STUDIES ON PRODUCTION OF XYLANASES FROM WHITE ROT FUNGI FOR BIOBLEACHING OF WHEAT STRAW PULP

A THESIS

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

DOCTOR OF PHILOSOPHY

by

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CANDIDATE'S DECLARATION

I hereby certify that the work which is being presented in the thesis entitled **STUDIES ON PRODUCTION OF XYLANASES FROM WHITE ROT FUNGI FOR BIOBLEACHING OF WHEAT STRAW PULP** in partial fulfilment of the requirement and for the award of the Degree of Doctor of Philosophy and submitted in the Department of Paper Technology of the Indian Institute of Technology Roorkee, Roorkee, is an authentic record of my own work carried out during a period from January, 2004 to November, 2008, under the supervision of Dr. C.H. Tyagi, Associate Professor, Dr. J.S. Upadhyaya, Professor and Dr. Dharm Dutt, Associate Professor, Department of Paper Technology, Indian Institute of Technology Roorkee, Roorkee.

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

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

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The Ph.D. Viva-Voce Examination of **Shalini Singh**, Research Scholar, has been held on.....

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ABSTRACT

Indian pulp and paper industry is facing multifarious issues and challenges like, tough competition from imports, obsolete technology, soaring energy and environmental costs, minimum profit level and inadequate supply of low-cost fiber to survive in the global competitive market. The dearth of wood fiber sources has compelled the paper technocrats to search for alternative and hitherto unexploited sources of fast growing plants which may fulfill the demand of pulp and paper industry. Agricultural residues are emerging out as a significant alternative raw material resource for pulp and paper industry. Of these, bagasse and straws are the most important. Although the availability of bagasse is likely to improve due to the expected increase in sugarcane production, yet due to an increase in its demand from sugar mills for producing steam in the boilers, its availability to paper industry would be limited. Thus, looking into the availability and cost, the straws look to be a promising raw material for paper industry.

The scarcity of raw materials for paper industry has grown in parallel with environmental pollution, where pulping and bleaching processes cause major pollution problems. The untreated effluent from bleaching section of pulp and paper industry without chemical recovery, considerably pollute the receiving waters since they have high BOD, COD, AOX and suspended solids. It was found that in chlorine-based pulp bleaching the C-stage is generally the first point in which 2,3,7,8-TCDD (tetrachlorodibenzodioxin), 2,3,7,8-TCDF (tetrachlorodibenzofuran) and 1,2,7,8-TCDF congeners are always present. The E-stage filtrate contained the highest concentrations

of dioxins. The chlorinated aromatic derivatives are toxic and persistent, constituting a potential environmental hazard if untreated before discharge to natural water bodies. Thus, strict legislation and environmental concerns regarding imposition of the effluent discharge norms with reference to AOX in Indian paper industry is forcing the paper mills to adopt alternative techniques to reduce or eliminate the use elemental chlorine in bleaching sequences.

Biotechnology has a high potential in pulp bleaching sector since it allows the development of more sustainable and environment friendly products and processes by reducing chemical usage, saving energy and water, and minimizing waste products. Several workers have approached biological bleaching with lignolytic and hemicellulolytic enzymes (mainly xylanases). The most important application of xylanase enzymes is in the prebleaching of pulps. Xylanases are being tested as bleaching agents for different raw materials and bleaching sequences. Xylanases also help to increase pulp fibrillation and reduce beating time for achieving the targeted beating level. This has focused the attention of researchers towards xylanases obtained from newer microbial isolates, for the use in pulp and paper industry. Microorganisms including bacteria, yeast, actinomycetes and filamentous fungi have been reported to produce xylanolytic enzymes.

Keeping the huge potential of xylanases in view, the present study aims at conducting laboratory studies on enzymatic bleaching of wheat straw soda-AQ pulp using crude xylanases obtained from indigenously isolated white rot fungal strains. The present study has been focused to produce bleached pulp of good mechanical strength by adopting environment friendly bleaching sequences, through enzymatic treatment.

The main objectives of the present study are as follows:

- to isolate and screen for potential xylanase producers
- to produce xylanases from the best of the isolated strains under optimum conditions of solid state fermentation (S.S.F.) using low-cost substrate for subsequent application in biobleaching experiments
- to study morphological, anatomical and chemical characterization of wheat straw
- to prepare soda-AQ pulp of wheat straw at optimized pulping conditions
- to optimize various operating parameters of enzymatic treatment for soda-AQ wheat straw pulp
- to study the impact of enzymatic bleaching, using xylanases obtained from the test isolates, on different bleaching sequences with regard to pulp viscosity, optical and mechanical strength properties and pollution load generated during bleaching

Studies on xylanase production

Isolation and screening of potential white rot xylanase producers was done. Of the seven wood-rotting basidiomycetous isolates that exhibited xylanase activity, *Coprinellus disseminatus* SH-1 (NTCC 1163) and *Coprinellus disseminatus* SH-2 (NTCC 1164) were selected for further studies as they exhibited the highest and the second highest xylanase activities, with very low cellulase activities. The effect of various factors (incubation period, temperature, initial pH, complex nitrogen source, lignocelluloses as solid substrates, moisture level, simple sugars i.e. glucose and lactose, and fermentation conditions) on xylanase production by the test strains

(*C.disseminatus* SH-1 and SH-2) under S.S.F. were studied. Plate assays for lacasse and amylase were done. The test strains exhibited higher xylanase activities under S.S.F. as compared to L.S.F. SDS-PAGE protein profiling of the crude xylanases and zymogram analysis of xylanases was done. Under optimized conditions, *C.disseminatus* strains SH-1 and SH-2 showed a xylanase activity of 727.78 and 227.99 IU/mL, cellulase activity of 0.925 and 0.660 IU/mL, laccase activity of 0.640 and 0.742 U/mL and protein concentration of 5.480 and 4.900 mg/mL, respectively.

Morphological, anatomical, and chemical characterization of wheat straw

In order to assess the suitability of wheat straw for pulp and papermaking, anatomical, morphological and chemical characterization of wheat straw was done. The morphological characteristics included fiber length, fiber diameter, cell wall thickness, lumen diameter and its derived values (slenderness ratio, flexibility coefficient and Runkel ratio). Chemical characterization included water solubility, 1% NaOH solubility, alcohol-benzene solubility, lignin, holocellulose, α-cellulose, pentosan, ash and silica content.

Preparation of wheat straw soda-AQ pulp

Wheat straw was delignified at different operating conditions (temperature, time and alkali doses). Wheat straw produced 45.05% screened pulp yield of kappa number 18.25 by soda-AQ pulping at optimum cooking temperature 150 ^{0}C, maximum cooking time 60 minutes and active alkali dose 12% (as Na_2O) and fixed AQ dose (0.1%) at liquor to raw material ratio 4:1. Bauer-McNett fiber classification of soda-AQ pulp produced at optimum cooking conditions was done using screens of 20, 48, 100 and 200 mesh sizes. The pulp was beaten at various beating levels in PFI mill to optimize various mechanical strength properties.

Xylanase-aided bleaching of soda-AQ pulp of wheat straw

The effect of crude xylanases, respectively obtained from the test strains *C. disseminatus* SH-1 and SH-2 on the bleachability of soda-AQ wheat straw pulp wa studied using conventional (CEHH), ECF (ODED and ODEP) and TCF (OQPP bleaching sequences. The effect of crude xylanases obtained from both the test strain on pulp viscosity, optical and mechanical strength properties and pollution loa generated during non-oxygen-delignified soda-AQ wheat straw pulp bleached b XECEHH bleaching sequence at a chlorine demand of 4.5 and 2.25%, respectively, wa studied and compared with CEHH bleaching sequences at total chlorine demand o 4.5%. The effect of crude xylanases obtained from both the test strains was also studie on pulp viscosity, optical and mechanical strength properties and pollution loa generated for soda-AQ wheat straw pulp bleached by two ECF bleaching sequences i.e OXEDED and OXEDEP and compared with ODED and ODEP bleaching sequences respectively. The soda-AQ pulp of wheat straw was bleached by single TCF bleachin sequence i.e. OQPP.

For both the test strains, kappa number of enzyme treated pulps was lower tha that of respective control pulps. In all bleaching sequences, an increase in brightnes and viscosity of enzyme treated pulps was observed compared to respective controls fo crude xylanase preparations obtained from both the test strains.

In order to attain a beating level of 40 ^{0}SR, enzyme treated pulps (for both th strains SH-1 and SH-2) in CEHH and OQPP bleaching sequences required lesser PF mill revolutions but ODED and ODEP bleaching sequences required slightly highe PFI revolutions, as compared to their respective controls.

The tensile and burst indices of enzyme treated pulps (for both the strains SH- and SH-2) in all the bleaching sequences slightly decreased as compared to thei

respective controls. The double fold of enzyme treated pulps (for both the strains SH-1 and SH-2) in CEHH and ODEP bleaching sequences increased but decreased for those in ODED and OQPP bleaching sequences, as compared to their respective control pulps. The enzyme treated pulps (for both the strains SH-1 and SH-2) in all bleaching sequences showed an increase in tear index as compared to respective controls.

The COD and color for enzyme treated pulps increased in all the bleaching sequences, compared to their respective controls (except in case of CEHH bleaching sequence at a total chlorine demand of 2.25%). The AOX of combined bleach effluents generated, was significantly lower for enzyme treated pulps in CEHH, ODED and ODEP bleaching sequences, as compared to their respective controls.

Fiber surface morphology of the xylanase treated and untreated pulps was studied by scanning electron microscopy (SEM). Significant changes on the fiber surface of the xylanase treated pulps, due to xylan hydrolysis, were observed, while no such changes were observed in the untreated pulps. In oxygen-delignified pulps, the crude xylanases obtained from the test strains seemingly produced greater fiber fibrillation, compared to non-oxygen-delignified pulps. The observations supported the view that xylanase treatment improved the accessibility of the pulps to bleaching chemicals, decreased the diffusion resistance to outward movement of the degraded lignin fragments and allowed the removal of less degraded lignin fragments from the fiber wall, thereby increasing brightness of the enzyme treated pulps.

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Shalini

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ABBREVIATIONS

SSF	Solid State Fermentation
LSF	Liquid State Fermentation
O.D.	Oven Dry
g	Gram
m	Metre
L	Litre
TCDD	Tetrachlorodibenzodioxin
TCDDF	Tetrachlorodibenzofuran
BOD	Biological Oxygen Demand
COD	Chemical Oxygen Demand
AOX	Adsorbable Organic Halogens
AQ	Anthraquinone
EDTA	Ethylenediaminetetraacetate
SEM	Scanning Electron Microscopy
ECF	Elemental Chlorine Free
TCF	Total Chlorine Free
ClO_2	Chlorine Dioxide
SDS-PAGE	Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis
kDa	Kilo Dalton
UV	Ultra Violet
CMC	Carboxymethylcellulose
DNS	3, 5 dinitrosalicylic acid
BSA	Bovine Serum Albumin
N.S.S.	Nutrient Salt Solution
ONP	Office Newsprint
WBA	Wheat Bran Agar
PDA	Potato Dextrose Agar
TOCl	Total Organic Chlorine

LCC	Lignin-Carbohydrate Complex
POPs	Persistent Organic Pollutants
CED	Cupriethylenediamine
O	Oxygen stage
C	Chlorination stage
E	Alkali Extraction stage
H	Hypochlorite stage
D	Chlorine Dioxide stage
P	Peroxide stage
Q	Chelation stage
HexA	Hexenuronic acid

CHAPTER 1
INTRODUCTION

Pulp and paper industry is considered as a vital and core industry and per capita consumption of paper may be taken as an index of a nation's development. Therefore, per capita consumption of paper may be related to growth in areas like, industrial, cultural and educational developments of a country. But it is very much disappointing that per capita consumption of paper in India is globally one of the lowest i.e. 7 kgs/person compared to average world consumption i.e. 50 kgs/person [42]. As can be seen in Figure 1.1, the consumption of paper is directly related to population and literacy rate [31]. The GDP and paper demand per capita has also been correlated with economic growth (Figure 1.2) [32]. Indian population is expected to grow by 1.2% per year reaching 1.3 billion by 2020. The share of urban population will grow steadily. Literacy rate is expected to grow to over 70% by 2020 [31]. Demand for paper in India is projected to grow at a compounded annual growth rate of 6.10% from 2004-05 to an estimated 7.4 million tons in 2008-09 and 11.87 million tons in 2020 [4]. The demand forecast for various grades of paper in India is summarized in Table 1.1. According to an estimate, the demand for paper and board in India is expected to be over 14 million tons by 2020 as shown in Figure 1.3 (to 12 million tons with the conservative scenario) [33].

The future paper demand should be determined by taking into considerations the factors like, level of national income, level of industrial production, level of literacy and education, size of population, price of paper, and other related factors such as, government expenditure on education, student population, and per capita income, etc.

Some assumptions have to be made regarding the rate of change of these determinants making demand predictions vulnerable to these assumptions being realistic and correct [39].

Over the past 40 years, the world demand for paper has increased at an average annual rate of 4.7%, which is expected to reduce by 2-3% in the future. Still, the existing wood resources may not meet this growing demand for paper, especially in the Asia-Pacific region and Eastern Europe [6]. According to a recent study on paper demand and supply, the world demand for paper and paperboard is forecast to grow by 2.1% annually in the long term to reach an estimated 490 million tons by the year 2020 from 380.28 million tons [42] in 2006. Depending on the product area, 35-70% of the global incremental demand will be accounted for by China, India and Russia in 2004-2020. Within Asia, China and India will be the most rapidly growing production areas, accounting for 39% and 8% respectively, of the world's incremental production through 2020. The world and Indian paper and paper board demand at a growth rate of 2.4% and 6.7% respectively from 2000-2015 is given in Figure 1.4 [34].

Indian pulp and paper industries are facing multifarious problems like tough competition from imports, obsolete technology, soaring environmental cost, depreciation of money and inadequate supply of low-cost fibre to survive in the globally competitive market. This has compelled the Indian pulp and paper industry to spend heavily on imported long wood fibre each year. If India cannot overcome its lack of domestic wood fibre as well as revamp and improve its end products to match imports, then the Indian pulp and paper industry will stagnate and decline. Increases in productivity through the adoption of more efficient and cleaner technologies in the manufacturing sector will be the most effective in merging economic, environmental, and social development objectives.

The paper industry's wood demand is expected to grow from 5.8 million tons to 9 million tons by 2010 and 13 million tons by 2020 (Figure 1.5). As can be seen in Figure 1.6, the current forest plantations in India are estimated at 32.5 million tons, of which 90% is based on hardwoods (mainly eucalyptus and acacia) [33]. The tropical hardwood species are estimated to occupy 31 million ha or approximately 57% of the tropical plantation forest area worldwide [44]. The hardwood pulp production is expected to be 1.5 million tons by 2015 and 1.8 million tons by 2020 from 0.9 million tons in 2000. The growth can be higher or lower, depending on the policies regarding the development of plantations in India. The cellulosic raw material forecast is shown in Table 1.2 [10]. Only 7% of the total world pulp production is represented by non wood fibres [7], but at over 10 million tons per annum, it is still a substantial quantity. About 70% of this non-wood pulp production occurs in India and China where, domestic wood fibre fails to sustain a continuous supply of fibres to pulp and paper industry. Straws constitute about 50% of the estimated total of 2300 million tons of available non-woody fibrous plants. Waste paper based industry accounts for about one third of the Indian paper capacity. Although the recovery of waste paper has increased substantially, yet due to alternative uses the recovery rate for paper industry is still about 20%. This is low when compared with international standards [30] and needs more attention.

Agricultural residues are emerging as a significant alternative raw material resource for the pulp and paper industry. The main agricultural residues utilized by the paper industry include bagasse, cereal straws, kenaf/mesta, jute sticks, grasses and cotton stalks, with bagasse and straw being the most important ones. Even if the theoretical availability of bagasse and straw is high there are limitations in their use that

include seasonal availability, high silica content (straw), high transportation cost for long distances and investments in pollution control equipment. The frequent use of agro-residue pulps in many parts of the world is due to the lack of an adequate pulp-wood supply, but it certainly shows that the problems associated with their use can be at least partly overcome once equipment, processing methods and quality expectations are according to the circumstances [19]. In fact, agricultural residues provide excellent speciality paper [18]. Agro waste/residues such as rice straw, wheat straw and bagasse are relatively short cycled, regenerative and abundant, as compared to forest based raw materials that are rather limited [39]. Although bagasse is found to be the best alternative, meeting the quality requirements for newsprint and fine paper manufacture and is also available in plenty, yet it is opined that it would not be possible to utilize the entire quantity of potentially available bagasse in the paper industry. This is because of the scattered nature of sugar industry and total captive consumption of bagasse as fuel in sugar mills and also high cost involved in collection, transportation and storage. Although, the availability of bagasse is expected to improve due to the expected increase in sugar production, yet there will be an increase in its demand from sugar mill, for the generation of power [10]. Thus, straws will become more important as a raw material for paper industry.

Indian paper mills utilizing rice and wheat straws have capacities up to 70-80 t/d or 24 000 t/a, which are very small. Wheat straw constitutes the major part of cereals' residues. A sizeable portion of wheat straw can be made available to the Indian paper industry. According to an IAPMA estimate, 9.1 million tons of surplus straw will be available for the paper industry by 2010-2011 (Table 1.3) [12]. Properties of papermaking fibres from wheat straw, like other fibre sources, can vary with location,

genetics and growth conditions [49]. The presence of different cells and different cell distributions in wheat straw affect the paper properties. The utilization of this fibre source also has some potential limitations. The foremost include small fibre dimensions and high content of non-fibrous cells that limit the strength of paper products [25] and paper machine operating speed. The high inorganic content of straw, typically silica, creates potential problems in conventional chemical recovery systems [40]. Even then, wheat straw is seemingly the most suitable among cereal straw materials for manufacturing paper [17]. Blends of straw and wood pulps can provide useful paper properties [24].

The scarcity of paper raw materials has grown in parallel with environmental pollution problems, where pulping and bleaching processes cause serious contamination problems. The pulp and paper industry is considered to be the sixth largest polluter (after oil, cement, leather, textile, and steel industries), discharging a variety of gaseous, liquid, and solid wastes into the environment [2]. Most of the agro based mills in India are using CEH, CEHH and CHH bleaching sequences. The chlorine consumption of agro-based and wood based mills is 130-200 and 60-100 kg/t of pulp [43, 45]. However, chlorine-based bleaching of pulp generates a complex mixture of degradation products of residual lignin, such as, organic halogens (i.e. AOX-adsorbable organic halogens), which prevent their recycling to the chemical recovery system [1, 11]. It has been shown that AOX formation mainly depends on the quantity of molecular chlorine used in the bleaching process [41]. Moreover, chlorinated aromatic derivatives are toxic and persistent, constituting a potential environmental hazard if untreated before discharge to natural water bodies [14]. Polychlorinated compounds like, dioxins are known to change the blood chemistry and cause liver damage, skin disorders, lung lesions and tumor types at numerous sites within the body,

liver and thyroid [35, 23]. The effluent discharged from the bleaching section contributes to about 65% of the total BOD (biological oxygen demand) and 90% of the total colour discharge and also has a high COD (chemical oxygen demand) value. Moreover, the biodegradability of the effluent i.e. BOD, COD ratio is also very low thereby making the effluent very difficult to treat by end of pipeline treatment [2]. Some of the mills are using oxygen delignification as a prebleaching stage. However, in spite of these limitations the Indian paper mills are managing to produce paper of brightness above 80% (ISO) which is at the cost of increased chlorine consumption resulting in high level of AOX generation. The strict legislation and the environmental concerns regarding imposition of the effluent discharge norms in respect of AOX in Indian paper industry is forcing the mills to look after for alternative techniques to reduce or eliminate the use of elemental chlorine in bleach sequence. The limit of total organic chloride (TOCl) is fixed to 2 kg/t in India [3]. A very promising technology to fulfill the above objective is the use of biotechnology in developing environment friendly technologies for pulp and paper industry.

Biotechnology has rapidly emerged as an area of activity having a marked realized as well as potential impact on virtually all domains of human welfare, ranging from food processing, protecting the environment, to human health. It may be defined as "the use of living organisms in systems or processes for the manufacture of useful products; it may involve algae, bacteria, fungi, yeast, cells of higher plants and animals or subsystems of any of these or isolated components from living matter" [13]. The attraction of applying biotechnology lies in its potential:

- to provide processes / products where non biological processes are impractical
- to increase specificity in reactions

- to provide less environmentally deleterious processes
- to save energy
- and by virtue of the foregoing, to decrease cost

The possibilities for employing biotechnology in the pulp and paper industries are numerous as one of nature's most important biological processes is the degradation of lignocellulosics to carbon dioxide, water and humic substances. It has the potential to increase the quality and supply of feed stocks for pulp and paper, reduce manufacturing costs, and create novel high-value products. Novel enzyme technologies can reduce environmental problems and alter fibre properties [16, 51]. Consequently, the use of micro-organisms and their enzymes to replace or supplement older chemical methods in the pulp and paper industry is gaining utmost interest. The major applications of enzymes in paper industry include:

(1) Enzymes for wood debarking – Enzymes like, polygalacturonase can be used to hydrolyze cambial layer [36].

(2) Biopulping – Pretreatment of wood chips with fungal cultures can modify lignin and extractives [22]. Biopulping has proved to use less energy than conventional pulping and to increase the paper strength [5].

(3) Retention in papermaking – Cationic demand can be reduced in peroxide brightened mechanical pulp furnishes by adding pectinase enzyme [48] that hydrolyze pectins present in cationic demand.

(4) Extractives' control – Lipases can hydrolyze triglycerides and esters found in extractives. Blue stain fungi can be very useful due to their ability to degrade pitch from wood [9]. Laccases can also be used to oxidize fatty acids and their triglycerides [54].

(5) Stickies control – Esterases can be used for stickies control, as they hydrolyze polyvinyl acetate, a component of stickies, to less sticky polyvinyl alcohol [27].

(6) Improving drainage – Cellulases and xylanases are being employed to improve the drainage on a pulp or paper machine. Amylase has also been used to target starch viscosity in tissue mills [26].

(7) Boil-outs and slime control – Using amylase in combination with lipase and protease in paper machine boil-out has provided unprecedented results compared to traditional caustic treatment. These enzymes also help to remove slime and control the growth of bacteria in paper machine systems [26].

(8) Catalases in paper industry – Catalases have found some application to deactivate residual peroxide after mechanical pulp bleaching to avoid the use of strong acid [47].

(9) Deinking – Cellulases are widely reported to facilitate deinking of mixed office waste but appear to be less effective for ONP (old newsprint) deinking [53].

(10) Effluent treatment – Biological processes like, aerated stabilization basins, activated sludge, oxygen-activated sludge, trickling filters, rotating biological contactors and anaerobic fluidized beds, are usually employed in secondary or polishing treatments that follow sedimentation or other primary treatment [2].

(11) By-products – Products like, xylitol, an artificial sweetener, and those having therapeutic value can be obtained as by-products [26], thereby improving profitability of kraft mills.

(12) Biobleaching – Biotechnology has a high potential in the pulp bleaching sector as it allows the development of more sustainable and environment friendly products and processes by reducing chemical usage, saving energy and water,

and minimizing waste products [28, 37]. Several workers have approached biological bleaching by use of lignolytic enzymes [8, 50] and hemicellulolytic enzymes [20, 46], with the focus being mainly on the xylanases [37].

Because the pulp and paper industry is capital-intensive with facilities specific to the tasks, new technology must either reduce expenses or fit easily into the existing process design [16, 52]. Keeping in mind the huge potential of xylanases in biobleaching, one of the appropriate approaches would be to search for powerful producers of xylanases [15] that can be used in biobleaching.

On account of its abundance and of the facts that wheat straw seemingly provides pulp of better quality than do other abundant agricultural residues [38], and of the fact that bleaching of wheat straw is so far less documented [29, 21] we chose wheat straw to evaluate the efficacy of xylanases as biobleaching agents.

In view of the above, it is proposed to conduct laboratory studies on enzymatic prebleaching of soda-AQ pulp of wheat straw in different bleaching sequences using crude xylanases obtained from indigenously isolated white rot fungal strains. The study is focused on following points to produce bleached pulp of good mechanical strength by adopting environment friendly bleaching sequences, through enzymatic treatment:

- to isolate and screen for potential xylanase producers
- to produce xylanases from the best of the isolated strains under optimum conditions of solid state fermentation (S.S.F.) using low-cost substrate for subsequent application in biobleaching experiments
- to study morphological, anatomical and chemical characterization of wheat straw.
- to prepare soda-AQ pulp of wheat straw at optimized pulping conditions

- to optimize various operating parameters of enzymatic prebleaching for soda-AQ wheat straw pulp
- to study the impact of enzymatic bleaching, using xylanases obtained from the test isolates, on different bleaching sequences with regard to pulp viscosity, optical and mechanical strength properties and pollution load generated during bleaching

The experimental results are expected to provide a basis for establishing suitable process sequences, optimizing operational parameters and integrating them with existing mill configurations. The environmental impacts are likely to be positive for such mills. It is expected that as a consequence of improved bleaching, savings are expected in terms of energy and chemicals in exact mill situations. All these are likely to provide significant benefits in terms of improved product quality and improved environmental management associated with improved economics.

Wheat straw pulp will likely to be used in blends with wood pulps in proportions consistent with paper and board cost and performance specifications. Industrial application of enzymes on a wider scale in the pulp and paper industry looks to be very promising. It seems to be a close reality that enzymes will be used in almost all the areas of the whole spectrum of the pulp and paper industry yielding numerous benefits like, reduction of energy consumption (i.e. refining), reduction of AOX and preservation of the environment.

Table 1.1 Paper supply and demand scenario for India (in 1000 tons)

Particulars		Growth					
		2000	2005	2010	2015	2000-2015	2020
		1000 tons					
Newsprint	Demand	844	1177	1552	1937	1093	2380
	Supply	456	700	1040	1390	934	1800
	Net trade	–388	–477	–512	–547		–580
WC printing/writing	Demand	40	61	80	99	59	110
	Supply					0	
	Net trade	–40	–61	–80	–99		–110
WF printing/writing	Demand	1490	2125	2870	3880	2390	5215
	Supply	1530	200	2580	3600	2070	4600
	Net trade	40	–125	–290	–280		–615
Tissue	Demand	38	75	130	185	147	235
	Supply	30	55	100	170	140	225
	Net trade	–8	–20	–30	–15		–10
Container board	Demand	814	1276	1942	2773	1959	3900
	Supply	806	1155	1840	2650	1844	3600
	Net trade	–8	–121	–102	–123		–300
Carton boards	Demand	798	1070	1468	1895	1097	2430
	Supply	828	1100	1300	1800	972	2200
	Net trade	30	30	–168	–95		–230
Others	Demand	191	222	249	276	85	315
	Supply	200	225	245	265	65	300
	Net trade	9	3	–4	–11		–15
Total base scenario	Demand	4215	6006	8291	11045	6830	14585
	Supply	3850	5235	7105	9875	6025	12725
	Net trade	–365	–771	–1186	–1170		–1860
Total conservative	Demand	4215	5660	7430	9435	5220	11870

Table 1.2 Raw material demand forecast for Indian paper industry

('000 tons)	Input-output norm (times)	2004-05 E			2009-10 F		
		Production	Demand for fibre	Share, %	Production	Demand for fibre	Share, %
Wood and bamboo	2.6	1,659	4,313	30.0	2,390	6,213	30.0
Agri residue (including bagasse)	14.2	17,70	7,432	32.0	2,469	10,370	31.0
Domestic wastepaper	1.3	938	1,219	17.0	1,249	1,624	15.7
Imported wastepaper	1.2	832	998	15.0	1,300	1,560	16.3
Imported pulp	0.9	332	299	6.0	558	502	7.0

E- Estimate; F- Forecast

Table 1.3 Estimated wheat straw based paper production: IAPMA 1996 (million tons/annum)

	1995-96	2000-01	2005-06	2010-11
Surplus straw available	7.9	8.3	8.7	9.1
Possible paper production	2.2	2.4	2.5	2.6

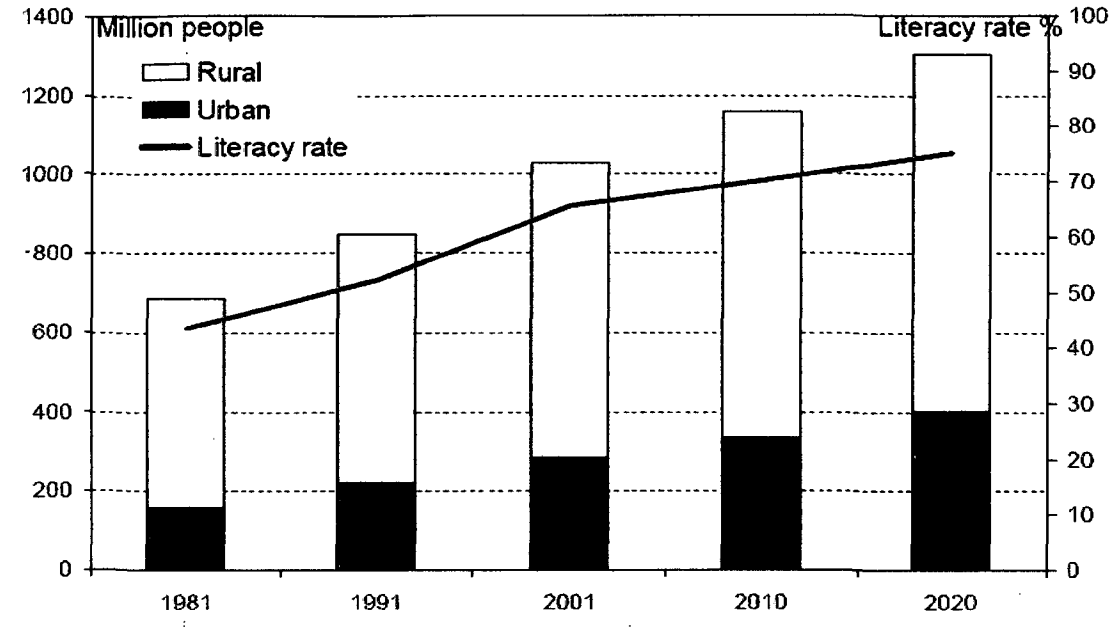

Figure 1.1 Indian population and literacy rate

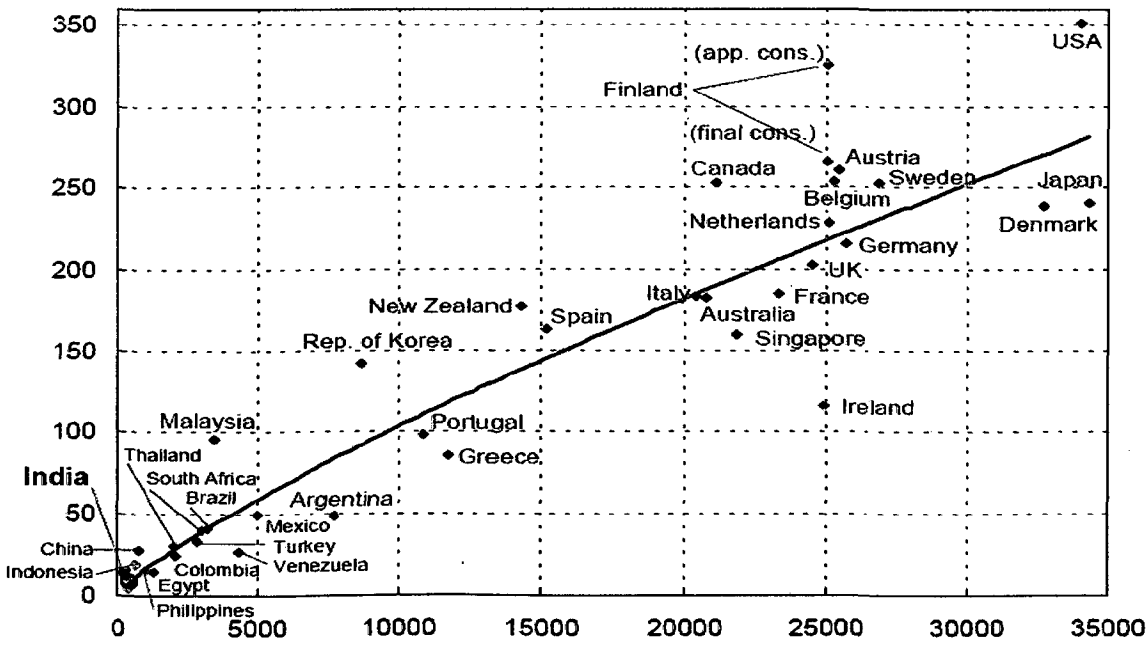

Figure 1.2 GDP and paper demand per capita

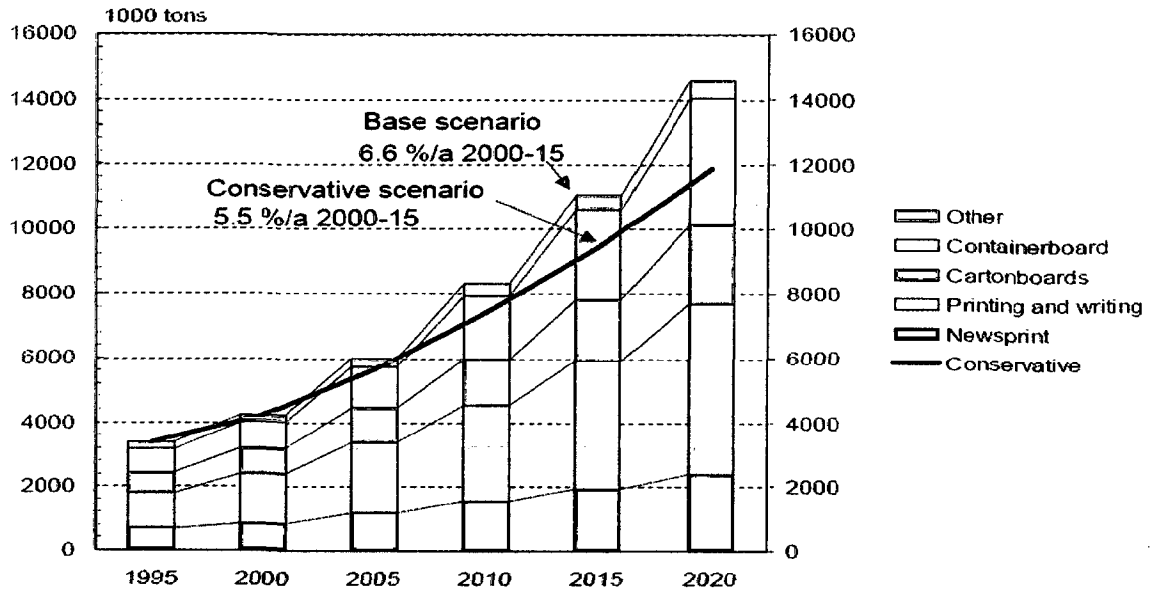

Figure 1.3 Total demand for paper and board in India up to 2020

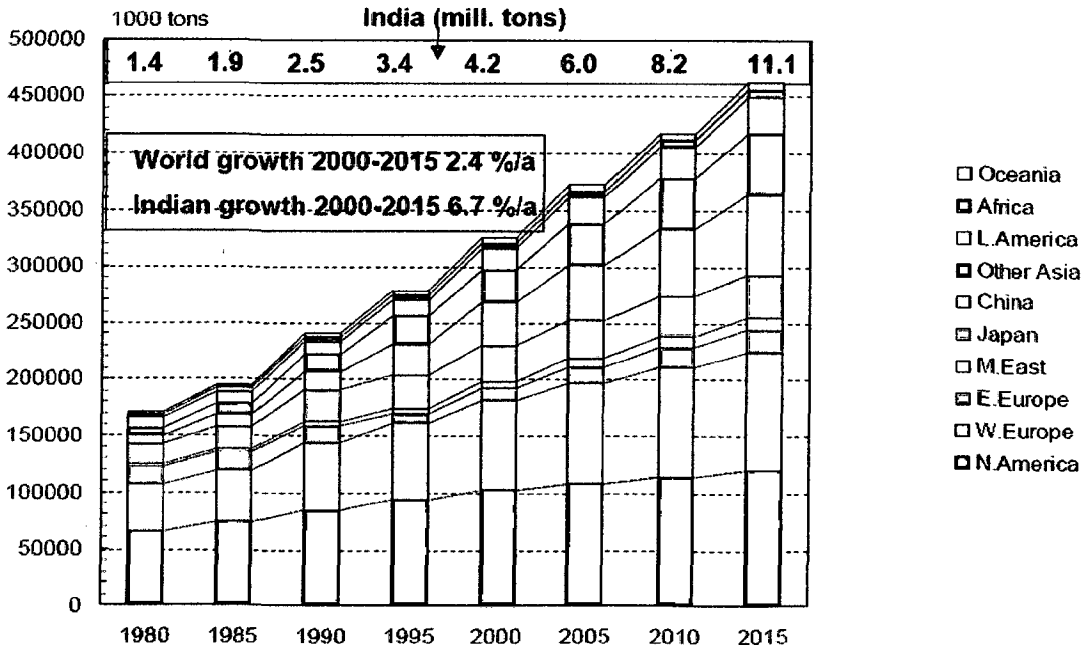

Figure 1.4 Expected world demand for paper and paper board up to 2015 (in 1000 tons)

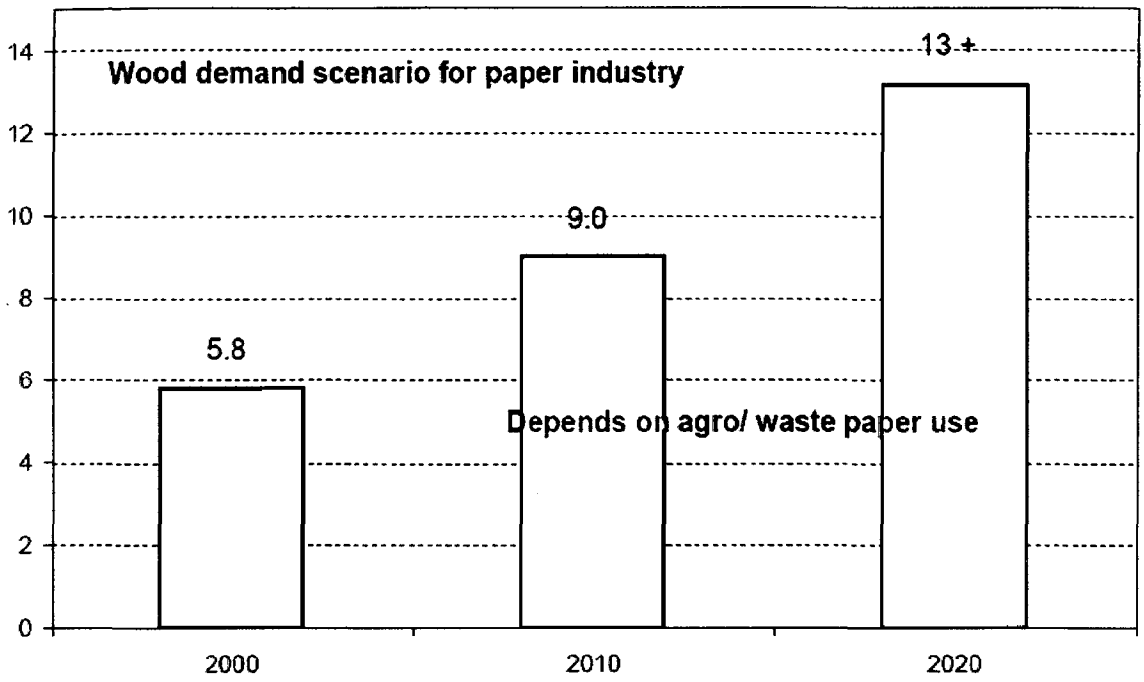

Figure 1.5 Wood demand scenario for paper industry (in 1000 tons)

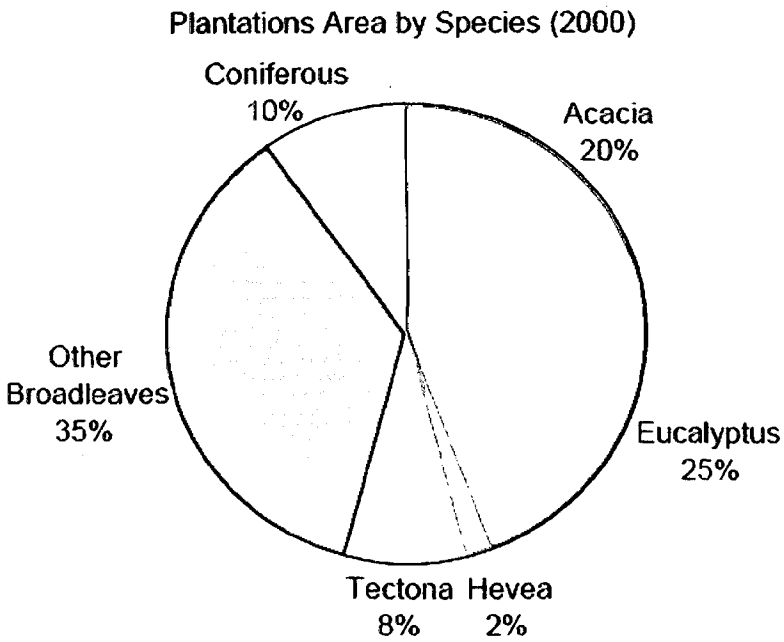

Figure 1.6 Forest plantation in India

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CHAPTER 2
STUDIES ON XYLANASE PRODUCTION

2.1 INTRODUCTION

Enzymes are non-living proteins or organic chemicals that exist in all living organisms. These "bio-catalysts" are designed by nature to either carry out the synthesis of different chemical compounds essential for the proper functioning of the organism and/or to facilitate the breakdown or alteration of such chemicals under a variety of environmental and physiological conditions [24].

Like all catalysts, enzymes work by lowering the activation energy for a reaction, thus, dramatically accelerating the rate of the reaction. They are not consumed by the reactions they catalyze, nor do they alter the equilibrium of these reactions [11]. The enzyme activity is affected by temperature, chemical environment (like pH), substrate concentration and presence of inhibitors or activators. Enzymes are generally globular proteins and their activities are determined by their three-dimensional structure [3]. Like all proteins, enzymes are made of long, linear chains of amino acids that fold to produce a three-dimensional product. Each unique amino acid sequence produces a specific structure, which has unique properties. Enzymes are usually very specific as to which reactions they catalyze and the substrates that are involved in these reactions. Complementary shape, charge and hydrophilic/hydrophobic characteristics of enzymes and substrates are responsible for this specificity. Enzymes can also show impressive levels of stereospecificity, regioselectivity and chemoselectivity [39].

Enzymes have played a central role in many manufacturing processes and have a wide array of applications in the chemical industry. There has been an unprecedented

expansion in our knowledge of the use of microorganisms, their metabolic products, and enzymes in a broad area of basic research and their potential industrial applications. Xylanases are one of the most important enzymes attracting the attention of researchers worldwide.

Xylanases are glycosidases (O-glycoside hydrolases, EC 3.2.1.x) which catalyze the endohydrolysis of 1, 4-β- D-xylosidic linkages in xylan. They are a widespread group of enzymes, involved in the production of xylose [67]. First reported in 1955 [101], they were originally termed pentosanases, and were recognized by the International Union of Biochemistry and Molecular Biology (IUBMB) in 1961 when they were assigned the enzyme code EC 3.2.1.8. Their official name is endo-1,4-β-xylanase, but commonly used synonymous terms include xylanase, endoxylanase, 1,4-β-D-xylan-xylanohydrolase, endo-1,4-β-D-xylanase, β-1,4-xylanase and β-xylanase.

Xylan, the substrate of xylanases, constitutes the major component of hemicelluloses [83], which together with cellulose and lignin make up the major polymeric constituents of plant cell walls [47]. Xylan is found in hardwoods (15–30% of the cell wall content), softwoods (7–10%), as well as in annual plants (<30%) [85]. It is typically located in the secondary cell wall of plants, but is also found in the primary cell wall, in particular in monocots [104]. A complex, highly branched heteropolysaccharide, it varies in structure between different plant species, and the homopolymeric backbone chain of 1,4-linked β-D-xylopyranosyl units can be substituted to varying degrees with glucuronopyranosyl, 4-O-methyl-D-glucuronopyranosyl, α-L-arabinofuranosyl, acetyl, feruloyl and/or p-coumaroyl side-chain groups. Wood xylan exists as O-acetyl-4-O-methylglucuronoxylan in hardwoods and as arabino-4-O-methylglucuronoxylan in softwoods, while xylans in grasses and

annual plants are typically arabinoxylans [47]. Linear unsubstituted xylan has also been reported [15, 23, 61]. The three-dimensional structure of the xylan molecules has been described by Atkins [9].

Due to its heterogeneity and complexity, the complete hydrolysis of xylan requires a large variety of cooperatively acting enzymes (Figure 2.1) [90]. Endo- 1,4-β-D-xylanases (EC 3.2.1.8) randomly cleave the xylan backbone, β-D-xylosidases (EC 3.2.1.37) cleave xylose monomers from the non-reducing end of xylo-oligosaccharides and xylobiose while removal of the side groups is catalysed by α-L-arabinofuranosidases (EC 3.2.1.55), α-D-glucuronidases (EC 3.2.1.139), acetylxylan esterases (EC 3.1.1.72), ferulic acid esterases (EC 3.1.1.73) and p-coumaric acid esterases (EC 3.1.1.-).

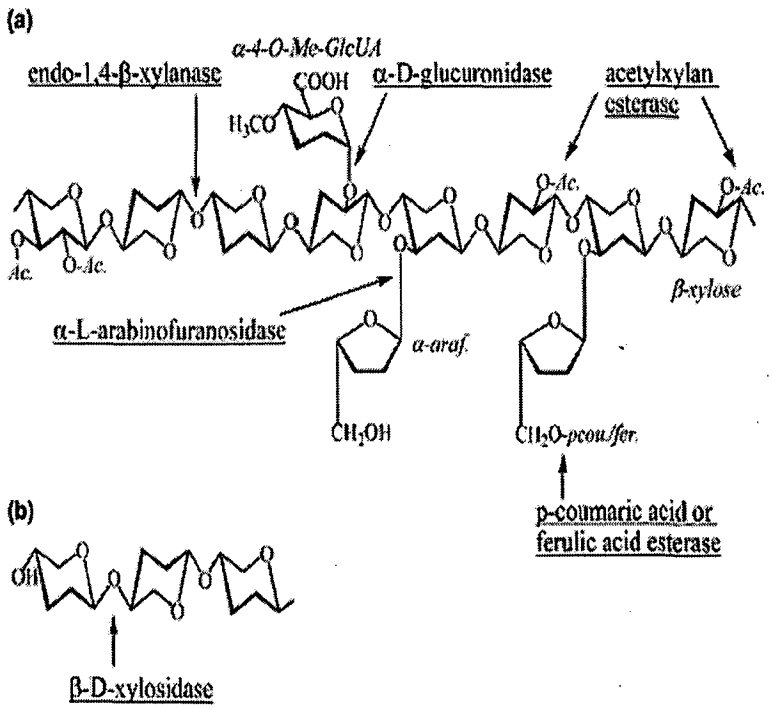


Figure 2.1 (a) Structure of xylan and the sites of its attack by xylanolytic enzymes. The backbone of the substrate is composed of 1, 4-β-linked xylose residues. Ac., Acetyl group; α-araf., α-arabinofuranosidase; α-4-*O*-Me-GlcUA, α-4-O-methylglucuronic acid; pcou., p-coumaric acid; fer., ferulic acid. (b) Hydrolysis of xylo-oligosaccharide by β-xylosidase [92].

Xylanases carry out hydrolytic reactions to break down xylan polymers, where all xylanases retain the anomeric configuration of the glycosidic oxygen following hydrolysis. This indicates that they use a double-displacement mechanism in which the reactive intermediate is bound to the enzyme. This enables them to carry out transglycosylation reactions [42].

In addition to the production of a variety of xylanolytic enzymes, many micro-organisms produce, multiple xylanases [33]. These may have diverse physico-chemical properties, structures, specific activities and yields, as well as overlapping but dissimilar specificities, thereby increasing the efficiency and extent of hydrolysis, but also the diversity and complexity of the enzymes. This multiplicity may be the result of genetic redundancy [104], or differential post-translational processing [12]. The isoenzyme genes may be found as polycistronic or non-polycistronic multiple copies within the genome, and in some cases several xylanases are expressed as a distinct gene product. Moreover, as well as multiple catalytic domains, many xylanases are also characterized by the presence of various supplementary domains [18].

The heterogeneity and complexity of xylan has resulted in an abundance of diverse xylanases that has led to limitations with the classification of these enzymes by substrate specificity alone. Wong *et al.* [104] classified xylanases on the basis of their physico-chemical properties and proposed two groups: those with a low molecular weight (<30 kDa) and basic pI, and those with a high molecular weight (>30 kDa) and acidic pI. However, several exceptions to this pattern have been found [92]. Later, a more complete classification system was introduced [36] which allowed the classification of not only xylanases, but glycosidases in general (EC 3.2.1.x), and which has now become the standard means for the classification of these enzymes. This

system is based on primary structure comparisons of the catalytic domains only and groups enzymes in families of related sequences. Enzymes within a particular family have a similar three-dimensional structure [35] and similar molecular mechanism [30] and it has also been suggested that they may have a similar specificity of action on small, soluble, synthetic substrates [17]. Furthermore, divergent evolution has resulted in some of the families having related three-dimensional structures and thus, the grouping of families into higher hierarchical levels, known as clans, has been introduced [14]. Within this classification system, xylanases are normally reported as being confined to families 10 (formerly F) and 11 (formerly G) [90]. Interestingly, sequences classified in families 5, 7, 8 and 43 are also found to contain truly distinct catalytic domains with a demonstrated endo-1, 4-β-xylanase activity as in families 10 and 11. Thus, the current view that enzymes with xylanase activity are solely restricted to families 10 and 11 is not entirely correct and should be expanded to include families 5, 7, 8 and 43 [18].

Xylanolytic enzymes have attracted a great deal of attention, particularly because of their biotechnological potential in various industrial processes. The major applications of xylanases are in manufacturing of bread, food and drinks, in the improvement of nutritional properties of agricultural silage and grain feed, in the textile industry to process plant fibres, in pharmaceutical and chemical applications and in cellulose pulp and paper [46, 103].

Recently, the interest in xylanases has focused on bleaching processes [98, 99] Enzymes began to be used in this sector during the last two decades, ever since peroxidases were applied to the degradation of lignin [95, 102]. Enzyme application improves pulp fibrillation and water retention, reduces beating time in virgin pulps, restores bonding and increased freeness in recycled fibres, and selectively removes

xylans from dissolving pulps. Xylanases are also useful in yielding cellulose from dissolving pulps for rayon production and biobleaching of cellulose pulps [88].

Xylanases occur in both prokaryotes and eukaryotes [20]. They have been demonstrated in higher eukaryotes, including protozoa, insects, snails and germinating plant seeds [93]. Amongst the prokaryotes, bacteria and cyanobacteria from marine environments produce xylanases [21]. Extracellular and intracellular xylanases have been studied extensively. Intracellular xylanases occur in rumen bacteria and protozoa [20]. A variety of microorganisms including bacteria [32], yeast [50], actinomycetes [29] and filamentous fungi [54] have been reported to produce xylanolytic enzymes.

The most potent producers of xylanases are considered to be the fungi [81], especially, wood-rot fungi may serve as good producers of xylanases as well as other extracellular polysaccharides-degrading enzymes since, they excrete the enzymes into the medium and their enzyme levels are much higher than those of yeast and bacteria [68]. Both the so-called brown and soft rot fungi, belonging to the basidiomycetes and ascomycetes respectively, decompose principally the polysaccharides. A third group, the wood-rotting basidiomycetes causing white-rot decay is able to degrade all the components of the wood cell wall including the highly recalcitrant polymer, lignin [45].

Like most fungi, white-rot fungi exist primarily as branching threads termed hyphae, usually 1 to 2 μ in diameter, which grow from the tips. Originating from spores or from nearby colonies, hyphae rapidly invade wood cells and lie along the lumen walls. From that vantage, they secrete the battery of enzymes and metabolites that bring about the depolymerization of the hemicelluloses and cellulose and fragmentation of the lignin. The white-rot fungi exhibit two gross patterns of decay. Firstly, a simultaneous decay, in which the cellulose, hemicelluloses, and lignin are removed more or less simultaneously, and secondly, the delignification, in which the lignin and

hemicelluloses are removed ahead of the cellulose. Fungi vary greatly in causing the two types of decay - among species and among strains within a species. Indeed, sometimes a single fungus causes simultaneous decay in one part of wood, and delignification close by. The factors responsible for the occurrence of one versus the other pattern of decay are as yet unclear [44]. The polymer-fragmenting enzymes and required metabolites are secreted into a fungal polysaccharide matrix (a β-1-3 glucan) that extends from the hyphae onto the lumen wall surface, sometimes over the entire inner surface [13]. This matrix is thought to direct the enzymes to the site of action - the exposed surfaces of the wall polymer composite. Enzymes are too large to penetrate the sound, intact wood cell [27].

