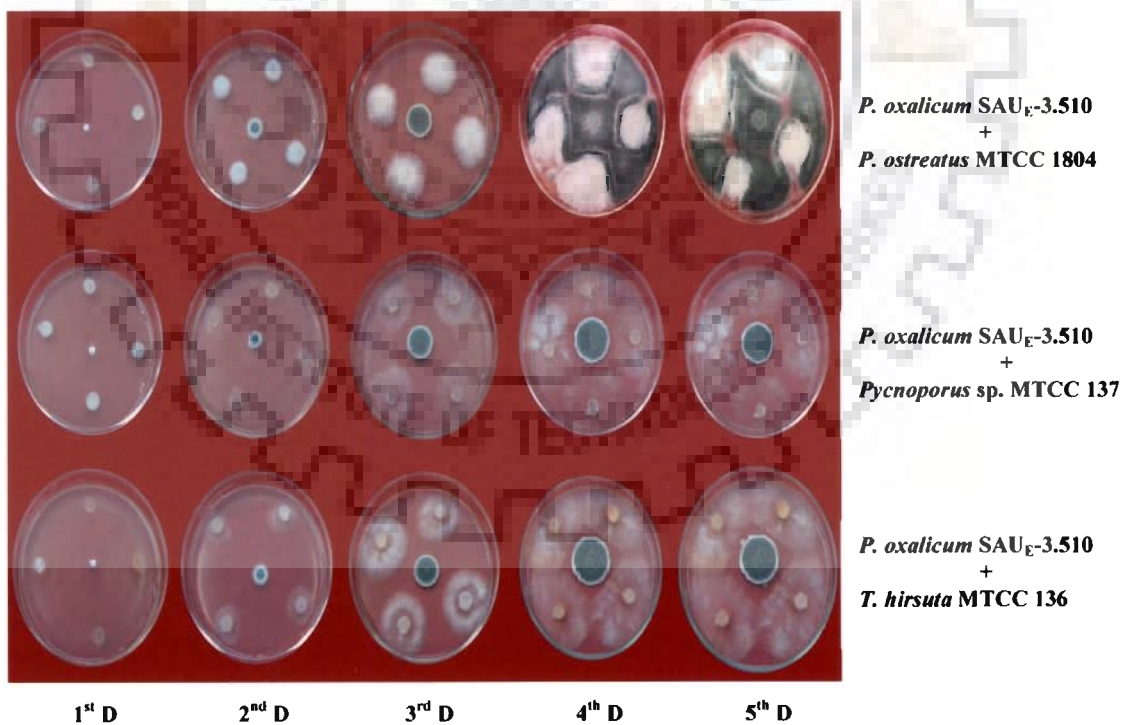
Interaction between *P. ostreatus* MTCC 1804 and mutant *P. oxalicum* SAU_E-3.510 during their co-culture on PDA plates was also enumerated by scanning electron microscopy. As observed, hyphae of mutant *P. oxalicum* SAU_E-3.510 are thick, flattened with rougher and peeled surface (Fig. 16a) while that of *P. ostreatus* MTCC 1804 are thinner, smooth surfaced, shiny and whitish in colour (Fig. 16b). Interaction of both fungal hyphae is shown in fig. 16c, 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 that had become thick and globous while no change was observed in its surface. This suggests that the interaction between two fungi is 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

| S. No. | Incubation Period (days) | Colony diameter (cm) | | | | | | | | | |
|--------|--------------------------|----------------------|-----|-----|-----|----------------|---------|---------|---------|---------|---------|
| | | Monoculture | | | | Co-cultivation | | | | | |
| | | M | Plu | Pyc | Tra | M : Plu | M : Pyc | M : Tra | M : Plu | M : Pyc | M : Tra |
| 1. | 1 | Point | 0.8 | 0.8 | 0.8 | Point | 0.8 | Point | 0.8 | Point | 0.8 |
| 2. | 2 | 1.7 | 1.9 | 1.6 | 1.1 | 1.8 | 1.9 | 1.9 | 1.1 | 1.9 | 1.2 |
| 3. | 3 | 2.3 | 2.5 | 2.8 | 1.9 | 2.5 | 2.8 | 2.3 | 2.3 | 2.2 | 1.6 |
| 4. | 4 | 3.3 | 3.5 | 3.4 | 2.2 | 3.4 | 4.0 | 3.6 | 3.2 | 3.1 | 1.9 |
| 5. | 5 | 4.1 | 4.4 | 3.8 | 3.3 | mix | mix | 4.1 | 3.6 | 3.8 | 2.2 |

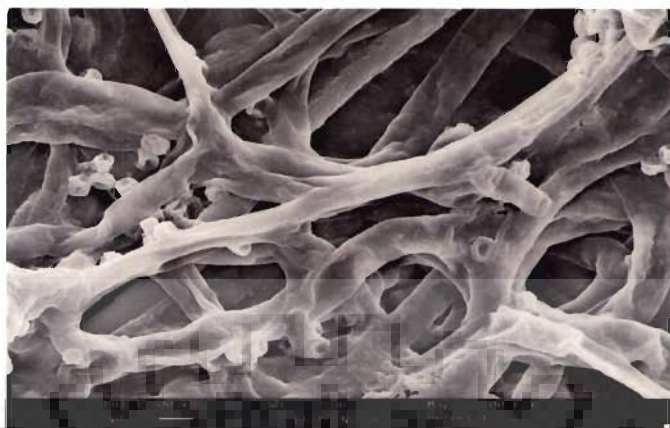


(a)



(b)

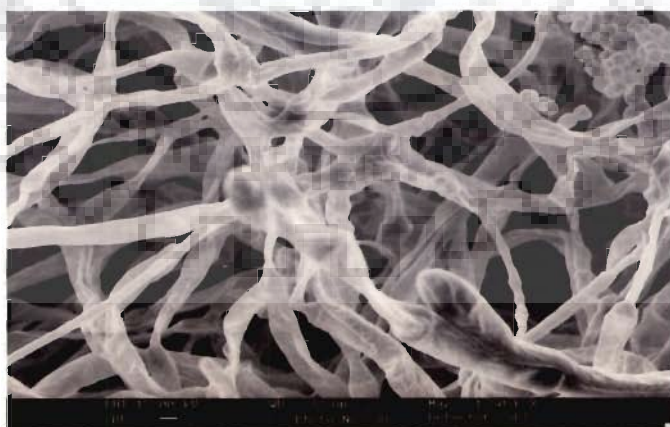
Fig. 15 Growth compatibility of mutant *P. oxalicum* SAU_E-3.510 with *P. ostreatus* MTCC 1804, *Pycnoporus* sp. MTCC 137 and *T. hirsuta* MTCC 136 under single (a) and paired culture (b) conditions



(a)



(b)



(c)

Fig. 16 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 ($714.13 \pm 15.40 \text{ IU ml}^{-1}$) xylanase activity after 6th day of incubation under derived conditions but no laccase activity was observed under submerged conditions. *P. ostreatus* MTCC 1804, *T. hirsuta* MTCC 136 and *Pycnoporus* sp. MTCC 137 were cultured in glucose peptone media and laccase activity was measured up to 20 days. Among three selected fungi, *P. ostreatus* MTCC 1804 had resulted into maximum laccase activity ($13.02 \pm 1.53 \text{ IU ml}^{-1}$) following 12 days of incubation (Fig. 17). Besides this, lower level of xylanase ($16.00 \pm 2.84 \text{ IU ml}^{-1}$) was also produced by *P. ostreatus* MTCC 1804 after 6 days of incubation period. Notable levels of enzyme biosynthesis ability of *P. ostreatus* MTCC 1804 along with its compatibility with mutant *P. oxalicum* SAU_E-3.510 had therefore indicated the former strain to be superior with respect to the remaining two strains (*T. hirsuta* MTCC 136, *Pycnoporus* sp. MTCC 137) analysed, hence the same was selected for further studies.

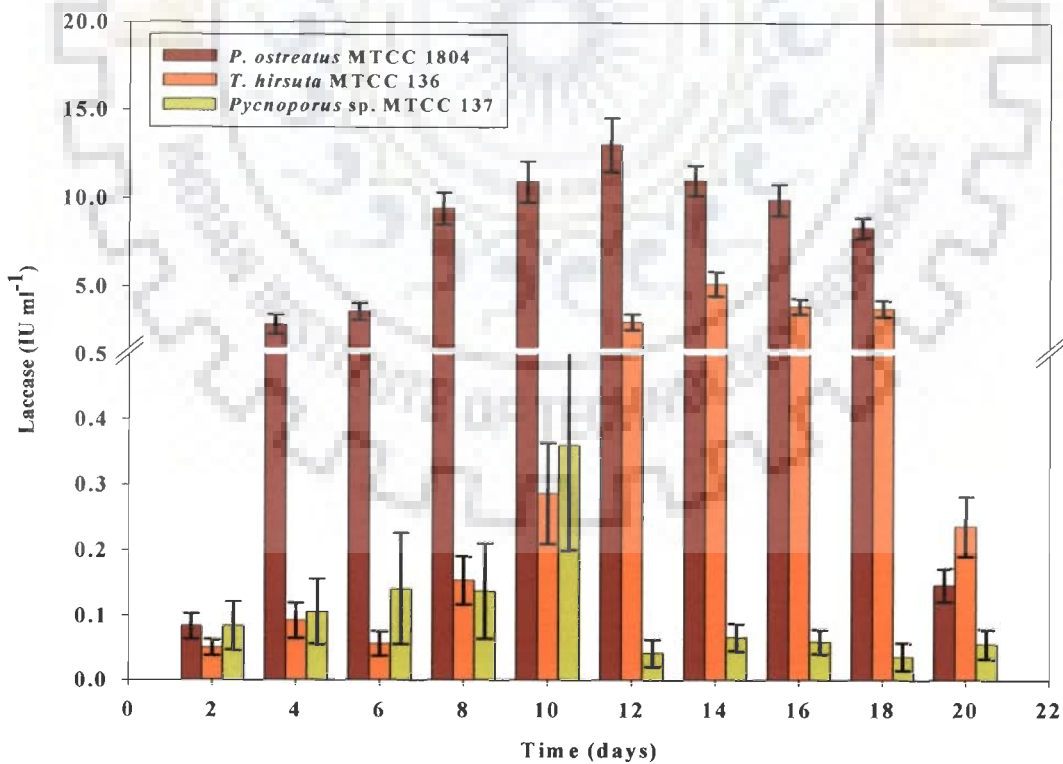


Fig. 17 Production of laccase by axenic cultures of white rot fungi at varying time intervals

4.5.3 Analysis of enzyme production by mixed culture

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 6th day, had resulted into maximum levels of xylanase (1208.00 ± 15.01 IU ml⁻¹) and that of laccase (19.40 ± 2.40 IU ml⁻¹) production (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. 18).

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

| S. 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 6 th day of incubation | 735.80 ± 17.37 | 27.63 ± 3.37 | 1208.00 ± 15.01 | 58.2 |

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

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

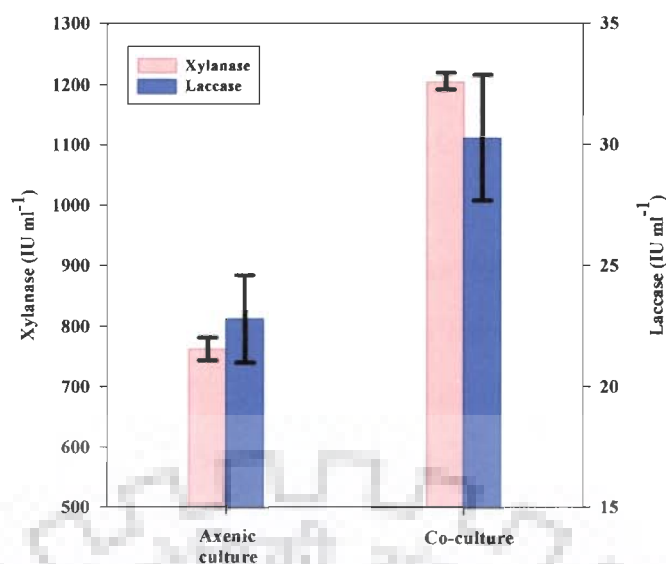


Fig. 18 Analysis of xylanase and laccase activity during co-cultivation of mutant *P. oxalicum* SAU_E-3.510 with *P. ostreatus* MTCC 1804 under derived conditions

4.5.4 Analysis of fermentation conditions

Enzyme production using paired culture of the mutant *P. oxalicum* SAU_E-3.510 and *P. ostreatus* MTCC 1804 was evaluated in different fermentation set-ups mainly surface, submerged and solid state fermentation conditions. Highest level of xylanase (1301.15 ± 12.73 IU ml⁻¹) and laccase (43.70 ± 3.72 IU ml⁻¹) was obtained when fungi were co-cultivated under solid-state fermentation condition (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

| S. No. | Fermentation conditions | Activity (IU ml ⁻¹) | |
|--------|--------------------------|---------------------------------|--------------|
| | | Xylanase | Laccase |
| 1. | Surface fermentation | 976.55 ± 12.20 | 15.00 ± 1.90 |
| 2. | Submerged fermentation | 1201.84 ± 11.72 | 20.83 ± 2.94 |
| 3. | Solid-state fermentation | 1301.51 ± 12.73 | 43.70 ± 3.72 |

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

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

4.6 CO-CULTIVATION UNDER SOLID-STATE FERMENTATION CONDITION

Co-cultivation under 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 during solid-state fermentation, which was then subsequently inoculated with *P. oxalicum* SAU_E-3.510 at different time periods. *P. oxalicum* SAU_E-3.510 when inoculated 48 hrs after fermentation had resulted into maximum levels of xylanase (6210.76 ± 56.79 IU g⁻¹) and laccase (247.16 ± 32.47 IU g⁻¹) production, however, contrary to this, inoculation with mutant after 144 hrs had resulted into maximum levels of production under submerged fermentation (Fig. 19). This discrepancy in the time of inoculation of mutant may be due to the fact that the growth of white rot fungus is comparatively faster during solid-state cultivation as compared to submerged condition because conditions in SSF are close to the natural environmental conditions.

