STUDIES ON PRODUCTION OF MICROBIAL ENZYMES AND THEIR USE IN BIODELIGNIFICATION OF BAGASSE

A THESIS

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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 MICROBIAL ENZYMES AND THEIR USE IN BIODELIGNIFICATION OF BAGASSE"** in partial fulfilment of the requirements 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 the period from January, 2004 to October, 2008 under the supervision of Dr. Dharm Dutt, Associate Professor, Dr. C.H. Tyagi, Associate Professor, and Prof. J.S. Upadhyaya, Prof. & Head, Department of Paper Technology, Indian Institute of Technology Roorkee, Roorkee.

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

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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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ABSTRACT

The pulp and paper industry is intensive in terms of consumption of raw materials, chemicals, energy, water and capital requirements besides environmental emissions. The energy cost as a percentage of manufacturing cost has increased from 15% in 1979-80 to about 33% in 2007-08. The current need in the pulp and paper industry is the energy conservation i.e. reduction of the net energy per unit of product. Therefore, use of energy-efficient equipments, processes and productivity are few of the immediate steps which should be given due thoughts. The benefits of the above mentioned options include lower production cost, a more competitive edge, an improved ability to withstand future fluctuations in energy cost and an improvement in productivity and environmental benefits. Therefore, the global concerns about energy, preservation of forests and elimination of pollution from pulping and bleaching processes has led us to explore alternate fibrous resources other than wood and new pulping and bleaching processes that are environmentally benevolent without sacrificing product quality. The pulp and paper industry is also under constant pressure to reduce and modify environmental emissions to air and water due to stringent rules of the governments. So the present investigation aims at developing an environmental benign technology for production of pulp and paper using a cheap agricultural residue i.e. sugarcane bagasse. The objective of the study is to evaluate and estimate the viability of reducing chemical consumption with preserving mechanical strength properties of sugarcane bagasse soda-AQ pulp through enzymatic treatment.

Sugarcane bagasse has been given mechanical treatments i.e. dry and wet depithing followed by intensive screening to remove most of the non-fibrous pith cells. The detailed morphological and anatomical features of sugarcane bagasse are determined and chemical characterization of depithed sugarcane bagasse is carried out to assess its suitability for pulp and papermaking. The studies indicate that depithed sugarcane bagasse can satisfactorily be used for pulp and paper production and will be proved as a good raw material for papermaking process.

Dry and wet depithed sugarcane bagasse is delignified by soda pulping process. It requires milder cooking conditions due to its open and looser structure and low specific gravity. The various operating process variables for soda pulping like, maximum cooking temperature, cooking time and active alkali dose are optimized for soda process and effect of anthraquinone (AQ) is also observed. An active alkali dose of 12% (as Na_2O), maximum cooking temperature 150 ^{0}C and cooking time of 60 min are found optimum for soda pulping of sugarcane bagasse. A small dose of 0.1% AQ accelerates the delignification rate with improvement in screened pulp yield. The mechanical strength properties are also optimized at different alkali doses as well as beating levels and are optimum at active alkali dose 12% (as Na_2O) and beating level of 45 ^{0}SR. The spent liquor generated during soda-AQ pulping is analyzed for various characteristics. Fiber length distribution of sugarcane bagasse soda-AQ pulp using Bauer-McNett fiber classifier shows the abundance of medium sized fibers in sugarcane bagasse soda-AQ pulp, therefore the sugarcane bagasse soda-AQ pulp obtained will be of good quality for paper making. The scanning

electron microscopic (SEM) study is carried out to understand the procedure of alkaline pulping and its affect on the fibers of sugarcane bagasse.

An investigation has been undertaken to isolate, screen and identify a potent microorganism having the potential ability to secrete xylanase. A thorough survey of various sites leads to isolation of two persuasive xylanase producing fungal strains. The strains are having notably higher xylanase activity along with lower cellulase contamination and identified to be the two different strains of white rot basidiomycete *Coprinellus disseminatus* from Forest Research Institute, Dehradun (India). Both of the strains have been designated as SW-1 and SW-2 respectively. The detailed morphological study of both of the test strains has been carried out using SEM which shows that the hyphae of fungal strain SW-1 are thin and elongated whereas, the hyphae of the fungal strain SW-2 are thick and compact. Xylanase production from SW-1 and SW-2 is evaluated under submerged and solid-state fermentation conditions; of these, the level of xylanase production observed is higher in solid-state fermentation (SSF) conditions. SSF is carried out for the optimization of various operating parameters i.e. incubation period, temperature, pH, carbon source, nitrogen source and moisture content using hyper-xylanase producing *C. disseminatus* SW-1 and SW-2 to achieve the maximum levels of xylanase secretion by both of the test strains. The biochemical characterization of crude xylanase produced by both of the isolates shows that the xylanase produced by *C. disseminatus* SW-1 is more alkali and thermotolerant in comparison to that produced by *C. disseminatus* SW-2. Therefore the test strain *C. disseminatus* SW-1 has been chosen for the further biobleaching studies of sugarcane bagasse soda-AQ pulp.

The xylanase produced by *C. disseminatus* SW-1 is analyzed and evaluated for its application in biobleaching of sugarcane bagasse pulp and to reduce the toxicity of effluents generated during various bleaching sequences, in terms of AOX. The various parameters for xylanase prebleaching i.e. xylanase dose, reaction time and pulp consistency are optimized and the pulp filtrates have been checked for the release of reducing sugars and chromophores from the sugarcane bagasse pulp. The xylanase preparation has been used in the biobleaching of sugarcane bagasse soda-AQ pulp during the conventional (CEHH, CEHHP and OCEHH), ECF (ODED and OD(E_{OP})DP) and TCF (O(E_{OP})P) bleaching sequences. The xylanase pretreatment reduces the total chlorine demand by 29.70% for CEHH and CEHHP and 36.53% for OCEHH bleaching sequence, while still achieving high degree of brightness and preserving mechanical strength properties of sugarcane bagasse soda-AQ pulp in comparison to control. Xylanase pretreatment also reduces the AOX formation by 28.16% for CEHH, 27.6% for CEHHP and 34.65% for OCEHH bleaching sequences. For ECF (ODED and OD(E_{OP})DP) and TCF (O(E_{OP})P) bleaching sequences; xylanase pretreatment increases the overall brightness ceiling of the pulp at the same chemical charge with a decrease in all the mechanical strength properties except tear index of sugarcane bagasse pulp. Xylanase pretreatment results in small gain in viscosity over control for all the bleaching sequences. ODED and OD(E_{OP})DP bleaching sequences reduce the AOX formation by 84.84 and 87.36%, COD by 23.8 and 21.16% and color by 33.11 and 22.2% respectively in combined bleach effluent compared to that of CEHH bleaching sequence. The introduction of xylanase stage before ODED and OD(E_{OP})DP

bleaching sequences reduces the AOX formation by 41.43 and 40% respectively. The reduction in copper number after xylanase pretreatment of all bleaching sequences shows that there is no brightness reversion with time while a small increase is noticed in effluent COD and color values after xylanase pretreatment of conventional (CEHH, CEHHP and OCEHH), ECF (ODED and OD(E_{OP})DP) and TCF (O(E_{OP})P) bleaching sequences. SEM study has been carried out in order to attain a better understanding of the effect of xylanase pretreatment on bagasse pulp bleachability which reveals that xylanase pretreatment helps in fiber surface modifications. So the xylanase pretreatment renders the fibers more susceptible for chemical bleaching, hence saving a fair amount of chemicals and reducing the effluent toxicity in terms of AOX. Additionally it improves the mechanical and optical properties of paper along with reduction in chlorine consumption which in turn mitigate the pollution load, therefore, denoting it to be an ecofriendly and environment benign bleaching technology.

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Swarnima Agnihotri

CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

AOX	Adsorbable Organic Halide
BOD	Biological Oxygen Demand
cm	Centimeter
COD	Chemical Oxygen Demand
cps	Centipoise
^0C	Degree Celsius
^0SR	Degree Schopper Riegler
g	Gram
GDP	Gross Domestic Products
h	Hours
ha	Hectare
IU	International Unit
ISO	International Organization of Standardization
kg	Kilograms
kJ	Kilo Joule
km	Kilometers
kPa	Kilo Pascal
kV	Kilo Volt
L	Litres
m	Meters
µ	Microns
µg	Micrograms
µm	Micrometers
mL	Millilitres
mm	Millimeters
mol	Molarity
mPa	Milli Pascal
nm	Nanometers
N	Newton

OD	Optical Density
O.D.	Oven Dried
Pa	Pascal
%	Percent
PDA	Potato Dextrose Agar
pI	Isoelectric Point
PTU	Platinum Cobalt Unit
rpm	Revolution per minute
SEM	Scanning Electron Microscope
t	Tonne
TOC	Total Organic Carbon
TPD	Tonnes Per Day
U.V.	Ultra Violet
v/v	Volume per Volume
Wt.	Weight
w/v	Weight per Volume

CHAPTER 1

INTRODUCTION

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The consumption of paper is an index of a country's development. Therefore, per capita consumption can be taken as a growth in areas related to industrial, cultural and educational developments, but it is rather disappointing that per capita consumption of paper in our country is one of the lowest compared to other developed countries of the world. The consumption of paper is directly related to population and literacy rate. 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 over 70% by 2020 (254) (Figure1.1). GDP and paper demand per capita are correlated with economic growth (254). Figure 1.2 shows that India has a huge growth potential. Still, India has a great paper market because of large population and we have to still import large amount of writing paper (Table 1.1).

Looking into the world demand for paper and paperboard, the forecast is that the market will grow from the current 300 million tonnes to over 420 million tonnes by the year 2010 with an average growth rate of 2.8% per annum (130). In India, the annual current production gap is 0.7 million tonne and will become 1½ times greater during 2010-15 (296). The per capita consumption of paper in India is 5.5 kg/annum compared to global average of about 50 kg (136). Table 1.2 comprises the per capita consumption of paper and board in various countries (329).

In the recent past, the Indian paper sector has maintained a growth rate of around 6%, which is a way above the world average of about 2.8% (136). With such growth prospects, the Indian paper industry has a vital role to play in the socio-economic development of the country. As on date there are more than 600 pulp and paper mills producing nearly 5.5 million tonnes of paper and paper board and 0.68 million tonnes of newsprint against the present installed capacity of 7.5 million tonnes of paper and paper board and about 1.30 million tonnes of newsprint. The projected demand for paper board and newsprint is expected to touch 8.3 million tonnes by the year 2010 (136).

Indian pulp and paper industry has a number of issues and challenges, particularly in the following thrust areas, such as:

- Non availability of good quality of cellulosic raw materials.
- Low scale of operation.
- Obsolescence of technology.
- Soaring environmental costs.
- High cost of basic inputs.

The Indian paper industry is primarily dependent on three major raw materials viz. forest, agricultural residues and secondary fibers (372). Pulp and paper industry uses 39% of forest based fiber, 31% agro residue based fiber and 30% fiber is derived from waste paper (170). The proportion of non-wood raw materials and waste paper is increasing over the year and at present about 60.8% of non-wood raw materials and 39.2% is based on wood (296). In India, forest and woodlands occupy around 20%, agricultural land 47% and uncultivated, non-agricultural and barren land 30% respectively of a total land

area of 328.8 million ha (254), of this, current forest plantations are estimated at 32.5 million ha (Figure 1.3). The Indian paper industry uses only 3.5% of the total wood produced in India. In the year 2000, the final demand for the wood by Indian paper industry was 5.8 million tonnes. It is projected that this figure will grow over 9 and 13 million tonnes by the year 2010 and 2020 respectively (Figure 1.4) (254), as the literacy rate in India has been steadily growing (52% in 2000) (254). Likely raw material supply, utilization and production of pulp and paper through 2010 are summarized in Tables 1.4 and 1.5 (324).

With depleting forest resources, the wood based segment of the paper industry has considerably shrunk, as in 1970, forest based segment of Indian paper industry was 84% which has been reduced to 31% in 2000 (329). In India, inadequate supply of wood fiber due to rapid depletion of forest wealth, forces the Indian pulp and paper industry to use various alternate fibrous resources such as non-woody plants, agricultural residues and waste paper. The share of agro based segment of the paper industry was only 9% in 1970 which has been increased up to 31% in 2000 (329). The main agricultural residues utilized by the paper industry include sugarcane bagasse, cereal straws (wheat and rice), kenaf/mesta, jute sticks, grasses and cotton stalks. The annual potential of agro fibers in India is given in Table 1.3 (254).

Bagasse, the sugarcane residue, is found to be the best alternative because of its low cost, longer fiber than straw, low refining energy consumption and good sheet formation and paper smoothness (254), which enabling the sugarcane bagasse to meet the quality requirements for newsprint and fine paper manufacture (274). Yet, there are certain inherent draw backs like high

storage cost and quality variation (pith), low brightness, wet strength and pulp yield (quite short fiber), very easy lumen collapse (optical properties, bulk and stiffness problems) and slow drainage i.e. high drying steam consumption (254) which need special treatment, by which the bagasse can replace the conventional forest based raw materials (274).

India is the largest consumer and the second largest producer of sugarcane next to Brazil with a production of more than 300 million tonnes of sugarcane in 2001–2002 (accounting for around 10–12% of world's sugar production) (214, 91). In India, about 4 million ha of land is under sugarcane farming with an average yield of 70 t/ha (214). The Indian sugar industry is the second largest agro processing industry in the country after cotton textiles (356, 221). With an estimated production of 18.6 million tonnes in sugar year or SY2006 (sugar year is from September to October), India's sugarcane cultivation area of 4–4.5 million ha accounts for 2.7% of India's cropped area. Sugar industry accounted for around 1% of GDP of the country during the financial year 2005–2006 (FY2005). The Indian sugar industry has a turnover of Rs. 500 billion per annum and it contributes almost Rs. 22.5 billion to the central and state exchequer as tax, cess, and excise duty every year (207). Figure 1.5 shows the sugar map of India. With 566 operating sugar mills in different parts of the country, Indian sugar industry has been a focal point for socio-economic development in the rural areas (268). At present about 50 million tonnes of bagasse is generated in the country per annum, of which only 10 million tonnes is available as surplus for the pulp and paper industry and the quantity of this surplus bagasse is difficult to be increased under the present operating

conditions of the sugar industry (329). It is expected that even if only 20% of the total quantity can be made available to Indian pulp and paper industry, then, there should be no difficulty in meeting the targeted demand of paper and board products (82). The bagasse based paper production estimated by the Development Council (average recovery of 1 tonne of paper from 6 tonne bagasse) is given in Table 1.6. The bagasse based paper production estimated by Indian Agropaper Mills Association (IAPMA) is given in Table 1.7. (325).

The pulp and paper industry is also under constant pressure to reduce and modify environmental emissions to air and water due to stringent rules of the government. Paper industry in India is on constant watch by the Ministry of Environment and Forest since it is an environment sensitive sector and falls under red category of industries (270). The pulp and paper industry is one of the oldest industries and is recognized as one of the seventeen industries causing extensive pollution and damage to the environment in India (165). Like any other large scale industry, the pulp and paper industry exerts its own impact on the environment. Organochlorine compounds formed during the bleaching of chemical pulp have attracted most attention in recent years. While not all AOX compounds are harmful to health or the environment, a number of these have been determined to be so (21). During a variety of steps of the chlorine bleaching, various chloroaromatic derivatives are released from the lignocellulosic biomass that are ecotoxic and cause significant damage to the environment. The need for the removal of hemicellulosic and lignin component is essential because these are the major chromophoric substances present in the plant cell wall and removal of these is a prerequisite for the processing of the cellulosic constituent of plant cell wall for pulp and paper industries.

The ever increasing pressure from environmental protection authorities has forced the pulp and paper industry to seek an environmentally benign bleaching process as the byproducts released during chemical bleaching processes are toxic, mutagenic, persistent, bioaccumulating and cause numerous harmful disturbances in biological systems. To reduce chlorine and chlorine compounds in bleaching which are the most polluting, industries/researchers have developed alternative bleaching methods (199) by the replacement of elemental chlorine with chlorine dioxide in bleaching process *giving elemental chlorine free* (ECF) *bleaching sequence, (49) and without* elemental chlorine or chlorine containing compounds, termed as totally chlorine free (TCF) bleaching process. These bleaching methods use oxygen, ozone and peroxide etc., but these are quite expensive to adopt, since, they require a lot of changes in the infrastructure and hence are only viable to large paper mills (199). These technologies may not be techno-economically feasible for agro residue pulp mills smaller than 100 TPD in developing countries like India (344).

So the new bleaching procedures have been adopted which replace the classical process based on chlorine, chlorinated compounds, hydrogen peroxide, oxygen, ozone, etc. with another method called the 'biobleaching process' (367, 33, 142). Biobleaching is one of the very promising alternatives for eliminating chlorine based chemicals in the pulp bleaching process (21). Biobleaching involves using microorganisms and enzymes to bleach pulp. Till date, biological bleaching of pulp has been approached mainly by the use of ligninolytic (20, 235, 302) and hemicellulolytic enzymes (108, 347). Among several enzymes, xylanase has tremendous potential for biobleaching.

Xylanases have been denoted as a major group of enzymes, having significant application in the pulp and paper industry. Viikari *et al.* was the first to demonstrate that xylanases are applicable for delignification in the bleaching process (363). The positive effect of xylanase is generally attributed to the elimination of the xylans, thereby breaking the existing link between the cellulose and the lignin, and since the lignin is therefore free, it is more rapidly eliminated in subsequent bleaching stages (355, 256) with minimal damage to the pulp. This process is less drastic, less expensive and especially less toxic than conventional chemical treatment. Besides, bleaching through lignin removal, the use of xylanases helps to increase pulp fibrillation, reduce beating time, and increase the freeness in recycled fibers (141, 140, 159). A treatment with xylanases can improve the chemical extraction of lignin from pulp. This leads to a significant reduction in the amount of chemicals required for bleaching and in the levels of toxic chlorine compounds released in to the environment (342), thus improving the characteristics of effluent like COD, BOD, color, AOX etc. generated during bleaching. The applicability of xylanases are not only limited to the pulp and paper industry but have found wide spread applications in the baking and textile industry, fruit and vegetable processing, brewing, wine production, starch-gluten separation, bioconversion and bioremediation of agro-residues.

Keeping this view the present communication aims at developing environmental benign technology for the production of pulp and paper using sugarcane bagasse. The objective of the study is to evaluate and estimate the viability of reducing chemical consumption and saving energy with preserving

mechanical strength properties of sugarcane bagasse pulp through enzymatic treatments.

The major outlines of the work are therefore defined as here under:

- Depithing (dry and wet), morphological studies, and a proximate chemical analysis of sugarcane bagasse.
- Optimization of soda pulping process and mechanical strength properties of sugarcane bagasse and use of anthraquinone (AQ) to reduce kappa number prior to bleaching and improve pulp yield.
- Isolation of fungal strains from lignocellulosic wastes, screening and evaluation of strains for higher xylanase and lower cellulase production.
- Analysis of xylanase production under submerged and solid-state fermentation systems. Selection of fermentation system in order to get high xylanase production and optimization of various operating parameters for achieving maximum xylanase production.
- The analysis and evaluation of the xylanase preparation for its application in biodelignification of sugarcane bagasse soda-AQ pulp and to reduce the toxicity of effluents generated during conventional, ECF and TCF bleaching sequences to make the process eco-friendly.

Table 1.1: Paper market in India in 2000 (Each figure is given in 1000 tonnes) (254)

Particulars	Production	Imports	Exports	Consumption
Newsprint	456	388	0	844
Printing/ writing	1530	60	60	1530
Uncoated mechanical	0	35	0	35
Coated mechanical	0	5	0	5
Uncoated wood free	1315	15	20	1310
Coated wood free	215	5	40	180
Tissue paper	30	8	0	38
Corrugating materials	806	8	0	814
Carton boards	828	0	30	798
Sack/ Kraft paper	50	0	0	50
Others	150	6	15	141
Total	3850	470	105	4215

Table 1.2: Per capita consumption of paper and paper board in different countries (329)

Country	Per capita consumption of paper & paper board, kg
USA	312
Singapore	228
Western Europe	160
Malaysia	89
China	20
Indonesia	14
Philippines	11
India	5.5

Table 1.3: Annual potential of agro based fibers in India in 2001 (254)

Agro residues	Availability, million tonnes	Tonnes needed for 1 tonne of pulp	Pulp potential (Theoretical)
Wheat straw	22	2.5-3.5	7
Rice straw	15	2.5-3.5	5
Bagasse	10	5.0-6.0	2
Jute, mesta, kenaf	2	–	–
Total	49	–	14

Table 1.4: Outlook 2010: Likely raw material supplies and production of pulp and paper (million tonnes) (324)

	Million tonnes		
	2000	**2005**	**2010**
Fibre source			
a) Bagasse			
1. Output	60.0	75.0	90.0
2. Availability to pulp and paper industry	6.0	9.0	12.0
3. Production	1.1	1.6	2.6
b) Recycled fiber*			
1. Output	–	–	–
2. Availability to pulp and paper industry	0.9	1.3	1.6
3. Production	0.7	1.0	1.2
c) Imported pulp			
1. Output	–	–	–
2. Availability to pulp and paper industry	0.9	0.9	0.9
3. Production	0.7	0.7	0.7
d). Wood and bamboo			
1. Output	64.0+5.2**	73.0+6.0**	82.0+6.8**
2. Availability to pulp and paper industry	–	–	–
3. Production	1.4	1.2	1.2
Estimated total production of paper and newsprint	4.1	5.0	6.1
Total production of fibers	7.3	8.2	9.7

*Chiefly domestic and imported waste paper
**Wood and bamboo

Table 1.5: Likely pattern of utilization of raw material through 2010 (324)

Raw material source	Likely production of paper, paperboards and newsprint, million tonnes			% Shares		
	2000	2005	2010	2000	2005	2010
Non wood fiber sources*	1.3	2.1	3.0	32	42	49
Bagasse	1.1	1.6	2.2	–	–	–
Others**	0.2	0.5	0.8	–	–	–
Recycled fiber	0.7	1.0	1.2	17	20	20
Imported pulp	0.7	0.7	0.7	17	14	11
Wood and bamboo	1.4	1.2	1.2	34	24	20
Total	4.1	5.0	6.1	100	100	100

*Bamboo not included

**Chiefly cereal straw and other assorted residues in small quantities

Table 1.6: Estimated bagasse based paper production: Development Council 1990 (325)

Year	Total bagasse available, million tonnes	Surplus bagasse, %	Quantity available for paper production, million tonnes per annum	Quantity of paper production, million tonnes per annum
1990	7.2	15	1.08	0.18
2000	7.2	50	3.60	0.60
2015	7.2	70	5.04	0.84

Table 1.7: Estimated bagasse based paper production: IAPMA 1996 (million tonnes per annum) (325)

Particulars	1994-95	2000-01	2005-06	2010-11
Sugar production	14.6	20	25	30
Cane crushed	146	200	250	300
Bagasse yield	43.8	60.0	75.0	90.0
% availability for paper making	7	10	12	13
Quantity -do-	3.1	6.0	9.0	12.0
Paper production	0.6	1.1	1.6	2.2

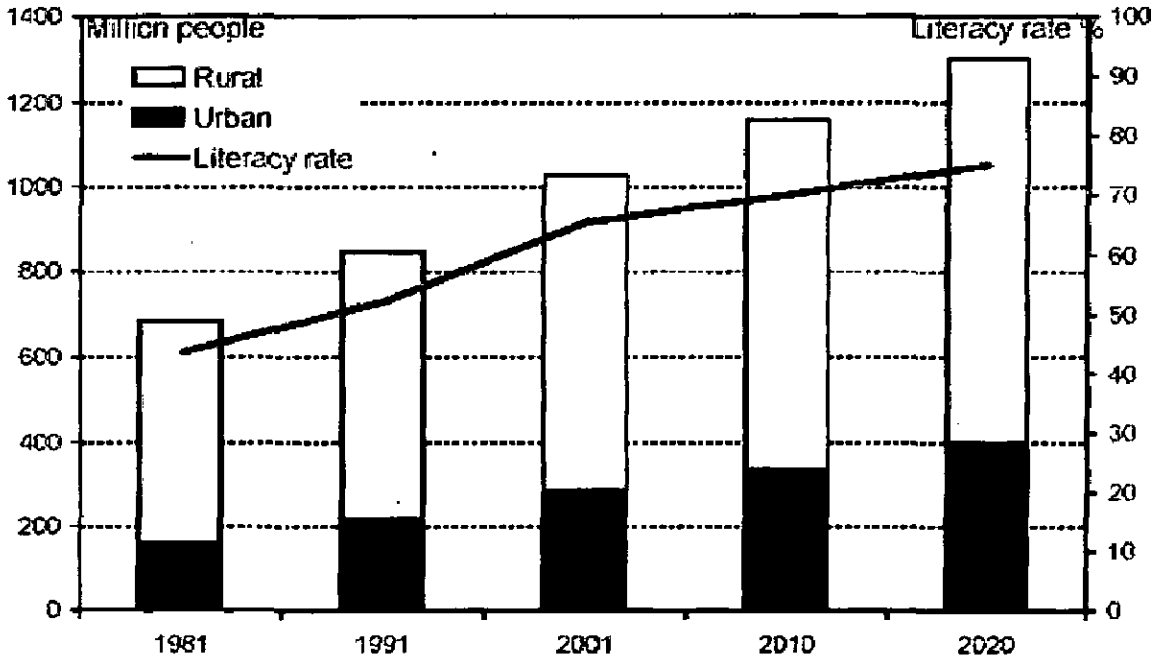

Figure 1.1: Indian population and literacy rate in both rural and urban area (254)

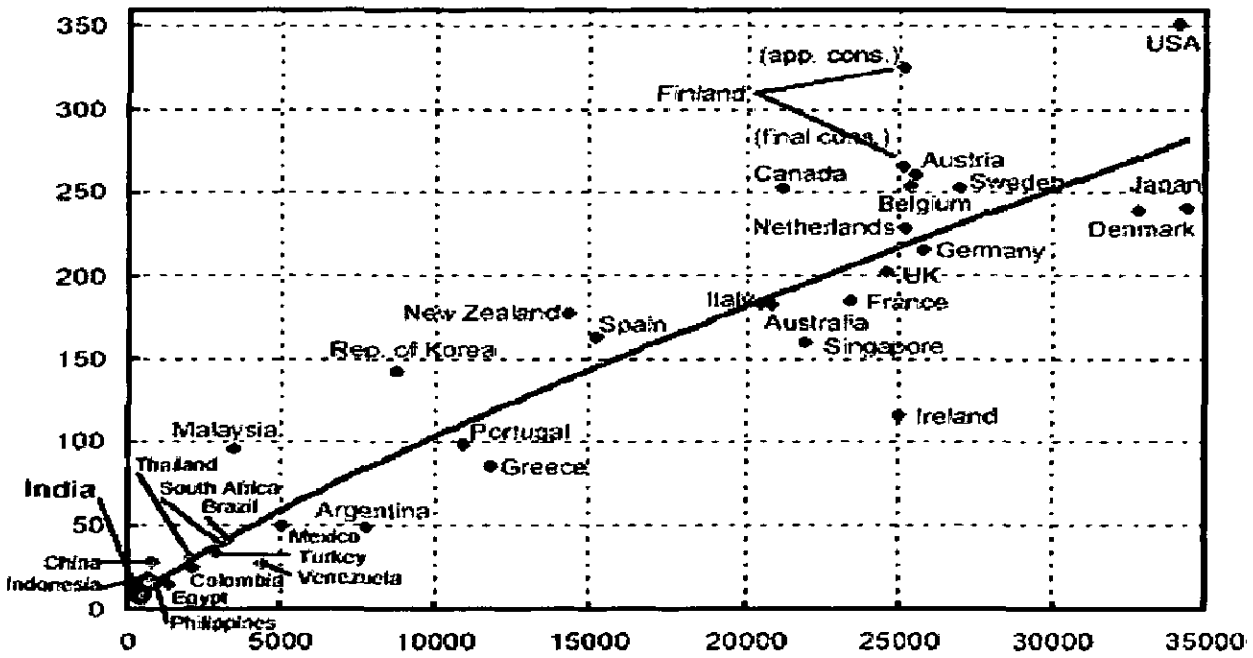

Figure 1.2: GDP and paper demand in the year 2000, kg per capita (254)

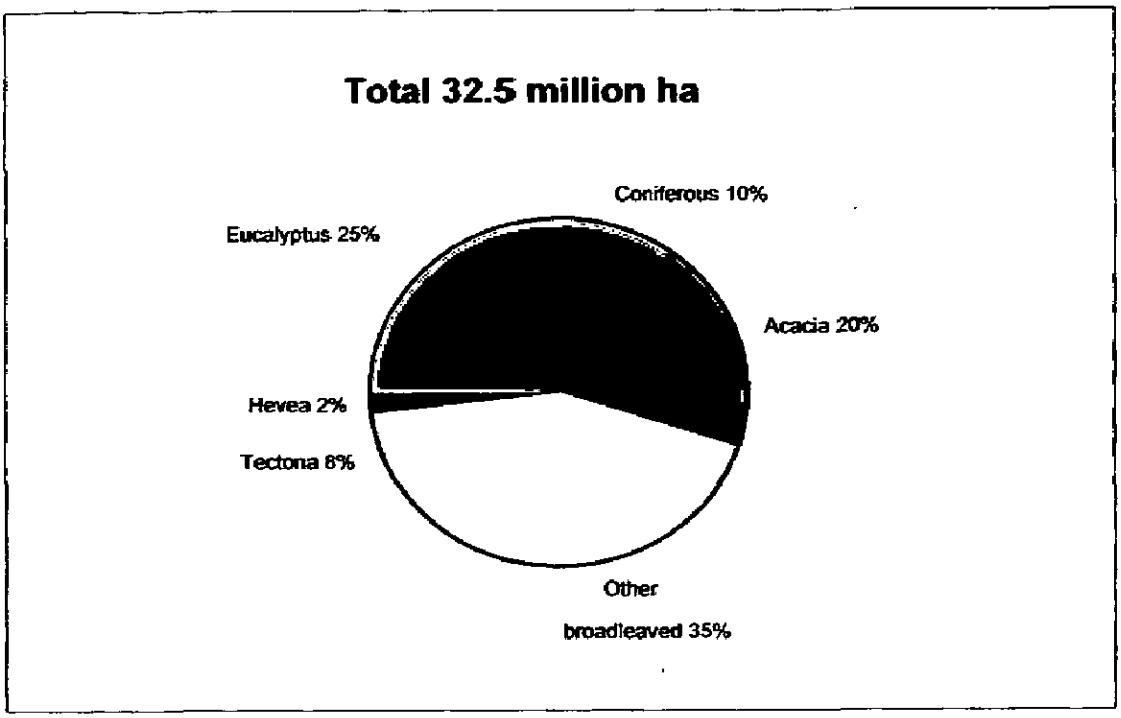

Figure 1.3: Forest plantation in India (254)

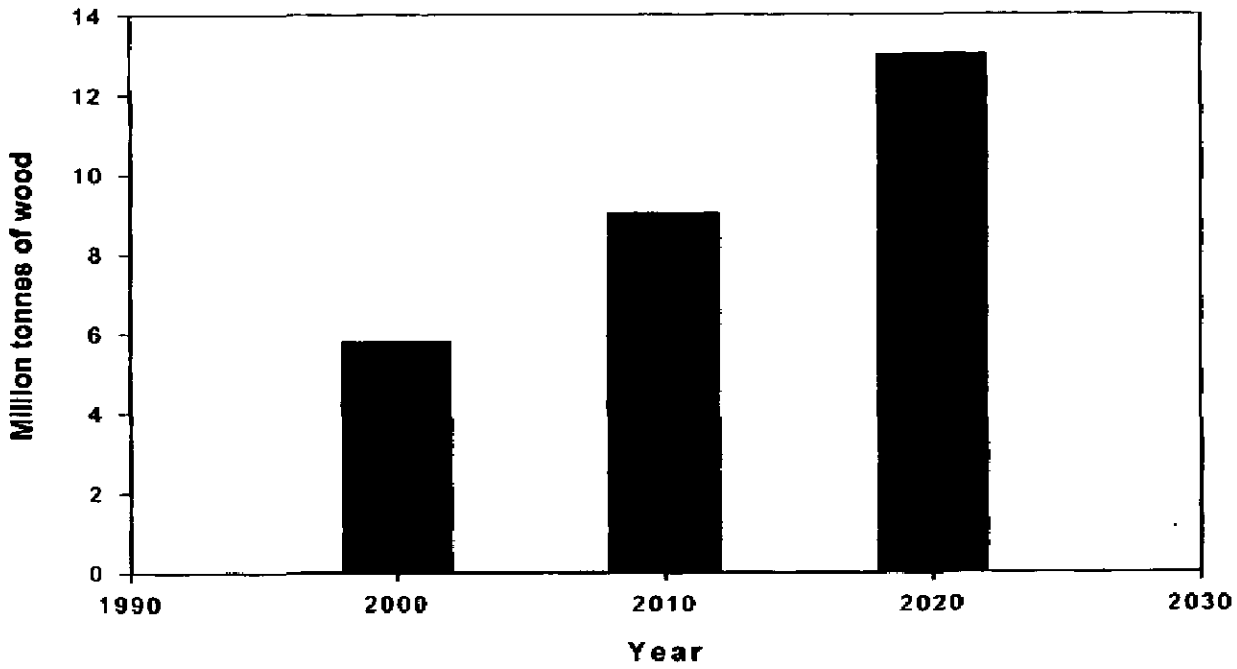

Figure 1.4: Industrial wood demand scenario for India (254)

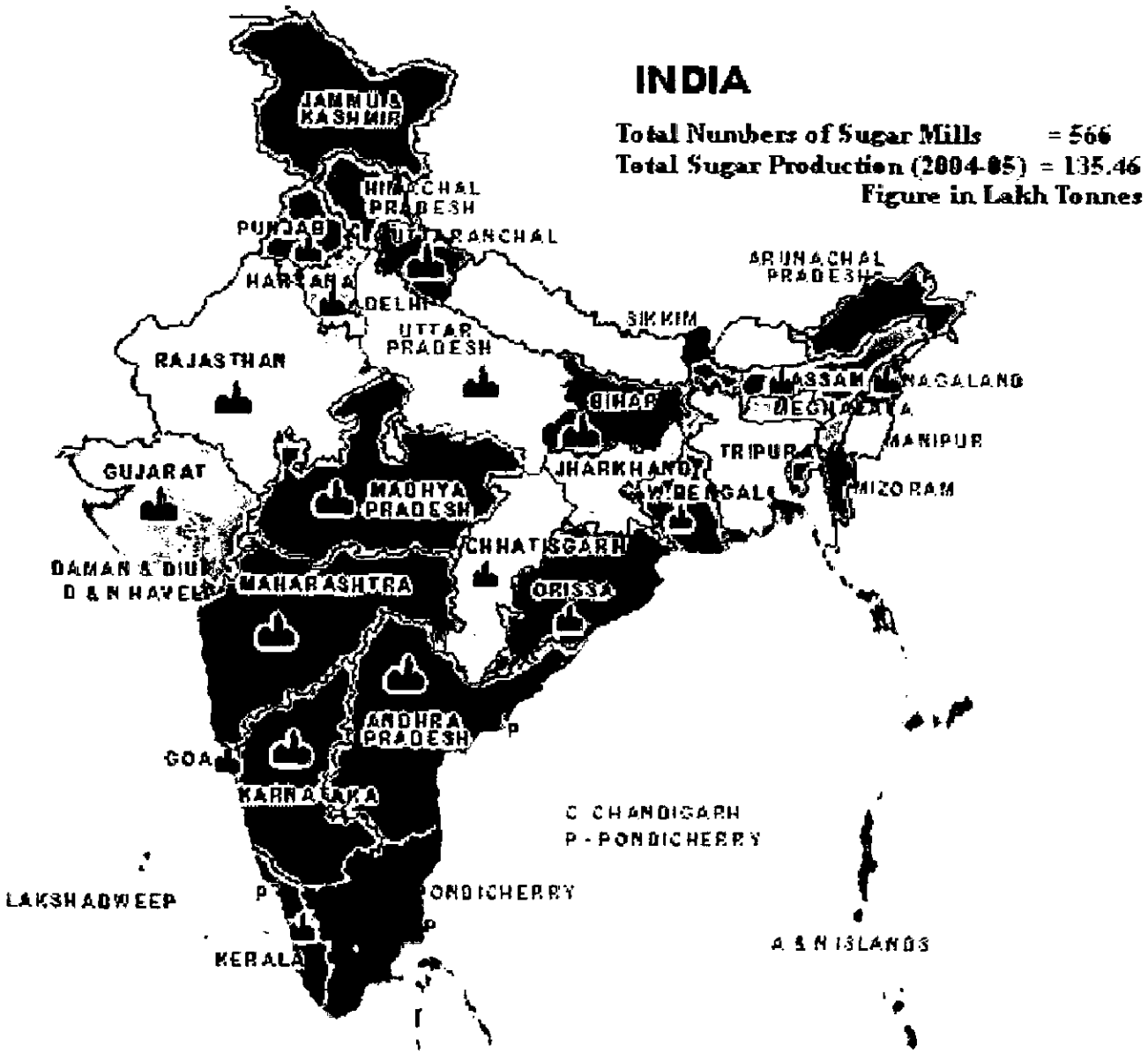

Figure 1.5: Sugar map of India (332)

CHAPTER 2

ANATOMICAL, MORPHOLOGICAL AND CHEMICAL COMPOSITION OF SUGARCANE BAGASSE

CHAPTER- 2

ANATOMICAL, MORPHOLOGICAL AND CHEMICAL COMPOSITION OF SUGARCANE BAGASSE

2.1 INTRODUCTION

A cell is the basic structural and functional unit of a plant which typically consists of protoplast and is surrounded by cell wall. In early stages of growth, the cell cavity contains protoplasm but soon after the cell is fully grown, the protoplasm disappears from the cell and the structure is known as the wood cell (239). The wood cells which are elongated, dead and hollow are classified as the plant fiber. They function to provide mechanical support and to transport photosynthetic products and water in the plant parts where these are located. They can occur as isolated cells, clusters or in large masses (tissues) depending upon the plant type or part (157). The fibers are divided into two large groups – xylem fibers and extraxylary fibers. The xylem fibers develop from meristematic tissue and constitute an integral part of xylem. On the other hand, some of the extraxylary fibers are related to the phloem. The fibers that form continuous cylinders in non-woody stems arise in the ground tissue are known as cortical fibers. The fibers forming sheaths around the vascular bundles in the non-woody stems arise partly from procambium and partly from the ground tissue (247).

A dark colored zone around the centre of the stem is known as heart wood. This portion is surrounded by light colored peripheral zone known as sapwood. Sapwood functions in sap conduction and storage of reserve food as

well as in the support of the tree crown. The sapwood has living parenchyma cells. In contrast to sapwood, heartwood is comprised of dead xylem cells. As the tree matures, all parenchyma cells of the sapwood die and other types of cells, such as trachieds or fibers become occluded with pigments composed of polyphenols and flavanoids, supplied mainly from ray parenchyma cells. In softwoods, the heartwood generally contains more extractives and lesser lignin and cellulose than the sapwood (112). Each year the wood cells grow fast early in the growing season (spring), producing springwood or early wood. Later in the season, as winter approaches, growth slows down producing summerwood or latewood. Early wood is generally characterized by fibers with large cross-sectional area and thin cell walls with wide lumen (for water conduction) while latewood fibers have a smaller cross-sectional area and thicker cell walls (for mechanical strength) with narrower lumen, higher cellulose and lower lignin (15, 282) than early wood fibers resulting in their different paper making properties (112).

In woody plants, softwoods are relatively uniform consisting of over 90% tracheid fibers and only 10% stubby ray cells and other fines (131). Softwood fibers (i.e. tracheids) are cells which combine the functions of conduction and support and, although those species with distinct growth rings show some quantitative differences, in general they are sausage shaped with abundant, fairly large, bordered pits (24). Hardwoods by comparison are more heterogeneous and contain only about 50% tracheid fibers and a large number of vessel cells and ray cells (131). Hardwood fibers on the other hand are principally structural cells, mostly spindle shaped with sharp pointed ends and

have pits which are smaller, not so abundant and may be either bordered or simple. Hardwood fibers with simple pits are referred to as libriform fibers and those with bordered pits as fiber-tracheids. The fiber-tracheid is considered to be a transitional form between tracheids and libriform fibers (89). Eucalypt fibers are classified as fiber-tracheids. In general non-wood fibers are longer and more slender than wood fibers and softwood fibers are longer and more slender than hardwood fibers. The non-wood fibers are commonly 100 to 200 times longer than they are wider (250); softwood fibers about 100 times (248) and eucalypt fibers are about 70 times (70). Average length of softwood fibers lies between 2.7 to 4.6 mm while that of hardwood and non-wood fibers (including hemp fibers) lies in between, 0.7 to 1.6 and 0.5 to 30 mm respectively (12). Comparison of fiber dimensions of softwood, hardwood and non-woody cellulosic raw materials is given in Table 2.1.