Only a few studies have been made on the hemicellulases of white-rot fungi and relatively little is known about them. This notwithstanding, it is clear from their ability to decay wood (i.e., to deplete all the structural components), and from studies demonstrating growth on hemicelluloses substrates, that white-rot fungi have effective hemicellulase systems [94].

The basic factors for efficient production of xylanolytic enzymes are the choice of an appropriate inducing substrate and an optimum medium composition. Since biotechnological applications require large amounts of low cost enzymes, one of the appropriate approaches for this purpose is the search for powerful xylanase producers and the utilization of the potential of lignocellulosic wastes/by-products, which may contain significant concentrations of soluble carbohydrates and inducers of enzyme synthesis, ensuring efficient production of lignocellulolytic enzymes, thereby exterminating the need of expensive pure xylans as substrate [76]. It is also worth mentioning that xylanase production has also been noticed along with CMCase

production using CMC as substrate. This might be due to xylan contamination of the CMC substrate [77]. Keeping in mind the huge potential of white rot fungi as xylanase producers, extensive studies need to be done to tap this promising source of xylanases.

Xylanase production has been carried out using different fermentation processes. Solid-state fermentation (S.S.F.) is gaining interest due to its potential advantages in manufacturing products such as enzymes in high yield at high concentration and with high specificity. It involves the growth and metabolism of microorganisms on moist solids in the absence or near absence of any free-flowing water. These fermentation systems, may prove more efficient for cultivation of filamentous fungi and production of lignocellulolytic enzymes because they grow under conditions close to their natural habitats due to which they may be more capable of producing certain enzymes and metabolites [10], which usually will not be produced or will be produced only at low yield in submerged cultures [62]. SSF offers distinct advantages over submerged fermentation including economy of the space, simplicity of the media, no complex machinery, equipments and control systems, greater compactness of the fermentation vessel owing to a lower water volume, greater product yields, reduced energy demand, lower capital and recurring expenditures in industry, easier scale up of processes, lesser volume of solvent needed for product recovery, superior yields, absence of foam build-up, and easier control of contamination due to the low moisture level in the system [51]. Surprisingly, biological parameters, such as the stability of the produced enzymes at high temperature or extreme pH, have also been reported to be better in SSF [22].

The present work aims at isolation and screening of potent xylanase producing white rot fungal strains for selecting the most efficient xylanase producers among the

isolates and their use in production of xylanases under optimum conditions of solid state fermentation conditions using cheap lignocellulosic substrates.

2.2 MATERIALS AND METHODS

2.2.1 Materials

Birchwood xylan and syringaldehyde were purchased from Sigma Chemical Co. (St. Louis, MO, USA), 3, 5-dinitrosalicylic acid (DNS) and bovine serum albumin (BSA) were procured from Loba Chemie, India, and standard protein markers were purchased from Bangalore Genei Pvt., India. All other reagents were of analytical grade made by known manufacturers. The solid substrates (wheat bran, sugarcane bagasse, rice bran and wheat straw) were purchased from local market of Saharanpur, India.

2.2.2 Methods

2.2.2.1 Isolation of xylanase producing basidiomycetes

Several species of basidiomycetes were isolated from decaying wood from various locations in Saharanpur and Meerut regions of Uttar Pradesh, India. Enrichment technique (with wheat bran as carbon source) was used to isolate xylan utilizing basidiomycetes. In this technique dead and decaying wood samples were collected and kept in glass Petri plates containing moist wheat bran. The plates were incubated at 37 °C for a period of 2 to 10 days. The plates were observed for the appearance of fungal growth and those exhibiting fruit bodies (indicative of growth of basidiomycetes) were isolated from these plates. The moisture level was carefully controlled with sterile tap water, so as to provide a solid substrate for fungal growth, with no free water available. The fungal species, in the form of fruiting bodies, were purified by aseptically transferring mycelia from the inner part of the fruiting bodies

onto wheat bran agar medium (2% w/v wheat bran and 2% w/v agar-agar) and incubating the plates at 37 °C. Purified cultures were routinely cultured on potato dextrose agar (PDA) slants with incubation at 37 °C for 72 h and subsequent storage at 4 °C. The cultures were maintained as a suspension of spores and hyphal fragments in 15% (v/v) sterile glycerol at – 20 °C for long term preservation.

2.2.2.2 Pretreatment of substrates

The lignocellulosic substrates were washed two to three times with warm water and then immediately dried in sunlight. This was done to remove any impurity like, dust, starch material or any other crop residue. The dried substrates were grinded in a laboratory grinder to obtain a fine powder. The fine powder was passed through 100μm mesh size screen and the fractions so obtained were stored in polyethylene bags for further use.

2.2.2.3 Preparation of culture medium

The culture medium used was wheat bran agar medium (WBA), consisting of 2% wheat bran (w/v) and 2% agar agar (w/v). The medium was prepared and then autoclaved at 15 Pa for 15 min. After autoclaving, the medium was cooled down to about 45 °C and 185 μg/mL of chloramphenicol [63] was added as an antibacterial agent. The medium was then poured aseptically in sterile Petri plates and stored at 4 °C, until used.

2.2.2.4 Sterilization of glassware

Glassware was washed with soap and thoroughly rinsed with running tap water followed by dry heating in a laboratory oven at 60 °C. The glassware was then autoclaved at 15 Pa for 15 min, followed by dry heating in oven at 60 °C for 5-6 h.

2.2.2.5 Disinfection of inoculation room and inoculation chamber

The inoculation chamber was first cleaned with 70% ethyl alcohol followed by exposure to UV radiation for 15 min. Inoculation room was disinfected by fumigation method, in which cotton wool was first spread on the bottom part of Petri plate and adequate amount of $KMnO_4$ (about one teaspoon) was kept on it. Formalin was then poured over $KMnO_4$ to wet the cotton completely. The fumes coming out in the air kill microbial spores in the surroundings.

2.2.2.6 Screening of isolates for potent xylanase producers

A set of highly selective procedures were used to select the fungal strains that were best among the isolates in terms of xylanase activity with poor or no cellulase activity.

2.2.2.6.1 Screening of xylanase producers by plate assay technique

The basidiomycetous isolates among the prevalent fungi recovered in the present work were screened for their abilities to produce extracellular xylanases during growth on xylan-agar medium. The medium contained 1 % w/v birch wood xylan (as sole carbon source) and 2% agar agar [1]. These xylan-agar plates were inoculated with isolates grown on wheat bran agar medium for 4 days. After 6 days of incubation period at 37 °C, the plates were stained with Congo red solution (0.5% w/v Congo red and 5% v/v ethanol in distilled water) for 15 min and then destained with 1M NaCl. The plates were then observed with naked eye for appearance of clear zones around the fungal cultures against the red background [96].

2.2.2.6.2 Screening of best xylanase producers among xylanase positive isolates

The isolates exhibiting areas of clear zones when grown on xylan-agar medium were further subjected to solid state fermentation (SSF) conditions to determine their actual xylanase activities. The enzymes from all the isolates were harvested on 6th day of incubation and the xylanase and cellulase activities of enzyme samples from each isolate were determined. The supernatant protein concentrations of the enzyme samples were also determined. The aim of screening was to select two isolates exhibiting the highest xylanase activities among the isolates with no or minimal cellulase activities.

2.2.2.7 Enzyme production

2.2.2.7.1 Solid state fermentation (S.S.F.)

For production of extracellular enzymes under S.S.F., the nutrient salt solution, (N.S.S.) prepared according to Vishniac and Santer [100] and as standardized by Singh and Garg [86], was used. It contained, as g/L, 1.5 KH_2PO_4, 4.0 NH_4Cl, 0.5 $MgSO_4.7.H_2O$, 0.5 KCl, 1.0 yeast extract and 0.04 trace element solution. The trace element solution contained, as g/L, 200 x 10^{-6} $FeSO_4.7H_2O$, 180 x 10^{-6} $ZnSO_4.7H_2O$ and 20 x 10^{-6} $MnSO_4.7H_2O$. The pH of the N.S.S., as desired, was first adjusted with NaOH /H_2SO_4 with the help of microprocessor controlled pH meter (Knick, Germany, Model-761 Calimatic) fitted with glass electrode. 5 g finely powdered wheat bran (100μm mesh size fraction) was added in Erlenmeyer flask (Borosil) of 250 mL capacity. 15 mL of nutrient medium was added thereby maintaining a solid to liquid ratio of 1:3 [43]. The flasks, covered with cotton plugs and aluminum foil, were autoclaved at 15 Pa for 15 min. On cooling, the flasks were inoculated aseptically with 2 discs of the test isolate, each of a diameter of 5mm. The discs were cut with a sterile cork borer from the periphery of a 3-day-old fungal culture. The inoculated flasks were incubated at desired temperature and harvested as and when required.

2.2.2.7.2 Liquid state fermentation (L.S.F.)

As in S.S.F., enzyme production under L.S.F. was also carried out in 250 mL Erlenmeyer flasks (Borosil). 0.8 g powder of wheat bran was added in each flask, to which 40 mL of N.S.S., prepared according to Vishniac and Santer [100] and as standardized by Singh and Garg [86], was added. Desired pH of nutrient salt solution was then adjusted as described above for S.S.F. The above-prepared flasks were plugged with cotton and sterilized at 15 Pa for 15 min. When cooled at room temperature, the pH of flasks were checked and readjusted aseptically. Two discs of 5 mm diameter from 4-day-old culture of test isolate were aseptically inoculated in each of the flask. These flasks were incubated in incubator shaker (Sanyo, Orbi-safe, UK) at 100 rpm at desired temperature and were harvested as and when required.

2.2.2.8 Harvesting and storage of enzyme

After desired growth of the test fungi under S.S.F, the enzyme was harvested. 15 mL of distilled water was added to each flask. The contents of the flask were crushed with the help of a glass rod and were then shaken on orbital shaker at 100 rpm for 10 min at room temperature. The contents were filtered through the four layers of cheese cloth. The filtrate was centrifuged at 5000x g (Sigma laboratory centrifuge model 3-18K) [59] for 10 min at 4 °C. The supernatant liquid was treated as crude enzyme and stored at – 20 °C in small volumes until use.

The enzymes produced under L.S.F. were harvested in the similar manner as in S.S.F, with the difference that no distilled water was added in the flasks and the contents of flasks were directly filtered through cheese cloth.

2.2.2.9 Analytical methods

2.2.2.9.1 Estimation of xylanase activity

The xylanase activity was determined by measuring the release of reducing sugars using birchwood xylan as the substrate. The amount of reducing sugars in the reaction tubes was measured using the 3, 5 dinitrosalicylic (DNS) acid reagent method [57]. 1.6 mL of enzyme preparation (the filtrate after removing pellets) was added in a sterile tube, which contained 0.4 mL of substrate suspension (10mg/mL birchwood xylan in potassium phosphate buffer, 0.1M, pH 6.0). Appropriate controls, in which substrate and/or enzyme preparation had been omitted, were also included. The assay mixtures were incubated at 55 °C for 15 min with constant shaking at 100 rpm on shaking water bath (Jindal water bath incubator shaker, SM Industry, New Delhi). 1 mL of this mixture was then taken separately in a fresh tube, and 3 mL of DNS reagent was added to it to stop the reaction. These well stirred assay mixtures were then boiled for 5 min, followed by quick cooling in cold water and the absorbance as optical density (O.D.) was measured at 540 nm [55] using a UV-Vis spectrophotometer (UV-Vis Spectrophotometer Cary 100 Bio, Varian-Australia). Blanks, containing distilled water in place of enzymatic reaction products but containing 3 mL of DNS reagent, were also prepared The amount of reducing sugars was calculated from the standard curve based on the equivalent xylose. The unit of activity was the amount of enzyme needed to release 1μmol of xylose released per min at 55 °C.

2.2.2.9.2 Estimation of cellulase activity

The cellulase activity, as carboxymethylcellulase (CMCase) activity, was determined using carboxymethylcellulose as a substrate [53]. The reducing sugars were measured by DNS acid reagent method by Miller *et al.* [57]. 2 mL of enzyme

preparation in appropriate dilutions as well as undiluted was thoroughly mixed in 20 mL screw capped universal sterile tubes containing 2 mL of 2% (w/v) carboxymethylcellulose (CMC), make Hi media, as a substrate prepared in 0.05 M citrate buffer (pH 4.8). The reaction mixture was incubated in a water bath at 50 °C for 30 min. Controls were routinely included in which enzyme or substrate were omitted and treated similarly. The tubes were kept in an ice bath to stop the reaction. 3 mL of dinitrosalicylic acid (DNS) reagent was added to 1 mL of the reaction mixture and the mixture was thoroughly mixed. This was followed by boiling of the reaction mixture on a vigorously boiling water bath for 5 min. The contents were immediately cooled under running tap water and O.D. was measured at 575 nm in a UV-Vis spectrophotometer. Reducing sugars released from CMC were determined by comparing the values with the standard curve prepared by D-glucose. The enzyme activity was expressed as μmoles of D-glucose equivalents released per min at 50 °C (IU).

2.2.2.9.3 Estimation of laccase activity

The laccase activity in the enzyme samples was determined by continuous spectrophotometric rate determination method given by Ride [75]. The enzyme assay is based on the oxidation of syringaldazine by laccase to form oxidized syringaldazine. In this method, sets of test samples containing 2.2 mL of reagent 'A' (100 mM potassium phosphate buffer, pH 6.5 at 30 °C) and 0.5 mL reagent 'C' (enzyme solution prepared at suitable concentration in cold deionized water) and blank solutions containing 0.5 mL deionized water and 2.2 mL reagent 'A' were pipetted into suitable cuvettes. These cuvettes were equilibrated to 30 °C and wavelength at 530 nm (A_{530} nm) was monitored until constant, using a thermostatted spectrophotometer. After equilibration, 0.3 mL of reagent 'B' (0.216 mM syringaldazine solution) was added to each test sample and the

blank solution. The contents in each cuvette were immediately mixed by inversion and the increase in absorbance at 530 nm ($A_{530\ nm}$) was recorded for about 10 min. The change in $A_{530\ nm}$ per min was obtained by using the maximum linear rate for both the test and blank. One unit is defined as unit that will produce $A_{530\ nm}$ of 0.001 per min at pH 6.5 at 30 °C in a 3 mL reaction volume using syringaldazine as substrate and the enzyme activity was expressed as unit per mL of the sample.

2.2.2.9.4 Estimation of supernatant protein concentration

The supernatant protein concentration was determined by the Lowry method [52]. The reagents used were: 2% Na_2CO_3 in 0.1N NaOH (Lowry 'A') and 0.5% $CuSO_4.5H_2O$ in 1% sodium or potassium tartarate (Lowry 'B'). Appropriate dilutions of the enzyme sample were prepared, with the total volume of each protein sample adjusted with distilled water to 200 µL. 1 mL of Lowry 'C' (50 mL of Lowry 'A' + 1 mL of Lowry 'B') reagent was added to the protein sample and the mixture incubated at room temperature for 10 min. 100 µL of Folin reagent (1 mL of phenol reagent + 3 mL distilled water) was then added and the final preparation was incubated for 30 min at room temperature. The colour developed was estimated at 750 nm using a UV-Vis spectrophotometer and the concentration of supernatant protein in each sample was estimated from a calibration curve constructed using bovine serum albumin (BSA) as a standard.

2.2.2.10 Identification of selected isolates

The screened isolates were sent to Pathology Division, Forest Research Institute, Dehradun (Uttarakhand), India, for identification. These isolates were used in further studies on xylanase production for their subsequent application in biobleaching experiments.

2.2.2.11 Microscopic examination of the test strains

Microscopic examination of the test fungi was carried out using microscopic slides of vegetative mycelium and fruit bodies that were prepared with lacto-phenol cotton blue stain. Lacto-phenol cotton blue stain contained lactic acid (20mL), phenol crystals (20 g), glycerol (40 mL), distilled water (20 mL) and 1% aqueous cotton blue (2 mL). The mycelia was picked and kept on a microscopic slide and a drop of lacto-phenol cotton blue stain was added to it. A cover slip was gently placed over the specimen so as to avoid appearance of air bubbles. The fruiting bodies were picked up and kept on a microscopic slide in a similar fashion and pressed with gentle pressure of the thumb after placing a cover slip over the specimen.

The microscopic features of the test fungi were also studied using microscopic slides containing a smear of PDA. PDA was first prepared and autoclaved. A smear of this medium was prepared on autoclaved microscopic slides. These slides were then point inoculated with the test fungi and incubated at 37 °C until desired growth appeared.

All the slides were viewed under light microscope and photomicrographs were taken under different magnifications.

2.2.2.12 Growth of test strains on different agar media

The effect of different media composition on the growth of test fungi was studied by comparing their growth on WBA, PDA and xylan-agar (1% w/v birchwood xylan) media. 5 mm discs from 4 day old cultures of the test fungi were inoculated in the centre of these plates. The plates were incubated at 37 °C and growth was measured after 5 days. The appearance was observed daily with naked eye.

2.2.2.13 Screening of test strains for production of laccase and amylase by plate assay technique

The test fungi were tested for their abilities to produce laccase by laccase plate assay, in which they were grown on guaiacol-agar medium (0.02% w/v guaiacol, 1% w/v yeast extract and 2% w/v agar agar). 5 mm discs from 4 day old cultures of the test fungi were inoculated in this medium and the plates were then incubated at 37 °C for 10 days, to get sufficient growth of the cultures on the medium.

The test strains were also grown on starch-agar plates (containing 1% w/v starch, 1% w/v yeast extract, and 2% w/v agar) to test for their ability to produce amylase. After an incubation period of 7 days at 37 °C, the plates were stained with iodine-potassium iodide (1% iodine and 0.2% potassium iodide) solution by flooding the plates with this solution. The staining was done to increase the visibility of the plates. After 5 min, the staining solution was poured into a waste beaker, to remove excess of the solution. Degradation of starch was evident based on "cleared" rings against a blue-black background [5].

2.2.2.14 Factors affecting extracellular enzyme production

Solid state fermentation was carried out to study the effect of different ecological and nutritional factors on xylanase production. This was done to determine the most favorable conditions for achieving enhanced levels of enzyme production conditions by the test isolates. Wheat bran was used as the substrate unless mentioned otherwise. The culture conditions were optimized by changing one independent variable at a time while keeping the other variables constant.

2.2.2.14.1 Effect of incubation period on xylanase production and supernatant protein concentration

250 mL Erlenmeyer flasks were prepared for both the test fungi with 5 g of wheat bran and 15 mL of N.S.S. added in each flask. The pH was adjusted as required, with the help of pH meter. The flasks were autoclaved as mentioned above. Two discs of 5 mm diameter from 4-day old culture of each test fungi were inoculated in different flasks. These were incubated at 37 °C and were harvested after 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 and 12 days of incubation. Xylanase activity and supernatant protein concentration were measured as previously described.

2.2.2.14.2 Effect of initial pH on xylanase production and supernatant protein concentration

For the optimization of initial pH, Erlenmeyer flasks were prepared as described above. The pH of the flasks, varying from 4.0 to 13, was adjusted using $NaOH/H_2SO_4$ separately, with the help of pH meter. These flasks were autoclaved and then inoculated as described above, with two discs of 5 mm diameter from 4-day old culture of each test fungi. Inoculated flasks were incubated at desired temperature (37 °C) and harvested after optimum incubation period (7 days). Xylanase activity and supernatant protein concentration were measured as already described.

2.2.2.14.3 Effect of incubation temperature on xylanase production and supernatant protein concentration

The effect of incubation temperature on the production of xylanases by the test fungi was studied by incubating the inoculated flasks at different temperatures varying from 27 to 47 °C for optimum incubation period (7 days) and pH 6.4. These flasks were harvested and the xylanase activity and supernatant protein concentration for enzyme samples from each test fungi were determined as described earlier.

2.2.2.14.4 Effect of different lignocelluloses as substrate on xylanase production and supernatant protein concentration

Natural lignocellulosic substrates, namely, sugarcane bagasse, wheat bran, rice bran, and wheat straw, singly and in different combinations, were used to study the effect of solid substrate on xylanase production by the test fungi. 5 g of each substrate was taken in Erlenmeyer flasks, to which 15 mL of N.S.S. was added. All these flasks were inoculated and then incubated under optimized conditions like, temperature 37 °C, pH 6.4 and incubation period 7 days. These flasks were harvested and the xylanase activity and supernatant protein concentration of the crude xylanases, so obtained, were measured as described earlier.

2.2.2.14.5 Effect of moisture level on xylanase production and supernatant protein concentration

The effect of moisture level (N.S.S.) on xylanase production by the test fungi was tested by varying the wheat bran-to-moisture ratio from 1:2 to 1:5. Accordingly, N.S.S. was added to wheat bran in each case. The prepared flasks were inoculated and then incubated under optimized conditions of temperature i.e. 37 °C, pH 6.4 and incubation period 7 days. After harvesting, the xylanase activity and supernatant protein concentration were determined as already described.

2.2.2.14.6 Effect of glucose and lactose on xylanase production and supernatant protein concentration

In order to investigate the effect of glucose and lactose on xylanase production by the test fungi, the production medium was supplemented with different concentrations (varying from 1 to 5 g/L) of glucose and lactose. These flasks were inoculated and then incubated at optimum conditions i.e. incubation temperature 37 °C,

pH 6.4 and incubation period 7 days. Xylanase activity and supernatant protein concentration were determined in each case after harvesting as previously described.

2.2.2.14.7 Effect of complex organic nitrogen sources on xylanase production and supernatant protein concentration

The effect of complex nitrogen sources on xylanase production by the test organisms was studied by taking peptone, beef extract, soybean meal, malt extract and yeast extract as a complex nitrogen source in the N.S.S at a concentration of 1.0 g/L. The flasks containing different nitrogen sources for each test fungi were inoculated and then incubated for optimized conditions of incubation temperature, period and pH, as described above. These were then harvested and assessed for xylanase activity and supernatant protein concentration as already explained.

2.2.2.14.8 Effect of fermentation conditions on xylanase and cellulase production, and supernatant protein concentration

The effect of culture conditions on xylanase production by the test fungi was studied by comparing the xylanase production under S.S.F and L.S.F. The flasks for each test fungi were inoculated and then incubated under optimized conditions of incubation temperature, period and pH, as already described. The enzymes were then harvested and the xylanase and cellulase activities and supernatant protein concentration of the same were determined as previously described.

2.2.2.15 Mass production of crude xylanases from the test strains, under optimized conditions of S.S.F., for biobleaching

After optimization of fermentation conditions, mass production of xylanases from both the fungal strains was carried out under optimized conditions of SSF for their use in biobleaching experiments. The mass production was carried out in flasks of

2 liter capacity. 30 g of wheat bran and 90 mL of N.S.S. were added in each flask. The pH of each flask was adjusted as described above. The flasks were autoclaved and twelve discs each of 5 mm diameter from 4-day old culture of each test fungi were inoculated in the flasks. These were incubated at optimum conditions of temperature, incubation period and pH, and harvested as described above. The xylanase, cellulase and laccase activities and supernatant protein concentration were determined as described above.

2.2.2.16 Characterization of crude xylanases produced by the test fungi under optimized conditions of S.S.F.

2.2.2.16.1 Effect of pH on xylanase activity

To determine the pH stability of the enzyme, the crude xylanases obtained from both the fungal strains were incubated in different buffers of 6.0, 6.4, 7.0, 7.4, 8.0, 8.4 and 9.0 pHs at temperature 55 °C for 15 min. Four different buffers (0.1 M) were used to maintain different pH levels. Citrate buffer was used for pH 4.0-6.0, phosphate buffer for pH 6.0-8.0, borax buffer for pH from 8.0-9.0 and glycine-NaOH buffer for pH 10.0-11.0. The residual xylanase activity was then measured by the standard assay procedure as described above.

2.2.2.16.2 Effect of temperature on xylanase activity

In order to assess the temperature stability of the enzymes, the crude xylanase preparations obtained from both the fungal strains were incubated at 55, 65 and 75 °C under the above mentioned conditions for determination of xylanase activity. The residual xylanase activities were measured following the standard assay procedure described above.

2.2.2.17 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis and xylanase activity detection by zymogram analysis

In order to determine the protein profile of the crude enzyme preparations obtained from the two test strains, SDS-PAGE analysis was done using 12.5% (w/v) acrylamide gel as described by Laemmli [48]. Biorad Apparatus was used to perform the experiment. Glass plates (1.5mm) were assembled according to the manufacturer's instructions and leakage was checked using distilled water. Resolving gel was poured into the gap between plates and was overlayed with isopropanol. After polymerization, the overlay was poured off and top of the gel was washed several times with water to remove any unpolymerised acrylamide. Stacking solution was poured directly onto the surface of the polymerized gel and a clean 10 well comb (1.5mm) was inserted. More gel solution was added to fill the spaces of the comb completely. Gel was placed in vertical position at room temperature and allowed to polymerize. While the stacking gel polymerized, samples were prepared by heating them to 100 °C for 5 min in 1X SDS gel loading buffer to denature the proteins. After polymerization was completed, comb was removed and wells were washed with distilled water to remove any unpolymerised acrylamide. The gel was mounted in the electrophoresis apparatus. Tris Glycine electrophoresis buffer was added to the top and bottom reservoirs. 30µL of pretreated samples were added to the wells and was electrophoresed. A voltage of 8 V/cm was applied to the gel, after the dye front moved into the resolving gel, the voltage was increased to 15 V/cm. The gel was run until the bromophenol blue reaches the bottom of the resolving gel. The power supply was turned off and gel was disassembled. Orientation of gel was marked by cutting a corner from the bottom of the gel. The gel was then stained overnight at room temperature with gentle shaking in Coomassie brilliant blue R-250. The staining solution contained 0.1% w/v Coomassie brilliant blue

R-250, 40% methanol and 10% acetic acid. The gel was then destained to get the stained bands over the clear background by washing with destaining solution containing 30% methanol and 10% acetic acid [72]. The gel was stored in 5% acetic acid until photographed. The molecular weight standard used was medium range marker (Bangalore Genei): phosphorylase b (97.4 kDa), bovine serum albumin (66 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa), soyabean trypsin inhibitor (20.1 kDa) and lysozyme (14.3 kDa).

For the zymogram analysis, the crude enzyme samples were electrophoresed as above on SDS-PAGE containing 0.1% xylan. After running, the gel was washed four times for 30 min in 100mM phosphate buffer (pH 6.4), the first two washes containing 25% (v/v) isopropyl alcohol, to remove SDS and renature protein in the gel. The gel was incubated for 20 min at 37 °C before soaking in Congo red solution for 5 min at room temperature and destained with 1 M NaCl until excess dye was removed from the active band. The zymogram was prepared after fixing the gel with 0.5% acetic acid solution. The background turned dark blue, and zymograms were observed for clear areas [60].

2.2.2.18 Statistical analysis

All cultures and analyses were replicated three times and the results are mean ± standard deviation (SD) of the values.

2.3 RESULTS AND DISCUSSION

2.3.1 Isolation of xylanase producing basidiomycetes

A total of twelve fungal strains were isolated from decaying wood samples by enrichment culture technique, in which selective culture media and incubation

conditions were used to isolate microorganisms directly from nature. In the present investigation moist wheat bran was used in the medium as a sole carbon source, as it has already been shown to be a promising carbon source for xylanase production [80]. Seven of the isolates exhibited white thread like mycelial network on the decaying wood and aerial fruit bodies, after successive degradation. This indicated growth of basidiomycetous strains (Photographs 2.1A-F). These were isolated and purified on wheat bran agar medium (Photographs 2.2A-D). The purified isolates produced fruiting bodies under laboratory conditions. Brown coloured basidiospores were observed when mature fruiting bodies were observed under light microscope. This confirmed the isolates as white rot basidiomycetes.

2.3.2 Screening of isolates for potent xylanase producers

The selected isolates, when screened for xylanase production using plate assay technique (Table 2.1), were found to be xylanase positive strains. The isolates exhibited variable xylanase activities, as indicated by the areas of clear zones (Photographs 2.3 A-B), around the fungal cultures, when viewed by Congo red staining method. The presence of clear zones indicated solubilization of xylan which would be due to the hydrolytic action of endoxylanases.

These isolates were further subjected to solid state fermentation (S.S.F.) conditions (Photographs 2.4A-D) for a fixed incubation period to determine the xylanase and cellulase activities and supernatant protein concentrations of the crude enzyme preparations obtained from them (Table 2.2). The aim was to select two isolates that would show the highest xylanase activities among the seven isolates, with minimal or no cellulase activities. As is evident from Figure 2.2, isolates C and E showed the highest and second highest xylanase activities, respectively. Both the isolates showed poor cellulase activity.

It is possible that other isolates might have different lag phase, and, if the substrate was sampled later, a higher enzyme concentration might have been achieved. However, it was in our interest to compare the enzyme activities on same day, as short incubation periods are desirable from an industrial point of view. Thus, isolates C and E, showing the highest and the second highest xylanase activities, were selected for further studies.

2.3.3 Identification of the selected isolates

The isolates C and E were identified at Pathology Division, Forest Research Institute, Dehradun, India, as different strains of white rot fungus, *Coprinellus disseminatus* (Pers.: Fr.) Lange (= *Coprinus disseminatus* (Per.: Fr.) Gray. The identification was based on the characters of the fruiting bodies and their microscopic details. The test strains were deposited at National Type Culture Collection, Forest Research Institute, Dehradun, India, as *Coprinellus disseminatus* SH-1 and *Coprinellus disseminatus* SH-2 with the deposition numbers NTCC 1163 and NTCC 1164, respectively.

2.3.4 Microscopic examination of the test strains

Microscopic examination of the test strains (*C. disseminatus* SH-1 and *C. disseminatus* SH-2) in a lactophenol-cotton blue mount (Photomicrographs 2.1 A-K) was done. It revealed the presence of basidium, basidiospores, pileocystidea, hymenial layers and chlamydospores (mitotic submerged spores). Clamp connections were also observed (Photomicrographs 2.1E and J), when test strains were directly inoculated on a smear of PDA medium on microscopic slides. This is a characteristic feature of class Hymenomycetes of Basidiomycotina, to which *C. disseminatus* belongs [41]. The mating system in *C. disseminatus* was determined by Lange [49].

C. disseminatus belongs to the family Coprinaceae of the order Agaricales [4]. This species had been placed in its own genus, *Pseudocoprinus* because it looked like a *Coprinus* (in the traditional sense) but did not autodigest completely like in case of *Coprinus* sp. [6]. In natural habitat, *C. disseminatus* typically fruits in clusters near the bases of stumps. Its cap is initially white, but soon begins to turn grayish brown, with a brownish center. It is easily recognized as a coprinoid mushroom by its cap shape and grayish black gills. It is an extremely fragile mushroom, and the caps quickly crumble when handled [56].

2.3.5 Growth of test strains on different agar media

The effect of media composition on the growth and appearance of the test strains was studied by observing their growth on three different culture media, namely, wheat bran agar (WBA), xylan agar and potato dextrose agar (PDA). It was observed that the growth of both the strains was the best on WBA, followed by PDA and the poorest on xylan agar (Table 2.3). In case of xylan agar medium, the growth was found to be sparse and irregular (Photographs 2.5A-D). This indicated that xylan agar was less favorable for the growth of the test strains and probably also not a good xylanase inducer for both the test strains. Previous studies have also shown that pure xylan tends to repress xylanase activity in case of many xylanase producers [65]. Also, the use of xylan agar medium is not cost effective from the industrial point of view. Though, PDA supported growth of the test strains yet not better than WBA. The frequency of contamination by competing fungal species was also higher in case of PDA as compared to WBA, simply because WBA acts as a selective medium. Thus, WBA was chosen for cultivation of the test strains.

2.3.6 Screening of test strains for production of laccase and amylase by plate assay technique

The test strains were screened for the production of laccase (Photographs 2.6A-B) and amylase (Photograph 2.7A-B) by plate assay technique. When the test strains were grown on guaiacol-agar medium, zones of dark brown colour were formed around the fungal cultures. This indicated the production of laccase by both the test strains. Areas of clear zones were observed when the test strains were grown on starch-agar plates for an incubation period of 4 days. These zones indicated starch hydrolysis by amylase produced by both the test strains. The zone was slightly larger in case of strain SH-2 than strain SH-1.This indicated slightly higher amylase production by strain SH-2.

2.3.7 Effect of incubation period on xylanase production and supernatant protein concentration

Table 2.5 shows the effect of incubation period on supernatant protein concentration and xylanase production by the test strains. The production of xylanases by the test strains varied depending on the duration of incubation. It was observed that for both the test strains, the xylanase production increased rapidly and was maximum for an incubation period of 7 days. The xylanase activity decreased thereafter. A decrease of 42.21% and 7.72% in xylanase activities for strain SH-1 (Figure 2.3) and strain SH-2 (Figure 2.4) respectively, was observed for an incubation period of 8 days. As can be seen, this decrease in activity was more drastic in case of strain SH-1 compared to that of strain SH-2. The supernatant protein concentration also increased up to 7 days of incubation period, and decreased thereafter.

Xylanases are primary metabolites. They are optimally expressed at the end of

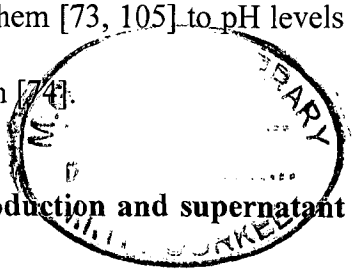
the exponential phase and the harvesting time of the xylanases must be correlated to the production of these enzymes [47]. The metabolic enzymes of the xylanase producers such as proteases [64] and transglycosidases have also been shown to affect the actual yield of the enzymes [38].

2.3.8 Effect of initial pH on xylanase production and supernatant protein concentration

Table 2.6 shows the effect of initial pH on xylanase production and supernatant protein concentration by the test strains. The optimum pH for xylanase production was found to be 6.4 for both the test strains, as the highest xylanase activity was obtained at this pH. The xylanase activity at pH 6.4 was 723.41 IU/mL for strain SH-1 (Figure 2.5) and 229.45 IU/mL for strain SH-2 (Figure 2.6). The supernatant protein concentration was also found to be the highest at pH 6.4. The supernatant protein concentration at pH 6.4 was 5.000 mg/mL for strain SH-1 (Figure 2.5) and 4.725 mg/mL for strain SH-2 (Figure 2.6).

The pH of the medium is an important parameter for enzyme production. A regulatory influence of pH shift on the production of both primary [97] and secondary metabolites [40] has been demonstrated. Any enzyme mediated reaction in the external medium, whether truly external or in the periplasm, is influenced by the culture pH which might result in possible changes in productivity. Structures such as, membranes in contact with the external environment can also undergo chemical changes in response to pH. Microorganisms may need to adapt their function in order to cope up with a change in hydrogen ion concentration. If this change is too abrupt, the response of microbes might lag behind or overshoot. Apart from affecting cell membrane permeability, pH may also determine the solubility of some components of the medium.

Thus, a modification in the pH might also cause some micronutrients to precipitate and become impossible to be assimilated [79]. Earlier studies have shown that most of the white-rot fungi grow best at slightly acidic pH. Many of these fungi have also been shown to acidify lignocellulosic substrates as they grow in them [73, 105] to pH levels that are probably at least partially inhibitory to further growth [74].



2.3.9 Effect of incubation temperature on xylanase production and supernatant protein concentration

The effect of incubation temperature on supernatant protein concentration and xylanase production by the test strains is shown in Table 2.7 and graphically represented in Figures 2.7 (for strain SH-1) and 2.8 (strain for SH-2). For both the test strains, the maximum xylanase production (724.45 IU/mL for strain SH-1 and 230.21 IU/mL for strain SH-2) was found to be at 37 °C. At 42 °C, a decrease of 79.86 and 73.23% in xylanase activity was observed for *C.disseminatus* strains SH-1 and SH-2, respectively. The maximum supernatant protein concentrations were observed at 37 °C, which were 5.119 mg/mL for strain SH-1 and 4.800 mg/mL for strain SH-2. This indicated that both the test strains are mesophiles. The literature also supports the results as it has been reported that most of the white-rot fungi are mesophiles [74].

2.3.10 Effect of different lignocelluloses as substrate on xylanase production and supernatant protein concentration

The effect of different lignocelluloses as substrate on supernatant protein concentration and xylanase production by the test strains is shown in Table 2.8 and graphically represented in Figures 2.9 (for strain SH-1) and 2.10 (for strain SH-2). The maximum xylanase production for both the strains, strain SH-1 (724.21 IU/mL) and strain SH-2 (229.81 IU/mL), was observed for wheat bran as a solid substrate. The order of the substrate suitability for xylanase production by strain SH-1 was: wheat

bran > rice bran + wheat bran> wheat bran+ bagasse > wheat bran+ wheat straw > rice bran + wheat straw > rice bran > wheat straw > bagasse > wheat straw+ bagasse. For strain SH-2, the xylanase activities recorded for different substrates were in the following descending order: wheat bran > wheat bran + wheat straw > wheat straw > rice bran > rice bran + wheat bran > rice bran +wheat straw > bagasse > wheat bran + bagasse > wheat straw + bagasse. The supernatant protein concentration for both the test strains using wheat bran as substrate was 5.098 mg/mL for strain SH-1 and 4.754 mg/mL for strain SH-2, which was the highest.

The use of pure xylan as substrate has been shown to be uneconomical for large scale production of xylanases. At the same time, lignocellulosic wastes offer cost effective substrate for xylanase production [46]. Studies have also indicated that lignocellulosic materials appear to be better substrates than xylan for producing xylanolytic enzymes [19, 34]. Keeping this in view, lignocellulosic residues, singly and in different combinations, were tested for their ability to support xylanase production.

The effect of a substrate on enzyme production is determined by many factors involved in the process, such as, the presence of activator or inhibitor, surface area, diffusion of catabolite, pretreatment, content and sugar composition of a substrate [84]. Xylanases have been shown to be inducible enzymes, with rare examples of constitutive xylanase expression [89]. Xylanolytic enzymes are generally induced by xylan, xylobiose, xylose and also by lignocellulosic residues that contain xylan [7, 26]. Xylan is a high molecular mass polymer and therefore cannot penetrate the cell wall. The low molecular mass fragments of xylan (including xylose, xylobiose, xylooligosaccharides, heterodisaccharides of xylose and glucose and their positional isomers) that are liberated from xylan by the action of small amounts of constitutively produced enzyme, play a key role in the regulation of xylanase biosynthesis. Cellulose

has also been shown to act as an inducer of the xylanase in a few cases, but it is not clear whether the inducing effect lies with cellulose or the contaminating xylan fraction. The low molecular mass substances that have been identified as xylanase inducers, need transferase enzymes for their translocation into the cytoplasm. Hence, the level of inducers and/or the required enzymes in the culture filtrate also affect the xylanase synthesis [47]. In general, the xylanase induction is a complex phenomenon and the level of response to an individual inducer varies with the organisms [37]. It is also dependent upon the nature and the concentration of the carbon sources. Generally, the slow release of the inducer molecules and the possibility of the culture filtrate converting the inducer to its non-metabolizable derivative are believed to boost up the level of xylanase activity [47].

Wheat bran was found to be the best substrate for xylanase production possibly because it contained sufficient nutrients and was able to remain loose in moist conditions, thereby, providing a large surface area [10]. The cell-wall polysaccharides of wheat bran contain about 40% xylans and about 28% protein. These might serve as the source of carbon and nitrogen for the microorganisms. Its lignin and silica contents are also been reported to be very low [80].

In the present investigation, despite of using different substrates, the fixed incubation period of 7 days (as found to be optimum for wheat bran as a substrate) was used. It is possible that the fungus might have a different lag phase, regarding to some of these substrates, and if that substrate was sampled later, a higher enzyme concentration might have been achieved. However, it was in our interest to compare the different substrates with the wheat bran, which had produced enzymes in 7 days of fermentation [84]. Previous studies have also shown that wheat bran is a promising carbon source for xylanase production [80].

2.3.11 Effect of moisture level on xylanase production and supernatant protein concentration

The effect of moisture level on supernatant protein concentration and xylanase production by both the test strains is shown in Table 2.9 and graphically represented in Figures 2.11 (for strain SH-1) and 2.12 (for strain SH-2). It was observed that the maximum xylanase production was achieved at a substrate to moisture (N.S.S.) ratio of 1: 3. The xylanase activity for strain SH-1 was 719.98 IU/mL and for strain SH-2 was 225.00 IU/mL at substrate to moisture (N.S.S.) ratio of 1: 3. The activity decreased thereafter. Supernatant protein concentration at substrate to moisture (N.S.S.) ratio of 1: 3 was 4.890 mg/mL for strain SH-1 and 4.587 mg/mL for strain SH-2, which was the highest.

Xylanase production and extracellular protein appeared to be sensitive to the initial moisture content of the wheat bran. The moisture content in S.S.F. is one of the crucial factors affecting enzyme activity [71]. Its influence on microbial growth and product biosynthesis might be attributed to its impact on the physical properties of the solid substrate. A higher than optimum moisture level might have caused a decrease in porosity, lower oxygen transfer and alteration in wheat bran particle structure, whereas, a lower than optimum moisture level might have caused a reduction in solubility and swelling of solid substrate [25, 69].

2.3.12 Effect of glucose and lactose on xylanase production and supernatant protein concentration

The effect of glucose on supernatant protein concentration and xylanase production by both the test strains is shown in Table 2.10 and graphically represented in Figures 2.13 (for strain SH-1) and 2.14 (for strain SH-2), while that of lactose is

shown in Table 2.11 and graphically represented in Figures 2.15 (for strain SH-1) and 2.16 (for strain SH-2). When different concentrations (1-5 g/L) of glucose or lactose were added to the wheat bran, the addition of these sugars resulted in repression of xylanase production in case of both the strains. Similarly, the supernatant protein concentration for both the test strains was found to decrease in presence of glucose as well as lactose. The repression was found to be concentration dependent as, higher was the concentration of glucose or lactose in the medium, higher was the repression in xylanase yield.

Smith and Wood [87] have also reported low titers of xylanases in presence of glucose and lactose. This phenomenon of xylanase repression in presence of easily metabolizable sugars has been observed by researchers in different microorganisms [16, 31, 80]. The repression in xylanase synthesis in the presence of easily metabolizable carbon sources in the growth medium, suggested that the synthesis of the enzyme is controlled by the transition state regulators and catabolite repression [47]. As mentioned earlier, xylanases have been shown to be inducible enzymes, with rare examples of constitutive xylanase expression [89]. As previously mentioned, the xylanase induction, in general, is a complex phenomenon and the level of response to an individual inducer varies with the organisms [37]. The substrate derivatives and the enzymatic end products might often play a key positive role in the induction of xylanases; they can also act as the end product inhibitors, possibly at much higher concentrations. The exact mechanism by which CREA (mediating factor for carbon catabolite repression) mediate glucose repression in filamentous fungi is not wholly understood [78]. It is possible that the mechanism in filamentous fungi has some similarity to that in *S.cerevisiae*, where glucose repression is mediated by a protein that involves nucleosome positioning [8].

2.3.13 Effect of complex nitrogen sources on xylanase production and supernatant protein concentration

Table 2.12 shows the effect of complex nitrogen sources on supernatant protein concentration and xylanase production for both the test strains and graphically represented in Figures 2.17 (for strain SH-1) and 2.18 (for strain SH-2). It was observed that maximum xylanase production was obtained with yeast extract as the source of complex nitrogen i.e. 723.78 IU/mL for strain SH-1 and 230.00 IU/mL for strain SH-2. The order of the suitability of complex nitrogen sources for xylanase production in the present study was: yeast extract > beef extract > malt extract > soybean meal > peptone, for *C. disseminatus* SH-1 and yeast extract > malt extract > soybean meal > peptone > beef extract, for *C. disseminatus* SH-2. The supernatant protein concentrations also showed a similar behavior, as the highest supernatant concentration (4.981 mg/mL in case of strain SH-1 and 4.888 mg/mL in case of strain SH-2) was also observed for yeast extract.

Many previous studies have proved that both the nature and concentration of nitrogen sources are powerful nutrition factors regulating lignocellulolytic enzyme production by wood-rotting basidiomycetes [91, 106]. The mechanisms that govern the formation of extracellular enzymes are influenced by the availability of precursors for protein synthesis [47] and the differences in enzyme activity obtained for media containing various complex nitrogen sources that were used in the present study could be caused by their varying contents of amino acids, peptides, vitamin, trace elements and/or mineral salts [66]. The highest xylanase production observed with yeast extract for both the test strains might be also attributed to a better absorption of amino acids of yeast extract directly through mycelia of the test strains [68].

2.3.14 Effect of fermentation conditions (S.S.F. vs L.S.F.) on xylanase and cellulase production, and supernatant protein concentration

Table 2.13 shows the effect of fermentation conditions on supernatant protein concentration, xylanase production and cellulase production by *C. disseminatus* strains SH-1 and SH-2, with its graphical representation in Figures 2.19 (for strain SH-1) and 2.20 (for strain SH-2). The fermentation conditions employed in enzyme production had a profound effect on it. The maximum xylanase production under S.S.F. was 725.03 and 224.88 IU/mL for strain SH-1 and SH-2, respectively. Under L.S.F., the xylanase production was 97.90 and 64.87 IU/mL for strain SH-1 and SH-2, respectively. Thus, xylanase production by both the test strains was much lower under L.S.F. than under S.S.F conditions. The CMCase activities of the test strains were also found to be lower in L.S.F. (0.117 IU/mL for strain SH-1 and 0.173 IU/mL for strain SH-2) as compared to S.S.F. (0.862 IU/mL for strain SH-1 and 0.599 IU/mL for strain SH-2). The supernatant protein concentration was also lower in L.S.F. (0.722 mg/mL for strain SH-1 and 1.391 mg/mL for strain SH-2) as compared to S.S.F. (5.340 mg/mL for strain SH-1 and 4.820 mg/mL for strain SH-2).

The greater xylanase production under S.S.F. compared to L.S.F. might be because S.S.F. provided the fungus with an environment closer to its natural habitat (wood and decayed organic matter). This might have stimulated these strains to produce more hemicellulolytic enzymes [84]. Generally, in submerged cultivation the growth form of filamentous fungi varies between pelleted and filamentous. Each form has its own characteristics, and can affect the rate of enzyme production by influencing the mass transfer rate [58]. In liquid state fermentation (L.S.F.), the fungus is exposed to hydrodynamic forces, while in SSF growth is restricted to the surface of the solid matrix, with no such negative effects [84]. Also, the hyphal mode of growth gives the filamentous fungi the power to penetrate into the solid substrates. The cell wall

structure attached to the tip and the branching of the mycelium ensures a firm and solid structure. The hydrolytic enzymes are excreted at the hyphal tip, without large dilution, as in the case of LSF. This makes the action of hydrolytic enzymes very efficient and allows penetration into moist solid substrates. Penetration increases the accessibility of all the available nutrients within particles [70] and thus, enzyme production is higher in SSF. Also, catabolite repression and protein degradation by proteases that are severe problems in SmF, have often been reported to be reduced or absent in SSF [2].

2.3.15 Mass production of crude xylanases from the test strains, under optimized conditions of S.S.F., for biobleaching

The mass production of crude xylanases for subsequent use in biobleaching of wheat straw soda-AQ pulp was done in 2000 mL flasks under production conditions as optimized above. Table 2.14 shows the xylanase, cellulase and laccase activities, and supernatant protein concentrations of the crude enzyme preparations obtained from *C. disseminatus* strains SH-1 and SH-2 under optimized conditions of S.S.F. Its graphical representation is shown in Figure 2.21. Under optimized conditions, the xylanase activity was 727.78 IU/mL for strain SH-1 and 227.99 IU/mL for strain SH-2. The cellulase and laccase activities in case of strain SH-1 were 0.925 IU/mL and 0.640 U/mL, respectively, while those for strain SH-2, were 0.660 IU/mL and 0.742 U/mL, respectively. The supernatant protein concentration for strain SH-1 was 5.480 mg/mL, while for strain SH-2 was 4.900 mg/mL.

As can be observed, a minor cellulase activity was found in the crude enzyme preparations obtained from both the test strains. Because the activity was very low, crude culture filtrate can be used for biobleaching experiments without further purification.

It was found that in all the cases, although an overestimation of soluble proteins in crude extracts due to interferences with other compounds was possible, its profile agreed with the increase of enzyme activities.

It is also to be worth mentioning that the xylanases bind tightly to the substrate and a part of the enzyme produced during the fermentation might be lost and discarded, as bound enzyme, along with the insoluble substrate [47].

2.3.16 Effect of pH on the activity of crude xylanases obtained from the test strains

The effect of pH on xylanase activity was tested between pH 6 and 9 using defined assay conditions and is shown in Table 2.15, with its graphical representation in Figure 2.22. The optimum pH for maximum xylanase activity was found to be 6.4 for both the test strains. At pH 6.4, the xylanase activity was 726.08 IU/mL for strain SH-1 and 230.81 IU/mL for strain SH-2. Above or below this pH, the xylanase activity was found to decrease. For strain SH-1, the enzyme retained 49.03, 32.64 and 5.70% of its activity, at pH 7.0, 8.0 and 9.0, respectively. For strain SH-2, the enzyme retained 95.30, 35.03 and 5.60% of its activity at pH 7.0, 8.0 and 9.0, respectively. At pH 6.0, the enzyme retained 97.47% of its activity for strain SH-1 and 89.48% of its activity for strain SH-2. The results showed that the xylanases obtained from *C.disseminatus* strains SH-1 and SH-2 have slightly acidic pH optima. Most fungal xylanases have also been shown to produce the highest activity at pH 6-6.5 [28]. These results suggested that although the xylanases obtained from the test strains were not highly pH stable yet show activity over a wide pH range of 6 to 9 and strain SH-2 xylanase seemed to have a slightly better pH stability than strain SH-1.

Enzyme activity is markedly affected by pH. This is because substrate binding and catalysis are often dependent on charge distribution on both substrate and enzyme

molecules [82]. All enzymes are proteinaceous in nature (except ribozymes) and they tend to lose their basic structure in presence of harsh conditions like, change in pH, etc. This might lead to loss in active site that translates into loss in enzyme activity. The pH activity profiles of enzymes are also highly dependent on the pK_as (ionization constant) of the catalytic residues which are themselves dependent on the local environment and hence, on the nature of the amino acids in the vicinity of the catalytic residues. Lower the pKa value, higher is the pH stability. Amino acid residues that contribute positive charges and hydrogen bonds serve to lower the pKa values with shorter bonds having a more definite effect. The chemical nature of the donor is also important, with COOH being more effective than OH and $CONH_2$ [18].

2.3.17 Effect of temperature on the activity of crude xylanases obtained from the test strains

Table 2.16 shows the effect of temperature on the xylanase activity of crude xylanases obtained from the test strains under defined assay conditions and is graphically represented in Figure 2.23. It was observed that the maximum xylanase activity was at a temperature of 55 °C for both the test strains. Above or below this temperature, the xylanase activity decreased. For strain SH-1, the xylanase retained 43.01 and 12.01% of its activity at 65 °C and 75 °C, respectively. In case of strain SH-2, the xylanase retained 25.00 and 20.22% of its activity at 65 °C and 75 °C, respectively. At 45 °C, the crude xylanases obtained from both the test strains showed 85% of their maximum activity. Many authors have suggested different temperature range of 40-70 °C for maximum activity of xylanases obtained from different microorganisms [28, 29].

While proceeding towards optimum temperature for enzyme activity, the major effect of temperature was an increase in the reaction-rate resulting from increased

kinetic energy of the reacting molecules. On further rise in temperature, another factor relating to protein denaturation came into play and consequently there was a loss of secondary and tertiary structures, leading to the loss of catalytic function of the enzyme proteins.

2.3.18 SDS PAGE analysis and xylanase activity detection by zymogram analysis

Photograph 2.8 shows the SDS-PAGE profile of the crude enzyme preparations obtained from the test strains, *C. disseminatus* SH-1 and *C. disseminatus* SH-2. It shows that the major protein bands for both the test strains were almost similar in number and molecular weights, corresponding to about 20 kDa, 29 kDa and 66 kDa, with an exception of presence of one more major band of molecular weight greater than 97 kDa in case of enzyme extract obtained from *C. disseminatus* SH-2. The concentration of some proteins in these extracts was low which caused weak staining of some proteins and difficulty in detection of some other proteins by polyacrylamide gel electrophoresis. It is to be noted that only an insight to the protein profile of the crude enzymes could be gained, as there is a high possibility that a number of proteins would exhibit similar molecular weights which might appear as overlapping bands in the gel. Thus, multiplicity of proteins in the crude extracts was demonstrated, but not their exact number.

Zymogram analysis (Photograph 2.8) of the crude enzyme samples obtained from the test strains was also done using birch wood xylan in the gel for detection of xylanase activity after PAGE. The xylanase activity was indicated by appearance of clear zones against dark blue background. For crude enzymes obtained from each strain, only a single active band corresponding to xylanase activity was shown as the hydrolysis (clearing) zone in the zymogram. This corresponded to a molecular weight

of about 66 kDa for *C. disseminatus* strain SH-1 and 29 kDa for *C. disseminatus* SH-2. Thus, zymogram analysis showing clearing zones corresponding to protein bands indicated xylanase activity.