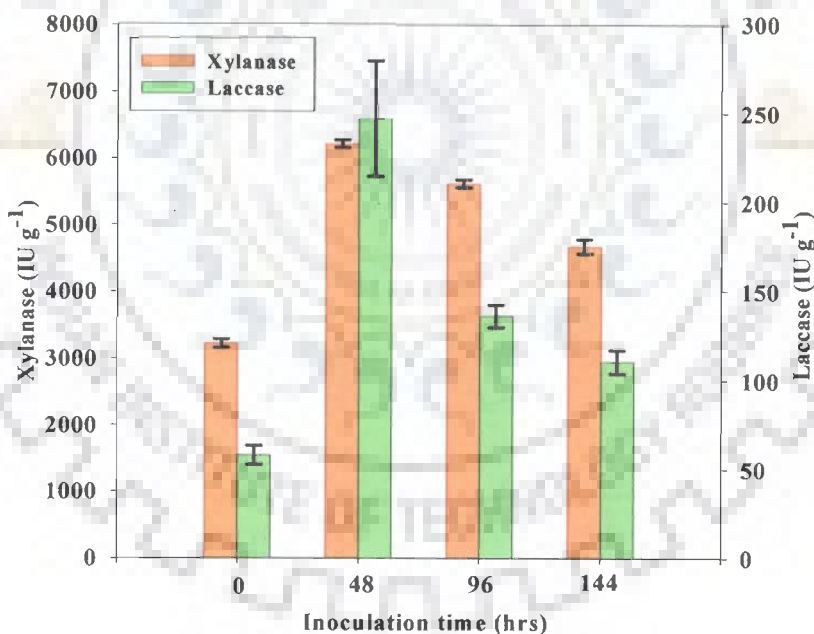


Fig. 19 Evaluation of inoculation time of mutant *P. oxalicum* SAU_E-3.510 with *P. ostreatus* 1804 for enzyme production under solid-state fermentation

4.6.2 Levels of enzyme production on various substrates

Solid-state fermentation led to comparatively higher levels of the xylanase and laccase production by co-culturing of fungi. Thus to obtain further improved levels of production and to

economize the process, solid-state fermentation was performed using a variety of agro-horticultural residues that are easily and abundantly available either at considerably low-price or with no cost (Fig. 21). Fig. 20 denotes the activity of xylanase and laccase by using various agroresidual materials. Almost similar levels of xylanase was obtained with bagasse ($6837.33 \pm 37.46 \text{ IU g}^{-1}$), black gram husk ($6551.31 \pm 81.61 \text{ IU g}^{-1}$), wheat bran ($6542.94 \pm 68.39 \text{ IU g}^{-1}$) and congress grass ($6305.64 \pm 48.9 \text{ IU g}^{-1}$) while saw dust had resulted into lower level of xylanase. However combination of bagasse with wheat bran and with black gram husk had demonstrated that later had led into maximum levels of both xylanase ($7121.11 \pm 67.74 \text{ IU g}^{-1}$) and laccase ($316.02 \pm 13.01 \text{ IU g}^{-1}$) production during co-culturing of mutant *P. oxalicum* SAU_E-3.510 and *P. ostreatus* MTCC 1804.

As observed through scanning electron microscopy, mixture of bagasse and black gram husk serve 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. Thus, it seems to be a suitable matrix supporting the metabolic activity of fungus (Fig. 22).

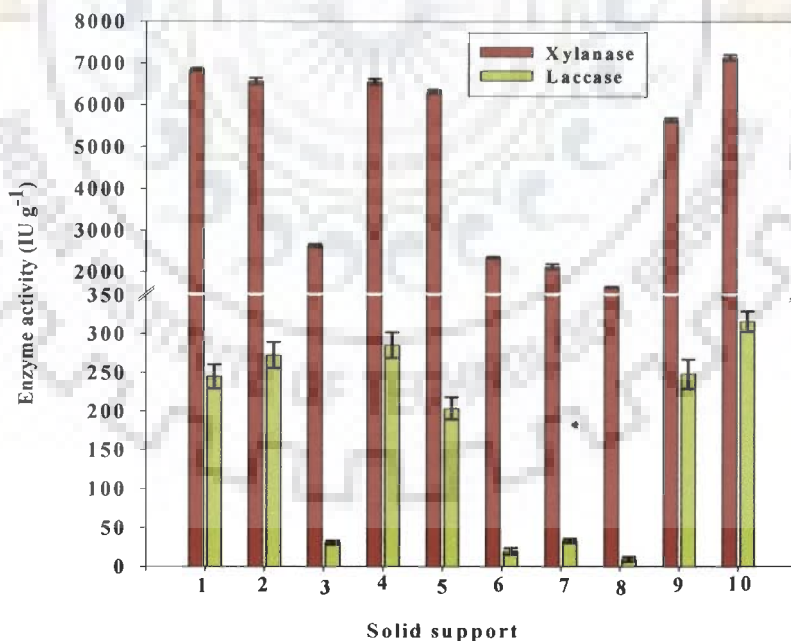


Fig. 20 Production of enzymes using different solid supports : 1, Bagasse; 2, Black gram husk; 3, Red gram husk; 4, Wheat bran; 5, Congress grass; 6, Rice bran; 7, Cotton hull; 8, Saw dust; 9, Bagasse + Wheat bran (1:1) and 10, Bagasse + Black gram husk (1:1)



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



(a)



(b)

Fig. 22 Scanning electron micrograph of bagasse and black gram husk in mixture as solid substrate (a) uninoculated and (b) entrapped with *P. oxalicum* SAU ϵ -3.510 and *P. ostreatus* MTCC 1804

4.6.3 Effect of substrate concentration and ratio of two substrates

Amount of substrate is of prime importance and 4g of substrate (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. 23a. 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 co-cultivation of *P. oxalicum* SAU_E-3.510 and *P. ostreatus* MTCC 1804. Estimation of xylanase and laccase activity had demonstrated that bagasse and black gram husk when used in the ratio of 3:1 had resulted into further higher levels of xylanase (7574.24 ± 118.99 IU g⁻¹) and laccase (381.33 ± 10.01 IU g⁻¹) (Fig. 23b).

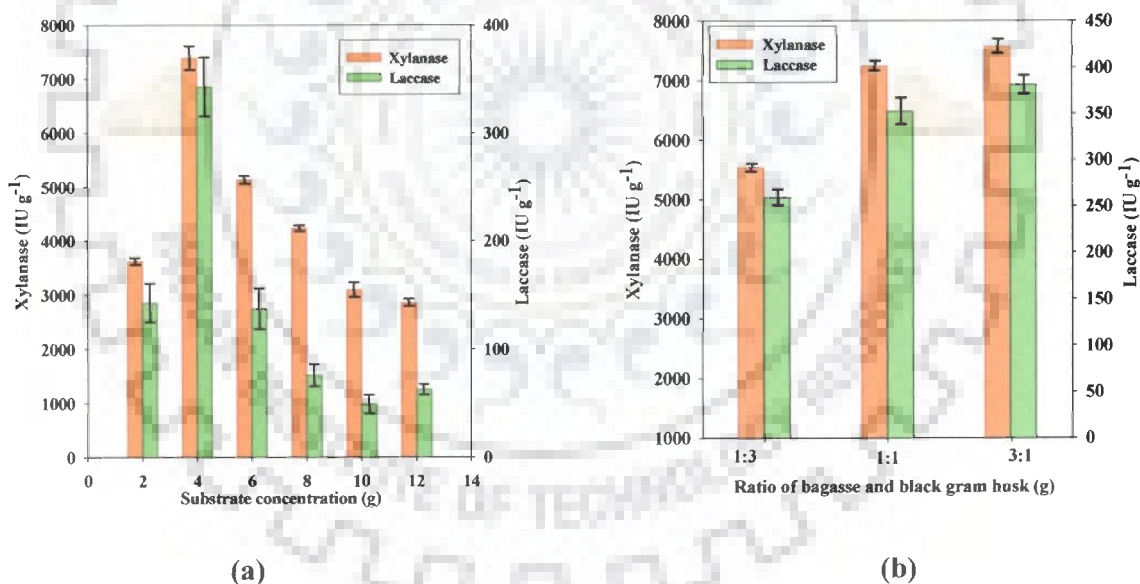


Fig. 23 Enzyme production at varying concentrations (a) and ratio (b) of bagasse and black gram husk under solid-state fermentation by co-cultivation of mutant *P. oxalicum* SAU_E-3.510 with *P. ostreatus* MTCC 1804

4.6.4 Enzyme production at different time period

Production of xylanase and laccase by binary 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 ($7904.27 \pm 52.36 \text{ IUg}^{-1}$) and notably higher levels of laccase ($348.42 \pm 32.27 \text{ IUg}^{-1}$) was observed after 8 days of incubation following inoculation with mutant (Fig. 24). However, marginally higher levels of laccase was observed after 6 days of incubation. Decreasing levels of enzymes were observed on further incubation; hence culture was harvested after 8th day of incubation to get maximum levels of both enzymes that was achieved following co-culturing.

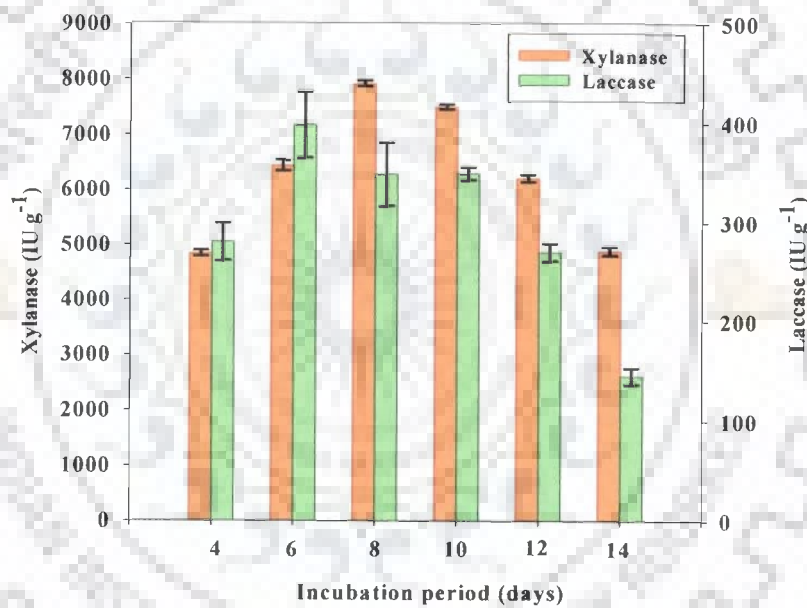


Fig. 24 Production of enzymes by paired culture of *P. oxalicum* SAU_E-3.510 and *P.ostreatus* MTCC 1804 at varying periods of incubation

4.6.5 Effect of moisture level

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 content ranging from 20% to 120% and as shown in fig. 25 moisture level of 80% was best suited for enzyme production and yielding $8205.31 \pm 168.31 \text{ IUg}^{-1}$ of xylanase and $375.53 \pm$

34.17 IUg⁻¹ of laccase under these conditions. Higher moisture content was inhibitory for production. Higher moisture level during SSF interferes with air circulation and heat transfer generated due to biological activities of the microbial strains.

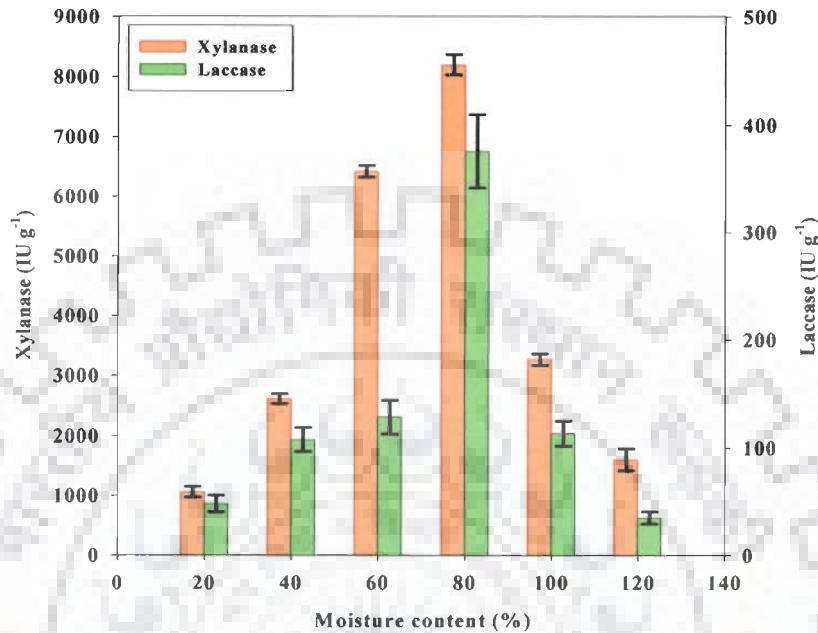


Fig. 25 Effect of moisture content on enzyme production by co-cultivation of mutant *P. oxalicum* SAU_E-3.510 with *P. ostreatus* MTCC 1804 under solid-state fermentation

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 (Table. 15).

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

| 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 |
| | With in 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 |
| | With in 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 |
| | With in 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 |
| | With in 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 |
| | With in 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 |
| | With in the Samples | 2 | 461896.6 | 230948.3 | 19.1 |
| | Error | 10 | 120415.1 | 12041.5 | |

Significant at 5% level

4.7 CHARACTERIZATION 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. 26a. The enzyme appeared stable and maintained 93% of the residual activity at pH 9.0 for 24 hrs, whereas 93% of the residual activity was reported following four hrs of incubation in the buffer at pH 11.0 while about 50% decrease in the activity was detected after 20 hrs. The effect of incubation temperature on xylanase activity was determined by incubating the enzyme with substrate at varying temperatures (25°C - 85°C) and 55°C was found to be most appropriate for achieving maximum activity (Fig. 26c). Influence of temperature on stability of xylanase from mutant *P. oxalicum* SAU_E-3.510 was evaluated at varying temperature (50°C-100°C, 2 hrs). Similarly, enzyme also appeared to be notably thermostable. Half life of enzyme was found to be two hrs at 80°C when 50% of the residual activity was estimated (Fig. 26b).