One of the first fiber properties related to paper strength is fiber length (127). Seth and Page, have shown that, under certain conditions, tearing resistance depends strongly on fiber length (308), whereas Horn, reported that increase in raw material fiber length enhances the tearing strength of hardwood pulps (128). Burst and tensile strengths are influenced primarily by a combined effect of fibril length and cell wall thickness as measured by the pulp fiber flexibility ratio index (L/T) (128). Runkel ratio and fiber to vessel ratio influences the basic density of wood (118, 69). It is directly related to the degree of fiber collapsibility, conformability, and the relative fiber bonded area. It, in turn, imparts its direct influence on surface and other mechanical strength properties of paper (195) and because of the necessity for the fiber walls to flatten and

ensure good conformability and fiber to fiber contact in paper, it is desirable to have a Runkel ratio less than 1 (24). Horn showed that the presence of high percentage of fines was detrimental to bursting and tensile strength (128). Vessel element, on the other hand, based on amount actually found in a typical hardwood furnish, has little effect on tensile strength. Fibril angle is the factor that has the greatest influence on stretch properties (extensibility) of a sheet from unbeaten fibers and multiple regressions showed that interaction of fiber length plus fibril angle could account for 76% of the variation in tearing strength for unbeaten hardwood pulp (128). The influence of fiber morphological properties on paper strength is summarized in Table 2.2.

Wood is a natural composite material and a chemical complex of cellulose, hemicellulose, lignin and extractives. Cellulose is a linear, unbranched homopolysaccharide, consisting of 10,000 to 15,000 D-glucose units which are linked by (β1→4) glycosidic linkages (Figure 2.1). Cellulose is the framework substance comprising 40 to 50% of wood in the form of cellulose microfibril. The smallest cellulosic strand, with an average width of 3.5 nm in the mature call wall, is termed an "elementary fibril". These fibrils, in turn, are organized into strands known as "microfibrils" (5-30 nm wide), which are visible under electron microscope. The diameter of microfibrils depends on the source of cellulose and the position of the microfibrils within the cell wall. The cellulose microfibrils are oriented in different directions in the different fiber wall layers, and they contain crystalline regions. In addition, their orientation has an influence on the physical properties of a wood fiber (112).

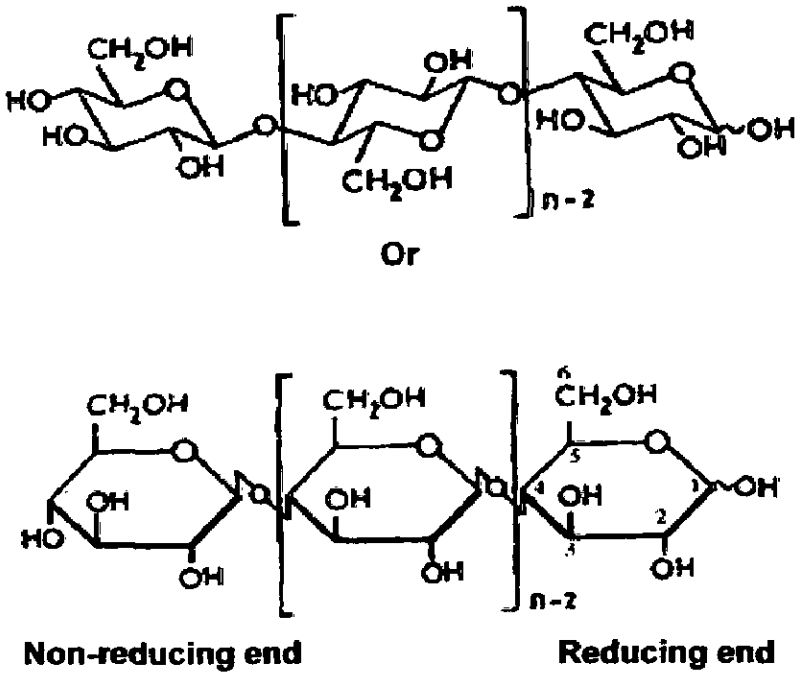

Figure 2.1: Molecular structure of cellulose (95)

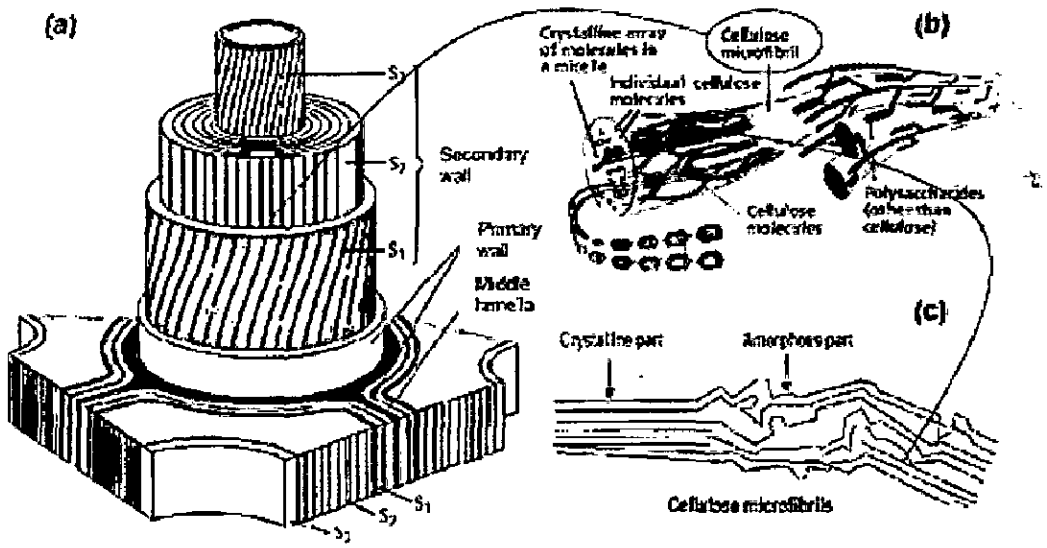

Figure 2.2: Simplified composition of the wood cell wall (a) The cell wall is divided into a primary (P) and secondary (S_1, S_2 and S_3 layers) cell wall. The lines in the secondary cell wall represent the microfibrillar alignment (338) (b) A schematic representation of the cellulosic microfibrils. Amorphous hemicellulose and some lignin are located between the crystalline cellulose microfibrils (c) Schematics of a cellulose microfibril (46)

The cell wall of all plant fibers is basically composed of two layers including the relatively thin "primary wall" (P) and the thick "secondary wall" (S) (Figure 2.2). Based on differences in the microfibrillar orientation, the latter layer is divided into three sub layers that are termed as follows: the outer layer of the secondary wall (S_1), the middle layer of the secondary wall (S_2), and the inner layer of the secondary wall (S_3) (Table2.3). The S_3 layer is sometimes also referred to as the "tertiary wall" (T). The microfibrils wind around the cell axis in different directions either to the right (Z helix) or to the left (S helix). In certain cases, for example, conifer tracheids and some hardwood cells, the inside of the S_3 layer is covered with a thin membrane called the "warty layer" (W). The central cavity of the hollow fiber is termed the "lumen" (L). The middle lamella (ML) is located between the P walls of adjacent cells and serves the function of binding the cells together. Since it is difficult to distinguish ML from the two P walls on either side, the term "compound middle lamella" (CML) is generally used to designate the combination of ML with the two adjacent P walls. In general, the S_2 layer increases with an increase in wall thickness, whereas S_1 and S_3 remain fairly constant. Thus, the S_2 layer is largely responsible for the physical properties of wood fiber. The S_2 layer exhibits a steep Z helix with a high degree of parallelism in the microfibrils. However, transition lamellae (S_{12} and S_{23}) occur on its outer and inner surfaces. The microfibrillar orientation in these lamellae gradually changes between S_1 and S_2 and between S_2 and S_3, with the change in microfibrillar angle being more abrupt in S_{23} than in S_{12} (110). Two main factors, cellulose microfibril angle (MFA) in S_2 layer (thickest layer) and chemical composition, govern functional properties of the cell wall such as

Young's modulus and growth-stress. Recently it was suggested that a change of MFAs resulted in the change of required mechanical properties such as stiffness or flexibility (278, 181, 88). Table 2.4 shows the general cell wall chemical composition of coniferous fibers (189).

Non-wood plants offer several advantages including short growth cycles, low lignin content and high pentosan or hemicellulose content than wood; resulting in reduced energy and chemicals consumption during pulping (130; 131). The chemical composition of non-wood fibrous plants varies somewhat from plant to plant, but on an average these contain around 40% cellulose, 15-22% lignin, little percentage of extractives and the rest is made up of a mixture of polymer non glucose carbohydrates, known as hemicelluloses. The chemical analysis of a plant is also known as proximate chemical analysis which provides important information pertaining to suitability of a plant to pulp and paper making. Water solubility provides a measure of tannins, gums, sugars, coloring matters and starches in wood and pulp. Water solubles affect the pulp yield to some extent. Therefore, water soluble in a plant should be as low as possible (8). The alcohol benzene solubility of wood is a measure of waxes, fats, resins, gums and phytosterols. The extractives influence the quality of pulp and paper making operation as they cause pitch problems (178). 1% NaOH solubility indicates that pulp is more susceptible to deterioration during storage as this indicates the degree of fungal decay or degradation by heat or light (217). Lignin represents what is called the "incrusting material" forming a part of the cell wall and middle lamella in wood. It is an aromatic, amorphous substance containing phenolic methoxyl, hydroxyl, and other constituent groups; its chemical structure

has not been fully elucidated. Wood contains about 20 to 30% lignin, removal of which is a main objective of pulping and bleaching processes. Determination of lignin content in wood and pulps provides information for evaluation and application of the processes. Hardness, bleachability, and other pulp properties, such as color, are also associated with the lignin content (299). The residual lignin present in the pulp influences the paper properties and causes the high stiffness in paper. The percentage of lignin in the wood is related with the chemical dose and time required for delignification, the higher the lignin content, the higher the chemical dose and longer the cooking cycle required for pulping. The cellulose is main component in chemical pulping so variation in pulp yield and economy in pulp production directly reflect the cellulose content of the wood. Cellulose is long chain polymer while the hemicellulose has a much lower molecular weight than the cellulose and has a low degree of crystallinity. The hemicellulose dissolves to a large extent in the chemical pulping; however, a substantial quantity of hemicellulose is always associated with the pulp. It also has a great influence on the swelling behavior of the fiber (4). Paper strength also depends on the cellulose content of the pulp and mechanical strength of the pulp (especially tensile strength) is directly proportional to the cellulose content (190). The ash content of wood or pulp gives an estimation of the content of mineral salts and other inorganic matter in the pulp, but it is not quantitatively equal to it. Silica impairs the burning and sedimentation operations in the recovery process. When the wood contains large quantities of silica, it may show damaging effect on the process of wood or on the paper quality (7).

2.2 EXPERIMENTAL METHODOLOGY

Sugarcane bagasse was collected from U.P. Sugar Cooperative Factory, Bidwi, 15 km away from Indian Institute of Technology Roorkee, Saharanpur Campus, Saharanpur located in the foothills of Shivalik hills in Western Uttar Pradesh (India). Sugarcane baggase was air dried and stored in polythene bags till further processing.

2.2.1 Depithing

Sugarcane bagasse was given mechanical treatments i.e. dry and wet depithing followed by intensive screening to remove most of the non-fibrous cells, means of course that part of the structure was already broken up before pulping. Dry depithing was done in dry depither (Bramco, BD 101, India). Further, the depithed bagasse was disintegrated in hydrapulper (wet depithing) to remove rest of the pith cells at 2.5% consistency and screened in WEVERK vibratory screen of mesh size –150 an approach similar to that followed by Rajesh (274). The useful fibers in dry and wet depithed sugarcane bagasse were determined as per Tappi UM-3, 1980. The results are reported in Table 2.5.

2.2.2 Anatomical and morphological studies

For fiber length determination, small slivers of sugar cane baggase were macerated with 10 mL of 67% HNO_3 and boiled in a water bath at 100 ^{0}C for 10 min (232). The slivers were then washed, placed in small flasks with 50 mL distilled water and the fiber bundles were separated into individual fibers using a small mixer with a plastic end to avoid fiber breaking. The macerated fiber suspension was finally placed on a slide (standard, 7.5 cm × 2.5 cm) by means

of a medicine dropper (121). All fiber samples were viewed under a calibrated microscope; a total of 100 randomly chosen fibers were measured. These derived values were also calculated using fiber dimensions: slenderness ratio as fiber length/fiber diameter, flexibility coefficient as (fiber lumen diameter/fiber diameter) × 100 and Runkel ratio as (2 × fiber cell wall thickness/lumen diameter) (291, 232). For fiber diameter, lumen diameter and cell wall thickness determination, cross sections of sugarcane were taken at base, middle and top of its height/length respectively, an approach similar to that followed by Dutt, (81) and were stained with 1:1 aniline sulphate–glycerin mixture to enhance cell wall visibility (cell walls retain a characteristic yellowish color). The anatomical structures of sugarcane are shown in Figure 2.3: Photomicrographs A-D. The morphological characteristics of sugarcane bagasse were compared to those of *Eucalyptus tereticornis*, *Eucalyptus robusta* and *Leucaena leucocephala* and are reported in Table 2.6.

A suspension of sugarcane bagasse fibers (0.02% consistency) was used for detailed anatomical features like fiber length, fiber width, curl index and kink index by using Hi-Resolution Fiber Quality Analyser (Optest Equipment Inc. model: LDA 2002) (Table 2.8).

2.2.3 Proximate chemical analysis

Depithed sugarcane bagasse was milled into 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 analysis of water solubility (T 207 cm-99), 1% caustic soda solubility (T 212 om−98), alcohol-benzene solubility (T 204 cm−97), holocellulose (T 249 cm−00), lignin (T 222 om−02),

ash (T 211 om-93), pentosan (T 223-cm-01), iron (T 242 wd-97), chloride (T 256 cm-97), and calcium (T 247 wd-98) as per Tappi Standard Test methods: 2000 and phosphorus (424 F) as per Standard methods for the examination of water and wastewater, American Public Health Association, 1985. Carbon, Hydrogen and Nitrogen were determined using Heraeus rapid C, H, N analyser. The results of proximate chemical analysis were compared with *Leucaena leucocephala* and *Eucalyptus globulus* and the results are reported in Table 2.9.

2.2.4 Statistical analysis

All experiments were carried out in triplicate and experimental results were represented as the mean ± standard deviation of three identical values.

2.3 RESULTS AND DISCUSSIONS

Table 2.5 shows the percentage of useful fiber, pith and solubles in dry and wet depithed *Saccharum officinarum* (sugarcane bagasse). Results show that even after dry and wet depithing; 15.48% pith is still associated with sugarcane bagasse fibers while useful fibers percentage is 71.36 along with 13.14% of solubles. A transverse section of depithed sugarcane bagasse suggests that minor bundles are concentrated close to the epidermis and form an almost continuous ring of fibrous tissue. Inside this outer circle the most valuable vascular bundles are found. This peripheral part of the stem called the rind, contains the main fibrous raw material for producing bagasse pulp. The conducting system is built up by two major complex tissues, xylem and phloem. The vascular bundles of sugarcane are collateral, which means that phloem is external to xylem (Figure 2.3: Photomicrograph A). The outermost part of the

stem is the epidermis, formed as a thin but very dense layer made up by various thick walled cells in a remarkably uniform pattern. The ground tissue is composed of different types of parenchymatous cells. These are primarily storage cells for solutes and food stuffs for the plant (122). The parenchymatous cells can be divided into four main groups: collenchyma, chlorenchyma, rind and pith parenchyma. The zone between epidermis and the vascular tissue is termed as cortex. There is a typical difference between the rind and pith parenchyma cells. Rind parenchyma cells are long, wide and quite thick walled (122) and present to a great extent in the pulp while pith parenchyma cells are barrel like and very thin walled and if present in the pulp, they will occur as fragments (Figure 2.3: Photomicrographs B, C). The pith parenchyma is readily broken to flakes but rind parenchyma is resistant to pulping which do not have bonding effect and act as fillers. If rind parenchymatous cells present on paper surface they cause linting during paper making or printing (122).

The fiber of sugarcane bagasse varies from 0.3 to 4.0 mm in length (average fiber length 1.51 mm) (Figure 2.3: Photomicrograph D) which is higher than that of *E. robusta* (average fiber length 1.07 mm) and *E. tereticornis* (average fiber length 0.70 mm) and *L. leucocephala* (average fiber length 0.96 mm). The fiber width of sugarcane bagasse varies from 10 to 40 μm (average fiber width 21.40 μm) which is almost comparable than that of *E. robusta* and *L. leucocephala* (average fiber widths 19 and 23.33 μm respectively) but higher than that of *E. tereticornis* (average fiber width 14.2 μm). Lumen diameter is 6.27 μm which is higher than that of *E. tereticornis* (average lumen diameter 3.4 μm) and much lower than that of *E. robusta* and *L. leucocephala* (average

lumen diameter 12.1 and 12.90 μm respectively). Cell wall thickness is 7.74 μm which is 20.23% higher than that of *E. tereticornis* and 56.07% than *E. robusta* but 25.93% lower than that of *L. leucocephala* (Table 2.6). Runkel ratio of sugarcane baggase is lower than that of *E. tereticornis* but higher than that of *E. robusta* and *L. leucocephala.* The flexibility coefficient is higher than that of *E. tereticornis* but much lower than that of *E. robusta* and *L. leucocephala* while rigidity coefficient is comparable to that of *E. tereticornis* and higher than that of *E. robusta*. However, the rigidity coefficient of sugarcane baggase fibers is comparable to *E. tereticornis* but sugarcane bagasse fibers are more flexible than that of *E. tereticornis.* The sugarcane bagasse fibers readily form ribbons on pressing and providing more surface contact area for bonding compared to *E. tereticornis.* The fibers of *E. robusta* and *L. leucocephala* are more flexible compared to sugarcane bagasse fibers. A decrease in rigidity coefficient and runkel ratio, which are measures of the flexibility and wet plasticity of fibers, results in a greater degree of fiber collapse and higher degree of conformability within the sheet, which gives rise to a sheet of a higher density or lower bulk (83, 84). The sugarcane bagasse fibers are wider, thick to thin walled usually with straight pointed tapering ends and relatively more numerous slit like or lenticular pits than that of bamboo. Transverse markings on sugarcane bagasse fibers are similar to those of bamboo which are quite common. The wider fibers are usually shorter and comparatively thin walled not frequently with blunt, oblique or forked ends.

Parenchyma cells are very abundant. They are up to 900 μm in length with an average of about 326.9 μm and up to 180 μm in width with an average of

53.4 μm. The parenchyma cells are small to medium sized, narrow rectangular and numerous. Vessels are ranging from 180 to 1600 μm (average 152.3 μm) fairly long and narrow. Length and width of parenchyma are much lower than those of wheat & rice straw while length of vessel is higher than that of wheat straw but much lower than that of rice straw. The width of vessels is on lower side when compared with both wheat and rice straw (Table 2.7). The dimensions of parenchymatous cells of sugarcane bagasse are lesser than that of rice and wheat straw; therefore the slot size of screen should be selected according to the morphology of sugarcane bagasse. The parenchyma and epidermal cells are non-fibrous cells that have large surface area and adversely affect the mechanical strength properties of paper (122, 128, 129). The epidermal cells are somewhat narrow and rectangular with undulating margins always present but not very common. The values of curl and kink index are shown in Table 2.8. Kinks are usually edge crack zones. They are identified as sudden slope changes during the fiber length calculation while curl is defined as the ratio of the arc to the developed length of the fiber (87). The mean fiber curl index in sugarcane bagasse is more compared to *E. terticornis*. On the other hand, kink index and kink per mm length of fiber are on lower side compared to *E. terticornis* but kink angle is more in sugarcane bagasse compared to *E. terticornis.* Defects like kinks and curls in fibers reduce paper strength because they give rise to weak points in the fiber that will reduce fiber strength. The introduction of curls and kinks reduces the modulus (240). The tensile strength of paper increases with beating or refining. Due to fiber swelling, the

internal and external fibrillation renders the fibers more flexible and it removes kinks and curls to some extent (215).

The chemical characteristics of sugarcane bagasse are shown in Table 2.9. The water solubles in bagasse are 7.42%, which are higher as compared to 2.21 and 5.98 % in *E. globulus* and *L. leucocephala* respectively. Bagasse has 1.8% alcohol benzene soluble when compared to 2.30, 2.55% in *E. globulus* and *L. leucocephala* respectively. All the soluble materials come under the category of extractives and these are totally undesirable for pulp and paper making. The water and alcohol benzene solubles affect the pulp yield, paper quality and drainage characteristics of paper machine. Sugarcane bagasse contains lower alcohol benzene solubles, so it will create lesser pitch problem and also more homogeneity in the paper sheet (150). 1% NaOH solubility in sugarcane bagasse is on higher side i.e. 32.29 % than that of 14.58, 13.26% in *E. globulus* and *L. leucocephala* respectively. It means that degradation of sugarcane bagasse due to light and heat and fungal decay is more compared to *E. globulus* and *L. leucocephala*. Hence adequate precaution should be taken for storing bagasse to avoid degradation. Pentosan content of sugarcane bagasse is 23.9%, which is on higher side compared to *E. globulus* and *L. leucocephala* (Table 2.9). Therefore, sugarcane bagasse will produce lower pulp yield compared to both the hardwoods. The α–cellulose in sugarcane bagasse is 42.34% in comparison to 48.66 and 58.70% in *E. globulus* and *L. leucocephala* respectively. It indicates that bagasse has the moderate quantities of α–cellulose. β and γ-cellulose content is more in sugarcane bagasse which ensures good bonding characteristics for sugarcane bagasse pulp (150),

because maximum quantity of hemicellulose is solubilized during pulping or bleaching processes. The lignin content in bagasse is 21.7% which is much lesser than that in bamboo (150) but comparable with the *E. globulus* and *L. leucocephala*. Of significance is the fact that the structure of lignin in bagasse is more open and so mild cooking conditions are adequate. Therefore, sugarcane bagasse will require less cooking chemicals and shorter cooking cycle with improved tensile, burst and tear strengths and double fold and sheet density. The holocellulose content is 71.03% in comparison to 73.27 and 76.58% in *E. globulus* and *L. leucocephala* respectively. The ash content of bagasse is 2.10%, which is higher than that of both of the hardwoods (Table 2.9) but still much lesser compared to straw (150).

2.4 CONCLUSIONS

Sugarcane bagasse contains 71.36% useful fibers, 15.48% pith cells and 13.14% solubles after dry and wet depithing. The pith cells are resistant to pulping and do not participate in bond formation. Improper depithing may cause fluff generation during paper making or printing due to larger surface area of pith cells. Wet end bonding additives should be added according to surface area of pulp. Morphological studies of sugarcane bagasse indicate that the fiber bundles are concentrated towards periphery. The vascular bundles of sugarcane are collateral. The fiber length of depithed sugarcane bagasse is higher than that of *E. tereticornis*, *E. robusta* and *L. leucocephala* and fiber diameter resembles to those of *E. robusta* and *L. leucocephala*. The fibers of depithed sugarcane bagasse are more flexible which are readily converted into double walled ribbons on pressing and provide more surface contact area for boding. Such type of

sheet will be denser, less porous and has high tensile and burst indexes, double fold and Z-direction strength except tear index. The dimensions of parenchyma cells are lesser than wheat straw and rice straw. On the other hand, vessels dimensions are more than wheat straw and lesser than rice straw. The slot size for screening of pulp must be taken according to morphological characteristics of sugarcane bagasse. Curl and kink are defects which can be removed by beating the bagasse fibers up to an optimum beating level. Proximate chemical analysis of depithed sugarcane bagasse shows that the solubles are on higher side compared to *E. globulus* and *L. leucocephala*. The lignin of depithed sugarcane bagasse is comparable to that of *E. globulus* and holocellulose is slightly lower than *E. globulus*. Depithed sugarcane bagasse requires less cooking chemicals and shorter cooking cycles due to less lignin content and more open and looser anatomy.

Table 2.1: Comparison of fiber dimensions of softwood, hardwood and non-wood cellulosic raw materials (12)

Particulars	Softwood	Hardwood	Non-wood
Fiber length, mm	2.7-4.6	0.7-1.6	0.5-30
Fiber diameter, μm	32-43	20-40	8-30

Table 2.2: Strength characteristics for morphological factors (321)

Sl. No.	Parameter	Tensile and bursting strength	Tearing strength	Folding strength	Sheet density
1	Fiber length	0 to +	++	0 to +	0 to –
2	Cell wall thickness late (summer) wood fraction (tube structure) rising	–	0 to +	– –	–
3	Cell wall thickness early (spring) wood fraction (ribbon structure) rising	+	0 to –	++	++
4	Fiber length to width (L/D) ratio rising			+	
5	Curling of fibers rising	– to –	+	+	–

0 No influence or no distinct influence
+ Marked positive influence
++ Decisive positive influence
- Marked negative influence
-- Decisive negative influence

Table 2.3: Average thickness of various cell wall layers and microfibrillar angle within the layers in typical wood fibers (112)

Wall layers[a]	Thickness, μm	Number of microfibrillar layers (lamellae)	Average angle of micrifibrils, degrees
P	0.05-0.1	–[b]	–[b]
S_1	0.1-0.3	3-6	50-70
S_2	1-8[c]	30-150[c]	5-30[d]
S_3	<0.1	<6	60-90
ML[e]	0.2-1.0	–	–

[a] P primary wall, S_1 outer layer of the secondary wall, S_2 middle layer of the secondary wall, S_3 inner layer of the secondary wall, and ML middle lamella.
[b] Cellulose microfibrils form mainly an "irregular network".
[c] Varies greatly between early wood (1-4 μm) and late wood (3-8 μm).
[d] The microfibrillar angle varies between 5-10^0 (latewood) and 20-30^0 (early wood).
[e] An intercellular layer bonding the cells together contains mainly non-fibrillar material.

Table 2.4: General cell wall chemical composition of coniferous fibers (189)

Wall layers	Designation	Approximate chemical composition, %		
		Cellulose	Hemicellulose and pectin	Lignin
P	Primary wall	10	20	70
S_1	Secondary wall	35	25	40
S_2	"	55	30	15
S_3	"	55	40	05
ML	Middle lamella	00	10	90

Table 2.5: Useful fiber, pith and soluble in dry and wet depithed bagasse

Useful fiber, %	Pith, %	Solubles, %
71.36±1.5	15.48±0.8	13.14±1.0

± refers standard deviation

Table 2.6: Morphological characteristics of depithed sugarcane bagasse

Sl. No.	Particulars	Depithed sugarcane bagasse	*Eucalyptus tereticornis* (263)	*Eucalyptus robusta* (249)	*Leucaena leucocephala* (195)
1	Fiber length (L), mm	1.51±0.08	0.70	1.07	0.96
2	Fiber width (D), µm	21.4±1.6	14.2	19	23.33
3	Lumen diameter (d), µm	6.27±0.4	3.4	12.1	12.90
4	Cell wall thickness (w), µm	7.74±0.2	5.4	3.4	10.45
5	Runkel ratio (2w/d)	2.46	3.18	0.56	1.65
6	Flexibility coefficient (dX100/D)	29.29	23.94	63.68	55.29
7	Slenderness ratio (L/D)	70.56	49.29	56.31	41.14
8	Rigidity coefficient (2w/D)	0.723	0.760	0.36	0.89

± refers standard deviation

Table 2.7: Morphological characteristics of parenchyma cells and vessels of sugarcane bagasse and their comparison with wheat and rice straws and hardwood

Particulars	Bagasse	Wheat straw (T259OM-93)*	Rice straw (T259OM-93)*	Hard wood (213)
Parenchyma				
Length, µm	326.9±4.2	450	350	-
Width, µm	53.4±2.9	130	82	-
%	-	30	-	3-21
Vessels				
Length, µm	152.3±2.5	100	650	-
Width, µm	28.1±1.0	60	40	-
%	-	5	-	5-58

± refers standard deviation
*Tappi Standard Test Methods: 2007

Table 2.8: Fiber dimensions of sugarcane bagasse using Hi-resolution Fiber Quality Analyzer and comparison with *Eucalyptus tereticornis*

Sl. No.	Dimensions	Depithed sugarcane Bagasse	*Eucalyptus tereticornis* (263)
1	Mean fiber length, mm (weight weighed) (L = 0.20-4.50 mm)	1.461	0.651
2	Mean fiber width, µm (w = 7-80 µm) (L = 0.30-6.0 mm)	20.8	14.2
3	Mean fiber curl index (Length weighed) (L = 0.20-4.5mm)	0.151	0.141
4	Mean fiber kink index (L = 0.5-5mm)		
	Kink index (1/mm)	1.89	2.15
	Total kink angle (degrees)	37.9	29.93
	Kink per mm (1/mm)	0.93	0.98
5	Fines (L = 0.07-0.20)		
	Arithmetic,%	30.29	25.59
	Length weighed, %	7.58	5.34

21-

Table 2.9: Proximate chemical analysis of depithed sugarcane bagasse

Sl. No.	Particulars	Depithed Bagasse	*Eucalyptus globulus* (279)	*Leucaena leucocephala* (195)
1	Cold water solubility, %	3.02±0.02	1.61	3.45
2	Hot water solubility, %	7.42±0.05	2.21	5.98
3	1% NaOH solubility, %	32.29±0.1	14.58	13.26
4	Alcohol benzene solubility, %	1.85±0.01	2.30	2.55
5	Ash content, %	2.10±0.03	0.30	0.85
6	Silica content, %	0.98±0.007	-	0.55
7	Lignin (acid insoluble), %	21.7±0.35	21.18	19.55
8	Pentosans, %	23.9±0.21	17.64	17.21
9	Holocellulose, %	71.03±0.5	73.27	76.58
10	α-cellulose, %	42.34±0.36	48.66	58.70
11	Hemicellulose, %	28.60±0.42	-	-
12	Phosphorus, %	0.44±0.007	-	-
13	Iron, %	0.013±0.001	-	-
14	Chloride, %	Nil	-	-
15	Hydrogen, %	6.51±0.2	-	-
16	Nitrogen, %	0.41±0.005	-	-
17	Carbon, %	37.72±0.3	-	-
18	Calcium, %	0.46±0.003	-	-

± refers standard deviation

All values on extractive free basis

CHAPTER 3

SODA AND SODA-AQ PULPING OF SUGARCANE BAGASSE

CHAPTER-3

SODA AND SODA-AQ PULPING OF SUGARCANE BAGASSE

3.1 INTRODUCTION

Pulping refers to any process by which wood (or other fibrous raw material) is reduced to a fibrous mass. Basically, it is the process by which the bonds are systematically ruptured within the wood structure (323). There are four broad categories of pulping processes: chemical, semi-chemical, chemi-mechanical and mechanical pulping. These are in order of increasing mechanical energy required to separate fibers and decreasing reliance on chemical action. Thus, chemical pulping methods rely on the effect of chemicals to separate fibers, whereas mechanical pulping processes rely completely on giving physical (mechanical) action to the fibers (41). The chemical pulping process of lignocellulosic materials can be generally categorized into alkaline-based (kraft and soda pulping); acid-based and solvent-based (organosolv pulping). The effectiveness of chemical pulping depends on its ability to penetrate and diffuse into the heterogeneous woody structure, and to break down the lignin macromolecules until the resulting molecular fragments become small enough to diffuse out to the aqueous pulping liquor (72).

In chemical pulping, wood pulp fibers are manufactured by chemically dissolving the components, mainly lignin, that keeps wood cells together to form the original wood structure (110). Enough lignin from the (lignin-rich) middle

lamellae has to be dissolved to separate the undamaged wood fibers from each other. Aqueous solutions of several alternative alkaline, neutral or acidic components at elevated temperature and pressure are used to dissolve lignin and some carbohydrates from wood chips. The aqueous solutions of cooking chemicals penetrate from the lumen through the cell wall towards the middle lamellae (110). Alkali is consumed by the rapid hydrolysis of acetyl groups of (galacto) glucomannans in softwoods and xylan in hardwoods (322, 101). The dissolution of hemicelluloses and the degradation of cellulose are the limiting factors in alkaline cooking. Substantial amounts of xylan and around 75% of glucomannan are lost from softwoods during cooking. The predominant degradation reactions of polysaccharides are alkali-induced stepwise eliminations of monomeric units (peeling reactions), starting from the reducing end of the cellulose chain. The reactions depend very much on alkali charge, concentration, cooking time and temperature, while a competitive stopping reaction, via a β-hydroxycarbonyl elimination that transforms the end-group of the cellulose chain to a stable D-glucometasaccharinic acid, hinders further peeling to take place (92). Rates of reactions (peeling and stopping) are determined by enolization, which is an intramolecular reaction and is dependent on the degree of ionization. The activation energies for the peeling and stopping reactions have been estimated to be 103 kJ/mol and 135 kJ/mol, respectively (117), which implies that the importance of the stopping reaction increases with temperature.

Alkaline delignification kinetics is generally divided into three stages (185): initial, bulk and residual delignification stages. The initial stage is characterized

with slow delignification, high carbohydrate dissolution and heavy consumption of alkali. Most of the lignin is removed in the bulk delignification stage. Residual delignification is slow and selectivity is poor in this stage. The yield loss and carbohydrate degradation make pulping in the residual stage detrimental to the process. In the splitting of lignin molecule by hydrolysis, formation of additional hydroxyl groups occurs during delignification, possibly from the hydrolysis of methoxyl from the furan or pyran or from the breaking of linkages between lignin and hydrates. At optimum temperature, aqueous alkali hydrolyzes away β-0-4 linkages of native lignin, cleaving the polymer chains and the decomposed products so formed are soluble and thus can be removed (158). Soda pulping process takes place in two phase system:

1. The solid phase, consisting of the wood chips.
2. The liquid phase, where moisture is inside the chips and the pulping liquor is outside the chips (early in the cook, these are actually two separate liquid phases).

The first industrial alkaline cooking process was soda cooking using sodium carbonate and sodium hydroxide as chemicals (110, 111). The soda process is particularly applicable to low lignin fibrous raw materials like agricultural residues (bagasse, straw), where the advantages of sulphidity are of a lower magnitude. The importance of soda process as a viable alternative was realized; but there are some basic drawbacks, such as: low pulp yield, relatively lower strength properties, longer cooking time, high temperature and caustic charges.

But on the other hand paper manufactured from soda pulps bear high bulk, opacity, absorbency and printability. Soda pulps are therefore best suited to paper grades where pulp strength requirements are not demanding. Soda pulps are too soft to be used alone and are strengthened by blending with longer and stronger fibers; they serve as the "filler fibers" and added to the paper (81). The chemical reactions occurred between wood components and alkali used for delignification, are extremely complicated.

Kraft process is the dominating pulping process today, the greatest disadvantage of kraft pulping is the release of malodorous reduced sulphur compounds to the atmosphere (359). Nowadays interest has been shown on sulfur free pulping. Therefore, studies on different pulping processes have been done. Among these processes, used to get improved quality pulp and higher yield, soda-anthraquinone (AQ) process has given encouraging result. It is believed that AQ is still the most cost-effective sulfur free accelerator for alkaline pulping. The soda-AQ process also offers a direct advantage of eliminating the air pollution associated with the kraft process (297). Many studies indicate that the addition of anthraquinone (AQ) or anthraquinone-2-sulfonate (AQ-S) in alkaline pulping can not only accelerate the delignification rate but also preserve pulp yield (42, 387, 28, 29). AQ is reduced to anthrahydroquinone (AHQ) by the polysaccharide end groups on fiber at moderate temperatures of around 50 ^{0}C or higher. The polysaccharide end groups are in turn oxidized to alkali-stable aldonic acid groups. AHQ then acts as an effective cleaving agent to the lignin β-aryl ether linkages in free phenolic phenylpropane units and is oxidized to AQ at the same time, which completes a reduction–oxidation cycle (380). Since AQ is

insoluble in caustic solutions, the effectiveness of AQ during the alkaline pulping process could be limited by many factors, mainly mass transfer (52).

3.2 EXPERIMENTAL METHODOLOGY

3.2.1 Pulping studies

Well depithed sugarcane bagasse was digested in WEVERK electrically heated rotary digester of 0.02 m^3 capacity having four bombs of 1 L capacity each. Sugarcane bagasse was cooked at different cooking conditions like, maximum temperature from 130 to 160 ^{0}C, cooking time from 15 to 150 min, active alkali from 10 to 14% (as Na_2O), and liquor to raw material ratio of 4:1. Based on experimental results 0.1% anthraquinone (AQ), a carbohydrate stabilizer was added at optimum soda cooking conditions to study its impact on pulp yield, screening rejects and kappa number at different alkali doses. 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 0.15 mm slits and the screened pulp was washed, pressed and crumbled. The pulp was 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: 2007. The results are shown in Tables 3.1 to 3.4 and Figures 3.1 to 3.5.

3.2.2 Preparation of laboratory hand sheets and testing

The unbleached pulp of sugarcane bagasse was disintegrated in PFI mill (T 200 sp-96) at different beating levels. Laboratory hand sheets of 60 g/m^2 were prepared (T 221 cm-99) and tested for various physical strength properties, like

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: 2007. The results are shown in Table 3.5 and Figures 3.6 to 3.9.

3.2.3 Spent liquor analysis

The spent liquor generated during soda-AQ pulping of depithed and whole bagasse at optimum pulping conditions i.e. active alkali 12% (as Na_2O), AQ dose 0.1%, time to reach maximum temperature 60 min, time at temperature 60 min, maximum cooking temperature 150 ^0C, liquor to raw material ratio 4:1 and digester pressure 5.0 kg/cm^2, was analyzed for pH, total solids, residual active alkali, inorganics, organics, silica, sodium, calcium (T 625 cm-85) and chloride (T 256 cm-97) as per Tappi Standard Test Methods: 2000. Potassium (322 A), total organic carbon (505 A) and color (204 A) values were determined as per Standard methods for the examination of water and wastewater, American Public Health Association, 1985. Carbon, hydrogen and nitrogen were determined using Heraeus rapid C,H,N analyzer. BOD was estimated by respirometric method using BOD bottles manufactured by WTW, Germany (**373**) and COD was determined by closed reflux titrimetric method using Thermoreactor CR 2010 (352, 378). The results are reported in Table 3.6.

3.2.4 Bauer-McNett fiber classification

Fiber fractionation (T 233 cm-06) of soda-AQ pulp of sugarcane bagasse at optimum pulping conditions was done by using the Bauer-McNett fiber classifier with mesh screen number 28, 48, 100 and 200 as per Tappi standard method 2006. The results are reported in Table 3.7.

3.2.5 Scanning electron microscopy

The detailed morphological studies of sugarcane bagasse soda-AQ pulp samples were carried out using scanning electron microscopy (SEM, Leo 435 VP, England). Pulp sample was taken and subjected for fixation using 3% (v/v) glutaraldehyde-2% (v/v) formaldehyde (4:1) for 24 h. Following the primary fixation, samples were washed thrice with double distilled water. The samples were then treated with the alcohol gradients of 30, 50, 70, 80, 90 and 100% respectively 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 (96). Electron photomicrographs were taken at 15.00 kV using detector SE1 and at desired magnifications and are shown in Figure 3.10: Photomicrographs A-F.

3.2.6 Statistical analysis

All experiments were carried out in triplicate and experimental results were represented as the mean ± standard deviation of three identical values.

3.3 RESULTS AND DISCUSSION

3.3.1 Influence of temperature and time

Figure 3.1 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 are pertaining to rapid solublization of bulk of lignin (bulk delignification), whereas the part of curves with more gentle slopes are

pertaining to the slow solublization of the residual lignin (residual delignification). Both parts of these curves are having different velocity constants. The bulk delignification corresponds to the removal of easily assessable lignin present in the middle lamella and the residual delignification corresponds 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 is also associated with the solubilization of significant amounts of hemicelluloses (155). These curves indicate that as the temperature is decreased from 160 to 130 ^{0}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 increase. Table 3.1 also reveals that at lower temperature range, the residual lignin contents decrease sharply, while at higher temperature, the magnitude of decrease in lignin content is not so pronounced. Figure 3.2 shows that pulp yield decreases with increasing time and temperature. At higher temperature, the degradation of carbohydrate fractions also increases, thereby results in reduced pulp yield (155). In other words, at the transition point, a lower pulp lignin contents are obtained at 150 ^{0}C. Beyond a temperature of 150 ^{0}C, degradation of carbohydrates contents occurs due to peeling reactions (124, 205), resulting in a lower pulp yield. The nature of curves after transition points are almost horizontal lines, clearly indicating that the bulk delignification are over up to these transition points and it is not economical to continue the cooking operation beyond this optimum temperature of 150 ^{0}C. Therefore, based on experimental data, a maximum cooking time of 1 h at 150 ^{0}C may be considered as an optimum cooking condition for the soda pulping of sugarcane bagasse.

3.3.2 Influence of alkali charge

Table 3.2 and Figure 3.3 reveal that screened pulp yield increases with increasing active alkali from 10 to 12% (as Na_2O) and then tends to decline sharply, whereas both kappa number and screening rejects decline sharply up to an alkali dose of 12% and beyond that both of these parameters practically remain constant. The screened pulp yield of sugarcane bagasse is found to be 42.18% at kappa number of 28.16 and an active alkali charge of 12% (as Na_2O), which may be considered as optimum cooking dose for sugarcane bagasse. During soda pulping, the consumption of active alkali is found to be constant over a wide range of alkali charge (184). It is found that the excessive active alkali charge which remains unconsumed during the course of pulping adversely decreases the pulp viscosity. As sugarcane bagasse contains slightly more soluble so the per digester pulp yield will be less.

3.3.3 Influence of time

Table 3.3 and Figure 3.4 show the effect of maximum cooking time on screened pulp yield, screening rejects and kappa number during soda pulping of sugarcane bagasse, while keeping all variables constant, like alkali dose 12% (as Na_2O), liquor to raw material ratio 4:1, digester pressure 5 kg/cm^2 and maximum cooking temperature 150 ^0C, when cooking time is increased from 30 min to 60 min, the screened pulp yield increases from 40.38 to 42.18% and kappa number drops from 41.42 to 28.16. Beyond that screened pulp yield drops sharply, while kappa number remains almost constant. Therefore, maximum cooking time of 60 min may be considered as an optimum cooking time for soda pulping of sugarcane baggase.