The molecular weight of xylanases obtained from *C. disseminatus* strains SH-1 and SH-2 are also supported by the literature, as microbial xylanases have been reported to be single subunit proteins with molecular masses in the range of 8-145 kDa [92].

Table 2.1 Screening of xylanase producers by xylan-agar plate assay showing xylanase activity after 6 days of incubation period

Sl. No.	Isolates	Xylanase activity
1.	A	+
2.	B	+++
3.	C	++++
4.	D	+++
5.	E	+++++
6.	F	++
7.	G	+

+	Very poor activity
++	Poor activity
+++	Average activity
++++	Good activity
+++++	Very good activity

Table 2.2 Screening of potent xylanase producers under solid state fermentation conditions

Sl. No.	Isolates	Day of harvest	Xylanase activity (IU/mL)	Cellulase activity (IU/mL)	Protein concentration (mg/mL)
1.	A	6	1.15±0.14	0.141±0.10	0.224±0.11
2.	B	6	80.41±1.19	0.700±0.26	1.892±0.43
3.	C	6	658.12±11.18	0.940±0.18	4.388±0.25
4.	D	6	91.48±2.87	0.925±0.13	2.014±0.19
5.	E	6	131.25±3.15	0.712±0.32	2.688±0.40
6.	F	6	54.89±4.01	1.324±0.21	1.010±0.43
7.	G	6	9.10±0.42	1.101±0.23	0.535±0.22

Fermentation conditions:		**Assay conditions:**	
Substrate: Nutrient salt solution	= 1:3	Temperature, °C	= 55
pH	= 6.0	pH	= 6.4
Temperature, °C	= 37	Incubation time, min	= 15

± Standard deviation from the mean

Table 2.3 Growth of test strains on different agar media

Sl. No.	Growth media	Strain SH-1		Strain SH-2	
		Growth (cm)	Appearance	Growth (cm)	Appearance
1.	Wheat bran agar (WBA)	Full plate growth	Concave, creamy white, aerial	Over growth	Creamy white, aerial
2.	Potato dextrose agar (PDA)	4.5	Dull white, lateral	5.6	Dull white, lateral
3.	Xylan agar	3.0	Dull white, irregular, sparse	6.2	Dull white, sparse, irregular
Temperature, °C = 37 Incubation period, days = 5					

Table 2.4 Screening of test strains for production of laccase and amylase by plate assay technique

Sl. No.	Assay	SH-1	SH-2
1.	Laccase (10 days of incubation period)	++	+++
2.	Amylase (4 days of incubation period)	+++	+++
++ Poor activity +++ Average activity ++++ Good activity			

Table 2.5 Effect of incubation period on xylanase production and supernatant protein concentration

Sl. No.	Incubation period (days)	Test isolates			
		Strain SH-1		Strain SH-2	
		Xylanase activity (IU/mL)	Protein concentration (mg/mL)	Xylanase activity (IU/mL)	Protein concentration (mg/mL)
1.	2	9.80±0.12	0.069±0.03	7.49±1.10	0.161±0.02
2.	3	13.60±0.10	0.099±0.05	19.43±1.45	0.400±0.07
3.	4	58.29±0.14	0.399±0.09	37.19±3.00	0.761±0.06
4.	5	319.23±0.09	2.139±0.18	85.35±2.12	1.732±0.21
5.	6	670.38±10.17	4.479±0.19	139.49±5.33	2.821±0.19
6.	7	722.98±11.05	4.829±0.24	228.62±4.98	4.621±0.34
7.	8	417.77±7.14	2.799±0.11	210.97±5.02	4.271±0.29
8.	9	408.06±6.50	2.729±0.13	185.98±4.10	3.761±0.33
9.	10	391.40±6.52	2.619±0.11	150.98±6.09	3.061±0.13
10.	11	157.67±9.13	1.059±0.12	128.87±4.87	2.600±0.03
11.	12	124.85±7.04	0.839±0.04	98.05±4.14	1.990±0.09

Fermentation conditions:		**Assay conditions:**	
Substrate: Nutrient salt solution	= 1:3	Temperature, °C	= 55
pH	= 6.0	pH	= 6.4
Temperature, °C	= 37	Incubation time, min	= 15

± Standard deviation from the mean

Table 2.6 Effect of initial pH on xylanase production and supernatant protein concentration

Sl. No.	pH	Test isolates			
		Strain SH-1		Strain SH-2	
		Xylanase activity (IU/mL)	Protein concentration (mg/mL)	Xylanase activity (IU/mL)	Protein concentration (mg/mL)
1.	4	582.92±6.22	4.029±0.10	160.62±4.06	3.308±0.06
2.	4.4	612.21±10.05	4.231±0.11	176.21±1.79	3.629±0.08
3.	5.0	631.00±4.45	4.361±0.08	199.63±4.98	4.111±0.19
4.	5.4	676.59±9.04	4.676±0.11	202.22±5.02	4.165±0.19
5.	6.0	709.90±9.11	4.907±0.52	218.30±3.47	4.490±0.25
6.	6.4	723.41±12.07	5.000±0.41	229.45±5.12	4.725±0.24
7.	7.0	573.21±5.14	4.792±0.12	173.01±4.18	3.563±0.09
8.	7.4	509.99±8.05	3.525±0.10	167.88±3.10	3.458±0.12
9.	8.0	437.20±10.10	3.022±0.12	162.69±2.77	3.351±0.13
10.	8.4	422.00±8.17	2.917±0.09	141.25±5.14	2.911±0.10
11.	9.0	417.77±7.86	2.888±0.08	139.97±3.00	2.883±0.12
12.	9.4	349.27±9.10	2.415±0.10	131.24±5.18	2.703±0.10
13.	10.0	238.72±4.06	1.650±0.15	125.93±5.20	2.594±0.11
14.	10.4	192.21±11.13	1.328±0.08	109.78±6.12	2.261±0.09
15.	11.0	163.77±5.41	1.132±0.04	88.85±5.78	1.830±0.11
16.	11.4	124.21±10.00	0.900±0.13	74.00±6.15	1.525±0.08

Fermentation conditions:		**Assay conditions:**	
Substrate: Nutrient salt solution	= 1:3	Temperature, °C	= 55
Temperature, °C	= 37	pH	= 6.4
Incubation period, days	= 7	Incubation time, min	= 15

± Standard deviation from the mean

Table 2.7 Effect of incubation temperature on xylanase production and supernatant protein concentration

Sl. No.	Incubation temperature (°C)	Test isolates			
		Strain SH-1		Strain SH-2	
		Xylanase activity (IU/mL)	Protein concentration (mg/mL)	Xylanase activity (IU/mL)	Protein concentration (mg/mL)
1.	27	65.78±3.10	0.512±0.08	32.75±4.45	0.684±0.05
2.	32	596.82±11.09	4.219±0.25	154.06±10.15	3.216±0.18
3.	37	724.45±12.08	5.119±0.31	230.21±9.80	4.800±0.25
4.	42	145.88±7.11	1.031±0.20	61.62±4.12	1.296±0.31
5.	47	79.86±2.74	0.566±0.05	11.20±1.10	0.240±0.04
6.	52	No growth	—	No growth	—

Fermentation conditions:		**Assay conditions:**	
Substrate: Nutrient salt solution	= 1:3	Temperature, °C	= 55
pH	= 6.4	pH	= 6.4
Temperature, °C	= 37	Incubation time, min	= 15
Incubation period, days	= 7		

± Standard deviation from the mean

Table 2.8 Effect of different lignocelluloses as substrate on xylanase production and supernatant protein concentration

Sl. No.	Solid substrate	Test isolates			
		SH-1		SH-2	
		Xylanase activity (IU/mL)	Protein concentration (mg/mL)	Xylanase activity (IU/mL)	Protein concentration (mg/mL)
1.	Wheat bran (WB)	724.21±13.02	5.098±0.21	229.81±7.94	4.754±0.25
2.	Wheat straw (WS)	29.81±2.81	0.211±0.03	38.71±3.61	0.832±0.24
3.	Bagasse (B)	21.71±2.25	0.153±0.01	19.91±2.00	0.413±0.13
4.	Rice bran (RB)	62.00±4.10	0.437±0.14	28.90±2.63	0.599±0.17
5.	Wheat straw + Bagasse (WS+B)	11.87±1.90	0.141±0.04	9.09±0.27	0.189±0.03
6.	Wheat bran + Bagasse (WB+B)	130.00±11.00	0.916±0.03	19.23±3.01	0.398±0.08
7.	Rice bran + Wheat bran (RB+WB)	271.00±10.76	1.909±0.06	21.40±1.90	0.444±0.16
8.	Rice bran + Wheat straw (RB+WS)	77.89±4.23	0.507±0.04	20.91±2.12	0.433±0.18
9.	Wheat bran + Wheat straw (WB+WS)	82.98±3.98	0.585±0.02	40.21±3.90	0.951±0.29

Fermentation conditions:		**Assay conditions:**	
Substrate: Nutrient salt solution	= 1:3	Temperature, °C	= 55
pH	= 6.4	pH	= 6.4
Temperature, °C	= 37	Incubation time, min	= 15
Incubation temperature, days	= 7		

± Standard deviation from the mean

Table 2.9 Effect of moisture level on xylanase production and supernatant protein concentration

Sl. No.	Solid substrate: moisture content (N.S.S.)	Test isolates			
		Strain SH-1		Strain SH-2	
		Xylanase activity (IU/mL)	Protein concentration (mg/mL)	Xylanase activity (IU/mL)	Protein concentration (mg/mL)
1.	1:2.0	555.98±7.23	4.107±0.18	161.06±7.15	3.229±0.10
2.	1:2.5	628.22±11.19	4.297±0.32	189.45±11.15	3.610±0.07
3.	1:3.0	719.98±13.08	4.890±0.21	225.00±10.08	4.587±0.25
4.	1:3.5	475.88±9.43	3.396±0.09	138.62±9.10	2.690±0.14
5.	1:4.0	449.86±11.21	3.127±0.07	122.20±6.13	2.230±0.11
6.	1:4.5	391.54±10.31	2.760±0.24	105.45±12.00	2.098±0.16
7.	1:5.0	318.27±5.12	2.321±0.10	89.21±10.21	1.521±0.12

Fermentation conditions:		**Assay conditions:**	
Substrate: Nutrient salt solution	= 1:3	Temperature, °C	= 55
pH	= 6.4	pH	= 6.4
Temperature, °C	= 37	Incubation time, min	= 15
Incubation period, days	= 7		

± Standard deviation from the mean

Table 2.10 Effect of glucose on xylanase production and supernatant protein concentration

Sl. No.	Concentration of glucose (g/L)	Test isolates			
		SH-1		SH-2	
		Xylanase activity (IU/mL)	Protein concentration (mg/mL)	Xylanase activity (IU/mL)	Protein concentration (mg/mL)
1.	0	724.00±7.90	5.210±0.24	228.99±11.15	4.710±0.21
2.	1	650.95±4.26	4.689±0.17	213.74±7.86	4.453±0.14
3.	2	592.66±10.41	4.265±0.12	208.19±7.04	4.397±0.16
4.	3	505.17±8.12	3.647±0.30	197.85±5.19	4.283±0.09
5.	4	495.50±7.45	3.569±0.29	162.24±6.15	3.584±0.22
6.	5	398.34±5.98	2.867±0.21	149.90±8.04	3.090±0.15

Fermentation conditions:		**Assay conditions:**	
Substrate: Nutrient salt solution	= 1:3	Temperature, °C	= 55
pH	= 6.4	pH	= 6.4
Temperature, °C	= 37	Incubation time, min	= 15
Incubation period, days	= 7		

± Standard deviation from the mean

Table 2.11 Effect of lactose on xylanase production and supernatant protein concentration

Sl. No.	Concentration of lactose (g/L)	Test isolates			
		SH-1		SH-2	
		Xylanase activity (IU/mL)	Protein concentration (mg/mL)	Xylanase activity (IU/mL)	Protein concentration (mg/mL)
1.	0	722.79±9.09	4.921±0.34	229.72±8.10	4.751±0.30
2.	1	716.18±10.06	4.877±0.40	216.55±7.15	4.478±0.19
3.	2	591.27±9.14	4.026±0.41	200.01±7.21	4.364±0.24
4.	3	541.30±11.11	3.194±0.20	172.42±8.07	3.764±0.15
5.	4	428.88±7.08	2.953±0.19	153.78±6.12	3.181±0.16
6	5	391.40±9.08	2.665±0.14	111.03±9.06	2.297±0.08

Fermentation conditions:

Substrate: Nutrient salt solution = 1:3
pH = 6.4
Temperature, °C = 37
Incubation period, days, = 7

Assay conditions:

Temperature, °C = 55
pH = 6.4
Incubation time, min = 15

± Standard deviation from the mean

Table 2.12 Effect of complex nitrogen sources on xylanase production and supernatant protein concentration

Sl. No.	Complex nitrogen source (0.1%)	Test isolates			
		SH-1		SH-2	
		Xylanase activity (IU/mL)	Protein concentration (mg/mL)	Xylanase activity (IU/mL)	Protein concentration (mg/mL)
1.	Soya bean meal	639.68±11.28	4.411±0.11	228.16±10.23	4.772±0.27
2.	Peptone	622.82±11.07	4.287±0.10	195.09±9.56	4.089±0.26
3.	Beef extract	722.69±12.09	4.914±0.20	104.84±9.21	2.197±0.20
4.	Malt extract	681.73±10.60	4.617±0.16	228.71±10.54	4.784±0.41
5.	Yeast extract	723.78±10.95	4.981±0.21	230.00±11.76	4.888±0.40

Fermentation conditions:

Substrate: Nutrient salt solution = 1:3
pH = 6.4
Temperature, °C = 37
Incubation period, days = 7

Assay conditions:

Temperature, °C = 55
pH = 6.4
Incubation time, min = 15

± Standard deviation from the mean

Table 2.13 Effect of fermentation conditions (S.S.F. vs L.S.F) on xylanase and cellulase production and, supernatant protein concentration

Sl. No.	Strain	Parameters	S.S.F.	L.S.F.
1.	SH-1	Xylanase activity (IU/mL)	725.03±12.36	97.90±8.97
		Cellulase activity (IU/mL)	0.862±0.14	0.117±0.02
		Protein concentration (mg/mL)	5.340±0.17	0.722±0.12
2.	SH-2	Xylanase activity (IU/mL)	224.88±13.01	64.87±7.60
		Cellulase activity (IU/mL)	0.599±0.18	0.173±0.04
		Protein concentration (mg/mL)	4.820±0.20	1.391±0.08

Fermentation conditions :

S.S.F. =

Substrate: Nutrient salt solution	= 1:3
pH	= 6.4
Temperature, °C	= 37
Incubation temperature, days	= 7

L.S.F. =

Substrate, %	= 2
pH	= 6.4
Temperature, °C	= 37
Incubation temperature, days	= 7

Assay conditions:

Temperature, °C	= 55
pH	= 6.4
Incubation time, min	= 1

± Standard deviation from the mean

Table 2.14 Mass production of crude xylanases from the test strains under optimized conditions of S.S.F, for biobleaching

Sl. No.	Strains	Xylanase activity (IU/mL)	Cellulase activity (IU/mL)	Laccase activity (U/mL)	Protein concentration (mg/mL)
1.	SH-1	727.78±12.21	0.925±0.14	.640±0.10	5.480±0.22
2.	SH-2	227.99±10.87	0.660±0.20	.742±0.0.12	4.900±0.18

Fermentation conditions:

Substrate: Nutrient salt solution	= 1:3
pH	= 6.4
Temperature, °C	= 37
Incubation period, days	= 7

Assay conditions:

Temperature, °C	= 55
pH	= 6.4
Incubation time, min	= 15

± Standard deviation from the mean

Table 2.15 Effect of pH on the activity of crude xylanases obtained from the test strains

Sl. No.	Buffer pH	Xylanase activity (IU/mL)	
		SH-1	SH-2
1.	6.0	707.69±11.21	206.51±9.10
2.	6.4	726.08±10.10	230.81±10.11
3.	7.0	355.93±12.34	219.94±8.04
4.	7.4	332.14±11.78	141.12±11.76
5.	8.0	236.92±10.45	80.83±10.54
6.	8.4	159.67±13.60	49.54±9.61
7.	9.0	41.32±12.62	12.91±2.02
Assay conditions:			
Temperature of incubation, °C = 55			
Substrate concentration = 10 mg xylan /mL potassium phosphate buffer			

± Standard deviation from the mean

Table 2.16 Effect of temperature on the activity of crude xylanases obtained from the test strains

Sl. No.	Incubation temperature (°C)	Xylanase activity (IU/mL)	
		SH-1	SH-2
1.	45	619.13±10.02	191.13±5.45
2.	55	728.39±6.54	224.86±2.07
3.	65	313.21±11.10	56.21±7.56
4.	75	87.41±11.84	45.46±5.21
Assay conditions:			
pH of buffer = 6.4			
Substrate concentration = 10 mg xylan /mL potassium phosphate buffer			

± Standard deviation from the mean

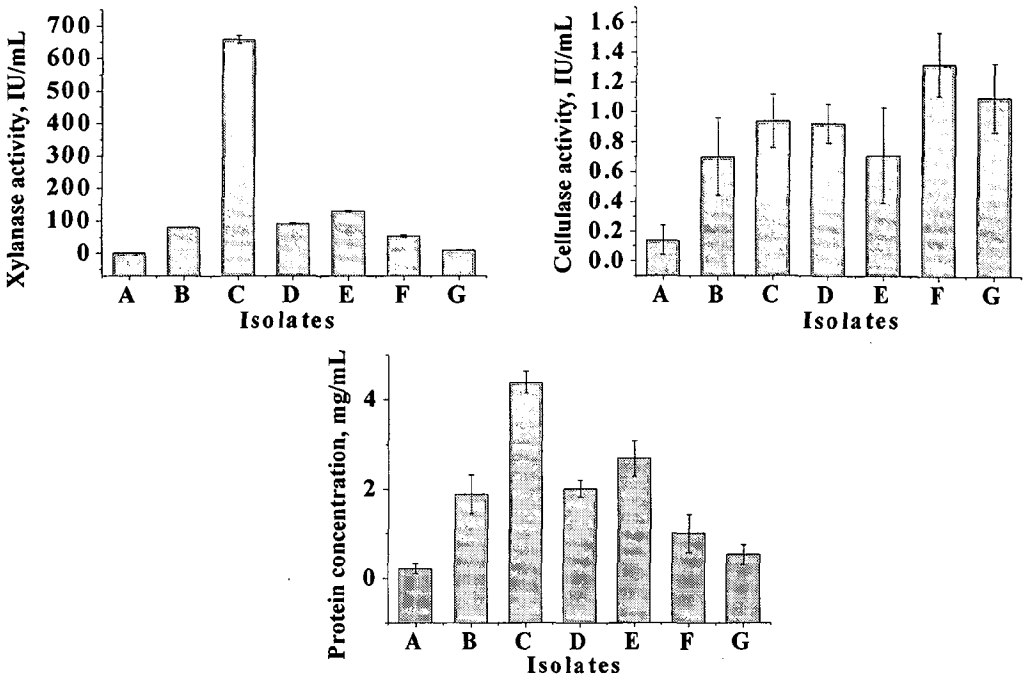


Figure 2.2 Screening of potent xylanase producers under solid state fermentation (S.S.F) conditions

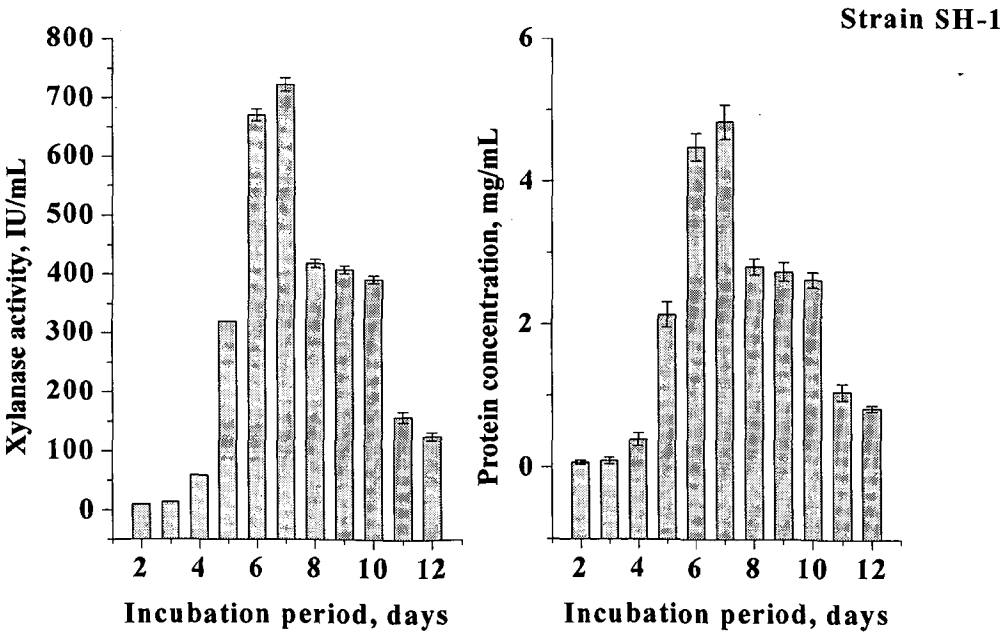


Figure 2.3 Effect of incubation period on xylanase production and supernatant protein concentration for *C. disseminatus* SH-1

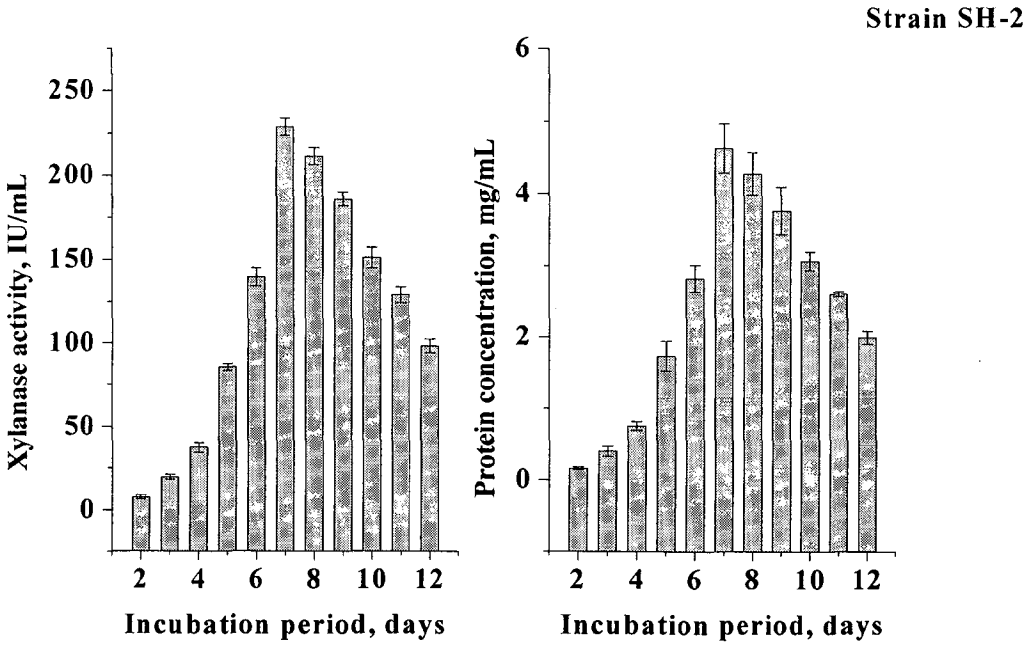


Figure 2.4 Effect of incubation period on xylanase production and supernatant protein concentration for *C. disseminatus* SH-2

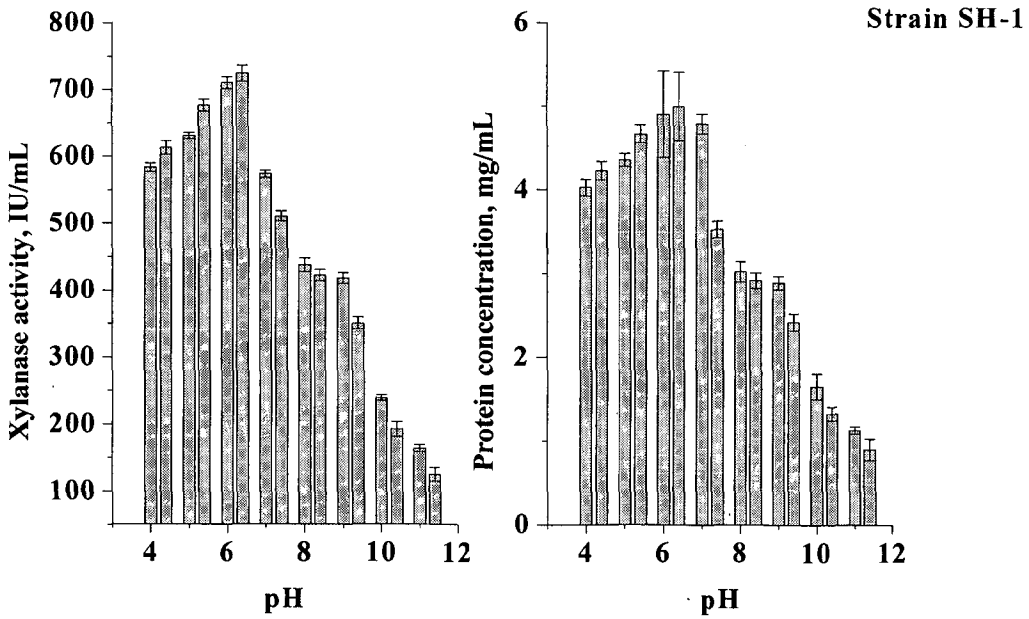


Figure 2.5 Effect of initial pH on xylanase production and supernatant protein concentration for *C. disseminatus* SH-1

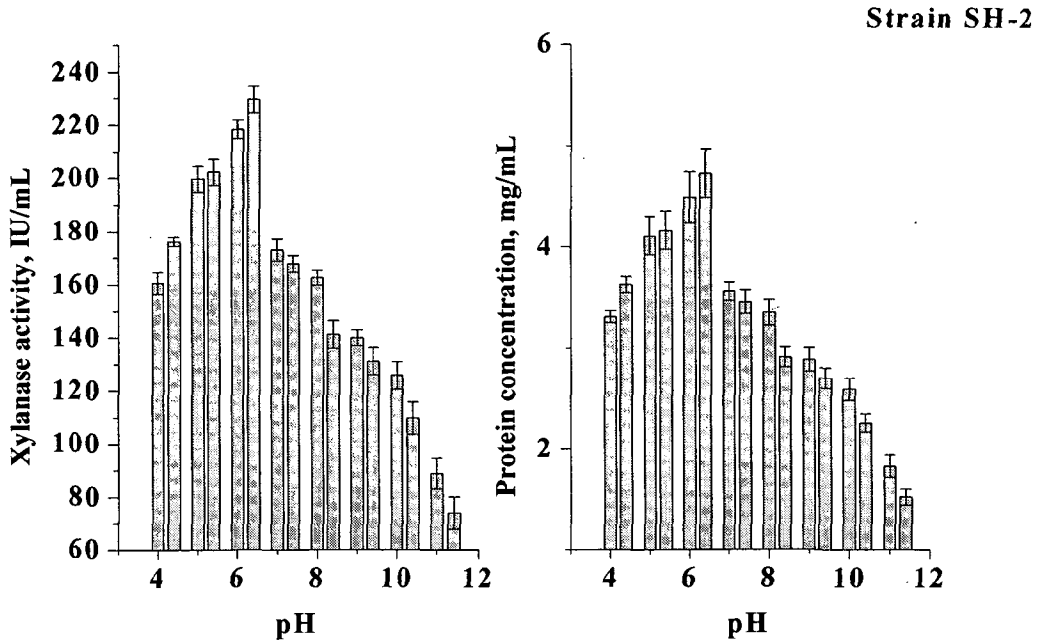


Figure 2.6 Effect of initial pH on xylanase production and supernatant protein concentration for *C. disseminatus* SH-2

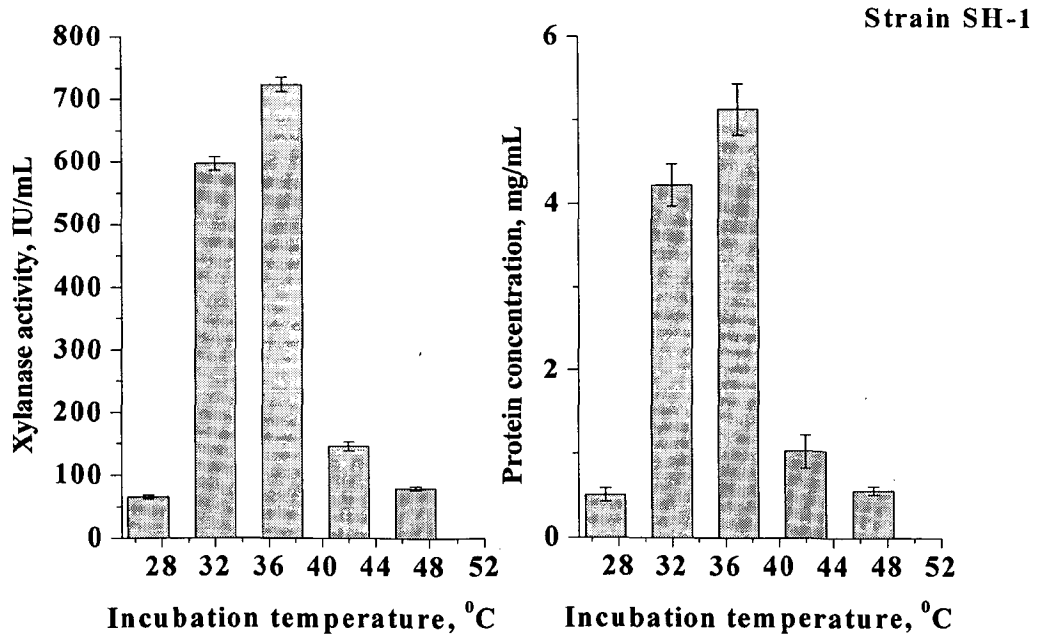


Figure 2.7 Effect of incubation temperature on xylanase production and supernatant protein concentration for *C. disseminatus* SH-1

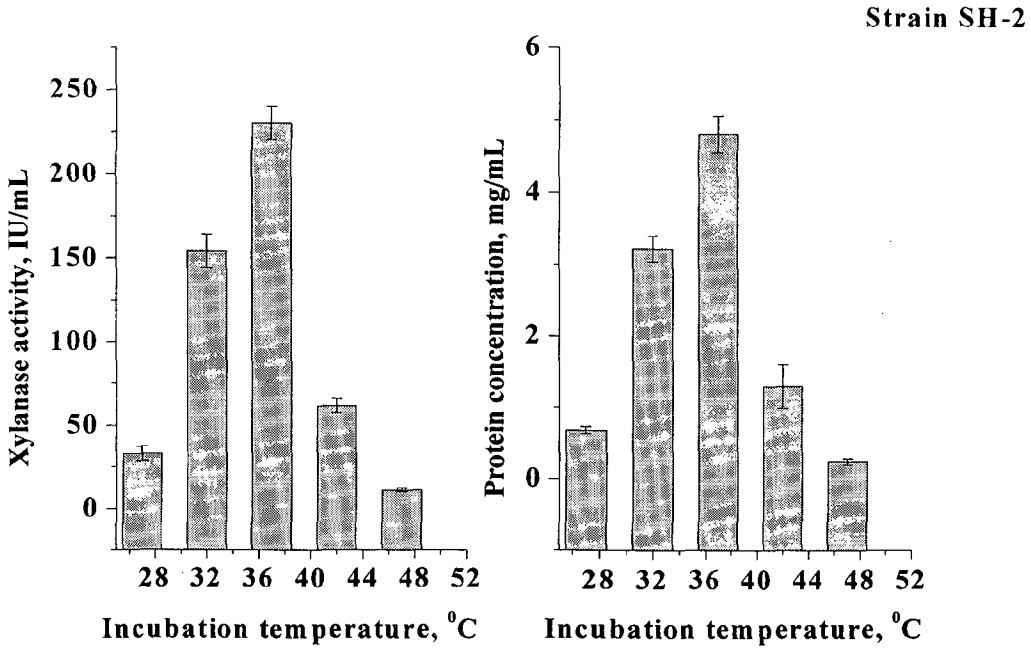


Figure 2.8 Effect of incubation temperature on xylanase production and supernatant protein concentration for *C. disseminatus* SH-2

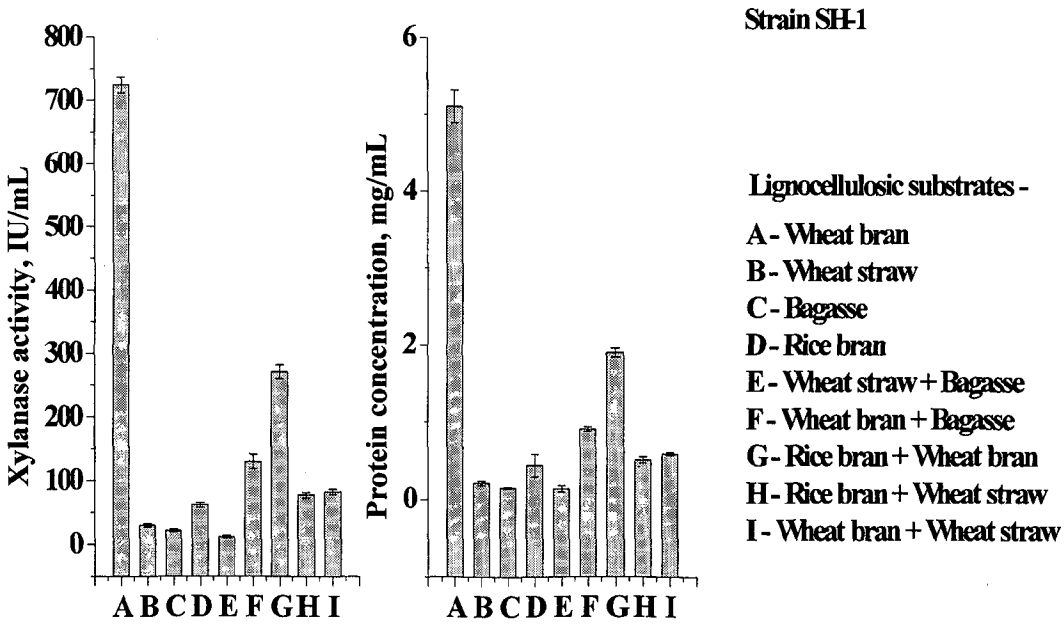


Figure 2.9 Effect of lignocelluloses as substrates on xylanase production and supernatant protein concentration for *C. disseminatus* SH-1

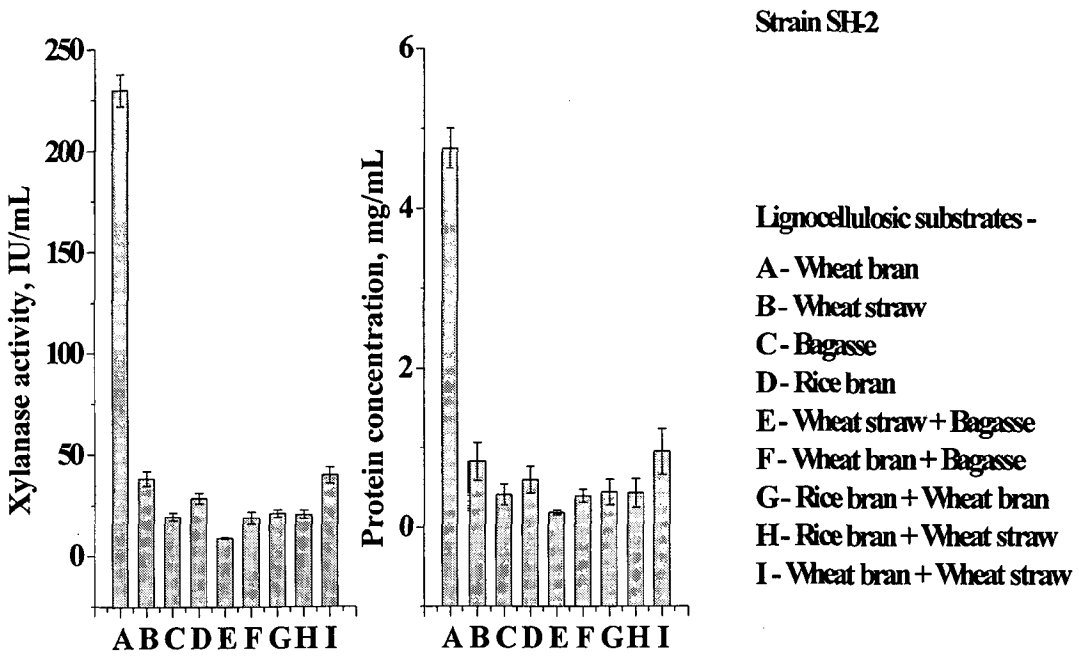


Figure 2.10 Effect of lignocelluloses as substrates on xylanase production and supernatant protein concentration for *C. disseminatus* SH-2

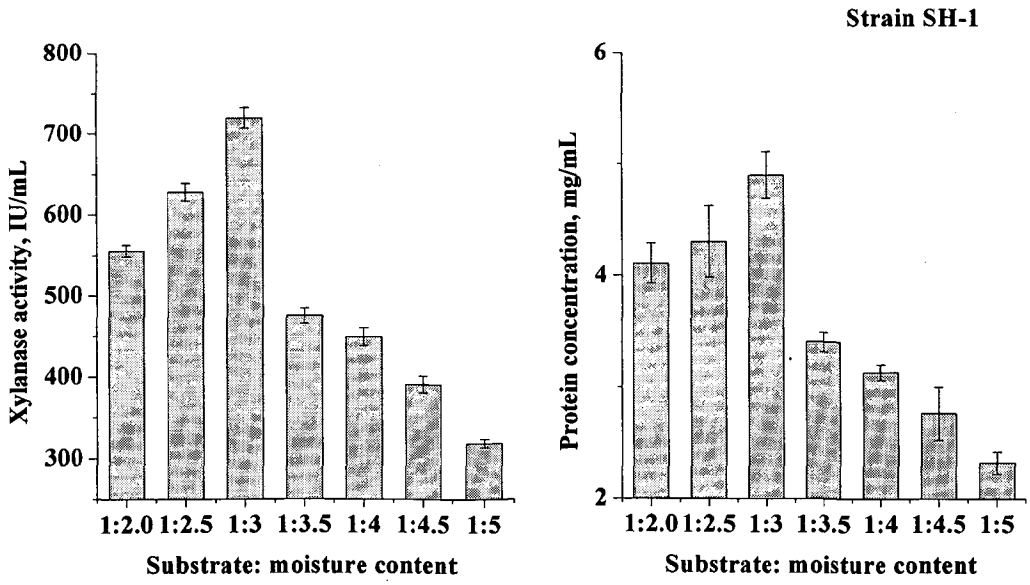


Figure 2.11 Effect of moisture level on xylanase production and supernatant protein concentration for *C. disseminatus* SH-1

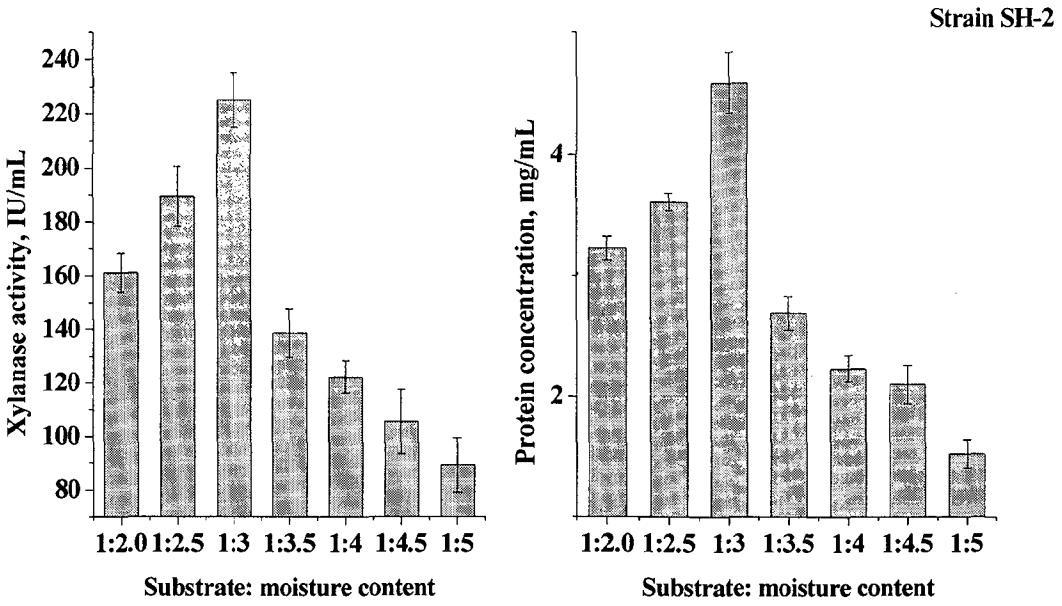


Figure 2.12 Effect of moisture level on xylanase production and supernatant protein concentration for *C. disseminatus* SH-2

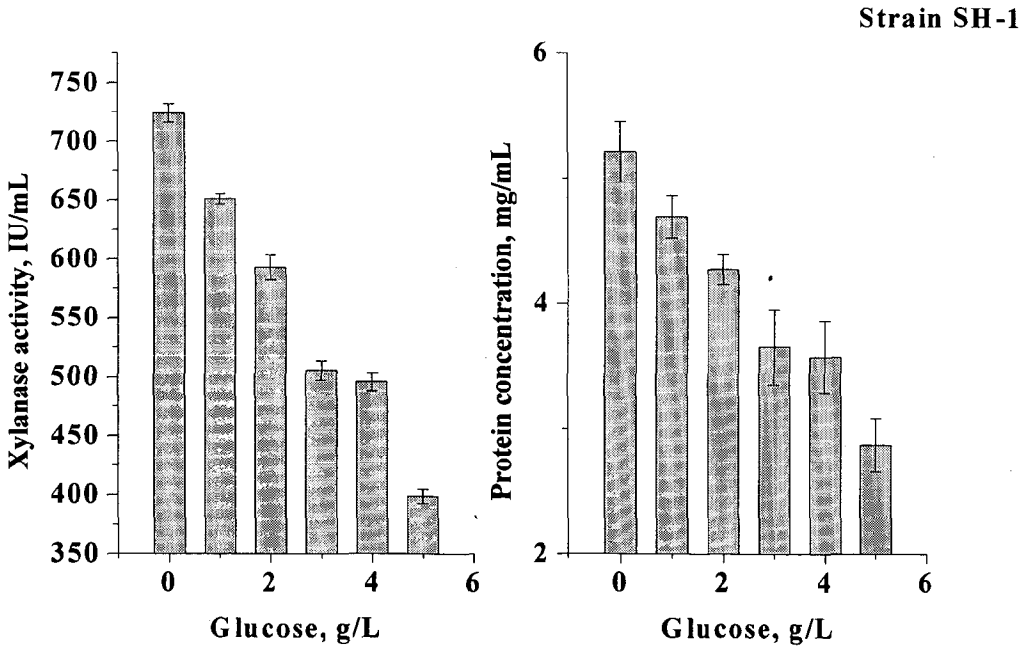


Figure 2.13 Effect of glucose on xylanase production and supernatant protein concentration for *C. disseminatus* SH-1

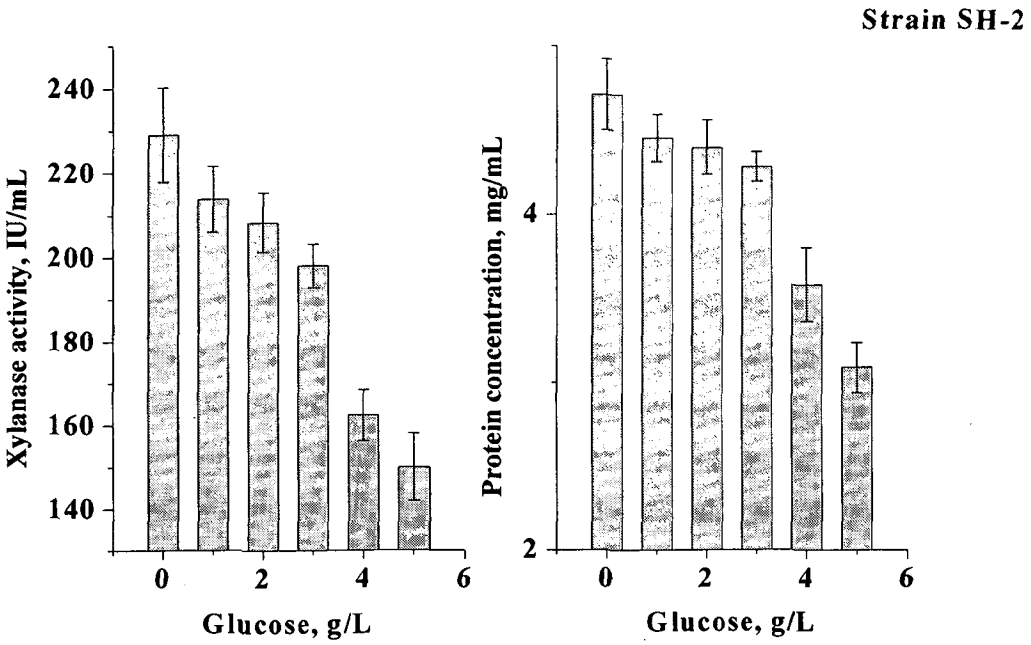


Figure 2.14 Effect of glucose on xylanase production and supernatant protein concentration for *C. disseminatus* SH-2

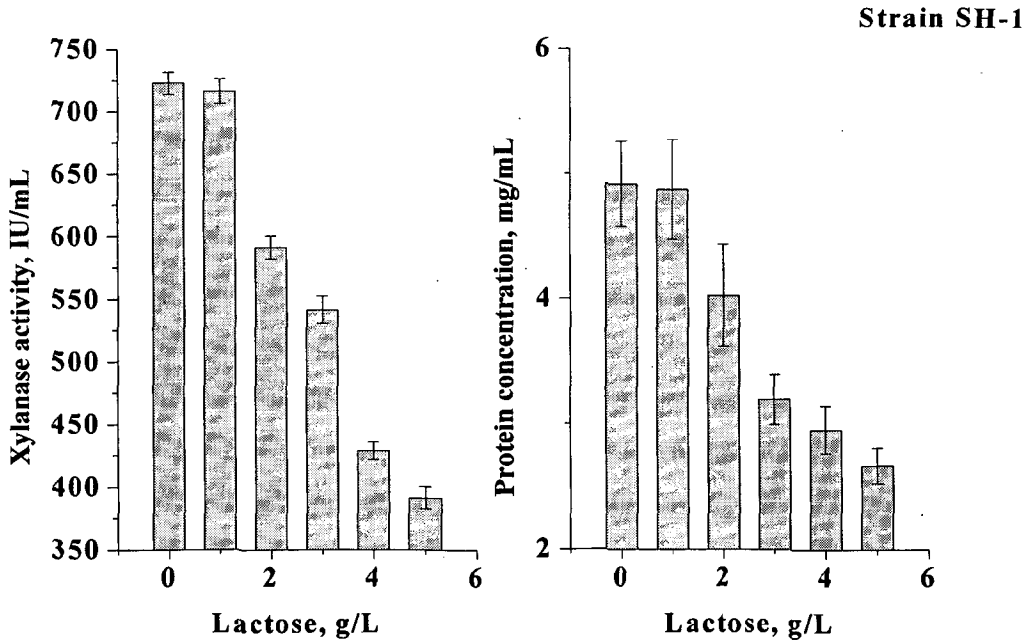


Figure 2.15 Effect of lactose on xylanase production and supernatant protein concentration for *C. disseminatus* SH-1

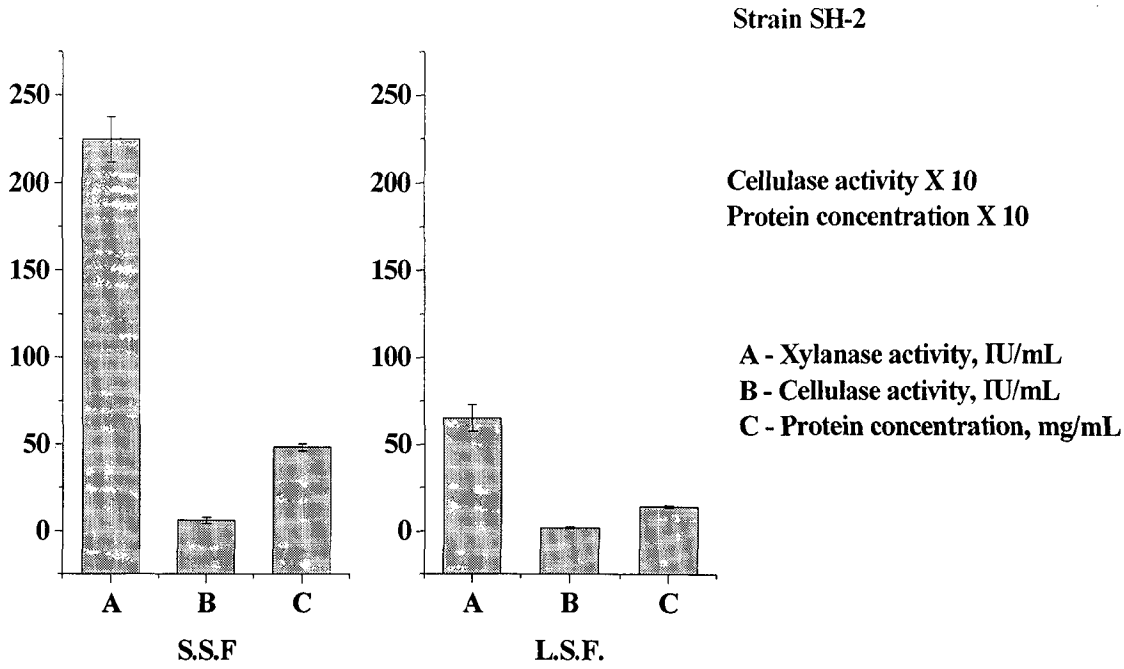


Figure 2.20 Effect of fermentation conditions on xylanase and cellulase production, and supernatant protein concentration for *C. disseminatus* SH-2

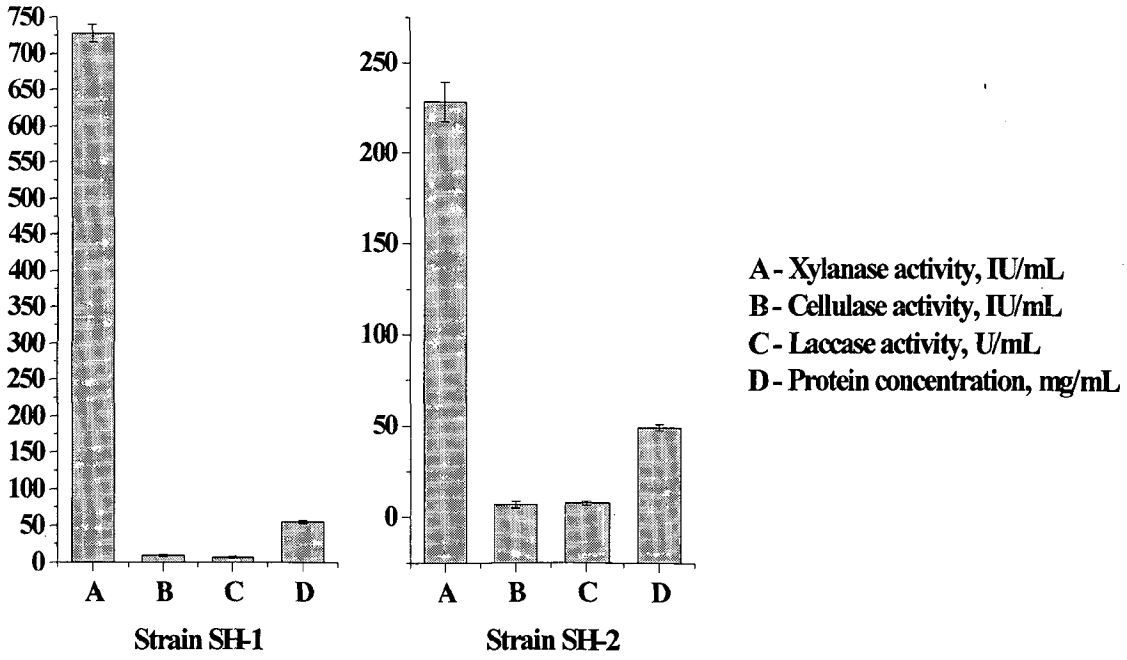


Figure 2.21 Mass production of crude xylanases from the test strains, *C. disseminatus* SH-1 and SH-2 under optimized conditions of S.S.F., for biobleaching