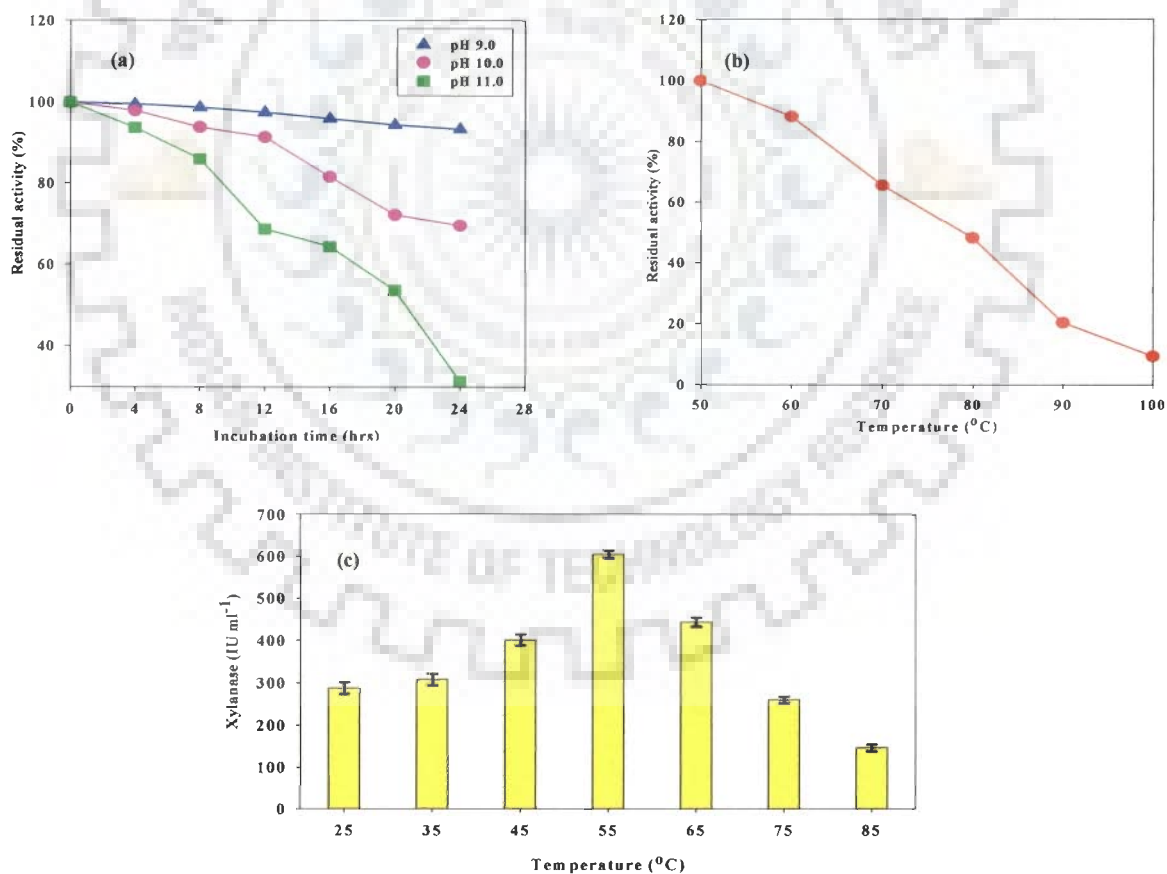


Fig. 26 pH (a), temperature (b) stability profile and optimum temperature (c) for xylanase activity from mutant *P. oxalicum* SAU_E-3.510

4.7.2 Molecular characterization

The protein profile of mutant *P. oxalicum* SAU_E-3.510 was analyzed through SDS PAGE and compared with that of the wild type *P. oxalicum* SA-8 (Fig 27 a). SDS-PAGE and zymogram analysis had indicated the presence of two isoforms of xylanase in both wild type and mutant strains and the molecular mass of these was deduced to be 121 kD and 77 kD respectively (Fig 27 a, b). The activity bands of mutant strain were pronounced as compared to wild type strain indicating there by the 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 analyzed by polyacrylamide electrophoresis and zymogram analysis (Fig. 28 a-c). Two xylanase isozymes of 121 kD and 77 kD which are specific to mutant *P. oxalicum* SAU_E-3.510 were present in the sample obtained by binary culture along with an extra xylanase of 40 kD position that corresponded to *P. ostreatus* MTCC 1804. Similarly, no laccase was detected in the axenic culture of mutant while laccase corresponding to 29 kD was detected for *P. ostreatus* MTCC 1804 during co-cultivation.

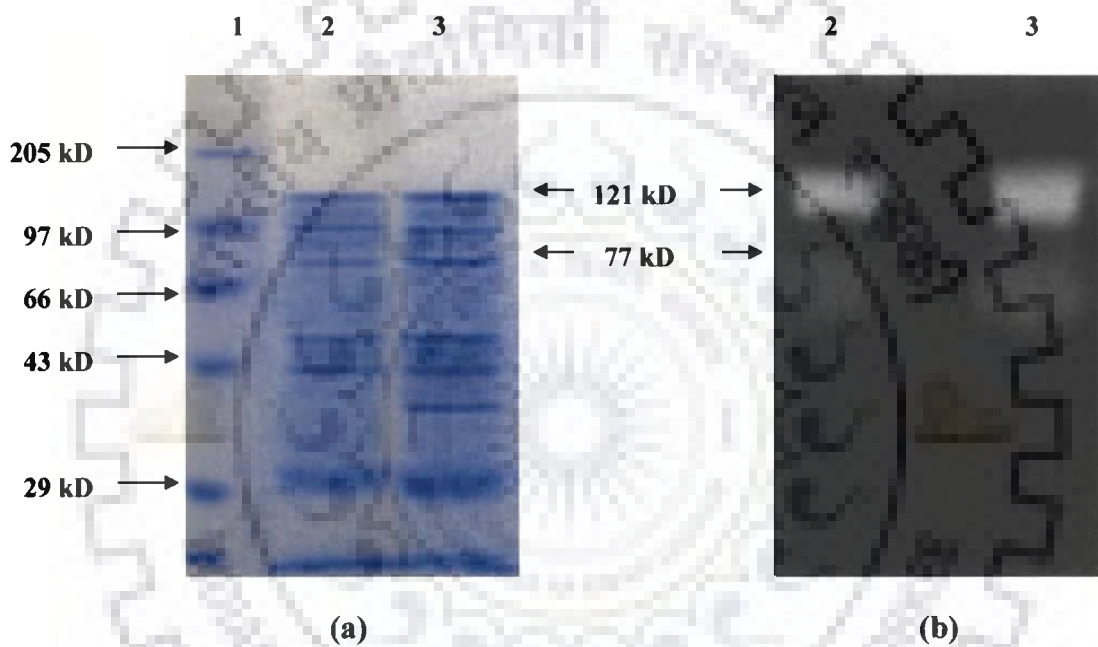


Fig 27 SDS-PAGE (a) and zymogram (b) analysis of proteins produced by wild type *P. oxalicum* SA-8 and mutant *P. oxalicum* SAUE3.510 following 6 days of incubation. Lane 1, protein molecular weight markers; 2 and 3, culture supernatant of wild type and mutant strains respectively

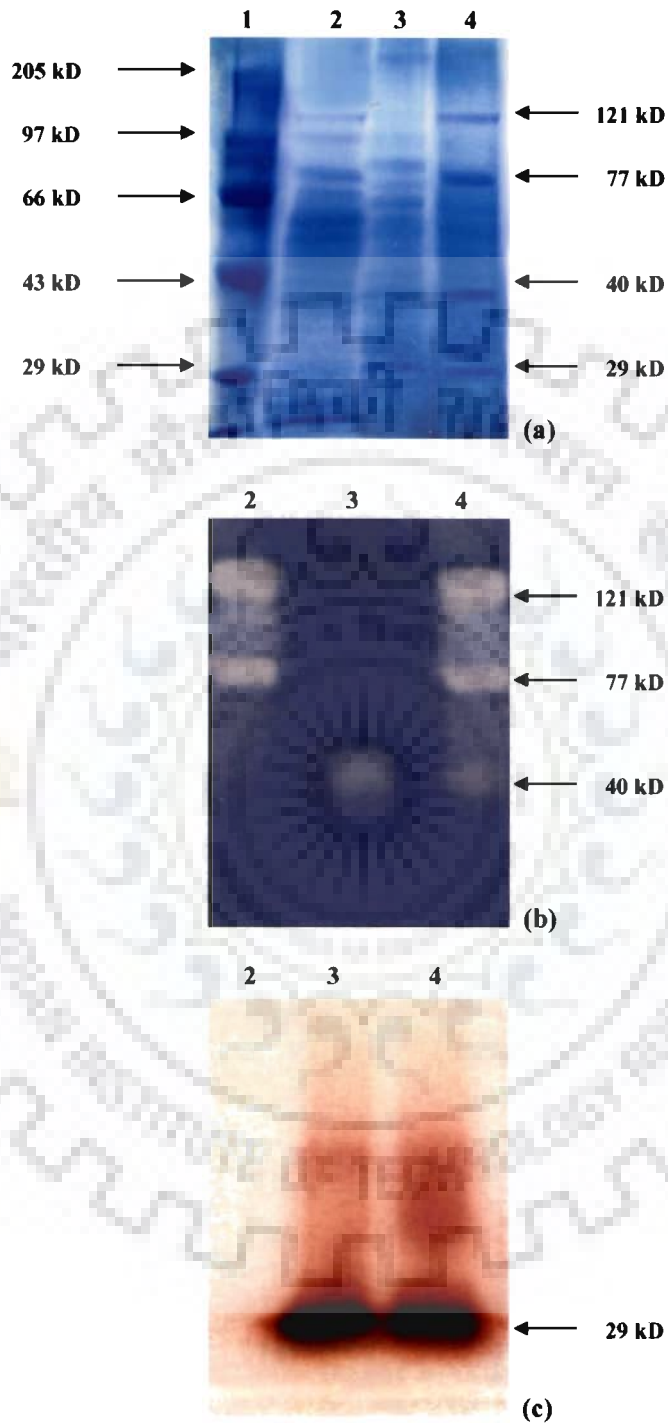


Fig. 28 SDS-PAGE (a), zymogram analysis for xylanase (b) and laccase (c) produced during mono and dual culture conditions. Lane 1, protein molecular weight markers; 2, culture supernatant of mutant *P. oxalicum* SAU_E-3.510; 3, *P. ostreatus* MTCC 1804 and 4, co-culture of mutant *P. oxalicum* SAU_E-3.510 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 from bacterial origin. A level of identity of 40-60% was observed from the xylanases from *Bacillus stearothermophilus*, *Geobacillus stearothermophilus*, *Clostridium sp.*, *Paenibacillus sp.* On the other hand, xylanase from *P. oxalicum* SAU_E-3.510 had depicted comparatively lower identity with xylanases of fungal origin viz *Aspergillus versicolor*, *Aspergillus fumigatus* and *Gibberella zeae* (Table 16).

4.7.4 Hydrolysis studies

Xylan extracted from *Parthenium sp.* (congress grass) was used as substrate to analyze 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 hrs at 55°C and hydrolysis products were analyzed by high performance liquid chromatography (HPLC). The peaks were identified by running the parallel standard of xylose with known concentration. As observed (Fig. 29), xylose is the major hydrolysis product released by the action of enzyme while presence of other oligosaccharides (xylobiose, xylotriose etc) were not detected. Higher concentration of xylose indicated the improved degree of substrate hydrolysis with xylanase in presence of laccase as compared to xylanase alone while no xylose was detected in the untreated samples (Fig. 29). These results showed that mutant secretes a set of enzymes (endo and exo acting) that synergistically promote efficient substrate degradation. Reported results also suggested that congress grass xylan could be effectively hydrolysed by xylanase and can be utilized for production of monosaccharides (xylose) which has wide set of industrial utility.

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

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

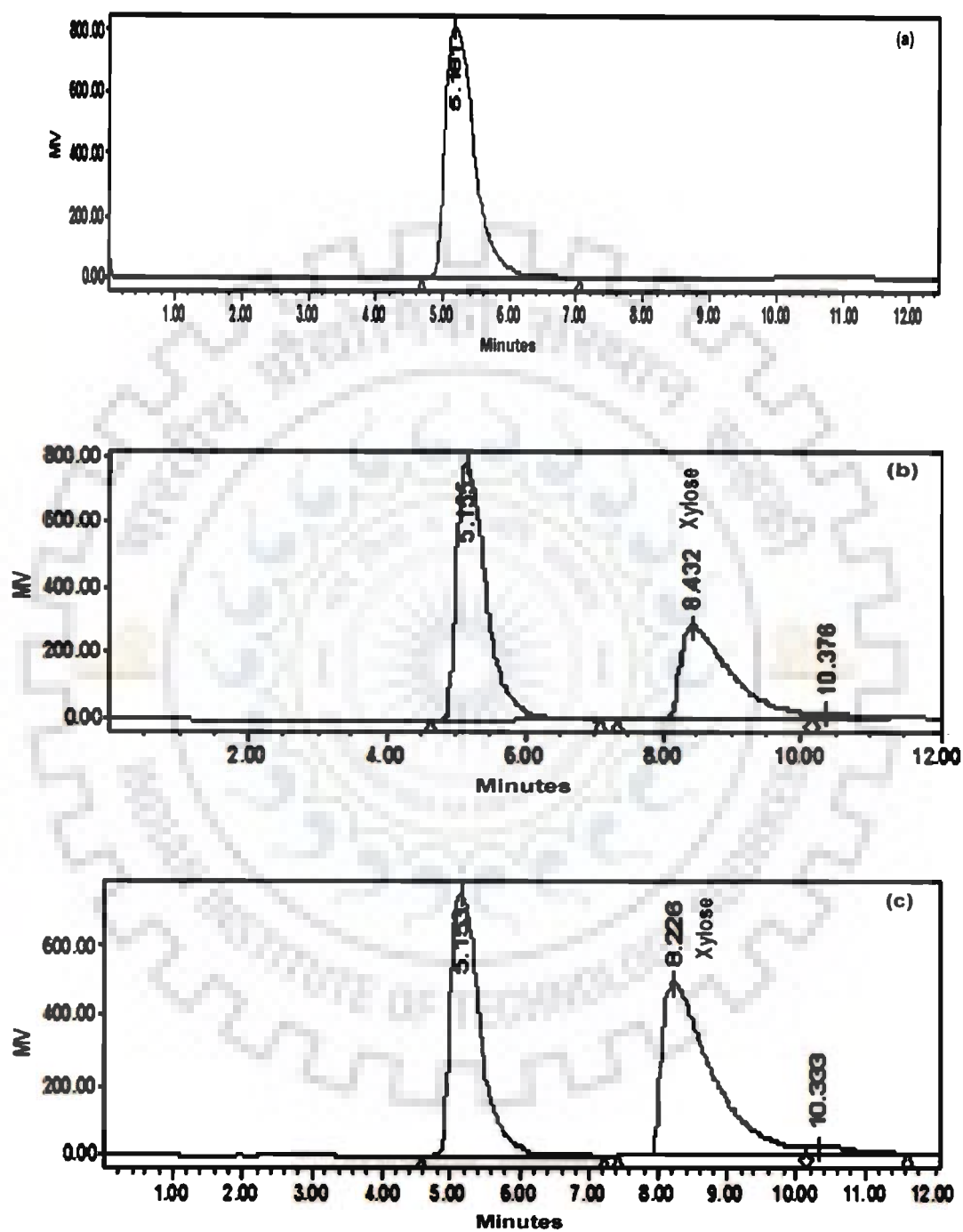


Fig. 29 Analysis of hydrolysis products released as a result of congress grass xylan hydrolysis (a) untreated (b) xylanase treated (c) xylanase-laccase mixture treated

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 xylanase- laccase mixture which was produced through co-cultivation of mutant *P. oxalicum* SAU_E-3.510 and *P. ostreatus* MTCC 1804 under solid-state fermentation condition. A significant decrease in kappa number and increase in brightness was achieved as result of enzymatic treatment due to removal of lignin as compared to untreated pulp. As compared to xylanase alone, mixed enzyme preparation had yielded better results in terms of kappa number and brightness (Fig. 30). Significantly higher lignin removal (kappa number decreased was 5 % higher as compared to xylanase alone) was obtained by using a mixture of xylanase and laccase produced through co-cultivation under solid-state fermentation. Similarly, brightness of the sheets that was treated with mixed enzyme preparation was also higher (38.3 ISO) as compared to xylanase alone (37.0 ISO). These results suggest mixed enzyme preparation as an effective and suitable alternative to xylanase alone for bleaching of mixed wood pulp, hence the same has been used in further experiments.