3.3.4 Influence of AQ

Table 3.4 and Figure 3.5 reveal the effect of AQ at different alkali doses i.e. 10-14% (as Na_2O) while keeping other conditions constant during soda pulping of sugarcane bagasse. The addition of 0.1% AQ dose at active alkali doses 10 and 12% (as Na_2O) improves the pulp yield by 3.97 and 2.62% respectively while keeping other parameters constant. On the other hand, 0.1% AQ reduces kappa number of sugarcane bagasse pulp by 10.29 and 3.9 units at active alkali doses of 10 and 12% (as Na_2O) respectively (Tables 3.2, 3.4). Table 3.4 shows that the screened pulp yield increases with increasing alkali dose up to 12% (as Na_2O) and then declines whereas both kappa number and screening rejects decline up to an alkali dose of 12% and then the decrease is not so pronounced. The increase in pulp yield and reduction in Kappa number can be explained based on redox catalytic activity of AQ. AQ is pulping additive used to increase delignification and decrease carbohydrates 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 AQ goes through a cyclic process, it is typically used at about 0.1% on O.D. wood basis and results in a 1-3% increase in pulp yield (126, 375, 85).

3.3.5 Optimization of mechanical strength properties

Table 3.5 reveals the mechanical strength properties of unbleached soda-AQ pulps of sugarcane bagasse cooked at different alkali doses i.e. 10-14 % (as

Na_2O) and beaten at different beating levels. Figures 3.6-3.9 indicate that tensile index, tear index, burst index and double fold increase with increasing alkali dose up to 12% (as Na_2O) and beating level of 45 ^{0}SR and beyond that there is no further increase in mechanical strength properties but a slight decrease. Initially, the mechanical strength properties depend upon fiber length. The degradation of carbohydrate fractions beyond optimum alkali dose causes the declinement of mechanical strength properties. All the mechanical strength properties are found to be increased with increasing beating level up to 45 ^{0}SR except tear strength. Removal of primary wall exposes secondary wall layers. However, primary wall is permeable to water but does not participate in bond formation. Therefore, tearing energy required to pull the fibers from the mesh will be 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 declines whereas all other properties, which depend upon hydrogen bonding except tear strength improve with pulp beating.

3.3.6 Soda-AQ spent liquor properties

Table 3.6 shows the characteristics of spent liquor generated during soda-AQ pulping of sugarcane bagasse. The whole sugarcane bagasse spent liquors have high total solid concentration than depithed bagasse due to solubilization of pith fraction. The total solid concentration in spent liquor is an important factor from energy conservation point of view due to presence of pith in it. In case of agricultural residues, spent liquors have low total solid concentrations than wood due to low chemical charge, higher liquor to raw material ratio and high dilution factor maintained during washing. The depithed bagasse spent liquor has higher

residual active alkali (RAA) content as compared to the whole bagasse spent liquor. This is due to higher amount of organic acids in whole bagasse which depletes RAA. Thus, it is always important to maintain certain level of RAA in spent liquor to keep the organic residue in colloidally stable state. Organic acids that are produced during storage by the degradation reactions get neutralized by the free alkali associated with sodium in the form of organically bound sodium. The distribution of alkali or sodium in the spent liquor is in the form of NaOH, Na_2CO_3 and organically bound sodium. It is observed that color of the effluent increases with increase in alkali concentration during pulping. BOD and COD values also increase in the same manner. The high COD/BOD ratio in the range shows the difficulty in providing effective biological treatment to the system. The TOC and COD values of the samples are directly linked to each other.

3.3.7 Fiber length distribution of sugarcane bagasse

Important quantitative information about the fiber length distribution of a bagasse pulp can be best achieved by fiber fractionation. Furthermore, using the Bauer-McNett fiber classifier with the screens 28, 48, 100 and 200 mesh, the fractionation not only separates the fibers according to the fiber length, but also, to a great extent, it separates the fractions of sclerenchyma fibers and parenchyma cells. Table 3.7 shows the fiber length distribution of soda-AQ pulp of sugarcane bagasse at 220 CSF. +28 fractions retain 9.0% of pulp which consists of mostly long sclerenchymatous fiber. +48 fractions involve 41.08% of pulp of the medium sized fiber. +100 fractions retain 34.22% of the total pulp with the shortened fibers, thick walled rind parenchyma cells; large vessel fragments while +200 fractions contain 8.24% of pulp having short rind

parenchyma cells and other short material; large fragments of pith parenchyma cells and fragments of the epidermal tissue. –200 fractions involve the fiber and cell debris, single epidermal and collenchyma cells and small pith cell fragments (122).

3.3.8 SEM studies of sugarcane bagasse fiber

The fibers in the soda-AQ pulp of sugarcane bagasse are uniform, straight and intact with a smooth, silky surface and bear an appearance of compactness (Figure 3.10: Photomicrographs A and C). Figure 3.10: Photomicrograph B shows the lumen of the sugarcane bagasse soda-AQ pulp fibers. In addition, fibers show no sign of external fibrillation or formation of fibrils. During alkaline pulping some part of hemicelluloses (mainly xylan) is solubilized and reprecipitated on to the fiber surface (Figure 3.10: Photomicrographs D and F). As pulping proceeds the connection between the fibers loosens, firstly along radial planes, and eventually the fibers stick together only along those edges where several cells meet and delignification is still incomplete (Figure 3.10: Photomicrograph E). The pith parenchyma is easily broken down to flexible flakes with some bonding effect which is shown in Figure 3.10: Photomicrograph C on the surface of the fibers. Finally after removal of most of the lignin, the fibers lose their rigidity and collapse (Figure 3.10: Photomicrograph E).

3.4 CONCLUSIONS

Depithed sugarcane bagasse produces screened pulp yield of 42.18% and kappa number 28.16 at optimized pulping conditions like; active alkali dose

12% (as Na_2O), maximum cooking temperature 150 ^0C, maximum cooking time 60 min, digester pressure 5 kg/cm^2 and liquor to wood ratio 4:1. The introduction of 0.1% AQ at optimized soda pulping conditions of sugarcane bagasse improves pulp yield by 2.62% and kappa number mitigates by 3.9 units. Unbleached soda-AQ pulp of sugarcane bagasse produces optimum mechanical strength properties at a beating level of 45 ^0SR. The total solids in spent liquor of soda-AQ pulp of whole sugarcane bagasse are more compared to that produced during pulping of depithed sugarcane bagasse. However, the total solid concentration in spent liquor of whole sugarcane bagasse may be important from energy conservation point of view as pith is converted into energy. Spent liquor of depithed sugarcane bagasse has comparatively lower BOD and COD values than that of whole sugarcane bagasse. Baur-McNett fiber classification shows that the soda-AQ pulp of sugarcane bagasse contains more medium pulp fraction (–20 to +48 and –48 to +100 contains 75.30%). The scanning electron microscopic (SEM) studies of sugarcane bagasse fiber of soda-AQ pulp show that these are smooth and flat. The fibers do not show external fibrillation and part of the xylan is reprecipitated on their surface.

Table 3.1: Effect of maximum cooking temperature on pulp yield, lignin and kappa number of sugarcane bagasse

Temperature, ^{0}C	Time at temperature, h	Sugarcane bagasse		
		Yield, %	Kappa number	Lignin, %
130	0.25	68.6±2.8	-	13.26±0.4
	0.50	63.4±1.2	-	10.45±0.32
	1.00	57.3±1.9	45.2±1.7	7.72±0.28
	1.50	52.2±1.7	-	6.45±0.50
	2.00	48.5±1.1	-	5.75±0.35
	2.50	46.2±1.5	-	4.92±0.72
140	0.25	65.2±1.3	-	11.35±0.5
	0.50	57.6±1.7	-	8.45±0.30
	1.00	49.3±2.0	37.3±1.5	5.15±0.35
	1.50	45.3±1.5	-	4.65±0.28
	2.00	42.3±1.2	-	4.12±0.25
	2.50	40.8±1.5	-	3.62±0.30
150	0.25	58.6±1.9	-	10.30±0.65
	0.50	50.2±1.5	-	7.62±0.80
	1.00	44.0±0.9	28.16±0.9	4.22±0.21
	1.50	42.2±1.8	-	4.02±0.30
	2.00	40.4±1.2	-	3.65±0.50
	2.50	38.8±1.5	-	3.25±0.50
160	0.25	55.7±2.4	-	9.50±1.1
	0.50	46.9±1.8	-	6.25±0.4
	1.0	41.3±1.5	22.3±0.3	3.45±0.29
	1.5	39.2±1.0	-	3.15±0.34
	2.0	38.2±1.8	-	2.75±0.25
	2.5	37.3±1.6	-	2.22±0.20

± refers standard deviation

Cooking conditions:

Liquor to wood ratio	:	4:1
Active alkali	:	12% (as Na_2O)
Digester pressure	:	5.0 kg/cm^2
Time from room temperature to 105±2 ^{0}C	:	30 min
Time from 105 to 150±2 ^{0}C	:	30 min

Table 3.2: Effect of active alkali (as Na_2O) on screened pulp yield, screening rejects and kappa number of sugarcane bagasse

Sl. No.	Active alkali, % (as Na_2O)	Total yield, %	Screened pulp yield, %	Screening rejects, %	Kappa number
1	10	47.23±3.1	39.23±1.6	8.00±0.80	40.41±0.5
2	12	44.00±2.0	42.18±1.1	1.82±0.20	28.16±0.9
3	13	41.22±0.8	40.42±1.2	0.80±0.08	25.12±0.8
4	14	38.80±1.3	38.14±1.5	0.66±0.10	22.82±0.6

± refers standard deviation

Cooking conditions:

Liquor to wood ratio	:	4:1
Digester pressure	:	5.0 kg/cm^2
Time from room temperature to 105 ±2 ^{0}C	:	30 min
Time from 105 to maximum temperature 150 ±2 ^{0}C	:	30 min
Time at maximum temperature, 150 ±2 ^{0}C	:	60 min

Table 3.3: Effect of maximum cooking time on screened pulp yield, screening rejects and kappa number of sugarcane bagasse

Sl. No.	Maximum cooking time, min	Total yield, %	Screened pulp yield, %	Screening rejects, %	Kappa number
1	30	49.50±2.5	40.38±1.9	9.12±1.3	41.42±0.80
2	60	44.00±3.0	42.18±2.1	1.82±0.9	28.16±1.10
3	90	42.20±1.6	41.55±1.6	0.65±0.18	24.58±0.53
4	120	41.30±3.2	40.92±1.5	0.38±0.06	22.10±0.83
5	150	38.80±1.3	38.68±2.5	0.12±0.05	20.80±0.62

± refers Standard deviation

Cooking conditions:

Liquor to wood ratio	:	4:1
Active alkali	:	12% (as Na_2O)
Digester pressure	:	5.0 kg/cm^2
Time from room temperature to 105 ±2 ^{0}C	:	30 min
Time from 105 ^{0}C to maximum temperature 150 ±2 ^{0}C	:	30 min
Maximum cooking temperature	:	150 ^{0}C

Table 3.4: Effect of AQ and alkali charges on pulp yield, kappa number and screening rejects

Active alkali, % (as Na_2O)	Total pulp yield, %	Screened pulp yield, %	Screening rejects, %	Kappa number	Spent liquor pH
10	50.51±3.0	43.20±2.3	7.31±0.95	30.12±0.40	8.9
12	46.12±2.5	44.85±1.6	1.27±0.12	24.26±0.38	10.0
13	43.00±1.9	42.12±2.1	0.47±0.05	19.52±0.51	10.4
14	42.21±2.9	42.00±1.5	0.21±0.02	16.05±0.30	10.6

± refers standard deviation

Cooking conditions:

Liquor to wood ratio	:	4:1
Digester pressure	:	5.0 kg/cm^2
Time from room temperature to 105±2 ^{0}C	:	30 min
Time from 105 ^{0}C to maximum temperature 150±2 ^{0}C	:	30 min
Time at maximum temperature 150±2 ^{0}C	:	60 min
AQ dose (on OD raw materials basis)	:	0.1%

Table 3.5: Mechanical strength properties of unbleached sugarcane bagasse soda-AQ pulp at different alkali doses and beating levels keeping all other conditions constant like temperature 150 ^{0}C, maximum cooking time 60 min and liquor to wood ratio of 4:1

Active alkali, % (as Na_2O)	Beating level, ^{0}SR	Tensile index, Nm/g	Tear index, mNm^2/g	Burst index, kPam^2/g	Double fold, number
10	16	18.23±1.20	4.35±0.22	0.89±0.09	4±2.56
	35	38.74±1.60	5.15±0.18	2.82±0.11	68±5.3
	45	57.56±2.20	4.22±0.20	4.22±0.15	91±2.6
	55	54.83±1.90	2.88±0.21	3.87±0.12	88±4.4
12	16	23.64±1.30	4.85±0.15	1.03±0.08	7±1.30
	35	57.64±2.10	5.45±0.20	3.56±0.20	86±3.5
	45	69.98±3.30	4.55±0.16	4.66±0.06	110±4.0
	55	66.72±1.80	3.12±0.30	4.34±0.18	100±6.3
13	16	22.91±0.76	4.65±0.25	0.96±0.03	6±1.9
	35	53.11±1.40	5.25±0.11	3.32±0.21	82±2.1
	45	66.47±3.60	4.35±0.14	4.49±0.17	102±8.0
	55	62.36±1.93	3.05±0.33	4.1±0.16	97±5.4
14	16	25.55±0.50	4.52±0.29	0.94±0.10	5±1.3
	35	55.32±1.66	5.02±0.16	3.14±0.22	77±5.3
	45	68.44±2.91	4.16±0.22	4.35±0.25	95±7.3
	55	65.05±3.03	2.76±0.13	3.75±0.15	95±4.0

± refers standard deviation

Table 3.6: Soda-AQ spent liquor properties at optimum pulping conditions

Sl. No.	Spent liquor properties	Sugarcane bagasse	
		Whole	Depithed
1	pH at 30^{0}C	11.90	11.88
2	Total solids, % (W/W)	9.1	8.4
3	Residual active alkali, g/L as NaOH	1.02	1.30
4	Inorganics % (W/W) as NaOH (expressed as sulphated ash)	25.85	23.85
5	Organics, % (W/W) (by difference)	72.96	74.88
6	Silica, % (W/W) as SiO_2	0.94	0.78
7	Colour, PTU	14308	13731
8	BOD, g/L	17.67	16.96
9	COD, g/L	73.78	71.26
10	TOC, %	40.61	40.25
11	COD:BOD	4.18	4.20
12	Carbon, % (W/W)	32.9	34.64
13	Hydrogen ,% (W/W)	4.85	4.02
14	Nitrogen, % (W/W)	0.88	0.94
15	Sodium, % (W/W)	9.12	9.90
16	Calcium, % (W/W)	0.15	0.13
17	Potassium, % (W/W)	0.28	0.36
18	Chlorides, % (W/W)	0.79	0.89

± refers standard deviation

Cooking conditions:

Liquor to wood ratio	:	4:1
Digester pressure	:	5.0 kg/cm^2
Time from room temperature to 105±2 ^{0}C	:	30 min
Time from 105 to maximum temperature 150±2 ^{0}C	:	30 min
Time at maximum temperature 150±2 ^{0}C	:	60 min
AQ dose (on OD raw materials basis)	:	0.1%

Table 3.7: Bauer-McNett fiber classification of soda-AQ pulp at optimum pulping conditions of sugarcane bagasse

Sl. No.	Mesh size	Pulp fraction, %
1	+20	9.0±0.98
2	-20 to +48	41.08±1.7
3	-48 to +100	34.22±1.0
4	-100 to +200	7.46±0.5
5	-200	8.24±0.41

± refers standard deviation

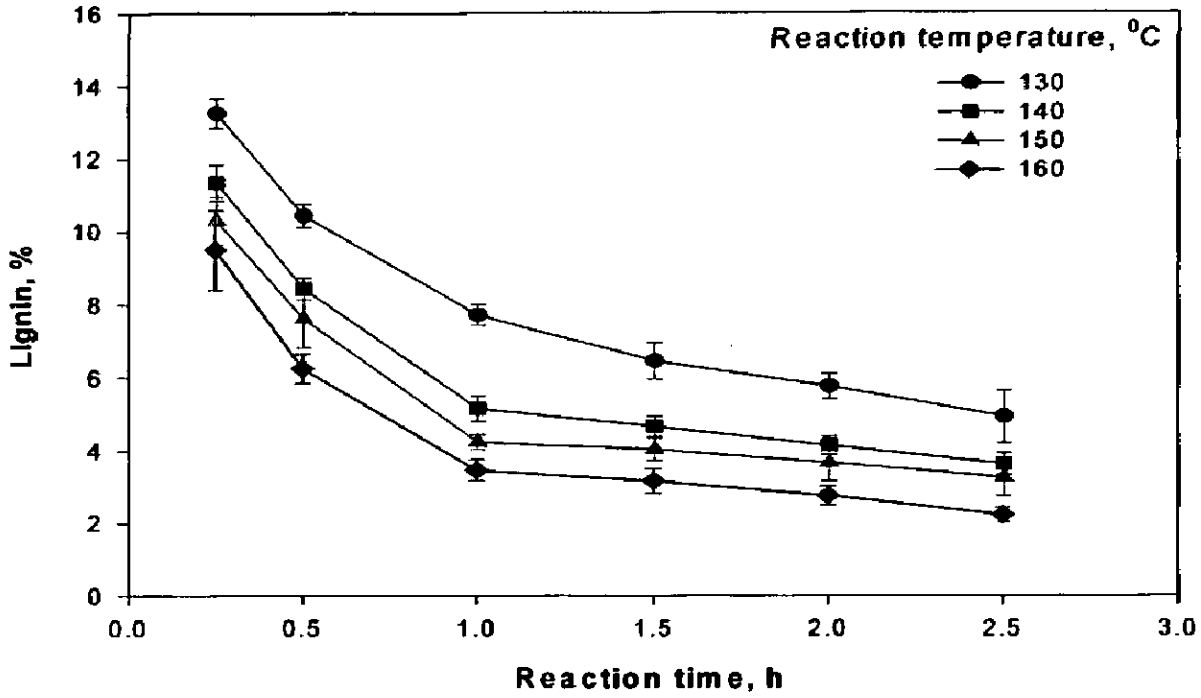

Figure 3.1: Curves of lignin vs different reaction times at maximum cooking temperature during soda pulping of sugarcane bagasse

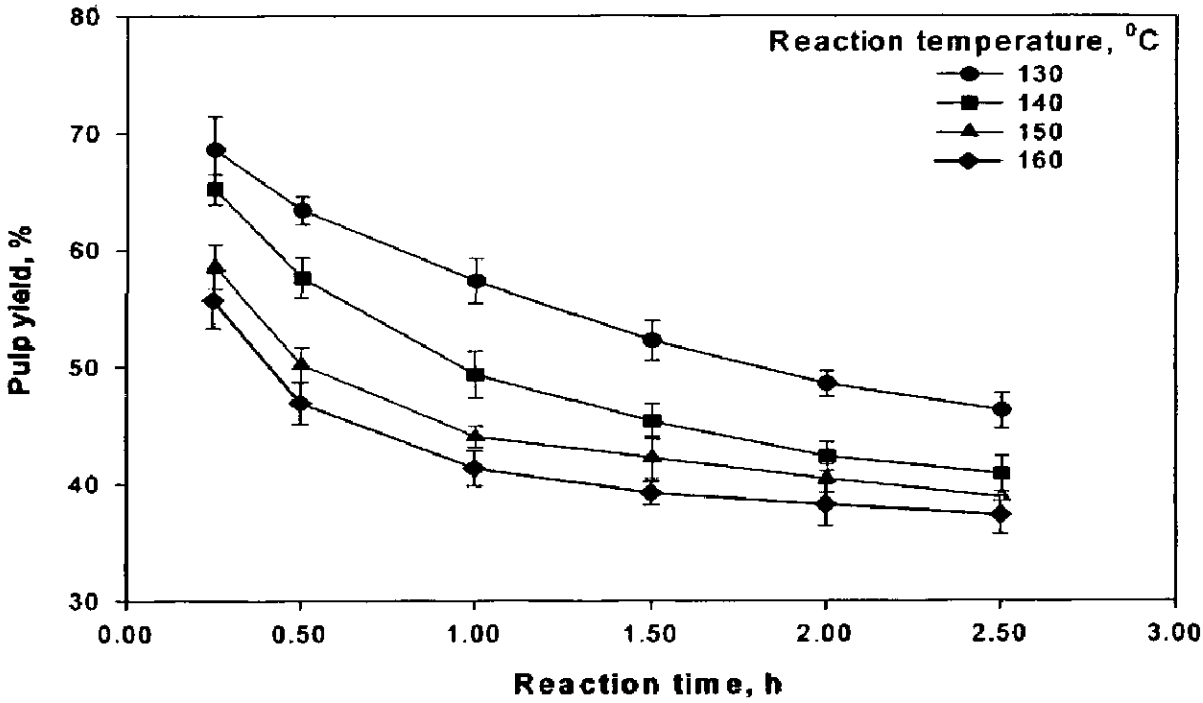

Figure 3.2: Curves of pulp yield vs different reaction times at maximum cooking temperature during soda pulping of sugarcane bagasse

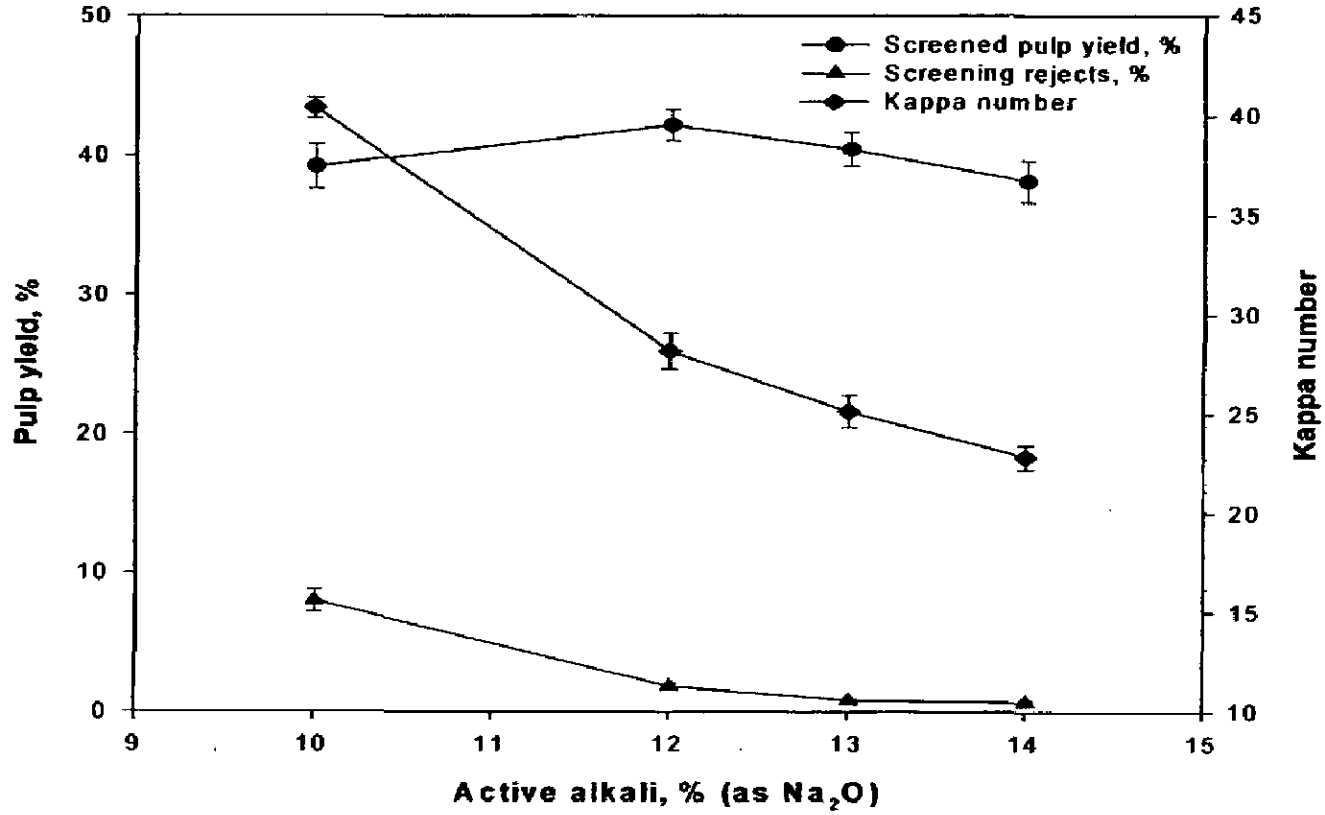

Figure 3.3: Effect of active alkali on screened pulp yield, screening rejects and kappa number during soda pulping of sugarcane bagasse

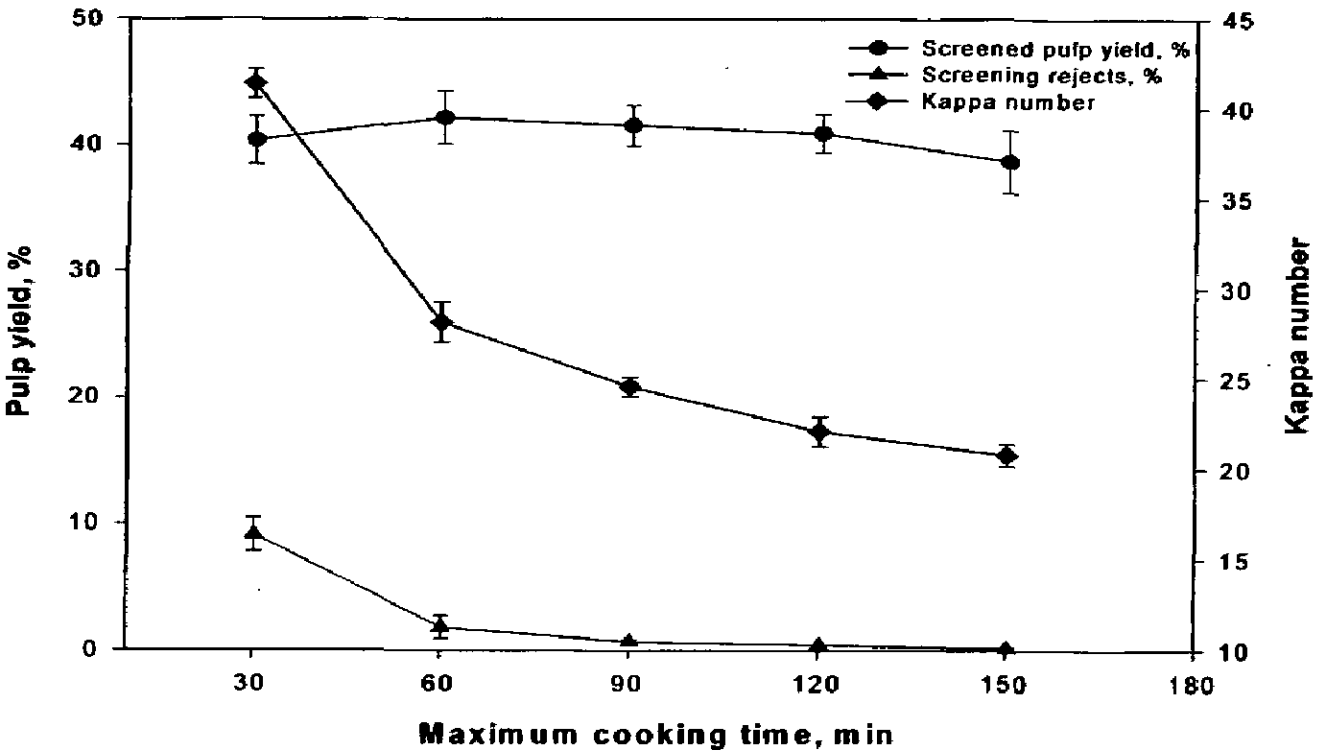

Figure 3.4: Effect of maximum cooking time on screened pulp yield, screening rejects and kappa number during soda pulping of sugarcane bagasse

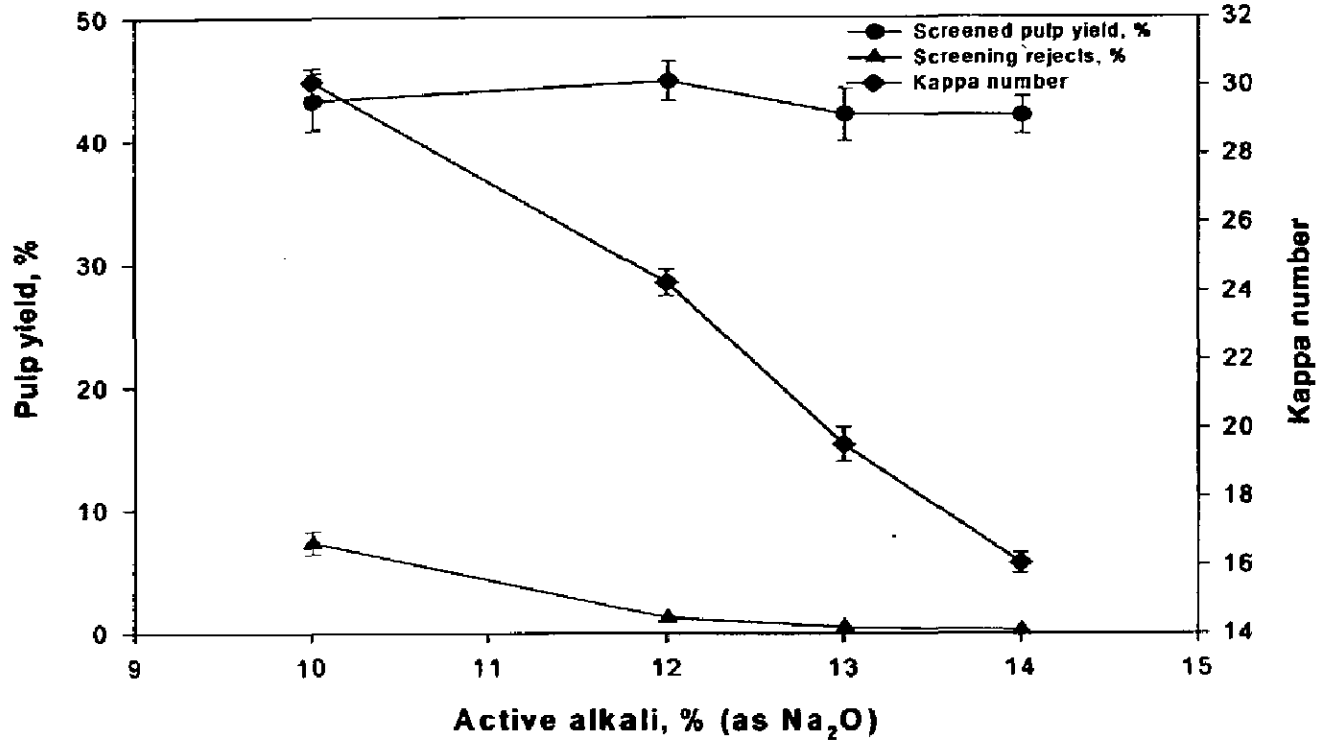

Figure 3.5: Effect of AQ at different active alkali doses on screened pulp yield, screening rejects and kappa number during soda pulping of sugarcane bagasse

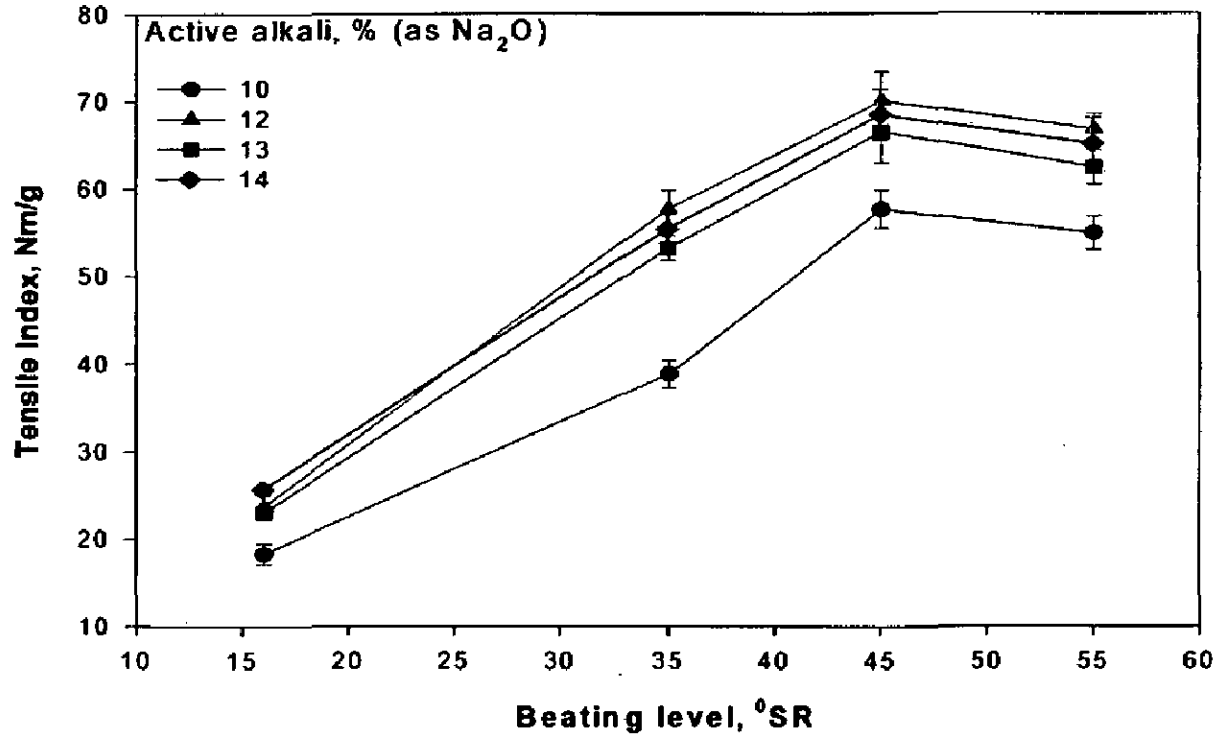

Figure 3.6: Effect of alkali dose and beating level (°SR) on tensile index of unbleached soda-AQ pulp of sugarcane bagasse

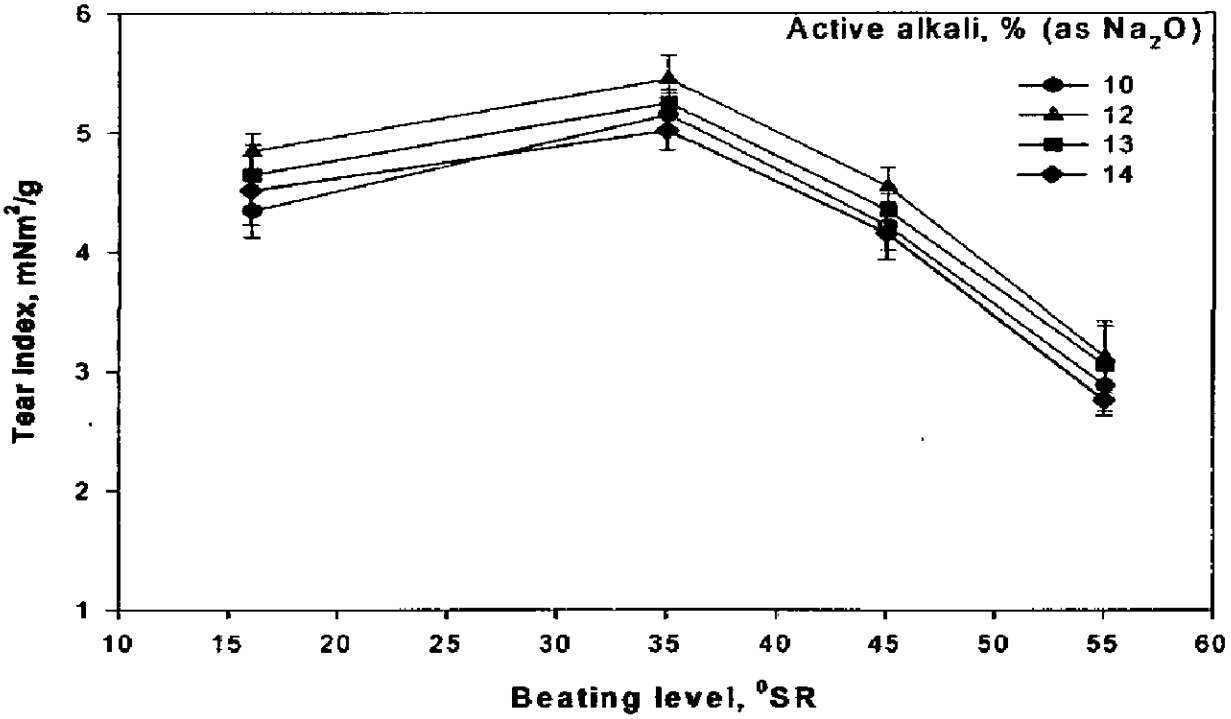

Figure 3.7: Effect of alkali dose and beating level (^{0}SR) on tear index of unbleached soda-AQ pulp of sugarcane bagasse

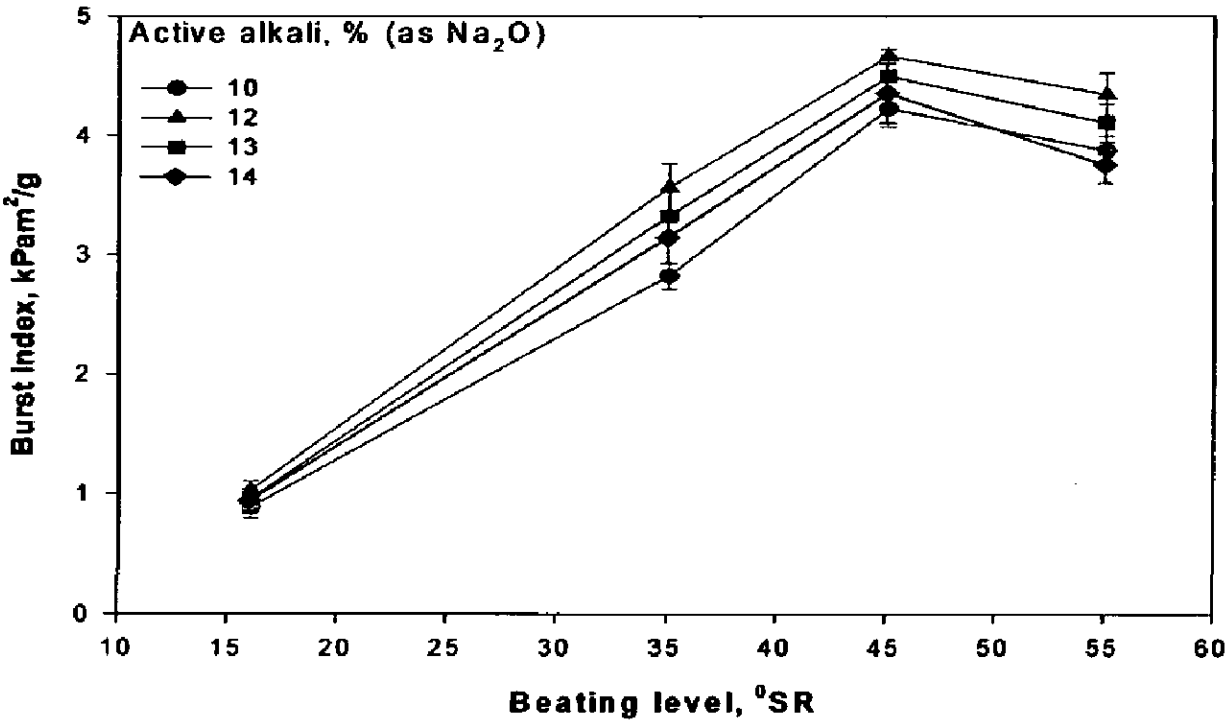

Figure 3.8: Effect of alkali dose and beating level (^{0}SR) on burst index of unbleached soda-AQ pulp of sugarcane bagasse

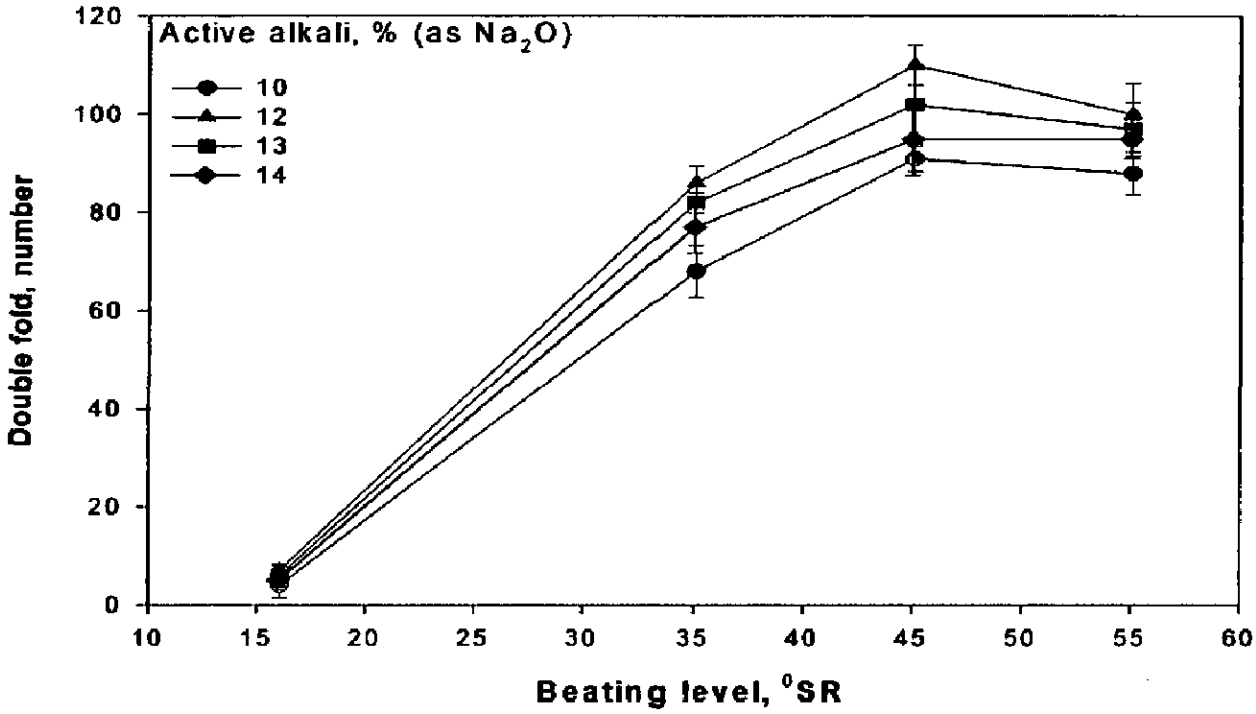

Figure 3.9: Effect of alkali dose and beating level (^{0}SR) on double fold of unbleached soda-AQ pulp of sugarcane bagasse

CHAPTER 4

STUDIES ON XYLANASE PRODUCTION

CHAPTER- 4
STUDIES ON XYLANASE PRODUCTION

4.1 INTRODUCTION

Enzymes are the catalytic cornerstone of metabolism, and as such are the focus of intense worldwide research, not only in the biological community, but also with process designers/engineers, chemical engineers, and researchers working in other scientific fields. Since, ancient times, enzymes have played a central role in many manufacturing processes, such as in the production of wine, cheese, bread and modification of starch etc. The latter half of the twentieth century has seen 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. In past two decades, however, microbial enzymes have been used in pulp and paper industry (34).