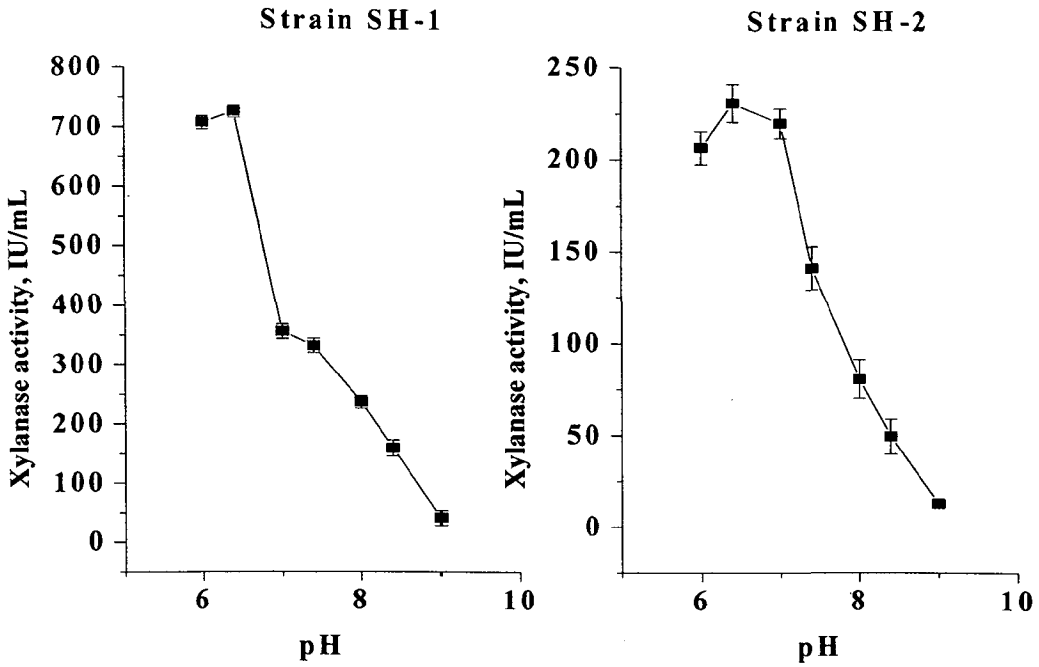


Figure 2.22 Effect of pH on the activity of crude xylanases obtained from the test strains, *C. disseminatus* SH-1 and SH-2

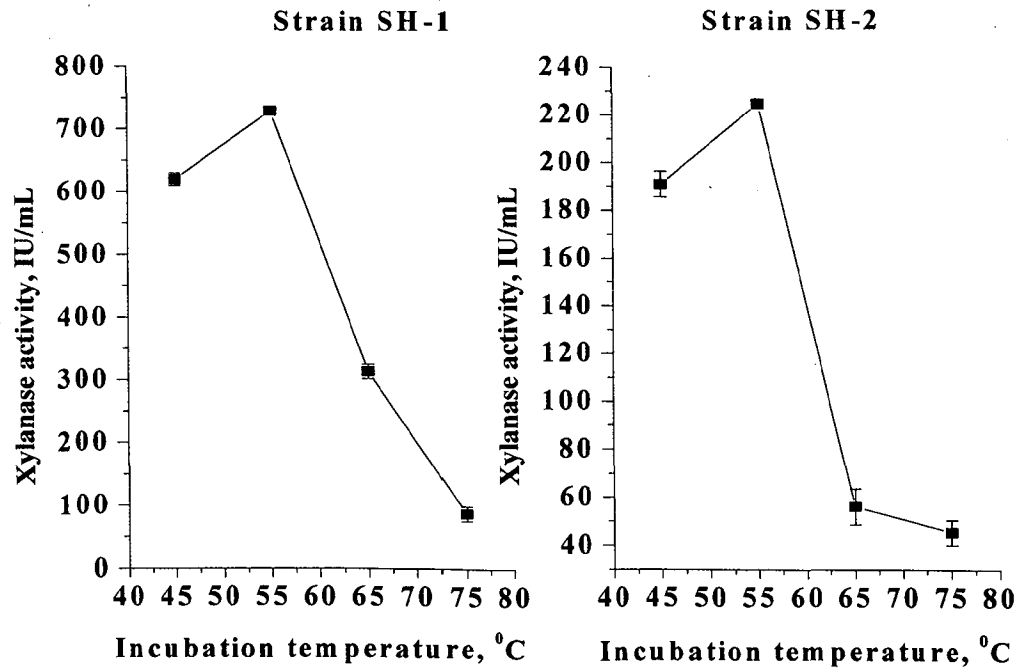
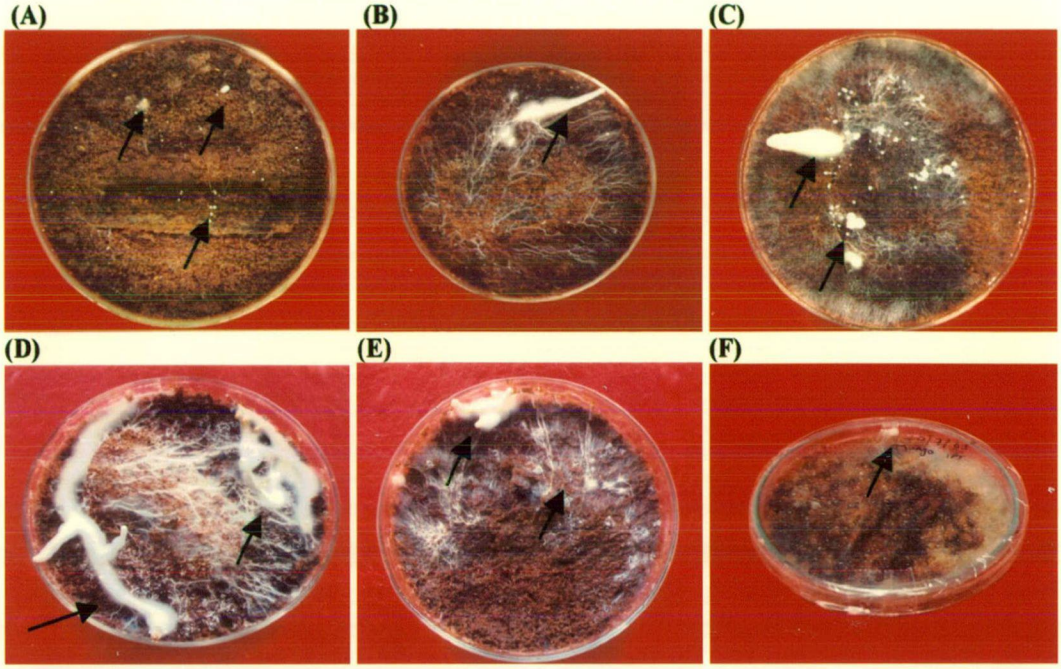
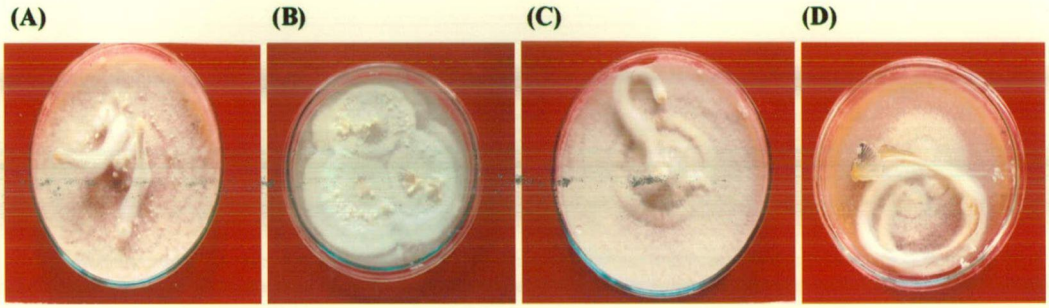


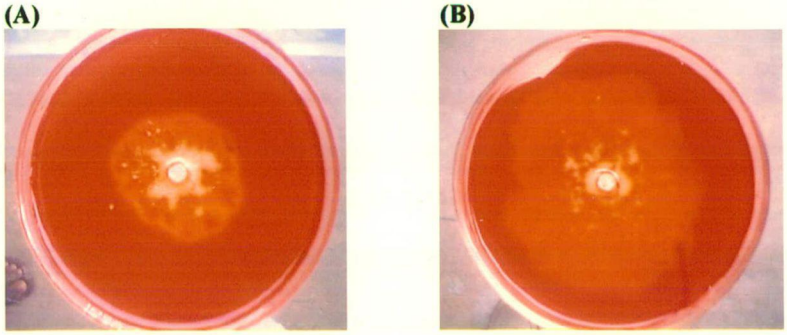
Figure 2.23 Effect of temperature on the activity of crude xylanases obtained from the test strains, *C. disseminatus* SH-1 and SH-2



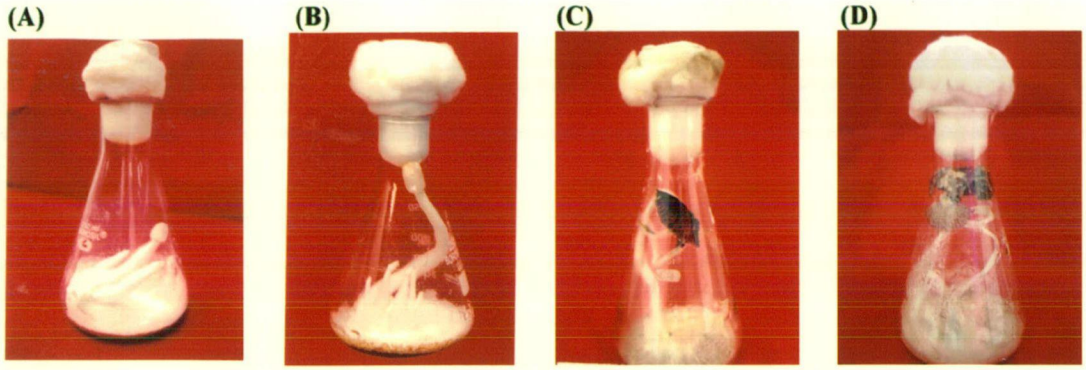
Photographs 2.1 For isolation of fungal xylanase producers by enrichment culture technique showing (A) to (F) fruit bodies at various stages of development and thread like mycelial network on the substrate



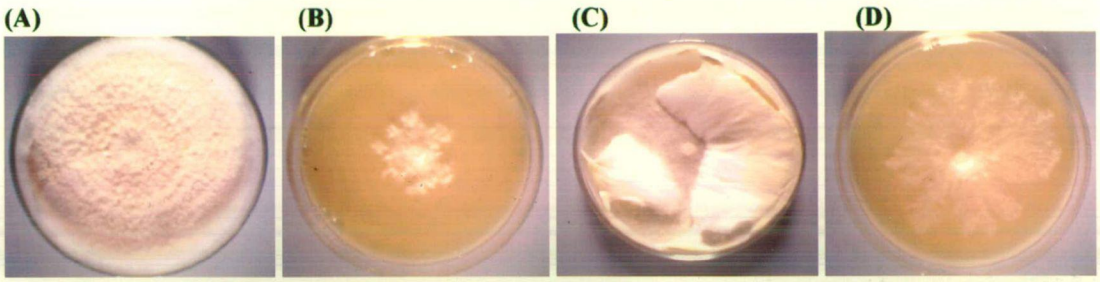
Photographs 2.2 Purification of fungal isolates on wheat bran agar medium showing (A) 7-day-old culture with fruit body primordia (B) 5-day-old culture in a 3-point inoculated plate with numerous fruit body primordia (C) 7-day-old culture with a fruit body (D) sporulation stage in a 9-day-old culture



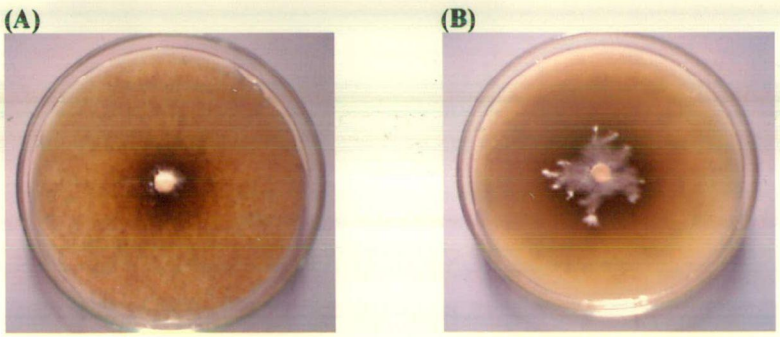
Photographs 2.3 Showing comparative growth on xylan agar using Congo red staining method for (A) strain SH-1 (6-day-old) (B) strain SH-2 (6-day-old)



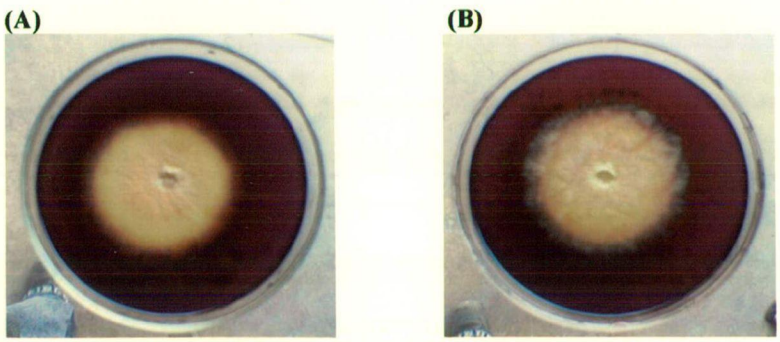
Photographs 2.4 Growth of isolates under solid state fermentation conditions showing (A) fruit body formation three fruit bodies (6-day-old) (B) a prominent fruit body touching the cotton plug (7-day-old) (C)&(D) sporulation stage (8-day-old)).



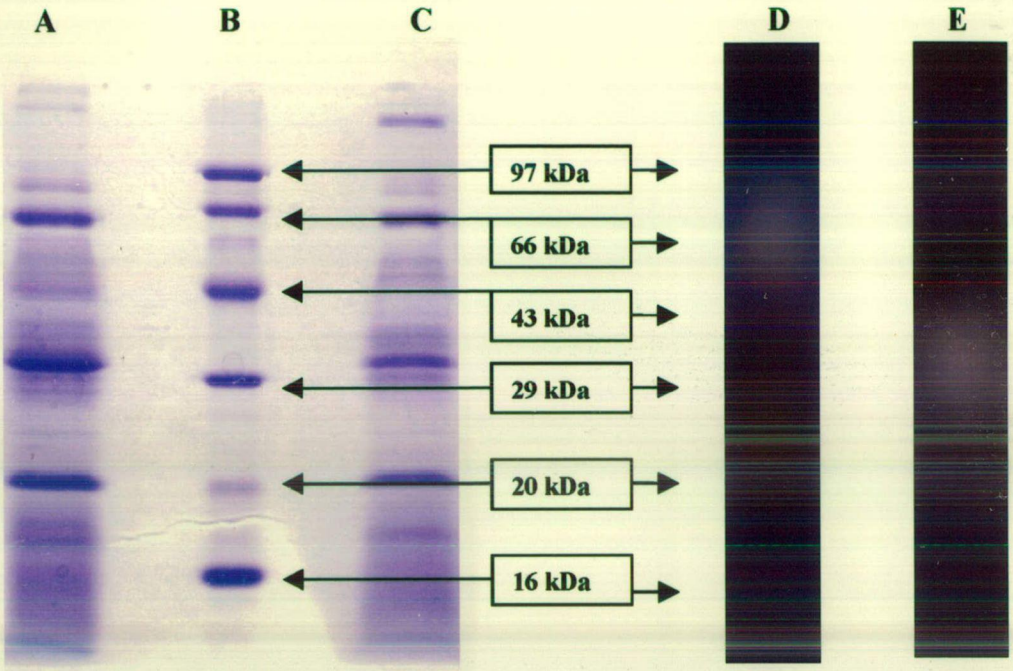
Photographs 2.5 (A) Growth of strain SH-1 (6-day-old) on wheat bran (B) growth of strain SH-1 (6-day-old) on birch wood xylan (C) growth of strain SH-2 (6-day-old) on wheat bran (D) growth of strain SH-2 (6-day-old) on birch wood xylan



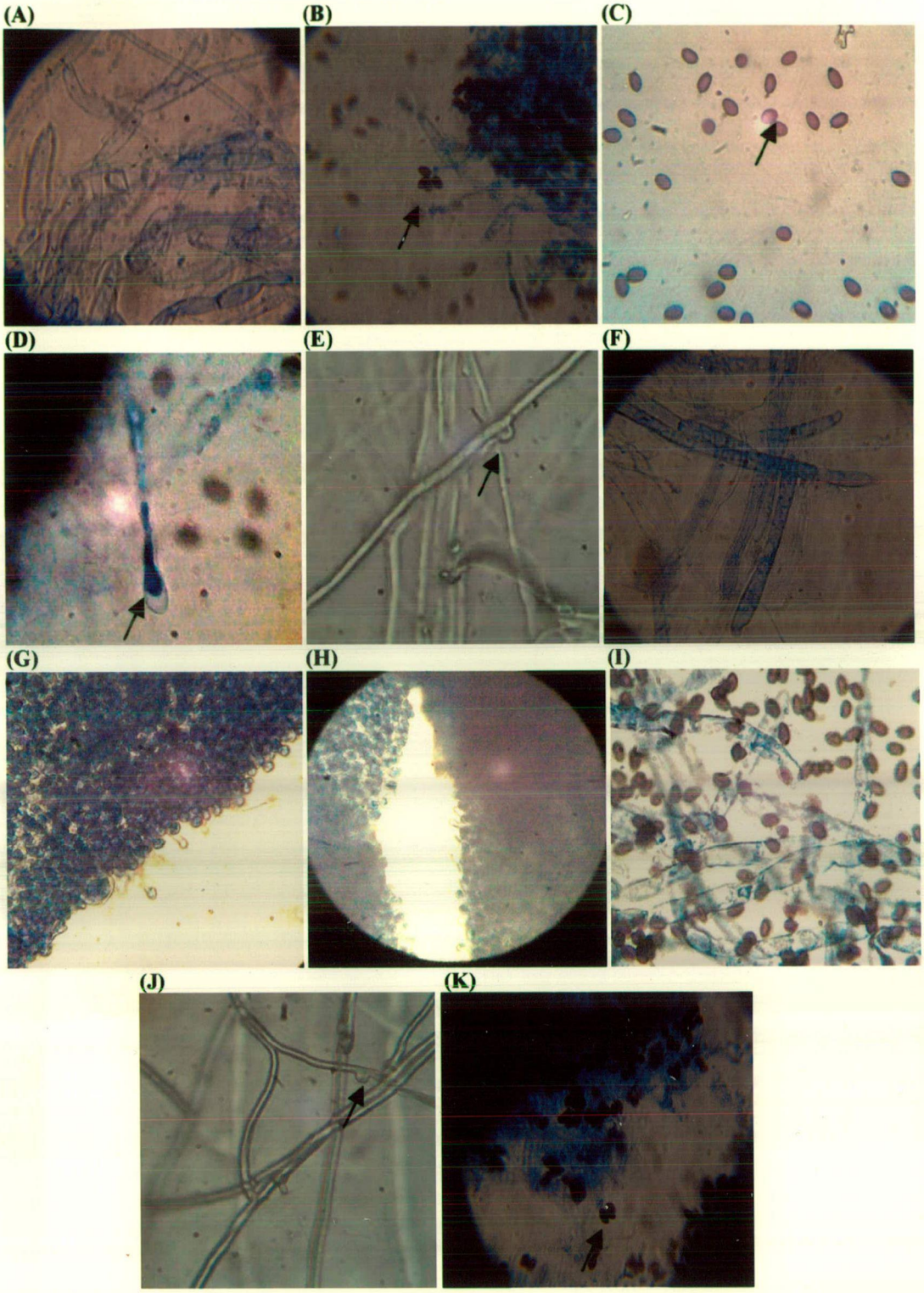
Photographs 2.6 Laccase plate assay for (A) strain SH-1 (10-day-old) (B) strain SH-2 (10-day-old), using guaiacol as substrate



Photographs 2.7 Amylase plate assay for (A) strain SH-1 (4-day-old) (B) strain SH-2 (4-day-old), using starch as substrate



Photographs 2.8 **12.5% SDS-PAGE and zymogram analysis of crude xylanases obtained from test strains, *C. disseminatus SH-1* and *C. disseminatus SH-2*. A = crude enzyme from strain SH-1; B = medium range protein marker (kDa); C = crude enzyme from strain SH-2; D = zymogram for strain SH-1; zymogram for strain SH-2**



Microphotographs 2.1 **Showing (A) pileocystidia in strain SH-1 (40x) (B) group of four basidiospores attached to basidium (40x) (C) basidiospores in strain SH-1 (40x) (D) a chlamydospore in strain SH-1(40x) (E) bipolar mating system (clamp connection) in strain SH-1 (40x) (F) pileocystidia in strain SH-2 (40x) (G)&(H) hymenial surfaces in strain SH-2 (40x) (I) basidiospores intermingled with mycelial network in strain SH-2 (40x) (J) bipolar mating system in strain SH-2 (40x) (K) basidiospores attached to basidium.**

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CHAPTER 3

MORPHOLOGICAL, ANATOMICAL AND CHEMICAL CHARACTERIZATION OF WHEAT STRAW

3.1 INTRODUCTION

Lignocellulosics in the form of wood and agricultural residues account for more than 60% of the total biomass produced [20]. Wood (softwoods and hardwoods) is the principle source of cellulosic fibre for papermaking. Apart from it, non-woods, mainly bagasse, cereal straws and bamboo are being used. Approximately $1/3^{rd}$ of all paper products are recycled into secondary fibre [54].

The basic structural and functional unit of a plant is a cell, which typically consists of protoplasm with a surrounding cell wall. In early stages of growth, the cell cavities contain protoplasm but soon after the cell wall is fully formed, the protoplasm disappears and the resultant structure is known as wood cell or 'fibre'. Wood cells are produced in the vascular cambium from two types of meristematic cells, fusiform and the ray initials. The cells derived from the fusiform initials are upright in the stem and occupy a major part of xylem. They play an important role in the chemical, physical and mechanical properties of the wood. The xylem tissues conduct water from roots to shoots, provide mechanical support to the plant and play physiological role such as, the storage of carbohydrates. The secondary xylem is formed by cell division in the vascular cambium after onset leaving a hollow tubular or quill shaped structure known as fibre, the xylem of which may be an important source for paper making fibres [42]. Wood is a natural complex material. Regardless of the source, lignocellulosic materials contain cellulose, hemicellulose and lignin, as major components.

At first step of differentiation of the wood cell, the living protoplasm produces a primary wall (P). The primary cell wall expands as the cell grows [8]. This layer has a high amount of lignin, hemicelluloses and pectin. The cellulose microfibrils in this layer show low crystallinity and do not exhibit any definite orientation [38]. The substance between the primary walls of adjacent cells is called the intercellular layer (I) or the middle lamella. The combined 'I' layer with the two adjacent P walls is called as compound middle lamella (CM). After the cell is fully grown, secondary cell wall (S), a thick layer, is formed inside the primary cell wall [8]. According to the generally accepted model, the secondary wall has at least three layers, a thin outer S_1 layer, inner S_3 layer and a relatively thick middle layer called S_2 [19]. The model of a typical cell wall structure [54] is given in Fig. 3.1.

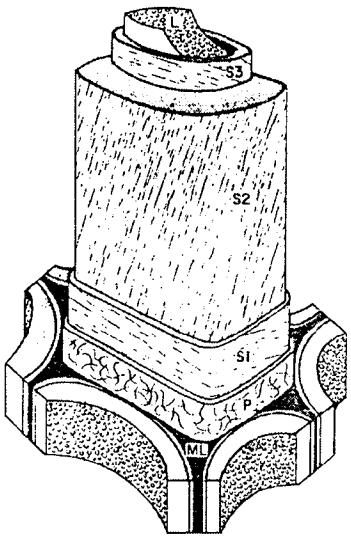


Figure 3.1 Idealized model of organization of a typical cell wall of a plant fibre. The cell wall consists of: P-primary wall; S_1, S_2, S_3-layers of the secondary wall; L-lumen; ML-middle lamella.

In the secondary wall layers, the amount of lignin is reduced and the cellulose microfibrils are deposited into sub-layers of varying orientation [4].

The three layers of the secondary wall are organized in a plywood type of construction [25, 61]. The orientation of cellulose microfibrils in wood cells is characterized by the microfibril angle (MFA), which is the angle between the microfibrils and the longitudinal cell axis (Figure 3.2). Based on early light microscopy studies [47, 4], the secondary cell wall is known to contain layers with varying MFA. The S_1 or S_3 layers, with a large microfibrillar angle, varying from 40 to 120 degrees [14], is designated as a flat helix, and the S_2, with a small angle, as a steep helix [25, 61]. The microfibrils in S_2 layer are, on average, nearly parallel to the cell axis [14]. The S_1 is composed of several lamellae with alternating S and Z helices of microfibril orientation [25, 61] and this structure in the S_1 layer is termed "crossed fibrillar texture" [61]. The S_1 and S_3 layers are so thin compared to the S_2 that some authors have discussed the possibility of all variation of MFA to be due to the S_2 layer [5]. A schematic drawing of a section of a fibre [51] is given in Figure 3.2

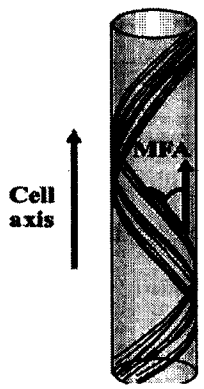


Figure 3.2 A schematic drawing of a section of a plant fibre and several microfibrils

The percentage of cellulose is higher in S_2 layer as compared to S_1 and S_3 layers. The S_3 layer is rich in hemicelluloses, while lignin is the dominant compound in

middle lamella [18]. There is a thin intermediate layer S_{12} between S_1 and S_2 layers and S_{23} layer between S_2 and S_3 layers [30].

The vertical structure of softwoods (gymnosperms) is composed of long tapering cells called tracheids. Resin canals are also present in some species. The horizontal system is composed of ray tracheids, present in certain softwood species and ray parenchyma cells present, in all softwood species. Softwoods have long fibres (3-7mm) with thin wall that collapse readily to double walled ribbon structure and provide more surface contact area for bonding during pressing of sheet. The vertical structure of hardwoods consists of long, narrow cells called libriform cells, shorter and wider vessels and parenchymatous cells. The average fibre length in hardwoods is 1-2 mm [54]. Non-woods contain a wide variety of fibre and cell types of varied dimensions. Monocots such as, cereal straws and sugarcane bagasse are much more similar to hardwoods. They contain a large proportion of parenchymatous cells, vessels (protoxylem and metaxylem) and epidermal cells in a wide range of dimensions.

Dicots such as, flax straw and kenaf consist of two distinct fibre types; the softwood-like bast fibres and the hardwood-like core fibres [9]. The bast fibres contain low lignin content compared to inner core fibres which is reflected in lower demand of pulping chemicals, energy consumption and bleaching chemicals to make it suitable for various grades of papers [24].

While 96% of softwood cells may be considered as fibres, only about 35-39% of cells in straw are fibres [59]. The average fibre length varies greatly with botanical parts. Table 3.1 shows the physical content and fibre ratio of different botanical parts of wheat straw [59].

The dimensions and arrangement of unit cells in a fibre determine the structure and also influence the properties of the fibres. The dimensions of individual cells in natural fibres are dependent on the species, growth stages and location of the fibres in the plant and also on the fibre extraction conditions [23, 37]. Fibre length is a fundamental property of a pulp that directly or indirectly affects many properties exhibited by a pulp sample like, under certain conditions it strongly affects tearing resistance [53]. There have been indications [11, 12] that, if the coarseness of the fibres (weight per unit length of the fibres) remains constant, the tensile strength of a sheet made from relatively unbeaten pulp (other factors being the same) will vary as $L^{1/2}$, the burst will vary as L, the fold as L^5 and the tear as $L^{3/2}$, where L is the weighted average length of the fibre. These relationships will be true, independent of the uniformity of lengths of the fibres, whether they are a mixture of various lengths or all of one length, but the numerical values of the exponents will decrease, as the pulp is beaten. It is well known that fibre morphology with high length, high length to width ratio (>33) and /or low runkel ratio (cell wall thickness to lumen diameter ratio, <1) are essential in papermaking [63]. The importance of plant materials' fibre dimensions and their derived values (slenderness ratio, flexibility coefficient and Runkel ratio) on pulp and paper mechanical properties, is well documented [13, 27, 34]. The derived values have successfully been used to assess the suitability of various non-wood fibre raw materials for pulp and paper manufacture [41, 50].

The proximate chemical analysis provides important information regarding the suitability of raw material for pulp and papermaking. The important tests include water solubility, 1% NaOH solubility, alcohol-benzene solubility, lignin, holocellulose, α-cellulose, pentosans, extractives, ash and silica content, etc.

The cold-water treatment removes a part of extraneous components, such as inorganic compounds, tannins, gums, sugars, and colouring matter present in wood. The hot-water treatment removes, in addition, starches.

The 1% NaOH solubility of wood indicates the degree of fungal decay or degradation by heat, light, oxidation, etc. [36, 45]. As the wood decays or degrades, the percentage of the alkali soluble material increases. It is often desirable to ascertain to what extent low cellulose value is due to attack by fungi, because in general, the greater is such an attack, lower is the pulp yield.

The alcohol-benzene solubility of wood is a measure of extractives (waxes, fats, resins, phytosterols, etc.). The extractives may inhibit pulping and create a lot of deposit problems on process equipments in the pulp mill and in the paper machine. They cause pitch problems and the homogeneities in the paper are affected by resin particles.

The ash content gives an estimation of mineral salts and other inorganic matters. Silica impairs the burning and sedimentation operations in the recovery process. Large quantities of silica in wood show damaging effect during processing of wood and affects paper quality adversely. If excessive amounts are present and carried out into the finished paper of some 'fine' or specialty grades, they may cause undesirable abrasion of metal repeatedly applied to the paper, such as dies, punches or knives [56].

The principle constituent of a plant fibre is the cellulose, comprising of 40-60% wood in the form of cellulose microfibrils. Cellulose consists of covalently bound cellobiose units, which are formed by two molecules of D-glucose, linked together by a (1, 4) β-glycosidic bond [7]. Most papermaking fibres have a weighted-average degree of polymerization (DP) in the 600-1500 range [55]. The variations in the cellulose

content in wood are directly reflected by variations in pulp yield, which in turn, strongly affects the pulp properties. The higher the cellulose content, the higher will be the pulp yield and higher will be the load bearing capacity of the individual fibres of the pulp. The mechanical strength of the pulp, especially the tensile strength is directly proportional to cellulose content [31].

Hemicelluloses are the amorphous compounds and are polymers of hexoses like, glucose, mannose and galactose, and pentoses like xylose and arabinose [28]. Hemicelluloses are the matrix substances present between cellulose microfibrils. Depending on the plant species, these sugars, along with uronic acids, form various polymeric structures; some are associated with the cellulosic portion of the plant while others are more closely associated with lignin. During chemical pulping, the hemicelluloses are dissolved, deacetylated, and hydrolyzed. Hemicelluloses interact strongly with water and greatly contribute to the swelling of fibres. They act as lubricating agents and also promote fibre-fibre bonding in paper. The carboxyl groups on xylan glucuronic acid groups are the principal source of negative fibre charge [2].

Pentosan content in softwoods is about 7 to 10%, and in hardwoods about 19 to 25%. Pentosan content in pulp indicates the amount of pentose sugars in hemicellulose in general during pulping and bleaching processes, and since, hemicellulose contributes to the strength of paper pulps, so high pentosan content is desirable.

The term 'holocellulose' is used to describe the total carbohydrate content of fibres that includes cellulose and hemicelluloses. The holocellulose content of wheat straw is approximately equal to that of hardwoods and higher than that of softwoods, because of high hemicellulose content (mainly pentosans) [59].

Lignin acts as glue between individual cells and between fibrils forming the cell wall [35]. Its principle role is to constitute the middle lamella. Wood contains 20-30%

lignin, removal of which is the main objective of chemical pulping and bleaching operations. The lignin content of the fibres influences the structure, properties, morphology, and flexibility of the fibres [57]. The time, temperature and chemical dose required for chemical pulping is directly proportional to the lignin content of the raw material.

The lignin content of softwood is generally higher than that of hardwoods, whereas the hemicellulose content of hardwoods is higher than that of softwoods. The lignin content of straws is lower than that normally found in both softwoods and hardwoods [48]. With a few exceptions, straw species are more uniform in composition than wood species. Generally, straws have lower cellulose content than wood, but, in spite of this, the holocellulose fraction is approximately equal to that of wood [59].

The present study aims at morphological, anatomical and chemical characterization of wheat straw.

3.2 MATERIALS AND METHODS

3.2.1 Materials

Wheat straw was procured from the vicinity of Saharanpur, India, in the month of May (harvesting time). Reagents used were of laboratory grade from Qualigens Fine Chemicals, Mumbai.

3.2.2 Methods

3.2.2.1 Pretreatment of the raw material

The procured straw was washed with water to remove extraneous dirt, sand, nodes and other foreign materials. The washed material was air dried and then stored in polyethylene bags under dry conditions for experimental work.

3.2.2.2 Morphological and anatomical characterization of wheat straw

In order to get representative results of anatomical and morphological studies, three randomly selected samples of wheat straw from storage bag were taken. For fibre length determination, small slivers were obtained and macerated with 10 mL of 67% HNO_3 and boiled in a water bath (100±2 °C) for 10 min [41]. The slivers were then washed, placed in small flasks with 50 mL of distilled water and the fibre bundles were separated into individual fibres using a small mixer with a plastic end to avoid fibre breaking. The macerated fibre suspension was finally placed on a slide (standard, 7.5 cm X 2.5 cm) by means of a dropper [22]. For fibre diameter, lumen diameter and cell wall thickness determination, cross-sections were cut on Lietz base sladge microtome 1300. These cross-sections were stained with 1:1 aniline sulphate-glycerin mixture to enhance cell wall visibility (cell walls retain a characteristic yellowish colour).

All fibre samples were viewed under a calibrated microscope; a total of 75 randomly chosen fibres were measured. Three derived values were also calculated using fibre dimensions: slenderness ratio as fibre length/fibre diameter, flexibility coefficient as (fibre lumen diameter/fibre diameter) × 100 and Runkel ratio as (2 × fibre cell wall thickness)/ lumen diameter [50]. Microscopic slides were also prepared in order to enumerate various cell types present and their frequency of occurrence. The dimensions of different cell types were also determined microscopically. The results were compared with rice straw and bagasse, as per Tappi standard test method, T 259 sp-98) [3]. The results for morphological characteristics were compared with *Populus deltoides* [1] and bamboo [16].

3.2.2.3 Chemical characterization of wheat straw

100 g of wheat straw was milled into a powder in a laboratory Wiley mill (Weverk, A- 47054, Sweden) and a fraction passing through −48 mesh size but retained on +80 mesh size was used for chemical analysis. The chemical characterization of wheat straw was carried out as per Tappi Standard Test methods [3], except test for silica that was carried out as per SCAN Test methods [52]. The tests that were carried out included: cold and hot water solubility (T 207 om-99), 1% NaOH solubility (T 212 om-98), alcohol-benzene solubility (1:2 v/v) (T 204 cm-97), lignin (T 222 om-02), pentosan (T 223 cm-01), holocellulose (T 249 cm-00), α-cellulose (T 203 om-88), ash (T 211 om-93) and silica (SCAN-C9:62). Three determinations were made for each test and the results are mean ± standard deviation (SD) of the values. The results were compared with *Populus deltoides* [1], bagasse [16] and rice straw [21].

3.2.2.4 Statistical analysis

Three experimental values were taken in each case and the results are the mean ± standard deviation (SD) of the values.

3.3 RESULTS AND DISCUSSION

3.3.1 Morphological and anatomical characterization of wheat straw

Photomicrograph 3.1 shows T.S. of wheat straw internode showing its anatomical features at a magnification of 10X. The epidermis (A) forms the outermost part of wheat straw stem. The epidermal cells vary in size and form, are sparsely pitted, have more or less serrated margins and appear in either groups or singly. The epidermis of wheat straw contain large amount of silica. This layer is completely undesirable for pulp and papermaking as the epidermal cells dissolve very slowly and incompletely

during chemical pulping processes. The ground tissue is composed of parenchyma cells (D). The parenchymas are barrel shaped cells. They gradually change their chemical composition and dimensions during growth that vary from plant to plant. The epidermal and parenchymal cells form a major part of fine fraction in the pulp. These cells pose the problem of fluff at dryer or printing machine due to larger surface area of the non-fibrous cells [17]. The majority of non-fibrous cells is short in length and cause poor drainage in wheat straw pulp [26]. The parenchymas are easily deformed and also reduce drainage [10]. This complexity of the ground tissue is a great problem to the paper makers. Therefore, processing and efficient screening systems must be developed for each raw material exclusively, taking into consideration its specific morphology. Even though, wheat straw fines reduce freeness and increase the water retention, they improve the tensile strength of unbeaten pulp as the thin-walled parenchyma collapse, thereby aiding in bonding and contributing to the tensile strength [62]. The epidermis is followed by 2 to 3 layers of rectangular collenchyma cells that have unevenly thickened cell wall and function as a supporting tissue. The primary wall of collenchymatous cells is composed of cellulose and pectin compounds. At maturity, the collenchymatous cells become lignified and thickened, to form sclerenchymatous cells (B), which give rigidity to the stem. Sclerenchymatous cells are thick walled and lignified but sometimes in the phloem region a bundle of sclerenchymatous cells are found, then these cells are called bast fibres or phloem fibres. The conductive tissues or vascular bundles (C) near periphery are fused with each other by a strong sheath of sclerenchyma cells or bast fibres. The larger bundles in the inner circle are embedded into a large ground tissue of parenchymatous cells. These two circles of vascular bundles have a considerable amount of extra vascular fibres which have good paper-

making value. Under microscope, most of the useful fibres were located at the outer part of the stem (near the skin) with a little area of the fibres being present in the vascular bundle. The fibres originating from the outer part of internode are thick walled and those coming from the inner part are called thin walled. However the bulk of the fibres (80%) are from the outer part of stem.

The dimensions of various non-fibrous cells of wheat straw pulp were compared with rice straw [3] and bagasse [3] in Table 3.2. The parenchyma cells were 445 μm long and 124 μm wide, vessels 96 μm long and 57 μm wide and epidermal cells 390 μm long and 38 μm wide. The dimensions of parenchymatous cells of wheat straw lied between rice straw and bagasse. Therefore, the slot size of screen should be selected according to the morphology of wheat straw. This might help to solve the problem of fluff during offset lithographic printing for paper made from wheat straw pulp. Photomicrographs 3.2 A-D show wheat straw fibres, along with parenchymal and epidermal cells. The protoxylem has strong lignified cell wall which is resistant to pulping. Similarly, thick walled parenchymatous cells are also resistant to chemical treatment and difficult to screen out due to its larger cell dimensions. The protoxylem vessels have an extreme length and a very undesirable shape which might form coarse tangles in the pulp and the phloem fibres form the characteristic shape of the vascular bundle.

Table 3.3 shows the morphological characteristics of wheat straw fibres and their comparison with those of *P. deltoides* [1] and bamboo [16]. The total fibres in wheat straw were about 39.20% compared to 50% in *P. deltoides*. Therefore, wheat straw produced lesser pulp yield compared to *P. deltoides*. Parenchyma and epidermal cells accounted for about 32.10 and 23.56% of the total cells, respectively. The

parenchyma and epidermal cells have large surface area [33] and might act as fillers. They might adversely affect the mechanical strength properties of paper. The vessels accounted for about 5.14% of the total cells compared to 32.40% in *P. deltoides*. Vessels have blunt ends, perforated end walls and have pits of various shapes. The fibres of wheat straw were slender with sharply pointed ends and with an average fibre length of 1.18 mm compared to 0.984 mm for *P. deltoides* and 1.70 mm for bamboo. The average fibre width of wheat straw fibre was 13.60 μm compared to 25.60 μm for *P. deltoides* and 23.60 μm for bamboo. The lumen of wheat straw fibre was narrow having a diameter of 5.68 μm compared to 17.60 μm for *P. deltoides* and 9.50 μm for bamboo. The cell wall thickness of wheat straw fibres was 3.96 μm which almost resembled to that of *P. deltoides* whereas, it was lower than that for bamboo fibres. The slenderness ratio of wheat straw fibres was 41.76 against a slenderness ratio of 72.03 for bamboo and 38.43 for *P. deltoides*. The slenderness ratio (L/D), which is also termed as felting power, is inversely proportional to the fibre diameter. Fibres having high slenderness ratio have a low degree of collapsing and conformability and such types of paper gives more tear, porosity, bulk, and opacity [46]. A poor slenderness ratio means a reduced tearing resistance that is partly because the short and thick fibres do not produce good surface contact and fibre to fibre bonding [41].

When used for applications such as paper, the slenderness ratio of individual cells in a fibre affects the flexibility and resistance to rupture of the fibres [32]. The flexibility coefficient of wheat straw fibres was 41.76 which almost resembled to that of bamboo fibres but was much less than that of *P. deltoides* fibres. Such types of fibres do not collapse readily to form double walled ribbons and tend to retain their tubular structure on pressing and thus, provide lesser surface contact area for bonding. The

sheet made from such types of fibres give more tear, porosity, bulk, and opacity [49]. The Runkel ratio of wheat straw was almost equal to that of bamboo. Runkel ratio is directly affected by cell wall thickness but not by lumen diameter and is related to fibre density [6, 44]. The breaking length, bursting strength, and double fold are determined by fibre density. Though the runkel ratio of wheat straw almost resembled to that of bamboo yet due to less fibre diameter, the flexibility and the degree of collapseness of fibre, both of which control the degree of conformability within the paper sheet and, as such the size and number of inter-fibre bonds, was improved in the case of wheat straw. The mechanical properties along with other properties of paper related to wet plasticity might be increased by fibrillation and high hemicellulose content (28.95%) (Table 3.4). The flexibility coefficient and Runkel ratio of wheat straw was also in close proximity with bamboo. Based on morphological characteristics the wheat straw was quite comparable to bamboo. Large amount of hemicellulose might result in a decrease in tensile and bursting strength not because of the bonding effect but possibly because the individual fibre strength might be reduced as a result of the decrease in the average molecular weight of the polymer system [60].

3.3.2 Chemical characterization of wheat straw

Table 3.4 shows the results for chemical characterization of wheat straw which were compared with rice straw [21], bagasse [16] and *P. deltoides* [1]. The cold and hot water solubles in wheat straw were on higher side in comparison to bagasse, rice straw and *P. deltoides*, which was due to the presence of higher inorganic compounds, tannins, gums, sugars, and colouring matter present in wheat straw. The higher is the water solubility; lower will be the pulp yield. The high NaOH solubility of wheat straw was due to the presence of low molar mass carbohydrates and other alkali soluble

materials. Compared to wheat straw, the degradation of rice straw due to fungal decay, heat and light was more but less than bagasse and *P. deltoides*. The alcohol benzene solubles in wheat and rice straw were almost same but was on a higher side in bagasse and a lower side in *P. deltoides*. It indicated that rice straw and wheat straw contained more of substances like waxes, fats, resins, photosterols and non-volatile hydrocarbons, low-molecular-weight carbohydrates, salts, and other water-soluble substances. Higher content of extractives in pulp have shown an adverse effect on the runnability of process equipments and paper made from such type of fibres show slow water absorbency [29]. Wheat straw has a total carbohydrate fraction (holocellulose) approximately equal to that of hardwood. This is due to the high hemicellulose (mainly pentosans) and low lignin content compared to wood, which is a characteristic feature of agro-residues. This characteristic directly influenced the fibrillation of fibres during refining operations. It has been shown that higher is the hemicellulose content, better is the swelling behavior of the pulp, which leads to an increase in mechanical strength properties like tensile, burst indices and double fold [39]. This also makes this raw material more amenable to the action of enzymes, like xylanases. The α-cellulose content of whole straw was 43.20% which more or less resembled to that of bagasse (42.00%). The α-cellulose of *P. deltoides* was higher than of wheat straw. A pulp with high α-content shows better strength properties. According to the rating system designated by Nieschlag [40], plant materials with 34% and over α-cellulose content are characterized as promising for pulp and paper manufacture from a chemical composition point of view. Holocellulose, as a whole, adds to the overall strength of the paper.

The lignin content in whole straw was 21.12%, which resembled to that of bagasse (20.30%) and *P. deltoides* (21.80%). The amount of lignin is directly related to

consumption of cooking liquor and cooking cycle. Also, higher is the lignin content, more is the stiffness of the fibres [15].

The ash content of wheat straw was 7.5%, which was much higher than that of *P.deltoides* (0.63%) and bagasse (3.80%) but much lower than that of rice straw (13.58%). The silica content in wheat straw was relatively high as compared to wood. Silica causes rather serious difficulties during recovery and poor drainage of straw pulp during papermaking [58]. At the same time, silica can play a role of inhibitor for O_2 delignification and bleaching with H_2O_2, thereby, exterminating need for additional inhibitors to mask transition metals ions during pulping/ bleaching [43].

Table 3.1 Physical content and fibre ratio of different botanical parts of wheat straw

Sl. No	Botanical part	Mass percent	Fibre ratio, %	Other cells (parenchyma, epitel cells, vessels and spirals), %
1.	Internodes	68.8±1.9	35.5±0.6	64.5±0.6
2.	Leaves and sheaths	20.0±1.0	33.8±0.9	66.2±0.9
3.	Glumes	5.6±0.8	30.2±0.7	69.2±0.7
4.	Nodes	4.5±0.9	38.3±3.4	61.7±3.4

± Standard deviation from the mean

Table 3.2 Dimensions of parenchyma cells, epidermal cells and vessels of wheat straw

Sl. No.	Cell type	Wheat straw	Rice straw [3]	Bagasse [3]
1.	Parenchyma cells, Length, μm Width, μm	 445±1.09 124±2.02	 — 10.2 to 20.5	 850 140
2.	Epidermal cells, Length, μm Width, μm	 96±2.09 57±1.01	 350 82	 1350 150
3.	Vessels, Length, μm Width, μm	 390±1.08 38±1.10	 650 40	 — —

± Standard deviation from the mean

Table 3.3 Morphological characterization of wheat straw

Sl. No.	Parameters	Wheat straw	*Populus deltoides* [1]	Bamboo [16]
1.	Length (L), mm	1.18±0.08	0.984	1.70
2.	Width (D), μm	13.60±1.7	25.60	23.60
3.	Lumen (d), μm	5.68±1.09	17.60	9.50
4.	Thickness (w), μm	3.96±0.08	4.10	7.00
5.	Slenderness ratio (L/D)	86.76	38.43	72.03
6.	Flexibility coefficient (d/DX100)	41.76	68.75	40.56
7.	Runkel ratio (2w/d)	1.39	0.465	1.47
8.	Fibre, %	39.20±0.06	50.00	—
9.	Parenchyma, %	32.10±0.05	—	—
10.	Vessels, %	5.14±0.05	32.40	—
11.	Epidermis, %	23.56±0.04	—	—

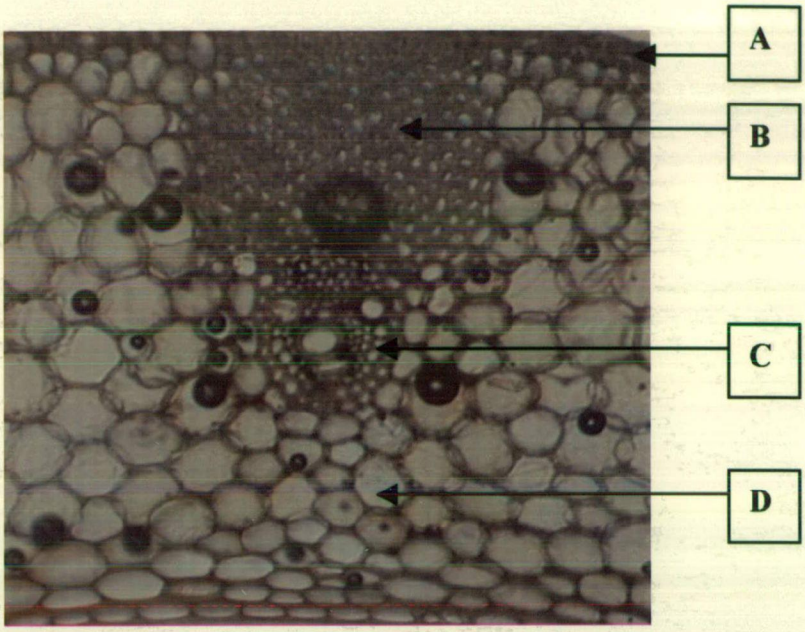
± Standard deviation from the mean

Table 3.4 Chemical characterization of wheat straw

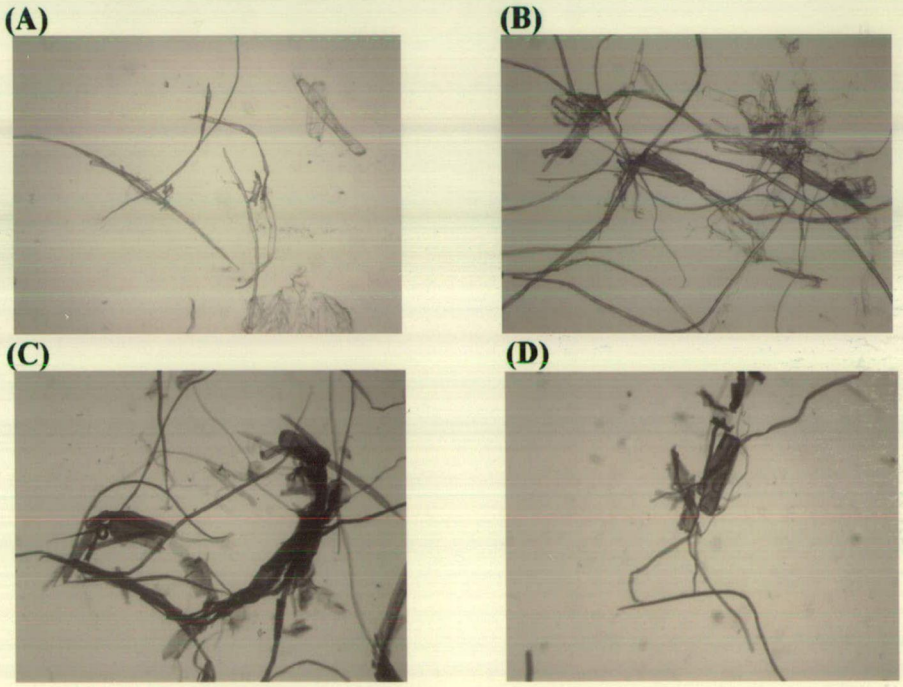
Sl. No.	Particulars	Wheat straw	*Populus deltoides* [1]	Bagasse [16]	Rice straw [21]
1.	Cold water solubility, %	14.46±0.03	3.80	5.91	4.83
2.	Hot water solubility, %	16.59±0.03	4.50	7.85	15.21
3.	1% NaOH solubility, %	39.90±0.07	19.60	33.60	49.82
4.	Alcohol-benzene solubility, %	4.95±0.02	2.80	6.30	4.87
5.	Holocellulose, * %	72.15±0.02	69.40	70.60	71.96
6.	Alpha-cellulose, * %	43.20±0.02	47.40	42.00	—
7.	Pentosans, * %	24.50±0.08	17.30	23.85	22.00
8.	Hemicellulose, * %	28.95	—	28.45	—
9.	Lignin, * %	21.12±0.06	21.80	20.30	25.22
10.	Ash, %	7.50±0.02	0.63	3.80	13.58
11.	Silica, %	4.50±0.08	—	2.10	12.18

± Standard deviation from the mean

* Extractive free basis, without ash correction, expressed on O.D. raw material basis



Microphotograph 3.1 Transverse section (T.S.) of wheat straw internode showing different types of cells (A) Epidermis (B) Sclerenchymas (bast fibres) (C) Vascular bundle (D) Parenchyma (ground tissue) (10x)



Microphotographs 3.2: (A) Wheat straw fibres, parenchyma cells and flakes of thin walled parenchyma (4x) (B) Wheat straw fibres with vessels, thick and thin walled parenchyma cells (10x). (C) Wheat straw fibres with flakes of epidermal cells and vessel bundles (10x) (D) Wheat straw fibres with a prominent parenchyma cell and flakes of epidermal cells (10x)

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CHAPTER 4

PREPARATION OF SODA-AQ PULP OF WHEAT STRAW

4.1 INTRODUCTION

The utility and value of pulp and paper fibre resources to the society begins with an understanding how fibre resources are converted into value added products through advanced pulping and papermaking processes. Generally, pulping refers to various industrial processes used to convert cellulosic raw materials or recycled fibre into pulp, which is used primarily to make paper or paperboard products (and, to a smaller extent, other products derived from cellulose like, synthetic rayon). Cellulosic raw materials such as wood, straws, or bamboo, generally contain cellulosic fibres together with lignin, a natural binding material that holds together cellulose fibres in wood or in the stalks of plants [17]. The primary aim of pulping is to separate cellulosic fibres and to modify a fibre surface suitable for bonding in the papermaking process. Many processes have been developed over the years to convert wood or non-woody cellulosic raw materials into pulp for paper and paperboard making. The choice of the process depends primarily on the nature of the cellulosic raw materials to be pulped and the grade of paper or paper products to be manufactured from it. The two principal alkali processes used for pulping of wood are the soda and the sulphate pulping processes. In both these processes sodium hydroxide is the principal cooking chemical. In the sulphate pulping process Na_2S is involved. Soda pulping process is particularly applicable to low lignin cellulosic fibrous raw materials like agricultural residues and hardwoods where the advantages of sulphidity are of lower magnitude than for softwoods and where the generation of odorous sulphur compounds is also more pronounced with softwoods [16].