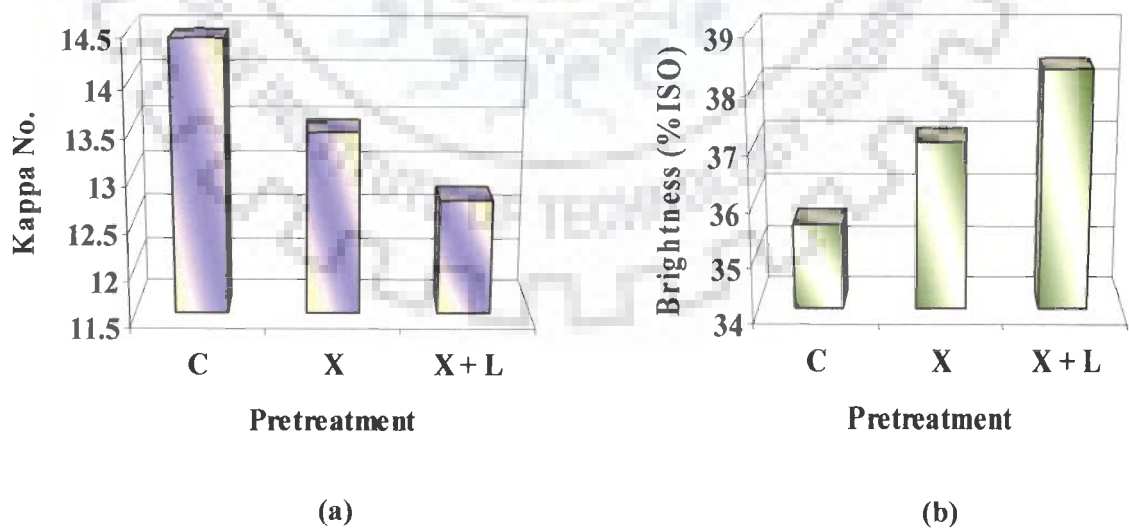


Fig. 30 Alteration in pulp kappa no (a) and brightness (b) following enzymatic treatment (C, untreated; X, xylanase; X+ L, xylanase-laccase concoction)

4.8.1.2 Optimization of enzyme dose

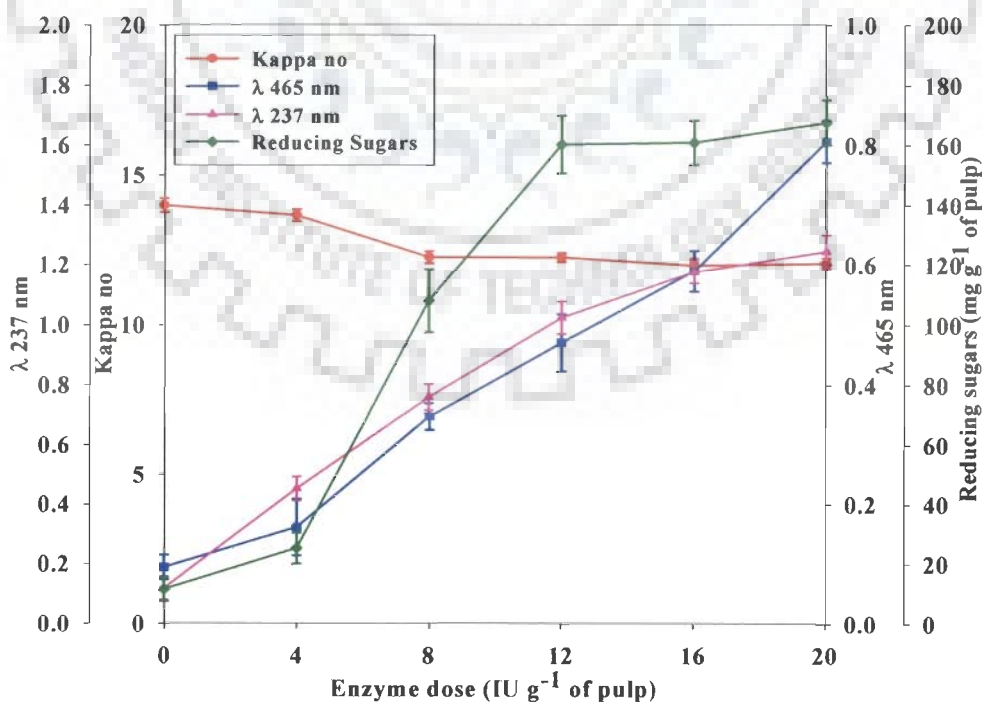
The biobleaching efficacy of xylanase-laccase preparation was evaluated at varying doses ranging from 0-20 IU g⁻¹ of pulp. Maximum decrease in kappa no (12.8%) was achieved by employing 8 IU g⁻¹ of enzyme. The kappa no. of pulp remained constant even after further increase in enzymatic dose which suggested that 8 IU g⁻¹ of enzyme concentration was most effective for achieving maximum degree of lignin removal. Release of reducing sugars, phenolic and hydrophobic compounds in the bleaching effluent also increased along with increasing dose of enzyme (Fig 31a).

4.8.1.3 Optimization of reaction time

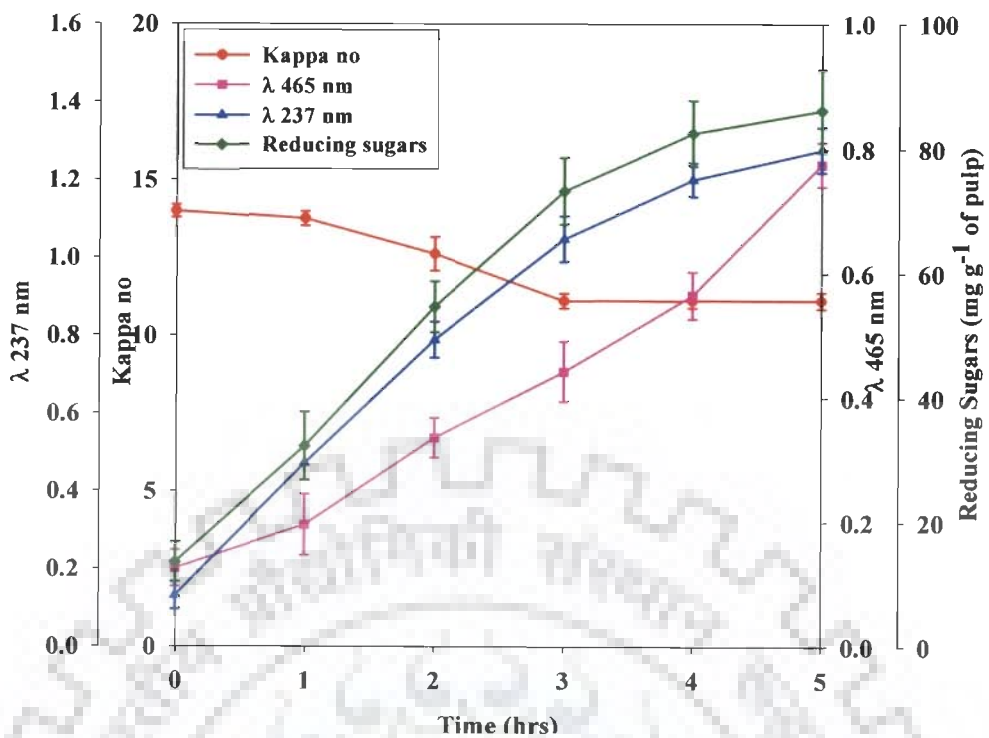
A treatment time of 3 hrs using mixed enzyme preparation was found to be the most suitable leading to maximum decline in kappa no. (20.7 %), release of reducing sugars, phenolic and hydrophobic compounds (Fig. 31b).

4.8.1.4 Effect of mediator concentration

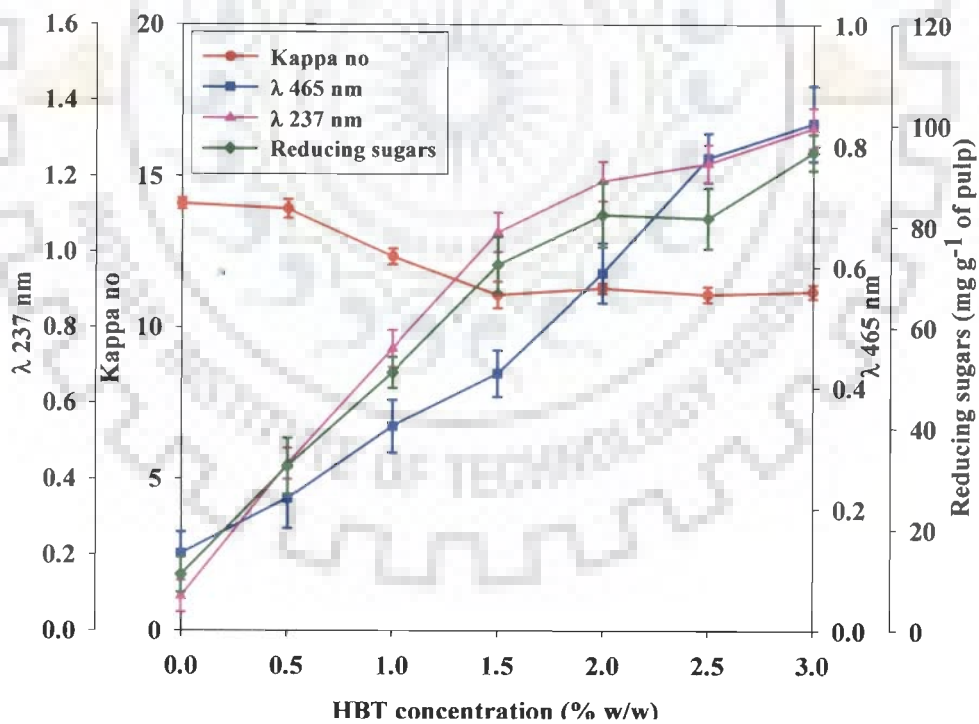
Increased level of lignin removal was achieved when a mediator 1-hydroxybenzotriazole hydrate (HBT) was added to the mixed enzyme preparation. Maximum decrease in pulp kappa no (21.4%) was observed by using 1.5 % of HBT and no further change in kappa no was observed by increasing the mediator concentration (Fig. 31c).



(a)



(b)



(c)

Fig. 31 Derivation of (a) enzyme dose (b) reaction time (b) and (c) mediator concentration for enzyme mediated prebleaching

Maximum level of delignification was achieved with 3 hrs of treatment of xylanase-laccase mixture (8 IU g⁻¹ of pulp) in presence of 1-hydroxybenzotriazol (HBT, 1.5% w/w) which acted as a mediator for laccase at 10 % pulp consistency (Fig. 31 a-c). After enzymatic treatment of pulp under derived conditions, 21% reduction of kappa number, 8% increment in brightness and 5% increase in pulp viscosity was obtained (Table 17). Fig 32 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 increase in release of reducing sugars was observed in the derived bleaching conditions (Fig. 31 a-c). The correlation between releases of chromophores (λ 237 nm), hydrophobic compounds (λ 465 nm) and reduction in kappa number coupled to the release of reducing sugars suggested the dissociation of lignin carbohydrate complex from pulp fibers.