The most abundant and renewable biomass available on the earth is lignocellulose, which contains three major groups of polymers i.e. cellulose, hemicellulose and lignin (167). Schulze introduced the term hemicellulose for the fractions isolated or extracted from plant materials with dilute alkali (300). The classification of these hemicellulosic fractions depends on the types of sugar moieties present. Xylan constitutes the major component of hemicellulose. It is the major structural polysaccharide in plant cells and is the second most abundant polysaccharide in nature, accounting for approximately one-third of all renewable organic carbon on earth (262). Xylan is found in large quantities in

hardwoods of angiosperms (15–30% of the cell wall content) and softwoods of gymnosperms (7–10%), as well as in annual plants (<30%) (320). It is typically located in the secondary cell wall of plants, but is also found in the primary cell wall, particularly in monocots (376).

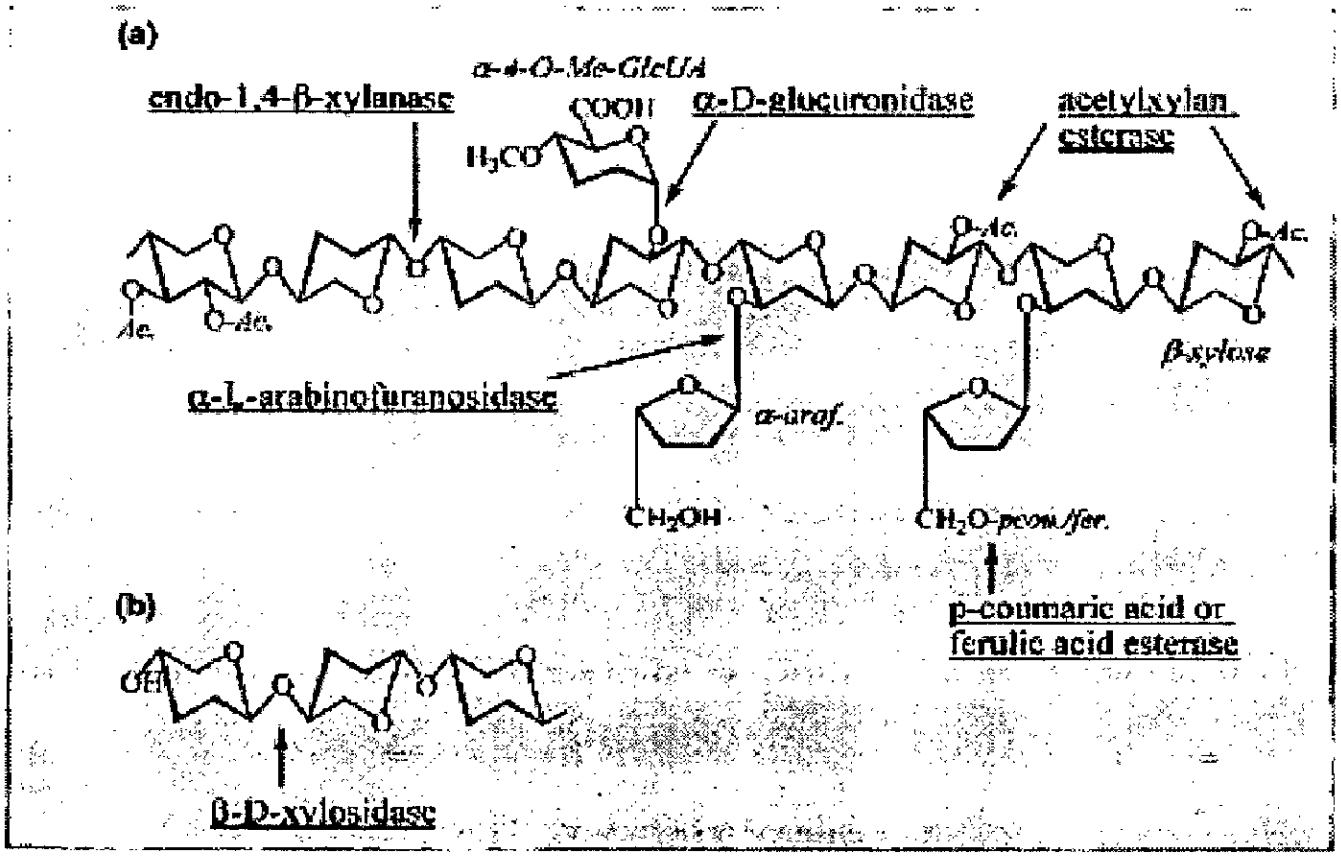

Figure 4.1: Hydrolysis of xylan (a) Structure of xylan and the sites of its attack by xylanolytic enzymes. The backbone of the substrate is composed of 1,4-β-linked xylose residues. Ac.=Acetyl group; α-araf.=α-arabinofuranose; α-4-*O*-Me-GlcUA=α-4-*O*-methylglucuronic acid; *p*cou. = *p*-coumaric acid; fer.= ferulic acid. (b) Hydrolysis of xylo-oligosaccharide by β-xylosidase (333).

Due to its heterogeneity and complexity, the complete hydrolysis of xylan requires a large variety of cooperatively acting enzymes (266, 40, 331) (Figure 4.1). 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

catalyzed by α-L-arabinofuranosidases (EC3.2.1.55), α-D-glucuronidases (EC 3.2.1.139), acetylxylan esterases (EC 3.1.1.72), ferulic acid esterases (EC3.1.1.73) and *p*-coumaric acid esterases (EC 3.1.1.-). Indeed, complete xylanolytic enzyme systems, including all of these activities, have been found to be quite widespread among fungi (35, 333), actinomycetes (86) and bacteria (333), and some of the most important xylanolytic enzyme producers include the *Aspergillus*, *Trichoderma*, *Streptomycetes*, *Phanerochaetes*, *Chytridiomycetes*, *Ruminococci*, *Fibrobacteres*, *Clostridia* and *Bacilli* (331, 333, 379, 201). Filamentous fungi are particularly interesting, since, these secrete xylanases in the medium (extracellular xylanase) and their enzyme levels are much higher than those of yeast and bacteria. Intracellular xylanases occur in rumen bacteria and protozoa (77).

Endoxylanases have been identified in *Aspergillus fischeri* (307); *Aspergillus foetidus* (312); *Bacillus* sp. (186; 113), *Cellulomonas* NCIM 2353 (55), *Cryptococcus albidus* (219), *Streptomyces* sp. (33, 34), *Staphylococcus* sp. SG-13 (115), *Trichoderma reesei* (346; 175) and many other microorganisms (37, 160, 183). The white rot fungus *Phanerochaete chrysosporium* has been shown to produce multiple endoxylanases (154). β-xylosidase appears to be mainly cell associated in bacteria and yeast but is extra-cellular in moulds (40). β-xylosidase have been reported in bacteria and fungi that include *Humicola grisea* var. *thermoidea* (6), *Cellulomonas flavigena* (13), *Bacillus thermantarcticus* (173), *Melanocarpus albomyces* IIS 68 (294), *Thermoascus* sp. (200), *Trichoderma koningii* G-39 (179) and *Aspergillus phoenicis* (283). Wong *et al.* 1988 suggested that endoxylanases could be grouped into those

that are low molecular weight (below 30 kDa) basic protein with high p*I* values and high molecular weight (above 30 kDa) acidic protein with low p*I* values (376). However, several exceptions to this pattern have been found (333, 201) and approximately 30% of presently identified xylanases, in particular fungal xylanases, cannot be classified by this system (66). β-D-xylosidases are the larger enzymes whose molecular weight lie between 60 to 360 kDa and may be monomeric or dimeric proteins (40).

Xylanases are produced by various microorganisms i.e. *Bacillus* NCIM 59 (168), *Streptomyces* sp. QG-11-3 (33), *Streptomyces cyaneus* SN32 (228), *Streptomyces thermoviolaceous* (97), *Aspegillus niger* (197), *Aspergillus Oryzae* (337), *Aspergillus caespitosus* (292), *Thermomyces lenuginosus* strain SSBP (319), and *Thermomyces lanuginosus* (59) etc. that have great potential for commercial application in pulp and paper industry. The importance of xylanases is not bound to the paper industry alone but these are equally employed for other industries as well (Table 4.1). The interest in xylanases has been raised markedly in the recent years, mainly because of their use in the pulp and paper industry, particularly in the bleaching process (367, 34, 331). Xylanases that are active and stable at elevated temperatures and alkaline pH, are needed to satisfy the specific conditions of the bleaching process in pulp and paper mills. Also, xylanases are mostly contaminated with cellulases, which degrade the structure of cellulose and destroy pulp quality. Therefore, the application of an alkali and heat stable cellulase-free xylanase for large-scale pulp bleaching biotechnology requires efforts that are aimed at process optimization, simplification and cost reduction (317). A considerable amount of worldwide

research has focused on the search for novel microbial isolates having the ability to produce thermotolerant and alkalophilic xylanases (226, 319, 341). Owing to the increasing biotechnological importance of thermostable xylanases, many thermophilic fungi had been examined for xylanase production (193, 320). Thermophilic fungi, a unique group of microorganisms, that thrive at high temperatures are often associated with piles of agricultural and forestry products and other composting materials (193). Since, these fungal strains function in amelioration of xylan substrate present in lignocellulosic waste, each xylanase produced may be biotechnologically important and show specialized function. Some of the thermophilic fungi, *Chaetomium thermophile*, *Humicola insolens*, *Thermomyces lanuginosus* and *Thermoascus aurantiacus* have been reported to produce biotechnologically important, thermostable xylanases (104). The temperature optima of most xylanases ranges from 55-65 ^{0}C. Xylanases of some strains of *T. aurantiacus* and *T. lanuginosus* are optimally active at 70 to 80 ^{0}C (193). The purified xylanase produced from *T. lanuginosus* CBS 288.54 exhibits its optimal activity at 70-75 ^{0}C. It is stable up to 65 ^{0}C for 30 min and retains more than 50% of its activity after heating at 85 ^{0}C for 30 min (180). The optimum temperature for xylanase production by *Penicillium oxalicum* under SSF is 45^{0}C (222) which is similar with *A. fumigates* and *A. nidulans* (54) while *A. niger* NKUC$_{N}$-3.40 has the temperature optima of 34 ^{0}C for xylanase production. The crude xylanase produced from *Aspergillus oryzae* has the temperature optima between 60 to 65 ^{0}C. After 10 and 30 min incubation at 60 ^{0}C, the residual activity is 40% and 10% respectively of the original (337). A very thermostable xylanase described is that of an extremely thermophilic

Thermotoga sp. with a half life of 20 min at 105 ^{0}C (47). Xylanases with half lives from a few min up to 90 min at 80 ^{0}C have been produced by *Thermoascus auranticus*, *Bacillus stearothermophilus*, *Caldocellum saccharolyticum*, *Clostridium stercorarium* and *Thermomonospora* spp. (384, 188). It has been reported that *Aspergillus sydowii* MG 49 produces two xylanases with optimal activity at 60 ^{0}C and a stability in the range of 40 to 70 ^{0}C. The enzyme activity is found declining sharply around temperature at 70 ^{0}C (105).

The majority of xylanases have pH optima ranging from 4.5 to 6.5 (193). The optimum pH for xylan hydrolysis is around 5 for most fungal xylanases, and they are normally stable between pH values of 2 to 9. The pH optima of bacterial xylanases are generally slightly higher than that of the fungal xylanases (48). The pH stability of the xylanase produced by *Thermomyces lanuginosus* strains DSM 5826 and ATCC 46882 was in the pH range of 5.0 to 9.0 (51, 36). The xylanase produced by *Bacillus* sp. Sam-3 was found to be most stable at pH 8, retaining 80% of its activity. At pH 9 and 6 the enzyme retained 57 and 52% of its activity (310). The crude xylanase obtained from *Aspergillus carneus* M34 was stable in the pH range of 3-10 (>70% relative activity) (90) while the partially purified xylanase from *A. nidulans* KK-99 was optimally active at pH 8.0 and was stable over a broad pH range (4.0-9.5) at 55 ^{0}C (339).

The use of crude cellulase-free xylanase in pulp processing is cost effective since purification of xylanase is not necessary in pulp processing (9). Numerous investigators have confirmed the observation and various companies have commercialized the technology. Xylanases have been studied using a wide variety of carbon sources including pure xylan (342). The use of purified xylan is

uneconomical for industrial use while agricultural residues offer coast effective alternatives for xylanase production. In order to make the enzyme applications more cost effective at industrial level, its production using low cost substrates (agro wastes) has been recommended by many workers such as sawdust (383), corn cob (36, 107, 267), wheat bran (381), sugar beet pulp (354), and sugarcane bagasse (56, 260). Solid state fermentation (SSF) which is closer to natural system is an attractive alternative process to produce microbial enzymes and metabolites due to its lower capital investment and lower operating cost (245, 164, 119, 139). SSF is generally defined as the growth of microorganisms on solid materials in the absence or near absence of free water. The substrate however must contain enough moisture, which exists in the absorbed form within the solid matrix (59). SSF holds tremendous potential for the production of xylanases (345) and can be of special interest in those processes where the crude fermented product may be used directly as enzyme source (245). SSF is considered as the most appropriated method for filamentous fungi (307, 327) and actinomycetes (227) cultivation and lignocellulosic enzyme production. As they grow under conditions close to their natural habitats i.e. moist substrates with less moisture content due to which they may be more capable of producing certain enzymes and metabolites, which usually will not be produced or will be produced only at low yield in submerged cultures (244). Bacteria need high moisture contents to grow (11) and there are only fewer reports related to successful utilization of bacteria for SSF (11, 370, 103). SSF has been evaluated successfully using *Trichoderma reesei* (346); *Trichoderma harzianum* (281); *Fusarium oxysporum* (242); *Streptomyces* sp. QG-11-3 (33); *Aspergillus oryzae*

(337); *Thermomyces lanuginosus* (59) and *Thermoascus aurantiacus* (208) for xylanase production. The use of SSF as a production method of xylanase can offer some apparent economic and engineering advantages over the classical SmF. These include high concentration of the product (greater yields), simple fermentation equipments as well as low effluent generation and low requirements for aeration and agitation (energy saving) during enzyme production (246, 369, 245, 208). Due to the considerably lower production costs, the SSF xylanase proved more cost-efficient for biobleaching when compared to commercial liquid enzyme products (336).

Xylanases have been isolated from basidiomycetes (166, 353, 67, 211, 133), but relatively little is known about patterns of proteins of white and brown rot fungi. Brown rot fungi depolymerize cellulose and degrade all polysaccharides early in the decay process (45), hemicellulose being apparently degraded prior to cellulose (123). White-rot fungi, in contrast, show different patterns of cell wall attack, depending on their species (237, 238). 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 nearly 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 lignin. The white-rot fungi exhibit two gross patterns of decay: (1) a simultaneous decay, in which the cellulose, hemicellulose, and lignin are removed more or less simultaneously, and (2) delignification, in which the lignin and hemicelluloses are removed ahead of the cellulose (382). Nature has

plentiful variety of micro-flora and fauna with various vital functions. A stable microbe having distinctive production ability of the compound of interest is a precondition for any triumphant fermentation process. Screening and selection of potent microbial strain producing the compound of interest is a critical and exhaustive step for accomplishing the production of a particular compound. A common practice among the researchers is to look for and select the microorganism, which secretes the required enzymatic component for a specific process. Many workers have employed the stepwise screening approaches for selecting the xylanase producing microorganism. The xylanase production is thought to be carried out by various bacterial and fungal organisms but fungal system is found to be effective due to multiple enzyme system, which enhances the degradation of the hemicelluloses (147).

Present study aims at isolating, screening and indentifying the white-rot basidiomycetes which are capable of producing the extracellular xylanases with little or no cellulase activity. The optimization of various operating physico-chemical parameters is done to achieve the higher xylanase activity from the screened strains. The xylanase was biochemically characterized to check its temperature and pH stability for its successful utilization in biobleaching experiments further.

4.2 EXPERIMENTAL METHODOLOGY

4.2.1 Materials

Birchwood xylan and ABTS (2, 2'-azino-bis-3-ethylbenz-thiazoline-6-sulphonic acid) were purchased from Sigma Chemical Company (USA); D-glucose and D-xylose (AR grade) from Qualigens Chemicals (India); di nitro

salicylic acid and bovine serum albumin (AR grade) from Loba Chemie (India); agar-agar from High Media Chemicals (USA); standard protein markers used for electrophoresis from Bangalore genei (India) and wheat bran, rice bran and soybean meal from the local market of Roorkee, U.P. (India). All other chemicals were of analytical grade and purchased from standard commercial manufacturers.

4.2.2 Strain isolation

Different fungal strains were isolated from lignocellulosic wastes (dead and decaying woods), decomposing manure, sugarcane dumping site, fruiting body and paper industry waste by enrichment technique. Samples were collected from different sites at Department of Paper Technology, I.I.T. Roorkee, Saharanpur campus (UP), Star Paper Mills Ltd. Saharanpur, local sugar units and main campus of I.I.T. Roorkee (UA) located in the foothills of Shivalik hills in Northern India, buried in the moist wheat bran in Petri dishes and incubated at 35 ^{0}C. Growth of various fungal strains was observed between 2-10 days. 12 numbers of fungal colonies with different morphological features appeared in different Petri dishes. The wheat bran agar plates were prepared by dissolving 4 g wheat bran and 2 g agar (4% wheat bran, 2% agar) in 1 L of double distilled water and autoclaved at 15 psi for 15 min. These different fungal hyphae and fruiting bodies were transferred into these plates. These plates were checked for fungal growth after 2-3 days and were further purified by sub culturing. Purified cultures were transferred to potato dextrose agar (PDA) slants, incubated at 35 ^{0}C for 5 days and further stored at 4 ^{0}C for future usage. Results are reported in Table 4.2 and Figure 4.15: Photographs A-J.

4.2.3 Screening of xylanase producing strains

Primary screening of all the 12 isolated strains for xylanase production was carried out on xylan agar plates as described (340). The xylan agar medium contained 1% xylan and 2% of agar dissolved in 1 L of double distilled water and autoclaved at 15 psi for 15 min. The crude enzyme extract (50 µL), obtained by solid state fermentation (SSF) of each fungal isolates, was placed separately into 2-3 mm diameter well cut into the solidified medium in each Petri dish and the plates were incubated at 30 ^{0}C for 48 h. The plates were then stained with Congo Red solution composed of 0.5% (w/v) Congo Red and 5% (v/v) ethanol in distilled water for 15 min and destained with 1 M NaCl. The xylanase producing micro-organisms were selected by observing yellow zones around the colonies against the red background. Enzymatic hydrolysis of the surrounding xylan resulted into clear zones in the medium. Controls with heat killed (140 ^{0}C, 20 min) supernatant did not produce any clear zones. The strains were primarily screened for their xylanase production ability on the basis of clear zone diameter obtained on the xylan-agar (XA) plate. Secondary screening of the 9 selected strains was carried out on the basis of higher xylanase (210) and lower cellulase (196) activity of crude enzyme extract produced by SSF of selected fungal strains and finally two strains were selected for further studies. Results are reported in Table 4.2-4.3, Figure 4.2 and Figure 4.15: Photographs K-L.

4.2.4 Scanning electron microscopy

The detailed morphological study of the fungal strains was carried out using scanning electron microscopy (SEM, Leo 435 VP, England). Fungal mat

was taken and subjected for fixation using 3% (v/v) glutaraldehyde-2% (v/v) formaldehyde (4:1) for 24 h. Following the primary fixation, samples were washed thrice with double distilled water. The samples were then treated with the alcohol gradients of 30, 50, 70, 80, 90 and 100% for dehydration. Samples were kept for 15 min each up to 70% alcohol gradient, thereafter treated for 30 min each for subsequent alcohol gradients. After treating with 100% alcohol, samples were air dried and examined under SEM using gold shadowing technique (96). Electron photomicrographs were taken at desired magnifications. Results are reported in Figure 4.16: Photomicrographs A-H.

4.2.5 Identification of strains

Finally, two selected fungal strains were sent to Plant Pathology Division, Forest Research Institute, Dehradun, Uttarakhand (India) for further identification up to species level.

4.2.6 Fermentation medium

4.2.6.1 Nutrient salt solution

Submerged and solid state fermentation techniques were followed for the production of xylanase. Nutrient salt solution (NSS) was prepared according to Vishniac and Santer (371) and standardized as per method adopted by Singh and Garg (318). The medium contained KH_2PO_4, 1.5 g/L; NH_4Cl, 4.0 g/L; $MgSO_4.7H_2O$, 0.5 g/L; KCl, 0.5 g/L and yeast extract, 1.0 g/L in distilled water with 0.04 mL/L trace element solution having $FeSO_4.7H_2O$, 200 µg/L; $ZnSO_4.7H_2O$, 180 µg/L and $MnSO_4.7H_2O$, 20 µg/L. The desired pH (Knick, Germany, Model-761 Calimatic) of the solution was adjusted with $NaOH/H_2SO_4$.

4.2.6.2 Pretreatment of solid substrate

Lignocellulosic substrate (wheat bran) was thoroughly washed in hot and cold distilled water subsequently until the starch material was completely removed. It was immediately dried in sunlight, grinded up to +100 mesh size and was stores in sealed polythene bags for further use.

4.2.6.3 Inoculum

Fungal cultures were transferred to Petri-plates (wheat bran agar medium) from PDA slants and incubated at 35 ^{0}C for 3 days. From this actively growing culture, 2 disks of size 5 mm were cut with the help of a borer and used as the inoculum for the biotransformation process.

4.2.7 Xylanase production by fungal strains

4.2.7.1 Submerged fermentation (SmF)

Submerged fermentation was carried out in 40 mL of NSS with 2% of wheat bran as carbon source in Erlenmeyer flasks (250 mL) (318). Medium was inoculated with fungal cultures following incubation at 35 ^{0}C for 8 days in an orbital incubator shaker (Sanyo, Orbi-safe, UK) with constant shaking (120 rpm). The crude enzyme was harvested and assayed for xylanase (210), and cellulase (196) activities as per standard protocols. Results are reported in Table 4.4 and Figure 4.3.

4.2.7.2 Solid-state fermentation (SSF)

Solid-state fermentation was performed as described by Beg *et al.* (33). Slurry of the fermentation medium containing 5 g of wheat bran and 15 mL of NSS was prepared in Erlenmeyer flasks (250 mL) and inoculated with fungal

culture. The culture flasks were incubated at 35 ^{0}C for 8 days. The crude enzyme was harvested and assayed for xylanase (210), and cellulase (196) activities as per standard protocols. Results are reported in Table 4.4 and Figure 4.3.

4.2.7.3 Enzyme harvesting

In submerged fermentation process enzyme was directly filtered through four layers of cheese cloth while in solid state fermentation enzyme was harvested using 15 mL of distilled water. The contents of the flask were crushed with the help of a glass rod and were shaken at least for 30 min to harvest the enzyme from the fungal cells. The whole content was then filtered through the four layers of cheese cloth as in the submerged fermentation. The filtrate obtained was centrifuged (Sigma centrifuge model 2K15) at 5000 g for 10 min at 4 ^{0}C. The clear brown colored supernatant was used as the crude enzyme sample and was stored at –20 ^{0}C until used.

4.2.8 Analytical methods

4.2.8.1 Estimation of xylanase activity

Xylanase activity was estimated by analysis of the xylose released by DNS method (210). DNS reagent was prepared by dissolving DNS acid, 1 g; phenol, 0.2 g; sodium sulphite, 0.05 g and sodium potassium tartarate, 20.0 g sequentially in 100 mL of 1% sodium hydroxide solution. Mixture was shaken for 5 min and filtered through Whatman filter paper (No.1). The regent was stored in dark at 4 ^{0}C for future use. 0.4 mL of 1% Birchwood xylan solution was mixed with 1.6 mL of suitably diluted culture filtrate in 50 mM potassium phosphate

buffer (pH 6.4) and incubated at 55 ^{0}C for 15 min. 0.3 mL of solution was taken from the incubated mixture in a test tube and 0.9 mL of dinitrosalicylic acid (DNS) reagent was then added, heated on boiling water bath for 5 min and the xylose released was estimated calorimetrically at 540 nm using UV-Vis spectrophotometer (Cary 100 Bio Varian-Australia) at 25 ^{0}C. Blank was included which contained distilled water in place of enzymatic reaction products but similarly contained 3 mL of DNS reagent. One unit of enzyme corresponded to one µmol of xylose released per min per mL under the assay conditions.

4.2.8.2 Estimation of cellulase (CMCase) activity

Carboxymethyl cellulase (CMCase) activity was determined as described by Mandels (196). The assay mixture, in a total volume of 2 mL, contained 0.5 mL of 1 mM of carboxymethyl cellulose (CMC) in 50 mM citrate buffer (pH 4.8) and 0.5 mL of the supernatant obtained from fermentation broth as the source of enzyme. Controls were routinely included in which enzyme or substrate were omitted and treated similarly. The mixture was incubated at 50 ^{0}C for 30 min. The reducing sugars released were measured optically at 575 nm using UV-Vis spectrophotometer (Cary 100 Bio, Varian-Australia) at 25 ^{0}C using DNS reagent (210). One unit of enzyme activity was expressed as one µmol of glucose liberated per min per mL of the reaction under above defined conditions.

4.2.8.3 Estimation of protein concentration

Protein concentration was determined as described by Lowry *et al.* with bovine serum albumin (BSA) as a standard (187). A total volume of 200 µL of a protein sample was taken and 1 mL of Lowry 'C' reagent was added which was

made freshly to add 50 mL of Lowry 'A' (2% Na_2CO_3 in 0.1N NaOH) and 1 mL of Lowry 'B' (0.5% $CuSO_4.5H_2O$ in 1% Sodium or Potassium Tartarate) and discarded after 1 day. The mixture was put for 10 min at room temperature. After 10 min 100 µL of Foline's reagent (1 mL of phenol reagent was mixed with 3 mL of distilled water) was added and this final preparation was incubated for 30 min at room temperature. Color developed was estimated at 750 nm using UV-Vis spectrophotometer (Cary 100 Bio, Varian-Australia) at 25 ^{0}C. The concentration of protein was determined by comparing the absorbance of the sample protein with that of a standard (BSA) using a standard curve.

4.2.8.4 Estimation of laccase activity

Laccase assay (75) was performed using ABTS (2, 2'-azino-bis-3-ethylbenz-thiazoline-6-sulphonic acid) as substrate. Reaction was carried out by taking 100 mM citric acid buffer at pH 5.0, enzyme extract and 1.0 mM of ABTS. Reaction was monitored at 420 nm using UV-Vis spectrophotometer (Cary 100 Bio, Varian-Australia) at 25 ^{0}C. The enzyme activity was expressed as the amount of enzyme which produced an increase of 1.0 absorbance unit per 30 seconds.

4.2.9 Optimization of various physicochemical parameters under SSF

4.2.9.1 Optimization of incubation period

For the optimization of incubation period, a set of Erlenmeyer flasks (250 mL) was prepared containing 5 g of wheat bran and 15 mL of NSS (pH 6.0). These flasks were autoclaved at 15 Pa for 15 min and inoculated aseptically with fungal cultures as described above. These were incubated at 35 ^{0}C and were

harvested after 2nd, 3rd, 4th, 5th, 6th, 7th, 8th, 9th, 10th and 11th day. The estimation of the protein concentration (187) and xylanase (210) and cellulase activity (196) was done as per standard protocols. Results are reported in Table 4.5-4.6 and Figure 4.4-4.5.

4.2.9.2 Optimization of incubation temperature

A set of Erlenmeyer flasks (250 mL) containing 10 g of wheat bran and 15 mL of NSS (pH 6) was prepared and after autoclaving inoculated with fungal cultures. The effect of temperature on the production of xylanases produced by both the strains was studied by incubating the inoculated flasks at 27, 32, 37, 42, 47 and 52 ^{0}C for 7 days. The xylanase (210) and cellulase activities (196) were determined as per the standard protocol. Results are reported in Table 4.7 and Figure 4.6.

4.2.9.3 Optimization of initial pH

For the optimization of pH, a set of 250 mL Erlenmeyer flasks was prepared as described above and pH was adjusted at 4.6, 5.2, 5.8, 6.4, 7.0, 7.6, 8.2, 8.8, 9.4, 10.0, 10.6 and 11.2 with 1 N $NaOH/H_2SO_4$ separately. These flasks were autoclaved at 15 Pa for 15 min, inoculated with fungal cultures aseptically and incubated at 37 ^{0}C. Flasks were harvested after 7 days and the xylanase (210) and cellulase activities (196) were measured as per the standard protocol. Results are reported in Table 4.8 and Figure 4.7.

4.2.9.4 Effect of carbon source

Erlenmeyer flasks (250 mL) containing 5 g each of various agricultural by-products/residues like wheat bran, sugarcane bagasse, wheat straw and rice

straw (grinded up to +100 mesh size) were used as substrates for xylanase production by both of the strains. The substrates were moistened with NSS, autoclaved, inoculated and incubated at 37 ^{0}C. The crude enzyme was extracted on 7th day and assayed as per standard protocol mentioned for xylanase (210) and cellulase activities (196). Results are reported in Table 4.9 and Figure 4.8.

4.2.9.5 Effect of nitrogen source

A set of 5 Erlenmeyer flasks (250 mL) was prepared as described above and each flask was supplemented with five different nitrogen sources including yeast extract, malt extract, peptone, soya bean meal and beef extract. The pH of NSS was set at 6.4. Fermentation was carried out for 7 days at 37 ^{0}C. Crude enzyme was harvested and xylanase (210) and cellulase activities (196) were estimated as per the standard protocols described. Results are reported in Table 4.10 and Figure 4.9.

4.2.9.6 Optimization of moisture level

The influence of moisture level on the xylanase titer was evaluated by varying the ratio (w/v) of wheat bran to NSS (1:2, 1:2.5, 1:3, 1:3.5 and 1:4.0). The fermentation was carried out for 7 days at 37 ^{0}C. The xylanase (210) and cellulase activities (196) in crude enzyme extract were checked as per standard protocols. Results are reported in Table 4.11 and Figure 4.10.

4.2.9.7 Effect of glucose and lactose concentration on xylanase production

Different levels of glucose/lactose (1-5 g/L) were incorporated into wheat bran moistened with NSS in a ratio of 1:3 and its effect on xylanase titer of both the strains was studied. Fermentation was carried out under optimum conditions

mentioned in Table 4.12 and 4.13. Xylanase activity (210) was determined as per standard protocol. Results are reported in Table 4.14 and Figure 4.11-4.12.

4.2.10 Characterization of crude xylanase

4.2.10.1 Optimum pH and pH stability

The pH stability was determined in the pH range of 6.0-9.0 by incubating the enzyme in buffers of different pH (potassium-phosphate; pH range: 6.0-7.4 and borax-boric acid; pH range: 7.6-9.0). After 15 min incubation, the residual xylanase activity (210) of the crude enzyme samples was determined, under standard assay conditions. Results are reported in Table 4.16 and Figure 4.13.

4.2.10.2 Optimum temperature and thermostability

The thermostability of the enzyme was determined by incubating the crude enzyme preparations at temperatures ranging between 55-85 ^{0}C for up to 15 min. Samples were withdrawn after 15 min and analyzed for residual xylanase activity (210) under standard assay conditions. Results are reported in Table 4.17 and Figure 4.14.

4.2.10.3 Sodium-dodecylsulphate Polyacrylamide Gel Electrophoresis

SDS-PAGE of the samples was performed according to Laemmli (172). Polyacrylamide gels (12%) of 1.5 mm thickness were prepared for electrophoretic analysis.

Reagents

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

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

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

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

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

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

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

4.2.10.3.1 Preparation of resolving and stacking gels

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

4.2.10.3.2 Casting of gel

A sandwich of glass plates (16 x 18 x 0.3 cm) was assembled by plastic spacers (1.5 mm) and the plates were held together by plastic clamps. The base of the gel mould was sealed. Resolving gel mixture was prepared by mixing all the components except ammonium persulphate and TEMED. Mixture was degassed for 15 min; ammonium persulphate and TEMED were added to it. The mixture was gently mixed and poured between the plates, and leaving the required space at the top for pouring the stacking gel. After polymerization of the resolving gel, the assembly was tilted to pour off the over layer, washed with

sterile distilled water and wiped gently to dry the empty space. Stacking gel mixture was then similarly degassed and layered on the resolving gel; comb was immediately inserted into the mixture to form the wells. After polymerization of stacking gel, comb was carefully removed, wells formed were rinsed with reservoir buffer and the polymerized gel was used for electrophoresis.

4.2.10.3.3 Sample preparation

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

4.2.10.3.4 *Electrophoresis*

Samples (100 μg of the protein) were gently loaded on the gel using a micropipette. Electrophoresis was carried out at constant voltage (stacking at 60 V, resolving at 90 V) until the tracking dye reached to about 1 cm above from the bottom of the gel. Gel after electrophoresis was removed and stained overnight with gentle shaking in coomassie brilliant blue R-250 0.1% in methanol: glacial acetic acid: water (5:2:5 v/v/v) at room temperature. Gel was then destained by washing in 10% isopropanol-7% acetic acid solution (374). Destaining was done so as to get the stained bands over the clear background. Gel was stored in 7% acetic acid until photographed.

4.2.10.3.5 Zymogram analysis of xylanase components

Native polyacrylamide gel electrophoresis (PAGE) using 12% gel was performed for visualization of enzyme activities in situ as described (301, 280). Substrate i.e. xylan to the final concentrations of 0.1% was incorporated into separating gel before adding the ammonium persulphate and TEMED for polymerization. The enzyme samples were mixed with sample buffer lacking SDS and β-merceptoethanol. After electrophoresis, the gel was soaked with 0.1% (w/v) Congo red for 5 min with mild shaking. Excess dye was decanted and gel was washed with 5% (w/v) NaCl until excess stain was totally removed and the background was clear. Results are reported in Figure 4.18.

4.2.11 Mass production of xylanase

The mass production of xylanase was carried out by SSF technique for use in biobleaching. 40 g of wheat bran was taken in a 2 L flask and 120 mL of NSS (pH 6.0) was added to it. From the actively growing culture of strain SW-1, 16 disks of size 5 mm were cut with the help of a borer and used as the inoculum for the biotransformation process. The flask was incubated at 37 ^{0}C and harvested after 7 days of incubation. The crude enzyme extract obtained was used for the determination of xylanase (210), cellulase (196), laccase activity (75) and protein estimation (187) as per standard protocols. Results are reported in Table 4.18.

4.2.12 Statistical analysis

All experiments were carried out in triplicate and experimental results were represented as the mean ± standard deviation of three identical values.

4.3 RESULTS AND DISCUSSION

4.3.1 Isolation, purification and screening of fungal strains

Table 4.2 reveals the morphological characteristics of 12 fungal strains collected from different lignocellulosic sources. The fungal strains were isolated on wheat bran agar plates, incubated at 35 ^{0}C and color of mycelia, spores and colony appearance was observed (Figure 4.15: Photographs A-F). The formation of fruiting bodies in some Petri-plates indicates the growth of basidiomycetes (Figure 4.15: Photographs G-J). The xylanase production ability of these strains on xylan-agar (XA) plate was observed after primary screening. Nine of these isolates have resulted into zone formation onto xylan-agar (XA) plates, of which SW-1 and SW-2 have resulted in to the maximum clear zone diameter after Congo Red staining (Figure 4.15: Photographs K-L). These isolates were selected as xylanase producers. These nine strains are further subjected to secondary screening for their xylanase production ability in solid state condition (Table 4.3). Three fungal strains namely, SW-1, SW-2 and SW-10 out of nine produce maximal of xylanase activity i.e. 362.1, 315.6 and 303.11 IU/mL respectively with minimum of cellulase activity i.e. 0.64, 0.88 and 1.03 IU/mL respectively. Two fungal isolates namely SW-1 and SW-2 were selected for further studies based on their higher xylanase and lower cellulase activities.

4.3.1.1 Identification of selected isolates

Both of these two isolates i.e. SW-1 and SW-2 were identified as different strains of white rot basidiomycete *Coprinellus disseminatus* (Pers.: Fr.) Lange (174) *(=Coprinus disseminatus* (Per.:Fr.) Gray), from Forest Research Institute (FRI), Dehradun, Uttarakhand (India). Both of these strains have been deposited in the National Type Culture Collection, Forest Pathology Division, FRI,

Dehradun and were allotted the NTCC Culture No. 1165 and 1166 respectively. *Coprinus disseminatus* is scientifically classified as Fungi, Basidiomycota, Agaricomycotina, Agaricomycetes, Agaricomycetidae, Agaricales, Coprinaceae, *Coprinus*.

4.3.1.2 Morphological characteristics of *Coprinellus disseminatus*

The characteristic features of both of the strains of *C. disseminatus* namely SW-1 and SW-2 are shown in Figure 4.15: Photographs G-J. The mycelia of both the fungal strains are of white colored. Spores produced by fungal strains SW-1 and SW-2 are of blackish and brownish black in color respectively (Figure 4.15: Photographs H and J). Fungal strain SW-2 produces distinct growth rings when cultured (Figure 4.15: Photograph I) while SW-1 shows the smooth colony without any distinct growth rings (Figure 4.15: Photograph G). Both of the strains produce the fruiting bodies indicating that these fungi are related to basidiomycetes family which ultimately lead to the spore formation on 10^{th} day in SW-1 and 12^{th} day in SW-2 (Figure 4.15: Photographs H, J). The finer structural details of the mycelia and spores as observed through the scanning electron microscopy (SEM) are shown in Figure 4.16: Photomicrographs A-H. The hyphae of fungal strain SW-1 are thin, elongated and straight (Figure 4.16: Photomicrographs A-B) whereas, the hyphae of the fungal strain SW-2 are thick and compact (Figure 4.16: Photomicrographs E-F). The basidiospores produced by both the fungal strains are club shaped (Figure 4.16: Photomicrographs C-D, G-H) which is a characteristic feature of all the members of basidiomycotina group.

4.3.2 Comparative analysis of different fermentation conditions

Table 4.4 and Figure 4.3 reveal a comparison between solid state and submerged fermentations on xylanase production ability of both the strains of *C. disseminatus*. The xylanase activity of fungal strains i.e. SW-1 and SW-2 is 196.3 and 166.4 IU/mL respectively while cellulase activity in both the cases is 0.28 and 0.38 IU/mL respectively under SmF. On the other hand, the xylanase activity of SW-1 and SW-2 is 370.2 and 320.6 IU/mL and cellulase activity is 0.64 and 0.81 IU/mL respectively. Results show that xylanase activity of strains SW-1 and SW-2 under SSF conditions is respectively 88.59 and 92.67% higher than that of SmF conditions. So the xylanase production in SSF is much higher than that of SmF. In the same way, cellulase activity of both the strains increases accordingly in SSF compared to SmF. The aim of SSF is to bring the cultivated fungi or bacteria into tight contact with the insoluble substrate and thus to achieve the highest substrate concentrations for fermentation (125), so the increased yield of xylanase produced by both of the fungal strains observed in SSF may be attributed to close contact between mycelium and substrate (134) which is not possible during SmF. A higher xylanase yield using SSF in comparison to the SmF with wheat straw and sugarcane bagasse had been reported from thermophilic *Melanocarpus albomyces* IIS-68 (134). Malarvizhi *et al.* observed 30-fold enhancement of xylanase production in solid state fermentation than liquid culture when wheat bran was used as the substrate for a culture of *Ganoderma lucidum* (194). Therefore, SSF has been chosen for further optimization studies for crude xylanase production by both of the strains.