Soda pulping is an important pulping process but there are some basic drawbacks associated with it. These include low pulp yield, relatively lower strength properties, longer cooking time and high temperature and caustic charges compared to kraft pulping. Soda pulps have lower tear resistance [31, 11], which is more due to the reduced fibre strength rather than a decrease in the bonding ability of the fibre [25]. At the same time, paper manufactured from soda pulps has high bulk, opacity; absorbency and good printability. Therefore, soda pulps are best suited to paper grades where pulp strength demands are not of prime importance. Soda pulps can be strengthened by blending with longer and stronger fibres.

The reactions that occur between wood/non-wood and alkali during pulping are extremely complicated. In the splitting of lignin molecule by hydrolysis, formation of additional hydroxyl groups occur during delignification, possibly from the hydrolysis of methoxyl group from the furan or pyran ring by the breaking of linkages between lignin and carbohydrates. At optimum temperature, aqueous alkali hydrolyzes away β-O-4 linkages of native lignin, cleaving the polymer chains. The decomposed products so formed are soluble and thus can be removed. Soda pulping process occurs in two-phase system [14, 16, 20], which are as follows:

- the solid phase, consisting of the cellulosic fibrous raw material
- the liquid phase, where moisture is inside the cellulosic fibrous raw material and the pulping liquor is outside the cellulosic fibrous raw material (early in the cook, these are actually two separate liquid phase).

A large number of additives have been used to catalyze soda pulping via, redox cycle. These can be divided into two categories [7]:

- Those, which are known to be converted into anthraquinones (AQ) in the strongly alkaline, oxido-reductive pulping liquor.

- Those, which, while unrelated to AQ, are alkali stable and undergo reversible redox reactions.

Anthraquinone is widely used as a catalyst in pulping processes. During soda-AQ pulping, the delignification can be divided into two phases. In initial phase, delignification increases quickly, while in the later phase it increases gradually [36]. The strength properties of soda-AQ pulp are found to be slightly lower but comparable to kraft pulp [26, 27]. AQ has zero toxicity, no adverse environmental impact, is stable to alkali and is cost effective to use [5].

The proposed mechanism for the action of anthraquinone in alkaline pulping [2] is shown in Figure 4.1.

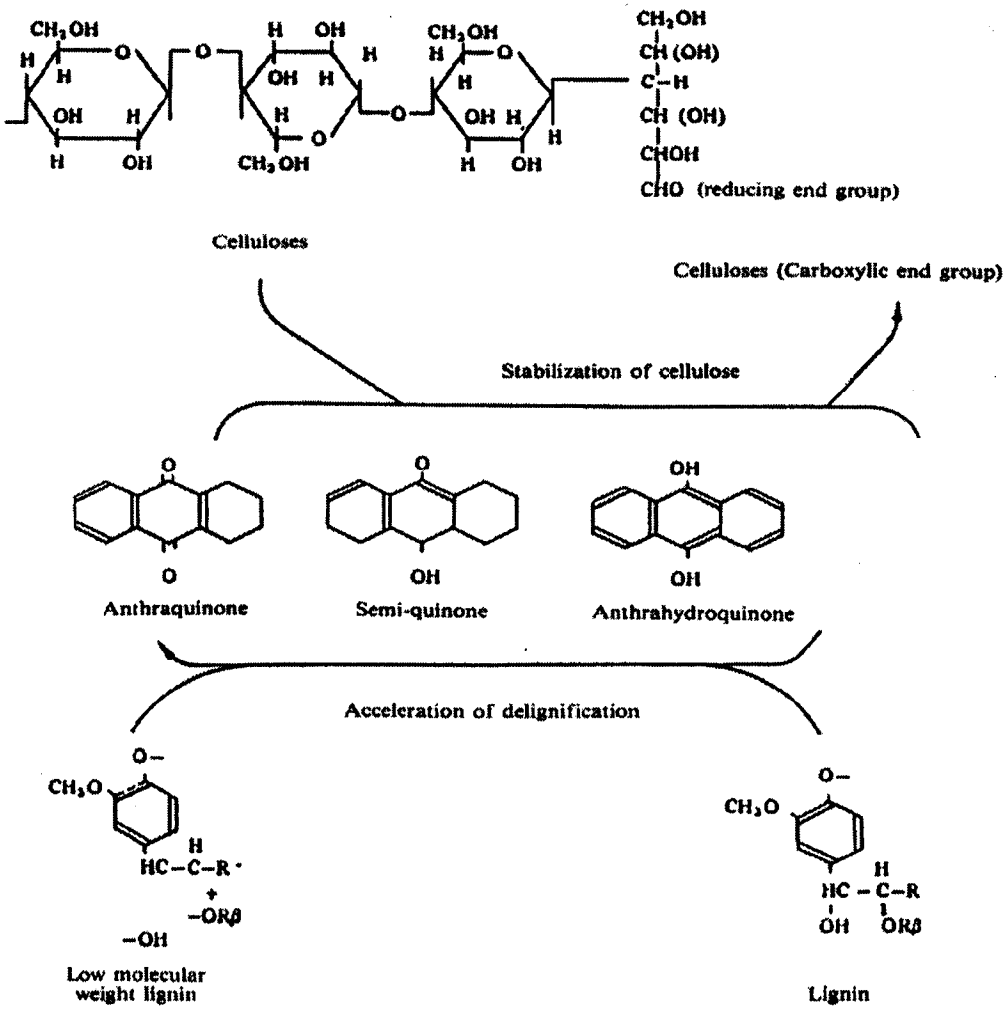


Figure 4.1 Proposed mechanism for the action of anthraquinone in alkaline pulping

Wood carbohydrates are degraded in alkali and convert anthraquinone into its soluble form. The anthraquinone functions as a redox catalyst by transferring electrons from carbohydrates to lignin [6]. These reduced lignin moieties promote faster rate of delignification. An efficient transfer of electrons occurs from source (carbohydrates) to a sink (lignin). Thus, the degradation and solubilization of lignin are accelerated (faster pulping) by end group oxidation and carbohydrates are stabilized against the attack of alkali (higher yield). The first step in the sequence is the reaction of anthraquinone with the reducing group of a carbohydrate, thus stabilizing the carbohydrate against alkaline peeling and producing the reduced form anthrahydroquinone which is soluble in alkali. The anthrahydroquinone reacts with quinonemethide segment of anthrahydroquinone is converted back to anthraquinone, which can then participate again in the redox cycle [18, 20].

The loose and open structure and low lignin content of wheat straw make it suitable to perform soda-AQ pulping [9].

In industrial applications, fibre deformation is a very important fibre characteristic, along with fibre length and width. [28]. Pulp and fibre defects are defined by various types of deformations like, fibre curl and kinks. Fibres in the plant stem are straight but they become curly during pulping, and mixing operations because of being subjected to bending and axial compressive stresses [33]. In Figure 4.2 the effects of various types of deformations and their effect on the fibre stress-strain curve are depicted.

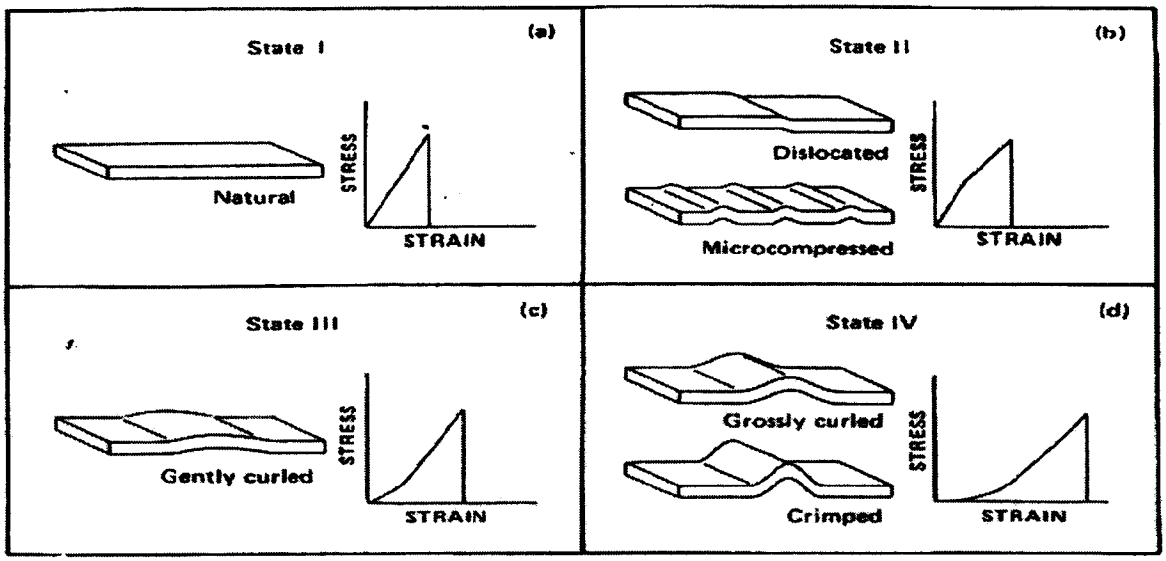


Figure 4.2 Various plant fibre deformation types, described as fibres being in different states, and the corresponding pulp sheet stress-strain curves [29].

Fibre curliness mostly affects the bonding ability of fibres in a fibre network. A sheet formed from such fibres has a low tensile index but can have high tear strength [30]. This has been explained by the uneven distribution of stress along the length of a curled fibre in a fracture zone, curly fibres transferring therefore larger stresses to the bonds which during breaking consume greater energy [35]. Fibre curl, in the absence of other effects reduces the drainage resistance of pulps [30]. It may also result in more scattering of reflected light and slightly higher brightness and opacity [13]. The fibre kinks have been reported to affect the wet strength of the pulp. The more kinked the fibres, the higher is the wet rupture energy [32]. In the literature much consideration has also been given to shives, that are particles or fibre bundles large enough, or in enough quantity, to produce a paper and board quality or productivity problems. Shives in bleached pulps show up as dirt in paper or board, and in unbleached pulps reduce print quality; reduce end strength, decrease runnability, and present visual defects [1, 15, 21, 23, 34]. Thus, we see that the inclusion of fibre deformation in pulp evaluation

improves the understanding of the relationship among fibre properties, pulping and papermaking processes, and paper quality.

4.2 MATERIALS AND METHODS

4.2.1 Materials

Wheat straw was used as raw material for preparation of soda-AQ pulp. The reagents used were of laboratory grade from Qualigens Fine Chemicals, Mumbai.

4.2.2 Methods

4.2.2.1 Pulping studies

The wheat straw was digested in WEVERK electrically heated rotary digester of 0.02 m^3 capacity having four bombs, each of 1 L capacity. It was cooked at different cooking conditions like, maximum temperature ranging from 130 to 160 °C, cooking time from 15 to 150 min, active alkali from 9 to 16% (as Na_2O), and liquor to raw material ratio of 4:1. Based on experimental results at optimum cooking conditions, 0.1% anthraquinone (AQ), a carbohydrate stabilizer, was added (based on O.D. raw material) to study its impact on pulp yield, screening rejects and kappa number, while keeping the other operating parameters constant. Wheat straw was also delignified at varying active alkali doses ranging from 10 to 12 % (as Na_2O) in the presence of 0.1% AQ while keeping other operating parameters constant. After completion of cooking, the pulp was washed on a laboratory flat stationary screen having 300 mesh wire bottom for the removal of residual cooking chemicals. The pulp was disintegrated and screened through WEVERK vibratory flat screen with slot size of 0.15 mm and the screened pulp was washed, pressed and crumbled. The pulp was then analyzed for kappa number (T 236 cm-85), pulp yield and lignin (T 222 om-88) and screening rejects as per Tappi Standard Test Methods [4].

4.2.2.2 Fibre classification studies

The fibre fractionation of soda-AQ pulps of wheat straw was carried out with the help of Bauer-McNett fibre classifier (T 233 cm-06) as per Tappi Standard Test Methods [4]. The mesh screen numbers were 20, 48, 100 and 200.

4.2.2.3 Preparation of laboratory hand sheets and evaluation of paper properties

The unbleached pulp was beaten in PFI mill (T 200 sp-96) to different beating levels. Laboratory hand sheets of 60 g/m^2 were prepared (T 221 cm-99) and tested for tear index (T 414 om-98), tensile index (T 494 om-01), burst index (T 403 om-97) and double fold (T 423 cm-98) as per Tappi standard test methods [4].

4.2.2.4 Studies on fibre deformation

A pulp suspension at 0.02% consistency was prepared and used for analysis of various fibre characteristics (curl index, number of shives per mm and kink index) by using Hi-Resolution Fibre Quality Analyser, Model V1.3REB19CV-M 4.

4.2.2.5 Microscopy

4.2.2.5.1 Light microscopy

The morphological features of the fibres were studied using light microscopy. Pulp fibres were simply boiled in lacto-phenol for 10 min and then microscopic slides were prepared. These slides were then observed under light microscope (WILL-OPTIK Wetzlar-Nbn, Germany) at different magnifications.

4.2.2.5.2 Scanning electron microscopy

Scanning electron microscopy (SEM) of the soda-AQ pulp samples was carried out at using Scanning electron microscope, Model SEM, Leo 435 VP, England.

Samples for microscopy were prepared by subjecting the pulp samples to fixation using 3% (v/v) glutaraldehyde-2% (v/v) formaldehyde (4:1) for 24 h. Following the primary fixation, samples were washed thrice with double distilled water. The samples were then treated with the ethyl alcohol of different concentrations i.e. 30%, 50%, 70%, 80%, 90% and 100% for dehydration. Samples were kept for 15 min each up to 70% alcohol gradient, thereafter treated for 30 min each, for subsequent alcohol gradients. After treating with 100% alcohol, samples were air dried and examined under SEM using gold shadowing technique [10]. Electron photomicrographs were taken at 15 kV using detector SE1 and at desired magnifications.

4.2.2.6 Statistical analysis

For determination of kappa number, three experimental values were taken in each case and the results are the mean ± standard (SD) of the values.

4.3 RESULTS AND DISCUSSION

4.3.1 Influence of temperature and time

Figure 4.3 reveals the curves plotted between residual lignin and reaction time at different reaction temperatures. The curves indicate that each curve can be approximated by two straight lines at each temperature investigated. The curves with steeper slopes pertained to rapid solublization of bulk of lignin (bulk delignification), where as the part of curves with more gentle slopes pertained to the slow solublization of the residual lignin (residual delignification). Both parts of these curves had different velocity constants. The bulk delignification corresponded to the removal of easily assessable lignin present in the middle lamella and the residual delignification corresponded to the removal of lignin present in the primary wall, secondary wall

layers i.e. S_1, S_2 and S_3 layers and the central inter connections cavity etc. The delignification of wood in alkaline pulping was also associated with the solubilization of significant amounts of hemicelluloses [19]. These curves indicated that as the temperature was decreased from 160 to 130 °C, the reaction time to reach transition from bulk to residual delignification phase and the lignin content of the pulp, corresponding to this transition point, both increased. Table 4.1 and Figure 4.4 also reveal that at lower temperature range, the residual lignin contents decreased sharply, while at higher temperature, the magnitude of decrease in lignin content was not so significant. Moreover, at higher temperature, the degradation of carbohydrate fractions also increased, thereby resulting in a reduced pulp yield [19]. In other words, at the transition point, lower pulp lignin contents were obtained at 150 °C. Beyond a temperature of 150 °C degradation of carbohydrates contents occurred due to peeling reactions [14, 24]. The nature of curves after transition points were almost horizontal lines which clearly indicated that the bulk delignification were over up to these transition points and it was not economical to continue the cooking operation beyond this optimum temperature of 150 °C. Therefore, based on experimental data, a maximum cooking time of 1 h and maximum cooking temperature of 150 °C might be considered as an optimum cooking condition for the soda pulping of wheat straw.

Table 4.4 and Figure 4.7 reveal the effect of maximum cooking temperature ranging from 140 to 160 °C, alkali dose 12% (as Na_2O), cooking time 1 h and liquor to raw material ratio 4:1, on screened pulp yield, screening rejects and kappa number of pulp. Figure 4.7 indicates that screened pulp yield increased with increasing temperature from 140 to 150 °C and then followed a declining trend, while both kappa number and screening rejects dropped sharply up to a cooking temperature of 150 °C

and then remained practically constant. Based on above observation, a cooking temperature of 150 °C might be considered as an optimum.

4.3.2 Influence of alkali charge

Table 4.2 and Figure 4.5 reveal that screened pulp yield increased with increasing active alkali from 9 to 12% (as Na_2O) and then tended to decline sharply, where as both kappa number and screening rejects declined sharply up to an alkali dose of 12%. Beyond an alkali dose of 12% the decrease in kappa number and screening rejects was not so significant. The screened pulp yield of wheat straw was found to be 44.30% and kappa number 24.70 at an active alkali charge of 12% (as Na_2O), which might be considered as optimum cooking dose for wheat straw. During soda pulping, the consumption of active alkali was found to be constant over a wide range of alkali charge [22]. It was found that the excessive active alkali charge which remained unconsumed during the course of pulping adversely affected the pulping viscosity. The wheat straw contained slightly more solubles, therefore, pulp yield per digester would be less.

4.3.3 Influence of time

Table 4.3 and Figure 4.6 show the effect of cooking time on screened pulp yield, screening rejects and kappa number during soda pulping of wheat straw, while keeping all other variables constant, like alkali dose 12% (as Na_2O), liquor to raw material ratio of 4:1, digester pressure 5 kg/cm^2 and maximum cooking temperature 150 °C. When cooking time was increased from 30 min to 60 min, the screened pulp yield increased from 40.25 to 44.30% and kappa number dropped from 28.30 to 24.70. Beyond that, the screened pulp yield dropped sharply, while kappa number remained

almost constant. Therefore, maximum cooking time of 60 min might be considered as an optimum cooking time for soda pulping of wheat straw.

4.3.4 Influence of AQ

Table 4.5 reveals the effect of AQ at different alkali doses i.e. 10-12% (as Na_2O), while keeping other conditions constant during soda pulping of wheat straw. The addition of AQ at a dose of 0.1% increased the screened pulp yield and decreased screening rejects and kappa number when applied at different alkali doses i.e. 10-12% (as Na_2O). AQ improved the pulp yield by 0.75% and mitigated the kappa number by 26.1% at optimum cooking alkali charge of 12% (as Na_2O) for soda pulping. The increase in pulp yield and reduction in kappa number can be explained on the basis of redox catalytic activity of AQ. AQ is a pulping additive that accelerates delignification and protect carbohydrates against degradation. It works through a cycle which leads to the reduction of lignin and the oxidation of the reducing end group of cellulose from an aldehyde to a carboxylic acid. In the latter case the carbohydrates are stabilized against the alkaline peeling reactions, so called stopping reactions, leading to increase in pulp yield. Because anthraquinone goes through a cyclic process, it was typically used at about 0.1% on O.D. raw material basis and resulted in an increase in pulp yield [8, 16].

Table 4.6 reveals the mechanical strength properties of unbleached soda-AQ pulps of wheat straw cooked at different alkali doses i.e. 10-12% (as Na_2O) and beaten at different beating levels i.e. 19-55 °SR. All the mechanical strength properties were in improving trend up to an alkali dose of 12% (as Na_2O) and beating level of 45 °SR, beyond which, there was a slight decrease in mechanical strength properties. Figures 4.9 and 4.10 show that the burst index, tensile index and double fold improved with increase in beating level up to a certain beating level, but the tear index first improved

and then declined sharply. The reason is that the work done on pulling the short fibres out of a sheet is less than that required to break the fibres. Thus, as the inter-fibre bonding increases and the proportion of fibre which is fractured rather than pulled out rises, tear index increases to a constant value i.e. 3.90 mNm^2/g for soda pulp and 4.25 mNm^2/g for soda-AQ pulp, which presumably represents the work done in fracturing the fibres.

Initially, the mechanical strength properties depended upon the fibre length. The degradation of carbohydrate fractions beyond optimum alkali dose caused the decline of mechanical strength properties. All the mechanical strength properties were found to increase with increasing beating level up to 45 ºSR except for tear strength. The removal of primary wall exposed secondary wall layers. However, primary wall is permeable to water but does not participate in bond formation. Therefore, tearing energy required to pull the fibres from the mesh was slightly more due to hydrogen bonding after removal of primary wall. Further, due to cutting action, external and internal fibrillation and brushing action, tear strength declined but all other properties which were dependent upon hydrogen bonding, were improved with pulp beating.

4.3.5 Fibre length distribution of wheat straw

Table 4.7 shows the fibre length distribution of soda–AQ pulp of wheat straw at 220 CSF (Canadian Standard Freeness). Important quantitative information about the fibre length distribution of a wheat straw pulp can be best achieved by fibre fractionation. Furthermore, while using the Bauer McNett fibre classifier with screens of 20, 48, 100 and 200 mesh size, the fractionation not only separated the fibres according to the fibre length, but also, to a great extent, it separated the fractions of sclerenchyma fibres and parenchyma cells. +20 fractions showed 12.16% which

consisted of mostly long sclerenchymatous fibre. +48 fractions involved 40.31% of pulp of the medium sized fibre. +100 fractions retained 21.21% of the total pulp with the shortened fibres, parenchyma cells, large vessel fragments while +200 fractions contained 0.92% of pulp having short parenchyma cells and other short material and fragments of the epidermal tissue. -200 fractions involved 25.40% of the pulp and included the fibre and cell debris, single epidermal cells and collenchyma cells [12].

4.3.6 Studies on fibre deformation

The soda-AQ wheat straw pulp samples were also studied for fibre deformations and the results are reported in Table 4.8. While the kink index of wheat straw was about 4.4% higher than that reported for *Eucalyptus tereticornis*, the curl index was found to be similar in both cases at 0.141. Kinks per mm reported for wheat straw fibres was a little higher (by 7.5%) as compared to *Eucalyptus tereticornis*. The fine content of wheat straw pulp was higher than *Eucalyptus tereticornis* by about 45% arithmetically and by about 66% when length weighted. Shives per mm for wheat straw pulp were reported to be 9.78. Thus, though the fine content of wheat straw pulp was much higher, it was found to be more or less comparable in quality to *Eucalyptus tereticornis* in terms of curl index and kinks per mm.

4.3.7 Microscopy

Photomicrograph 4.1A shows a smooth slender wheat straw pulp fibre having a thick cell wall and a narrow lumen. Such fibres easily conformed during beating and contributed to paper strength. As the pulping proceeded, the connections between the fibres were broken up and the fibres gradually separated out. The presence of different types of cells in the wheat straw pulp gave it a heterogeneous character. These different

types of cells included parenchyma cells (Photomicrographs 4.1C and E), vessels (Photomicrograph 4.1C), useful fibres with narrow lumen (Photomicrographs 4.1B and D), and others with comparatively wider lumen (Photomicrographs 4.1A and B). As a result of pulping, partial delignification took place. The fibres lost their rigidity and collapsed, thereby acquiring a ribbon shaped conformation (Photomicrographs 4.2A and C). There was also no sign of external fibrillation after pulping (Photomicrograph 4.2B).

Table 4.1 Effect of temperature and maximum cooking time on pulp yield kappa number and lignin of wheat straw

Temperature, °C	Time at temperature, h	Wheat straw		
		Yield, %	Kappa number	Lignin, %
130	0.25	56.25	-	12.85
	0.50	54.25	-	10.35
	1.00	52.10	45.3±0.28	7.92
	1.50	50.30	-	6.72
	2.00	48.20	-	5.75
	2.50	46.25	-	4.72
140	0.25	53.80	-	10.35
	0.50	51.75	-	8.06
	1.00	49.30	38.4±0.31	5.56
	1.50	47.15	-	4.76
	2.00	44.80	-	3.98
	2.50	43.00	-	3.5
150	0.25	50.15	-	8.45
	0.50	47.50	-	6.15
	1.00	45.10	24.70±0.17	4.05
	1.50	42.85	-	3.62
	2.00	40.25	-	3.35
	2.50	38.20	-	3.05
160	0.25	48.25	-	7.85
	0.50	45.40	-	5.78
	1.0	43.10	22.8±0.30	3.67
	1.5	40.30	-	3.15
	2.0	37.80	-	2.82
	2.5	35.70	-	2.62

Cooking conditions:

Liquor to raw material ratio	= 4:1
Active alkali, % (as Na_2O)	= 12
Time from ambient to 105 °C, min	= 30
Time from 105 to maximum temperature, min	= 30

Table 4.2 Effect of active alkali (as Na_2O) on screened pulp yield, screening rejects and kappa number of wheat straw

Sl. No.	Active alkali, % (as Na_2O)	Total pulp yield, %	Screened pulp yield, %	Screening rejects, %	Kappa number
1.	9	46.29	37.25	9.04	35.78±0.28
2.	10	45.23	40.25	5.98	31.29±0.32
3.	11	44.17	42.75	3.42	27.50±0.36
4.	12	45.10	44.30	0.80	24.70±0.31
5.	13	40.10	42.35	0.65	22.16±0.36
6.	14	36.30	39.8	0.50	20.40±0.19
7.	15	33.05	36.55	0.30	19.15±0.22
8.	16	29.76	32.56	0.20	18.40±0.27

Cooking conditions:

Liquor to raw material ratio = 4:1
Digester pressure, kg/cm^2 = 5.0
Time from room temperature to 105 °C, min = 30
Time from 105 to 150±2 °C, min = 30
Time at 150±2 °C, min = 60

± Standard deviation from the mean

Table 4.3 Effect of maximum cooking time on screened pulp yield, screening rejects and kappa number of wheat straw

Sl. No.	Maximum cooking time, min	Total yield, %	Screened pulp yield, %	Screening Rejects, %	Kappa number
1	30	47.50	40.25	7.25	28.30±0.39
2	60	45.10	44.30	0.80	24.70±0.42
3	90	42.85	42.25	0.60	22.20±0.25
4	120	40.25	39.65	0.50	19.80±0.29
5	150	38.20	37.90	0.30	18.40±0.32

Cooking conditions:

Liquor to raw material ratio = 4:1
Active alkali, % (as Na_2O) = 12
Time from ambient to 105 °C, min = 30
Time from 105 to maximum temperature, min = 30
Maximum cooking temperature, °C = 150

± Standard deviation from the mean

Table 4.4 Effect of maximum cooking temperature on screened pulp yield, screening rejects and kappa number of wheat straw

Sl. No.	Maximum Temperature, °C	Total pulp yield, %	Screened pulp yield, %	Screening rejects, %	Kappa number
1.	140	49.30	43.95	5.35	38.40±0.36
2.	150	45.10	44.30	0.80	24.70±0.27
3.	160	43.10	42.50	0.60	22.80±0.25
Cooking conditions:					
Liquor to raw material ratio			= 4:1		
Alkali charge, % (as Na_2O)			= 12		
Time from ambient to 105 °C, min			= 30		
Time from 105 to maximum temperature, min			= 30		
Time at maximum temperature, min			= 60		

± Standard deviation from the mean

Table 4.5 Effect of anthraquinone on screened pulp yield, screening rejects and kappa number of wheat straw

Sl. No.	Alkali charge,% (as Na_2O)	AQ dose, %	Total pulp yield	Screened pulp yield, %	Screening Rejects, %	Kappa number
1.	10	0.0	45.23	40.25	5.98	31.29±0.25
2.	10	0.1	45.40	42.50	2.73	25.50±0.20
3.	11	0.0	44.17	42.75	1.42	27.50±0.31
4.	11	0.1	44.30	44.10	0.20	20.30±0.21
5.	12	0.0	45.10	44.30	0.80	24.70±0.29
6.	12	0.1	45.20	45.05	0.15	18.25±0.19
Cooking conditions:						
Liquor to raw material ratio			= 4:1			
Maximum temperature, °C			=150			
Time from ambient to 105 °C, min			= 30			
Time from 105 to maximum temperature, min			= 30			

± Standard deviation from the mean

Table 4.6 Mechanical strength properties of soda and soda-AQ unbleached wheat straw pulps at different alkali doses and beating levels

Active alkali, % (as Na_2O)	Beating level, °SR	Tensile index, Nm/g	Tear index, mNm^2/g	Burst index, $kPam^2/g$	Double fold, no.
10	19	18.85	3.07	0.75	5
	35	45.32	4.21	2.55	35
	45	64.84	3.42	3.35	40
	55	64.12	3.12	3.45	42
10*	19	19.1	3.15	0.90	6
	35	46	4.35	2.72	32
	45	65.75	3.21	3.42	43
	55	64.9	3.10	3.51	45
11	19	20.15	3.65	0.85	6
	35	47.21	4.41	2.65	37
	45	66.9	4.06	3.47	67
	55	66.2	3.70	3.55	70
11*	19	21.35	3.75	1.02	5
	35	47.9	4.55	2.72	41
	45	67.12	4.15	3.92	71
	55	66.9	3.50	4.02	75
12	19	22.35	3.86	1.10	8
	35	48.71	4.96	3.52	70
	45	68.25	4.12	3.90	81
	55	67.25	3.24	3.86	85
12*	19	23.75	3.55	1.15	7
	35	50.08	5.10	3.76	71
	45	69.75	4.02	4.25	89
	55	68.12	3.15	4.21	85

Cooking conditions:

Liquor to raw material ratio	= 4:1
Maximum temperature, °C	=150
Time from ambient to 105 °C, minutes	= 30
Time from 105 to maximum temperature, minutes	= 30
Time at maximum temperature, minutes	= 60

* 0.1 % AQ dose (on O.D. raw material basis)

Table 4.7 Bauer McNett fibre classification of soda-AQ wheat straw pulp at optimum pulping conditions

Sl. No.	Mesh size	Fractions, %
1.	+ 20	12.16
2.	– 20+48	40.31
3.	–48+100	21.21
4.	–100+200	0.92
5.	– 200	25.4

Table 4.8 Analysis of wheat straw pulp for fibre deformations by Fibre Quality Analyzer (FQA)[a]

Sl. No.	Properties	Wheat straw soda-AQ pulp[b]	*Eucalyptus tereticornis* [3]
1.	Mean curl index		
	Arithmetic	0.141	0.141
	Length weighted	0.152	-
2.	Mean kink index		
	Kink index (1/mm)	2.25	2.15
	Total kink angle (degrees)	38.75	29.93
	Kinks per mm (1/mm)	1.06	0.98
3.	Percent fines		
	Arithmetic	-	25.59
	Length weighted		5.34
4.	Number of shives/mm	9.78	-

[a] Fibre Quality Analyzer Model V1.3REB19CV-M 4
[b] Total fibre count = 8965.00

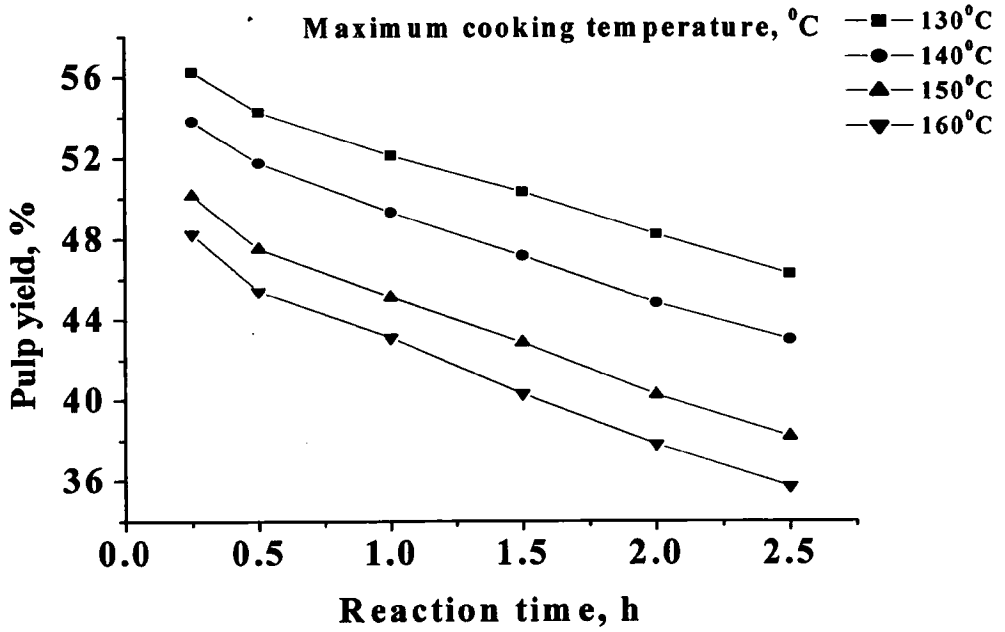


Figure 4.3 Curves of lignin vs different reaction times at maximum cooking temperature during soda pulping of wheat straw.

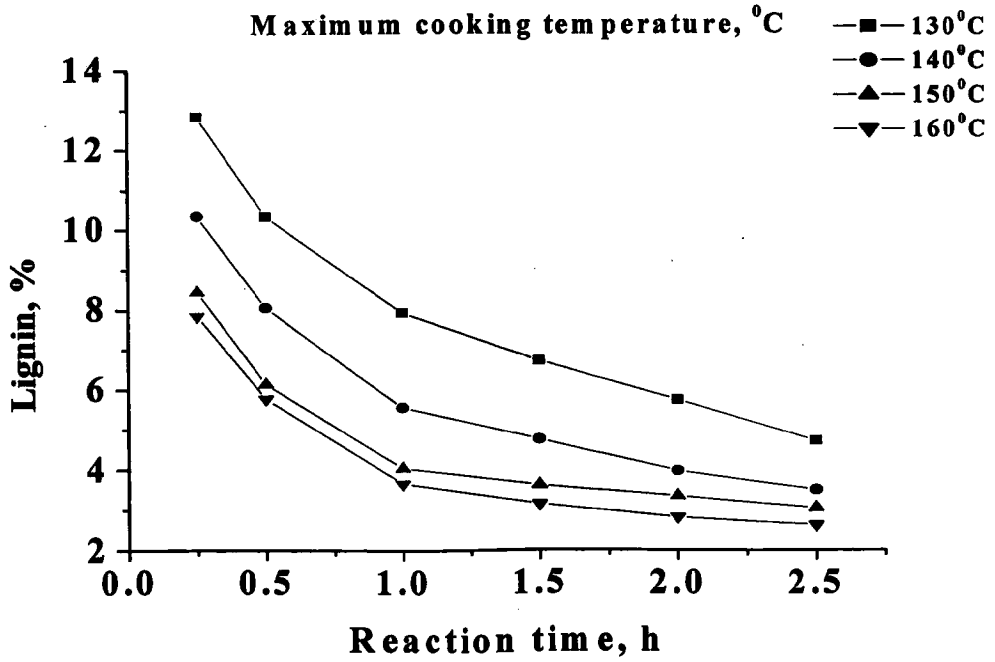


Figure 4.4 Curves of pulp yield vs different reaction times at maximum cooking temperature during soda pulping of wheat straw.

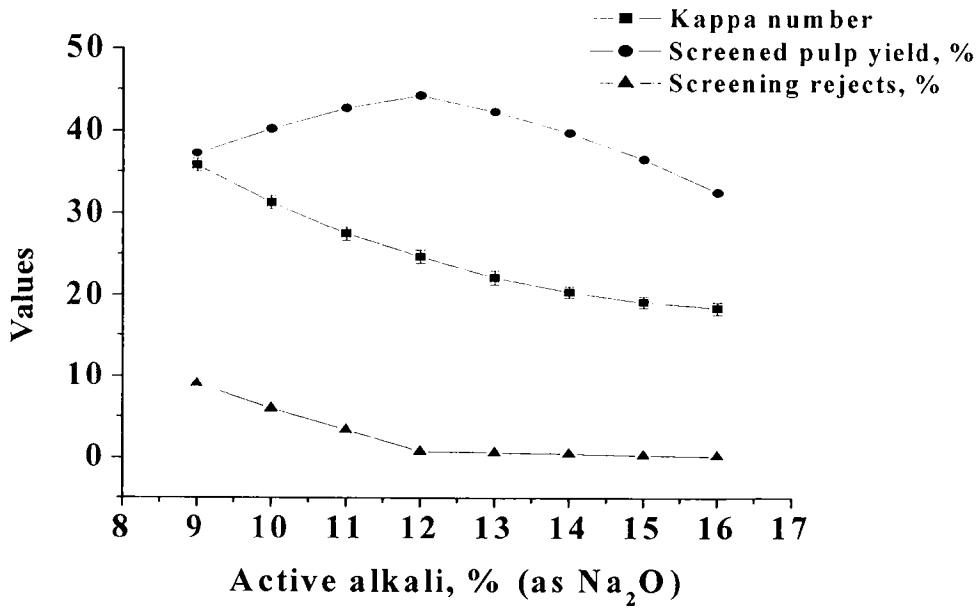


Figure 4.5 Effect of alkali dose on screened pulp yield, screening rejects and kappa number during soda pulping of wheat straw

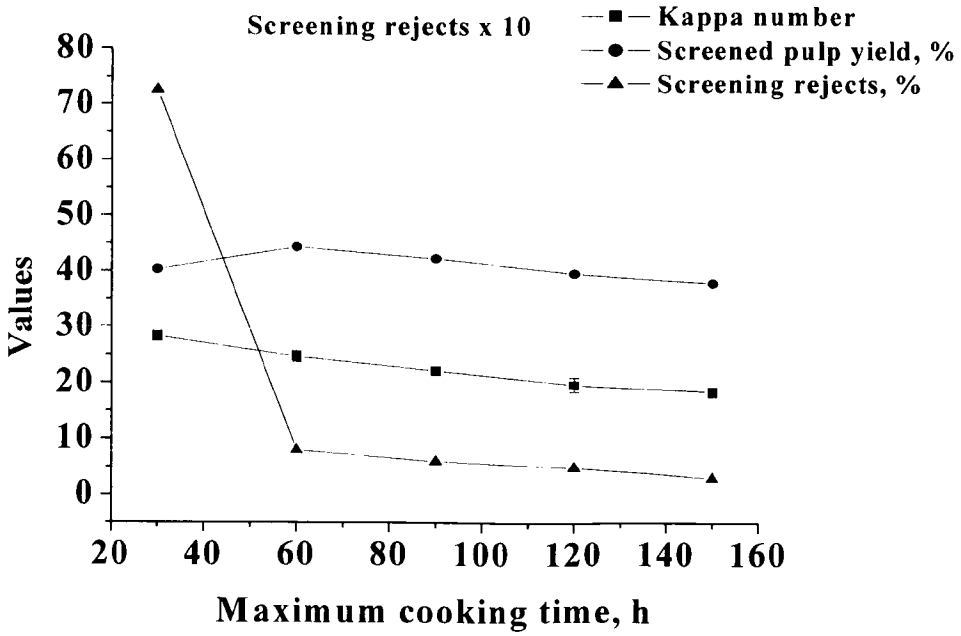


Figure 4.6 Effect of maximum cooking time on screened pulp yield, screening rejects and kappa number during soda pulping of wheat straw

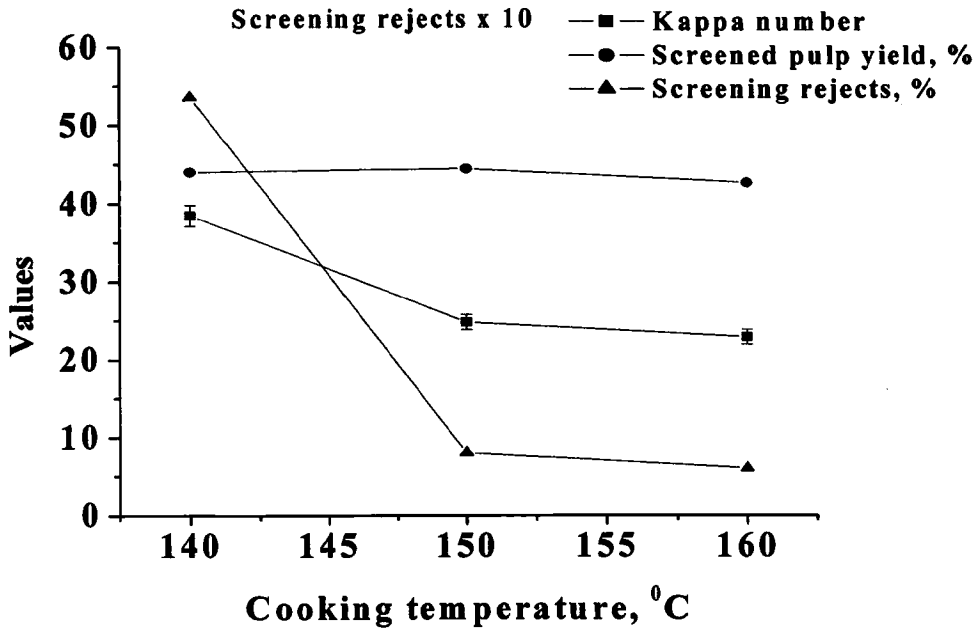


Figure 4.7 Effect of maximum cooking temperature on screened pulp yield, screening rejects and kappa number during soda pulping of wheat straw

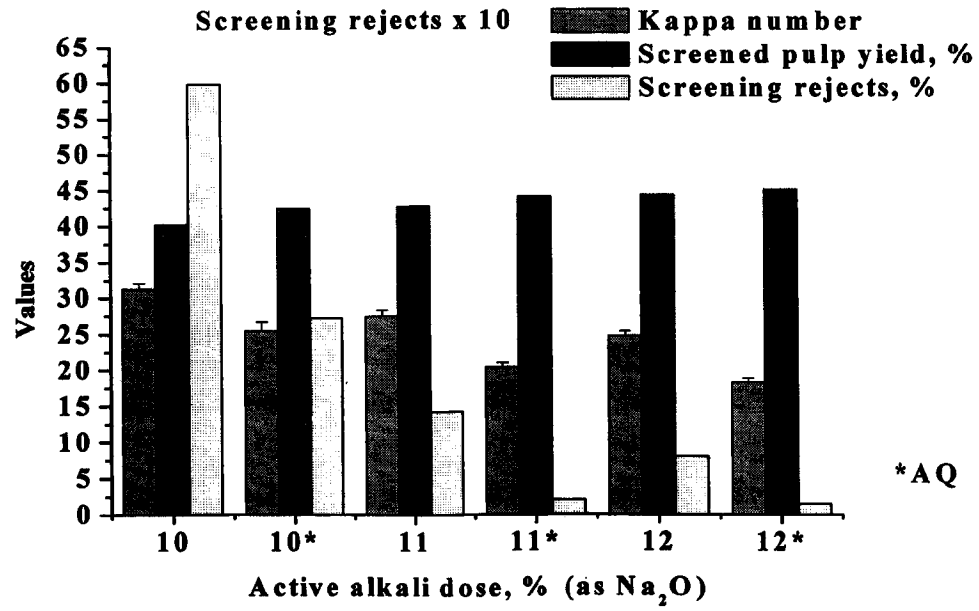


Figure 4.8 Effect of anthraquinone on screened pulp yield, screening rejects and kappa number at different alkali doses at optimum pulping conditions during soda pulping of wheat straw

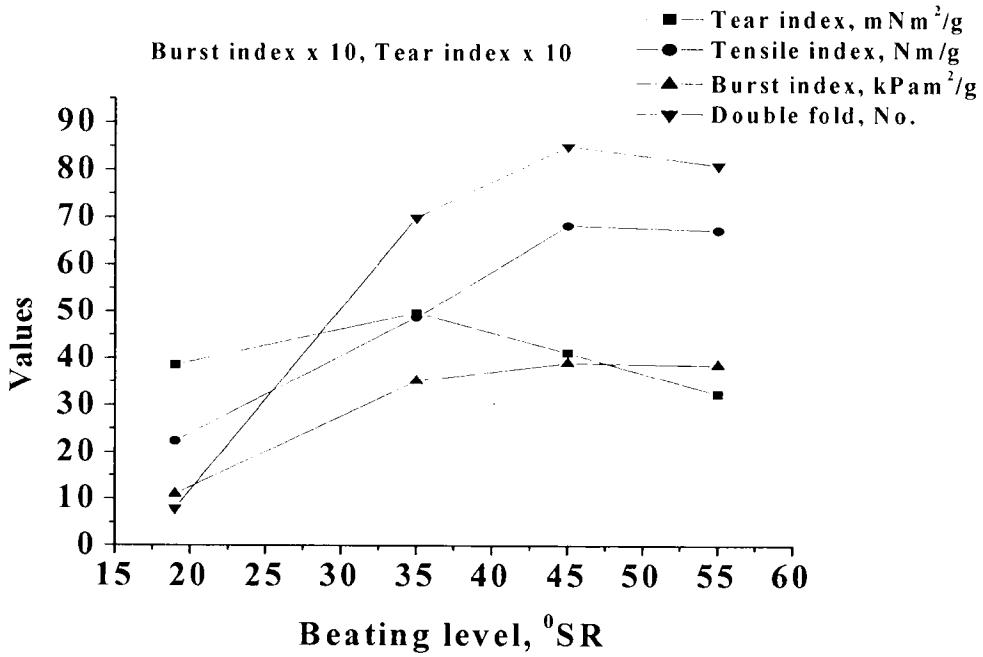


Figure 4.9 Plots of mechanical strength properties vs beating levels (°SR) of soda pulp of wheat straw at 12 % active alkali dose

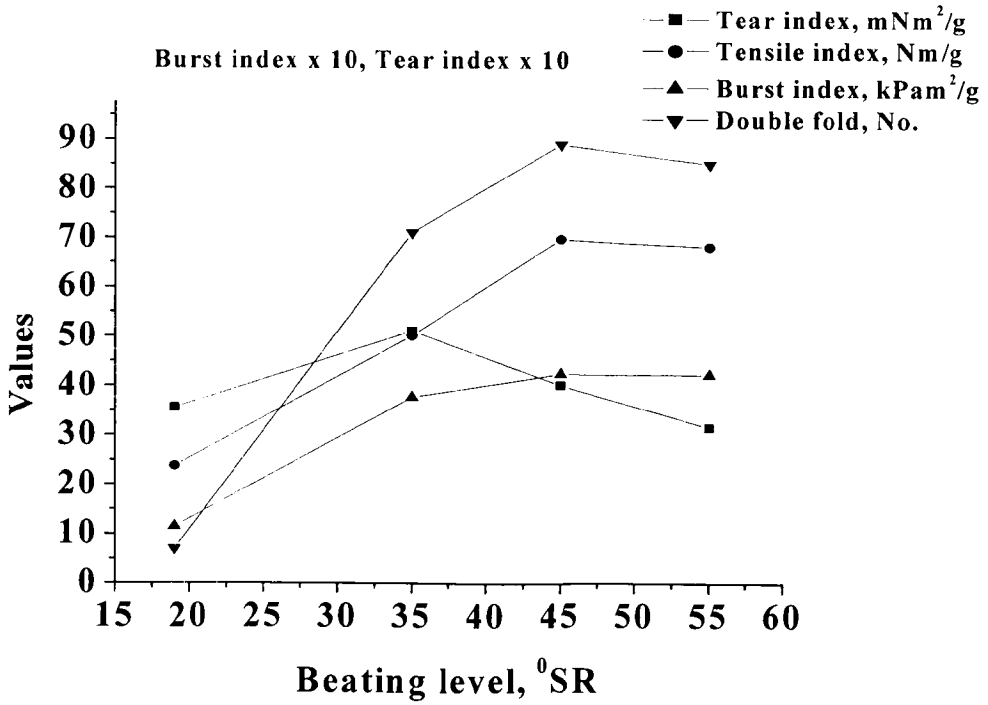
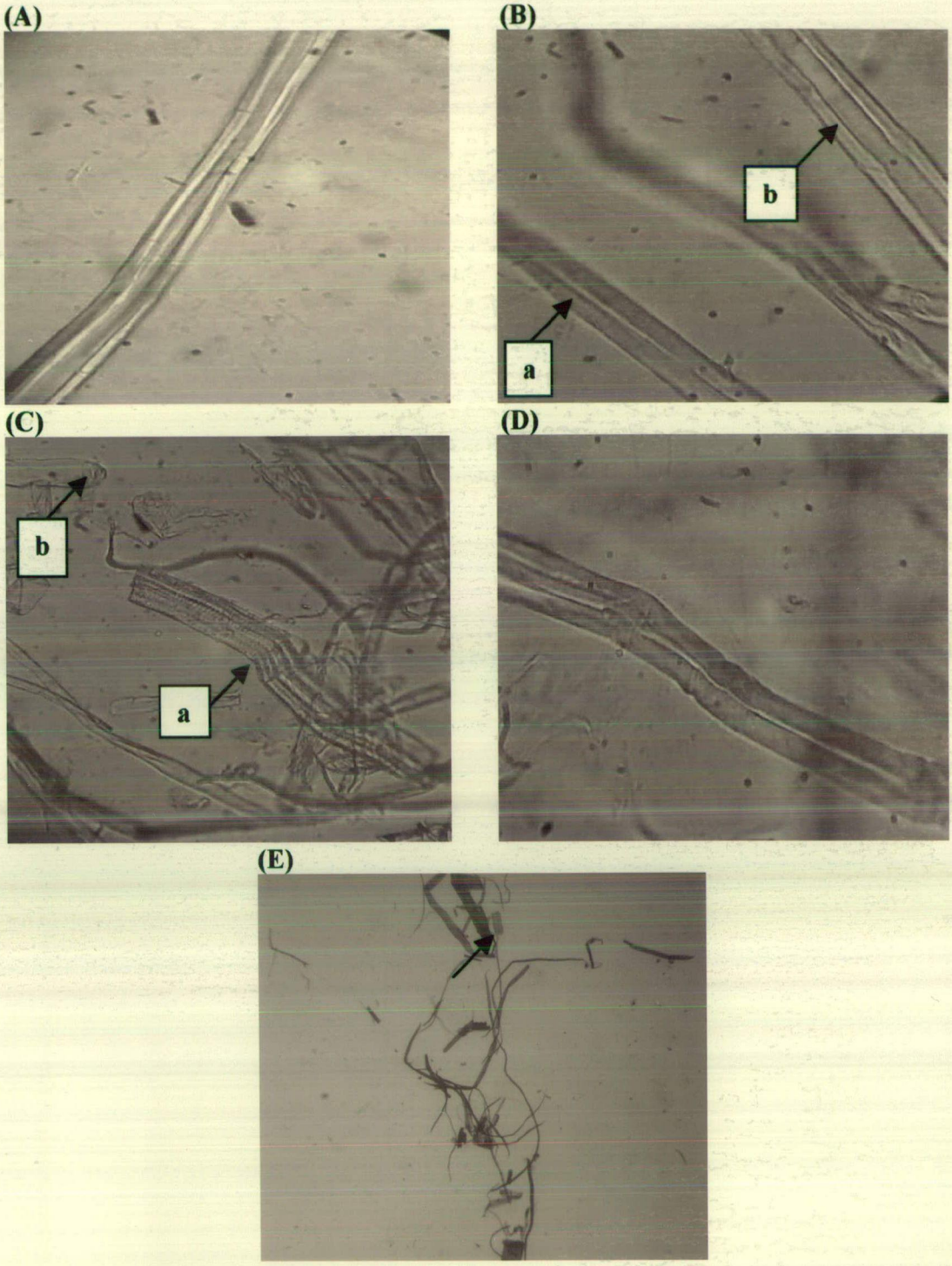
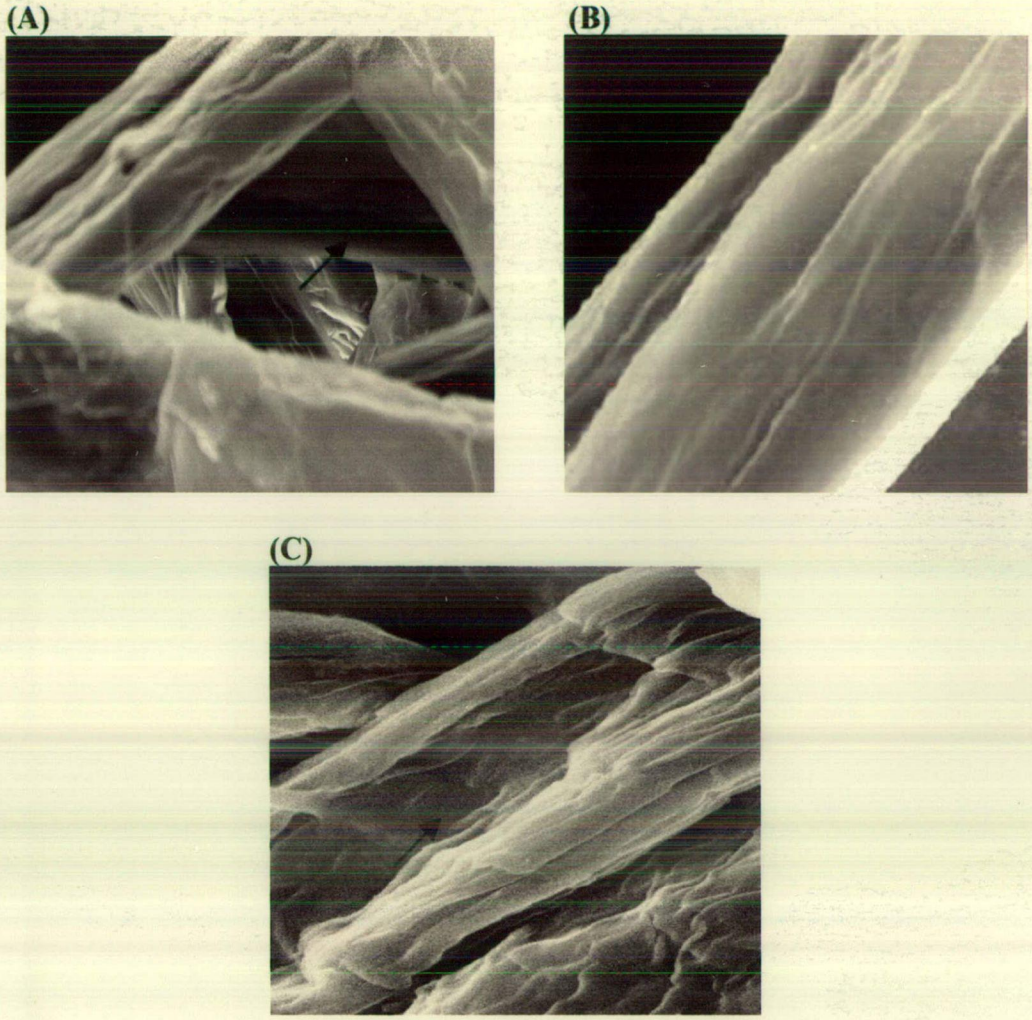


Figure 4.10 Plots of mechanical strength properties vs beating levels (°SR) of soda-AQ pulp of wheat straw at 12 % active alkali dose



Photomicrographs 4.1 Showing (A) wheat straw fibre with a wide lumen (40x) (B) wheat straw fibres with lumen of variable thickness (40x) (C) (a) a vessel element (b) a parenchyma cell (40x) (D) wheat straw fibre with extremely narrow lumen (40x) (E) tangles of wheat straw fibres and other cell types, a parenchyma cell is also visible(4x).