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

| S. No. | Properties | Initial | Final | % improvement |
|--------|--------------|-------------|-------------|---------------|
| 1. | Kappa number | 14.0 ± 0.17 | 11.0 ± 0.44 | 21.0 |
| 2. | Brightness | 35.5 ± 0.34 | 38.3 ± 0.26 | 8.0 |
| 3. | Yellowness | 30.3 ± 0.14 | 29.3 ± 0.14 | 3.0 |
| 4. | Viscosity | 10.1 ± 0.08 | 10.6 ± 0.08 | 5.0 |

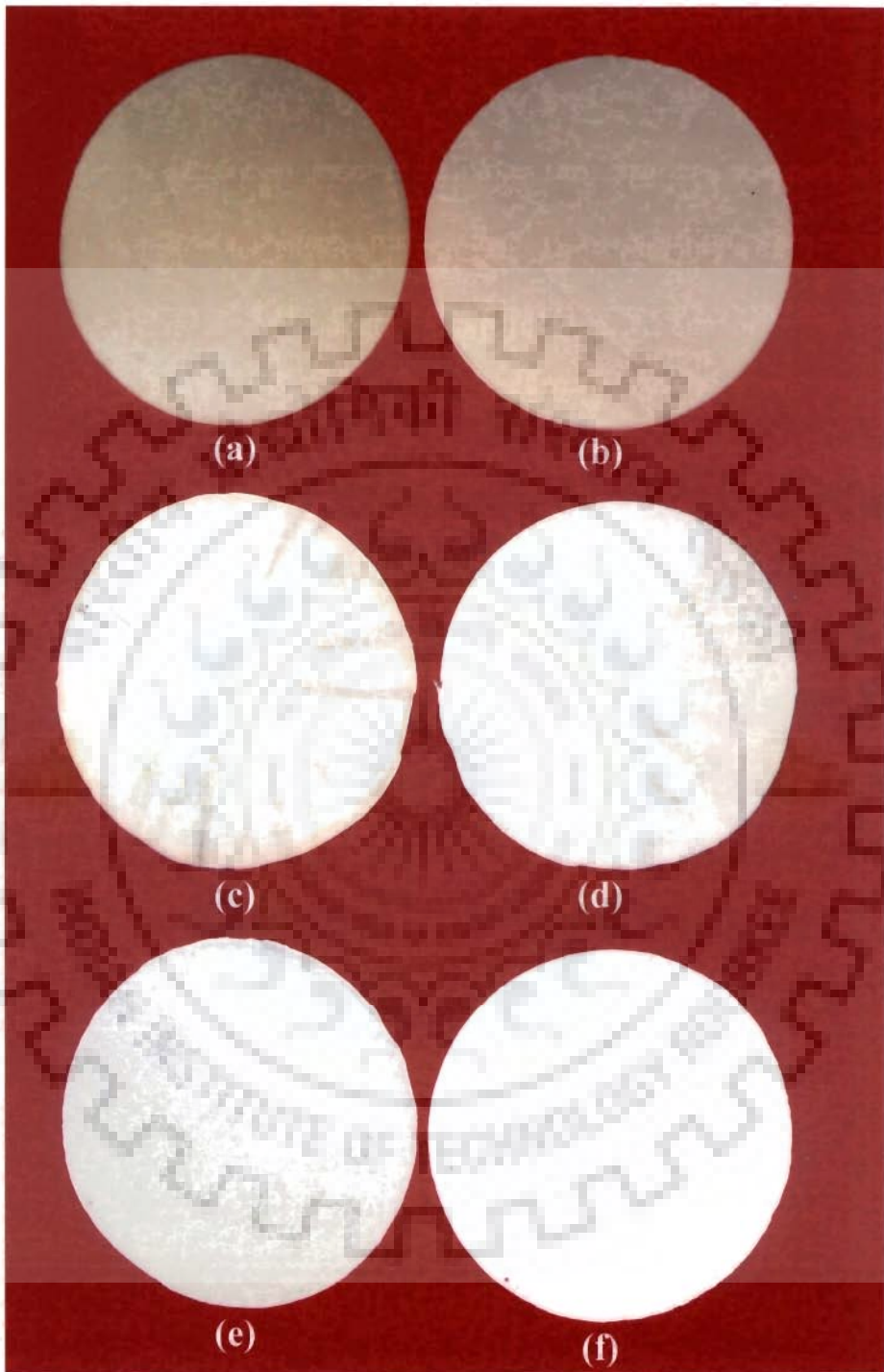


Fig. 32 Colour of the handsheets made from mixed wood pulp (a), untreated; (b), enzyme treated; (c), employing CEPHHP; (d), employing XCEPHHP; (e), employing ODED and (f), employing XODED bleaching sequences

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 lesser amount of bleaching chemicals to achieve a particular brightness standard ($\geq 70\%$ ISO). Following enzymatic treatment 22% less chlorine and 26% less chlorine dioxide was 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 into 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 deterioration.

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

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

| S.No. | Bleaching Sequences | Brightness (%ISO) | Chemical consumption (%) | AOX (Kg t ⁻¹) | COD (mg l ⁻¹) |
|-------|---------------------|-------------------|--------------------------|---------------------------|---------------------------|
| 1a. | CEPHHP | 71.5 ± 0.53 | 95.0 | 5.8 | 921 ± 12.7 |
| 1b. | XCEPHHP | 81.2 ± 0.53 | 74.0 | 4.0 | 849 ± 7.7 |
| 2a. | ODED | 73.9 ± 0.20 | 92.0 | 4.1 | 673 ± 15.0 |
| 2b. | 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 mixed enzyme preparation and its subsequent bleaching with ODED procedure had enhanced various physical properties viz tensile strength, tear strength and burst strength while comparatively less superior strength properties were observed following conventional bleaching sequence. These results indicate that mixed enzyme preparation is more effective and preserve paper strength during elemental chlorine free bleaching sequence.

Table. 19 Effect of enzyme treatment on paper properties

| S.No. | Bleaching Sequence (N mg ⁻¹) | Tensile Strength (m N m ² g ⁻¹) | Tear Strength (k pam ² g ⁻¹) | Burst Strength |
|-------|--|--|---|----------------|
| 1. | CEPHHP | 36.6 ± 0.17 | 10.3 ± 0.77 | 3.7 ± 0.05 |
| 2. | XCEPHHP | 33.4 ± 0.17 | 4.6 ± 0.02 | 2.5 ± 0.08 |
| 3. | ODED | 34.6 ± 0.13 | 5.1 ± 0.08 | 1.7 ± 0.05 |
| 4. | 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 collected were 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 the increase in enzyme dose (Fig. 33).

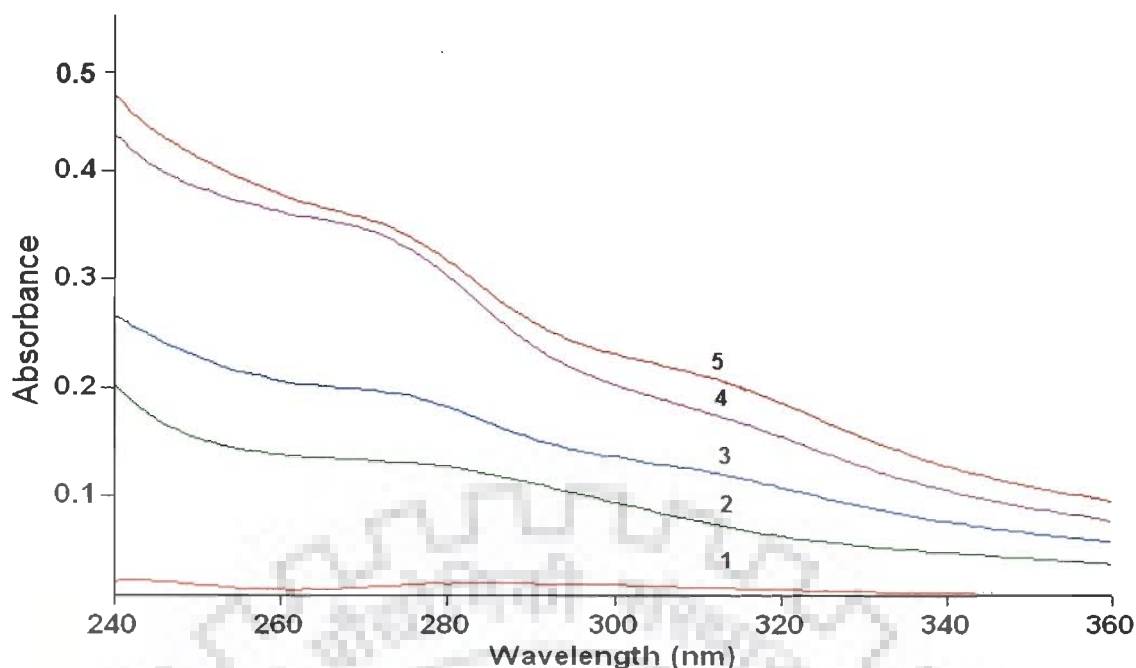


Fig. 33 UV spectra of coloured compounds released during enzymatic treatment at different enzyme doses (1, untreated; 2, 4 IU g⁻¹; 3, 8 IU g⁻¹; 4, 12 IU g⁻¹; 5, 16 IU g⁻¹ of oven dried pulp)

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 mixed enzyme preparation, while marginally lower levels of the same was found in the effluents of pulp treated with the xylanase (Fig. 34a-c). These results had indicated that presence of laccase may have synergistically enabled the xylanase attack on the xylan layer yielding the xylooligomers (xylopentose, xylohexaose, xyloheptaose, xylooctaose, xylononaose and xylodecaose) which was further degraded into 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 had led into increased release of catechol, syringaldazine and some extra phenols (Fig. 35a-c). Lower levels of catechol and syringaldazine was observed in the laccase treated samples.

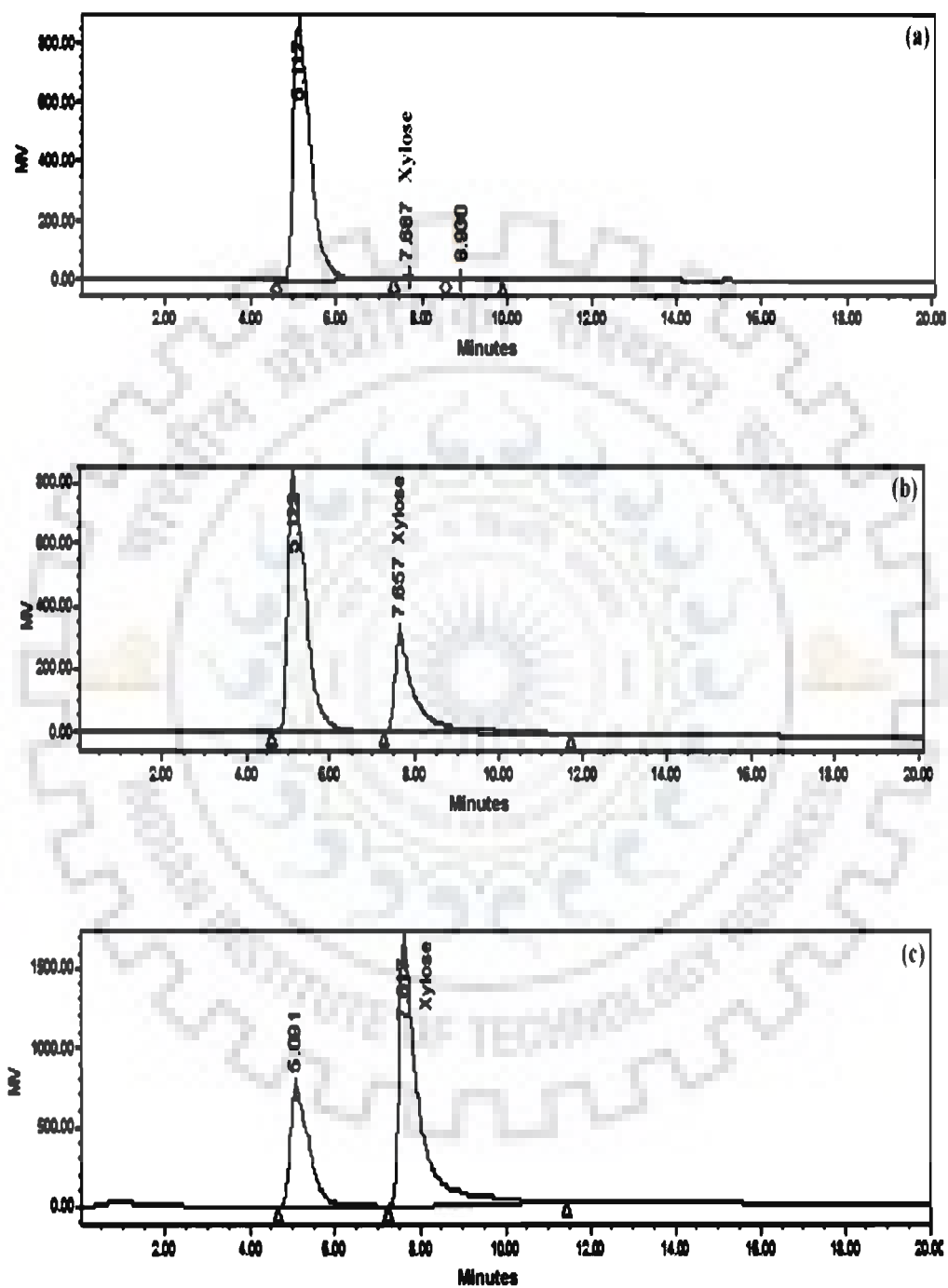


Fig. 34 High performance liquid chromatography of xylooligomers released (a, untreated; b, xylanase treated and c, xylanase-laccase treated)

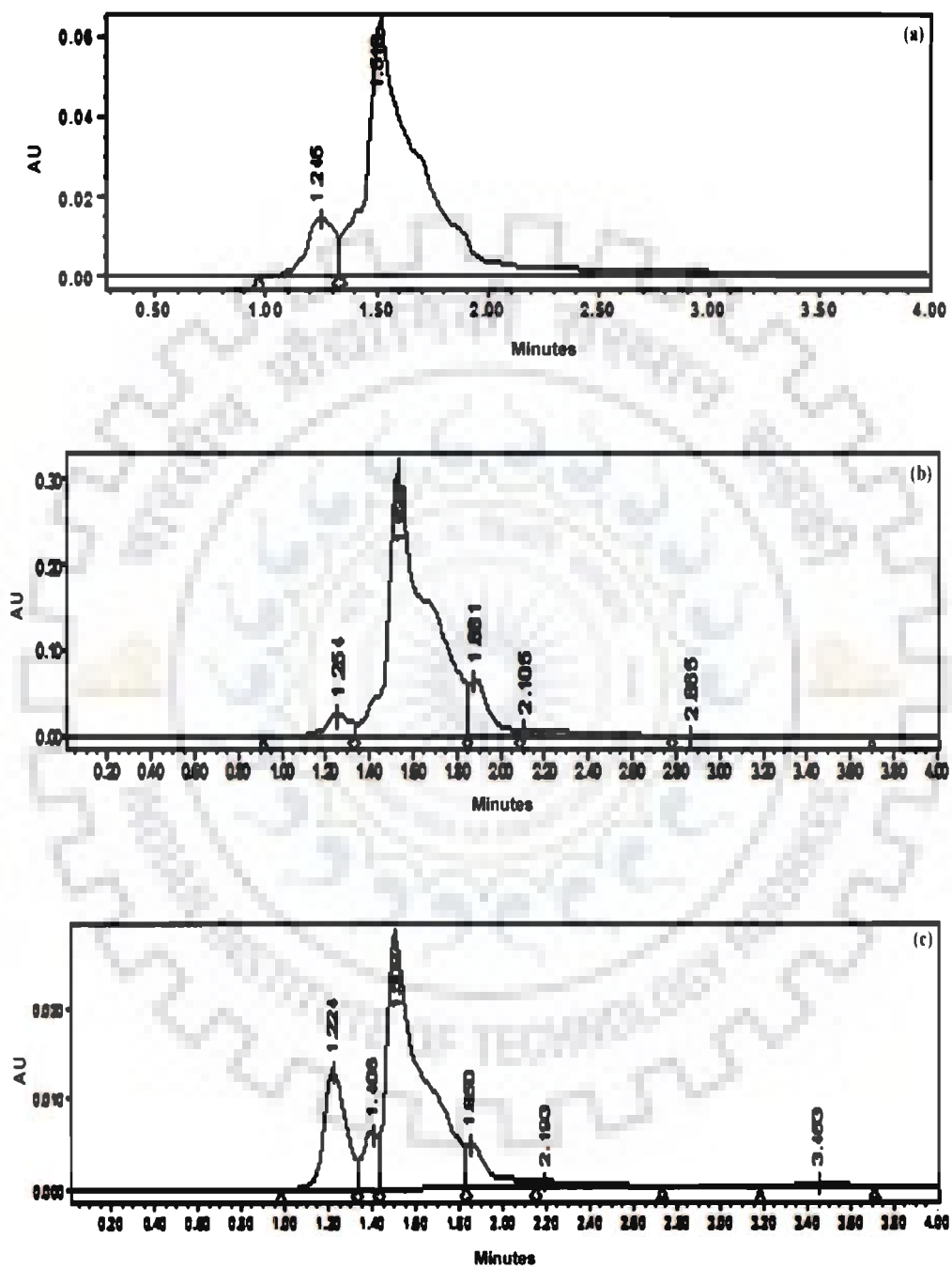


Fig. 35 High performance liquid chromatography of lignin degradation products released (a, untreated; b, xylanase treated and c, xylanase-laccase treated)