4.3.3 Analysis of critical parameters for improving the xylanase production

SSF was carried out for optimizing various operating parameters i.e. incubation period, temperature, pH, carbon source, nitrogen source and moisture content using hyper-xylanase producing *C. disseminatus* SW-1 and SW-2 (Figure 4.17: Photographs B-C). Using SSF conditions, critical physicochemical and biological factors were analyzed for deciphering the most favorable conditions for achieving enhanced levels of enzymatic production.

4.3.3.1 Optimization of incubation period for xylanase production

The influence of incubation period on enzymatic production by *C. disseminatus* in a batch process is depicted in Table 4.5 and 4.6. The xylanase activity of *C. disseminatus* SW-1 and SW-2 increases steadily with increasing incubation period and attains maximum levels on 7^{th} day of incubation i.e. 401.82 and 341.73 IU/mL respectively which corresponds to mycelial biomass in terms of protein concentration (Figure 4.4 and 4.5). Further on increasing incubation period beyond 7^{th} day the xylanase activity of both the fungal strains i.e. SW-1 and SW-2 deceases. Cellulase production increases to its maximum levels after 8 days of incubation for SW-1 (0.69 IU/mL) and 7 days for SW-2 (0.79 IU/mL). On longer incubation; enzyme activities of both the strains decrease while protein biomass continues to increase from day 2^{nd} to 8^{th} for both the strains and then nearly becomes constant with a small decrease after 10^{th} day. It suggests that the enzyme production is dependent on biomass but only during the exponential phase of growth of fungi. Because, cellulase and xylanase are a part of primary metabolites thus, these are produced during

exponential phase of growth and at the onset of death phase, the enzyme secretion starts decreasing. Xylanase production is maximum at the end of exponential phase (7^{th} day) and then slowly starts decreasing on the onset of death phase. It suggests that the enzyme production is dependent on fungal biomass but up to some extent (54). A similar trend was reported for production of xylanase by *Thermoactinomyces thalophilus* (159) *Aspergillus carneus* M34 (90) and Trichoderma reesei SAF3 (149). Similar incubation periods for xylanase production have been reported for other fungal systems. Basidiomycetes are slow growing fungi so xylanase production was maximum after 7 days in *Pleurotus ostreatus* (269) and *Trichoderma harzianum* 1073 D3 (309), 6 days in *Phanerochaete chrysosporium* (335) and 8 days in *Volvariella diplasia* (257). While Garzillo *et al.* indicated that xylanase activity by *Pleurotus ostreatus* reached a maximum after 16 days incubation (98). Other fungi such as *Thermomyces lanuginosus* and *Thermomyces auranticus* had produced maximum xylanase on 7^{th} day of incubation (5) while maximum xylanase production was observed on 6^{th} day in *Penicillium oxalicum* in SSF (222).

4.3.3.2 Optimization of incubation temperature for xylanase production

Temperature is well known to affect the growth, development and overall metabolic activities of an organism (216) hence; it was felt desirable to optimize the temperature under SSF using wheat bran as carbon source for the xylanase production by both of the strains of *C. disseminatus*. Table 4.7 shows that both of the strains produce the highest xylanase activity i.e. 428.51 IU/mL for SW-1 and 377.2 IU/mL for SW-2 at 37 ^{0}C. Both the fungal strains yield the lowest xylanase activity at 27 ^{0}C which increases with increasing temperature up to 37

^{0}C and decreases later on (Figure 4.6). The xylanase activity of strain SW-1 at temperature 42 and 47 ^{0}C is 345.08 and 210.8 IU/mL. However, the xylanase activity for SW-2 decreases drastically after 37^{0}C and is observed to 21.6 IU/mL at 47 ^{0}C. The growth of strain SW-2 is inhibited at 52 ^{0}C, while strain SW-1 is even productive at this temperature with a xylanase activity of 43.8 IU/mL. The maximum xylanase activity at 37 ^{0}C for both the fungal strains SW-1 and SW-2 suggests that both the fungi are mesophilic in nature. On the other hand, fungal strain SW-1 is more thermotolerant in comparison with fungal strain SW-2. The decrease in xylanase production level may be possible due to lower transport of substrate across the cells at lower temperature causing lower yield of the product. At higher temperature, the maintenance energy requirement of cellular growth is high due to thermal denaturation of enzymes of the metabolic pathway resulting in lower production of the metabolites (3). The productivity decline observed at high temperature could be due to the reversible denaturation of enzyme formed on optimized-medium as described previously (68). The cellulase activity of both the fungal strains also increases with increasing incubation temperature up to 37 ^{0}C and then declines. Temperature regulation is one of the crucial factors for solid-state fermentation. Stoichiometric global equation and respiration is highly exothermic and heat generation was due to higher levels of fungal activity within the solids leads to thermal gradients due to the limited heat transfer in the solid substrates. The thermal gradation can be controlled by maintaining the desired level of humidity (147).

Similarly, a thermostable and cellulase free xylanase has been produced by *Streptomyces* sp. QG-11-3 in the solid state fermentation using wheat bran as

the carbon source at 37^{0}C (33). *S. cuspidosporus* had also been reported to grow rapidly and produce maximum xylanase under SSF using wheat bran at a temperature of 37 ^{0}C (192). Xylanase production was found to be optimum at 37 ^{0}C under SSF for *Bacillus pumilus* ASH using wheat bran (31) while the highest xylanase production by *B. licheniformis* A99 was observed at 50 ^{0}C under SSF using wheat bran as carbon source (10).

4.3.3.3 Optimization of pH of the medium for the production of xylanases

Table 4.8 reveals that xylanase activity of both of the *C. disseminatus* strains increases with increasing pH from 4.6 to 6.4 while keeping other fermentation conditions constant and further on increasing pH the xylanase activity decreases gradually. Figure 4.7 illustrates three distinct phases as a function of pH, i.e. at low pH (4.6 to 5.8) there is an increase in enzyme activity, at high pH (above 7.6), there is a decrease and at an intermediate range, the pH optimum (6.4-7.0), the activity is maximal, leading to characteristic bell-shaped curve. The maximal xylanase activity of both of the fungal strains i.e. SW-1 and SW-2 is 441.5 and 397.84 IU/mL respectively at pH 6.4. A further increase in pH steadily reduces the xylanase titres of both of the fungal strains; however, considerable high amounts of xylanase have also been produced at lower and higher pH values. The cellulase activity of both of the isolates is found higher at acidic pH which gradually decreases with increasing the pH of medium. The maximum cellulase activity of strain SW-1 (0.97 IU/mL) and SW-2 (1.2 IU/mL) is observed at pH 4.6 while minimum is noted at pH 11.2 (0.05 and 0.09 IU/mL respectively). The H^+ concentration in the fermentation medium has profound effect on the xylanase production. The initial pH influences much enzymatic

system and the transport of several species of enzymes across the cell membrane (11). Fermentation at lower and higher pH proved to be detrimental perhaps because of the inactivation of the enzyme system. Since, enzymes are proteins, the ionic character of the amino and carboxylic acid groups on the protein surface are likely to be affected by pH changes and the catalytic property of the enzyme is markedly influenced. The reason for poor production at higher pH was probably due to proteolytic inactivation of the xylanase. It suggests that slightly acidic pH values favor the xylanase production but both the strains are tolerant to alkaline pH values as well. The isolates were considered as alkali-tolerant fungi according to the definitions of Nagai et al. (223). These results are in agreement with those reported for other fungal species. The optimum pH for the xylanase production by *A. versicolor* was 6.5 (50) under SmF at 30 ^{0}C and 6.0 by aquatic hypomycetes under submerged fermentation at 20-25 ^{0}C (1); while *A. carneus* M34 grew well in the pH range of 4.5 to 5.5 in the temperature range of 33-36 ^{0}C under response surface methodology (RSM) (90).

The alkali tolerant property makes the enzyme available for direct application on the alkaline pulp without any intervening pH adjustment. Thus; our enzyme preparation fulfills the current paper industry requirements.

4.3.3.4 Effect of carbon source on xylanase production

Table 4.9 shows that the xylanase activity of *C. disseminatus* SW-1 and SW-2 is investigated on several cheap agricultural wastes including sugarcane bagasse, wheat straw, rice bran and wheat bran (Figure 4.17: Photograph A), which are used as the sole carbon source. Figure 4.8 depicts that wheat bran is the most suitable substrate which has induced the xylanase production by both

of the strains up to the highest levels on which strains SW-1 and SW-2 produce 469.45 and 384.4 IU/mL of xylanase activity respectively followed by rice bran, wheat straw and bagasse. Wheat straw has induced the xylanase production of SW-2 (54.64 IU/mL) more in comparison with that of SW-1 (32.86 IU/mL). A combination of 50% of wheat bran and 50% of rice bran also induces the xylanase production from both of the species i.e. SW-1 (244.64 IU/mL) and SW-2 (198.13 IU/mL) up to a good extent followed by bagasse+wheat bran, wheat straw+wheat bran, wheat straw+rice bran, bagasse+rice bran and bagasse+wheat straw for SW-1 and wheat straw+wheat bran, bagasse+wheat bran, wheat straw+rice bran, bagasse+wheat straw and bagasse+rice bran for SW-2. In the same way the cellulase activity of both of the strains is also the highest when wheat bran is used as the carbon source (SW-1: 0.73 and SW-2 0.85 IU/mL) followed by rice bran, wheat straw and bagasse respectively. Hence, based on results wheat bran was selected and used as solid substrate for further optimization studies under SSF. Wheat bran is considered to be the best substrate for xylanase induction, as it contains sufficient nutrients and is able to remain loose in moist conditions, thereby providing good aeration and large surface area (251, 10), which can be used by microbes for growth and metabolic activity (243). The biochemical composition of wheat bran (177) indicates that wheat bran contains considerable amount of soluble sugars like glucose (42.5% dry wt.), xylose (15.4% dry wt.), arabinose (3.1% dry wt.) and galactose (2.7% dry wt.) required for the initiation of growth and replication of the microorganism. The degree of substitution of the main xylan chains by arabinose is higher in wheat bran (177). It contains 45% hemicellulose (40% xylans), which

may fulfill the role of inducers, and organic nitrogen sources (23%) that are essential for protein synthesis (14). In addition, it contains about 28% protein which might serve as the source of carbon and nitrogen for the microorganisms. Further, its lignin and silica contents are very low (293). Wheat bran was used as the substrate for the xylanase production under SSF by *Streptomyces cyaneus* SN32 (228), *Aspergillus fischeri* (53), *Cephalosporium* sp. (25), *Bacillus pumilus* (11), *Bacillus subtilis* (293) and *Bacillus pumilus* ASH (32) etc.

4.3.3.5 Effect of nitrogen source on xylanase production

Table 4.10 shows the effect of different organic nitrogen sources (yeast extract, peptone, malt extract, soya bean meal and beef extract) on xylanase production by both of the strains of *C. disseminatus*. In an attempt to maintain low fermentation costs during enzyme production, relatively inexpensive organic nitrogen source i.e. soya bean meal is tested together with other organic nitrogen sources. Figure 4.9 shows that all tested nitrogen sources have stimulated the production of xylanases by strains SW-1 and SW-2. Soya bean meal is the best nitrogen source for xylanase production by fungal strain SW-1 (478.5 IU/mL) while peptone increases the xylanase production of SW-2 (392.23 IU/mL) up to the maximum level in comparison to other nitrogen sources. The maximum cellulase activity of fungal strain SW-1 is 0.79 IU/mL on soya bean meal and 0.90 IU/mL for strain SW-2 on peptone. The productivity of xylanases is greatly influenced by both the source and concentration of nitrogen (169). In the presence of more available nitrogen, the mycelium grows better and its activity also increases (132). The use of soya bean meal as a cheap nitrogen source had been reported earlier for the xylanase production under SSF from

Aspergillus oryzae (337), *Bacillus megaterium* (317) and under SmF from *Trichoderma harzianum 1073 D3* (309). Peptone was the best nitrogen source to achieve optimal xylanase production by white-rot basidiomycetes under SmF from *Trichoderma harzianum* (2), *Pleurotus ostreatus* (269) and under SSF from *Lentinus edodes* IBB 363 (145).

4.3.3.6 Optimization of moisture content for xylanase production

Table 4.11 shows the effect of moisture content on xylanase production by *C. disseminatus* SW-1 and SW-2. Figure 4.10 depicts that both the basidiomycetes fungi produce maximum xylanase titer (SW-1: 479.7 and SW-2: 404.3 IU/mL) when wheat bran is moistened with NSS in a ratio of 1:3 and it declines with further increase in substrate to moisture ratio. Cellulase activity for both of the fungal strains too is maximum i.e. 0.80 IU/mL for SW-1 and 0.96 IU/mL for SW-2 at solid substrate to moisture content ratio of 1:3 and decreases further on increasing substrate to moisture content ratio. The moisture content in SSF is a crucial factor that determines the success of the process (93). The importance of moisture level in SSF media and its influence on microbial growth and product biosynthesis may be attributed to the impact of moisture on the physical properties of the solid substrate (273, 225, 93). Moisture level above level causes decreased porosity, alteration in wheat bran particle structure, gummy texture, lower oxygen transfer (as void space of the solid support is filled with moisture) and enhancement of the formation of the aerial mycelia (102; 100, 273, 93). Likewise, moisture level below optimum leads to reduced solubility of the nutrients of the solid substrate and lower degree of swelling of substrate (93). In SSF using wheat bran and eucalyptus kraft pulp as the primary solid

substrates, *Streptomyces sp.* QG-11-3 (33) produces maximum xylanase yield at substrate-to-moisture ratio of 1:2.5 and 1:3, respectively. In contrast, a lower solid substrate to moisture level of 1:1 has been reported for maximum xylanase production by *Bacillus* sp. A-009 (103).

Based on above discussion, the various optimized physico-chemical parameters for crude xylanase production by fungal strains SW-1 and SW-2 are: incubation period 7 day, incubation temperature 37 ^{0}C, pH 6.4, solid substrate (wheat bran) to moisture ratio 1:3 and carbon source wheat bran except nitrogen source which is soya bean meal for strain SW-1 and peptone for SW-2 (Table 4.12). Table 4.13 shows the enzyme production by both of the fungal strains under optimized conditions. The xylanase activity of SW-1 and SW-2 is 499.60 and 410.21 IU/mL while cellulase activity is 0.86 and 1.02 IU/mL respectively. The laccase activity is 25.3 IU/mL for SW-1 and 14.3 IU/mL for SW-2 while protein biomass is 4.7 and 3.2 mg/mL for SW-1 and SW-2 respectively.

4.3.3.7 Effect of glucose and lactose on xylanase production

Table 4.14 and 4.15 show the effect of glucose and lactose concentration on xylanase production by *C. disseminatus* SW-1 and SW-2. Figures 4.11 and 4.12 show that xylanase production by both of the strains decreases with increasing glucose and lactose concentration from 1 to 5 g/L. Figure 4.11 shows that in absence of glucose; strains SW-1 and SW-2 produce 482.28 and 396.90 IU/mL of xylanase activity respectively which decreases later on with increasing the concentration of glucose. In the same way, the xylanase activity of fungal strains SW-1 and SW-2 is 460.83 and 386.61 IU/mL respectively which decrease further with addition of lactose during solid state fermentation (Figure

4.12). The addition of glucose and lactose had repressed the xylanase production, which may be due to catabolite repression (370). Catabolite repression of xylanase production by glucose was also observed by Sanghi *et al.* (293) in *Bacillus subtilis* ASH and Hamzah *et al.* (120) in *Bacillus pumilus*. While Srivastava observed that xylanase produced from the bacterium *Thermomonospora* was resistant to catabolite repression by glucose and xylose (328).

4.3.4 Biochemical characterization of xylanase

4.3.4.1 Effect of pH on the activity and stability of xylanase

Table 4.16 shows the effect of pH on the xylanase activity of *C. disseminates* SW-1 and SW-2 at temperature 55 ^{0}C for 15 min of reaction time. Figure 4.13 shows that the crude xylanase produced by fungal strains SW-1 and SW-2 are active in the pH range of 6.0 to 9.0 with the maximum xylanase activity obtained at pH 6.4 i.e. 479.60 IU/mL and 389.80 IU/mL respectively. Xylanase produced by fungal strains SW-1 and SW-2 maintains 79 and 75% respectively of its activity at pH 6.0 in comparison to xylanase activity obtained at optimum pH of 6.4. Xylanase activity of both the strains starts decreasing when buffer pH is increased above 6.4. It is observed that at neutral pH (7.0) strain SW-1 retains about 80% of its optimum xylanase activity while strain SW-2 retains only 60% of its optimum xylanase activity. Up to a pH level of 7.6; xylanase from SW-1 retains about 53% of its optimum activity while xylanase produced by strain SW-2 retains 37% of its optimum activity. Xylanase activity of both the strains sharply decreases above pH 8.0 which is 25 and 15%

of its optimum activity for strains SW-1 and SW-2 respectively which decreases to 8.4 (SW-1) and 3.0% (SW-2) at pH 9.0. The xylanase produced by strain SW-1 maintains its high stability (more than 50% of its optimum xylanase activity) over a pH range of 6.0 to 7.6, while less than 50% of its optimum xylanase activity is verified above pH 7.6. In contrast, xylanase produced by SW-2 maintains its stability in the pH range of 6.0 to 7.0 (above 50% of its optimum xylanase activity) while less than 50% of its optimum xylanase activity is noted above pH 7.0.Therefore, xylanase produced by strain SW-1 is more alkali-tolerant than that of strain SW-2. Enzymes are protein in nature. In the harsh conditions such as change in pH, high temperature or in presence of high concentration of metal ions, proteins tend to lose their basic structure (denaturation), subsequently losing active site that in turn results in loss of enzyme activity. Besides this, the pH activity profiles of enzymes are highly dependent on the pKa value 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. pH stability increases with decreasing the pKa value (144). The optimum pH for the xylanase produced by *Aspergillus fumigatus* AR1 was 6.0-6.5 (9). The xylanase produced by white-rot fungus *Pleurotus ostreatus* was active over a broad range of pH (3.0-7.0), with optima at 6.0 (269). The xylanase produced by *Penicillium purpurogenum* had the two pH optima i.e. 3.5 and 7.0 and was active in the pH range of 6-7.5 (35). Studies carried out with other fungal species (290, 65) also concluded that the most suitable pH value for xylanase activity was within acidic region which support our findings.

4.3.4.2 Effect of temperature on the activity and stability of xylanase

Table 4.17 shows the effect of temperature on the activity of crude xylanase produced by both of the strains of *C. disseminatus* at their optimum pH (6.4) for 15 min of reaction time. Figure 4.14 depicts that the optimum temperature for the crude xylanase activity of both the strains is 55 ^{0}C (SW-1: 484.21 IU/mL and SW-2: 395.2 IU/mL) and beyond that xylanase activity goes on decreasing. At temperature 65 ^{0}C xylanase produced from strain SW-1 retains about 50% of its optimum xylanase activity while that of strain SW-2 exhibits about 35% of its optimum xylanase activity. When assayed at higher temperatures; both of the strains lose most of their xylanase activity; SW-1 maintains 13% and 10% of its optimum xylanase activity at 75 and 85 ^{0}C respectively while SW-2 retains only 6.33 and 2.6% of its optimum xylanase activity at 75 and 85 ^{0}C respectively. SW-1 is found slightly more thermo-tolerant in comparison to SW-2 as it resists the more temperature increase compared to that of SW-2. Thermostability of enzymes seems to be a property acquired by a protein through a combination of many small structural modifications that are achieved with the exchange of some amino acids. The variation of the canonical forces e.g. hydrogen bonds, ion-pair interactions and hydrophobic interactions provide thermozymes resistance at high temperature (298). The optimal temperature for the crude xylanase activity of both the strains is 55 ^{0}C. This result substantiates with the finding that the optimum temperature for the xylanase produced by most of the fungi is in the range of 40-60 ^{0}C (169). 55 ^{0}C was considered as the optimum temperature for xylanase activity of *Aspergillus nidulans* KK-99 (339) while xylanase from *Aspergillus fumigatus* AR1 exhibited maximum xylanase activity at temperature 60-65 ^{0}C.

Due to high temperature of the pulp bleaching process, it is desirable to use enzymes under alkaline pH (8-10) and high temperatures (55-70 ^{0}C) conditions. The crude xylanase produced by strain SW-1 is active in the pH range of 6 to 9 and temperature variation from 55 to 85 ^{0}C whereas, high xylanase stability is observed in pH range of 6-7.6 and temperature variation of 55-65 ^{0}C. Therefore, crude xylanase produced by strain SW-1 is more tolerant to higher temperature and pH and has lower cellulase contamination in comparison with to strain SW-2 and thus, *C. disseminatus* SW-1 was selected for further biobleaching experiments. The mass production of crude xylanase by SW-1 under optimized conditions is summarized in Table 4.18. SW-1 exhibits 513.21 IU/mL of xylanase activity, and 21.6 IU/mL of laccase activity. A negligible cellulase contamination (0.83 IU/mL) is observed in crude enzyme extract obtained from strain SW-1 while the protein concentration of crude enzyme sample is 4.4 mg/mL which explains that crude enzyme obtained from strain SW-1 is concentrated solution of a variety of proteins.

4.3.4.3 Molecular characterization

The protein profile of *C. disseminatus* SW-1 was analyzed through SDS PAGE using concentrated crude culture filtrate as samples (Figure 4.18). SDS-PAGE and zymogram analysis indicates the presence of single isoform of xylanase. Molecular weight analysis of the respective bands indicates that xylanase has a molecular weight of 43 kDa. According to the classification given by Wong *et al.* (376), the xylanase isolated from strain SW-1 belongs to the high molecular weight category. The white rot fungus *Phanerochaete chrysosporium* produced three xylanases with molecular weights of 52, 30 and 50 kDa

respectively (76) while molecular weight of xylanase produced by *Ceriporiopsis subvermispora* was 29 kDa (209). *Chaetomium cellulolyticum* produced three xylanases with molecular weights of 25, 47 and 57 kDa and *pIs* of 8.9, 8.4 and 5.0 respectively (26).

4.4 CONCLUSIONS

Among twelve fungal strains isolated from different lignocellulosic sources, nine isolates have been selected as xylanase producers on the basis of clear zone-formation on xylan-agar (XA) plates. These strains have distinct morphological features, color appearance and growth patterns. Finally, two strains i.e. SW-1 and SW-2 have been selected, resulting into maximum xylanase along with minimum cellulase production and were identified to be the different strains of *C. disseminatus* from Forest Research Institute, Dehradun (India). SEM studies reveal that *C. disseminatus* SW-1 has thin, elongated and straight mycelium, whereas mycelium of the *C. disseminatus* SW-2 is thick and compact. Moreover in identical fermentation conditions, the *C. disseminatus* SW-1 produces notably higher levels of xylanase and lower level of cellulase in comparison to the *C. disseminatus* SW-2. The xylanase production by both of the strains of *C. disseminatus* is assessed under SmF and SSF conditions. It is found that SSF is the preferred mode of xylanase production by both of the strains. The various physico-chemical and biological parameters for achieving enhanced level of crude xylanase by *C. disseminatus* SW-1 and SW-2 are evaluated in SSF condition. Using *C. disseminatus* SW-1 and SW-2, maximum production of xylanase is obtained on 7^{th} day of fermentation (at 37 ^{0}C, pH 6.4) under SSF condition. An attempt has been made to further economize the process of xylanase production by evaluating the SSF using agro-industrial

residues as the carbon source. Among four different carbon sources used, wheat bran is found to be the most effective for xylanase production by both of the strains. It is seen that all four different nitrogen sources tested have stimulated the xylanase production by both of the strains but soya bean meal is the best nitrogen source for xylanase production by *C. disseminatus* SW-1 which is a cheap agricultural residue and peptone for strain SW-2. A solid substrate to moisture content ratio of 1:3 is suitable for the xylanase production by both of the test strains. It is seen that both glucose and lactose have repressed the xylanase production by *C. disseminatus* SW-1 and SW-2 as a result of catabolite repression. The crude xylanase produced by both of the strains has been biochemically characterized by checking their pH and temperature stability. It is found that the crude xylanase produced by *C. disseminatus* SW-1 is more thermo and alkali-tolerant in comparison to that produced by *Coprinellus disseminatus* SW-2. Hence white rot strain *C. disseminatus* SW-1 has been chosen for further biobleaching studies. The xylanase produced by *C. disseminatus* SW-1 following characterization is found to be a 43 kDa protein. The mass production of the crude xylanase produced by *C. disseminatus* SW-1 has been carried out under SSF condition for its use in biobleaching studies. The test fungus grows luxuriously and produces a high level of xylanase on the low cast substrate i.e. wheat bran. For commercial applications, the use of cheap agricultural byproducts like wheat bran to replace purified xylan makes it possible to reduce the cost of enzyme considerably. Considering the optimum conditions for xylanase production; it is clear that *Coprinellus disseminatus* SW-1 has a potential role in the development of a bioprocess for the mass production of xylanase using low-cost media.

Table 4.1: Applications of xylanases (34, 66)

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

Table 4.2: Morphological analysis of fungal isolates

Sl. No.	Isolated strains	Source	Mycelia color	Spore color	Zone diameter, mm
1	SW-1	Dead and decaying wood	White	Black	20.2
2	SW-2	Dead and decaying wood	White	Brown	20.2
3	SW-3	Decomposing manure	White	Black	12.7
4	SW-4	Dead and decaying wood	Green	Green	—
5	SW-5	Paper industry waste	White	Brown	11.0
6	SW-6	Paper industry waste	Black	Black	—
7	SW-7	Fruiting body	White	No spore formation	6.2
8	SW-8	Sugarcane dumping site	Yellowish white	No spore formation	—
9	SW-9	Dead and decaying wood	White	Brown	10.8
10	SW-10	Dead and decaying wood	White	Brown	18.3
11	SW-11	Decomposing manure	Dirty white	Black	3.5
12	SW-12	Mango tree bark	Creamish white	No spore formation	1.2

Table 4.3: Enzyme production from the fungal isolates under SSF conditions

Sl. No.	Fungal strain	Xylanase activity, IU/mL	Cellulase activity, IU/mL	Zone diameter, mm
1	SW-1	362.1±10.7	0.64±0.08	20.2
2	SW-2	315.6±7.6	0.88±0.10	20.0
3	SW-3	222.07±8.2	2.96±0.09	12.7
4	SW-5	150.22±9.1	1.21±0.12	11.0
5	SW-7	59.96±4.4	2.22±0.18	6.2
6	SW-9	144.37±7.0	0.92±0.09	10.8
7	SW-10	303.11±8.3	1.03±0.05	19.3
8	SW-11	11.10±4.2	0.185±0.01	3.5
9	SW-12	1.66±1.9	1.48±0.09	1.2

± refers standard deviation

Fermentation conditions:

Wheat bran, g : 5
Nutrient salt solution, mL : 15
pH : 6
Temperature, ^{0}C : 35
Incubation period, days : 8

Table 4.4: Comparison of solid state and submerged fermentation conditions for xylanase production by *C. disseminatus* SW-1 and SW-2

Sl. No.	Fermentation condition	SW-1		SW-2	
		Xylanase activity, IU/mL	Cellulase activity, IU/mL	Xylanase activity, IU/mL	Cellulase activity, IU/mL
1	Submerged fermentation	196.3±11.3	0.28±0.02	166.4±8.6	0.38±0.04
2	Solid state fermentation	370.2±9.2	0.64±0. 05	320.6±12.5	0.81±0.05

± refers standard deviation

Fermentation conditions :

Submerged fermentation:

Wheat bran, g : 0.8
Nutrient salt solution, mL : 40
pH : 6
Temperature, ^{0}C : 35
Incubation period, days : 8

Solid state fermentation:

Wheat bran, g : 5
Nutrient salt solution, mL : 15
pH : 6
Temperature, ^{0}C : 35
Incubation period, days : 8

Table 4.5: Optimization of incubation period for xylanase production by *C. disseminatus* SW-1

Sl. No.	Day	Xylanase activity, IU/mL	Cellulase activity, IU/mL	Protein concentration, mg/mL
1	2	8.9±2.1	0.074±0.002	0.09±0.005
2	3	23.9±2.5	0.179±0.030	0.38±0.032
3	4	67.41±5.7	0.334±0.035	0.94±0.050
4	5	139.11±8.4	0.389±0.010	1.60±0.080
5	6	319.5±12.7	0.490±0.012	2.70±0.140
6	7	401.82±10.3	0.660±0.030	3.50±0.260
7	8	367.83±12.3	0.69±0.020	3.56±0.150
8	9	312.8±9.8	0.465±0.010	3.55±0.200
9	10	261.2±9.22	0.386±0.025	3.51±0.100
10	11	222.4±10.6	0.302±0.013	3.47±0.170

± refers standard deviation

Fermentation conditions:
Wheat bran, g : 5
Nutrient salt solution, mL : 15
pH : 6
Temperature, ^{0}C : 35

Table 4.6: Optimization of incubation period for xylanase production by *C. disseminatus* SW-2

Sl. No.	Day	Xylanase activity, IU/mL	Cellulase activity, IU/mL	Protein concentration, mg/mL
1	2	4.23±2.1	0.09±0.004	0.03±0.001
2	3	13.54±1.9	0.21±0.020	0.23±0.020
3	4	48.23±5.6	0.38±0.015	0.49±0.050
4	5	80.92±7.8	0.56±0.034	1.02±0.140
5	6	223.06±8.5	0.68±0.050	1.79±0.200
6	7	341.73±13.8	0.79±0.017	2.60±0.200
7	8	306.24±11.7	0.74±0.030	2.62±0.170
8	9	276±9.7	0.73±0.050	2.59±0.350
9	10	168.54±15.02	0.51±0.032	2.46±0.210
10	11	154.4±13.3	0.39±0.020	2.42±0.100

± refers standard deviation

Fermentation conditions:
Wheat bran, g : 5
Nutrient salt solution, mL : 15
pH : 6
Temperature, ^{0}C : 35

Table 4.7: Optimization of incubation temperature for xylanase production by *C. disseminatus* SW-1 and SW-2

Sl. No.	Temperature, ^{0}C	SW-1		SW-2	
		Xylanase activity, IU/mL	Cellulase activity, IU/mL	Xylanase activity, IU/mL	Cellulase activity, IU/mL
1	27	17.66±3.5	0.18±0.030	21.45±2.9	0.27±0.046
2	32	132.9±10.8	0.39±0.020	111.46±9.3	0.50±0.040
3	37	428.51±15.4	0.71±0.080	377.2±14.21	0.82±0.100
4	42	345.08±10.9	0.26±0.040	133.6±9.7	0.23±0.040
5	47	210.8±7.6	0.13±0.018	21.6±6.5	0.08±0.012
6	52	43.8±7.9	0.05±0.003	Growth inhibited	Growth inhibited

± refers standard deviation

Fermentation conditions:
Wheat bran, g : 5
Nutrient salt solution, mL : 15
pH : 6
Incubation period, days : 7

Table 4.8: Optimization of initial pH for xylanase production by *C. disseminatus* SW-1 and SW-2

Sl. No.	pH	SW-1		SW-2	
		Xylanase activity, IU/mL	Cellulase activity, IU/mL	Xylanase activity, IU/mL	Cellulase activity, IU/mL
1	4.6	176.8±9.3	0.97±0.09	165.6±15.2	1.20±0.07
2	5.2	295.53±12.1	0.86±0.08	187.96±8.5	1.04±0.05
3	5.8	347.13±10.6	0.85±0.02	257.1±6.6	0.97±0.08
4	6.4	441.5±16.4	0.76±0.04	387.84±9.0	0.92±0.05
5	7.0	401.01±13.72	0.62±0.04	225.93±10.5	0.83±0.04
6	7.6	386±8.3	0.53±0.02	248.62±12.1	0.65±0.04
7	8.2	336.73±9.5	0.60±0.06	254.46±8.8	0.61±0.03
8	8.8	228.5±7.3	0.48±0.01	225.4±7.2	0.55±0.06
9	9.4	227±12.6	0.42±0.03	195.7±10.4	0.49±0.02
10	10.0	154.7±6.8	0.21±0.015	220.5±13.0	0.37±0.04
11	10.6	188.5±7.6	0.06±0.001	194.7±9.7	0.20±0.02
12	11.2	189.7±9.4	0.05±0.001	145.84±8.6	0.09±0.003

± refers standard deviation

Fermentation conditions:
Wheat bran, g : 5
Nutrient salt solution, mL : 15
Temperature, ^{0}C : 37
Incubation period, days : 7

Table 4.9: Effect of carbon source on xylanase production by *C. disseminatus* SW-1 and SW-2

Sl. No.	Carbon source	SW-1		SW-2	
		Xylanase activity, IU/mL	Cellulase activity, IU/mL	Xylanase activity, IU/mL	Cellulase activity, IU/mL
1	Bagasse	19.36±3.9	0.09±0.001	12.08±2.1	0.13±0.008
2	Wheat straw	32.86±2.5	0.20±0.020	54.64±7.3	0.33±0.020
3	Rice bran	124.03±9.7	0.32±0.020	104.11±9.2	0.47±0.010
4	Wheat bran	469.45±19.3	0.73±0.050	384.4±11.7	0.85±0.070
5	Bagasse+wheat straw	34.1±3.6	0.16±0.010	52.93±5.8	0.39±0.025
6	Bagasse+rice bran	62.81±5.5	0.35±0.040	35.6±2.6	0.27±0.050
7	Bagasse+wheat bran	134.67±10.2	0.41±0.032	106.9±9.1	0.54±0.03
8	Wheat straw+rice bran	72.63±4.4	0.34±0.060	56.13±6.3	0.40±0.06
9	Wheat straw+wheat bran	105.9±7.6	0.29±0.020	151.7±8.7	0.58±0.05
10	Rice bran+wheat bran	244.64±12.2	0.37±0.030	198.13±15.2	0.65±0.04

± refers standard deviation

Fermentation conditions:

Nutrient salt solution, mL : 15
pH : 6.4
Temperature, ^{0}C : 37
Incubation period, days : 7

Table 4.10: Effect of nitrogen source for xylanase production by *C. disseminatus* SW-1 and SW-2

Sl. No.	Nitrogen source	SW-1		SW-2	
		Xylanase activity, IU/mL	Cellulase activity, IU/mL	Xylanase activity, IU/mL	Cellulase activity, IU/mL
1	Yeast extract	458.5±18.5	0.72±0.051	380.08±10.2	0.82±0.020
2	Peptone	354.88±13.7	0.50±0.020	392.23±8.5	0.90±0.021
3	Malt extract	387.2±8.8	0.65±0.025	355.36±13.3	0.88±0.011
4	Soya bean meal	478.5±21.3	0.79±0.030	304.4±9.70	0.63±0.020
5	Beef extract	388.7±15.5	0.70±0.050	318.7±12.0	0.76±0.020

± refers standard deviation

Fermentation conditions:

Wheat bran, g : 5
Nutrient salt solution, mL : 15
pH : 6.0
Temperature, ^{0}C : 37
Incubation period, days : 7

Table 4.11: Optimization of solid substrate: moisture content for xylanase production by *C. disseminatus* SW-1 and SW-2

Sl. No.	Solid substrate: moisture content	SW-1		SW-2	
		Xylanase activity, IU/mL	Cellulase activity, IU/mL	Xylanase activity, IU/mL	Cellulase activity, IU/mL
1	1:2.0	257±10.2	0.52±0.040	233.42±8.3	0.65±0.020
2	1:2.5	359.01±14.7	0.75±0.043	319±12.1	0.84±0.025
3	1:3.0	479.7±8.3	0.80±0.020	404.3±17.3	0.96±0.040
4	1:3.5	319.8±9.6	0.50±0.028	256.9±10.11	0.80±0.029
5	1:4.0	235.2±15.5	0.42±0.020	181.36±8.9	0.44±0.030

± refers standard deviation

Fermentation conditions:

Wheat bran, g : 5
pH : 6.4
Temperature, ^{0}C : 37
Incubation period, days : 7

Table 4.12: Derivation of various physico-chemical parameters for xylanase production by *C. disseminatus* SW-1 and SW-2

Sl. No.	Parameters	SW-1	SW-2
1	Incubation period, days	7	7
2	Incubation temperature, ^{0}C	37	37
3	Carbon source	Wheat bran	Wheat bran
4	Nitrogen source	Soya bean meal	Peptone
5	pH	6.4	6.4
6	Solid substrate: moisture content	1:3	1:3

Table 4.13: Production of extracellular enzymes by *C. disseminatus* SW-1 and SW-2 under optimized conditions

Strain	Xylanase activity, IU/mL	Cellulase activity, IU/mL	Laccase activity, IU/mL	Protein concentration, mg/mL
SW-1	499.60±9.3	0.86±0.05	25.5±5.7	4.7±0.50
SW-2	410.21±7.6	1.02±0.09	14.3±4.4	3.2±1.40

± refers standard deviation

Table 4.14: Effect of glucose on xylanase production by *C. disseminatus* SW-1 and SW-2

Sl. No.	Glucose concentration, g/L	SW-1	SW-2
		Xylanase activity, IU/mL	Xylanase activity, IU/mL
1	0	482.28±15.2	396.90±10.0
2	1	341.2±10.01	220.86±8.30
3	2	325.9±13.85	213.91±11.5
4	3	306.4±9.40	170.84±7.86
5	4	268.95±8.9	183.13±14.31
6	5	244.2±11.0	151.53±9.9

± refers standard deviation

Fermentation conditions:

Wheat bran, g : 5
Nutrient salt solution, mL : 15
pH : 6.4
Temperature, ^{0}C : 37
Incubation period, days : 7

Table 4.15: Effect of lactose on xylanase production by *C. disseminatus* SW-1 and SW-2

Sl. No.	Lactose concentration, g/L	SW-1	SW-2
		Xylanase activity, IU/mL	Xylanase activity, IU/mL
1	0	460.83±11.5	386.61±7.1
2	1	372.8±10.3	294.30±13.7
3	2	301.1±15.5	224.40±9.9
4	3	278.68±7.81	220.33±8.6
5	4	257±6.7	197.65±12.3
6	5	243.1±10.33	173.37±8.4

± refers standard deviation

Fermentation conditions:

Wheat bran, g : 5
Nutrient salt solution, mL : 15
pH : 6.4
Temperature, ^{0}C : 37
Incubation period, days : 7

Table 4.16: pH stabilization of xylanase produced by *C. disseminatus* SW-1 and SW-2

Sl. No.	pH	SW-1	SW-2
		Xylanase activity, IU/mL	Xylanase activity, IU/mL
1	6.0	377.10±7.6	292.60±10.6
2	6.4	479.60±11.1	389.80±8.52
3	7.0	384.8±8.9	234.31±12.9
4	7.4	303.11±6.1	166.55±8.6
5	7.6	255.41±10.7	145.73±4.6
6	8.0	120.77±9.0	59.39±9.4
7	8.4	71.3±8.1	25.53±3.7
8	9.0	40.33±6.6	12.43±5.2

± refers standard deviation

Assay conditions:
pH of buffer : 6.4
Substrate concentration for xylanase activity : 10 mg/mL potassium phosphate buffer

Table 4.17: Temperature stabilization of xylanase produced by *C. disseminatus* SW-1 and SW-2

Sl. No.	Temperature, ^{0}C	Time, min	SW-1	SW-2
			Xylanase activity, IU/mL	Xylanase activity, IU/mL
1	55	30	484.21±13.4	395.2±9.0
2	65	30	240.6±9.3	136.22±7.3
3	75	30	63.87±5.2	25.0±4.1
4	85	30	48.81±5.5	10.4±2.6

± refers standard deviation

Assay conditions:
pH of buffer : 6.4
Substrate concentration for xylanase activity : 10 mg/mL potassium phosphate buffer

Table 4.18: Mass production of extracellular enzymes by *C. disseminatus* SW-1 under optimized conditions

Strain	Xylanase activity, IU/mL	Cellulase activity, IU/mL	Laccase activity, IU/mL	Protein concentration, mg/mL
SW-1	522.21±12.70	0.93±0.072	21.6±4.8	4.4±1.25