Photomicrographs 4.2 **Scanning electron micrograph showing, (A) unfibrillated wheat straw fibres with flattened surface (3.5 kx) (B) wheat straw fibres without external fibrillation, after soda-AQ pulping, (2000x) (C) flattened wheat straw fibre, showing ribbon shaped conformation, 2.5 kx.**

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CHAPTER 5

XYLANASE-AIDED BLEACHING OF SODA-AQ PULP OF WHEAT STRAW

5.1 INTRODUCTION

Pulp and paper industry is considered as a vital and core industry. Historically, the pulp and paper industry has been considered to be a major consumer of natural resources (wood, water) and energy (fossil fuels, electricity) and a significant contributor of pollutant discharges to the environment [99]. This industry is considered to be the sixth largest polluter (after oil, cement, leather, textile, and steel industries) discharging a variety of gaseous, liquid, and solid wastes into the environment [2]. Paper production is a multi-variant process. In the process of paper production, pulping is a step in which fibres are separated by chemical means and most of the lignin that is associated with the middle lamella, is removed. The residual lignin is then removed by a multistage bleaching process [7]. Chemical pulping, which is frequently used around the world, is mainly achieved by sulphate (kraft) pulping process. Non-woods are generally delignified by soda or soda-AQ pulping. These processes remove most of the lignin, and dissolve and partially degrade hemicelluloses. Residual lignin is covalently bound to carbohydrate moieties in the pulp [118]. As the cook progresses, xylan precipitates onto the surfaces of the cellulosic fibres [26, 118], trapping degradation products in the matrix. These degradation products impart a characteristic brown colour to the pulp. Usually, one or more bleaching sequences are needed to remove the dark brown colour caused by the deposition of lignin. Most of the Indian pulp and paper

industries bleach the pulp by conventional bleaching sequence where chlorination stage has traditionally been considered as the 'workhorse' of post pulping processing steps. Chlorination followed by extraction stage can selectively remove 75-90% of the lignin remaining on the fibre after pulping. Further, hypochlorite or chlorine dioxide is used to oxidize rest of the lignin. Since the pulp produced corresponds to only approximately 40-45% of the original weight of the wood, the effluents are heavily loaded with organic matter. These effluents cause considerable damage to the receiving water if discharged untreated since they have a high biochemical oxygen demand (BOD), chemical oxygen demand (COD), chlorinated compounds (measured as adsorbable organic halides, AOX), suspended solids (mainly fibres), fatty acids, tannins, resin acids, lignin and its derivatives, sulphur and sulphur compounds, etc. While some of these pollutants are naturally occurring wood extractives (tannins, resin acids, stilbenes, lignin), others are xenobiotic compounds that are formed during the process of pulping and paper making (chlorinated lignin, resin acids and phenols, dioxins, furans), thereby turning pulp and paper mill effluents into 'a Pandora's box of waste chemicals' [69].

It has been found that the use of chlorine as a chemical bleach in the pulp and paper industry generates toxic chlorinated organic byproducts. In India, the permissible AOX range in final discharge of agro based mills with and without chemical recovery process is 7.0-11.0 and 14.2-21.5 mg/L, while in wood based mills is 0.60-9.0 mg/L. It was found out that the C stage was generally the first point in which 2,3,7,8-TCDD, 2,3,7,8-TCDF and 1,2,7,8-TCDF congeners were always present [90, 94]. Some of the pollutants, notably, polychlorinated dibenzodioxins and dibenzofurans (dioxins and furans), are recalcitrant to degradation and tend to persist in nature [2]. They are thus known as persistent organic pollutants (POPs). It is well established that many of these

contaminants are acute or even chronic toxins. Chlorinated organic compounds have the ability to induce genetic changes in exposed organisms [59]. In particular, DNA damaging agents have been shown to induce inherited genetic defects and cancer [20]. Dioxins have been named as 'known human carcinogens' by the World Health Organization [29]. This has resulted in a growing concern about the potential adverse effects of genotoxicants on aquatic biota and public health through the contamination of drinking water supplies, recreational waters, or edible organic species [51]. The AOX measurement has been accepted as the quantitative measure of the organochlorine compounds formed in the bleaching process. AOX is very closely related to total organic chlorine (TOCL). In response to the growing concerns for the environment, regulations on the release of waste bleach waters from the pulp and paper industry are becoming stringent. The dioxins and furans have been classified as 'priority pollutants' by the United States Environmental Protection Agency [104] and they figure in the 'dirty dozen' group of persistent organic pollutants (POPs) identified by United Nations Environment Program [103]. In India, the "Charter of Corporate Responsibilities for Environmental Protection" (CREP) introduced by Central Pollution Control Board, India (CPCB), has forced the mills to reduce the AOX level in a fixed time frame. The current environmental standards of AOX is 2 kg/t which will be progressively reduced to 1.5 kg/t and 1 kg/t in the next 5 years as per the recent charter on environment declared by the Government of India [58, 68]. Faced with market, environmental and legislative pressures the pulp and paper industry is modifying its pulping, bleaching and effluent treatment technologies to reduce the environmental load of mill effluents. Rightly or wrongly "Chlorophobia"' has played a major role in the drive to find alternative bleaching technologies [19]. The focus on reduction in adsorbable organic

halides (AOX) and total organic chlorides (TOC1) in bleach plant effluents has promoted elemental chlorine free (ECF) and totally chlorine-free (TCF) bleaching processes [14, 57, 73, 74]. The principal bleaching agent used in ECF processes is chlorine dioxide. TCF bleaching processes significantly reduce the effluent loadings and allow total system closure. Oxygen, ozone, hydrogen peroxide, and various other peroxygens are the principal bleaching agents for these processes. Though these compounds help to cut out on the discharge of pollutants yet these compounds have some disadvantages. The major disadvantage being that the paper produced by these processes generally loses quality during the bleaching [71]. Also, chemicals like, chlorine dioxide are expensive to use. The commitment to remove chlorine from the production of the pulps (ECF) and subsequently to eliminate chlorine compounds totally, producing TCF pulps, requires the study and optimization of new bleaching sequences so that the chemical consumption as a whole can be decreased. This has shifted the focus on the use of biological means for environment friendly bleaching processes that can work in combination with different bleaching sequences.

Biotechnology has attracted considerable attention and achieved interesting results in bleaching of pulps [6, 63]. Biobleaching involves using microorganisms and enzymes to bleach pulp. It relies on the ability of some microorganisms to depolymerize lignin directly or uses microorganisms or enzymes that attack hemicelluloses, thus facilitating subsequent depolymerization [8]. Many enzymes have been put to trial for use in biobleaching processes, but two enzymes, namely, xylanases and ligninases, have been predominantly employed for this purpose [2]. Still, xylanases are considered to be the most important enzyme needed for enhancing the bleaching of pulp, [35, 107]. Though there is a great promise in using oxidative enzymes in the

bleaching of pulp [13], the process is not yet economical. This is due to the limited availability of the enzyme and the cost of the mediators needed for lignin destruction [63].

Xylanases are the enzymes that degrade xylan. Xylan forms an interface between lignin and cellulose in wood and is bound by covalent and non-covalent interactions [38]. Over the years the number of possible applications of xylanases in the pulp and paper industry has increased steadily [9]. The application of xylanases in prebleaching of pulps is gaining importance as alternatives to toxic chlorine-containing chemicals [67, 75, 88]. Xylanases can offer an attractive and commercially viable option to eliminate chlorine in bleaching and reduce chlorinated organic compounds in bleach plant effluents, reduce the kappa number (residual lignin content in the pulp) and increase the brightness of the pulp [91, 96]. The first scientific report of pulp biobleaching using xylanases was published by Viikari *et al.* [109]. Since then, there have been many reports on the efficacy of xylanase pretreatment for biobleaching. Xylanases are being tested as bleaching agents for a variety of wood and non-wood raw materials [78, 79, 95, 98]. The action of xylanase on pulp bleachability has been studied by different methods [5]. In the first phase, enzymes are usually characterized by isolated substrates, which are used in the determination of their activities. These substrates, however, vary extremely with respect to their origin and composition. Furthermore, comparison of enzyme activities is complicated by the utilization of different analysis methods. The action of different enzymes can also be compared using the actual substrate, the pulp. In these tests, the solubilization of carbohydrates has usually been the key parameter. In addition, an increase in the liberation of the lignin-derived compounds after the enzymatic treatment or after alkali extraction has been

used for evaluation of different enzymes. The enzymatic treatment can be combined to different bleaching sequences [34, 50], as reviewed by Suurnäkki *et al.* [93]. Xylanases can be applied in elementary chlorine and chlorine dioxide containing bleaching sequences, as well as in combination with oxygen, ozone and hydrogen peroxide [96, 106]. Crude enzyme filtrates of hemicellulases produced by different microorganisms were tried in the first delignification experiments and most of the reports published on application of enzymes are still based on unpurified enzymes. The culture filtrates contain xylanase as their main activity and the enzyme preparations are generally dosed according to the xylanase activity [108]. Studies have also demonstrated that although, the main enzyme needed to enhance the delignification of pulp is endo-β-xylanase, yet enrichment of other enzymes such as, mannanase, lipase, and α-galactosidase also improve the effect of enzymatic treatment of kraft pulp [27, 116]. Enzymes like, cellulases, laccases, lipases, mannanases and manganese peroxidases, have also been tested for bleaching of pulp or improving specific aspects of the pulp and papermaking processes such as pitch, drainability and refinability [78]. Xylanases are being used, primarily for the removal of the lignin carbohydrate complexes (LCC) generated during the pulping process that act as physical barriers to the entry of bleaching chemicals [64]. The bleaching efficiency of xylanase is measured either as the reduction in the amount of chemicals used for bleaching of pulp or the brightness gain induced by the enzyme [52]. Besides bleaching through lignin removal, xylanases also helps to increase pulp fibrillation and reduce beating time in the original pulp [32, 33], which result in better bonding and saving of energy.

The presence of cellulases in such preparations has been considered detrimental to yield and strength properties in pulp treatments, as they can cause a rapid

depolymerization of cellulose [110]. However significant differences in the action of individual cellulases in enzyme prebleaching have been observed.

Much research has been focused on the mechanism by which xylanases enhance bleaching and several hypotheses have been given but the exact mechanism remains unclear. Paice *et al.* [64] reported that xylanase prebleaching "results primarily from depolymerization but not necessarily solubilization of xylan-derived hemicellulose". Kantelinen *et al.* [36] proposed that re-precipitated xylan forms an insoluble barrier that interferes with pulp bleaching and that removal of the surface xylan thus enhances extraction. During the heating period of the pulping process, when the alkali concentration is comparatively high, part of the xylan is dissolved in the pulping liquor. As the cooking process proceeds, the alkali concentration decreases, and short-chain xylan precipitates in a more or less crystalline form on the surface of cellulose microfibrils. This decreases the accessibility of the fibre walls [54]. The configuration of the xylose units allows close contact of the xylan chains with the cellulose. It is likely that part of the xylan, after removal of its substituent, tends to co-crystallize with, or become adsorbed on, the cellulose of the pulp. A considerable part of the xylan is reabsorbed or reprecipitated onto the cellulose, although a part remains undissolved at its original location in the fibre. The reprecipitation of xylan is preceded by reprecipitation of dissolved lignin during pulping [45, 92]. These redeposited polymers have suggested to be chemically linked to each other. Furthermore, hemicelluloses seem to physically restrict the passage of high molecular mass lignin to come out of the pulp fibre. Clark *et al.* [17] suggested that xylanases loosen the hemicellulose structure to facilitate extraction of lignin. Saake *et al.* [82] concluded that various interactions of the enzyme with the pulp surface determine the efficacy of the treatment. Xylanases

hydrolyze some precipitated xylan onto the fibres, increasing their permeability and giving the bleaching chemicals an easier and smoother penetration and access to lignin. This results in an increase in pulp brightness and a decrease in the consumption of chemicals [36, 102]. Wong *et al.* [114, 115] noted that the exact mechanism by which bleach boosting occurs is not clear because the "carbohydrate degrading enzymes are not expected to act directly on the residual lignin in pulp", and developed the following hypothesis:

i. After alkaline cooking, some xylan will contain chromophoric carbohydrate units. A xylanase treatment could remove these xylan-derived chromophores, thus increasing the pulp brightness. Recent studies [21, 111] have shown that the majority of the 4-*O*-methylglucuronic acid side groups in xylan are converted to hexenuronic acid (HexA) in the early phases of the kraft cooking. These HexA contribute to the kappa number and to the yellowing of pulp.

ii. During pulping, lignin–xylan complexes are known to be formed. Xylanases may hydrolyze xylan in lignin–carbohydrate complexes. This facilitates delignification, either by releasing lignin compounds or by decreasing the size of lignin containing macromolecules.

iii. Reprecipitated xylan present during pulping may physically entrap residual lignin on the fibre surfaces. The hydrolysis of xylan facilitates the removal of residual lignin and also increases the accessibility of lignin to bleaching reagents.

iv. The interaction between cellulose and co-crystallized xylan gives rise to a closed surface. The removal of xylan by xylanase may modify the fibres porosity, and thus allows the passage of lignin and lignin–carbohydrate

molecules in higher amounts and of higher molecular masses in the subsequent chemical extraction. Xylanases are also found to increase the fibre swelling which facilitates refining.

The first mill trial for enzyme prebleaching was done in 1988. Most of the trials use chlorine based bleaching sequences, though some TCF trials are also reported [31, 41, 85, 89, 100, 102]. The addition point of the enzyme in a mill is after the brown stock washing tower. To achieve good results, mixing must be efficient, both after pH adjustment and after enzyme addition. To obtain the best results for enzyme use, enzyme dosage must be optimized. In addition, the pulp consistency must be optimized to obtain effective dispersion of enzyme for improving the efficiency of enzyme pretreatment. Retention time should also be optimized as very long incubation periods might adversely affect pulp quality, especially if cellulases are present in the enzyme preparation. Typically, enzyme is added as an aqueous solution in the last brown stock washer. The enzyme makes the pulp easier to bleach in the subsequent bleaching stages. This results in a decrease in bleaching costs and an increase in operational flexibility. The xylanase treated pulp reaches a higher brightness for a given chemical charge. In a mill, these benefits can be taken care of as a decrease in chemical usage to achieve target brightness or an increase in brightness at a given chemical charge or a combination of these benefits, a reduction in toxicity and energy saving for pulp beating. Xylanase treatment can be implemented with little capital investment [12].

The present study aims at analyzing the effect of crude xylanases obtained from *Coprinellus disseminatus* strains SH-1 and SH-2 on optical properties, mechanical strength properties and pollution load generated during bleaching of soda-AQ pulp of wheat straw in conventional, ECF and TCF bleaching sequences.

5.2 MATERIALS AND METHODS

5.2.1 Materials

Birchwood xylan was purchased from Sigma Chemicals Co. (USA). All the other chemicals used were of analytical grade from reputed companies of Himedia (Himedia Laboratories, Pvt. Ltd) and Qualigens Fine Chemicals, Mumbai. Crude xylanases, produced by both the test strains (strains *C. disseminatus* SH-1 and SH-2) under optimized conditions of solid state fermentation (S.S.F.), were used in biobleaching experiments. Unbleached wheat straw pulp produced under optimized conditions of soda-AQ pulping process was used in the present study. The unbleached pulp kappa number was 18.25, brightness 27.41 % (ISO) and viscosity 26.04 cps.

5.2.2 Methods

5.2.2.1 Optimization of reaction conditions for enzyme treatment of soda-AQ pulp

The optimization of various operating parameters *viz.*, enzyme doses, reaction times and pulp consistencies for enzyme treatment of soda-AQ pulp of wheat straw was carried out with the crude xylanases obtained from the test strains SH-1 and SH-2. The soda-AQ pulp of wheat straw was disintegrated as per SCAN test methods, SCAN-C 18:65 [84] to open fibre bundles and lumps without fibre cutting prior to commencement of bleaching experiments. Pulp bleaching trials were carried out in temperature controlled water bath. Crude xylanases were used for enzymatic bleaching of soda-AQ pulp of wheat straw because accessory enzymes (like, mannanase, lipase and α-galactosidase) present in crude enzyme preparations might also aid in the bleaching process [27, 116]. The optimum conditions for enzyme treatment were: incubation temperature 55 °C and pH 6.4 for both the test strains, as optimized and described in chapter 2.

5.2.2.1.1 Optimization of enzyme dose for xylanase treatment

Disintegrated, unbleached soda-AQ pulp samples, of 10 g each on oven dry pulp basis, were taken in polyethylene bags maintaining a consistency of 5% with 0.1 M potassium hydroxide buffer at pH 6.4. These pulp samples were separately treated with different doses of crude xylanases obtained from strains SH-1 and SH-2, varying from 0 to 30 IU/g (on o.d. pulp basis) at incubation temperature of 55 °C for reaction time of 2 h. The pulp samples were kneaded by hands after every 15 min to ensure uniform distribution of the enzyme in the pulp samples. Now, the enzyme treated pulps were filtered through cheese cloth and respective filtrates were collected. The pulp samples were then washed with 1 L of tap water and squeezed with hands. The respective filtrates were analyzed for release of reducing sugars by the DNS method [55] at wavelength of 540 nm and for the release of chromophoric compounds by measuring the absorption at wavelengths of 237, 280 and 465 nm [66]. The pulp filtrates collected from the enzyme treated and control pulps were analyzed for the release of chromophores and reducing sugars (as xylose release) to establish a correlation between the release of chromophores (at wavelengths 237, 280 and 465 nm), the reduction in kappa number and the release of reducing sugars. It is worth mentioning, that control experiments (with phosphate buffer in place of enzyme) might also cause some change in pulp properties [87]. The exclusive effect of xylanase action was therefore assessed through comparison with control samples. The enzyme treated pulp was extracted with 2% NaOH (as Na_2O) at temperature 70±2 °C for 90 min and pH 11.0. The alkali extracted pulp samples were then washed with 1 L of tap water, squeezed with hands and analyzed for kappa number (T 236 cm-85), viscosity (T 230 om-04) and brightness, % (ISO) (T 452 om-02) as per Tappi standard test methods [3].

The thick pulp pads for brightness determination were prepared on büchner funnel (T 218 sp-02) as per Tappi standard methods [3]. The same was repeated for blanks by replacing the crude xylanases with an equivalent volume of phosphate buffer.

5.2.2.1.2 Optimization of reaction time for xylanase treatment

In the same way, soda-AQ pulps of wheat straw (10 g each on oven dry pulp basis) were treated with optimized dose of 10 IU/g of crude xylanases obtained from strains SH-1 and SH-2, separately, for different reaction times varying from 1 to 5 h at incubation temperature of 55 °C, pH 6.4 and pulp consistency of 5%. The pulp samples were filtered through cheese cloth and analyzed for release of reducing sugars and chromophores as per methods described above. Similarly, pulp samples were washed with 1 L of tap water and squeezed with hands. The pulp samples were then extracted with 2% NaOH (as Na_2O) at temperature 70±2 °C, pulp consistency 10%, pH 11.0 for 90 min. The alkali extracted pulps were washed with 1 L of tap water, squeezed with hands and subsequently analyzed for kappa number, viscosity and pulp brightness as per Tappi standard test methods [3].

5.2.2.1.3 Optimization of pulp consistency for xylanase treatment

The soda-AQ pulps of wheat straw (10 g each on oven dry pulp basis) were separately treated with crude xylanases obtained from strains SH-1 and SH-2 at optimized dose of 10 IU/g separately at optimum reaction time of 3 h, incubation temperature of 55 °C and pH 6.4 while varying pulp consistencies from 2 to 20%. The pulp samples were washed and filtered through cheese cloth. The collected filtrates were analyzed for release of reducing sugars and chromophores as per methods described above. Similarly, the pulps were extracted with 2% NaOH (as Na_2O) at

temperature 70±2 °C, pulp consistency 10% and pH 11.0 for 90 min. The pulp samples were further washed, squeezed and evaluated for kappa number, viscosity and pulp brightness as per Tappi standard test methods [3].

5.2.2.2 Xylanase treatment in multi-step bleaching process

The effect of xylanase treatment on soda-AQ pulp of wheat straw was studied under multistage conventional, ECF and TCF bleaching sequences. 60 g disintegrated, unbleached soda-AQ pulp of wheat straw was taken for each bleaching sequence.

5.2.2.2.1 Conventional bleaching

The effect of enzyme treatment on soda-AQ pulp of wheat straw was studied in following conventional bleaching sequences i.e., X*ECEHH (using strain SH-1) and X**ECEHH (using strain SH-2).

The soda-AQ pulp of wheat straw was separately prebleached with crude xylanases (X stage) obtained from strains SH-1 and SH-2 at enzyme dose of 10 IU/g, temperature 55 °C, pH 6.4 and reaction time 3 h. The pulp consistency was maintained at 10% for strain SH-1 and 5% for strain SH-2 with 0.1 M potassium hydroxide buffer at pH 6.4. The enzyme treated pulps were extracted with 2% NaOH (as Na_2O) at pH 11.0, consistency 10%, reaction time 90 min and temperature 70±2 °C (E_1 stage). The alkali extracted pulps were filtered through cheese cloth and filtrate was collected for further analysis. The pulps were washed with 2 L tap water, squeezed with hands and evaluated for kappa number as per Tappi standard test methods [3].

The enzyme treated pulps were bleached by CEHH bleaching sequences using different chlorine charges, i.e., 4.5 and 2.25%. In the first case, the total chlorine demand was calculated as (0.25 x kappa number of the pulp), while in the second case,

bleaching was done at 50% reduced chlorine charge (0.5 x kappa number x 0.25). In the similar way, the untreated soda-AQ pulp (without enzyme treatment) was bleached by CEHH bleaching sequence at a total chlorine charge of 4.5%. 70% of the total chlorine charge was charged in 'C' stage and the remaining 30% was charged in two distinct hypochlorite stages 'H' (15% in each H stage).

The chlorination stage (C) was carried out in capped plastic bottles. The disintegrated pulp suspension of consistency 3% was bleached with different doses of molecular chlorine and pH of the suspension was maintained at 2.0 with dilute H_2SO_4 before adding bleach liquor to it. The bottles were immediately capped after addition of bleach liquor so as to prevent loss of chlorine gas. The contents were thoroughly mixed and the chlorination stage was performed at ambient temperature for 45 min. The pulp was then filtered through cheese cloth and the filtrates were collected. The residual chlorine in the collected filtrates was immediately determined and the remaining filtrates were stored at 4 °C for further analysis. The pulp was washed with 2 L of tap water and squeezed with hands.

The chlorinated pulp was extracted with alkali (E_2 stage) to extract chlorolignins. The alkali (NaOH, as Na_2O) was taken as half of the total charge applied in the previous stage in each bleach sequence. The required amount of NaOH and water were mixed with the pulp suspension to achieve a final pulp consistency of 10%. The extraction was carried for 90 min in polyethylene bags at 70 ±2 °C. The pulp was properly mixed from time to time. The pulps were then filtered through cheese cloth and the filtrates were collected for further analysis. The pulp was then washed with 2 L of tap water and squeezed with hands

The alkali extraction stages were followed by hypochlorite, 1st and 2nd stages, respectively. The pulp was mixed with requisite amount of bleach liquor and water so as to maintain a 10% pulp consistency. Both the H-stages were carried out in polyethylene bags at pH 11.0, reaction time 90 min, pulp consistency 10% and temperature 70 ±2 °C. The bags were then removed from the water bath and the pulps filtered through cheese cloth. The filtrates were collected and residual chlorine in the collected filtrates was immediately determined. The remaining filtrates were stored at 4 °C for further analysis. The pulps were washed with 2 L of tap water and squeezed with hands.

In the similar manner the control pulps (without enzyme treatment) were bleached by CEHH bleaching sequence for comparison.

5.2.2.2.2 ECF bleaching

The effect of enzyme treatment on soda-AQ pulp of wheat straw was studied using following ECF bleaching sequences: OX*EDED (using strain SH-1), OX**EDED (using strain SH-2) and OX*EDEP (using strain SH-1), OX**EDEP (using strain SH-2).

Oxygen delignification (O stage) of soda-AQ pulp was performed in autoclaves heated by ethylene glycol bath (CCL Digester-Feronics, Roorkee, India). 2% NaOH (as Na_2O) and 0.1% $MgSO_4$ (on O.D. pulp basis) – as carbohydrate stabilizer, were mixed with the disintegrated pulp and the pulp consistency was maintained at 15%. The pulp was then transferred into autoclaves mounted on stands. Oxygen at a pressure of 5 kg/cm^2 was fed into the autoclaves through the top valve. The autoclaves were checked for leakage and then placed in glycol bath. The pulps were delignified at a temperature

of 110 °C for 90 min. The pulp was then washed through cheese cloth with 2 L of tap water and squeezed with hands. The kappa number of the pulp was determined as per Tappi standard test method [3].

The enzyme treatment (X stage) was given after oxygen delignification, as has also been reported by Blanco *et al.* [11]. Oxygen-delignified pulp was treated with crude xylanases obtained from strains SH-1 and SH-2, separately, and then subjected to alkali extraction stage as already described above for conventional bleaching.

In chlorine dioxide (D stage) bleaching, the enzyme treated pulps were mixed with calculated amount of the $NaClO_2$ solution (of known strength) [42] and water in order to maintain the initial pH at 2.5. The 'D' stage was carried out in polyethylene bags at a pulp consistency of 7% at 70±2 °C. Each chlorine dioxide (D) stage was performed for a reaction time of 1.5 h during ODED bleaching of wheat straw soda-AQ pulp. On the other hand, single chlorine dioxide stage in ODEP bleaching sequence was performed at a reaction time of 3 h. The first stage of chlorine dioxide in ODED bleaching sequence was then followed by 2^{nd} alkali extraction stage (E_2). The conditions for E_2 stages were: NaOH 2% (as Na_2O), temperature 70±2 °C, consistency 10% and reaction time 90 min. The pulp samples were filtered through cheese cloth. Residual chlorine was immediately determined in the collected filtrates and the remaining filtrates were stored at 4 °C for further analysis. The pulps were washed with 2 L of tap water and squeezed with hands.

For the peroxide stage (P), the concentration of peroxide added to the pulp was determined by iodometric titration [42]. Calculated amount of hydrogen peroxide solution and water was added to the disintegrated pulp in polyethylene bags to maintain a consistency of 10%. The peroxide stage was performed at temperature 90±2 °C,

reaction time 2 h and pH 11.8. The pulps were filtered through cheese cloth. The filtrates were immediately analyzed for residual peroxide and the remaining filtrates were stored at 4 °C for further analysis. The pulps were washed with 2 L of tap water and squeezed with hands.

The control soda-AQ pulps (without enzyme stage) were bleached in a similar way using ODED and ODEP bleaching sequences, for comparison.

5.2.2.2.3 TCF bleaching sequence

Soda-AQ pulp of wheat straw was bleached using TCF bleaching sequences i.e., OQPP, OX*EQPP (using strain SH-1) and OX**EQPP (using strain SH-2).

The soda-AQ pulp of wheat straw was oxygen delignified in autoclaves heated by ethylene glycol (CCL Digester-Feronics, Roorkee, India). The oxygen delignification was conducted with 2% NaOH (as Na_2O) and 0.1% $MgSO_4$ (on O.D. pulp basis) - as carbohydrate stabilizer, at O_2 pressure 5 kg/cm^2, pulp consistency 15%, reaction temperature 110 °C, pH 11.0 and reaction time 90 min. The pulps were washed through cheese cloth with 2 L of tap water and squeezed with hands. The oxygen delignified soda-AQ pulps was treated with a xylanase dose of 10 IU/g for 120 min at pH 6.4 for both the strains, separately. The pulp consistency was maintained at 10% for strain SH-1 and 5% for strain SH-2 as described above. The pulps were filtered through cheese cloth and the collected filtrates were stored at 4 °C for further analysis. The pulps were washed with 2 L of tap water and squeezed with hands. The enzyme treated pulps were extracted with 2% NaOH (as Na_2O) at temperature 70±2 °C for 90 min and pH 11.0. The pulps were filtered through cheese cloth and the collected filtrates were stored at 4 °C for further analysis. The pulps were washed with 2 L of tap water and squeezed with hands. The alkali extracted pulps were then subjected to chelation (Q)

stage so as to remove metal ions present in the pulp which may cause decomposition of H_2O_2 and liberate OH radicals. 1% EDTA (on O.D. pulp basis) was applied at a pulp consistency of 3%, reaction time 30 min, pH 5.5 and temperature 50±2 °C. The pulps were filtered through cheese cloth and the filtrates were stored at 4 °C for further analysis. The pulps were further subjected to two consecutive peroxide stages (P_1 and P_2) in which 1.5% peroxide charge was given in each peroxide stage. The reactions were carried out at pulp consistency 10%, temperature 90±2 °C, reaction time 2 h and pH 11.8. The pulps were filtered through cheese cloth. The filtrates were immediately analyzed for residual peroxide and the remaining filtrates were stored at 4 °C for further analysis. The pulps were washed with 2 L of tap water and squeezed with hands. Similarly, the control pulps (without enzyme) were bleached by OQPP bleaching sequence for comparison.

All the pulp samples were stored in the dark at 4 °C until further pulp analysis was carried out.

5.2.2.3 Physical and chemical characterization of pulps

The pulps obtained for various bleaching sequences were evaluated for different pulp characteristics using Tappi standard test methods [3]. The kappa number was determined by $KMnO_4$ titration of the pulp as per Tappi standard test method, T 236 cm-85. For brightness measurement, pulp samples were prepared by Büchner funnel procedure as T 218 sp-02. The brightness (ISO) of these samples was then determined (T 452 om-02) using Technibrite Eric 950 from Technibrite Corporation, USA [97]. The viscosity (CED) of the pulps was estimated by Ostwald viscometer as per Tappi standard test method, T 230 om-04.

The pulps were beaten at a beating level of 40 °SR in a PFI mill according to Tappi standard method (T 248 sp-00). Laboratory hand sheets of 60 g/m^2 were prepared on British sheet former according to Tappi standard method, T 205 sp-02. The hand sheets were conditioned at relative humidity of 65%±2 and temperature of 27±1 °C and evaluated for various physical strength properties like, burst index (T 403 om-02) (Burst tester, Lorentzen & Wettres, Stockholm, Sweden), tensile index (T 494 om-01) (Tensile index tester, Lorentzen & Wettres, Stockholm, Sweden), double fold (T 423 cm-98) (Folding endurance tester, Lorentzen & Wettres, Stockholm, Sweden) and tear index (T 414 om-04) (Tear tester, Lorentzen & Wettres, Stockholm, Sweden). Fifteen hand sheets were prepared for each bleaching sequence for determination of different physical strength properties.

5.2.2.4 Characterization of bleaching effluents

The filtrates collected from various stages of each bleaching sequence were separately mixed in equal amounts and the combined bleach effluents were analyzed for various effluent characteristics. The COD was determined by closed reflux titrimetric method using Thermoreactor CR 2010 [101, 117]. The colour was determined by cobaltiplatinate method at wavelength 465 nm using UV-VIS spectrophotometer (Systronics UV Visible 118). One unit equals to the absorbance produced by 1 mg/mL of platinum present in the form of cobaltiplatinate ion at 465 nm. AOX was measured by AOX Analyzer Dextar ECS 1200.

5.2.2.5 Statistical analysis

For brightness measurement six experimental values, in each case, were taken and the results are mean ± standard deviation (SD) of the values. For kappa number and

viscosity three experimental values, in each case, were taken and the results are mean ± (SD) of the values.

5.2.2.6 Scanning electron microscopy

Samples of control and enzyme-treated pulps were processed for scanning electron microscopy (SEM). The fibres were suspended in water, placed on a cover glass, and allowed to dry [25]. The samples were examined under SEM using gold shadowing technique [23]. Electron photomicrographs were taken at 15 kV using detector SE1 at desired magnifications.

5.3 RESULTS AND DISCUSSION

The xylanase, cellulase (CMCase) and laccase activities of crude enzyme preparations produced under optimized S.S.F. conditions from *Coprinellus disseminatus* strain SH-1 used in biobleaching of soda-AQ pulp of wheat straw were 727.78 IU/mL, 0.925 IU/mL and 0.640 U/mL respectively, and from strain SH-2 were 227.99 IU/mL, 0.660 IU/mL and 0.742 U/mL respectively.

5.3.1 Optimization of reaction conditions for enzyme treatment

5.3.1.1 Optimization of enzyme dose for xylanase treatment

Table 5.1 reveals the optimization of dose of crude xylanases obtained from strains SH-1 and SH-2 during enzymatic treatment of wheat straw soda-AQ pulp at different xylanase doses varying from 5 to 30 IU/g at 55 °C, pulp consistency 5%, pH 6.4 and reaction time 2 h. It was observed that as the dose of crude xylanase (obtained from strain SH-1) increased from 0 to 10 IU/g, the kappa number decreased by 2.13 units and brightness improved by 16.33% compared to control. Beyond it, both kappa number and brightness remained almost constant. Similarly, the kappa number

decreased by 3.98 units and brightness improved by 17.94%, compared to control, when the dose of crude xylanase (obtained from strain SH-2) was increased from 0 to 10 IU/g (Figures 5.1 and 5.3). The decrease in kappa number and increase in pulp brightness up to a xylanase dose of 10 IU/g might be explained on the basis of rapid release of chromophores from the pulp treated with crude xylanases obtained from both the test strains. The release of chromophores decreased beyond a xylanase dose of 10 IU/g (Figures 5.2 and 5.4). This indicated that on further increase in xylanase dose, only small amount of additional LCC (lignin-carbohydrate complex) was attacked. This confirmed that xylanase acts on LCC, releasing degraded chromophoric groups into effluent which is measured at different wavelengths i.e. 237, 280 and 465 nm. However, the amount of reducing sugars released by the crude xylanases from wheat straw soda-AQ pulp increased with increasing xylanase doses for both the test strains (Figure 5.2). The kappa number of soda-AQ pulp of wheat straw at optimum cooking conditions was 18.25. The buffer solution (without enzyme addition) also slightly reduced the kappa number of soda-AQ pulp of wheat straw which was taken as control (Table 5.1). The viscosity of soda-AQ pulp decreased with increasing xylanase doses, where decrease in viscosities at xylanase dose of 10 IU/g was by 0.11 and 0.12% for strain SH-1 and SH-2, respectively (Figures 5.1 and 5.3). Thus, for both the strains xylanase dose of 10 IU/g was found to be optimum for pulp prebleaching. Various studies have indicated that enzyme treatment followed by alkali extraction improved the pulp brightness [53]. The decrease in kappa number after XE stages (enzyme treatment followed by alkali extraction) facilitated the dissolution of lignin-carbohydrate fragments that were previously modified by these enzymes but still remain in pulp because of their large molecular weight [15].

5.3.1.2 Optimization of reaction time for xylanase treatment

Table 5.2 reveals the optimization of reaction time during enzymatic treatment of soda-AQ pulp of wheat straw with crude xylanases obtained from strains SH-1 and SH-2 by varying the reaction time from 1 to 5 h at an optimum enzyme dose of 10 IU/g, pH 6.4, incubation temperature 55 °C and pulp consistency of 5%. The optimum reaction time for both the crude xylanases was found to be 3 h, as decrease in kappa number or increase in pulp brightness was maximum at 3 h of reaction time. For strain SH-1, the kappa number decreased by 4.05 units and pulp brightness improved by 16.17% compared to kappa number and pulp brightness of unbleached soda-AQ wheat straw pulp at incubation period of 3 h. Similarly, the kappa number decreased by 4.25 units and pulp brightness improved by 16.59% compared to kappa number and pulp brightness of unbleached soda-AQ wheat straw pulp, at incubation period of 3 h for strain SH-2 (Figures 5.5 and 5.7). The release of chromophores was also maximum at 3 h of reaction time for crude xylanases obtained from both the test strains. Beyond an incubation period of 3 h the release of chromophoric groups declined (Figures 5.6 and 5.8). The release of reducing sugars increased with increasing reaction time (Figures 5.6 and 5.8). The pulp viscosity slightly decreased with increasing retention time in case of both the test strains. A decrease of 2.35 and 1.37 units in viscosity for strain SH-1 and SH-2 respectively, was observed after 3 h of reaction time (Figures 5.5 and 5.7). Long incubation period might adversely affect pulp viscosity without any appreciable reduction in kappa number or gain in brightness of the pulp.

5.3.1.3 Optimization of pulp consistency for xylanase treatment

Table 5.3 shows the optimization of pulp consistency during enzymatic treatment of soda-AQ wheat straw pulp with crude xylanases obtained from the test strains SH-1 and SH-2 by varying pulp consistency from 2 to 20% at optimum enzyme

dose of 10 IU/g, pH 6.4, temperature 55 °C and reaction time 3 h. Figure 5.9 reveals that as a result of xylanase treatment, the kappa number of wheat straw soda-AQ pulp decreased by 5.05 units and brightness improved by 17.27 units with increasing pulp consistency up to 10% for crude xylanase obtained from strain SH-1. Similarly, the kappa number decreased by 4.92 units and brightness improved by 16.48 units up to 5% pulp consistency for crude xylanase obtained from strain SH-2 (Figure 5.11). Several factors determine the efficiency of enzymatic bleaching of pulps. The extent of reaction product removal is largely a function of substrate characteristics, but enzyme specificity also plays an important role [40]. The interaction of the enzyme with the pulp is also important, including the effective molecular weight, net ionic properties and specific pattern [28]. Thus, the response of any pulp or parameter will vary according to the characteristic features of the enzyme. Also, the cellulosic fibres when merged in water contain mobile and immobile layers surrounding the fibres [62]. As the consistency of pulp is increased, the mobile layer is progressively eliminated leaving only the thin immobile layer enveloping the fibre. This considerably decreases the diffusion path length of reactant to the fibre [37, 76]. Water layer thickness now becomes the rate-determining step. The liquor to raw material ratio is also important because amount of water required depends upon surface area of the raw material because each fibre must receive the same treatment at the same time [18]. It is difficult to get results at high consistency due to limitations in pulp mixing equipment needed to break physical barriers for good mixing. For the decrease in kappa number above pulp consistency of 10%, the pulp is to be finely shredded to separate fibre aggregates to the greatest extent possible before contacting the fibre with reactant [37, 61, 62].The maximum quantity of chromophores was released at pulp consistency of 10% for crude

xylanase obtained from strain SH-1 and 5% for crude xylanase obtained from strain SH-2. Beyond this, there was a decrease in release of chromophores (Figures 5.10 and 5.12). As noticed earlier, the release of reducing sugars kept on increasing with increasing pulp consistency (Figures 5.10 and 5.12). At higher consistency, no appreciable gain in brightness or reduction in kappa number was observed. This might be attributed to the fact that higher consistency kept the enzymes from physically reaching further substrate [24]. The pulp viscosity also slightly decreased with increasing pulp consistency for both the strains (Figures 5.9 and 5.11). For strain SH-1, there was a drop of 2.5 units in viscosity at a pulp consistency of 10%, while for strain SH-2, the viscosity dropped by 1.63 units at a pulp consistency of 5%.

The xylanase dose, reaction time and pulp consistency revealed the correlation between the release of chromophores (at wavelengths 237, 280 and 465 nm) and the reduction in kappa number, which suggested the dissociation of LCC from the fibres [60]. It is known that absorbance at 237 nm is indicative of presence of phenolic groups while absorbance at 465 nm is indicative of presence of hydrophobic compounds [39]. The lignin is believed to absorb strongly at wavelength of 280 nm [47]. Thus, these data suggest that there was a significant decrease in the aromaticity of residual lignin which was confirmed by the decrease in kappa number. The reducing sugars continued being produced as a result of xylanase attack on xylooligomers released by the initial hydrolysis of the xylan coating the fibre surface. This led to the continuous increase in the release of reducing sugars from the pulp. These results are in agreement with earlier observation made by *Garg et al.* [25], that the release of chromophores is a better indicator of xylanase effect on the lingo-cellulose than the liberation of reducing sugars.

Thus, xylanase dose of 10 IU/g, reaction time of 3 h, pulp consistency of 10%, pH 6.4 and incubation temperature of 55 °C were optimum conditions for enzyme treatment using crude xylanase obtained from strain SH-1. All the conditions of xylanase treatment for crude xylanases obtained from strain SH-2 were similar to those for strain SH-1, except pulp consistency which was 5%.

5.3.2 Effect of enzyme treatment on different bleaching sequences

The effect of crude xylanases obtained from both the test strains, strains SH-1 and SH-2 on viscosity, optical and mechanical strength properties and pollution load generated during conventional, ECF and TCF bleaching of soda-AQ wheat straw pulp was studied. Non-oxygen-delignified soda-AQ pulp of wheat straw was bleached by XECEHH bleaching sequence using crude xylanases obtained from both the test strains and results were compared with CEHH bleaching sequence. The soda-AQ pulp of wheat straw was bleached by two ECF bleaching sequences i.e. OXEDED and OXEDEP and compared with ODED and ODEP bleaching sequences. The soda-AQ pulp of wheat straw was bleached by single TCF bleaching sequence i.e. OQPP.

5.3.2.1 Effect of enzyme treatment on kappa number and brightness of pulp

The effect of enzyme treatment on kappa number of non-oxygen-delignified soda-AQ pulp of wheat straw is shown in Table 5.4. Crude xylanases obtained from strains SH-1 and SH-2 mitigated kappa number of unbleached wheat straw soda-AQ pulp by 24.38% and 27.94%, respectively. An oxygen pressure of 5 kg/cm^2 reduced the kappa number of soda-AQ pulp of wheat straw by 45.20%. Both the fungal strains (strains SH-1 and SH-2) reduced the kappa number of oxygen delignified soda-AQ pulps by 20.20 and 24.10%, respectively (Table 5.5).

Direct delignification of pulp would be expected if xylanase attacked (LCC) or xylan that was closely associated with lignin, thus, liberating the lignin component. A role for LCC in the mechanism of xylanase prebleaching has been advocated by many researchers [75, 119].

It was observed that the decrease in kappa number of enzyme treated alkali extracted pulps was more in case of non-oxygen-delignifed pulps (Table 5.4) as compared to oxygen-delignified pulps (Tables 5.5, 5.6, 5.7). When enzyme dose for both non-oxygen-delignified and oxygen-delignified pulps was kept same, the decrease in kappa number of oxygen delignified pulp was less compared to non-oxygen-delignified pulp. The possible reason is that a major part of hemicelluloses was already removed during oxygen delignification and hence, the efficiency of crude xylanases was directly affected.

Table 5.4 shows the effect of enzyme treatment on brightness of soda-AQ pulp of wheat straw in CEHH bleaching sequence. The X*ECEHH (using strain SH-1) and X**ECEHH (using strain SH-2) bleached pulps showed an increase in pulp brightness by 5.17 and 2.58% (ISO), respectively at total chlorine demand of 4.5% compared to pulp brightness of CEHH bleached pulp at same chlorine demand (Figure 5.13). On the other hand, the brightness of X*ECEHH (using strain SH-1) and X**ECEHH (using strain SH-2) bleached pulps was reduced by 5.32 and 7.52% respectively, at total chlorine demand of 2.25% compared to the brightness of CEHH bleached pulp at total chlorine demand of 4.5% (Figure 5.17).

The OX*EDED (using strain SH-1) and OX**EDED (using strain SH-2) bleached soda-AQ pulps of wheat straw showed an improvement in pulp brightness by 7.58 and 10.06%, respectively at a total ClO_2 charge of 2% over brightness of ODED

bleached pulp at the same ClO_2 charge (Table 5.5 and Figure 5.21). The brightness of OX^*EDEP (using SH-1 strain) and $OX^{**}EDEP$ (using SH-2 strain) bleached soda-AQ pulps improved by 6.29 and 10.78% respectively over brightness of ODEP bleached pulp at a total ClO_2 charge of 2% (Table 5.6 and Figure 5.25). The effect of enzyme treatment on pulp brightness in an OQPP bleaching sequence is shown in Table 5.7. The brightness of enzyme treated pulps was improved by 6.07 and 3.34% for strain SH-1 and strain SH-2, respectively, compared to pulp brightness of OQPP bleaching sequence (Figure 5.29).

It is observed that the brightness gain in oxygen delignified enzyme treated pulp was higher as compared to that in case of non-oxygen-delignified pulp. During oxygen delignification the pulp xylan as well as residual lignin content decreased and oxygen delignification made more xylan accessible to the enzyme action. Thus, the enzyme doses for oxygen-delignified pulp would actually be lower compared to non-oxygen-delignified pulp [113]. But as the enzyme dose during bleaching of both non-oxygen-delignified and oxygen-delignified pulps was kept constant, the brightness of oxygen-delignified pulp was higher compared to the brightness of non-oxygen-delignified pulp. This was due to the fact that the final brightness of pulp actually depended upon the initial colour (brightness) of unbleached pulp. Similar observations have also been reported by Jeffries *et al.* [113].

Washing of pulps was done after each bleaching stage, including enzyme pretreatment. Many studies indicate that washing of pulp should be done after enzyme treatment [70, 121], possibly because without washing impregnation of subsequent bleaching chemicals would not be proper as the diffusion and porosity of the pulp would decrease. This in turn would be due to incomplete dissolution of water-soluble

products with low molecular weight substances resulting from partial hydrolysis of xylan in enzymatic pretreatment stage [1, 10].

5.3.2.2 Effect of enzyme treatment on pulp viscosity

Table 5.4 shows the effect of enzyme treatment on pulp viscosity of soda-AQ pulp of wheat straw in a CEHH bleaching sequence. The X*ECEHH (using strain SH-1) and X**ECEHH (using strain SH-2) bleached pulps at a total chlorine demand of 4.5% showed an improvement in viscosity by 6.75 and 6.0%, respectively (Figure 5.13), as compared to CEHH bleached pulp. The X*ECEHH (using strain SH-1) and X**ECEHH (using strain SH-2) bleached pulps at a total chlorine demand of 2.25% showed an increase in pulp viscosity by 7.25% and 7.12%, respectively, when compared with CEHH bleached pulp (total chlorine demand 4.5%) (Figure 5.17). The increase in viscosity of enzyme treated pulps in CEHH bleach sequence, at a total chlorine demand of 2.25% was a little higher as compared to CEHH bleaching at a total chlorine demand of 4.5%. This could be attributed to the lesser degradation of carbohydrates owing to the application of reduced chemical charge in the former case.

Table 5.5 shows the effect of enzyme treatment on pulp viscosity of wheat straw soda-AQ pulp in ODED bleaching sequence. OX*EDED (using strain SH-1) and OX**EDED (strain SH-2) bleached soda-AQ wheat straw pulp at total ClO_2 charge of 2% showed an increase in pulp viscosity by 6.82 and 4.93%, respectively, as compared to ODED bleached pulp (Figure 5.21).

The effect of enzyme treatment on pulp viscosity of wheat straw soda-AQ pulp in ODEP bleaching sequence is shown in Table 5.6. OX*EDEP (using strain SH-1) and OX**EDEP (using strain (SH-2) bleached pulps at total ClO_2 charge of 2% showed an improvement in viscosity by 6.33 and 4.22%, respectively, as compared to ODEP bleached pulp (Figure 5.25).

The effect of enzyme treatment on pulp viscosity of soda-AQ pulp of wheat straw in OQPP bleaching sequence is shown in Table 5.7. OX*EQPP (using strain SH-1) and OX**EQPP (using strain (SH-2) bleached pulps showed an improvement in viscosity by 6.07 and 4.58% respectively, as compared to OQPP bleached pulp (Figure 5.29). Thus, it is observed that there was an increase in pulp viscosity of enzyme treated pulp as compared to controls in all the bleaching sequences. However, a moderate loss of viscosity was observed after enzyme treatment (Tables 5.1 to 5.3). This may be due to some other reason than cellulose-degrading enzyme activity. Similar observations have been made by Medeiros *et al.* [53], while working on biobleaching of eucalyptus kraft pulp with xylan degrading enzyme preparations from *Acrophialophora nainiana*, *Humicola grisea var. thermoidea* and two *Trichoderma harzianum* strains.

The increase in viscosity might be a result of the selective removal of lower DP (degree of polymerization) xylan and enrichment of high molecular weight polysaccharides. Similar observations have also been reported by Kantelinen *et al.* [36], while they worked on xylanase bleaching of kraft pulp to study its mechanism, and Paice *et al.* [65], while they worked on biological bleaching of hardwood kraft pulp with the fungus *Coriolus versicolor*.

The increase in viscosity also indicated that there was no adverse effect of small amount of cellulases (CMCases) present in crude enzyme samples. The viscosity is known to decrease when cellulases cleave cellulose chains, lowering the degree of cellulose polymerization and destroying fibre integrity [40]. CMCases are known to act upon amorphous region of cellulose [48]. In fact, a mild cellulase activity might prove beneficial, considering the fact that mild cellulase activity in hemicellulase preparations could improve pulp fibrillation and induce better bonding of fibres [49]. Researchers

have also reported that endo-glucanases might play a crucial role in the improvement of drainage [46, 112].

5.3.2.3 Effect of enzyme treatment on pulp beating level

Table 5.4 shows the effect of enzyme treatment on beating response of soda-AQ wheat straw pulp in CEHH bleaching sequence. X*ECEHH (using strain SH-1) and X*ECEHH (using strain SH-2) bleached pulps required 66.66% and 64.64% (Figure 5.14) lesser PFI mill revolutions to reach a beating level of 40 °SR as compared to CEHH bleached pulp at a total chlorine demand of 4.5%. X*ECEHH (using strain SH-1) and X**ECEHH (using strain SH-2) pulps bleached at total chlorine demand of 2.25% required 30.30% and 28.28% lesser number of PFI revolutions to reach beating level of 40 °SR as compared to CEHH bleached pulp (Figure 5.18) .

It is a well-known fact that the hemicellulose content influences the beating behavior of pulp [4]. The improvement in drainage is attributed to the removal of fibre materials with a high hydrophilic property, change of the fibre structure and liberation of free-water by enzymatic treatment. Straw pulp, as such, has a poor drainage property due to its higher fines content when compared to wood pulp. It is also characterized by a high pentose content, low crystallinity, and low viscosity. As a result of xylanase treatment the drainage property of straw pulp improved due to decrease in the fine content. The experimental results showed that enzymatic treatment in a CEHH bleaching sequence could decrease the Schopper Reigler of pulp for a targeted °SR. Jhao *et al.* [120] has also reported that enzymatic treatment can improve the drainage of wheat straw pulp, on its treatment with crude enzymes produced by *Penicillium A10* and *Aspergillus L22* in CEH bleaching sequence.