4.8.5 Analysis of fiber 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 fibers (Fig. 36 b) as compared to the smoother surface of untreated pulp (Fig. 36a). The swelling, separation and loss in compactness in the pulp fibers render them more susceptible for chemical bleaching as it facilitates the entry of various chemicals during subsequent bleaching stages. Grooves and cracks appeared to have formed in distinct regions of the fiber, although they were not evenly distributed. It is well known that during pulping process short xylan chains precipitated on the surface of pulp fibers and act 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 leads 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. 37), it is highest for xylanase-laccase treated (XL) pulp, hence it is least crystalline. Thus bleaching ability of mixed enzyme preparation was superior compared to xylanase alone as it had enabled in higher extent of lignin removal from the lignocellulosic materials.



(a)



(b)

Fig. 36 Scanning electron microscopy of the pulp (a) before and (b) after treatment with mixed enzyme preparation

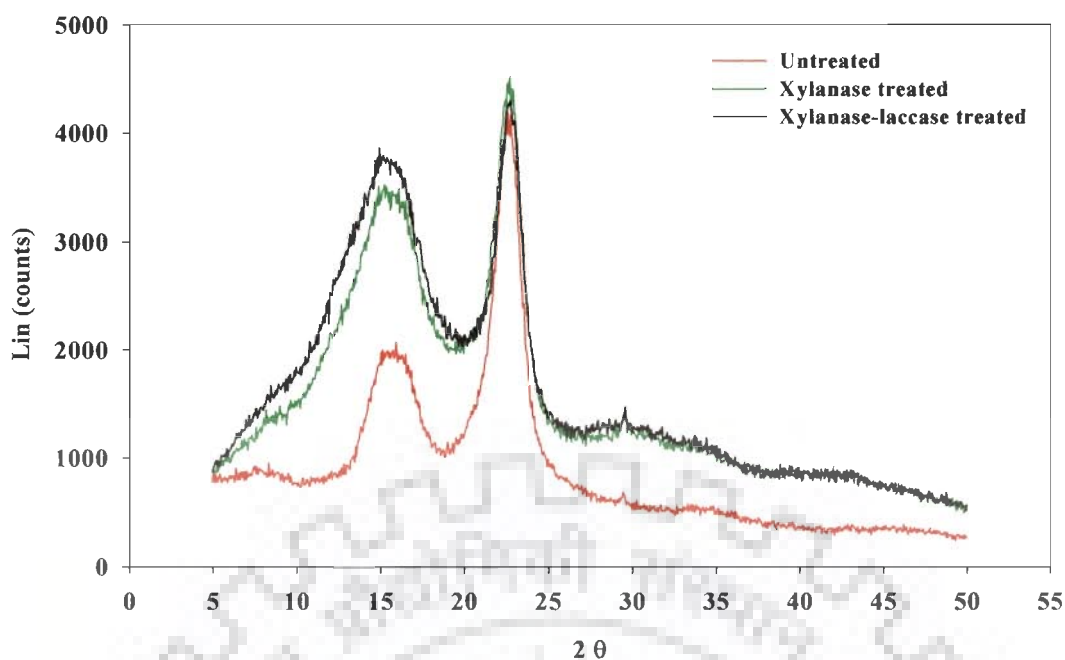


Fig. 37 X-ray diffraction analysis for crystallinity of pulp samples

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 the 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} denote 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 one (Fig 38 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 few more peaks had appeared in this region (Fig. 38c). The peak intensity at this wave number is attributed due to presence of methyl/methylene (-CH) groups. The new, strong absorption peak at 1720 cm^{-1} in XL and X treated pulp had appeared, however, its intensity was lower in X treated pulp and it was totally absent in untreated pulp. This peak is attributed due to carboxylic acid and unconjugated carbonyl groups (C=O) due to action of enzyme on lignin's aromatic ring. In untreated samples these carbonyl groups remain associated with aromatic rings, hence effected into no absorption. Besides this more peaks had appeared at wave number $1600\text{--}1400\text{ cm}^{-1}$ and $1300\text{--}1200\text{ cm}^{-1}$ due to the changes in the ratio of syringyl and guaiacol groups.