± refers standard deviation

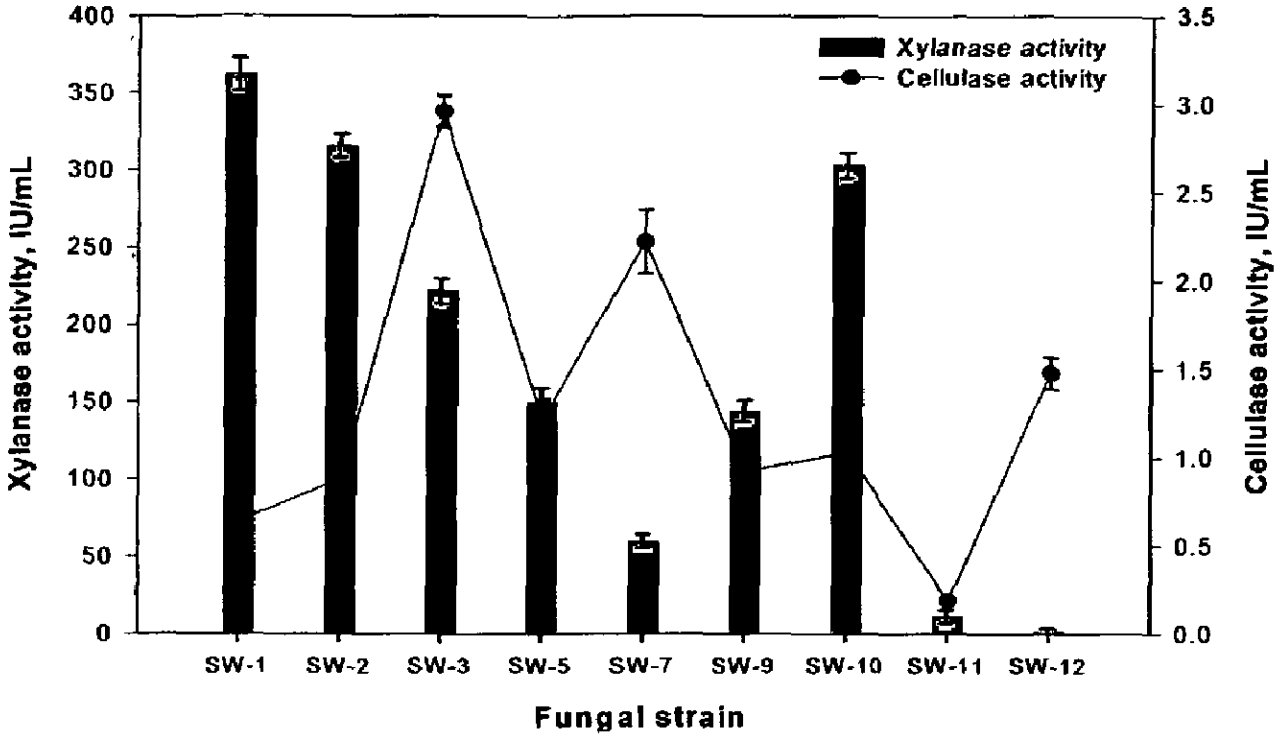

Figure 4.2: Enzyme production from the fungal isolates under SSF conditions

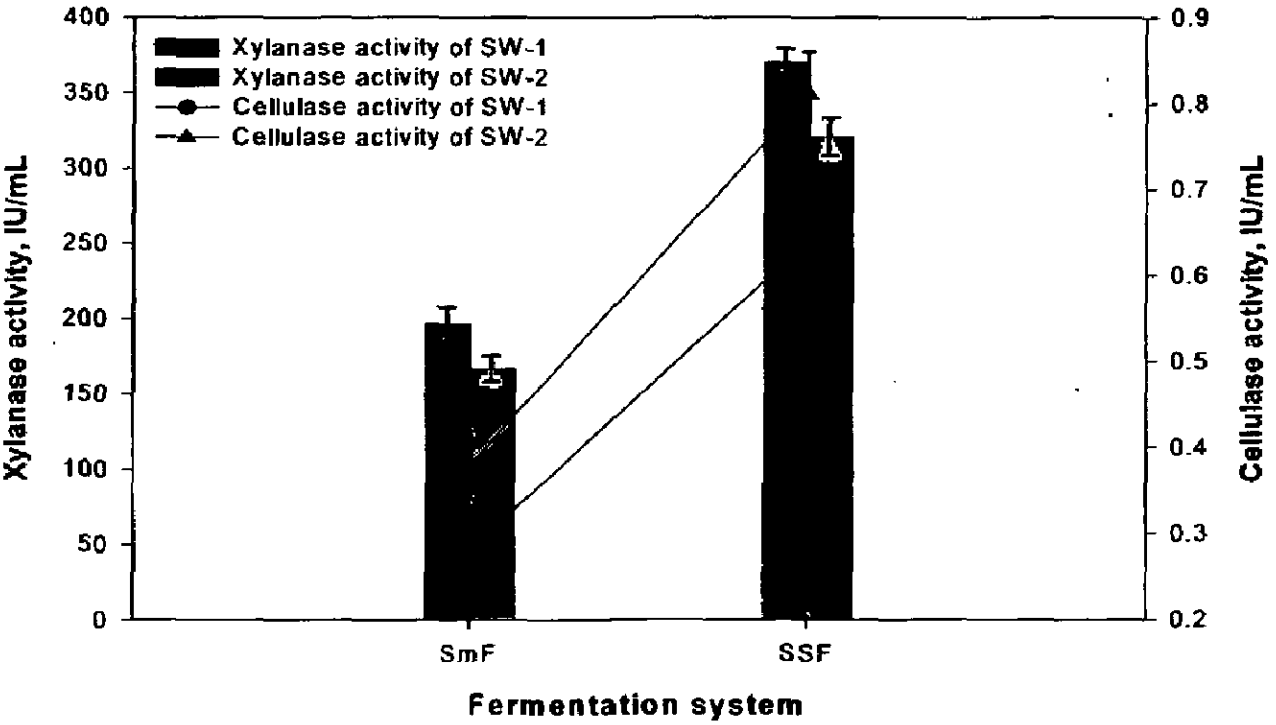

Figure 4.3: Comparison of fermentation systems for the enzyme production by *C. disseminatus* SW-1 and SW-2

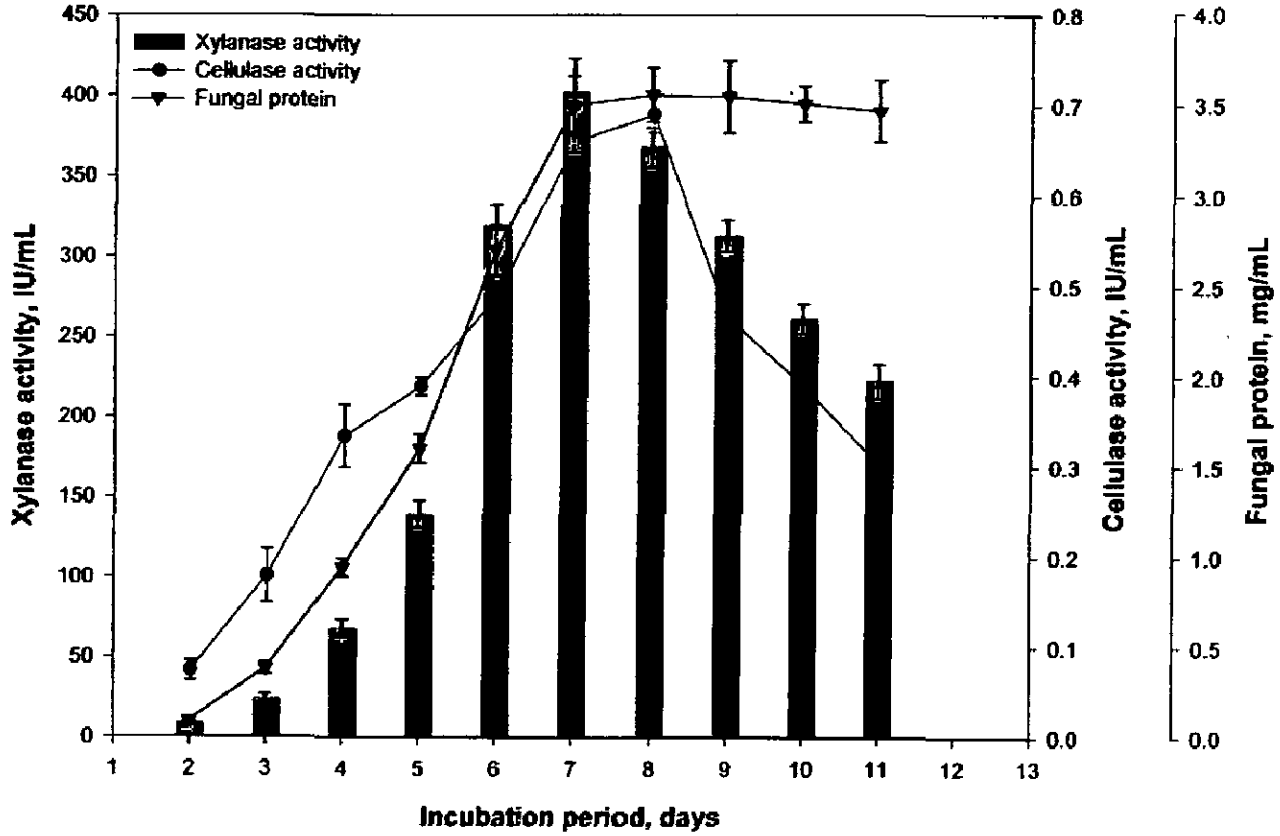

Figure 4.4: Optimization of incubation period for xylanase production by *C. disseminatus* SW-1

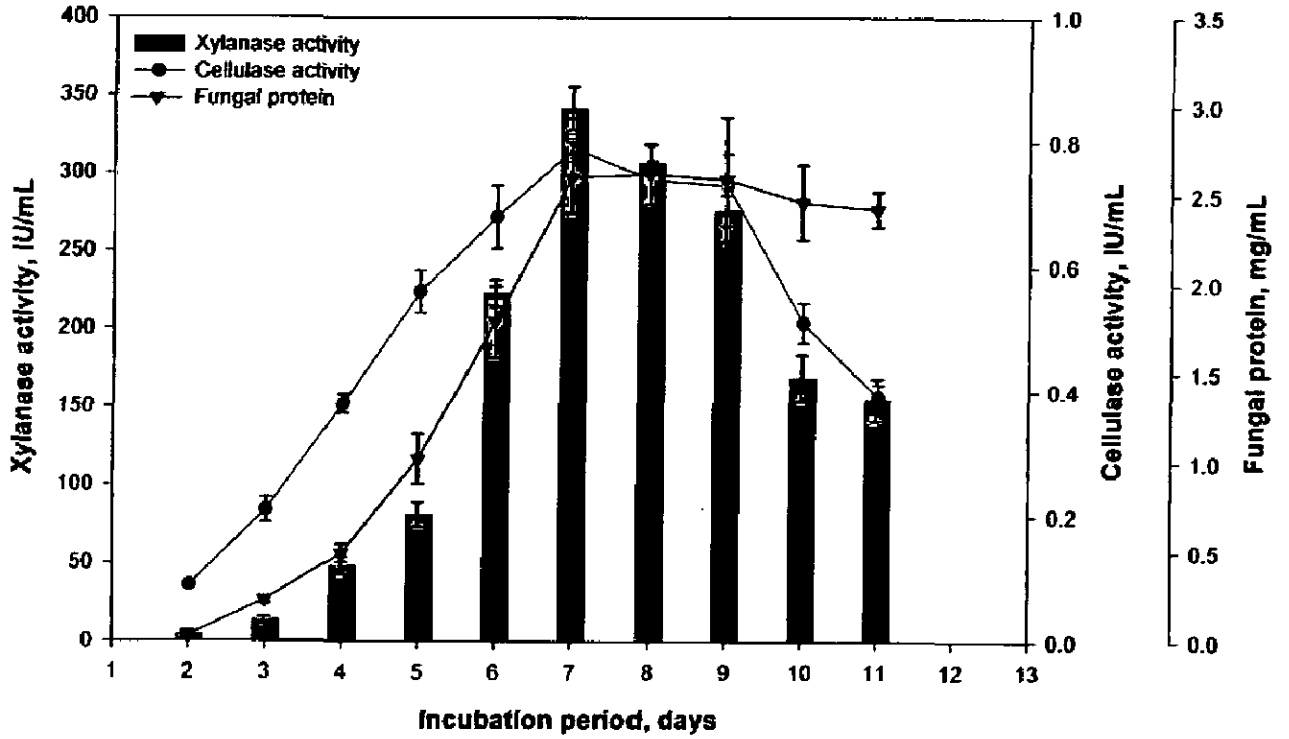

Figure 4.5: Optimization of incubation period for xylanase production by *C. disseminatus* SW-2

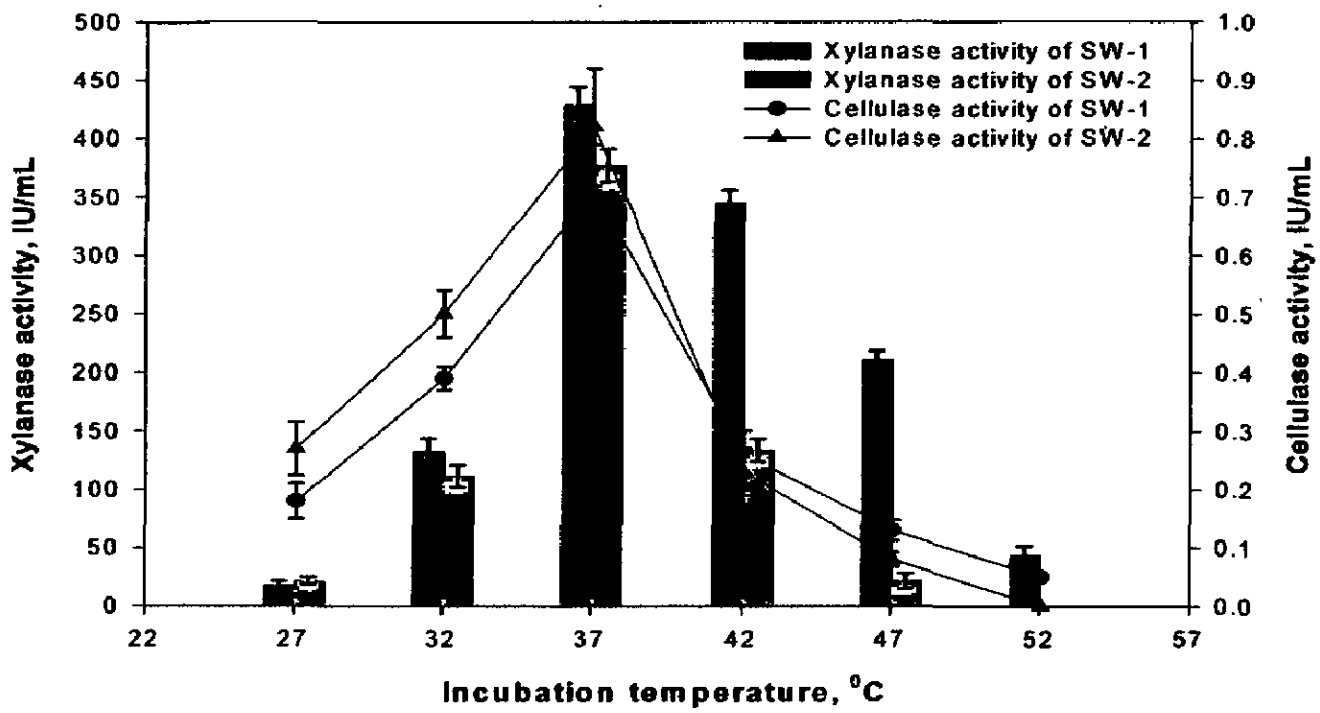

Figure 4.6: Optimization of incubation temperature for xylanase production by *C. disseminatus* SW-1 and SW-2

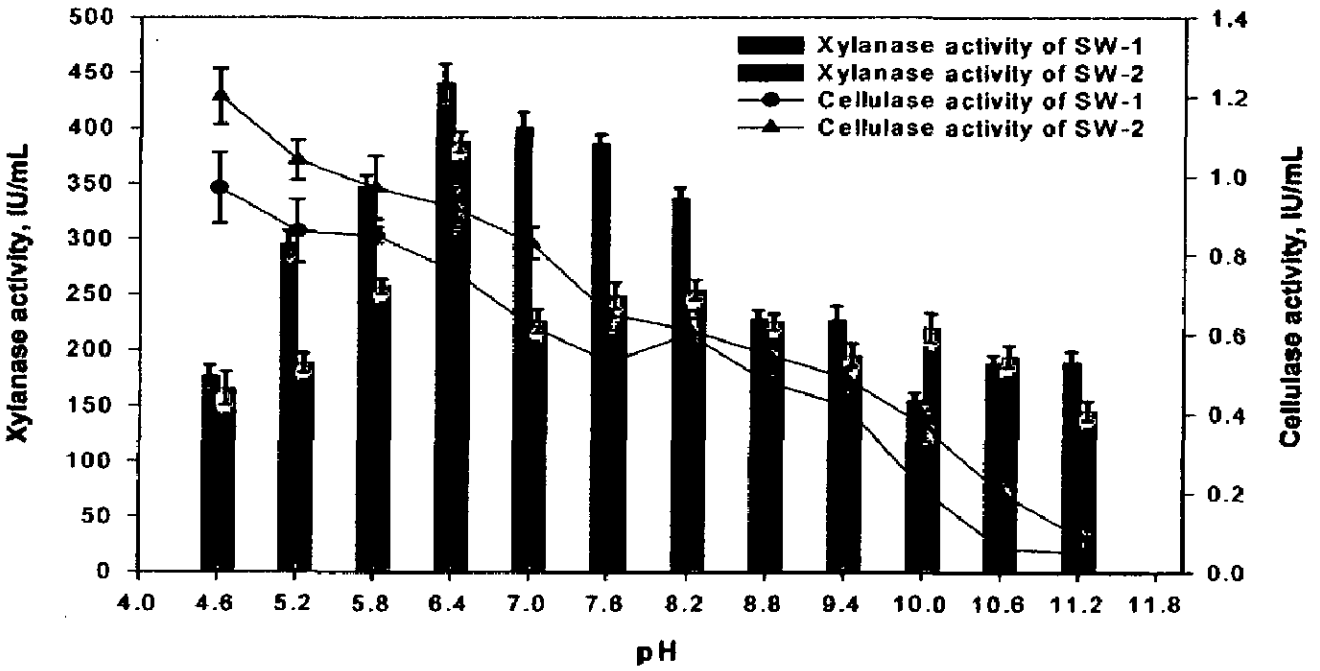

Figure 4.7: Optimization of initial pH for xylanase production by *C. disseminatus* SW-1 and SW-2

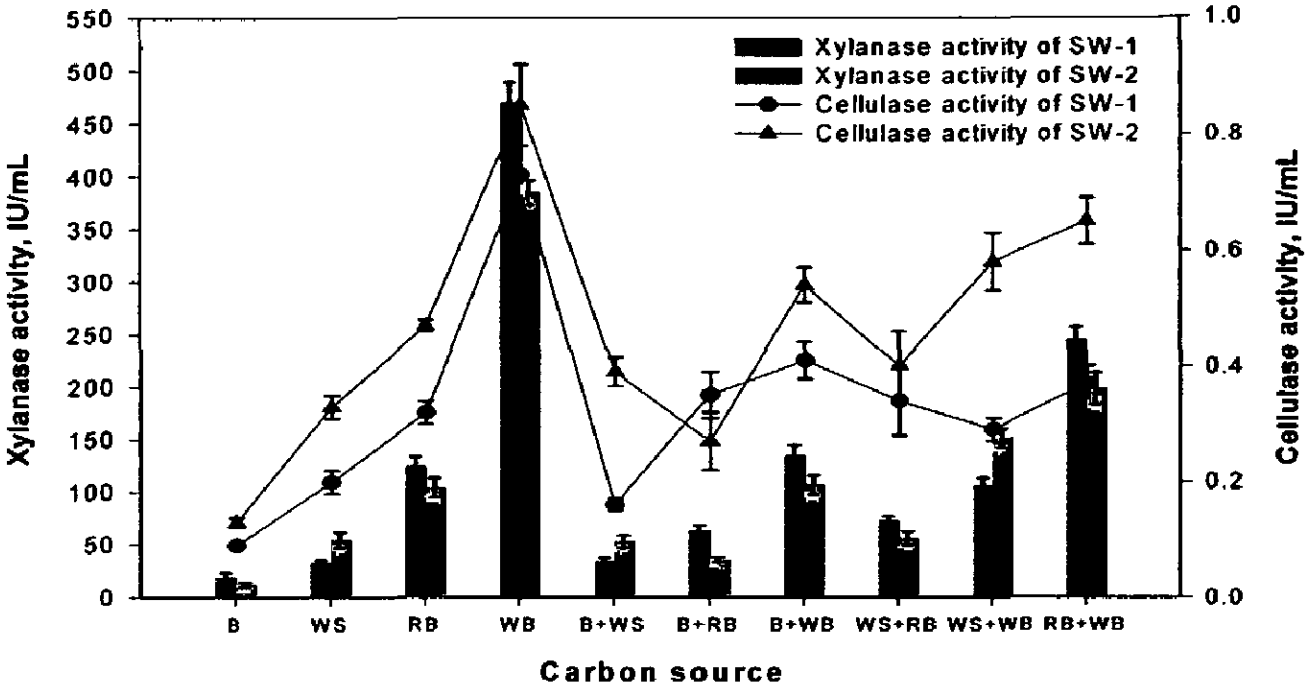

Figure 4.8: Effect of carbon source on xylanase production by *C. disseminatus* SW-1 and SW-2 (B: Bagasse, WS: Wheat straw, WB: Wheat bran, RB: Rice bran)

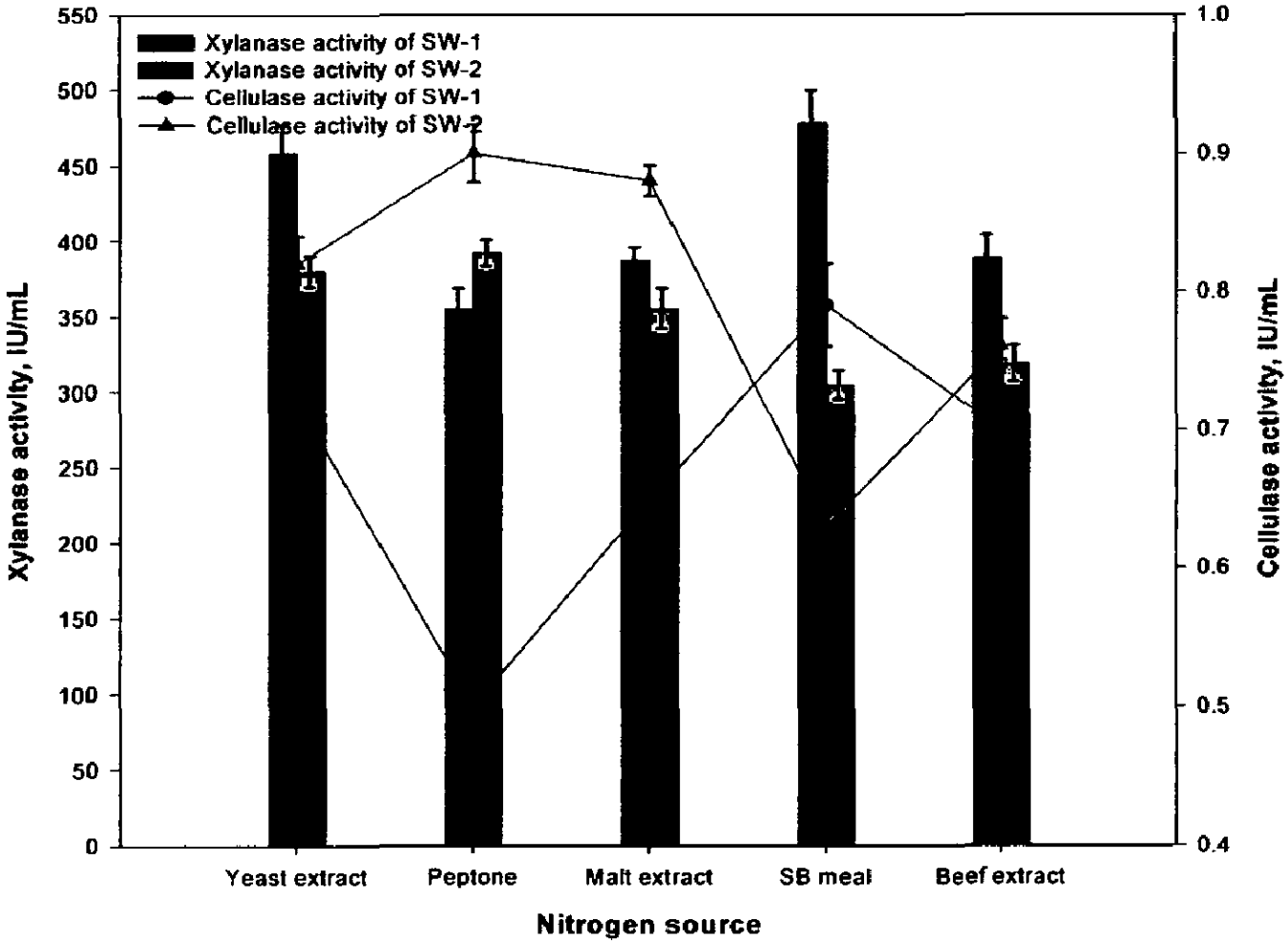

Figure 4.9: Effect of nitrogen source on xylanase production by *C. disseminatus* SW-1 and SW-2

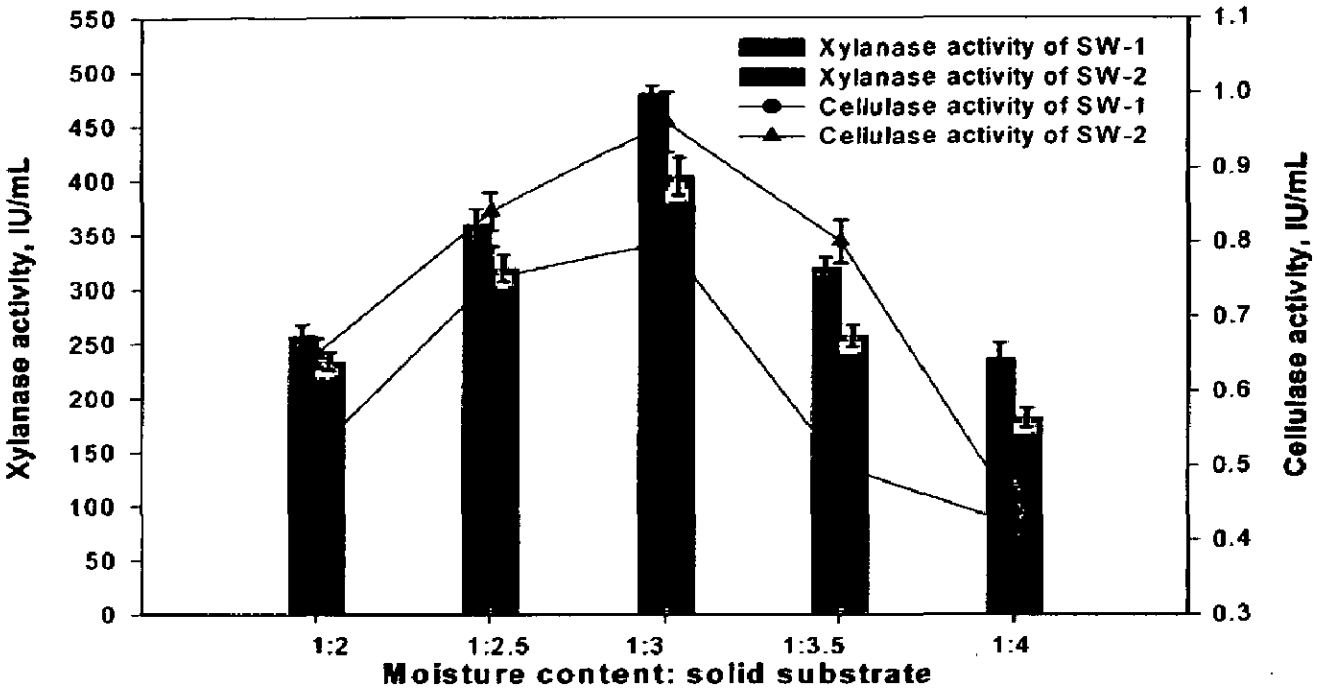

Figure 4.10: Optimization of moisture content for xylanase production by *C. disseminatus* SW-1 and SW-2

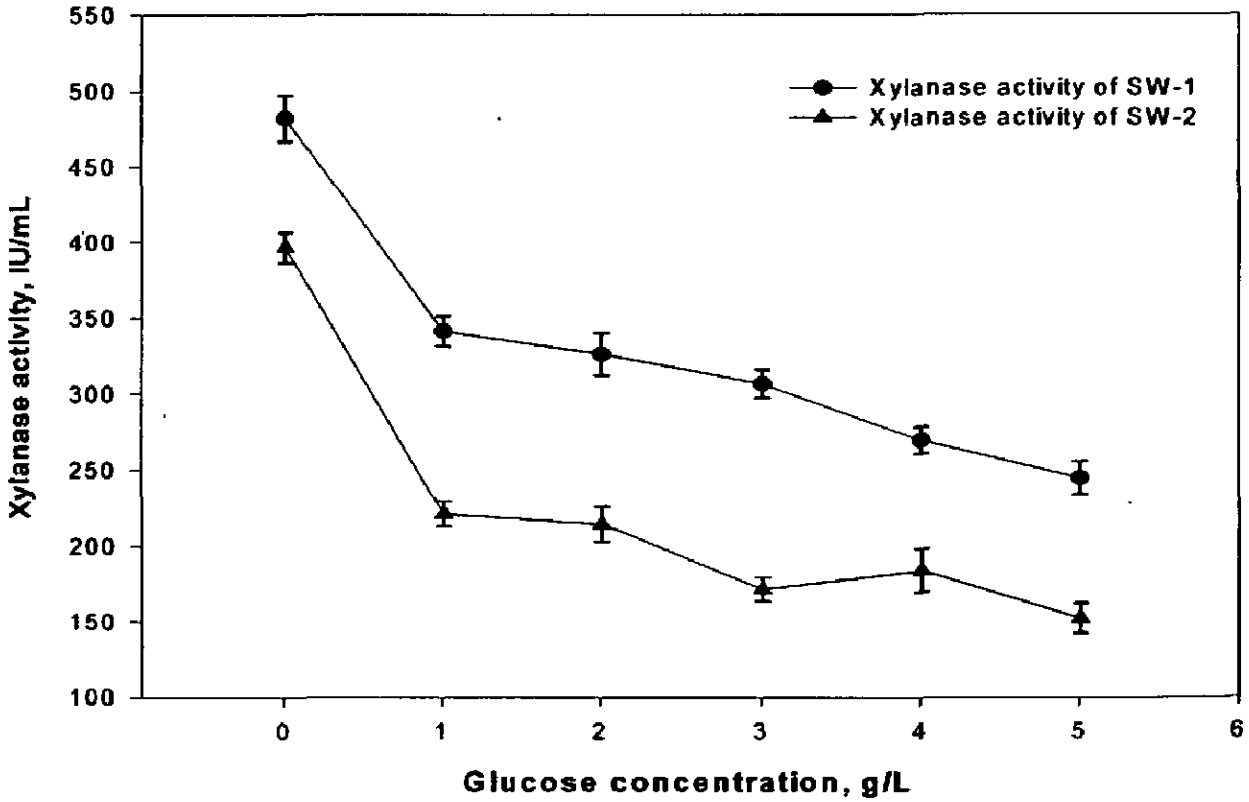

Figure 4.11: Effect of glucose concentration on crude xylanase production by *C. disseminatus* SW-1 and SW-2

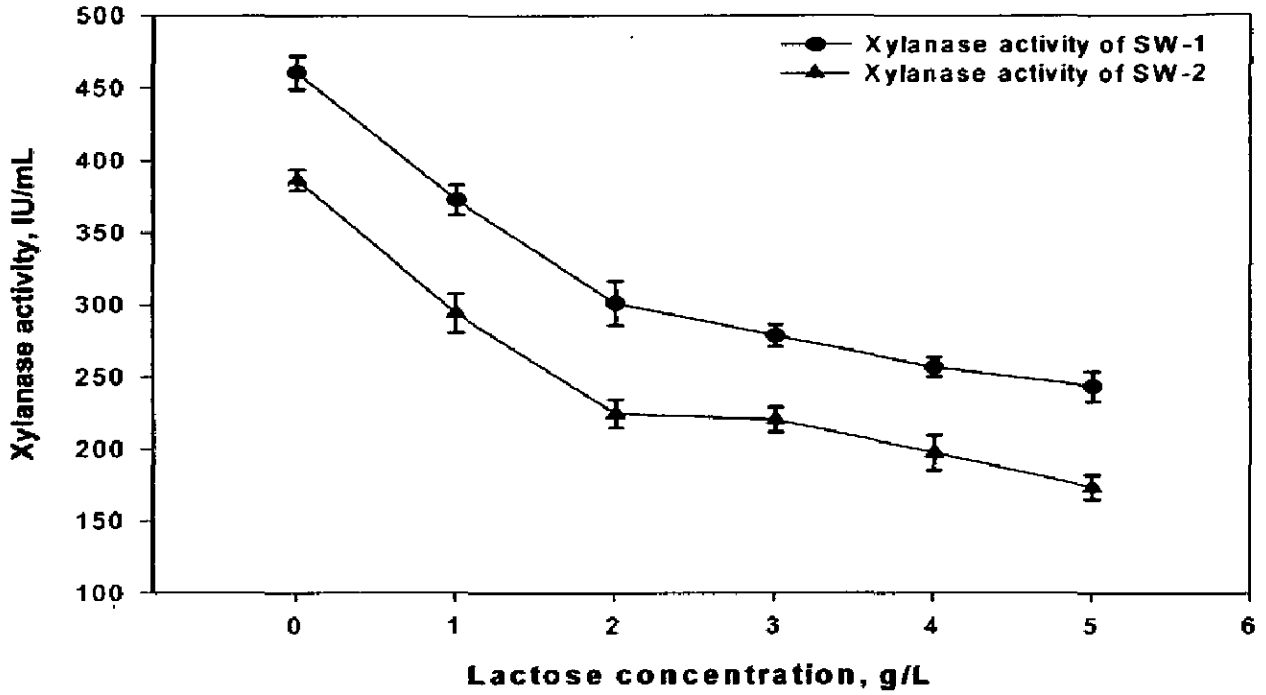

Figure 4.12: Effect of lactose concentration on xylanase production by *C. disseminatus* SW-1 and SW-2

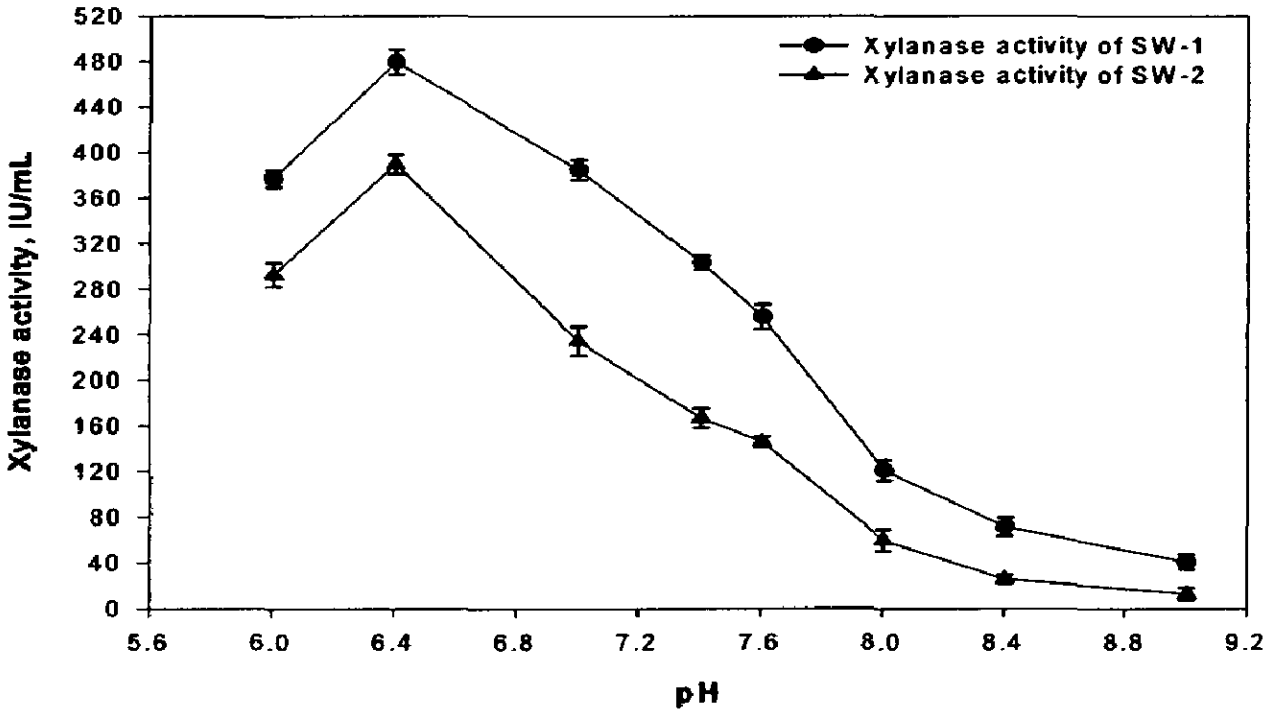

Figure 4.13: pH stability of xylanase produced by *C. disseminatus* SW-1 and SW-2

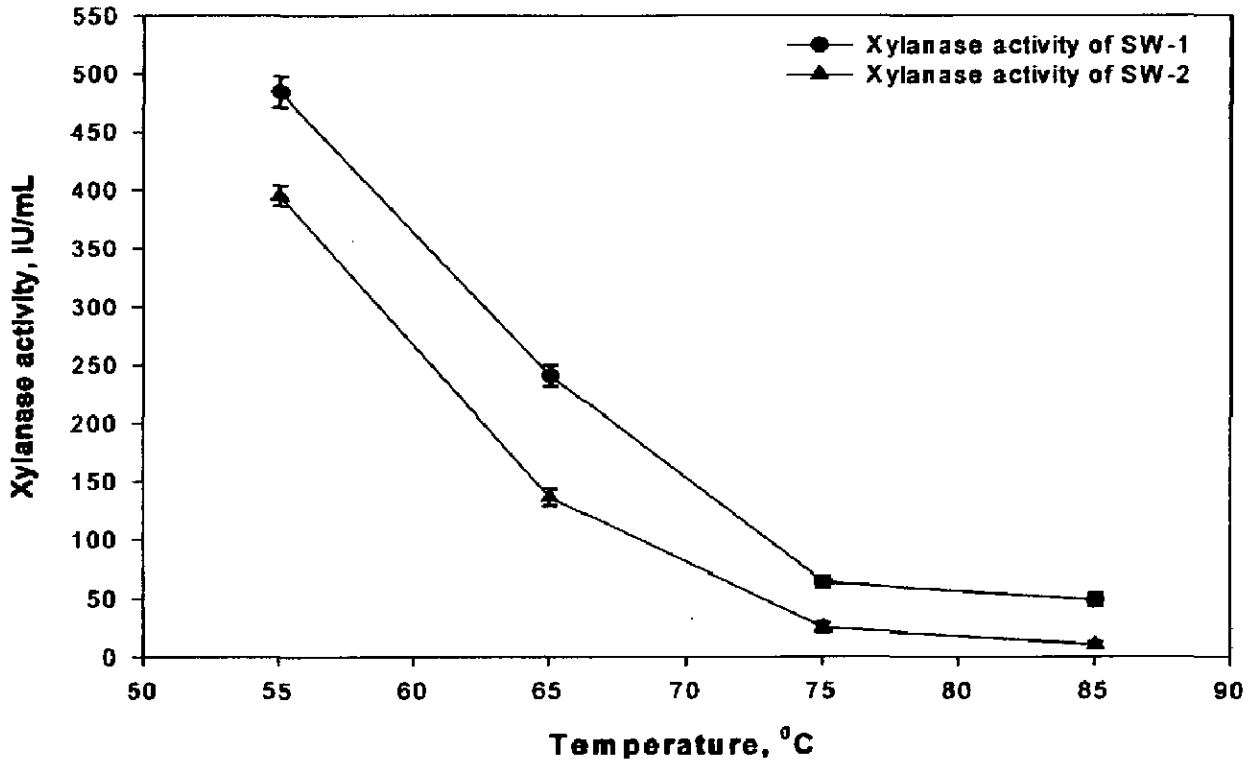

Figure 4.14: Temperature stability of xylanase produced by *C. disseminatus* SW-1 and SW-2

CHAPTER 5

BLEACHING STUDIES ON SUGARCANE BAGASSE SODA-AQ PULP

CHAPTER-5

BLEACHING STUDIES ON SUGARCANE BAGASSE SODA-AQ PULP

5.1 INTRODUCTION

Bleaching is a chemical process applied to cellulosic materials to increase their brightness. Brightness is the reflectance of visible light from cellulosic cloth or pulp fibers formed into sheet. It also serves the purpose of purifying pulp, thereby extending its application, increasing its stability, and enhancing some of its properties. Bleaching is also effective in removing unwanted particles that contaminate pulp fibers. Bleaching processes increase brightness by lignin removal or lignin decolorization. The chemicals commonly used for pulp bleaching include oxidants (e.g., chlorine, chlorine dioxide, oxygen, ozone and hydrogen peroxide), alkali (sodium hydroxide), and for mechanical pulp bleaching only, a reducing agent, sodium hydrosulfite (sodium dithionite) and hydrogen peroxide (H_2O_2). These chemicals are mixed with pulp suspensions and the mixture is retained at prescribed pH, temperature, and concentration conditions for a specified time period (78). The progress of bleaching reactions is monitored by measuring pulp lignin content, pulp brightness, and residual chemical. Bleaching chemicals are frequently applied sequentially with intermediate washing between treatments (stages), because it is not possible to achieve sufficient removal or decolorization of lignin by the action of any one chemical in a single treatment or stage. The principal objective of pulp bleaching

is to achieve a high brightness with secondary objectives, depending upon the end uses e.g. high brightness stability, high pulp cleanliness (freedom from colored particles), and a high cellulose content. These objectives must be achieved without compromising the strength of the final product; cellulose degradation during bleaching can lead to significant loss of strength in product paper sheets (78).

In India 181 mills are agro based and 28 mills are wood based. The kappa number of wood based mills vary from 14-25 and kappa number of agro based mills vary from 18-30. Most of the mills in India are using CEH, CEHH and CHH bleaching sequences. The bleaching of pulp with chlorine and chlorine based chemicals generate various chlorinated compounds (58, 138) which include chlorinated phenolics, chlorinated resin and fatty acids, dioxins and furans which are found to have high toxic effects on receiving environment. The adsorbable organic halide (AOX), extractable organic halide (EOX) and purgeable organic halide (POX) are used normally to indicate the level of organochlorine compounds present in bleaching plant effluents.