OX*EDED (for strain SH-1) and OX**EDED (for strain SH-2) bleached wheat straw soda-AQ pulp at total ClO_2 charge of 2% required 10.00% and 5.55% more PFI mill revolutions to reach a beating level of 40 °SR compared to ODED bleached pulp (Table 5.5 and Figure 5.22). Similarly, OX*EDEP (for strain SH-1) and OX**EDEP (for strain SH-2) bleached wheat straw soda-AQ pulp at total ClO_2 charge of 2% required 13.04% and 8.69% more number of PFI mill revolutions as compared to ODEP bleached pulp (Table 5.6 and Figure 5.26). The increase in PFI mill revolutions after enzymatic treatment to reach a given °SR might be attributed to the fact that the latter pulps contained lesser quantity of low DP xylan (favorable for pulp beating) due to removal of xylan during oxygen delignification [80].

Table 5.7 shows the effect of enzyme treatment on beating level of wheat straw soda-AQ pulp in an OQPP bleaching sequence. OX*EQPP (for strain SH-1) and OX**EQPP (for strain SH-2) bleached pulp required 5.55% and 11.11% lesser number of PFI mill revolutions as compared to ODEP bleached pulp (Figure 5.30).

As discussed above, the improvement in drainage in OQPP bleaching sequence can also be attributed to the removal of fibre materials with a high hydrophilic property, change of the fibre structure and liberation of free-water by enzymatic treatment.

5.3.2.4 Effect of enzyme treatment on mechanical strength properties of the pulp

Table 5.4 shows the effect of enzyme treatment on mechanical strength properties of CEHH bleached wheat straw soda-AQ pulp. The tensile and burst indices were reduced by 11.36 and 8.09% (Figure 5.15), respectively, when soda-AQ wheat straw pulp was bleached by X*ECEHH (using strain SH-1) bleaching sequence, as compared to CEHH bleaching sequence at total chlorine demand of 4.5%. The tensile and burst indices of X*ECEHH (using strain SH-1) pulp bleached at a total chlorine

demand of 2.25% were reduced by 5.99 and 3.36% (Figure 5.19) respectively, when compared to CEHH pulp bleached at total chlorine demand of 4.5%. On the other hand, double fold and tear index of X*ECEHH (using strain SH-1) bleached pulp showed an improvement over double fold and tear index of CEHH bleached pulp at same chlorine demand i.e. 4.5% (Figure 5.15). There was a significant increase in double fold (15.71%) and tear index (13.96%) of X*ECEHH (using strain SH-1) bleached pulp over double fold and tear index of CEHH bleached pulp when total chlorine demand in case of X*ECEHH bleaching sequence was half of that used in CEHH bleaching sequence (Figure 5.19). Similarly, wheat straw soda-AQ pulp was bleached by X**ECEHH (using strain SH-2) bleaching sequences using total chlorine demand of 4.5 and 2.25% separately, and their tensile and burst indices were compared with the tensile and burst indices of CEHH pulp bleached at total chlorine demand 4.5%. The reduction in tensile index of X**ECEHH (using strain SH-2) pulps bleached at a total chlorine demand of 4.5 (Figure 5.15) and 2.25% (Figure 5.19) was on a lower side compared to tensile index of X*ECEHH (using strain SH-1) pulps bleached at a total chlorine demand of 4.5% and 2.25%, respectively. The reduction in burst index of X**ECEHH (using strain SH-2) pulps bleached at total chlorine demand of 4.5% (Figure 5.15) was also on a lower side compared to burst index of X*ECEHH (using strain SH-1) pulps bleached at same chlorine demand. The reduction in burst index of X**ECEHH (using strain SH-2) pulps bleached at a total chlorine demand of 2.25% (Figure 5.19) was slightly on a higher side compared to burst index of X*ECEHH (using strain SH-1) pulps bleached at the same chlorine demand. The double fold and tear index of X**ECEHH (using strain SH-2) pulps bleached at a total chlorine demand of 4.5 (Figure 5.15) and 2.25% (Figure 5.19) respectively, slightly improved over

double fold and tear index of X*ECEHH (using strain SH-1) pulps bleached at total chlorine demand of 4.5% and 2.25% respectively.

The tensile index, burst index, tear index and double fold of soda-AQ pulp of wheat straw pulp bleached by ODED bleaching sequence (Table 5.5 and Figure 5.23) showed marginal increase over tensile index, burst index, tear index and double fold of soda-AQ pulp of wheat straw pulp bleached by CEHH bleaching sequence (Table 5.4). The tensile index, burst index and double fold of soda-AQ pulp of wheat straw pulp bleached by OX*EDED (using strain SH-1) bleaching sequence decreased by 16.12, 10.47 and 10.60%, respectively, over tensile index, burst index and double fold of soda-AQ pulp of wheat straw pulp bleached by ODED bleaching sequence but the decrease in tensile index, burst index and double fold was lesser when pulp was bleached by OX**EDED (using strain SH-2) bleaching sequence. The tear index improved almost equally in both the bleaching sequences i.e. OX*EDED (using strain SH-1) and OX**EDED (using SH-2 strains) bleaching sequences over ODED bleaching sequence.

All the mechanical strength properties i.e. tensile index, burst index, double fold and tear index of soda-AQ pulp of wheat straw pulp bleached by ODEP bleaching sequence (Table 5.6 and Figure 5.27) showed a marginal improvement over mechanical strength properties of ODED bleached pulp (Table 5.5). The tensile and burst indices of OX*EDEP (strain SH-1) bleached pulp decreased by 16.30 and 9.46% respectively, over ODEP bleached pulp. This decrease in OX**EDEP (using strain SH-2) bleaching sequence was slightly on lower side compared to OX*EDEP (strain SH-1) bleaching sequence. On the other hand, double fold and tear index of OX*EDEP (strain SH-1) bleached pulp improved by 5.97 and 2.61% respectively over ODEP bleached pulp but improvement in double fold and tear index of OX**EDEP (using strain SH-2) bleached

pulp was more i.e. 7.46 and 3.17% respectively over OX**EDEP (using strain SH-2) bleached pulp.

The tensile and burst indices and double fold of OX*EQPP (using strain SH-1) bleached soda-AQ pulp of wheat straw showed a decrease by 16.44, 11.98 and 10.44%, respectively over tensile and burst indices and double fold of OQPP bleached pulp. This decrease in tensile and burst indices and double fold of OX**EQPP (using strain SH-2) was lesser compared to OX*EQPP (using strain SH-1) bleaching sequence. The tear index in both cases i.e. OX*EQPP (using strain SH-1) and OX**EQPP (using strain SH-2) bleaching sequences was more or less same but improved over OQPP bleaching sequences (Table 5.7 and Figure 5.31).

The tear index is greatly influenced by fibre length but the tensile and burst indices are influenced by both the fibre length and extent of hydrogen bonding. Studies on wheat straw have shown that the xylanase treated wheat straw pulps have longer fibre on average and lower fines contents than the control. Both of these factors seem to be disadvantageous for fibre bonding in the pulps. The average longer fibre average length has been shown to lead to higher tear index of a sheet prepared from the xylanase treated wheat straw soda pulp [121]. The retention of hemicelluloses has been shown to be favorable for the strength (mechanical) properties of paper, because of their positive effect on the interfibrillar bonding during paper-sheet formation [16]. The quantity, chemical structure, distribution, and the degree of polymerization of the hemicelluloses have also been shown to play an intricate role in determining the final paper strength [22]. The decrease in tensile and burst indices indicated that a large percentage of the hemicelluloses were hydrolyzed during biobleaching of soda-AQ pulp of wheat straw. The hydrolysis of hemicelluloses in case of biobleaching of soda-

AQ wheat straw pulp with enzyme preparations obtained from strain SH-2 was less compared to that obtained for strain SH-1. It is possible that both the removal of hemicelluloses and the concomitant reduction in the degree of polymerization of the residual hemicelluloses might have contributed to the reduced paper strength. The increase in tear index can be attributed to the fact that even though excess xylan removal can reduce burst and tensile strength by reducing inter-fibre bonding, yet it does not weaken the fibres themselves [77].

5.3.2.5 Effect of enzyme treatment on combined bleach effluent characteristics

Table 5.4 shows the effect of enzyme treatment on AOX, COD and colour of combined bleach effluent characteristics generated during X*ECEHH (using strain SH-1) and X**ECEHH (using strain SH-2) bleaching of soda-AQ wheat straw pulp and its comparison with conventional bleaching sequence. The AOX of combined effluent of X*ECEHH (using strain SH-1) wheat straw soda-AQ pulp bleached at total chlorine demand of 4.5 and 2.25% was reduced by 56.11 (Figure 5.16) and 68.34%, respectively (Figure 5.20), compared to CEHH bleaching sequence bleached at chlorine demand of 4.5%. The decrease in AOX of combined effluent of X**ECEHH (using strain SH-2) wheat straw soda-AQ pulp bleached at total chlorine demand of 4.5 and 2.25%, respectively, remained almost similar to that in X*ECEHH (using strain SH-1) bleaching sequence. The COD and colour of combined effluent of X*ECEHH (using strain SH-1) wheat straw soda-AQ pulp bleached at total chlorine demand of 4.5% increased by 10.91 and 38.00%, respectively (Figure 5.16), compared to CEHH bleaching sequence. When soda-AQ wheat straw pulp was bleached by X*ECEHH (using strain SH-1) bleaching sequence using total chlorine demand of 2.25%, the COD and colour of combined effluent decreased by 18.03 and 60.19%, respectively (Figure

5.20), compared to CEHH bleaching sequence of soda-AQ wheat straw pulp bleached at total chlorine demand of 4.5%.

The AOX, COD and colour of combined effluent of ODED bleached soda-AQ pulp of wheat straw were lesser by 75.53, 56.91 and 91.42%, respectively, compared to CEHH bleaching sequence. The AOX of combined effluent of OX*EDED (using strain SH-1) and OX**EDED (using strain SH-2) soda-AQ wheat straw pulp bleached at total ClO_2 charge of 2% showed a significant decrease in AOX by 25.00% and 22.05% respectively, compared to ODED bleached pulp (Figure 5.24). The COD and colour of combined effluent of OX*EDED (using strain SH-1) and OX**EDED (using strain SH-2) bleached soda-AQ wheat straw pulp increased by 43.15 & 51.99% and 43.33 & 62.99%, respectively, compared to ODED bleaching sequence (Figure 5.24).

As would be expected, the values for COD and colour (A 465 nm) of enzyme treated oxygen-delignified pulp were lower than for non-oxygen-delignified pulp. This was due to the fact that the oxygen bleached pulps show a yellowish colour and were already clear in comparison with the non-oxygen-delignified pulp. This result could be related to lignin depolymerisation, as was suggested by Patel *et al.* [66], while they treated kraft pulps with crude xylanase from *Streptomyces roseiscleroticus*.

The AOX of combined effluent in ODEP (Table 5.6) bleaching sequence was slightly on a higher side but COD and colour decreased by 27.07 and 15.52%, respectively, compared to ODED (Table 5.5) bleaching sequence. The combined effluent of OX*EDEP (strain SH-1) soda-AQ wheat straw pulp bleached at total ClO_2 charge of 2% showed an increase in COD and colour of 40.84 and 53.55%, respectively. The combined effluent of OX**EDEP (using strain SH-2) soda-AQ wheat straw pulp bleached at total ClO_2 charge of 2% showed an increase in COD and colour

of 48.00 and 66.89%, respectively. The AOX of the combined effluent of OX*EDEP (strain SH-1) and OX**EDEP (using strain SH-2) soda-AQ pulp wheat straw bleached at total ClO_2 charge of 2% decreased by 38.75 and 36.25%, respectively, in comparison to ODEP bleaching sequence (Figure 5.28).

The combined effluent of OX*EQPP (using strain SH-1) and OX**EQPP (using strain SH-2) bleached soda-AQ wheat straw pulp showed an increase in COD (40.0 and 38.00%, respectively) and colour (47.99 and 44.99%, respectively) compared to OQPP bleached pulp (Figure 5.32).

The increase in COD and colour of effluents from enzyme treated pulps might be due to the hydrolytic action of the enzyme leading to weakening of the carbohydrate bonds in the pulp and its dissolution into the media. Thus, the concentration of lignin and hydrolyzed xylan in the effluent was also significantly enhanced. Increase in COD and colour of effluents from enzyme treated pulps in different bleaching sequences has also been reported in other studies as well [81, 105].

The percent increase in COD and colour of enzyme treated oxygen-delignified pulp from their respective controls (without enzyme treatment), was slightly greater than that observed for enzyme treated non-oxygen-delignified pulp from their respective controls. It might be due to greater dissolution of carbohydrates and lignin in case of enzyme treated oxygen delignified pulps in comparison to enzyme treated non-oxygen-delignified pulps. Similar observation has also been made by Jeffries *et al.* for kraft pulp[113].

The reduction in AOX was remarkable as a reduction of 20-45% in AOX values of effluents from xylanase treated pulps has been reported in the literature for different bleaching sequences [30, 43, 56, 86].

Tables 5.4, 5.5 and 5.6 show the effect of enzyme treatment on residual chlorine in CEHH, ODED and ODEP bleaching sequences, respectively. Tables 5.6 and 5.7 show the effect of enzyme treatment on residual peroxide in ODEP and OQPP bleaching sequences, respectively. As can be observed, slightly less bleach chemicals were utilized by the enzyme treated pulps compared to their respective controls, suggesting that the treated pulp reached the chemical saturation point sooner than the control pulp [53]. This indicated that the charge of chemicals in the beginning of the stage can be reduced or that stage parameters can be changed in order to allow an increase in pulp brightness.

5.3.2.6 Scanning electron microscopy (SEM)

In order to obtain a better understanding of the effect of xylanase treatment on enhanced bleachability, the fibre surface morphology of the pulps was studied by SEM. The microphotographs 5.1A to F show significant changes on the fibre surface of the xylanase treated pulps as a result of the xylan hydrolysis (Microphotographs 5.1B, C, E, and F). No such effect was observed in the untreated pulps (Microphotographs 5.1A and D). Microphotograph 5.1A shows an unbleached non-oxygen-delignified control pulp while microphotographs 5.1B and C are the enzyme treated non-oxygen-delignified pulps for *C.* strain SH-1 and strain SH-2, respectively. Microphotograph 5.1D shows an oxygen delignified control pulp, while microphotographs 5.1E and F shows oxygen delignified enzyme treated pulps. It can be seen that treated pulps underwent a peeling process giving rise to flakes and filaments of material detached from the fibre surface. The surface of control pulps was smoother and cleaner as compared to enzyme treated pulps. The control pulps did not show fibrillation but they did show fibres of different thickness. On the other hand, the pulps after enzyme

treatment showed fibres that are rougher and more heterogeneous, with small filaments on the surface of the fibres. The enzyme treated pulps showed cracking and delamination of the cell wall. In all cases, enzymes produced changes in the surface of the fibre owing to the hydrolysis that they cause. This is in agreement with the findings of other researchers [25, 72] as well. In oxygen-delignified pulps (Microphotographs 5.1E and F), the enzymes seem to produce greater fibrillation when compared with the non-oxygen-delignified pulps (Microphotographs 5.1B and C). On the latter, the main morphological change visualized was peeling, as has also been observed earlier by Salles *et al.* [83]. The greater fibrillation in oxygen-delignified enzyme treated pulps supported the higher difference in brightness achieved in these pulps compared to non-oxygen-delignified enzyme treated pulps. Xylanase treatment seemed to be effective in opening the closed cell-wall-pores of the pulps. The cracks formed due to enzyme treatment facilitated the diffusion of larger lignin macromolecules out through the fibre cell wall, as reported earlier [36, 44]. Thus, the xylanase treatment improved the accessibility of the pulps for the bleaching chemicals, thereby decreasing kappa number and increasing brightness of the enzyme treated pulps.

Table 5 1 Optimization of enzyme dose for xylanase treatment

Enzyme	Particulars	Enzyme dose, IU/g						
		0	5	10	15	20	25	30
X*	Kappa no. (after NaOH extraction)	17.98 ±0.12	17.30 ±0.10	15.85 ±0.11	16.45 ± 0.12	16.68 ±0.14	17.00 ±0.10	17.20 ±0.08
	Viscosity, cps	25.65 ±0.12	25.30 ± 0.13	24.71 ± 0.11	24.33 ± 0.10	24.00 ± 0.14	23.51 ± 0.13	22.98 ± 0.15
	Brightness, % (ISO)	27.64 ±0.21	38.76 ±0.34	43.97 ±0.19	42.33 ±0.15	41.57 ±0.11	41.24 ±0.13	40.64 ±0.17
	Reducing sugars, mg/g	0.20	4.05	6.30	10.70	14.00	14.79	15.20
	Chromophore release, 465 nm	0.02	0.242	0.451	0.405	0.360	0.332	0.302
	Chromophore release, 280 nm	0.05	0.310	0.497	0.452	0.429	0.410	0.349
	Chromophore release, 237 nm	0.04	0.309	0.458	0.419	0.390	0.369	0.352
X**	Kappa no. (after NaOH extraction)	—	15.20 ± 0.10	14.00 ± 0.15	14.98 ± 0.20	15.34 ± 0.19	16.47 ± 0.15	16.70 ± 0.21
	Viscosity, cps	—	25.51 ± 0.13	24.89 ± 0.12	24.60 ± 0.11	24.24 ± 0.12	23.78 ± 0.14	23.10 ± 0.10
	Brightness,% (ISO)	—	44.44 ±0.29	45.58 ±0.30	44.46 ±0.18	43.98 ±0.20	43.45 ±0.17	42.98 ±0.19
	Reducing sugars, mg/g	—	6.14	7.35	12.85	14.89	15.40	16.59
	Chromophore release, 465 nm	—	0.294	0.489	0.421	0.380	0.348	0.276
	Chromophore release, 280 nm	—	0.321	0.514	0.480	0.454	0.431	0.398
	Chromophore release, 237 nm	—	0.318	0.464	0.421	0.410	0.389	0.367

Conditions for enzyme treatment:		**Conditions for alkali extraction:**	
Reaction pH	= 6.4	Reaction pH	= 11.0
Reaction time, h	= 2	Reaction time, min	= 90
Temperature, °C	= 55 ± 2.0	Temperature, °C	= 70±2
Initial kappa number	= 18.25	Consistency, %	= 10%
Initial brightness, % (ISO)	= 27.41		
Initial viscosity, cps	= 26.04		
Consistency, %	= 5		

X* Crude xylanases from strain SH-1
X** Crude xylanases from strain SH-2
± Standard deviation from the mean

Table 5.2 Optimization of reaction time for xylanase treatment

Enzyme	Particulars	Reaction time, h				
		1	2	3	4	5
X*	Kappa no. (after NaOH extraction)	16.39 ± 0.19	15.24 ± 0.21	14.20 ± 0.14	15.16 ±0.11	15.67 ±0.23
	Viscosity, cps	24.81 ±0.20	24.57 ±0.23	23.69 ± 0.18	23.12 ± 0.13	22.54 ± 0.20
	Brightness, % (ISO)	39.79 ±0.31	42.14 ±0.26	43.58 ±0.19	42.50 ±0.24	42.12 ±0.25
	Reducing sugars, mg/g	4.12	8.60	13.44	15.95	18.42
	Chromophore release, 465 nm	0.151	0.329	0.522	0.411	0.402
	Chromophore release, 280 nm	0.265	0.501	0.725	0.698±	0.624
	Chromophore release, 237 nm	0.242	0.459	0.612	0.565	0.560
X**	Kappa no. (after NaOH extraction)	15.18 ± 0.14	14.80 ±0.13	14.00 ±0.12	14.88 ±0.10	15.08 ±0.10
	Viscosity, cps	25.60 ±0.22	25.04 ±0.20	24.67 ±0.15	23.95 ±0.11	23.21 ±0.17
	Brightness, % (ISO)	41.81 ±0.22	43.10 ±0.25	44.00 ±0.10	42.77 ±0.18	41.98 ±0.12
	Reducing sugars, mg/g	5.80	9.20	14.89	17.50	21.02
	Chromophore release, 465 nm	0.280	0.460	0.721	0.642	0.600
	Chromophore release, 280 nm	0.310	0.520	0.776	0.721	0.700
	Chromophore release, 237 nm	0.268	0.478	0.629	0.610	0.604

Conditions for enzyme treatment:		**Conditions for alkali extraction:**	
Enzyme dose, IU/g	= 10	Reaction pH	= 11.0
Reaction pH	= 6.4	Reaction time, min	= 90
Temperature, °C	= 55 ± 2.0	Temperature, °C	= 70±2
Initial kappa number	= 18.25	Consistency, %	= 10%
Initial brightness, % (ISO)	= 27.41		
Initial viscosity, cps	= 26.04		
Consistency, %	= 5		

X* Crude xylanases from strain SH-1
X** Crude xylanases from strain SH-2
± Standard deviation from the mean

Table 5.3 Optimization of pulp consistency for xylanase treatment

Enzyme	Particulars	Pulp consistency, %				
		2	5	10	15	20
X*	Kappa No. (after NaOH extraction)	15.63 ± 0.17	14.18 ± 0.11	13.20 ± 0.13	13.54 ± 0.15	13.92 ± 0.21
	Viscosity, cps	24.85 ± 0.12	24.48 ± 0.16	23.54 ± 0.20	23.00 ± 0.18	22.16 ± 0.15
	Brightness, % (ISO)	40.53 ±0.31	43.58 ±0.27	44.68 ±0.24	43.54 ±0.17	42.77 ±0.22
	Reducing sugars, mg/g	5.50	11.78	17.21	17.58	18.14
	Chromophore release, 465 nm	0.430	0.540	0.797	0.712	0.674
	Chromophore release, 280 nm	0.321	0.642	0.786	0.722	0.700
	Chromophore release, 237 nm	0.347	0.458	0.620	0.602	0.576
X**	Kappa No. (after NaOH extraction)	15.00 ± 0.15	13.33 ± 0.11	13.89 ± 0.19	14.02 ± 0.12	14.29 ± 0.11
	Viscosity, cps	24.78 ± 0.24	24.41 ± 0.27	23.97 ± 0.31	23.34 ± 0.29	22.89 ± 0.21
	Brightness, % (ISO)	42.72 ±0.19	43.89 ±0.17	43.31 ±0.22	42.77 ±0.20	40.33 ±0.13
	Reducing sugars, mg/g	7.71	15.50	16.40	16.81	17.91
	Chromophore release, 465 nm	0.448	0.801	0.756	0.743	0.701
	Chromophore release, 280 nm	0.564	0.821	0.789	0.758	0.721
	Chromophore release, 237 nm	0.500	0.631	0.600	0.576	0.543

Conditions for enzyme treatment:		**Conditions for alkali extraction:**	
Enzyme dose, IU/g	= 10	Reaction pH	= 11.0
Reaction pH	= 6.4	Reaction time, min	= 90
Temperature, °C	= 55 ± 2.0	Temperature, °C	= 70±2
Initial kappa number	= 18.25	Consistency, %	= 10%
Initial brightness, % (ISO)	= 27.41		
Initial viscosity, cps	= 26.04		
Reaction time, h	= 3		

X* Crude xylanases from strain SH-1
X** Crude xylanases from strain SH-2
± Standard deviation from the mean

Table 5.4 Effect of enzyme treatment on soda-AQ pulp of wheat straw in CEHH bleaching sequence

Particulars	Bleaching sequences				
	CEHH	X*ECEHH	X*ECEHH	X**ECEHH	X**ECEHH
Enzyme dose, X*, IU/g§	—	10	10	—	—
Enzyme dose, X**, IU/g§	—	—	—	10	10
Extraction (E_1), NaOH applied§, %	—	2	2	2	2
Kappa number	—	13.80 ± 0.14	13.80 ± 0.14	13.15 ± 0.11	13.15 ± 0.11
Total Chlorine charge§, %	4.5	4.5	2.25	4.5	2.25
Chlorination (C) stage§					
% Cl_2 applied as available Cl_2	2.25	2.25	1.12	2.25	1.12
Cl_2 consumed as available Cl_2	2.24	2.23	1.11	2.22	1.10
Extraction (E_2), NaOH applied§, %	1.12	1.12	0.56	1.12	0.56
Hypochlorite (H_1) stage§					
% $Ca(OCl)_2$ applied as available Cl_2	1.57	1.57	0.78	1.57	0.78
% Ca $(OCl)_2$ consumed as available Cl_2	1.55	1.53	0.77	1.54	0.76
Hypochlorite (H_2) stage§					
% $Ca(OCl)_2$ applied as avail. Cl_2	0.675	0.675	0.337	0.675	0.337
% Ca $(OCl)_2$ consumed as available Cl_2	0.674	0.672	0.334	0.671	0.334
Final brightness, % (ISO)	81.10±0.17	85.30±0.11 (+4.2)	76.78±0.20 (-4.32)	83.20±0.19 (+2.10)	75.00±0.23 (-6.1)
Pulp viscosity, cps	8.0 ± 0.05	8.54 ± 0.04 (+6.80)	8.58 ± 0.04 (+7.35)	8.48 ± 0.02 (+6.0)	8.57 ± 0.12 (+7.18)
Pulp beating					
Beating level, °SR	40	40	40	40	40
PFI mill revolutions	990	330 (—66.66)	690 (—30.30)	350 (—64.64)	710 (—28.28)
Mechanical strength properties					
Tensile index, Nm/g	49.22	43.63 (—11.36)	46.27 (—6.00)	43.94 (—10.74)	47.06 (—4.40)
Burst index, kPa m^2/g	3.27	3.01 (—8.09)	3.16 (—3.55)	3.05 (—6.94)	3.15 (—3.73)
Double fold, no.	64	69 (+7.52)	74 (+15.71)	70 (+8.63)	75 (+16.50)
Tear index, mNm^2/g	5.27	5.30 (+0.59)	6.00 (+13.96)	5.31 (+0.73)	6.13 (+16.46)
AOX, kg/t (Combined bleach effluent)	2.78	1.22 (—56.11)	0.88 (—68.34)	1.23 (—55.75)	0.89 (—67.98)
COD, mg/L (Combined bleach effluent)	3170	3516.00 (+10.93)	2598.20 (—18.04)	3471.00 (+9.50)	2517.00 (—20.60)
Colour, PCU (Combined bleach effluent)	1743	2405.34 (+38.00)	693.72 (—60.20)	2422.77 (+39.00)	744.44 (—57.29)

Bleaching conditions	**X***	**X****	**E_1**	**C**	**E_2**	**H_1**	**H_2**
pH	6.4	6.4	11.0	2	11.0	11.0	11.0
Consistency, %	10	5	10	3	10	10	10
Retention time, min	180	180	90	45	90	90	90
Temperature, °C	55±2	55±2	70±2	ambient	70±2	70±2	70±2

Unbleached pulp kappa number = 18.25; Unbleached pulp brightness, % (ISO) = 27.41; Unbleached pulp viscosity, cps = 26.04

*X Crude xylanases from strain SH-1
**X Crude xylanases from strain SH-2
§ Chemical charge on O.D. pulp basis
(+/—) % difference compared to control pulp
± Standard deviation from the mean

Table 5.5 Effect of enzyme treatment on ODED bleaching sequence of soda-AQ pulp of wheat straw

Particulars	Bleaching sequences		
	ODED	OX*EDED	OX**EDED
Oxygen (O) stage§			
Pressure, kg/cm^2	5	5	5
NaOH, %	2	2	2
MgSO4, %	0.1	0.1	0.1
Kappa number after oxygen delignification	10.00 ± 0.14	10.00 ± 0.14	10.00 ± 0.14
Enzyme dose, X*, IU/g§	—	10	—
Enzyme dose, X**, IU/g§	—	—	10
Extraction (E_1), NaOH applied§, %	—	2	2
Kappa number	—	7.98± 0.13	7.59 ± 0.11
Total chlorine dioxide charge§, %	2	2	2
D_1 stage			
% ClO_2 applied as available Cl_2	1	1	1
% ClO_2 consumed as available Cl_2	0.96	0.97	0.99
D_2 stage			
% ClO_2 applied as available Cl_2	1	1	1
% ClO_2 consumed as available Cl_2	0.99	0.97	0.95
Extraction (E_2), NaOH applied§, %	0.5	0.5	0.5
Final brightness, % (ISO)	73.85 ±	79.45± (+7.58)	81.28 ± (+10.06)
Final viscosity, cps	9.53 ± 0.10	10.18 ± 0.13 (+6.82)	10.00 ± 0.11 (+4.93)
Pulp beating			
Beating level, °SR	40	40	40
PFI mill revolutions	900	990 (+10.00)	950 (+5.55)
Mechanical strength properties			
Tensile index, Nm/g	49.60	41.60 (—16.12)	43.60 (—12.09)
Burst index, kPa m^2/g	3.34	2.99 (—10.47)	3.01 (—9.88)
Double fold, no.	66	59 (—10.60)	60 (—9.09)
Tear index, mNm^2/g	5.30	5.46 (+3.01)	5.44 (+2.64)
AOX, kg/t (Combined bleach effluent)	0.68	0.51(—25.00)	0.53 (—22.05)
COD, mg/L (Combined bleach effluent)	1365.72	1955.16 (+43.15)	1957.62 (+43.33)
Colour, PCU (Combined bleach effluent)	149.40	227.08 (+51.99)	243.52 (+62.99)

Bleaching conditions	**X***	**X****	**E_1**	**O**	**D_1**	**E_2**	**D_2**
pH	6.4	6.4	11.0	11.0	2.5	11.0	2.5
Consistency, %	10	5	10	15	7	10	7
Retention time, min	180	180	90	90	120	90	120
Temperature, °C	55±2	55±2	70±2	110±2	70±2	70±2	70±2

Unbleached pulp kappa number = 18.25; Unbleached pulp brightness, % (ISO) = 27.4; Unbleached pulp viscosity, cps = 26.04

*X Crude xylanases from strain SH-1
**X Crude xylanases from strain SH-2
§ Chemical charge on o.d. pulp basis
(+/—) % difference compared to control pulp
± Standard deviation from the mean

Table 5.6 Effect of enzyme treatment on ODEP bleaching sequence of soda-AQ pulp of wheat straw

Particulars	Bleaching sequences		
	ODEP	O X*EDEP	OX**EDEP
Oxygen (O) stage§			
Pressure, kg/cm^2	5	5	5
NaOH, %	2	2	2
$MgSO_4$, %	0.1	0.1	0.1
Kappa number after oxygen delignification	10.00 ± 0.14	10.00 ± 0.14	10.00 ± 0.14
Enzyme dose, X*, IU/g§	—	10	—
Enzyme dose, X**, IU/g§	—	—	10
Extraction (E_1), NaOH applied§, %	—	2	2
Kappa number		7.98 ± 0.13	7.59 ± 0.11
Chlorine dioxide (D) stage§			
% ClO_2 applied as available Cl_2	2	2	2
% ClO_2 consumed as available Cl_2	1.99	1.96	1.98
Extraction (E_2), NaOH applied§, %	1	1	1
Peroxide (P) stage§			
H_2O_2 applied, %	3	3	3
H_2O_2 consumed, %	2.99	2.96	2.96
NaOH, %	2	2	2
Sodium silicate, %	5	5	5
MgSO4, %	0.05	0.05	0.05
Final brightness, % (ISO)	70.50±0.22	74.94 ±0.18 (+6.29)	78.1±0.13 (+10.78)
Final viscosity, cps	9.47± 0.06	10.07± 0.03 (+6.33)	9.87± 0.05 (+4.22)
Pulp beating			
Beating level, °SR	40	40	40
PFI mill revolutions	690	780 (+13.04)	750 (+8.69)
Mechanical strength properties			
Tensile index, Nm/g	49.97	41.82 (—16.30)	42.61(—14.72)
Burst index, kPa m^2/g	3.38	3.06 (—9.46)	3.08 (—8.87)
Double fold, no.	67	71(+5.97)	72 (+7.46)
Tear index, mNm^2/g	5.35	5.49 (+2.61)	5.52 (+3.17)
AOX, kg/t (Combined bleach effluent	0.80	0.49 (—38.75)	0.51(—36.25)
COD, mg/L (Combined bleach effluent)	996.00	1402.86 (+40.84)	1474.08 (+48.00)
Colour, PCU (Combined bleach effluent)	126.20	193.79 (+53.55)	210.62 (+66.89)

Bleaching conditions	**X***	**X****	**E_1**	**O**	**D**	**E_2**	**P**
pH	6.4	6.4	11.0	11.0	2.5	11.0	11.8
Consistency, %	10	5	10	15	7	10	10
Retention time, min	180	180	90	90	120	90	120
Temperature, °C	55±2	55±2	70±2	110±2	70±2	70±2	90±2

Unbleached pulp kappa number = 18.25; Unbleached pulp brightness, % (ISO) = 27.41; Unbleached pulp viscosity, cps = 26.04

*X Crude xylanases from strain SH-1
**X Crude xylanases from strain SH-2
§ Chemical charge on O.D. pulp basis
(+/—) % difference compared to control pulp
± Standard deviation from the mean

Table 5.7 Effect of enzyme treatment on OQPP bleaching sequence of soda-AQ pulp of wheat straw

Particulars	Bleaching sequences		
	OQPP	OX*EQPP	OX**EQPP
Oxygen (O) stage[§]			
Pressure, kg/cm^2	5	5	5
NaOH, %	2	2	2
MgSO4, %	0.1	0.1	0.1
Kappa number after oxygen delignification	10.00 ± 0.14	10.00 ± 0.14	10.00 ± 0.14
Enzyme dose, X*, IU/g[§]	—	10	—
Enzyme dose, X**, IU/g[§]	—	—	10
Extraction (E), NaOH applied[§], %	—	2	2
Kappa number after alkali extraction	—	7.98 ± 0.11	7.59± 0.10
Chelation (Q) stage, EDTA applied[§], %	1	1	1
Peroxide (P) stage			
Total peroxide charge[§], %	3	3	3
P_1 stage			
H_2O_2 applied, %	1.50	1.50	1.50
H_2O_2 consumed, %	1.49	1.47	1.46
P_2 stage			
H_2O_2 applied, %	1.50	1.50	1.50
H_2O_2 consumed, %	1.49	1.46	1.46
NaOH, %	2	2	2
Sodium silicate, %	5	5	5
$MgSO_4$, %	0.05	0.05	0.05
Final brightness, % (ISO)	65.80 ±0.3	69.80 ±0.24 (+6.07)	68.00 ±0.22 (+3.34)
Final viscosity, cps	10.04 ± 0.04	10.65± 0.07 (+6.07)	10.50± 0.03 (+4.58)
Pulp beating			
Beating level, °SR	40	40	40
PFI mill revolutions	900	850 (—5.55)	800 (—11.11)
Mechanical strength properties			
Tensile index, Nm/g	50.60	42.28 (—16.44)	42.51 (—15.98)
Burst index, kPa m^2/g	3.42	3.01 (—11.98)	3.04 (—11.11)
Double fold, no.	67	60 (—10.44)	61(—8.95)
Tear index, mNm^2/g	5.36	5.53 (+3.17)	5.57 (+3.91)
COD, mg/L (Combined bleach effluent)	1200	1680.00 (+40.00)	1656 (+38.00)
Colour, PCU (Combined bleach effluent)	100.10	148.14 (+48.00)	145.14 (+45.00)

Bleaching conditions	**X***	**X****	**E**	**O**	**Q**	**P_1**	**P_2**
pH	6.4	6.4	11.0	10	5.5	11.8	11.8
Consistency, %	10	5	10	15	3	10	10
Retention time, min	180	180	90	90	30	120	120
Temperature, °C	55±2	55±2	70±2	110±2	50±2	90±2	90±2

Unbleached pulp kappa number = 18.25; Unbleached pulp brightness, % (ISO) = 27.41; Unbleached pulp viscosity, cps = 26.04

*X Crude xylanases from strain SH-1
**X Crude xylanases from strain SH-2
§ Chemical charge on o.d. pulp basis
(+/—) % difference compared to control pulp
± Standard deviation from the mean

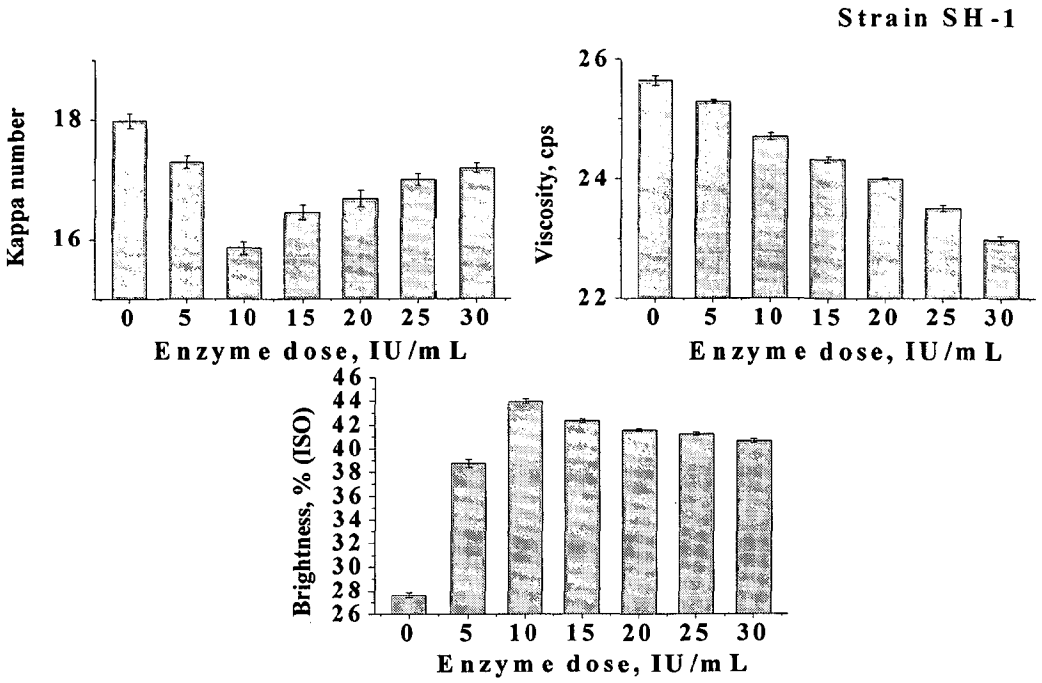


Figure 5.1 Effect of enzyme dose on kappa number, viscosity and brightness of soda-AQ pulp of wheat straw for crude xylanase from strain SH-1

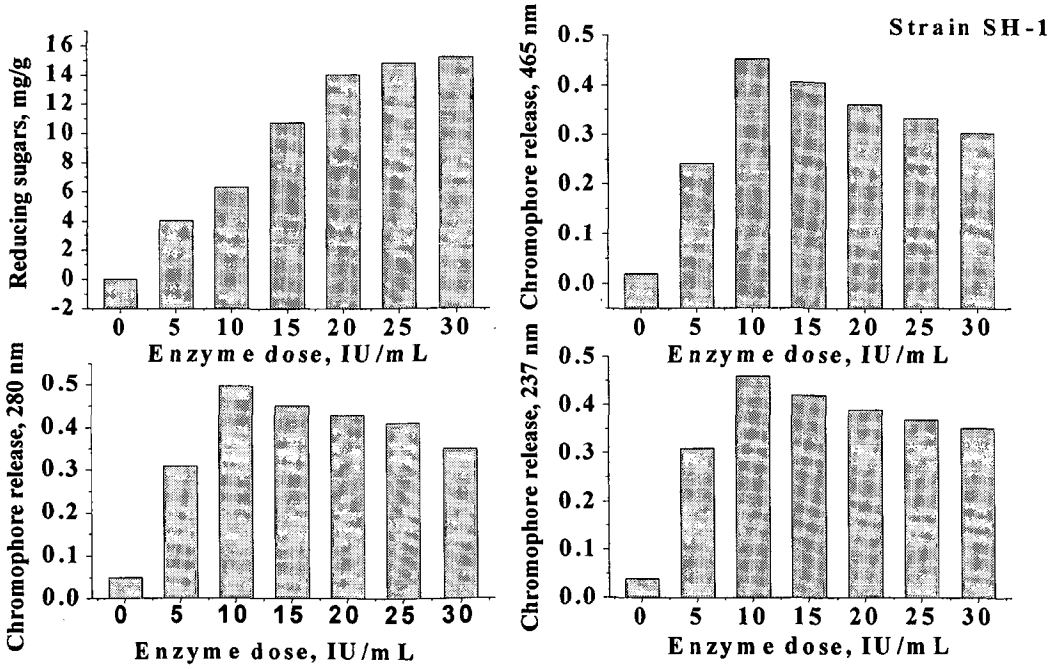


Figure 5.2 Effect of enzyme dose on release of reducing sugars and chromophoric groups from soda-AQ pulp of wheat straw for crude xylanase from strain SH-1

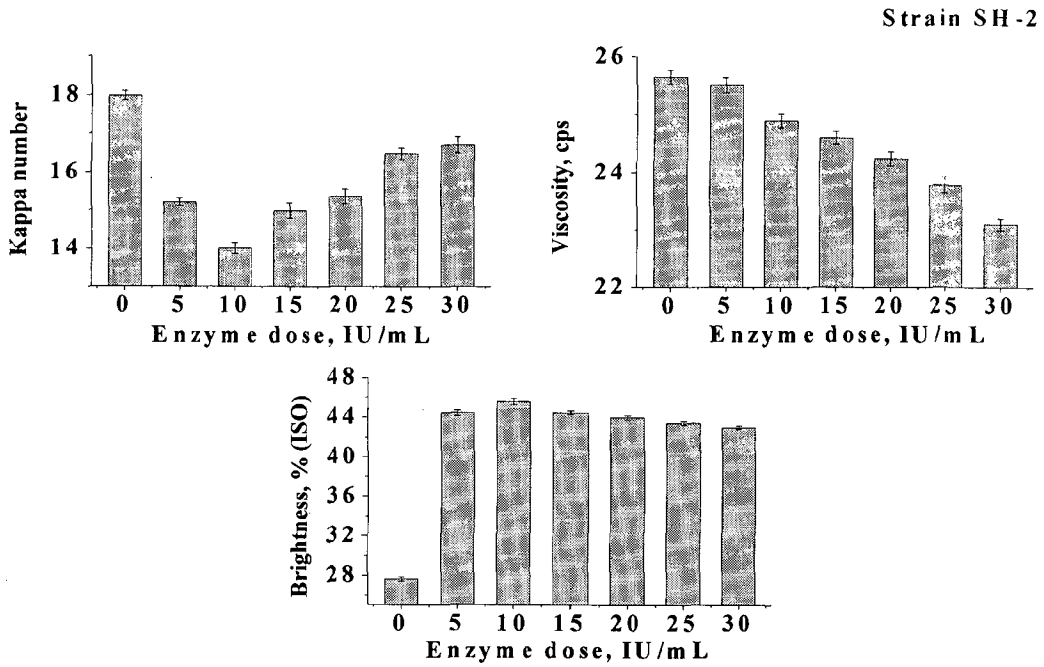


Figure 5.3 Effect of enzyme dose on kappa number, viscosity and brightness of soda-AQ pulp of wheat straw for crude xylanase from strain SH-2

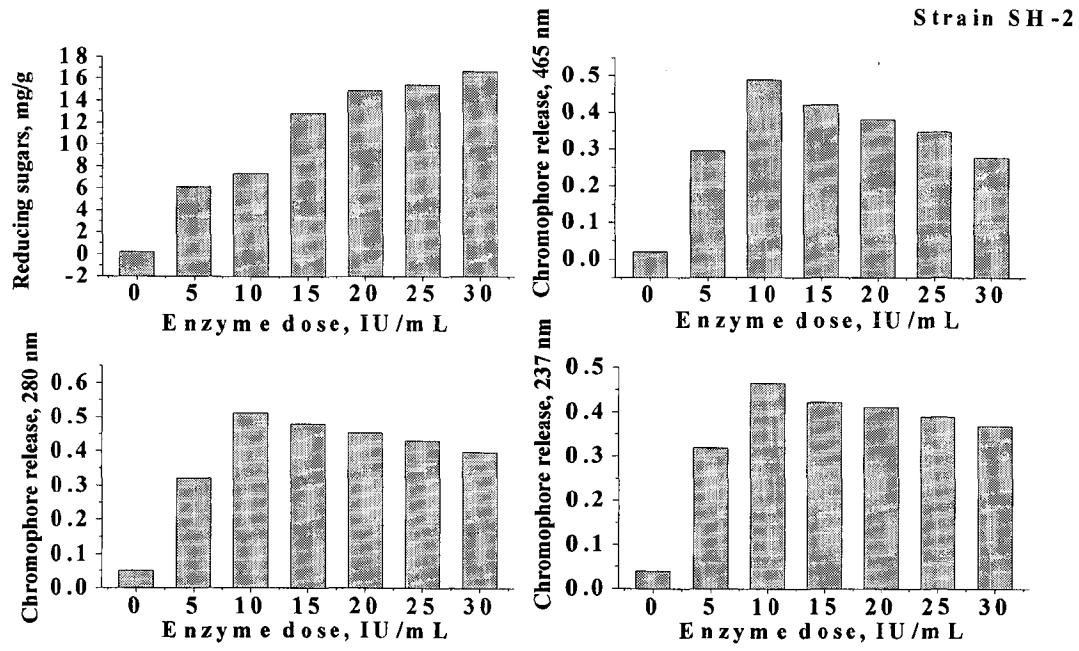


Figure 5.4 Effect of enzyme dose on release of reducing sugars and chromophoric groups from soda-AQ pulp of wheat straw for crude xylanase from strain SH-2

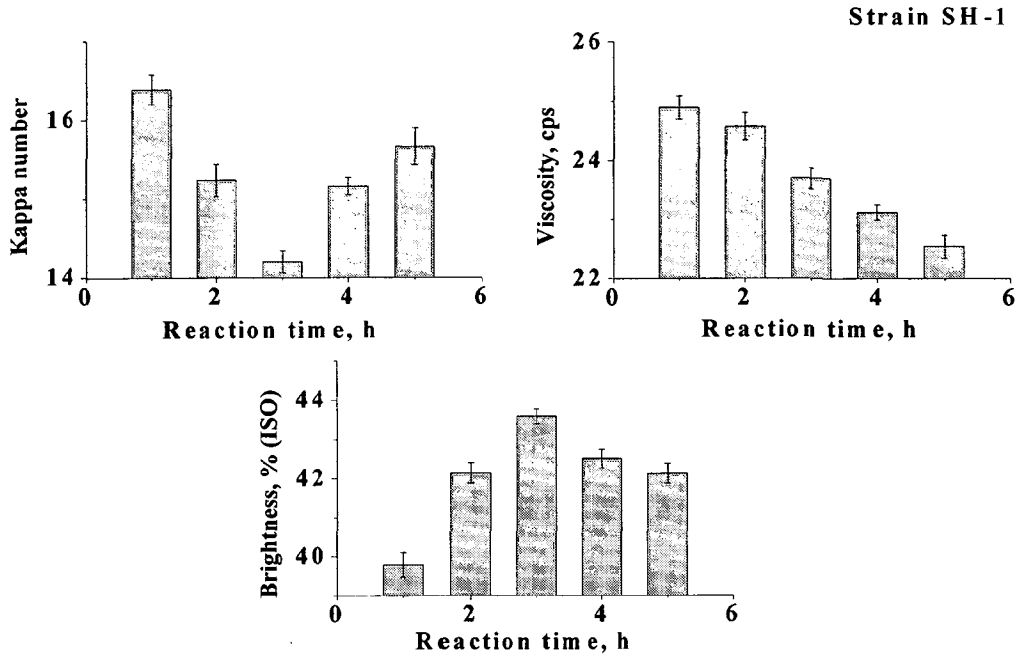


Figure 5.5 Effect of reaction time on kappa number, viscosity and brightness of soda-AQ pulp of wheat straw for crude xylanase from strain SH-1

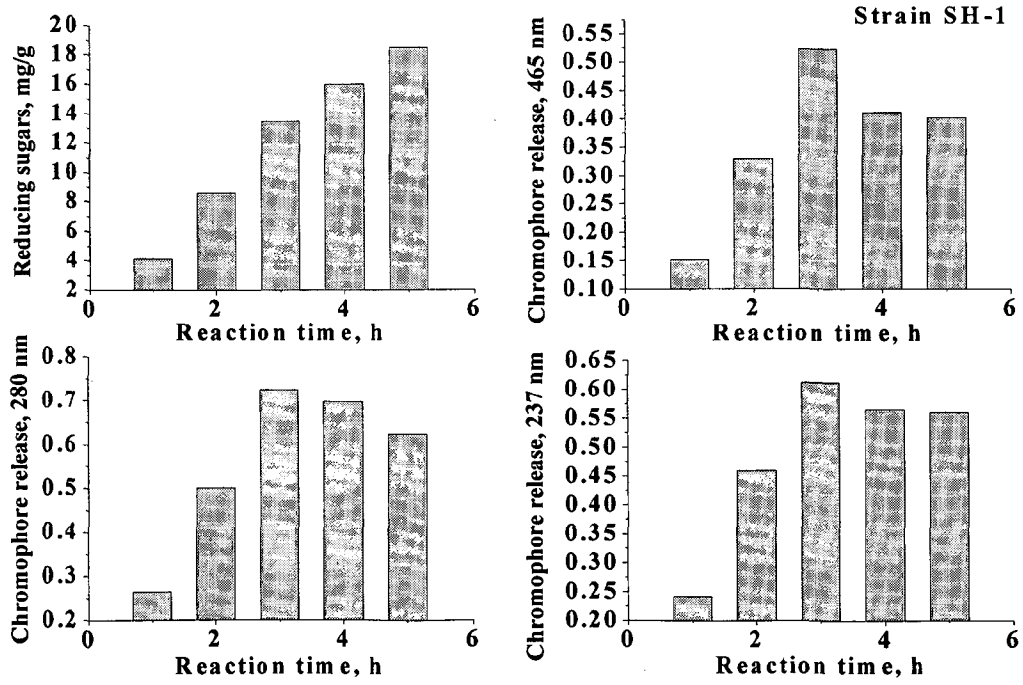


Figure 5.6 Effect of reaction time on release of reducing sugars and chromophoric groups from soda-AQ pulp of wheat straw for crude xylanase from strain SH-1

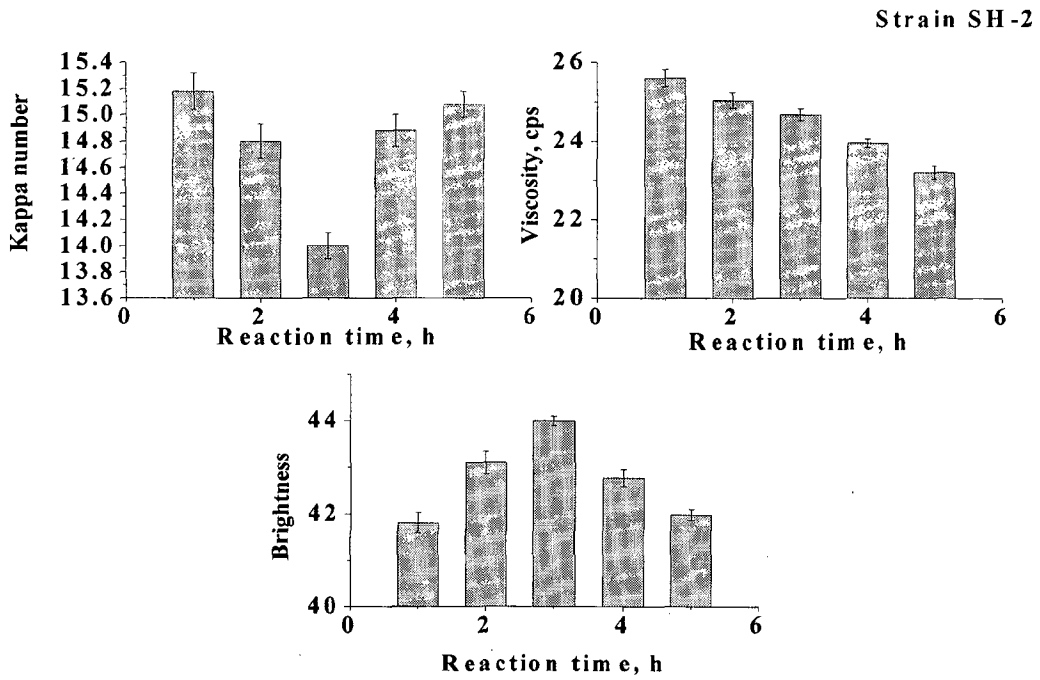


Figure 5.7 Effect of reaction time on kappa number, viscosity and brightness of soda-AQ pulp of wheat straw for crude xylanase from strain SH-2