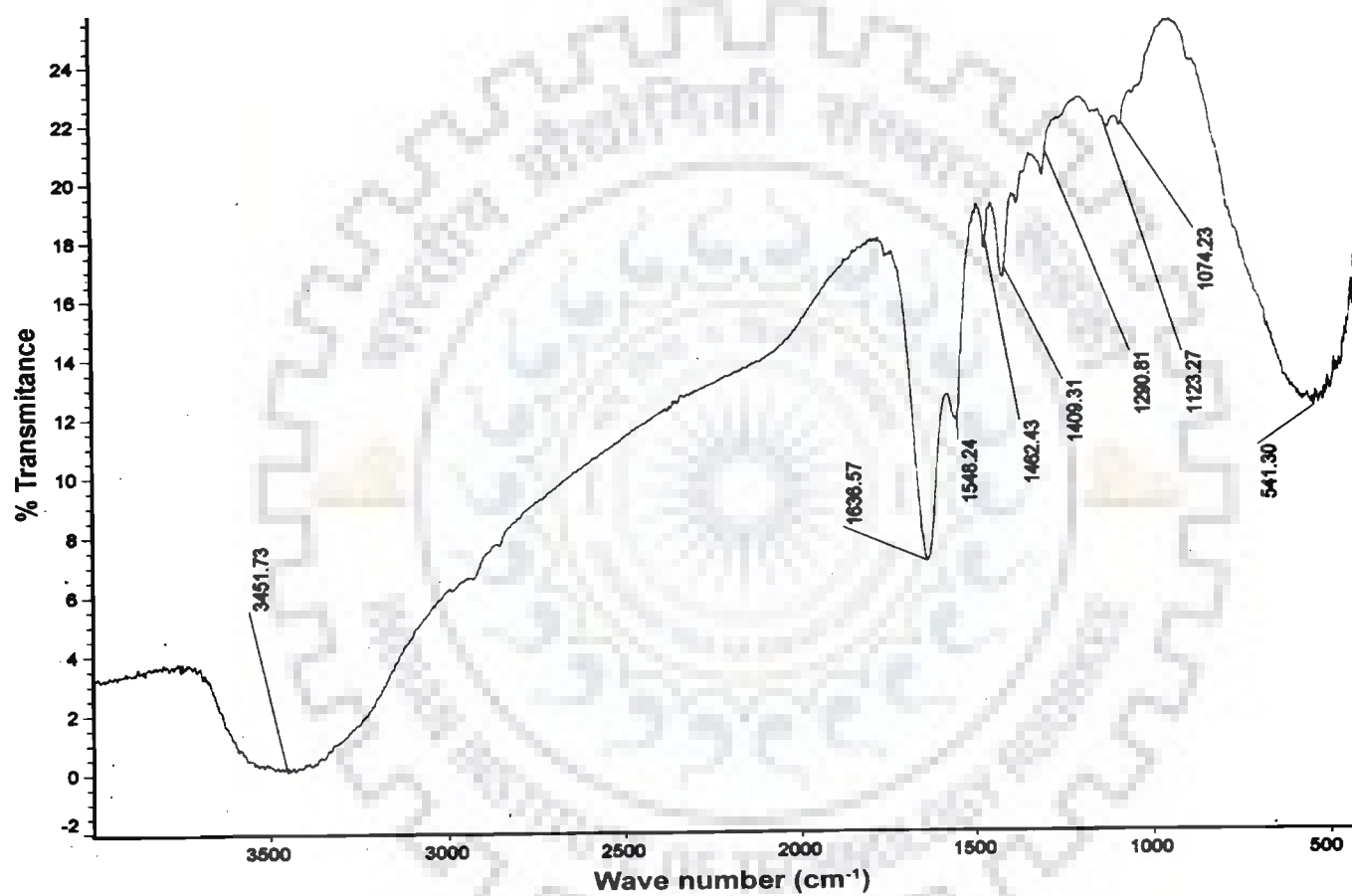


Fig. 38a FTIR spectra of lignin extracted from untreated pulp

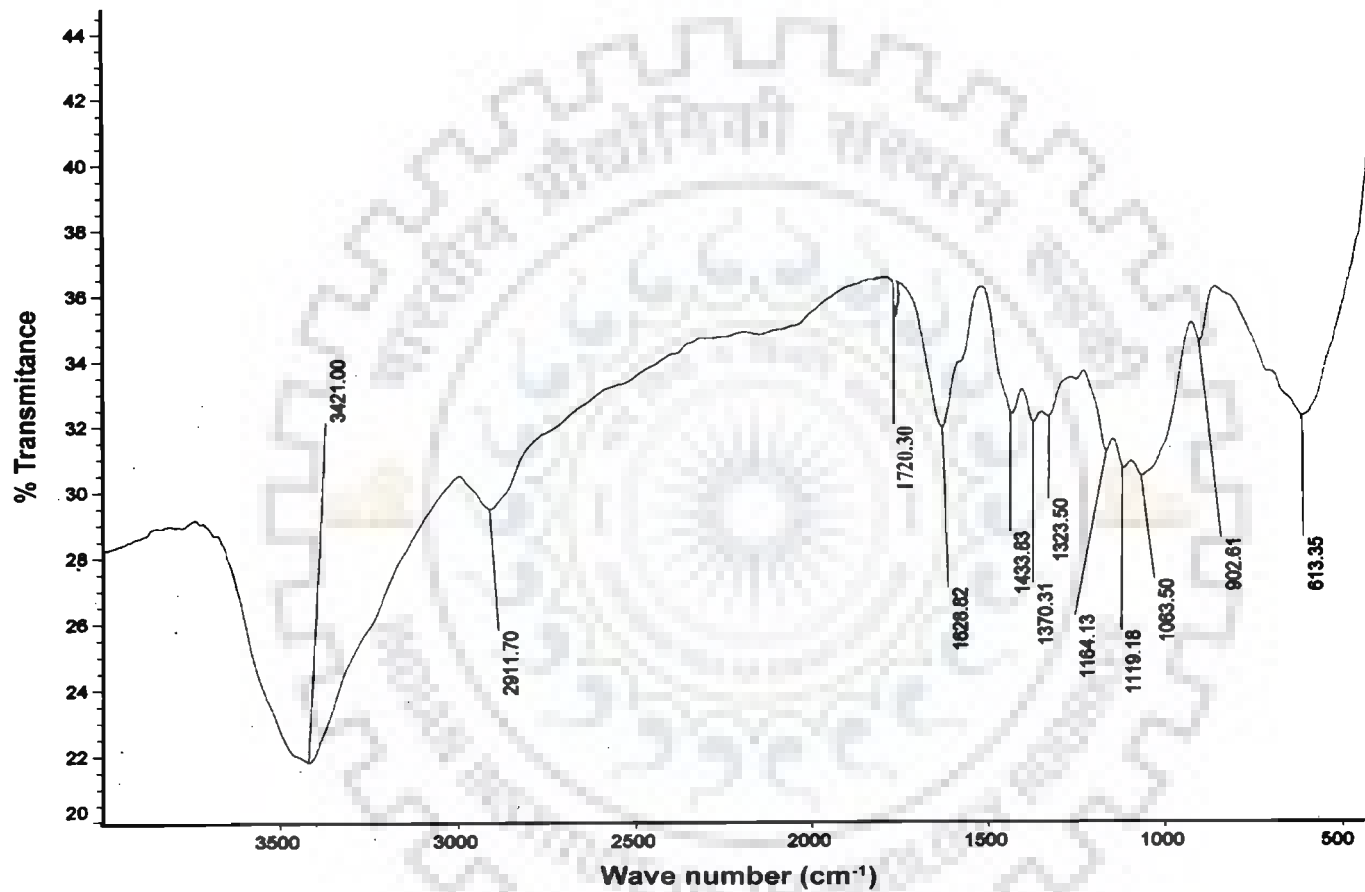


Fig. 38b FTIR spectra of lignin extracted from xylanase treated pulp

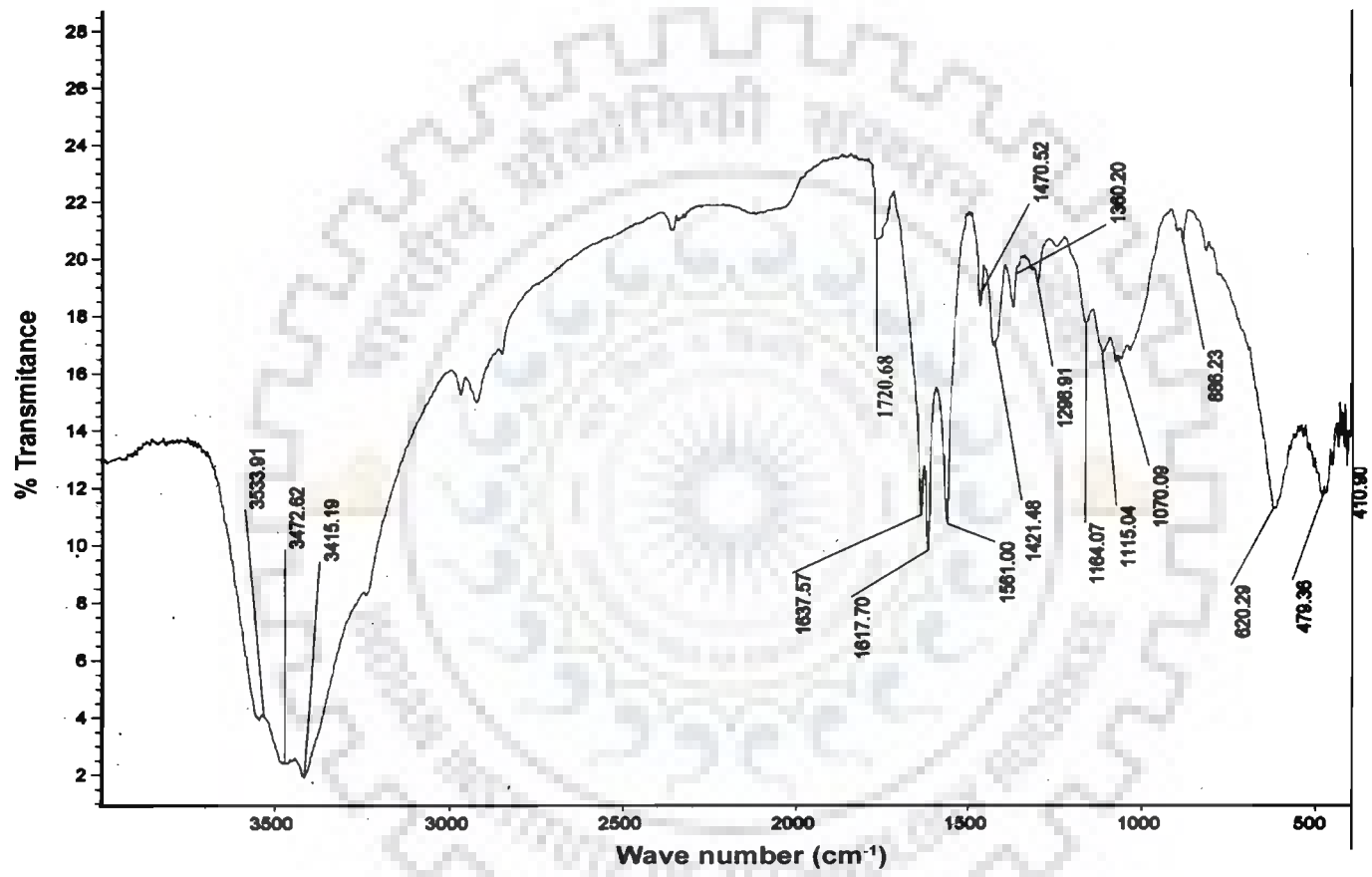


Fig. 38c FTIR spectra of lignin extracted from xylanase-laccase mixture treated pulp

4.9 BIOPROCESS DESIGNING

Scaling-up of 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 as 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 was added with 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 80% of initial moisture content. A typical growth associated production of enzymes was observed by using the optimized culture medium under derived growth conditions of 80% initial moisture and 30°C temperature (Fig. 39). Production of xylanase and laccase begun after 2nd day of incubation following inoculation with mutant *P. oxalicum* SAU_E-3.510 which increased progressively thereafter and reached to maximum level of xylanase (7832.46 ± 353.71 IU g⁻¹) and laccase (280.76 ± 19.05 IU g⁻¹) following 6 days of incubation (Fig. 39). Significant levels of enzymes were observed up to 8th day but activity declined thereafter. Similar pattern of enzyme production was also observed for β -xylosidase but maximal levels of cellulase activity (1.33 ± 0.02 IU g⁻¹) was achieved following 2 days of incubation that decreased after further incubation.

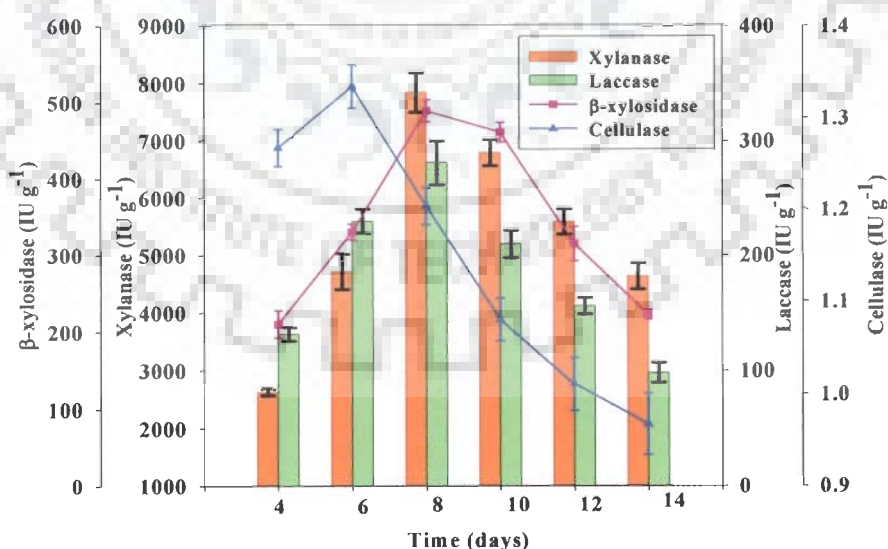


Fig. 39 Simultaneous production of xylanase and laccase in laboratory scale intermittent rotating drum bioreactor by co-cultivation of mutant *P. oxalicum* SAU_E-3.510 and *P. ostreatus* MTCC 1804



(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

To assess the efficacy of bioreactor, the levels of production of enzymes obtained in the bioreactor were compared with that the production levels obtained in the smaller scale laboratory experiments. Significantly comparable levels of production of enzymes were obtained in the bioreactor (Table. 20), xylanase yield obtained in the bioreactor was around 12% lower than that obtained in laboratory scale flask set ups.

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

| S.No. | 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) |
| 1. | Xylanase | 7832.46 ±353.71 | 1304.60 ± 44.61 | 8965.66 ±194.54 | 1494.27 ±32.42 |
| 2. | Laccase | 280.76 ±19.05 | 46.00 ±2.08 | 390.00 ±15.01 | 65.00 ± 2.50 |
| 3. | β-xylosidase | 488.28 ±14.42 | 81.44 ±5.44 | 591.33 ± 4.91 | 98.55 ±0.81 |
| 4. | Cellulase | 1.20 ±0.02 | 0.18 ±0.009 | 1.21 ±0.06 | 0.20 ±0.01 |

These findings therefore denote that solid-state process can be effectively scaled-up and the designed intermittent rotating bioreactor system can be successfully utilized for scaling-up the production level. Minor modifications in this system may further enable improvements in the levels of production.



CHAPTER V

DISCUSSION

5.1 IDENTIFICATION OF ALKALITOLERANT XYLANASE PRODUCING MICROORGANISM

Microorganisms are considered as “miniature cell factories” and are exploited for the production of enzymes of commercial significance. Enzymes are recognized as potential alternatives to harsh chemical technologies which have led to intensive efforts to explore natural microbial diversity to discover enzymes that could be used for environment 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 utilize these for industrial applications.

Many workers have employed the stepwise screening approaches for selecting the xylanase producing microorganism (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 mainly, waste of soda ash industry (pH 11.0) and effluents from 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 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 maximum level of xylanase (242.05 ± 3.91 IU ml⁻¹) with noteworthy amount of β -xylosidase (16.20 ± 2.00 IU ml⁻¹) under alkaline conditions of growth (pH-9.0) and also due to its having 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 Indian Type Culture Collection Bank, Indian Agricultural Research Institute, New Delhi, India. Attempts also have been made to isolate alkalophilic strains from alkaline lake (Mamo et al., 2006,

Gessesse and Mamo, 1999), effluent of pulp and paper industry (Anthony et al., 2003) and decomposed plant samples (Swaroop Rani and Nand, 2000) for obtaining 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 although had shown significant levels of xylanase production but in order to explore the possibilities for commercial exploitation of the enzyme, attempts were made for achieving further increase in the enzyme production levels. Strain was subjected to mutagenesis for achieving an improvement in production level and the process was further economized by evaluating the low cost agro-industrial residual materials as substrate in the production media.

5.2 MUTAGENESIS OF *P. OXALICUM* SA-8

Isolated strain *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 has 87% higher xylanase levels as compared to wild type strain. Induced mutagenesis and strain selection has 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 (1.2-2.5 folds higher) but also had improved bioconversion efficiency.

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

5.3 ANALYSIS OF XYLANASE PRODUCTION AT VARYING pH

Production of xylanase in media having wide range of pH indicate towards alkalitolerant nature of the fungus since higher level of xylanase production was reported in alkaline condition (pH 9.0). A specific pattern of xylanase activity was observed for both mutant and wild type strains, when activity was measured at varying pH from 4 to 11. Maximum xylanase activity i.e. 487.44 ± 3.57 IU ml⁻¹ and 252.50 ± 6.27 IU ml⁻¹ was obtained at pH 9.0 followed by the subsequent decrease in activity along with increasing pH. However, interestingly at pH 5.0 again a notably higher xylanase activity was observed in 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 from *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 as observed with *Trichoderma reesei* C-30 had also resulted into 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. Xylanase activity at different pH were 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 reported from genus *Penicillium* are optimally active in acidic range (pH 5.0). Recently, a cellulase free, alkaliphilic 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 pulp and paper industry due to its environmental and technological benefits. During paper manufacturing, the fibers 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 frequently used around the world and requires cooking of wood chips at high temperature and pH. This process removes around 95% of total lignin but remaining 5% lignin which is responsible for 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 use of thermostable and alkalophilic xylanase would prove to be more suitable. Very few reports are available regarding alkalophilic xylanases from fungi like *Aspergillus fischeri* (Chandra Raj and Chandra, 1996), *Cephalosporium* 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 UNDER SUBMERGED FERMENTATION

The carbon source used for the production medium is one of the major factor 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 had resulted into maximum level of xylanase (488.56 ± 6.53 IU ml⁻¹), which was followed by xylose. It is therefore apparent that xylan acts as inducer for xylanase biosynthesis because it releases mono, di and largely oligosaccharides on enzymatic degradation and however xylose appear to act as marginal inducer due to apparently its quick utilization by microbe which results into lower level 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 into remarkably higher xylanase activity (475.23 ± 6.05 IU ml⁻¹) followed by the activity (397.05 ± 7.53 IU ml⁻¹) from the treated water hyacinth (*Eichhornia crassipes*). Xylanase activity, obtained from the congress grass was about at similar levels to that obtained with commercial oat spelt xylan. Thus it can be inferred that this replacement, for xylanase production, implies environmental and economic advantages. Since, 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. Utilization of these plant biomass materials will not only address the hazards due to the pollution menace but would also authenticate this menace to potential providers.

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

Increased production of xylanase was observed in presence of veratryl alcohol and ABTS. Improved rate of xylan hydrolysis was also reported in presence of lignin or lignin model compounds at lower concentrations. The affinity of xylanase for lignin or lignin model compounds has earlier been observed (Kaya et al., 2000). This interaction may possibly regulate the molecular conformation of xylanase resulting into increase in xylanase activity as observed. Soluble lignin-xylanase complex apparently accelerate hydrolysis of soluble xylan possibly by introducing some change in 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 biosynthetic abilities as compared to respective monoculture, hence used for obtaining improved activities mainly for biodegradation of compounds like 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), cyanide (Ezzi and Lynch, 2005). Mixed fungal cultivation appears to have a

number of advantages over a pure culture. As compared to monoculture, paired fungal culture affects into better substrate utilization, increased productivity, improved adaptability to changing conditions and superior resistance to contamination by unwanted microbes. It impacts into 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 reduction in 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 of antagonistic, parasitic or synergistic type. Mixed fungal cultures could lead into higher enzyme production through synergistic interaction, but final outcome seems to depend on particular combination of species, mode of interaction between them and on the microenvironment or nutritional conditions provided by the substrate under colonization (Gutierrez-Correa and Tengerdy, 1998). Interaction between *Laccaria laccata* and *Trichoderma virens* in co-culture is of 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 contact zone (Werner et al., 2002). Similar kind of antagonistic interaction was also observed by Stepanova et al. (2003) during co-cultivation of *T. reesei* with three basidiomycetes. The mold *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 colonize 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 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 had resulted into stimulation of the enzyme production ability. 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) had 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) had 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, when 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 (1.5-2.1 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). 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 UNDER SOLID-STATE FERMENTATION

The advantages of mixed culturing may be even more evident in solid-state fermentation because the colonization 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, 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). Utilization of these residual materials for bioprocesses also eases the pollution menace, which otherwise is a major concern due to their disposal (Pandey et al., 2000). 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 under solid-state fermentation condition.

To obtain further improvement in production level, SSF was performed using a variety of solid supports of agricultural origin. Medium constituents particularly the substrate to a greater extent affect the cost of production, the same can be further economized by using low cost and easily available agro-industrial residual materials (Sandhya et al., 2005). A mixture of

bagasse and black gram husk in a ratio of 3:1 was most productive for both xylanase ($7574.24 \pm 118.99 \text{ IU g}^{-1}$) and laccase ($381.33 \pm 10.01 \text{ IU g}^{-1}$). Sugar cane bagasse and black gram husk both are abundantly available in India due to the agricultural practices and serve as a solid substrate during SSF. 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, support 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 varying moisture content 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 level up to 85% and had resulted into 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 hrs. A 93% of residual activity was observed after four hours of incubation in the respective buffer at pH 11.0, while about 50% decrease in the activity was detected after 20 hrs. Similarly, enzyme was also detected to be appreciably thermostable. Maximal activity of the enzyme was obtained at 55°C and half life of enzyme was found to be two hrs at 80°C when 50% of the activity remained following incubation.