The chlorine consumption of agro based and wood based mills is 130-200 and 60-100 kg/t of pulp. It is estimated that production of 1 tonne of pulp is reported to contribute about 100 kg of color imparting substances and 2-4 kg of organochlorines to bleach plant effluents (224). The AOX range in final discharge of agro based mills with and without chemical recovery process is 7-11 and 14.2-21.5 mg/L. Where, AOX in final discharge of wood based mills is 0.60-9 mg/L. It was found out that the C stage was generally the point in which 2,3,7,8-TCDD, 2,3,7,8- TCDF congeners were always present (334, 326, 163).

The extraction (E) stage filtrate was found to have the highest concentration of dioxins (295) known as changing the blood chemistry and causing lever damage, skin disorders, lung lesions and tumor types at numerous sites within the body, liver and thyroid included (258, 259, 156). Hence, bleach plants are considered to be the major source of polluting discharges accounting for 60-70% of BOD and 80-90% color load of the entire mill (with chemical recovery) (275).

Modification of bleaching process is generally the least costly and simplest means of reducing environmental load of bleach plants (182). With the replacement of bleaching sequences using elemental chlorine (Cl_2) by chlorine dioxide (ClO_2), the pulp and paper industry has reduced considerably the formation and discharge of chlorinated organic material into the aquatic environment (330). The introduction of elemental chlorine free (ECF) process has resulted in a decrease of AOX in kraft bleaching ranging from 48% to 65% (162). Moreover, since 1990, total chlorine free (TCF) bleaching has been introduced, largely in response to market demands for non-chlorine bleached pulp (162). Modern bleaching sequences of both ECF and TCF types can meet the tough environmental demands of best available technology (BAT) set up by European Commission (272).

ECF bleaching, based on ClO_2, offers a number of fundamental benefits over the traditional methods. ClO_2 may be a better bleaching agent than Cl_2. ECF proponents say that ClO_2 is 2.5 times as powerful an oxidizer as Cl_2, and that it preserves cellulose and attacks lignin more selectively. Therefore, pulp produced by ClO_2 bleaching is brighter and stronger than that produced by Cl_2 bleaching itself (271). By substituting chlorine dioxide at levels of 70 to 100%,

mills can apparently reduce the level of chlorinated organics found in mill effluent by 80 to 90% and reduce dioxins to "non-detect" levels. In fact ClO_2 is a superior bleaching agent and will therefore have a prominent place in production of high brightness and high quality pulp during the next decades (271). ClO_2 substitution decreases effluent color in proportion to the percent substitution; one estimate is that a 1% decrease in color occurs for each 2% increase in percent substitution (202, 63, 203). BOD changes very little up to 90% ClO_2 substitution and then declines slightly thereafter. Effluent COD decreases approximately 10% as ClO_2 substitution increases from 0-100% although there is considerable scatter in data (202, 203, 182). The addition of ClO_2 in the first stage also helps in reducing the toxicity of the effluent by virtually eliminating dioxins and 12 priority chlorophenols proposed by the U.S. Environmental Protection Agency (EPA) for regulation to non-detect levels (23) The other benefits of ECF bleaching are that it decreases the chloroform formation and total chlorinated organic compound (AOX) by 90%; efficiently utilizes forest resources; contributes to eco-system recovery; and is compatible with emerging minimum-impact mill technologies (264). Therefore, the environmental regulations have made the use of ClO_2 important and moreover it is economic too, even if total replacement of Cl_2 by ClO_2 is done. For bagasse pulp the replacement of greater percentage of Cl_2 for the equivalent of ClO_2 causes the greater brightness, greater viscosity, lesser *brightness reversion, greater strengths and a light increment in the yield* (385).

For any bleaching method, environmental and/or economic considerations demand that the lignin content of the pulp should be reduced as much as possible before bleaching. Molecular oxygen in alkaline medium allows extended

delignification of chemical pulps without a serious loss in pulp yield and with positive environmental impacts in presence of carbohydrate stabilizer i.e. Epsom salt ($MgSO_4$) (285, 109, 220). An oxygen stage before bleaching stage reduces the doses of bleaching chemicals and the NaOH required in the first extraction stage, roughly in proportion to the delignification achieved in the oxygen stage. Oxygen delignification (ODL) is used to reduce the value of kappa number of the pulp by only 50% without affecting much, the viscosity of pulp (252, 38, 64), because an attempt in greater reduction in kappa number is expected to lead to unexpected degradation of carbohydrates in the pulp and loss of pulp strength (41). However, ODL does not affect the pulp yield as negatively as other methods of extended cooking do. The chemicals applied to the pulp in the oxygen stage and the resultant reaction products are removed and sent to chemical recovery process. ODL decreases the formation of chloroorganics (AOX) in bleach plant effluents when chlorine-based chemicals are used in subsequent bleaching of the pulp. Regardless, of the bleaching chemicals used, ODL decreases the BOD, COD and color of the effluents (348).

As an oxygen based chemical, hydrogen peroxide (H_2O_2) could have a major role in ECF, TCF and TEF (total effluent free) bleaching strategies of chemical and mechanical pulps (143, 358, 265, 79), being used both as an alkaline bleaching stage and as reinforcement for alkaline extraction. H_2O_2 acts as a true bleaching agent. Because of its specific and efficient action on carbonyl and conjugated carbonyl groups, the hydroperoxide anion (HOO^-) can destroy many of chromophoric groups present in pulp, including those created by the other bleach chemicals applied in previous bleaching stages. Compared with

oxygen delignification, H_2O_2 delignification appears to provide better color abatement because of its specific action on chromophores (78). The use of this chemical agent on the bleaching of non-woody chemical pulps has been reported as a successful bleaching agent in TCF bleaching sequences (313, 152, 288, 27, 74, 198). A final peroxide bleaching stage is quite useful to improve pulp brightness stability (233, 344). The effectiveness of this bleaching agent is limited by a poor selectivity, which is reflected by a severe viscosity loss. It is generally admitted that the free radicals generated during the peroxide decomposition are responsible for this cellulose degradation (106). Use of chelating agent (EDTA) and stabilizer ($MgSO_4$) prevents the formation of hydroxyl radicals ($HO^{\cdot}$) as these compounds may form a complex with the transition metals that catalyze the decomposition of peroxide and lower their catalytic activity (30).

Ozone ranks with elemental chlorine in terms of its reactivity towards lignin and is thus capable of delignifying any chemical pulp. The problem in ozone bleaching is the high reactivity of ozone; ozone bleaching is therefore not sufficiently selective and results in lower viscosity and poorer pulp strength properties (276).

In present practice, bleaching of pulp uses large amounts of chlorine based and other chemicals which cause several effluent-based problems in the pulp and paper industries (34). Byproducts from using these chemicals are chlorinated organic substances, some of which are toxic, mutagenic, persistent, bioaccumulating and cause numerous harmful disturbances in biological systems (235). In response to government and environmental protection groups,

paper industries are currently changing practices to minimize the use of chlorine-based chemicals. The available options are oxygen delignification, extended delignification, substitution of ClO_2 for Cl_2, H_2O_2 and ozone (34). But most of these methods involve high capital investment for process change. Thus, an alternative and cost effective method, i.e., use of enzymes, has provided a very simple and economic way to reduce the use of chlorine and other bleaching chemicals. Biobleaching involves using microorganisms and enzymes for bleaching pulps. It relies on the ability of some microorganisms to depolymerize lignin directly and on the use of microorganisms or enzymes that attack hemicellulose and hence favor subsequent depolymerization (141).

The biotechnological approach for pulp bleaching is new and is gaining momentum all over the world. Bleaching with white rot fungi and the enzyme ligninases (314, 16, 368) and hemicellulases (151, 104, 228, 59, 292, 339, 108, 141, 347) has been explored. These enzymes are used commercially for pulp bleaching. The original concept of using hemicellulases to improve bleachability of kraft pulps was first published in 1986 (363; 364). The brightness of the pulp was substantially increased by xylanase treatment, and this improved brightness can be exploited for reduction of chlorine chemical consumption (363; 364, 367). Pretreatment of cellulase-free, neutral xylanase from *Thermomyces lanuginosus* CBS 288.54 improves the brightness of wheat straw pulp by 1.8-7.79% (ISO) (180). Pre-bleaching of wheat straw pulp by crude xylanase produced by *Aspergillus niger* An76 reduces chlorine charge by 20-30%, or increases final brightness by approximately 4-5% (ISO), with improved paper strength properties (386). Currently, the application of xylanases to wood pulp can lead to a substantial reduction (25-40%) in chlorine input to achieve the target

brightness (386, 362, 199, 350, 361, 97, 94). The release of reducing sugars and the release of lignin and phenolic compounds are interrelated phenomena. When xylan is degraded by the xylanase, in addition to xylose, it also results in the release of lignin and phenolic compounds from the pulp fibers that ultimately cause the enhancement in absorbance (λ 237 nm) of pulp free samples compared to the control (151, 97). The strength properties of enzymatically-treated pulps have been reported to be rather similar to those of the reference pulps, both in laboratory scale and in mill trials (363; 364; 365; 241; 255; 161). The treatment of kraft pulp with cellulase free xylanases increases pulp viscosity due to partial hydrolysis of xylan in the pulp (241, 63). Xylanases from *Staphylococcus* sp. SG-13 has been shown to bring about 30% reduction in kappa number of hardwood kraft pulp and 11, 1.8, 10 and 17% increase in brightness, viscosity, tensile index and burst factor, respectively (114). Similarly, biobleaching benefits to different extent have also been reported by Silva *et al.* for xylanases from *Humicola* sp (316). The biotreatment of bagasse pulp with xylanase from thermophilic *Bacillus* sp. NCIM59 results in the reduction of kappa number by 21% and increase in brightness by 2.5%, whereas viscosity of enzyme treated samples has been unaltered (168). The use of xylanase from *S. cyaneus* SN32 has caused increase in tear index and burst factor, which in turn support that enzyme treatment might have rendered strength to the pulp fiber probably due to external fibrillations (228). According to Bajpai, the increase in pulp fibrillation, water retention, restoration of bonding in fibers and increased freeness in fibers are important factors to facilitate enzymatic bleaching action (20).

The use of enzyme enables the amounts of toxic compounds (chlorophenols and other forms of organically bound chlorine) in the spent bleach liquor to be reduced (135, 137, 199, 350, 16) while an increase is noted in effluent COD and color due to the hydrolysis of hemicelluloses (289, 199, 362). At 100% ClO_2 substitution, the extent of AOX reduction in effluent of xylanase pretreated softwood pulp is 40% (306). Effluent COD and BOD are increased by 32.19% and 107.89% after eucalyptus kraft pulp was pretreated by commercial xylanase while AOX is reduced by 15.09% after same treatment in comparison with control (137). Cellulase free nature, thermostability and pH stability are the prerequisite characteristics of xylanases for their utility in pulp and paper industry. Moreover, xylanases having low molecular weight are often an added advantage, as they can easily penetrate into the re-precipitated xylan on the surface of pulp fibers (228). The use of abundantly available and cost effective agricultural residues, such as wheat bran and other similar agro-wastes to achieve higher xylanase yields using solid state fermentation (SSF) and immobilized cell systems provide suitable means to reduce the manufacturing cost of bio-bleached paper. This in turn also facilitates the adaptation of this environment friendly technology in paper industry (33).

5.2 EXPERIMENTAL METHODOLOGY

5.2.1 Biobleaching of sugarcane bagasse soda-AQ pulp with xylanase produced by C. *disseminatus* SW-1

Various operating parameters for xylanase prebleaching were studied and are summarized here as under:

5.2.1.1 Optimization of xylanase dose, reaction time and pulp consistency

The sugarcane bagasse soda-AQ pulp was treated with varying doses of xylanase i.e. 0-25 IU/g while keeping other variables constant like, consistency 10%, reaction temperature 55 ^{0}C, reaction time 120 min and pH 6.4 and its effect was observed on, kappa number, pulp brightness and reducing sugars released during treatment. Control samples were treated under the same conditions but using buffer in place of xylanase. The treated and untreated pulp samples were filtered through muslin cloth. The filtrate after each xylanase treatment stage was collected for further analysis. The results are reported in Tables 5.1 and 5.2 and Figures 5.1 and 5.2.

In another set of experiment, the sugarcane bagasse soda-AQ pulp was treated with xylanase dose of 8 IU/g at pulp consistency of 10%, temperature 55 ^{0}C and pH 6.4 while varying reaction time from 30 to 240 min in order to observe its effect on kappa number, pulp brightness and reducing sugars released during treatment. The filtrate after each reaction time stage was collected for further analysis. The results are reported in Tables 5.3 and 5.4 and Figures 5.3 and 5.4. The sugarcane bagasse soda-AQ pulp was treated with xylanase dose of 8 IU/g at different pulp consistencies i.e. 2-12% while keeping other variables constant like, reaction temperature 55 ^{0}C, reaction time 120 min and pH 6.4. Kappa number, pulp brightness and reducing sugar released at each consistency stage were determined. The filtrate after each consistency stage was collected for further analysis. The results are reported in Tables 5.5-5.6 and Figures 5.5-5.6. Enzyme mediated release of chromophoric material was monitored in filtrates by measuring absorption spectra at 237, 254, 280 and

465 nm (253, 114). Reducing sugar concentrations in pulp filtrates were determined by dinitrosalisylic (DNS) acid method (210) and expressed as D-xylose equivalents. Xylanase treated pulp samples were followed by alkaline extraction as per conditions mentioned in Table 5.1. The kappa number (T 236 cm-85) of pulp samples was determined as per Tappi Standard Test Method: 2007. Brightness was determined by Technibrite Eric 950 from Technibrite Corporation, USA (343).

5.2.2 Application of xylanase in multi-stage bleaching process

Xylanase pretreated and untreated sugarcane bagasse soda-AQ pulp samples were bleached by multistage conventional (CEHH, CEHHP, OCEHH), ECF (ODED, OD(E_{OP})DP and TCF (O(E_{OP})P bleaching sequences. Unbleached sugarcane bagasse pulp equivalent to 50 g O.D. was taken in polythene bag and bleaching experiments were performed in temperature controlled water bath except chlorination that was performed in air tight plastic bottle at ambient temperature. The pulp and the chemicals were well kneaded at desired pulp consistency by shaking from time to time during bleaching. All bleaching experiments were conducted in triplicates. After each stage, the pulps were washed with water properly and pulp samples were collected for the further analysis.

5.2.2.1 Conventional bleaching

The soda-AQ pulp of sugarcane bagasse was bleached by CEHH; XECEHH, CEHHP; XECEHHP and OCEHH; XOCEHH bleaching sequences, where 'X' stands for xylanase stage, 'C' for chlorination, 'E' for alkaline

extraction, 'H_1' for hypochlorite 1st stage, 'H_2' for hypochlorite 2nd stage, P for hydrogen peroxide (H_2O_2) and 'O' for oxygen bleaching stage. The bleaching conditions and results are reported in Tables 5.7 and 5.8. The xylanase prebleaching stage (X) was conducted at an enzyme dose of 8 IU/g, pH 6.4, pulp consistency 10%, reaction time 120 min and temperature 55 ^{0}C. The bleach liquor was analyzed by taking 10 mL of bleach liquor and diluted it to 5 times with distilled water in Erlenmeyer flask of 250 mL capacity and mixed with 10 mL of 10% KI and 10 mL of 10% acetic acid. The solution was titrated with 0.1 N $Na_2S_2O_3$ using 2-3 drops of freshly prepared starch solution as indicator. The end point turned from blue to colorless (171) indicated the amount of $Na_2S_2O_3$ consumed and can be calculated by using the following formula:

$$\text{Bleach liquor g/L} = \frac{\text{Amount of } Na_2S_2O_3 \text{ consumed (mL)} \times 35.5}{\text{Volume of bleach liquor (mL)}}$$

The total chlorine demand (TCD) was calculated by using the following formula

Total chlorine demand, % = 0.25 X kappa number

Of the total chlorine demand, 50% of the molecular chlorine was charged in 'C' stage and remaining 50% was charged in hypochlorite 1st and 2nd stages respectively i.e. 75% in 'H_1' stage and 25% in 'H_2' stage respectively. The chlorination stage was conducted in sealed plastic bottles with vigorous mixing at the following bleaching conditions: consistency 3%, temperature ambient, pH 1.75 and reaction time 30 min. On the other hand, hypochlorite 1st and 2nd stages were conducted at following bleaching conditions: consistency 10%, temperature 45 ^{0}C, pH 11.5 and reaction time 60 min.

The P stage of CEHHP bleaching sequence was conducted at consistency 10%, temperature 90 ^{0}C, pH 10.7 and reaction time 60 min in polythene bag using 0.5% H_2O_2, 0.1% $MgSO_4$ (as carbohydrate stabilizer) and 0.5% EDTA (for masking the activities of d-block elements). All the chemicals were added on O.D. pulp basis. The strength of H_2O_2 was determined by taking 5 mL of H_2O_2 solution and diluted it to 5 times with distilled water in Erlenmeyer flask of capacity 250 mL. Now, poured 10 mL of 10% KI solution, 10 mL of 4N H_2SO_4 and 1 mL of 1% ammonium molybdate and titrated all the content of Erlenmeyer flask with 0.1 N $Na_2S_2O_3$ solution using 2-3 drops of freshly prepared starch solution as an indicator (373). The amount of $Na_2S_2O_3$ consumed was indicated by changing the color of end point from blue to colorless. The strength of H_2O_2 was calculated by using the following formula:

H_2O_2, g/L = Volume (mL) of 0.1 N $Na_2S_2O_3$ consumed × 0.34

Sugarcane bagasse soda-AQ pulp was further delignified with molecular O_2 in electrically heated WEVERK rotatory digester of capacity 2000 mL. The O_2 delignified pulp was bleached by OCEHH and XOCEHH bleaching sequences in order to observe its effect on kappa number, bleaching losses, pulp brightness and pulp viscosity. Pulp samples were mixed with 0.1% (O.D. pulp basis) $MgSO_4$, 1.5% NaOH (O.D. pulp basis) and water to maintain consistency of 10% and placed in a vessel at following conditions: oxygen pressure 5.0 kg/cm^2, temperature 90 ^{0}C, reaction time 45 min and pH 11.1.

The residual chlorine in the filtrate of chlorination stage and hypochlorite 1st and 2nd stages respectively was calculated as per method described above for analysis of bleach liquor except the volume of spent bleach liquor was

increased to 100 mL and titrated with 0.1N $Na_2S_2O_3$ solution (171). The results of CEHH; XECEHH, CEHHP; XECEHHP and OCEHH; XOCEHH bleaching sequences are reported in Tables 5.7-5.9 and Figures 5.7 to 5.11.

5.2.2.2 ECF bleaching

Sugarcane bagasse soda-AQ pulp samples were bleached by ODED; XODED and OD(E_{OP})DP; XOD(E_{OP})DP bleaching sequences where 'X' stands for xylanase stage, 'O' for oxygen delignification, 'D_1' and 'D_2' for chlorine dioxide 1st and 2nd stage respectively, 'E' for alkaline extraction stage, 'P' for hydrogen peroxide stage and 'E_{OP}' for oxygen and peroxide reinforced alkaline extraction stage. The results and bleaching conditions are reported in Table 5.10 and 5.11. The xylanase pretreatment stage was performed under the following bleaching conditions i.e. enzyme dose of 8 IU/g for 120 min, temperature 55 ^{0}C, pH 6.4 and pulp consistency of 10%. The xylanase pretreated and untreated (control) sugarcane bagasse soda- AQ pulp samples were oxygen delignified in WEVERK rotatory digester at a pressure of 5 kg/cm^2, temperature 90 ^{0}C for 45 min. A 20 g/L solution of sodium chlorite was prepared and this solution was titrated by same procedure as used for analysis of calcium hypochlorite (bleach liquor) solution (171). Oxygen delignified pulp samples were treated with 1.6% (O.D. pulp basis) chlorine dioxide in 'D_1' and 'D_2' stages (1% in 'D_1' and 0.6% in 'D_2' stages respectively) at a pulp consistency of 10% at temperature 70 ^{0}C for 180 min and pH 4.0. In E-stage 2.5% NaOH was applied at 10% consistency, temperature 60 ^{0}C for 60 min and pH 11.7. In OD(E_{OP})DP bleaching sequence, 0.5% H_2O_2, 3% NaOH, 0.1% $MgSO_4$ and 5.0 kg/cm^2 oxygen pressure in E_{OP} stage were applied by maintaining the following conditions: consistency 11.5%,

temperature 75 ^{0}C for 70 min and pH 11.3. In final stage 0.5% H_2O_2 was charged at conditions described in Table 5.11. All the chemicals were added on O.D. pulp basis. The results of ODED; XODED and OD(E_{OP})DP; XOD(E_{OP})DP bleaching sequences are reported in Tables 5.10 to 5.12 and Figures 5.12 to 5.15.

5.2.2.3 TCF bleaching

Sugarcane bagasse soda-AQ pulp was bleached using O(E_{OP})P, XO(E_{OP})P and OX(E_{OP})P bleaching sequences where 'X' stands for xylanase stage, 'O' *for oxygen delignification*, 'E_{OP}' *for oxygen and peroxide reinforced* alkaline extraction stage and 'P' for hydrogen peroxide stage. The bleaching results and conditions are summarized in Table 5.13 and 5.14. Sugarcane bagasse soda-AQ pulp was treated with a xylanase dose of 8 IU/g at following conditions like, consistency 10%, temperature 55 ^{0}C for 120 min and pH of 6.4. Sugarcane bagasse soda-AQ pulp after xylanase pretreatment was delignified with O_2 in WEVERK rotatory digester. Pulp samples were mixed with 0.1% $MgSO_4$, 1.5% NaOH and water to maintain consistency of 10% and placed in a vessel at following conditions, oxygen pressure 5.0 kg/cm^2, temperature 90 ^{0}C, reaction time 45 min and pH 11.8. In extraction stage 0.5% peroxide charge was given with 3% NaOH, 0.1% $MgSO_4$ and 5 kg/cm^2 O_2 pressure at consistency 10%, temperature 75 ^{0}C for 70 min and pH 11.6. In final stage 2.0% H_2O_2 was applied on O.D. pulp basis under conditions described in Table 5.13. All the chemicals were added on O.D. pulp basis. The results of O(E_{OP})P, XO(E_{OP})P and OX(E_{OP})P bleaching sequences are summarized in Tables 5.13 to 5.15 and Figures 5.16 and 5.17.

5.2.3 Preparation of laboratory hand sheets and evaluation of paper properties

The bleached pulp samples were evaluated for pulp yield (T 222 om-88), viscosity (T 230 om-04), and copper number (T 430 om-88) as per Tappi Standard Test Methods: 2007. The bleached sugarcane bagasse soda-AQ pulp was disintegrated in PFI mill (T 200 sp-96) at a beating level of 35 ^{0}SR. Laboratory hand sheets of 60 g/m^2 were prepared (T 221 cm-99) and tested for various physical strength properties like, 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: 2007. The brightness of pulp samples was determined by Technibrite Eric 950 from Technibrite Corporation, USA (343).

5.2.4 Analysis of combined bleaching effluent

Bleach plant effluent collected after each stage of bleaching were mixed in equal amounts (at the end of each bleaching sequence) and were analyzed for COD (closed reflux titrimetric method using Thermoreactor CR 2010) (352, 378), color (204 A) as per Standard methods for the examination of water and wastewater, American Public Health Association, 1985 and AOX by column method (357).

5.2.5 Scanning electron microscopy

The detailed morphological studies of unbleached sugarcane bagasse soda-AQ pulp samples (before and after xylanase treatment) were carried out using scanning electron microscopy (SEM, Leo 435 VP, England). Pulp samples were taken and subjected for fixation using 3% (v/v) glutaraldehyde-2% (v/v) formaldehyde (4:1) for 24 h. Following the primary

fixation, samples were washed thrice with double distilled water. The samples were then treated with the alcohol gradients of 30, 50, 70, 80, 90 and 100% for dehydration. Samples were kept for 15 min each up to 70% alcohol gradient, thereafter treated for 30 min each for subsequent alcohol gradients. After treating with 100% alcohol, samples were air dried and examined under SEM using gold shadowing technique (96). SEM microphotographs were taken at desired magnifications. Results of SEM are shown in Figure 5.18: Photomicrogrphs A-F.

5.2.6 Statistical analysis

All experiments were carried out in triplicate and experimental results were represented as the mean ± standard deviation of three identical values.

5.3 RESULTS AND DISCUSSIONS

5.3.1 Optimization of various operating parameters for xylanase pretreatment of sugarcane bagasse soda-AQ pulp

5.3.1.1 Influence of xylanase doses

Table 5.1 shows the effect of varying doses of xylanase i.e. 0-25 IU/g while keeping other variables constant like temperature 55 ^{0}C, reaction time 120 min and pH 6.4 on kappa number and brightness of soda-AQ pulp of sugarcane bagasse after alkaline extraction stage. Figure 5.1 shows that kappa number after XE stage reduces by 7.9 units (28.85%) with increasing enzyme dose up to 8 IU/g and further there is no significant decrease in kappa number on increasing enzyme dose. On the other hand, pulp brightness after XE stage increases by 9.2 units with increasing enzyme dose from 0.0 to 8 IU/g and after

that there is no significant gain in brightness. Literature showed that the brightening of eucalyptus pulp was enhanced by the enzyme preparations followed by subsequent alkaline extraction (206). An alkaline extraction stage was carried out after xylanase treatment of bagasse pulp to see the effect of xylanase on bagasse pulp kappa number and brightness as the effect of enzymatic attack on the removal of lignin from pulp was not detectable without a subsequent chemical treatment (146, 253, 168, 16, 61, 310). Previously alkaline extraction in conjunction with enzyme treatment was shown to improve pulp characteristics (60). The extraction is suggested to facilitate the dissolution of lignin-carbohydrate fragments in pulp that were previously modified by these enzymes but still remain in pulp because of their large molecular weight (61). The reducing sugars released after different doses of enzyme are shown in Figure 5.1. The curve can be approximated by two straight lines. The curve with steeper slope is pertaining to rapid release of sugars (up to an enzyme dose of 12 IU/g) whereas the part of curve with gentler slope is pertaining to the slow release of sugars. Both parts of the curve are having different velocity constants. Release of chromophores is probably a better indicator of the kinetics of the enzyme attack on the pulp as reducing sugar will continue to be cleaved by xylanase hydrolysis of the soluble oligosaccharides. Literature indicated that oligosaccharides are released by the initial depolymerization of the xylan coating on the fiber surface (97). Table 5.2 shows spectrophotometric analysis of filtrate generated during xylanase treatment of soda-AQ pulp of sugarcane bagasse at different doses of xylanase. Figure 5.2 reveals that the absorbance at a wave length of 237 nm increases up to xylanase dose of 8 IU/g due to release of

phenolic compounds or chromophores and beyond an enzyme dose of 8 IU/g there is no significant increase in absorbance. The absorbance at a wave length of 465 nm increases due to release of hydrophobic compounds (253, 114, 151, 33) up to an enzyme dose of 8 IU/g and then there is slight increase in absorbance. However, there is an increase in the absorbance at a wave length of 280 nm above enzyme dosage of 8 IU/g. The peak at 280 nm in the U.V. spectrum indicates the presence of lignin in the released coloring matter (168, 229). At higher enzyme dosage (above 8 IU/g), there is an increase in the absorbance at 280 nm supporting the observation by Ziobro that carbohydrate degradation products also attributes to the coloring matter (388, 389). The release of chromophores from the bagasse pulp indicated the degradation of lignin hemicellulose linkages (73). This release correlates with the reduction in kappa number found after the first extraction (116). The release of reducing sugars and the release of lignin and phenolic compounds are interrelated phenomenon. When the sugarcane bagasse soda-AQ pulp is pretreated with xylanase, the xylose and other reducing sugars are released from the hemicellulose layers that ultimately results in an increase in the free sugar content. Xylan is a part of hemicellulose which is sandwiched between lignin and cellulose layer. When xylan is hydrolyzed by the xylanase, lignin and phenolic compounds are also released in addition to xylose from the pulp fibers that ultimately cause the enhancement in absorbance of pulp free samples compared to the control (151).

5.3.1.2 Influence of reaction time

Table 5.3 shows the effect of different xylanase reaction time i.e. 0-240 min on kappa number and brightness of bagasse soda-AQ pulp while keeping other variables constant like, xylanase dose of 8 IU/g, temperature 55 ^{0}C, pH 6.4 and pulp consistency of 10%; after alkaline extraction of pulp. Figure 5.3 indicates that kappa number after XE stage decreases by 7.06 units (29.1%) on increasing the reaction time from 0 to 120 min and beyond a reaction time of 120 min, kappa number remains nearly constant. In the same pattern, pulp brightness of soda-AQ pulp after XE stage increases by 9.42 units and then there is insignificant increase in brightness. Figure 5.3 shows that the concentration of reducing sugars released after xylanase treatment is the maximum at a reaction time of 120 min and then it remains almost constant. Table 5.4 reveals the spectrophotometric analysis of filtrates generated during xylanase treatment of soda-AQ pulp of sugarcane bagasse at different reaction times. Figure 5.4 depicts the effect of reaction time on absorbance of filtrates of xylanase treated pulp at different wavelengths i.e. 237, 254, 280 and 465 nm. The curves reveal that the absorbance at all the wavelengths increases up to a reaction time of 120 min and then there is no significant gain in absorbance. Similar trend of chromophore release as a result of xylanase pretreatment was reported by Beg *et al.* (33), Ninawe and Kuhad (228), Khandeparkar and Bhosle (151), Shah *et al.* (310) and Kulkarni and Rao (168). A reaction time of 120 min was considered as optimum by many other workers too for achieving the highest biobleaching effect on pulp (386, 33, 97, 228, 116, 310). Enzyme dosage and holding time are inter-related. By increasing enzyme dosage, for example, the same bleach boosting effect may be achieved in shorter time (16).

5.3.1.3 Influence of pulp consistency

Table 5.5 describes the effect of pulp consistency on kappa number and brightness of soda-AQ pulp of sugarcane bagasse while keeping other conditions constant like, xylanase dose 8 IU/g, temperature 55 ^{0}C, reaction time 120 min and pH 6.4 after XE stage. Figure 5.5 indicates that kappa number decreases with increasing pulp consistency. The kappa number decreases by 7.07 units (29.14%) at a pulp consistency of 10% (medium consistency). There is no significant decrease in kappa number above consistency 10% (high consistency). The cellulosic fibers when merged in water; contain mobile and immobile layers surrounding the fibers (236). As the consistency of pulp is increased the mobile layer is progressively eliminated leaving only the thin immobile layer enveloping the fiber, thus decreasing considerably the diffusion path length of reactant to the fiber (176, 277, 148). Water layer thickness now becomes the rate-determining step. However, the higher pulp consistency provides a close contact between enzymes and pulp fibers (116, 61), probably because of the reduced volume of the liquid phase, thus facilitating enzyme adsorption to pulp and the sequential hydrolysis of hemicellulose (61). Good mixing is a critical requirement for effective pulp bleaching. For the decrease in kappa number above pulp consistency of 10%, the pulp is to be finely shredded to separate fiber aggregates to the greatest extent possible before contacting the fiber with reactant (231, 236, 148, 234, 99). In the similar pattern, the brightness increases by 9.6% (ISO) up to consistency of 10% and beyond that the increase in brightness is insignificant. Figure 5.5 shows the concentration of reducing sugar released after xylanase treatment at different pulp consistencies. The

curve shows that the increase in pulp consistency also increases the amount of reducing sugars released. The amount of reducing sugars increases up to consistency of 10% and beyond that curve becomes nearly stable. Table 5.6 depicts the spectrophotometric analysis of filtrates generated during xylanase treatment of soda-AQ pulp of sugarcane bagasse at different pulp consistencies. Figure 5.6 reveals that the absorbance at different wavelengths (237, 254, 280 and 265 nm), also increases with increasing pulp consistency up to 10% and beyond that the increase in absorbance is insignificant. Many researchers have used a pulp consistency of 10% for xylanase pretreatment of bagasse soda pulp (191, 59), wheat straw-rich soda pulp (228), eucalyptus kraft pulp (80, 206, 23, 292), bagasse soda pulp and oxygen delignified soda-AQ pulp of *Eucalyptus grandis* (337), bagasse pulp (310), eucalyptus kraft pulp and bagasse soda pulp (135), softwood kraft pulp (349), kraft pulps of Douglas fir, western red cedar and western hemlock (377) and oxygen bleached eucalyptus kraft pulp (43). High pulp consistencies help to stabilize the enzyme, allowing it to remain active under more severe conditions, e.g. higher temperature and higher pH values, than would normally be tolerated (16). In case of enzymatic treatment, several factors determine the efficiency of pulp prebleaching. The extent of product removal is largely a function of substrate characteristics, but enzyme specificity also plays an important role (153). The interaction of the enzyme with the pulp is also important, including the effective molecular weight, net ionic properties and specific action pattern (303). Thus, the response of any pulp or parameter will vary according to the characteristic features of any enzyme preparation.

5.3.2 Effect of xylanase pretreatment on conventional bleaching

The effect of xylanase pretreatment was studied on total chlorine demand (TCD), brightness, viscosity, strength properties and combined effluent properties of sugarcane bagasse soda-AQ pulp during CEHH, CEHHP and OCEHH bleaching sequences. Table 5.7 shows the effect of CEHH, CEHHP and OCEHH bleaching sequences on brightness, viscosity and bleach losses of soda-AQ pulp of sugarcane bagasse. The brightness and viscosity of soda-AQ pulp of sugarcane bagasse bleached by CEHH bleaching sequence are 80.1% (ISO) and 9.3 cps respectively. The introduction of H_2O_2 in the hypochlorite 2nd stage (CEHHP) increases the pulp brightness by 1.5% (ISO) with marginal decrease in pulp viscosity at the same chlorine demand compared to CEHH bleaching sequence. Introduction of O_2 (O_2 pressure 5 kg/cm^2) before chlorination stage reduces the kappa number by 38.17% and chlorine demand 38.12%. A drop of about 50% in kappa number of unbleached pulp of *Melocanna baccifera* (Muli bamboo) after ODL was denoted at an O_2 pressure of 5 bar, temperature 70 ^{0}C, alkali charge 2% and residence time 60 min (348) while 60% reduction in the kappa number of unbleached bagasse pulp and 59% of unbleached hardwood pulp after ODL was reported at an O_2 pressure 0.50 mPa, temperature 120 ^{0}C, alkali charge 2% and reaction time of 30 min (261). The OCEHH bleached pulp produces pulp of brightness 83.2% (ISO) and pulp viscosity comparable to CEHH bleaching sequence of sugarcane soda-AQ pulp. The bleaching losses in CEHH, CEHHP and OCEHH bleaching sequences are 9.0, 9.2 and 8.78% respectively.

Table 5.8 shows the effect of xylanase pretreatment of CEHH, CEHHP and OCEHH bleaching sequences on TCD, bleaching losses, brightness and pulp viscosity of sugarcane bagasse soda-AQ pulp. The xylanase pretreatment before CEHH and CEHHP bleaching sequences followed by alkaline extraction reduces the kappa number of sugarcane bagasse soda-AQ pulp by 7.23 units (29.80%) and chlorine demand by 29.70% in comparison with TCD of both CEHH and CEHHP bleaching sequences without xylanase pretreatment. In the similar manner, the kappa number after XO stage in XOCEHH bleaching sequence of sugar cane bagasse soda-AQ pulp reduces by 5.49 units (36.6%) and TCD by 36.53% in comparison with OCEHH bleaching sequence. Results from laboratory studies and mil trials showed about 35-41% reduction in active chlorine at the chlorination stage for hardwoods and 10-20% for softwoods (305, 349, 17). The pretreatment of wheat straw pulp with xylanase obtained from *A. niger* An76 prior to H, CH or CEH bleaching could reduce the chlorine consumption by 20-30% to attain the same brightness level (386). 18% decrease was noticed in kappa number of birch wood kraft pulp after XE stage when it was treated with cellulase free xylanases from *Streptomyces thermoviolaceus* (97). Pretreatment of eucalyptus kraft pulp with xylanases from *Staphylococcus* sp. SG-13 and its subsequent treatment with 8% hypochlorite had been shown to bring about 30% reduction in kappa number (114).

The brightness of soda-AQ pulp of sugarcane bagasse bleached by XECEHH, XECEHHP and XOCEHH bleaching sequences increases by 4.4%, 4.1% and 3.7% respectively in comparison with the brightness of sugarcane bagasse pulp bleached by CEHH, CEHHP and OCEHH bleaching sequences.

There is a positive gain in brightness of sugarcane bagasse pulp as xylanase improves the accessibility of bleaching chemicals by disrupting the xylan chain and thus facilitates the easier removal of lignin during bleaching (305). The crude xylanase produced from the cultural filtrates of *A. niger* An76 pre-treatment had increased the final brightness of wheat straw pulp by 4-5% (ISO) in comparison with control under the identical bleaching conditions (CEH) (386).

The xylanase pretreatment increases pulp viscosity of CEHH, CEHHP and OCEHH bleached sugar cane bagasse soda-AQ pulps by 0.65, 1.09 and 2.57% after XECEHH, XECEHHP and XOCEHH bleaching sequences respectively in comparison with control. The increase in viscosity reflects the hydrolysis of low DP xylan in the pulp (241, 63, 286, 315, 362). The crude xylanase extract used in the study is having negligible cellulase contamination so no loss in pulp viscosity is noted. The nonspecific endoglucanases had been reported to reduce the viscosity of softwood kraft pulp, indicating the degradation of cellulose chains (366, 316). The bleaching losses after XECEHH, XECEHHP and XOCEHH bleaching sequences are 8.4, 8.7 and 8.5% respectively compared to 9.0, 9.2 and 8.78% after CEHH, CEHHP and OCEHH bleaching sequences of sugarcane bagasse soda-AQ pulp respectively.

Table 5.9 shows a comparison of mechanical strength properties and combined effluent generated during CEHH and XECEHH; CEHHP and XECEHHP and OCEHH and XOCEHH bleaching sequences of sugarcane bagasse soda-AQ pulp. Primarily, the strength of paper will depend upon fiber length (127). The xylanase pretreatment increases the viscosity of pulp bleached by XECEHH, XECEHHP and XOCEHH bleaching sequences which

results in an increase in mechanical strength properties of paper when compared with mechanical strength properties of CEHH and CEHHP and OCEHH bleached pulp at the same freeness level. Figure 5.7 shows that xylanase pretreatment increases tear index by 5.47%, burst index by 18.98%, tensile index by 15.99% and double fold by 11.76% of XECEHH bleached pulp compared to control. Figure 5.8 shows that xylanase pretreatment causes an increase in tear index by 5.32%, burst index by 15.8%, tensile index by 13.48% and double fold by 5.6% of XECEHHP bleached pulp compared to mechanical strength properties of CEHHP bleached pulp. In the same manner, XOCEHH bleached pulp shows an improvement in burst index by 15.53%, tensile index by 10.63% and double fold by 8.1% while tear index remains unaltered in comparison with OCEHH bleaching sequence (Figure 5.9). Clark *et al.* suggested that xylanase prebleaching increases the fiber swelling which facilitates refining and in turn results in better physical properties (64). The results indicate that xylanase prebleaching could have facilitated an increase in pulp fibrillation, water retention, restoration of bonding and freeness in fibers (32, 20). Reduction of chlorine demand for xylanase pretreated pulps could also be a possible reason for improved strength properties as higher chlorine charge proves to be detrimental for paper strength. Enzyme treated wheat straw pulp had a high tear index and breaking length compared to the control pulp (386). Beg *et al.* had also reported improvement in tensile strength and burst factor of eucalyptus kraft pulp by 63 and 8% respectively by pretreatment with xylanase produced from *Streptomyces* sp. QG-11-3 in comparison with control pulp (33). Copper number after CEHH, CEHHP and OCEHH is 0.25, 0.22 and 0.13 which

reduces to 0.18 (–28%), 0.15 (–31.81%) and 0.11 (–15.38%) after XECEHH, XECEHHP and XOCEHH bleaching sequences respectively. Copper number shows the degree of damage to cellulose in paper (218) which has been reduced as a result of reduction in TCD after xylanase pretreatment of sugarcane bagasse soda-AQ pulp.

Figure 5.10 shows the effect of xylanase pretreatment on the AOX generated during CEHH, CEHHP and OCEHH bleaching sequences. The AOX level after CEHH, CEHHP and OCEHH bleaching sequences are 2.77, 2.21 and 2.02 kg/t respectively which decrease to 1.99 (–28.16%), 1.6 (–27.6%) and 1.32 (–34.65%) kg/t respectively after XECEHH, XECEHHP and XOCEHH bleaching sequences. Organochlorine compounds are produced mainly by the reactions between residual lignin present in wood fibers and chlorine used for bleaching (23). Reduction in TCD after xylanase pretreatment of bleaching sequences, results in lowering the toxicity of the bleach plant effluents also. So the xylanase pretreatment reduces the amount of chlorophenols and other forms of organically bound chlorine (AOX) in the spent bleach liquor (16, 350). AOX level is reduced by 27.07% after OCEHH in comparison with CEHH bleaching sequence as a result of reduced TCD after ODL of bagasse soda-AQ pulp before 'C' stage. Xylanase pretreatment of a hardwood kraft pulp under the same bleaching conditions led to a reduction of 35-40% of chlorination charge and 24% of E stage AOX while the BOD/COD ratio was increased (304). Effluent AOX was reduced by 15.09% after eucalyptus kraft pulp was pretreated by commercial xylanase after same treatment compared to control (137).