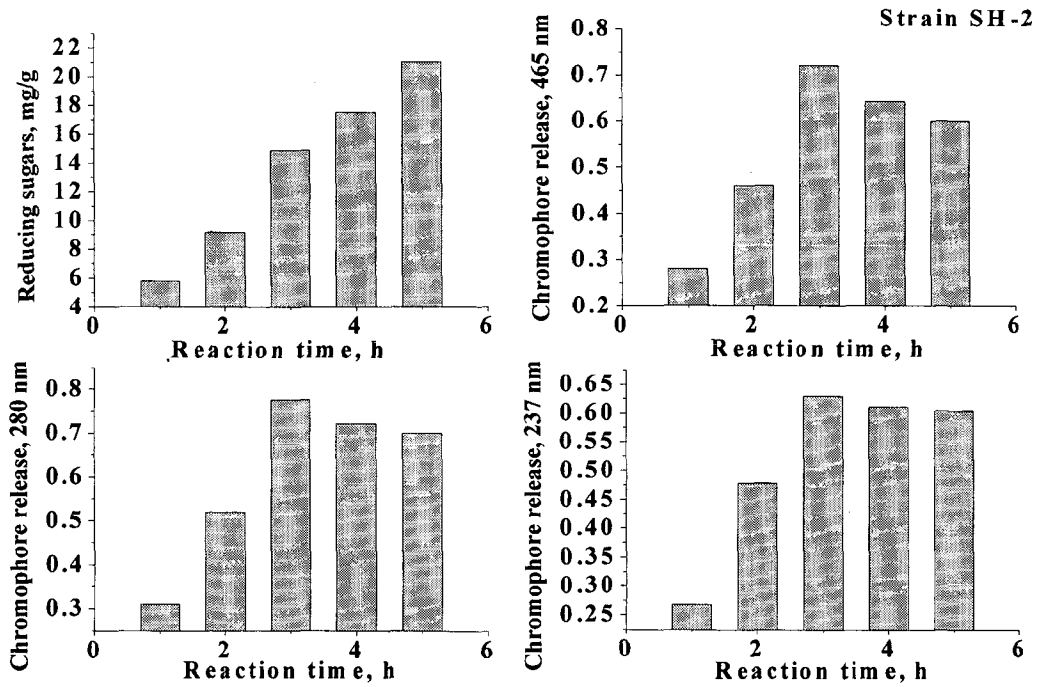


Figure 5.8 Effect of reaction time on release of reducing sugars and chromophoric groups from soda-AQ pulp of wheat straw for crude xylanase from strain SH-2

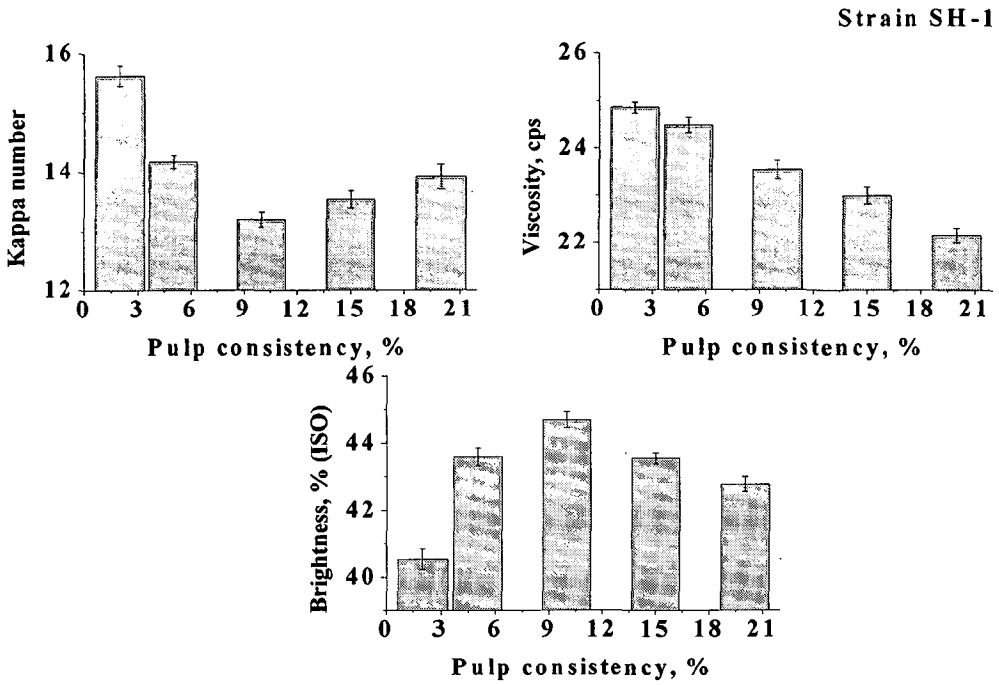


Figure 5.9 Effect of pulp consistency on kappa number, viscosity and brightness of soda-AQ pulp of wheat straw for crude xylanase from strain SH-1

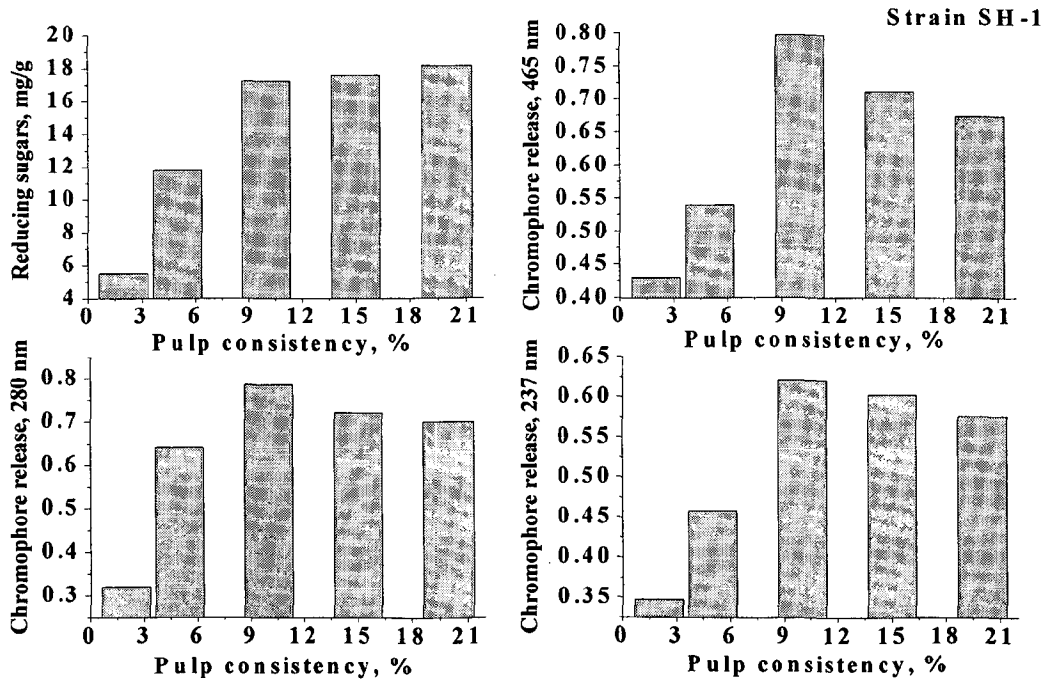


Figure 5.10 Effect of pulp consistency on release of reducing sugars and chromophoric groups from soda-AQ pulp of wheat straw for crude xylanase from strain SH-1

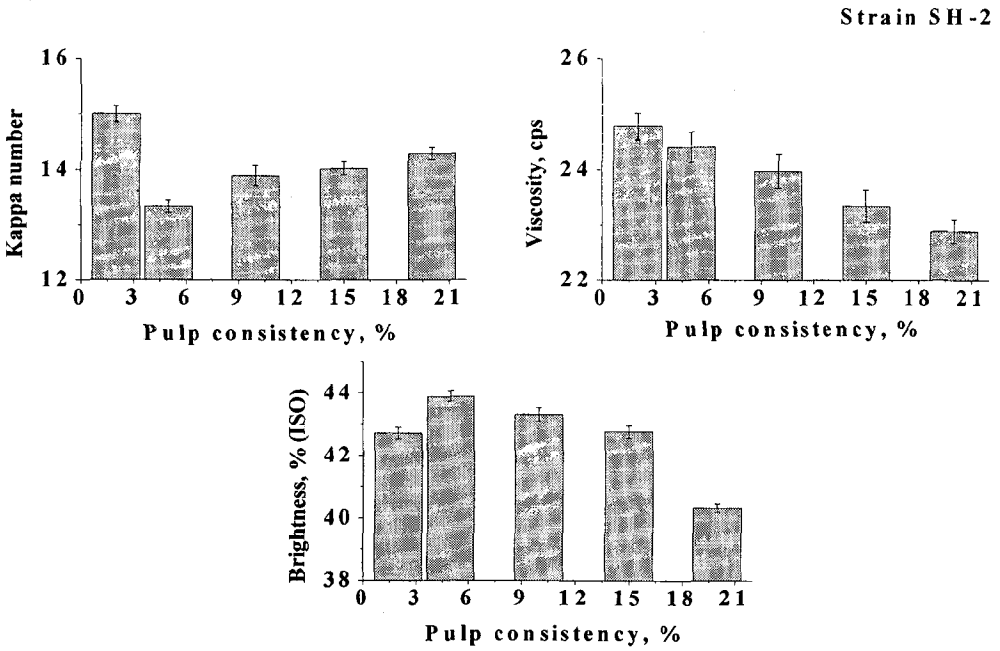


Figure 5.11 Effect of pulp consistency on kappa number, viscosity and brightness of soda-AQ pulp of wheat straw for crude xylanase from strain SH-2

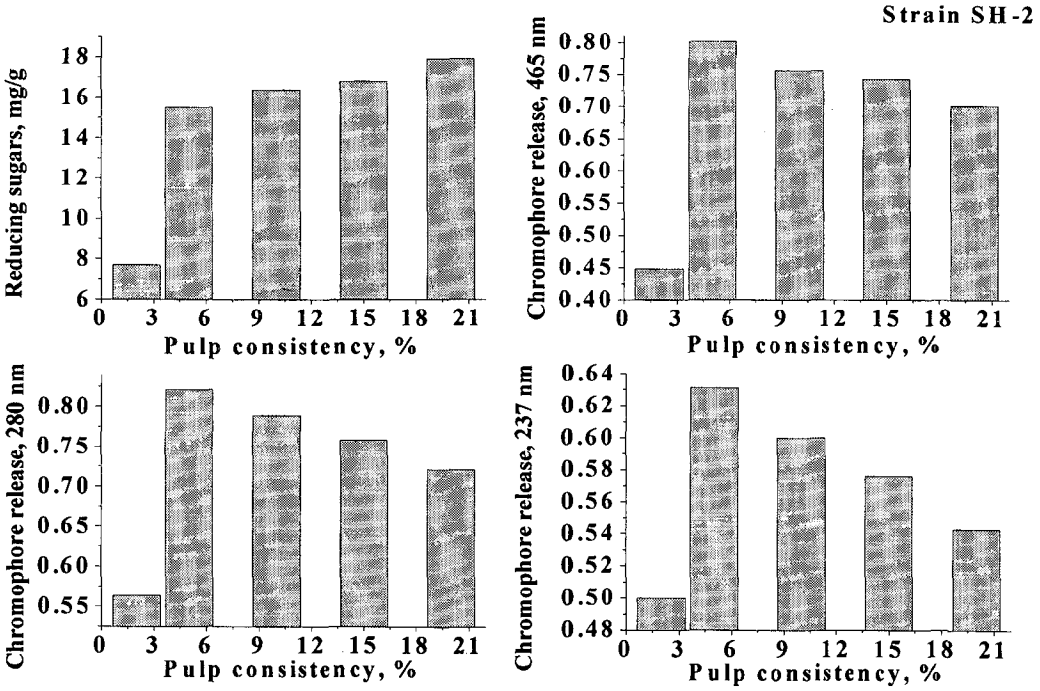


Figure 5.12 Effect of pulp consistency on release of reducing sugars and chromophoric groups from soda-AQ pulp of wheat straw for crude xylanase from strain SH-2

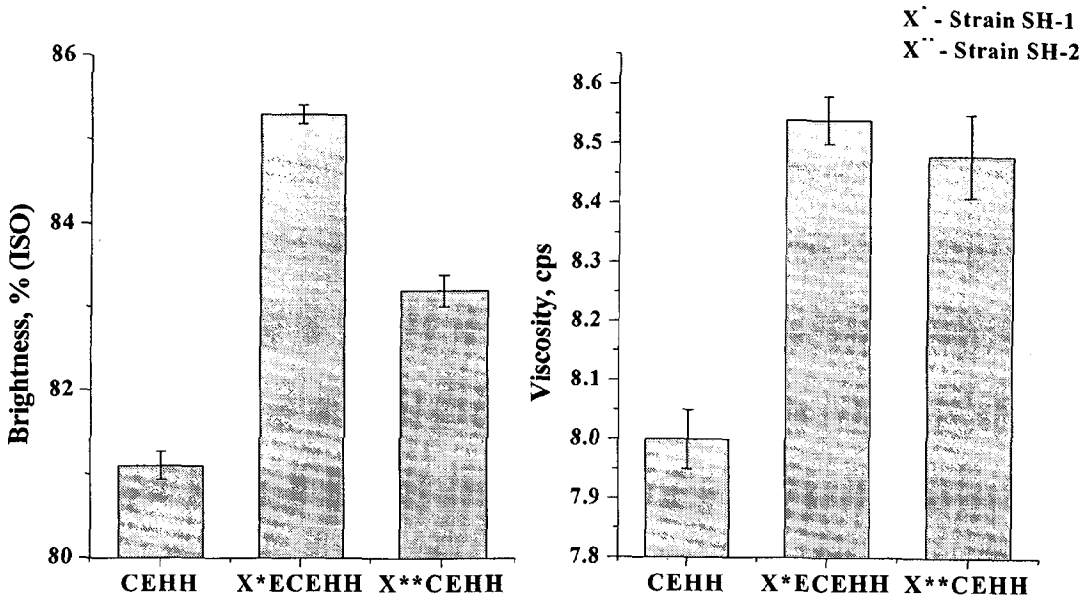


Figure 5.13 Effect of enzyme treatment on brightness and viscosity of soda-AQ pulp of wheat straw in a CEHH bleach sequence at same chemical charge

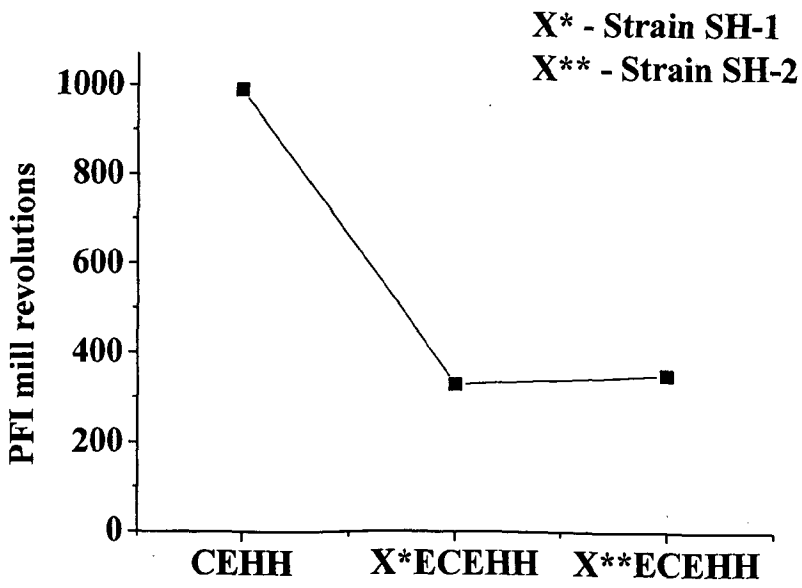


Figure 5.14 Effect of enzyme treatment on slowness of soda-AQ pulp of wheat straw in a CEHH bleaching sequence at same chemical charge

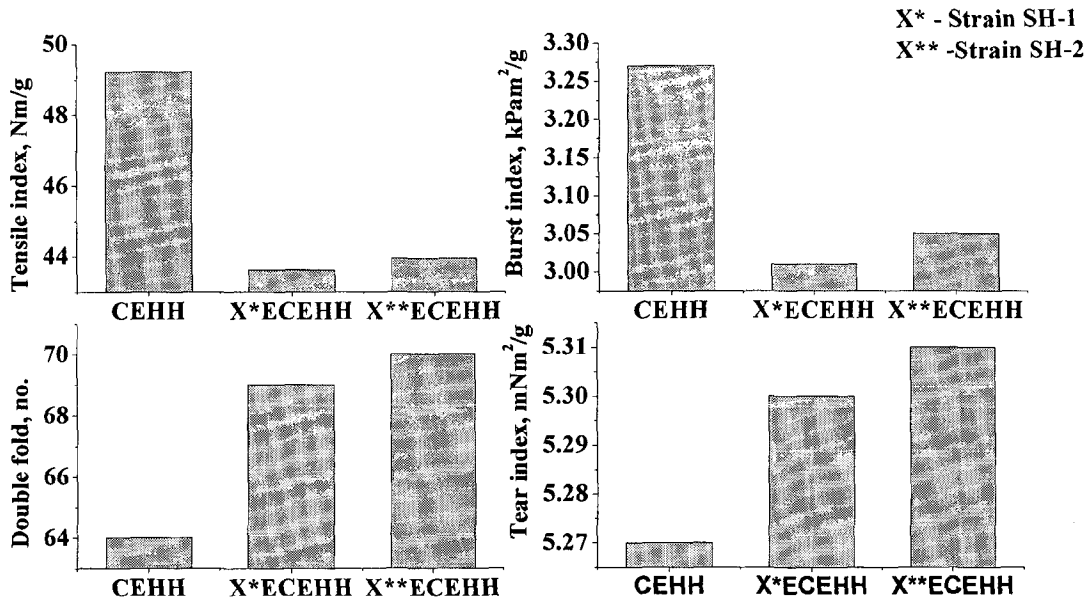


Figure 5.15 Effect of enzyme treatment on mechanical strength properties of the soda-AQ pulp of wheat straw in a CEHH bleach sequence at same chemical charge

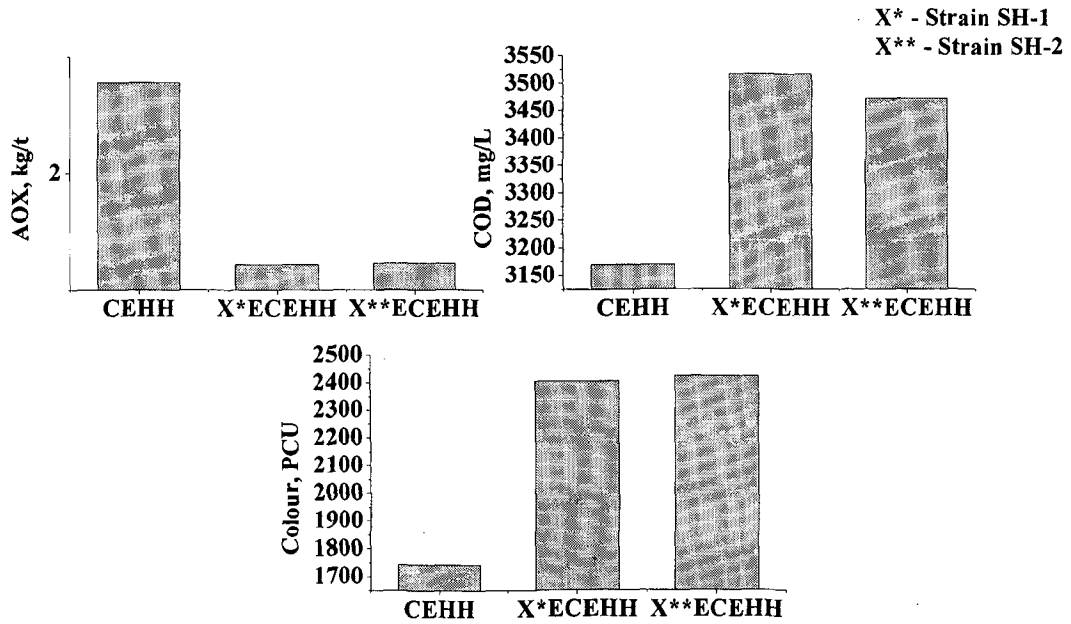


Figure 5.16 Effect of enzyme treatment on combined bleach effluent characteristics in a CEHH bleach sequence at same chemical charge

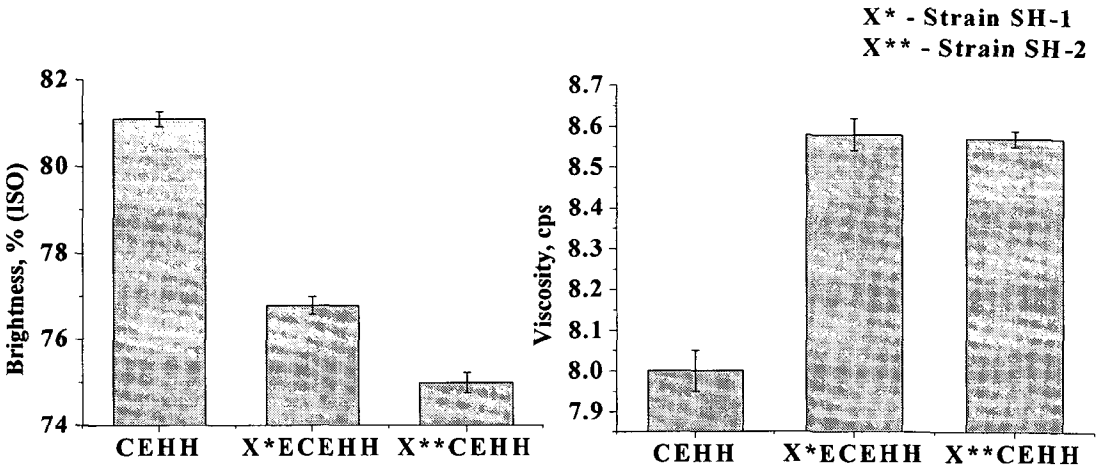


Figure 5.17 Effect of enzyme treatment on brightness and viscosity of soda-AQ pulp of wheat straw in a CEHH bleaching sequence at reduced chemical charge

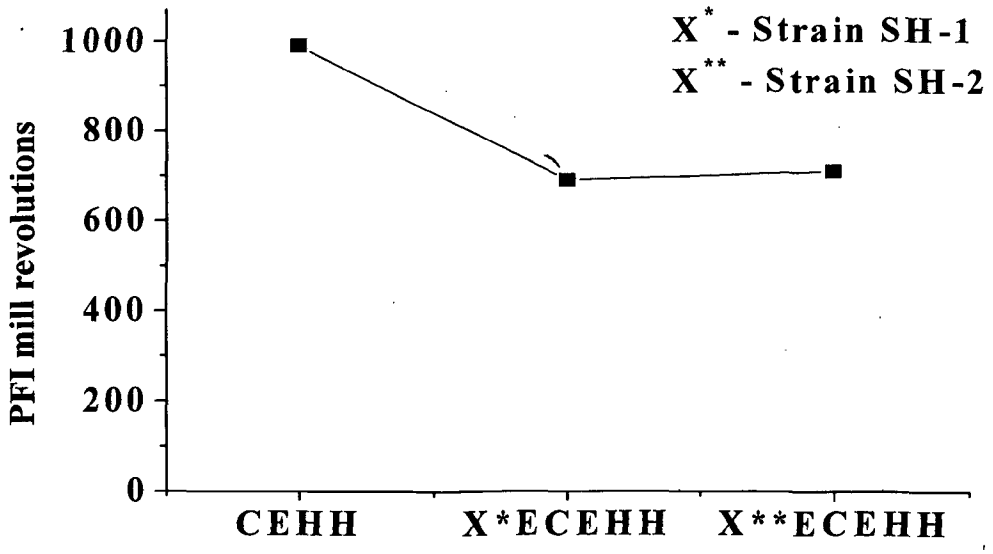


Figure 5.18 Effect of enzyme treatment on slowness of soda-AQ pulp of wheat straw in a CEHH bleaching sequence at reduced chemical charge

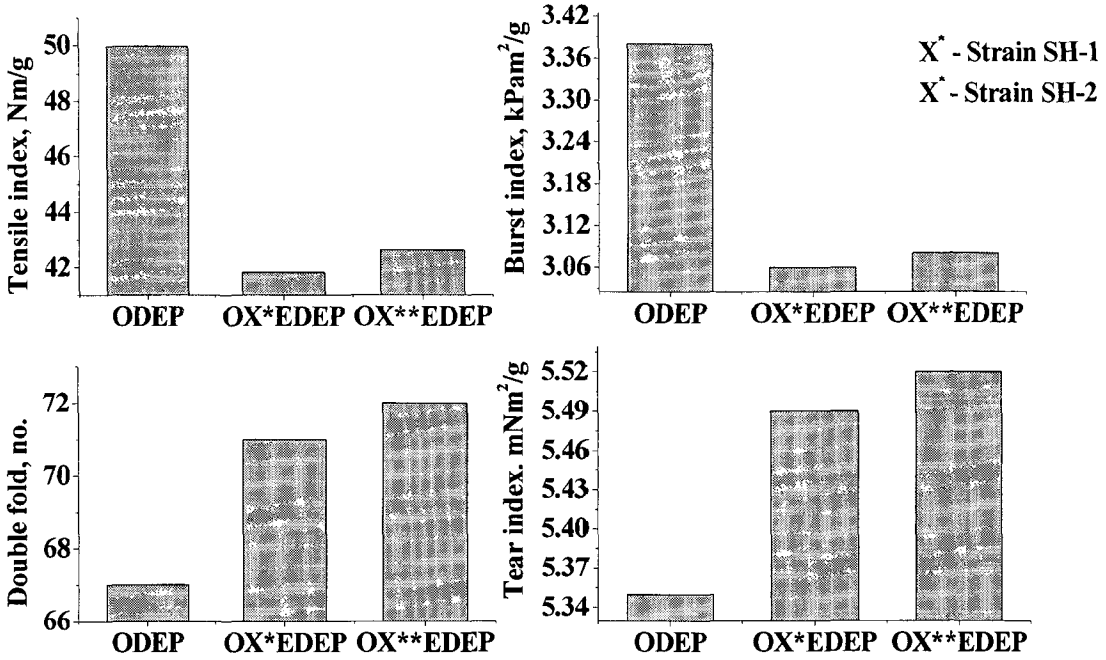


Figure 5.27 Effect of enzyme treatment on mechanical strength properties of soda-AQ pulp of wheat straw in an ODEP bleaching sequence

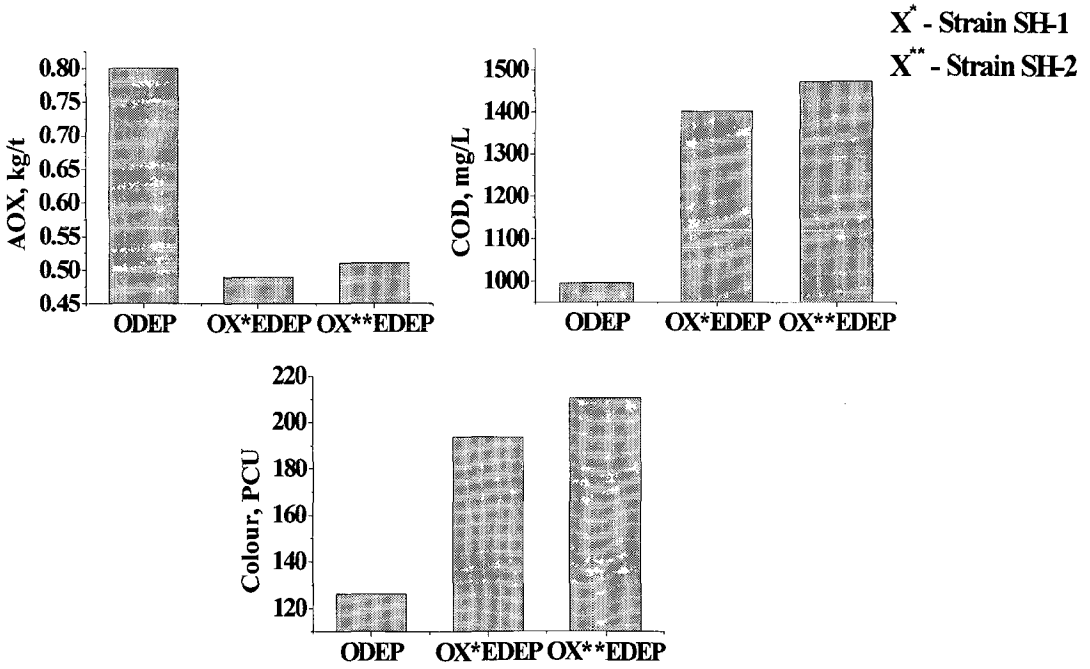


Figure 5.28 Effect of enzyme treatment on combined bleach effluent characteristics in an ODEP bleaching sequence

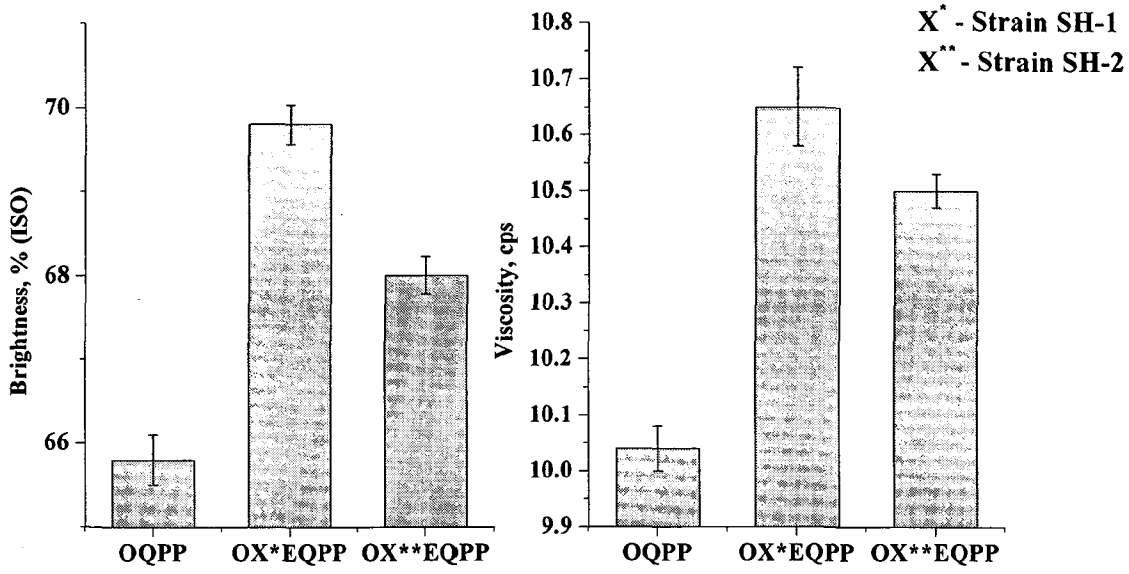


Figure 5.29 Effect of enzyme treatment on brightness and viscosity of soda-AQ pulp of wheat straw in an OQPP bleaching sequence

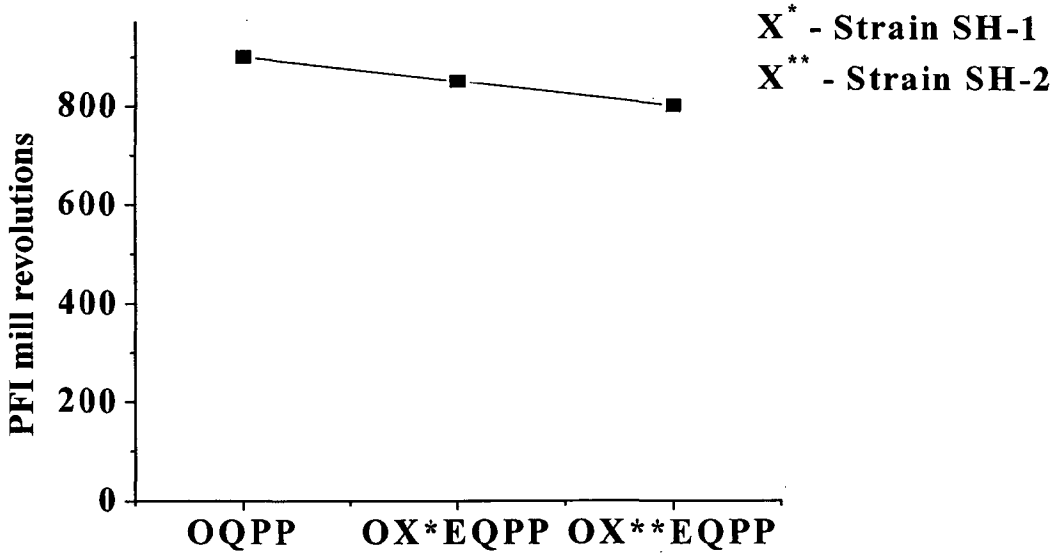


Figure 5.30 Effect of enzyme treatment on slowness of soda-AQ pulp of wheat straw in an OQPP bleaching sequence

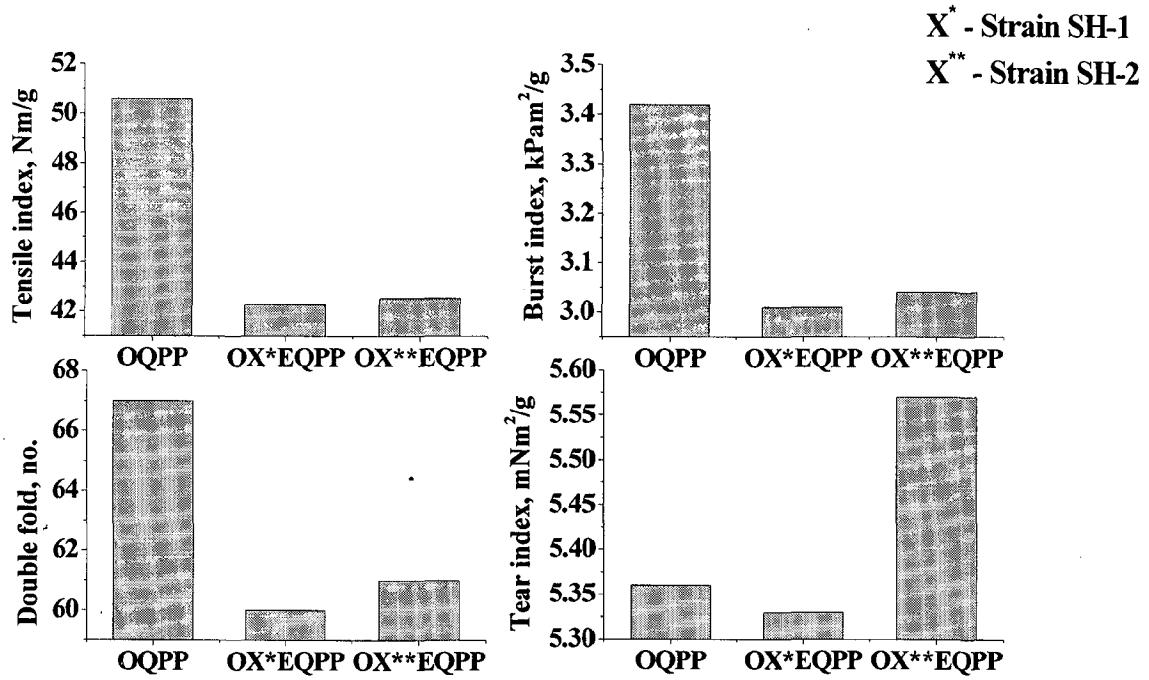


Figure 5.31 Effect of enzyme treatment on mechanical strength properties of soda-AQ pulp of wheat straw in an OQPP bleaching sequence

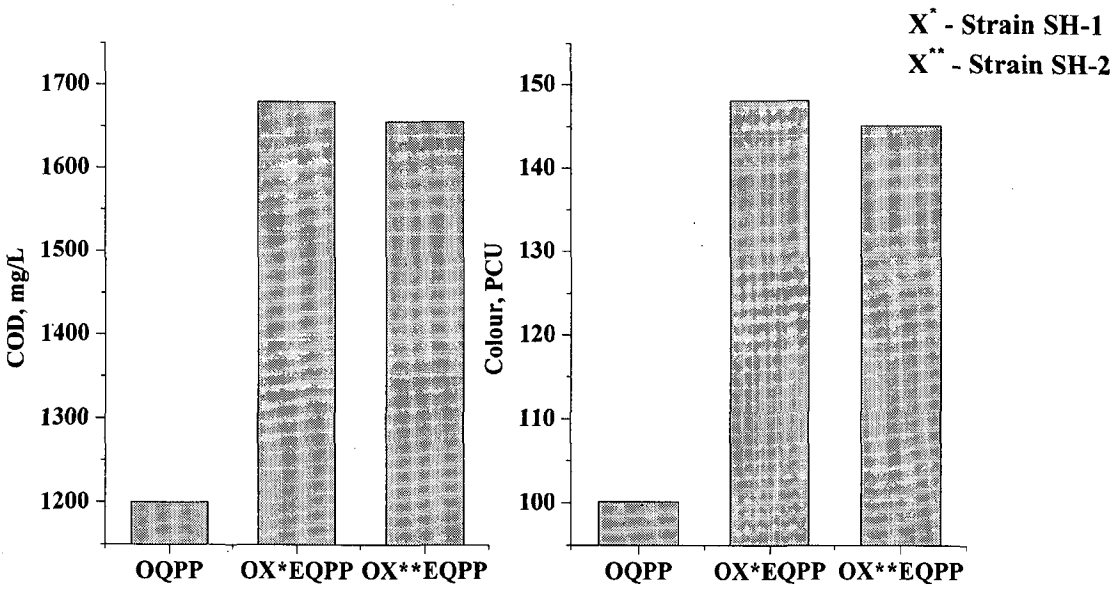
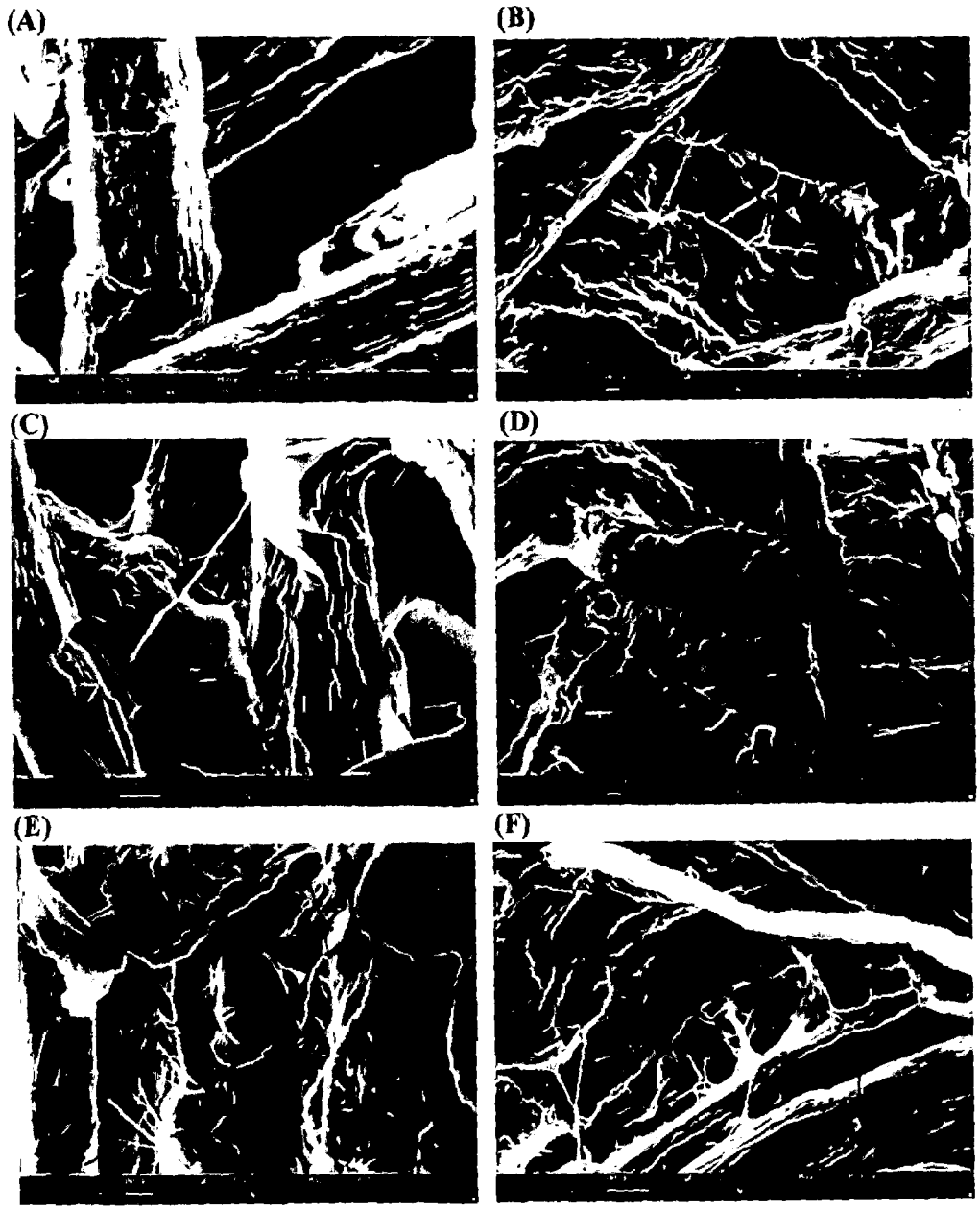


Figure 5.32 Effect of enzyme treatment on combined bleach effluent characteristics in an OQPP bleaching sequence



Photomicrographs 5.1 **Scanning electron micrograph showing (A) unbleached control pulp fibres (2.5 kx) (B) enzyme treated pulp fibres (strain SH-1) (3.5 kx) (C) enzyme pretreated pulp fibres (strain SH-2) (3.5 kx) (D) oxygen delignified control pulp fibres (3.5 kx) (E) oxygen delignified enzyme treated pulp fibres (strain SH-1) (3.5 kx) (F) oxygen delignified enzyme treated pulp fibres (strain SH-2) (3.5 kx).**

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CHAPTER 6
CONCLUSIONS AND RECOMMENDATIONS

6.1 CONCLUSIONS

The conclusions drawn from the present study on the production of xylanases from white rot fungi for biobleaching of soda-AQ pulp of wheat straw are summarized as below:

6.1.1 Studies on xylanase production

- Among the seven isolates, *Coprinellus disseminatus* strain SH-1 (NTCC 1163) and *Coprinellus disseminatus* strain SH-2 (NTCC 1164) were selected for biobleaching studies as they exhibited the highest and the second highest xylanase activities, with very low cellulase activities.
- The optimum conditions for the production of xylanases obtained from the test strains (*C. disseminatus* SH-1 and *C. disseminatus* SH-2) under S.S.F were: incubation period 7 days, temperature 37 °C and pH 6.4.
- Yeast extract was found to be the best complex nitrogen source and wheat bran the best lignocellulosic substrate for xylanase production by both the test strains.
- Glucose and lactose were found to repress xylanase activity in case of both the test strains.
- Plate assays showed that the test strains produced laccases and amylases as well.
- The test strains exhibited higher xylanase activities under S.S.F. as compared to L.S.F.

- For both the test strains, the highest xylanase activity was at temperature 55 °C and pH 6.4 and decreased thereafter.
- SDS-PAGE gave an insight to the protein profile of the crude enzyme samples. The multiplicity of proteins in the crude extracts was demonstrated, but not their exact number. During zymogram analysis for xylanases in crude enzyme samples obtained from the test strains clear zones corresponding to protein bands were observed. These indicated xylanase activity.
- Under optimized S.S.F. conditions, *C.disseminatus* strain SH-1 and SH-2 showed a xylanase activity of 727.78 and 227.99 IU/mL, cellulase activity of 0.925 and 0.660 IU/mL, laccase activity of 0.640 and 0.742 U/ml and protein concentration of 5.480 and 4.900 mg/mL, respectively.

6.1.2 Morphological, anatomical and chemical characterization of wheat straw

- The fibres of wheat straw were short compared to bamboo but larger than those of *Populus deltoides*. The fibre diameter was more than that of bamboo and *P. deltoides.* The narrow lumen fibres tended to produce more slenderness The flexibility coefficient of wheat straw fibres was slightly higher than bamboo, but much lower than *Populus deltoides*. The Runkel ratio of wheat straw fibres almost resembled to that of bamboo, yet due to less fibre diameter, the flexibility and the degree of collapseness of fibre, both of which control the degree of conformability within the paper sheet and, as such the size and number of inter-fibre bonds, is improved in the case of wheat straw. The Runkel ratio of wheat straw fibres was much higher in comparison to *Populus deltoides.*
- The fibre content of wheat straw was lesser in comparison to *Populus deltoides.* The wheat straw had high amount of non-fibrous cells, including parenchymas,

epidermal cells and vessels. The length and width of parenchyma cells in wheat straw was lower than sugarcane bagasse. The dimensions of epidermal cells in wheat straw were lower than that of rice straw and sugarcane bagasse. The dimensions of vessels in wheat straw were also lower than rice straw. The parenchyma cells are troublesome residues that are difficult to screen and act as filler and have no bonding effect.

- Water solubility of wheat straw was higher than *P. deltoides*, sugarcane bagasse and rice straw. 1% NaOH solubility of wheat straw was higher than *P. deltoides* and sugarcane bagasse, but lower than rice straw. The alcohol benzene solubility of wheat straw was lesser than sugarcane bagasse, but only slightly higher than rice straw. The alcohol benzene solubility of wheat straw was 4.95%. The holocellulose content of wheat straw was 72.15%. The high holocellulose content of wheat straw is favorable for better fibrillation and swelling of pulp. It also makes wheat straw more amenable to action of enzymes, like xylanases, which can successfully be applied to wheat straw at pulping or bleaching stages for improving paper quality and reducing pollution load occurring due to papermaking operations. The hemicellulose content of wheat straw was more or less similar to that of sugarcane bagasse. It is to be noted that the large amount of hemicellulose may result in a decrease in mechanical strength not because of bonding effect but possibly because the individual fibre strength may be reduced as a result of the decrease in the average molecular weight of the polymer system. The lignin content of wheat straw was 21.12%. Therefore, wheat straw required less cooking chemicals and short cooking cycle. The ash and silica content of wheat straw was 7.50 and

4.50%, respectively. The silica can play a role of inhibitor for O_2 delignification and bleaching with H_2O_2, thereby exterminating need for additional inhibitors to mask transition metals ions during pulping/ bleaching.

6.1.3 Preparation of wheat straw soda-AQ pulp

- Wheat straw produced 45.05% screened pulp yield of kappa number 18.25 by soda-AQ pulping process at optimum cooking conditions (maximum cooking temperature 150 °C, maximum cooking time 60 minutes and active alkali dose 12% (as Na_2O), AQ dose 0.1% and liquor to raw material ratio 4:1).
- Bauer-McNett fibre classification showed that soda-AQ pulp of wheat straw consisted of 40.31% medium sized fibres and 12.16% long fibres.
- Wheat straw produced optimal mechanical properties at 45 °SR, except tear strength. In terms of fibre quality (curliness), wheat straw pulp was found to be more or less comparable to that of *Eucalyptus tereticornis*.

6.1.4 Xylanase-aided bleaching of soda-AQ pulp of wheat straw

- The crude xylanases obtained from the test strains, *C. disseminatus* strain SH-1 and SH-2 successfully decreased the kappa number of unbleached wheat straw soda-AQ pulps. The kappa number of soda-AQ pulp of wheat straw also decreased when crude xylanases from both the test strains were applied after oxygen delignification. It was also observed that the decrease in kappa number of enzyme treated alkali extracted pulps was more in case of non-oxygen-delignifed pulps as compared to oxygen-delignified pulps.
- Xylanase treatment (for both the strains SH-1 and SH-2) improved the brightness of soda-AQ wheat straw pulp compared to their respective control

pulps in all the studied bleaching sequences i.e. CEHH at a total chlorine demand of 4.5%, ODED, ODEP and OQPP. The brightness of enzyme treated pulps (for both the test strains) in CEHH bleaching sequence at a total chlorine charge of 2.25%, was slightly lower as compared to control (without enzyme treatment) bleached at a total chlorine demand of 4.5%.

- The enzyme treatment improved the viscosity of soda-AQ pulp in all the bleaching sequences compared to their respective control, which might be due to selective removal of lower DP xylan and enrichment of high molecular weight polysaccharides.
- In all the bleaching sequences, the xylanase treatment of both non-oxygen-delignified and oxygen-delignified pulps slightly decreased the tensile and burst indices when beaten at 40 °SR, due to removal of large percentage of hemicelluloses and concomitant reduction in the degree of polymerization of residual hemicelluloses. However, the tear index of xylanase treated pulps (for both the test strains) was slightly higher than untreated pulps in all bleaching sequences. The double fold of enzyme treated pulps (for both the test strains) was higher in CEHH and ODEP bleaching sequences and lower in ODED and OQPP bleaching sequences.
- The xylanase treatment increased the COD and colour of the combined bleach effluents generated during CEHH bleaching at a chlorine demand of 4.5% as compared to control bleached at the same chlorine demand. The COD and colour was slightly lower in case of enzyme pretreated pulps (for both the test strains) bleached at a chlorine charge of 2.25% compared to CEHH bleaching at a chlorine demand of 4.5%. The COD and colour load in combined bleach

effluent of pulps in all the studied bleaching sequences was increased due to removal of lignin carbohydrate complex (LCC) as a result of enzyme treatment compared to their respective controls. The AOX of the combined bleach effluents obtained from enzyme treated pulps in CEHH, ODED and ODEP bleaching sequences were significantly reduced compared to their respective control pulps, thus making xylanase biobleaching all the more important for environment protection point of view.

- Scanning electron microscopy (SEM) of enzyme treated and untreated pulps also helped to better understand the effect of crude xylanases obtained from *C. disseminatus* strain SH-1 and SH-2 on pulp fibres. The fibres of enzyme treated pulps (for both the test strains) showed better fibrillation and they underwent peeling process. The surface of enzyme treated fibres was observed to be rougher, with prominent cracks and delamination. Thus, facilitating the diffusion of larger lignin macromolecules out through the fibre wall and made the fibres more accessible to the diffusion of bleaching chemicals.

The study clearly indicated that the crude xylanases obtained from *C. disseminatus* strain SH-1 and *C.disseminatus* strain SH-2 can successfully be used as prebleaching agent for wheat straw soda-AQ pulp in different bleaching sequences.

6.2 RECOMMENDATIONS

- The effect of mutations on the xylanase production by the test strains (*C. disseminatus* SH-1 and *C. disseminatus* SH-2) may be further studied. The performance of the enzymes obtained from the test strains might also be improved by employing gene manipulation techniques for improving enzyme

production by the test strains. This will make the fermentation process more cost effective and commercially viable.

- Further work on purification and characterization of the xylanases obtained from the test strains is suggested for a better understanding of their enzyme system.
- It is known that non-fibrous cells are less susceptible to enzyme action, so some methods should be devised to mechanically separate non-fibrous cells from wheat straw fibres. This would increase the efficiency of xylanases as prebleaching agent.
- Future studies should be carried out to see the effect of xylanases, obtained from the test strains, on wheat straw pulp in other bleaching sequences, on different raw materials and on pulps produced by different methods.
- Chromatographic analysis to study the change in molecular weight profiles of lignin and carbohydrates in the pulp should be carried out for a better understanding of the attack of xylanase on lignin-carbohydrate complexes (LCC).
- Realistic cost estimates for enzyme prebleaching should be made for better evaluation of the process economics.

The study brings about sequences that will ensure environmental compliances with respect to AOX generation for soda-AQ pulp of wheat straw, indicating the effectiveness of xylanase biobleaching for improving environmental performance of the bleach plant. Thus xylanase biobleaching must seriously be considered as an important step in pulp treatment, especially for bleaching of agricultural residues, so as to reduce the impact of AOX generation in chlorine/ECF bleaching sequences and also to improve the performance of TCF bleaching sequences.

Realistic cost estimates and improvement in process economics are the key factors for the commercial success of any technology. The enzyme production was designed in a way so as to keep the process as cost effective as possible, like, cheap lignocellulosic substrates were used for the enzyme production under S.S.F. and crude xylanases were used for enzyme biobleaching. Still, it must be clearly understood that no enzyme-based process for bleaching can be as inexpensive as using chlorine or even organic chlorine compounds. The added expenses incurred by the use of enzymes must be viewed in terms of their accrued indirect benefits like prevention of environmental derangement and reduced health hazards to mankind. At the same time, xylanase treatment can easily be applied to any traditional or modern bleaching sequence without significant investment in the infrastructure or major process changes.

RESEARCH PUBLICATIONS

1. Dharm Dutt, D., C.H. Tyagi, J.S. Upadhyaya, and **Shalini Singh,** Development of Speciality Papers is an Art: Playing Cards Base Paper from *Eucalyptus tereticornis*, *Leucaena leaucocephala* and *Bambusa aurandacea* – Part XV, *Ippta J.*, 19 (3): 153-155 (2007).

2. Dharm Dutt, J. S. Upadhyaya, C. H. Tyagi, Alok Kumar and **Shalini Singh,** Studies on *Ipomea carnea* and *Cannabis sativa* as an alternative pulp blend for softwood: Optimization of soda pulping process, *Journal of Industrial and Scientific Research*, **Communicated**, (2008).