Analysis employing SDS-PAGE and zymogram had indicated the presence of two isoforms of xylanase in both 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 synthesized 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 mutant while 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* till date while higher molecular mass isoforms of xylanases have been reported from *Aspergillus 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 mainly *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 from 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. 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 paired culture of mutant

P. oxalicum SAU_E-3.510 and *P. ostreatus* MTCC 1804 were analyzed 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 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. 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 from mutant *P. oxalicum* SAU_E-3.510, possessing both endo and exo degrading activities. These observations suggest that xylanase from mutant strain could be used for the production of monomeric xylose from congress grass which grows wildly and abundantly available. The release of xylose and xylobiose as the main hydrolysis product was also reported for xylanase 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 pulp and paper industry. The concoction of xylanase-laccase which was developed by co-culturing of fungi under solid-state fermentation, was evaluated for its biobleaching efficacy of mixed wood kraft pulp. Improved levels of delignification was achieved by using xylanase laccase mixture as it had resulted into 5% more

decrease in kappa number as compared to xylanase alone. Similarly, brightness of the sheets which was treated with mixed enzyme preparation was also higher (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 synergistic action of xylanase and laccase. The size of laccase enzyme is considered to be too large for penetration into the fiber wall (Goodell et al., 1998), but action of xylanase may affect the cell wall to be porous and lignin is exposed on the surface for the action of laccase enzyme. Xylanase attacks on xylan layer, and to reprecipitated xylan on fiber surface and break lignin carbohydrate complex. The reaction products that are released by the xylanase (xylooligomers and xylose) may have quenched the radicals created in the lignin structure by lignin modifying enzymes (Niku-paavola et al., 1994). Therefore, 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, 21% reduction of kappa number, 8% increment in brightness and 5% increase in pulp viscosity was obtained. The application of xylanases for improvement in pulp bleaching had been 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 8-20% reduction in pulp kappa number, 3.5-9.6% improvement in brightness, 10-29% less chemical load. The pulp viscosity remains unchanged or slightly increased which indicate the selective nature of enzyme that left cellulose fibers intact. Synergistic effect of xylanase and laccase for the bleaching of soda pulp was

studied by sequential addition of xylanase and laccase. A 17.6 % reduction in kappa number and 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 other). The findings reported here on derivation 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 lignin carbohydrate complex from the fibers. Release of chromophores as well as hydrophobic compounds was higher at optimized bleaching conditions (8 IU g⁻¹ of pulp, 3hrs, and 55°C). Release of reducing sugars continued at reduced rate due to xylanase mediated hydrolysis of soluble xylooligosaccharides which were released by the initial depolymerization of the xylan. Thus, 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 indicates 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 in the decreased requirement of bleaching chemicals. Thus requirement of chemicals for bleaching got decreased by 22-26% for enzymatically treated pulp as compared to the untreated one. In addition to this, enzymatic bleaching with xylanase-laccase concoction reduces the discharge of adsorbable organic halides (17-30%) and chemical oxygen demand (7-21%) respectively. These findings suggest the superiority of xylanase-laccase mixture for bleaching of mixed wood pulp in economic and environment friendly manner.

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

5.8.1 Analysis of 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, xylan remained intact, therefore, there was no release of degradation products. Detection of increasing levels of phenolic compounds in xylanase-laccase treated sample had indicated the delignification efficacy of mixed enzyme preparation. Niku-Paavola et al., (1994) had also observed the enzymatic delignification on pine kraft pulp and obtained the similar results.

5.8.2 Analysis of fiber morphology

Pulp fibers before and following enzymatic treatment revealed distinct changes in the surface architecture. Morphological changes such as “cracks” and “peeling” of fiber surface

were evident after enzymatic treatment and were observed by other groups (Pham et al., 1995; Torres et al., 2000 and Roncero et al., 2000, 2005). 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 Analysis of 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 decrease in pulp crystallinity. Thus mixed enzyme preparation as denoted by X-ray diffraction analysis had better bleaching ability as compared to xylanase alone. Decreased pulp crystallinity due to improvement in fiber fragility was also observed following xylanase from *Bacillus* sp. treatment of ramie fibers (Zheng et al., 2000). Ammonia pretreated soybean straw also showed decline in crystallinity following enzymatic treatment due to removal of lignin and hemicellulose (Xu et al., 2007).

5.8.4 Analysis of residual lignin

Due to enzymatic treatment there was 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. Appearance of a strong absorption peak at 1720 cm^{-1} in FTIR spectra of pulp samples treated with xylanase-laccase mixture had suggested lignin degradation as the same peak was not detected in the spectra of untreated pulp samples. This peak is attributed to carboxylic acid and unconjugated carbonyl groups ($\text{C}=\text{O}$) due to action of enzyme on lignin's aromatic ring (Buta et al., 1989). The peak therefore specifically denotes lignin degradation due to enzymatic action. In untreated samples these carbonyl groups remain associated with aromatic rings, hence result into no absorption. Oxidation of phenolic components and enrichment in carboxylic acid groups (absorption peak at 1719 cm^{-1}) was 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

Scaling-up of process for production of the mixed enzyme preparation having significant levels of xylanase and laccase through co-cultivation was accomplished through designing and operating of an intermittent rotating drum bioreactor (IRDB). In contrast to submerged fermentation, scaling-up of solid-state fermentation appears to be tedious due to difficulty in controlling the important process parameters such as heat transfer, aeration, mixing, temperature and moisture control. 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 submerged process due to poorer heat transfer ability of solid-support used (Ward, 1992). The problem of heat generation is more pronounced in tray bioreactors whereas in drum bioreactors, 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 hrs) of fermentation vessel facilitates the proper mixing of substrate and fungal inoculum during production period which had led into higher levels of xylanase, β -xylosidase and laccase production. Growth of the fungi in drum

bioreactor is 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 for other SSF bioreactors. Solid-state fermentation in a laboratory scale horizontal bioreactor using the derived medium showed the production of 320 U g^{-1} of xylanase by *Sporotrichum thermophile* (Topakas et al., 2003). Similarly higher 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). 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 promote proper heat transfer, aeration, distribution of inoculum and nutrients during cultivation, it may to some extent damage mycelia due to sheer 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, the designed system therefore has a provision of a cooling tray at the base of fermenter vessel to minimize the effect of heat build-up.



CHAPTER VI

SUMMARY AND CONCLUSIONS

SUMMARY AND CONCLUSIONS

An attempt was made to have an effective, ecofriendly bleaching technology by using industrially compatible enzyme preparation to minimize the pollution load due to paper industries. With an aim of developing an alkalitolerant, cellulase free xylanase, waste from soda ash industry (pH- 11.0) was explored for its microbial biodiversity. A potent xylanase producer fungal strain (SA-8) having notable xylanase activity at alkaline pH (242.05 ± 3.91 IU ml⁻¹) was isolated and identified as *Penicillium oxalicum* SA-8 (ITCC 6204) from Indian Agricultural Research Institute, New Delhi, India. Due to its alkalitolerant nature and negligible cellulase activity (0.18 ± 0.03) the strain was selected for further studies and subjected to mutagenesis in an attempt for achieving enhanced 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. Higher levels of xylanase (453.02 ± 5.70 IU ml⁻¹) was observed from *P. oxalicum* SAU_E-3.510. Mutant and wild type strains of *P. oxalicum* can 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 rough peeled surface whereas that of wild type *P. oxalicum* SA-8 were flattened, shiny, smooth surfaced and straight. Similarly, spores of mutant were smooth and crescent shaped while the wild type spores were rounded with rough surface.

A specific pattern of xylanase activity was observed for mutant as well as for wild type strains, considerably higher levels of xylanase 487.44 ± 3.57 IU ml⁻¹ and 252.50 ± 6.27 IU ml⁻¹ was observed at pH 9.0, the activity decreased although remained higher on further increase in pH. Interestingly, notably higher levels of activity from mutant as well as wild type strain 444.89 ± 7.60 IU ml⁻¹ and 197.81 ± 6.94 IU ml⁻¹ respectively was also observed at pH 5.0.

Production of xylanase was evaluated under submerged condition by using wildy growing and abundantly available plant biomass mainly *Parthenium* sp. (congress grass), *Eichhornia crassipes* (water hyacinth). A notably higher level of xylanase activity ($475.23 \pm 6.05 \text{ IU ml}^{-1}$) 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 ($488.56 \pm 6.53 \text{ IU ml}^{-1}$) 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 wide spread and wild occurrence but it would substantially contribute towards lowering the cost of xylanase production. The mutant *P. oxalicum* SAU_E-3.510 produced $714.13 \pm 15.40 \text{ IU ml}^{-1}$ of xylanase at 6th day of incubation under derived conditions along with significant levels of β -xylosidase.

Mixed culture fermentation, consisting of inoculums from two or more organisms is a promising system for 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 type which may lead into changes in enzymatic activities. As compared to monocultures, paired culture of fungi may lead into better substrate utilization, increased productivity and increased resistance to contamination. In addition to this, co-culturing of compatible microbial partners in same fermentation medium may result into 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 further broadens the horizon of enzymes in pulp and paper industry. Hydrolysis of xylan due to action of xylanases leads into exposure of lignin layer. The exposed lignin layer is effectively attacked by laccase resulting into improved delignification and therefore require lesser extent of bleaching chemicals in the subsequent

bleaching stages. Therefore, bleaching of pulp with comparatively superior xylanase-laccase concoction may be economically fruitful to paper industry in an ecofriendly manner.

A novel strategy of fungal co-cultivation was evaluated for developing a xylanase-laccase concoction. Growth compatibility analysis revealed the suitability of mutant *P. oxalicum* SAU_E-3.510 with *P. ostreatus* MTCC 1804 which is known as hyper laccase producer strain. *P. oxalicum* SAU_E-3.510 was observed to be a potent xylanase producer while laccase activity was not detected from the same. The scanning electron microscopy had indicated the closely associated contact regions for both the fungi and any type of branching, deformities or injuries were not observed. Dual culture of mutant *P. oxalicum* SAU_E-3.510 and *P. ostreatus* MTCC 1804 had yielded an over all increase of 58% and 33% in xylanase and laccase activities respectively under derived conditions.

Enzyme production during co-cultivation was also evaluated by employing different fermentation systems. Highest level of xylanase (1301.51 ± 12.73 IU ml⁻¹) and laccase (43.70 ± 3.72 IU ml⁻¹) was obtained when fungi were co-cultured under solid-state fermentation as compared to production under surface and submerged fermentation. The advantages of mixed culturing are more evident in solid-state fermentation due to the proper colonization of the substrate by the fungus. The SSF system had led to maximum levels of enzyme production and an attempt to further improve and economize the level of production was made by evaluating the 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 most promising for xylanase (7574.24 ± 118.99 IU g⁻¹) and laccase (381.33 ± 10.01 IU g⁻¹) production.

Xylanase of mutant *P. oxalicum* SAU_E-3.510 appeared to be quite stable at higher pH and temperature. The enzyme maintained 93% of its residual activity at pH 9.0 for 24 hrs. Similarly, enzyme was also detected to be appreciably thermostable. Half life of enzyme was

found to be two hrs at 80°C as 50% of its residual activity was detectable. Polyacrylamide gel electrophoresis and zymogram analysis had revealed the presence of two isoforms (121 kD and 77 kD respectively) from mutant *P. oxalicum* SAU_E-3.510 while paired culture of *P. oxalicum* SAU_E-3.510 with *P. ostreatus* MTCC 1804 had yielded an additional xylanase (40 kD) corresponding to *P. ostreatus* MTCC 1804 along with the laccase of 29 kD. The amino acid sequence of xylanase from *P. oxalicum* SAU_E-3.510 shares significant identity with xylanase sequences from *Bacillus stearothermophilus*, *Geobacillus stearothermophilus*, *Paenibacillus sp.* and *Clostridium sp.* It is noticeable that the amino acid sequences of xylanases are sufficiently diverse in composition and the degree of identity can be marked in various groups of organisms. Analysis of hydrolysis product of congress grass xylan with xylanase by high performance liquid chromatography had 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 xylanase-laccase preparation as well as xylanase alone. Improved level of bleaching was obtained with mixed enzyme preparation. An overall 21% reduction in kappa number and 8% increase in brightness and 5% increase in viscosity was obtained following 3 hrs of treatment with xylanase-laccase mixture (8 IU g⁻¹ of pulp) in the presence of mediator (1.5% HBT) at 10% pulp consistency. Thus an additional 5% decrease in pulp kappa number and comparatively higher level of brightness (3.6%) was obtained with 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 had resulted into 22-26% decrease in requirement of bleaching chemicals as compared to untreated pulp samples. The paper generated from treated pulp had shown superior mechanical and optical properties as compared to paper made from untreated pulp. Higher bleaching ability of xylanase-laccase concoction was also observed due to the increased release of xylose and monophenols in the effluent as compared to the treatment using xylanase alone or untreated one. During

scanning electron microscopy, cracks and peelings were observed in enzymatically treated pulp which had enabled swift action of bleaching chemicals thus leading into enhanced delignification and resulting into lower chemical consumption. As a result of enzyme prebleaching, crystallinity of pulp decreased due to removal and degradation of hemicellulose and lignin thereby leaving cellulose to be amorphous. Degradation of lignin was further evident following FTIR analysis of the treated mixed wood pulp as an extra peak had appeared at 1720 cm^{-1} wave number which indicates oxidative action of laccase on lignin. These findings suggest the efficacy of 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 by using the mixture of bagasse and black gram husk as solid support and using the conditions as derived earlier. Cultivation was performed in batch mode and levels of enzymes were achieved with almost similar yield as obtained in small scale laboratory experiments. Maximum level of xylanase ($7832.46 \pm 353.71\text{ IU g}^{-1}$) and laccase ($280.76 \pm 19.05\text{ IU g}^{-1}$) was attained on the 8th day of incubation. The observed levels of production for xylanase and laccase were significantly higher than those obtained earlier for other SSF bioreactors. Mixing by rotating the reactor vessel promote 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 xylanase-laccase concoction appears to be effective, eco-friendly and 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 soda ash industry was found to be a potential strain producing higher levels of xylanase, β -xylosidase with negligible amounts of cellulase at alkaline pH (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 had 87% higher levels of xylanase production.
- c. *Parthenium* sp. when added in production media as carbon source led to about comparative 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 had resulted into 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 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 concoction had resulted into significant increase in ISO brightness and improved paper quality. Enzymatic pre-bleaching had reduced the chlorine requirement by 26 %, thus the process can be considered as ecofriendly, effective and economically viable process.
- g. Solid-state process was scaled up by designing an intermittent rotating drum bioreactor using the earlier derived parameters. A comparable and higher level of xylanase (7832.46 ± 353.71 IU g⁻¹) and laccase (280.76 ± 19.05 IU g⁻¹) was obtained under these conditions.



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