Figure 5.11 shows the effect of xylanase pretreatment on combined bleach effluent generated after CEHH and XECEHH, CEHHP and XECEHHP and OCEHH and XOCEHH. The COD load of combined effluent, after CEHH, CEHHP and OCEHH bleaching sequences are 723, 701 and 598 mg/L which increase to 787 (+8.85%), 745 (+6.27%) and 650 (+8.7%) mg/L in the combined effluent of XECEHH, XECEHHP and XOCEHH bleaching sequences respectively. The COD load in combined effluent of OCEHH bleaching sequence decreases by 17.3% in comparison with CEHH bleaching sequence due to prebleaching with molecular O_2. Since the pentosans are released from the xylanase prebleaching, the COD of the bleach effluents is rather high after xylanase pretreatment compared to control (22, 199, 362). The COD and BOD load of combined bleach effluent were increased by 32.19 and 107.89% after pretreatment of eucalyptus kraft pulp with commercial xylanase in comparison to control (135, 137). These effluents are amenable to biological degradation, due to high proportion of degraded xylan as had been confirmed by Onysko (235), Senior and Hamilton (304).

The color values of combined effluent of CEHH, CEHHP and OCEHH bleaching sequences are 2250, 1600 and 1560 PTU respectively which increase to 2500 (+11.11%), 1840 (+15%) and 1825 (16.99%) PTU respectively after XECEHH, XECEHHP and XOCEHH sequences. A peroxide stage at the end of CEHH bleaching sequence (CEHHP) decreases the color formation by 28.88% in comparison with CEHH bleaching sequence. The hydroperoxide anion (HOO^-) destroys chromophoric groups present in pulp because of its specific and efficient action on carbonyl and conjugated carbonyl groups (78) which further

leads to color reduction of bleach plant effluents. A decrease of 30.66% in color of bleach plant effluent is observed after OCEHH bleaching sequence compared to CEHH bleaching sequence. Regardless, of the bleaching chemicals used, ODL decreases the BOD, COD and color of the effluents (348).

Effluent color is enhanced in xylanase pretreated pulps as xylanase alters the carbohydrate composition of pulps by reducing their xylan content. The dissolution of xylans produced by the xylanase gives rise to an increase in effluent color (351). This could also be explained by the increased amount of lignin dissolved from enzyme treated pulps (351, 286). An increase of 27.76% was noted in the color of bleaching effluent when *E. globulus* pulp was pretreated with xylanase in a bleaching sequence OD_1PD_2 (362). Color increase is the highest in case of XOCEHH as xylan hydrolysis is more when oxygen delignification is carried out after xylanase stage (289).

5.3.3 Effect of xylanase pretreatment on ECF bleaching

The effect of xylanase pretreatment was studied on brightness, viscosity, mechanical strength properties and combined effluent properties of sugarcane bagasse soda-AQ pulp in elemental chlorine free (ECF) bleaching sequences like ODED and $OD(E_{OP})DP$. Table 5.10 shows the effect of ODED and $OD(E_{OP})DP$ on sugarcane bagasse soda-AQ pulp brightness, viscosity and bleaching losses. The brightness and viscosity of soda-AQ pulp of sugarcane bagasse bleached by ODED bleaching sequence are 79.2% (ISO) and 9.36 cps respectively. The introduction of H_2O_2 and O_2 as reinforcement for alkaline extraction (E_{OP}) after chlorine dioxide 1st stage ($OD(E_{OP})DP$) increases the brightness of sugarcane bagasse soda-AQ pulp by 6.3% (ISO) in comparison to

pulp brightness of sugarcane bagasse after ODE stage of ODED bleaching sequence without any adverse effect on pulp viscosity. The bleaching losses after ODED and OD(E_{OP})DP bleaching sequences are 7.5 and 7.7% respectively.

Table 5.11 shows the effect of xylanase pretreatment during XODED and XOD(E_{OP})DP bleaching sequences on brightness, viscosity and bleaching losses of sugarcane bagasse soda-AQ pulp. Brightness of sugarcane bagasse soda-AQ pulp increases by 7.3 and 4.7% (ISO) respectively after XODED and XOD(E_{OP})DP bleaching sequences in comparison with ODED and OD(E_{OP})DP bleaching sequences at the same chlorine dioxide charge. Xylanase treatment improves the accessibility of the pulps for the bleaching chemicals. It decreases the diffusion resistance to the outward movement of the degraded lignin fragments and allows the removal of less degraded lignin fragments from the fiber wall. This is the reason for the higher brightness of the xylanase treated pulps at the same bleaching reagent consumption (351). Bissoon *et al.* reported that pretreatment of bagasse pulp with *T. lanuginosus* SSBP xylanase (DED bleaching sequence) increased pulp brightness by 4.5% over the control (44) while a commercial enzyme (Xylanase P) increased the brightness of bagasse pulp by 3.1% and softwood kraft pulp by 5.1% in comparison to control after ECF bleaching (191).

Residual chlorine dioxide increases by 26.6% after XODED and 37.2% after OD(E_{OP})DP in comparison with ODED and OD(E_{OP})DP bleaching sequences at same chlorine dioxide charge. The results show that the xylanase treated pulps use lesser chlorine dioxide than control and still reaches up to a

much better brightness level. This suggests that the treated pulp reaches the chlorine saturation point sooner than the control pulp (116). An increased level of residual chlorine dioxide and pulp brightness obtained from the bleaching sequences with crude xylanase pretreatment indicate that the chlorine dioxide charge may be reduced to a good extent to achieve a given target brightness (206).

Sugarcane bagasse soda-AQ pulp viscosity increases by 0.43% after XODED and 0.53% after XOD(E_{OP})DP in comparison with ODED and OD(E_{OP})DP bleaching sequences. The results indicate that the crude xylanase only hydrolyzes xylan (241, 63, 286, 315, 362) and not cellulose chains in pulp (366, 316). The bleaching losses after XODED and XOD(E_{OP})DP are slightly more in comparison with ODED and OD(E_{OP})DP bleaching sequences as both (control and xylanase pretreated) pulps have been charged with same chlorine dioxide charge.

Table 5.12 shows the comparison of mechanical strength properties at same beating level and combined effluent generated during ODED and XODED and OD(E_{OP})DP and XOD(E_{OP})DP bleaching sequences. Copper number decreases by 38.46 and 25% respectively after XODED and XOD(E_{OP})DP bleaching sequences in comparison with control, indicating that xylanase pretreatment has reduced degree of damage to cellulose of sugarcane bagasse soda-AQ pulp after full bleaching sequences. There is an overall improvement in mechanical strength properties and viscosity of bagasse pulp after ODED and OD(E_{OP})DP bleaching sequences in comparison with that of CEHH, CEHHP and OCEHH bleaching sequences because chlorine dioxide is known to be less

damaging to cellulose than chlorine (78). Figure 5.12 shows the effect of xylanase pretreatment on mechanical strength properties of sugarcane bagasse soda-AQ pulp bleached by ODED bleaching sequence. Tear index increases by 6.72% while burst index, tensile index and double fold are reduced by 6.76, 9.1 and 10.34% respectively after XODED in comparison with ODED bleaching sequence. Figure 5.13 shows that the tear index increases by 4.99% while burst index, tensile index and double fold are reduced by 14.17, 12.26 and 38.64% respectively after XOD(E_{OP})DP bleaching sequence of sugarcane bagasse soda-AQ in comparison with control. There is a decrease in strength properties except tear strength for both the sequences after the xylanase pretreatment in comparison with control. The fact that the tear index value is higher for the xylanase treated pulp could be due to a greater tendency to external fibrillation owing to the elimination of xylans deposited on the surface of fiber, which would limit this fibrillation (289, 228). The retention of hemicelluloses is favorable for the mechanical strength properties of papermaking fibers, because of their positive effect on the interfibrillar bonding during paper-sheet formation (62, 19). The partial hemicelluloses (xylan) removal during enzymatic stage can therefore affect adversely the pulp strengths (specially tensile and burst index which depend on interfiber bonding of paper). Reduction in interfiber bonding has been observed after treatment of pulps with xylanases (230, 284). The xylanase pretreated pulps are bleached using the same chemical charges during ECF bleaching sequences which may be the other reason for lower strength properties except tear index. After bleaching with the same chemical loadings, most reports claim little change in papermaking properties while some indicate

an improvement to the tear index (64, 57, 286). The xylanase pretreated wheat straw pulp showed a slightly lower tensile index and breaking length than the control (180) while a decrease was observed in all the strength properties of eucalyptus kraft pulp in comparison with control after pretreatment with xylanase produced by a thermophilic fungi (116). The pretreatment of xylanase produced by *Streptomyces cyaneus* SN32 on wheat straw pulp had increased the tear index and burst factor (228) while xylanase pretreatment has increased the tear index of *Eucalyptus globulus* kraft pulp leaving tensile index unaffected (289).

Table 5.12 shows that ECF bleaching sequences (ODED and OD(E_{OP})DP) reduce the COD, color and AOX formation in combined bleach effluents in comparison with that of CEHH bleaching sequence. In combined effluent of ODED and OD(E_{OP})DP bleaching sequences, COD decreases by 23.8 and 21.16% respectively while color decreases by 33.11 and 22.2% respectively in comparison with CEHH bleaching sequence. The AOX formation reduces by 84.84 and 87.36% respectively after ODED and OD(E_{OP})DP bleaching sequences in comparison with CEHH bleaching sequence. Use of chlorine dioxide in place of elemental chlorine reduces the amount of AOX in the effluents (204, 212). Pryke suggested that ECF bleaching decreases chloroform and total organic halide (AOX) formation by 90% (264). Chlorine dioxide is a stronger bleaching agent than chlorine. The reactions during bleaching are to a large extent oxidative with chlorine dioxide, which reduces the amount of AOX formed. The atomic chlorine content of chlorine dioxide is lower than chlorine, which further decreases AOX in effluents (311).

Figure 5.14 shows the effect of xylanase pretreatment on the AOX generation after ODED and OD(E_{OP})DP bleaching sequences. The AOX values after ODED and OD(E_{OP})DP bleaching sequences are 0.42 and 0.35 kg/t respectively which reduce to 0.24 (–41.43%) and 0.21 (–40%) kg/t respectively after XODED and XOD(E_{OP})DP bleaching sequences. The introduction of xylanase pretreatment before ODED and OD(E_{OP})DP bleaching sequences increases residual ClO_2 from 6.9 to 9.4% and 7.5 to 11.94% respectively which shows that the xylanase treated pulps use lesser chlorine dioxide than control. This suggests that the treated pulps reach the chlorine saturation point sooner than the control pulp (116). It explains the lower level of AOX obtained after xylanase pretreatment of bagasse pulp before ODED and OD(E_{OP})DP bleaching sequences.

Figure 5.15 shows the comparison of combined effluents generated after ODED & OD(E_{OP})DP and XODED & XOD(E_{OP})DP bleaching sequences. The COD after ODED and OD(E_{OP})DP bleaching sequences are 551 and 570 mg/L, which increase to 623 (+13.07%) and 655 (14.91%) mg/L after XODED and XOD(E_{OP})DP bleaching sequences. Same trend for increase in COD after xylanase pretreatment was reported by Bajpai (22), Mathur *et al.* (199) and Jain *et al.* (135) as a result of hemicellulose removal from pulps. An increase of 51.13% was noticed in the COD of bleaching effluent generated during the bleaching *of E. globulus pulp in* OD_1PD_2 *sequence as a result of* xylanase pretreatment (362).

The values of color in combined bleach effluent generated after ODED and OD(E_{OP})DP bleaching sequences are 1505 and 1750 PTU which increase to

1772 (+17.74%) and 1884 (+7.66%) PTU after xylanase pretreatment in XODED and XOD(E_{OP})DP bleaching sequences. An increase in color of bleaching effluents after xylanase pretreatment was also observed by Roncero *et al.* (286) and Vidal *et al.* (362) as a result of dissolution of xylan and more lignin from pulp in comparison with control.

5.3.4 Effect of xylanase pretreatment on TCF bleaching

Table 5.13 reveals the effect of O(E_{OP})P bleaching sequence on brightness, viscosity and bleaching losses of sugarcane bagasse soda-AQ pulp. The brightness, and viscosity of sugarcane bagasse soda-AQ pulp bleached by O(E_{OP})P bleaching sequence are 71.3% (ISO) and 9.8 cps respectively. The bleaching losses after O(E_{OP})P bleaching sequence are 7.1%. The pulp viscosity after O(E_{OP})P bleaching sequence is higher and bleaching losses are lower in comparison with that of CEHH, CEHHP & OCEHH (conventional) and ODED & OD(E_{OP})DP (ECF) bleaching sequences as no chlorine containing chemical is used during O(E_{OP})P (TCF) bleaching sequence.

Table 5.14 shows the effect of xylanase pretreatment before O(E_{OP})P bleaching sequence on brightness, viscosity and bleaching losses of sugarcane bagasse soda-AQ pulp. The efficiency of xylanase pretreatment on O(E_{OP})P is checked in two ways. Xylanase stage is applied before oxygen delignification (XO(E_{OP})P) and after oxygen delignification (OX(E_{OP})P). Brightness of sugarcane bagasse soda-AQ pulp increases by 6.1 and 8.2% (ISO) respectively after OX(E_{OP})P and XO(E_{OP})P bleaching sequences in comparison with O(E_{OP})P bleaching sequence. The increase in brightness after XO(E_{OP})P is more compared to OX(E_{OP})P bleaching sequence. It indicates that xylanase stage

prior to oxygen delignification is more effective than xylanase stage after oxygen delignification to produce the pulp of higher brightness. Xylanase pretreatment hydrolyzes the pulp xylan allowing better access of bleaching chemicals to the residual lignin and easier extraction of lignin from pulp fibers, which in turn improves the brightness of pulp (71). The xylan hydrolysis is more effective when the xylanase pretreatment is applied prior to oxygen stage rather than after oxygen stage as had also been suggested by Roncero *et al.* (289).

The pulp viscosity improves by 3.37 and 2.55% respectively after OX(E_{OP})P and XO(E_{OP})P bleaching sequences compared to control because of removal of low DP xylan as a result of xylanase pretreatment (241, 63, 286, 315, 362, 366, 316). Bleaching losses increase by 5.63 and 9.85% after OX(E_{OP})P and XO(E_{OP})P bleaching sequences compared to O(E_{OP})P bleaching sequence at the same chemical charge. The results also indicate that pulp yield is lesser when xylanase pretreatment is put before oxygen delignification because bleaching losses are more after XO(E_{OP})P in comparison with OX(E_{OP})P bleaching sequence as a result of more xylan removal after XO stage in comparison with OX stage.

Table 5.15 shows the comparison of mechanical strength properties and combined effluent generated during O(E_{OP})P, OX(E_{OP})P and XO(E_{OP})P bleaching sequences. Copper number after O(E_{OP})P bleaching sequence is 0.09 which decreases by 33.3% after OX(E_{OP})P and XO(E_{OP}) bleaching sequences. The reduction in copper number indicates that a xylanase pretreatment stage has reduced the degree of cellulose damage of sugarcane bagasse soda-AQ pulp in comparison with control sequence. Figure 5.16 shows the comparison of

mechanical strength properties of sugarcane bagasse soda-AQ pulp bleached by $O(E_{OP})P$, $OX(E_{OP})P$ and $XO(E_{OP})P$ bleaching sequences at 35 ^{0}SR. Figure 5.16 shows that tear index increases by 5.96 and 3.85% after $OX(E_{OP})P$ and $XO(E_{OP})P$ in comparison with $O(E_{OP})P$ bleaching sequence. While burst index, tensile index and double fold decrease by 16.56, 8.29 and 18.51% respectively after $OX(E_{OP})P$ bleaching sequence and 20.45, 18.08 and 11.11% after $XO(E_{OP})P$ bleaching sequence when compared with $O(E_{OP})P$ bleaching sequence. The decrease in strength properties is noticed probably due to extra removal of xylan after xylanase pretreatment of pulp which adversely affects the interfibrillar bonding of paper (62, 19). The xylan removal enhances the degree of external fibrillation on fiber surface, which improves the tear index after xylanase pretreatment of bagasse pulp (289). It is also observed that the mechanical strength properties are inferior after $XO(E_{OP})P$ bleaching sequence compared to $OX(E_{OP})P$ bleaching sequence. The reason is that xylan removal is more after $XO(E_{OP})P$ bleaching sequence than that of $OX(E_{OP})P$ bleaching sequence.

Figure 5.17 shows the comparison of COD and color among combined effluents generated during $O(E_{OP})P$, $OX(E_{OP})P$ and $XO(E_{OP})P$ bleaching sequences. COD after $O(E_{OP})P$ bleaching sequence is 710 mg/L which increases to 745 (+4.92%) and 776 (+9.3%) mg/L respectively after $OX(E_{OP})P$ and $XO(E_{OP})P$ bleaching sequences. Xylanase pretreatment causes an increase of COD in bleaching effluents due to the hydrolysis of hemicelluloses (362, 22, 199). Color of the combined effluent produced by $O(E_{OP})P$ bleaching sequence is 2692 PTU, which increases to 3250 (+20.72) and 3580 (+32.98%) PTU after

$OX(E_{OP})P$ and $XO(E_{OP})P$ bleaching sequences due to release of xylan and more lignin from pulp after xylanase pretreatment of control sequence (286, 362). The increase in COD and color after $XO(E_{OP})P$ bleaching sequence is more than that of $OX(E_{OP})P$ bleaching sequence which indicates that removal of xylan and lignin after XO stage is more compared to OX stage (289).

5.3.5 Scanning electron microscopy

Scanning electron microscopic (SEM) study is carried out in order to attain a better understanding of the effect of xylanase pretreatment on sugarcane bagasse pulp bleachability. The surface of untreated bagasse fibers is smooth and shows no signs of external fibrillation and swelling as shown by Figure 5.18: photomicrograph A. Figure 5.18: Photomicrograph B, C and E represent the xylanase pretreated fibers which bear cracks, peelings, swelling and external fibrillation on their surface as a result of xylanase action. Bagasse fibers that had undergone xylanase treatment have a rougher surface with striations and splits, i.e. a more open surface. These results confirm that xylanase acts by hydrolyzing the xylans deposited on the surface of fibers during alkaline pulping, which constitute a physical barrier to penetration by bleaching agents. Their elimination facilitates the flow of bleaching agents, which explains the bleach boosting effect of the xylanases (289, 305). Xylanase treatment improves accessibility of bleaching chemicals to the pulps, decreases diffusion resistance to outward movement of the degraded lignin fragments and allows the removal of less degraded lignin fragments from the cell wall. As a result, pulps treated with xylanase show lower kappa number and higher brightness and viscosity than pulps not treated with the xylanase (287). Beg *et al.*, have reported

that xylanase from *Streptomyces* sp. QG-11-3 introduced greater porosity, swelling up and separation of pulp microfibrils in eucalyptus pulp fibers compared to the smooth surface of untreated pulp fiber (33). Examination of xylanase pretreated wheat straw pulp revealed noticeable changes in the surface architecture of the pulp in comparison to the control. Morphological changes such as cracks and peelings of fiber surfaces were evident after xylanase pretreatment (180). The observations are in agreement with other authors too (44, 288). Figure 5.18: Photomicrograph E (untreated fibers) and F (xylanase treated fibers) show the unbleached bagasse fibers beaten up to 35 ^{0}SR. These photomicrographs depict that xylanase pretreated fibers are more fibrillated and show more roughness on their surface than untreated fibers. It can be concluded from these results that xylanase pretreated fibers need lesser rotations in a PFI mill to reach a targeted ^{0}SR in comparison with untreated fibers thus saving a certain amount of electrical energy too. The results are in accordance with the findings of Bharadwaj *et al.*, which showed that less beating of xylanase treated pulp seems to be required to reach a given freeness level (39). However, few reports indicated that xylanase pretreated bleached pulps were more difficult to refine, requiring more beating to achieve an equivalent tensile and freeness than control (286, 360, 18).

5.4 CONCLUSIONS

The optimum conditions for prebleaching of soda-AQ pulp of sugarcane bagasse with crude xylanase produced by *C. disseminatus* SW-1 are: enzyme dose of 8 IU/g, pH 6.4, reaction time 120 min, temperature 55 ^{0}C and pulp consistency 10%. The xylanase pretreatment also causes the release of

reducing sugars and chromophores from sugarcane bagasse soda-AQ pulp. The xylanase pretreatment reduces the total chlorine demand by 29.70% in CEHH and CEHHP bleaching sequences and 36.53% in OCEHH bleaching sequence. Xylanase pretreatment improves the pulp brightness of CEHH, CEHHP and OCEHH bleaching sequences by 4.4, 4.1 and 3.6% respectively with improvement in mechanical strength properties. The xylanase pretreatment also improves the viscosity of pulp by 0.69, 1.08 and 2.56% in CEHH, CEHHP and OCEHH bleaching sequences respectively whereas copper number reduces by 28% in CEHH, 31.81% in CEHHP and 15.38% in OCEHH bleaching sequences respectively. The AOX in combined effluent in CEHH, CEHHP and OCEHH bleaching sequences reduces by 28.16, 27.6 and 34.65% respectively with a slight increase in COD and color values in combined bleach effluent.

The xylanase pretreatment in ODED and OD(E_{OP})DP bleaching sequences improves the pulp brightness by 7.3 and 4.3% respectively at the same chemical dose whereas a decrease in mechanical strength properties is noticed except tear index which is improved by 6.72 and 4.99% in ODED and OD(E_{OP})DP bleaching sequences respectively. The AOX in combined beach effluent of ODED and OD(E_{OP})DP bleaching sequences reduces by 84.84 and 87.3% respectively compared to that of CEHH bleaching sequence. In the same way COD in the combined bleach effluent of ODED and OD(E_{OP})DP bleaching sequences reduces by 23.8 and 21.16% respectively and color reduces by 33.11 and 22.2% respectively compared to CEHH bleaching sequence. The xylanase pretreatment increases the viscosity by 0.43 and 0.45% while copper number decreases by 65.22 and 25% for ODED and OD(E_{OP})DP bleaching sequences.

A decrease of 41.43 and 40% is noted in AOX of combined bleach effluent generated during ODED and OD(E_{OP})DP bleaching sequences as a result of xylanase pretreatment while COD and color values increase slightly.

The pulp viscosity after TCF bleaching sequence O(E_{OP})P is higher while bleaching losses are lower compared to conventional (CEHH, CEHHP and OCEHH) and ECF (ODED, OD(E_{OP})DP) bleaching sequences. It is beneficial to put xylanase stage before oxygen delignification for a higher brightness gain as brightness increases by 8.2% after XO(E_{OP})P and 6.1% after OX(E_{OP})P compared to O(E_{OP})P bleaching sequence. A decrease is noticed in mechanical strength properties except tear index which increased by 3.85 and 5.96% after XO(E_{OP})P and OX(E_{OP})P bleaching sequences respectively with a slight increase in bleaching losses. Pulp viscosity is increased by 2.55 and 3.37% after XO(E_{OP})P and OX(E_{OP})P bleaching sequences respectively while copper number is decreased by 33.3% in both the cases. Xylanase pretreatment causes a slight increase in COD and color values of combined effluent generated during O(E_{OP})P bleaching sequence. The SEM studies reveal that xylanase pretreatment helps in fiber surface modifications as xylanase pretreated fibers show cracks, peelings, swelling and external fibrillation on their surface compared to smooth surface of untreated fibers.

Table 5.1: Effect of xylanase doses on reducing sugars released, kappa number and brightness of soda-AQ pulp of sugarcane bagasse

Sl. No.	Particulars	Test results						
1	Xylanase stage (X) Enzyme dose, IU/g	0	4	8	12	16	20	25
2	Reducing sugars released, mg/g	0	0.65±0.09	1.45±0.13	2.15±0.1	2.35±0.12	2.7±0.21	3.1±0.15
3	Alkali extraction stage (E) NaOH applied, % (O.D. pulp basis)	–	1.5	1.5	1.5	1.5	1.5	1.5
4	Kappa number	24.26±0.38	19.40±0.3	17.26±0.32	17.15±0.29	17.15±0.32	17.18±0.4	17.17±0.25
5	Brightness, % (ISO)	34.3±0.5	41.85±0.2	43.50±0.6	43.81±0.19	43.70±0.7	43.76±0.3	43.50±0.3
Conditions of treatment		X				E		
Consistency, %		10				10		
Temperature, ^{0}C		55±2				70±2		
Time, min		120				90		
pH		6.4				11.5		

± refers standard deviation

Table 5.2: Spectrophotometric analysis of filtrates generated during xylanase treatment of sugarcane bagasse soda-AQ pulp at different doses

Sl. No.	Particulars	Test results						
1	Xylanase stage (X) Enzyme dose, IU/g	0	4	8	12	16	20	25
Absorbance, nm								
2	237	0.184±0.004	0.338±0.008	0.449±0.007	0.451±0.008	0.471±0.02	0.480±0.013	0.500±0.015
3	254	0.123±0.003	0.202±0.008	0.268±0.01	0.272±0.005	0.275±0.01	0.281±0.008	0.300±0.013
4	280	0.088±0.001	0.159±0.004	0.206±0.002	0.254±0.02	0.269±0.01	0.333±0.015	0.360±0.02
5	465	0.108±0.001	0.166±0.002	0.215±0.015	0.223±0.02	0.235±0.008	0.251±0.01	0.280±0.006
Conditions of treatment		X						
Consistency, %		10						
Temperature, ^{0}C		55±2						
Time, min		120						
pH		6.4						

± refers standard deviation

Table 5.3: Effect of reaction time on reducing sugars released, kappa number and brightness during xylanase treatment of soda-AQ pulp of sugarcane bagasse

Sl. No.	Particulars	Test results					
1	Time, min	0	30	60	120	180	240
2	Reducing sugars released, mg/g	0	0.60±0.05	1.05±0.09	1.45±0.12	1.5±0.1	1.45±0.08
3	Alkali extraction stage (E) NaOH applied, % (O.D. pulp basis)	–	1.5	1.5	1.5	1.5	1.5
4	Kappa number	24.26±0.35	20.80±0.5	18.10±0.41	17.20±0.35	17.22±0.31	17.24±0.36
5	Brightness, % (ISO)	34.3±0.5	40.50±0.1	42.12±0.5	43.72±0.3	43.66±0.1	43.50±0.4
Conditions of treatment		X			E		
Xylanase dose, IU/g		8			–		
Consistency, %		10			10		
Temperature, ^{0}C		55±2			70±2		
Time, min		Variable			90		
pH		6.4			11.5		

± refers standard deviation

Table 5.4: Spectrophotometric analysis of filtrates generated during xylanase treatment of sugarcane bagasse soda-AQ pulp at different reaction times

Sl. No.	Particulars	Test results					
1	Time, min	0	30	60	120	180	240
Absorbance, nm							
2	237	0	0.020±0.006	0.427±0.03	0.448±0.021	0.450±0.03	0.457±0.015
3	254	0	0.147±0.015	0.249±0.02	0.264±0.011	0.270±0.024	0.265±0.018
4	280	0	0.103±0.001	0.193±0.01	0.206±0.014	0.210±0.02	0.208±0.019
5	465	0	0.151±0.008	0.210±0.04	0.218±0.02	0.223±0.03	0.228±0.032
Conditions of treatment		X					
Xylanase dose, IU/g		8					
Consistency, %		10					
Temperature, ^{0}C		55±2					
pH		6.4					

± refers standard deviation

Table 5.5: Effect of pulp consistency on reducing sugars released, kappa number and brightness during xylanase treatment of soda-AQ pulp of sugarcane bagasse

Sl. No.	Particulars	Test results					
1	Consistency, %	2	4	6	8	10	12
2	Reducing sugars released, mg/g	0	0.25±0.04	0.65±0.09	1.2±0.08	1.45±0.05	1.5±0.07
3	Alkali extraction stage (E) NaOH applied, % (O.D. pulp basis)	--	1.5	1.5	1.5	1.5	1.5
4	Kappa number	24.01±0.53	20.10±0.45	19.26±0.3	18.70±0.24	17.19±0.37	17.22±0.31
5	Brightness, % (ISO)	34.8±0.5	40.20±0.2	41.33±0.4	42.14±0.7	43.90±1.0	43.66±0.7
Conditions of treatment		X			E		
Xylanase dose, IU/g		8			–		
Consistency, %		Variable			10		
Temperature, ^{0}C		55±2			70±2		
Time, min		120			90		
pH		6.4			11.5		

± refers standard deviation

Table 5.6: Spectrophotometric analysis of filtrates generated during xylanase treatment of sugarcane bagasse soda-AQ pulp at different pulp consistency

Sl. No.	Particulars	Test results					
1	Consistency, %	2	4	6	8	10	12
Absorbance, nm							
2	237	0.198±0.012	0.256±0.015	0.335±0.008	0.435±0.008	0.449±0.01	0.451±0.015
3	254	0.123±0.007	0.156±0.005	0.203±0.018	0.262±0.009	0.270±0.01	0.271±0.017
4	280	0.088±0.003	0.113±0.007	0.157±0.016	0.205±0.01	0.209±0.02	0.209±0.02
5	465	0.073±0.002	0.128±0.005	0.180±0.009	0.210±0.02	0.219±0.018	0.223±0.011
Conditions of treatment		X					
Xylanase dose, IU/g		8					
Time, min		120					
Temperature, ^{0}C		55±2					
pH		6.4					

± refers standard deviation

Table 5.7: Effect of conventional bleaching on pulp shrinkage, brightness and viscosity of soda-AQ pulp of sugarcane bagasse

Particulars	Bleaching sequence		
	CEHH	CEHHP	OCEHH
Unbleached pulp kappa number	24.26	24.26	24.26
Unbleached pulp brightness, % (ISO)	34.3	34.3	34.3
Unbleached pulp viscosity, cps	26.5	26.5	26.5
Oxygen stage (O)			
O_2 pressure, kg/cm^2			5
$MgSO_4$ applied, % (O.D. pulp basis)	–	–	0.1
NaOH applied, % (O.D. pulp basis)	–	–	1.5
Final pH	–	–	11.1
kappa number of O_2 delignified pulp	–	–	15.00
Chlorination stage (C)			
Cl_2 applied as available Cl_2, % (O.D. pulp basis)	3.03	3.03	1.88
Cl_2 consumed, % (O.D. pulp basis)	3.02	3.026	1.878
Amount of Cl_2 consumed, %	99.7	99.86	99.9
Final pH	1.75	1.71	1.75
Alkali extraction stage (E)			
NaOH applied, % (O.D. pulp basis)	1.55	1.55	0.97
Initial pH	11.0	11.0	11.0
Final pH	11.2	11.6	11.1
Hypochlorite stage (H_1)			
Hypo applied as available Cl_2, % (O.D. pulp basis)	2.27	2.27	1.41
Hypo consumed as available Cl_2, % (O.D. pulp basis)	2.18	2.19	1.31
Hypo consumed, %	96.03	96.5	92.9
Final pH	11.5	11.2	11.2
Hypochlorite stage (H_2)			
Hypo applied as available Cl_2, % (O.D. pulp basis)	0.75	0.75	0.47
Hypo consumed as available Cl_2, % (O.D. pulp basis)	0.69	0.71	0.39
Hypo consumed, %	92.0	94.6	82.9
Final pH	11.2	11.3	11.0
Peroxide stage (P)			
H_2O_2 applied, % (O.D. pulp basis)	–	0.5	–
EDTA applied, % (O.D. pulp basis)	–	0.5	–
$MgSO_4$ applied, % (O.D. pulp basis)	–	0.1	–
Final pH	–	10.7	–
Total Cl_2 applied, % (O.D. pulp basis)	6.06	6.06	3.75
Total Cl_2 consumed, % (O.D. pulp basis)	5.89	5.93	3.58
Total Cl_2 consumed on Cl_2 basis, %	97.2	97.9	95.5
Total residual Cl_2, %	2.8	2.14	4.53
Bleaching losses, %	9.0	9.2	8.78
Bleached pulp yield, %	40.81±1.3	40.72±2.2	40.91±1.5
Pulp brightness, % (ISO)	80.1±0.5	81.6±0.5	83.2±0.38
Pulp viscosity, cps	9.3±0.021	9.18±0.04	9.32±0.034

Bleaching conditions	O	C	H_1	H_2	P
Consistency, %	10	3	10	10	10
Temperature, ^{0}C	90±2	Ambient	45±2	45±2	90±2
Time, min	90	30	60	60	60

± refers standard deviation

Table 5.8: Effect of xylanase pretreatment on pulp shrinkage, brightness and viscosity of sugarcane bagasse soda-AQ pulp during conventional bleaching

Particulars	Bleaching sequence		
	XECEHH	XECEHHP	XOCEHH
Unbleached pulp kappa number	24.26	24.26	24.26
Unbleached pulp brightness, % (ISO)	34.3	34.3	34.3
Unbleached pulp viscosity, cps	26.5	26.5	26.5
Xylanase stage (X)			
Amount of xylanase added (on O.D. pulp basis), IU/g	8	8	8
Final pH	6.4	6.4	6.4
Alkali extraction stage (E)			
NaOH applied, % (O.D. pulp basis)	1.5	1.5	–
Initial pH	11.4	11.5	–
Final pH	10.2	10.7	–
kappa number of xylanase treated pulp	17.03	17.03	–
Oxygen stage (O)			
O_2 pressure, kg/cm^2	–	–	5
$MgSO_4$ applied, % (O.D. pulp basis)	–	–	0.1
NaOH applied, % (O.D. pulp basis)	–	–	1.5
Final pH			11.2
Kappa number of xylanase and O_2 delignified pulp	–	–	9.51
Chlorination stage (C)			
Cl_2 applied, % (O.D. pulp basis)	2.13	2.13	1.18
Cl_2 consumed, % (O.D. pulp basis)	2.12	2.125	1.177
Amount of Cl_2 consumed, %	99.5	99.8	99.74
Final pH	2.5	2.5	2.5
Alkali extraction stage (E)			
NaOH applied, % (O.D. pulp basis)	1.09	1.09	0.62
Initial pH	11.4	11.5	11.4
Final pH	11.2	11.4	11.1
Hypochlorite stage (H_1)			
Hypo applied as available Cl_2, % (O.D. pulp basis)	1.6	1.6	0.89
Hypo consumed as available Cl_2, % (O.D. pulp basis)	1.51	1.52	0.81
Hypo consumed, %	94.4	95.0	91.01
Final pH	11.0	11.2	11.5
Hypochlorite stage (H_2)			
Hypo applied as available Cl_2, % (O.D. pulp basis)	0.53	0.53	0.30
Hypo consumed as available Cl_2, % (O.D. pulp basis)	0.48	0.49	0.26
Hypo consumed, %	90.56	92.45	86.6
Final pH	11.2	11.2	11.0
Peroxide stage (P)			
H_2O_2 applied, % (O.D. pulp basis)	–	0.5	–
EDTA applied, % (O.D. pulp basis)	–	0.5	–
$MgSO_4$ applied, % (O.D. pulp basis)	–	0.1	–
Final pH	–	10.5	–
Total Cl_2 applied, % (O.D. pulp basis)	4.26	4.26	2.38
Total Cl_2 consumed, % (O.D. pulp basis)	4.11	4.135	2.247
Total Cl_2 consumed, %	96.48	97.06	94.41
Total residual Cl_2, %	3.52	2.93	5.6
Bleaching losses, %	8.4	8.7	8.5
Bleached pulp yield, %	41.08±2.1	40.95±3.0	41.04±1.8
Pulp brightness, % (ISO)	84.5±0.7	85.7±0.36	86.9±0.71
Pulp viscosity, cps	9.364±0.01	9.28±0.011	9.56±0.026

Bleaching conditions	X	O	C	E	H_1	H_2
Consistency, %	10	10	3	10	10	10
Temperature, ^{0}C	55±2	90±2	Ambient	60±2	45±2	90±2
Time, min	120	90	30	60	60	60

± refers standard deviation

Table 5.9: Comparison of mechanical strength properties and combined effluent generated during conventional bleaching

Sl. No.	Particulars	CEHH	XECEHH	% difference	CEHHP	XECEHHP	% difference	OCEHH	XOCEHH	% difference
1	Total chlorine demand	6.06	4.26	–29.70	6.06	4.26	–29.70	3.75	2.38	–36.53
2	Pulp brightness, (ISO), %	80.1±0.5	84.5±0.7	+4.40	81.6±0.5	85.7±0.36	+4.1	83.2±0.38	86.9±0.71	+3.7
3	Beating level, °SR	35	35	–	35	35	–	35	35	–
4	Tear index, mNm^2/g	4.57±0.22	4.82±0.35	+5.47	4.51±0.34	4.75±0. 15	+5.32	4.82±0. 17	4.82±0.21	–
5	Burst index, $kPam^2/g$	2.95±0.25	3.51±0.18	+18.98	2.98±0.2	3.45±0.31	+15.8	3.22±0. 1	3.72±0. 6	+15.53
6	Tensile index, Nm/g	48.38±1.9	56.12±1.5	+15.99	48.57±3.1	55.12±2.2	+13.48	51.34±2.4	56.8±1.7	+10.63
7	Double fold, number	34±4.2	38±2.2	+11.76	36±2.8	38±3.3	+5.6	37±2.1	40±3.9	+8.1
8	Pulp viscosity, cps	9.30±0.011	9.36±0.009	+0.65	9.18±0.011	9.28±0.015	+1.09	9.32±0.024	9.56±0.026	+2.57
9	Copper number	0.25±0.003	0.18±0.005	–28	0.22±0.004	0.15±0.002	–31.81	0.13±0.004	0.11±0.002	–15.38
10	COD, mg/L	723	787	+8.85	701	745	+6.27	598	650	+8.7
11	Color, PTU	2250	2500	+11.11	1600	1840	+15	1560	1825	+16.99
12	AOX, kg/t	2.77	1.99	–28.16	2.21	1.60	–27.6	2.02	1.32	–34.65

± refers standard deviation

Table 5.10: Effect of ECF bleaching on pulp shrinkage, brightness and viscosity of soda-AQ pulp of sugarcane bagasse

Particulars	Bleaching sequence	
	ODED	OD(E_{OP})DP
Unbleached pulp kappa number	24.26	24.26
Unbleached pulp brightness, % (ISO)	34.3	34.3
Unbleached pulp viscosity, cps	26.5	26.5
Oxygen stage (O)		
O_2 pressure, kg/cm^2	5	5
$MgSO_4$ applied, % (O.D. pulp basis)	0.1	0.1
NaOH applied, % (O.D. pulp basis)	1.5	1.5
Final pH	11.4	11.6
Chlorine dioxide stage (D_1)		
ClO_2 applied as available Cl_2, % (O.D. pulp basis)	1.0	1.0
ClO_2 consumed as available Cl_2, % (O.D. pulp basis)	0.93	0.91
ClO_2 consumed on Cl_2 basis, %	93.0	91.0
Final pH	4.0	4.0
Alkali extraction stage (E)		
NaOH applied, % (O.D. pulp basis)	2.5	–
Initial pH	11.7	–
Final pH	9.9	–
Extraction stage (E_{OP})		
NaOH applied, % (O.D. pulp basis)	–	3.0
H_2O_2 applied, % (O.D. pulp basis)	–	0.5
O_2 pressure, kg/cm^2	–	5.0
$MgSO_4$ applied, % (O.D. pulp basis)	–	0.1
Final pH	–	11.3
Chlorine dioxide stage (D_2)		
ClO_2 applied as available Cl_2, % (O.D. pulp basis)	0.6	0.6
ClO_2 consumed as available Cl_2, % (O.D. pulp basis)	0.56	0.57
ClO_2 consumed, %	93.3	95.0
Final pH	4.1	4.0
Peroxide stage (P)		
H_2O_2 applied, % (O.D. pulp basis)	–	0.5
EDTA applied, % (O.D. pulp basis)	–	0.5
$MgSO_4$ applied, % (O.D. pulp basis)	–	0.1
Final pH		10.4
Total ClO_2 applied, % (O.D. pulp basis)	1.6	1.6
Total ClO_2 consumed, % (O.D. pulp basis)	1.49	1.48
Total ClO_2 consumed on Cl_2 basis, %	93.1	92.5
Total residual ClO_2 on Cl_2 basis, %	6.9	7.5
Bleaching losses, %	7.5	7.7
Bleached pulp yield, %	41.49±2.5	41.4±3.3
Pulp brightness, % (ISO)	79.2±0.47	85.5±1.0
Pulp viscosity, cps	9.36±0.022	9.38±0.034

Bleaching conditions	O	D_1	E	E_{OP}	D_2	P
Consistency, %	10	10-12	10	11.5	10-12	10
Temperature, ^{0}C	90±2	70±2	60±2	75±2	70±2	90±2
Time, min	90	180	60	70	180	60

± refers standard deviation

Table 5.11: Effect of xylanase pretreatment on pulp shrinkage, brightness and viscosity of soda-AQ pulp of sugarcane bagasse during ECF bleaching

Particulars	Bleaching sequence	
	XODED	XOD(E_{OP})DP
Unbleached pulp kappa number	24.26	24.26
Unbleached pulp brightness, % (ISO)	34.3	34.3
Unbleached pulp viscosity, cps	26.5	26.5
Xylanase stage (X)		
Amount of xylanase added (on O.D. pulp basis), IU/g	8	8
Final pH	6.2	6.5
Oxygen stage (O)		
O_2 pressure, kg/cm^2	5	5
$MgSO_4$ applied, % (O.D. pulp basis)	0.1	0.1
NaOH applied, % (O.D. pulp basis)	1.5	1.5
Final pH	11.6	11.5
Chlorine dioxide stage (D_1)		
ClO_2 applied as available Cl_2, % (O.D. pulp basis)	1.0	1.0
ClO_2 consumed as available Cl_2, % (O.D. pulp basis)	0.902	0.86
ClO_2 consumed, %	90.2	86.0
Final pH	4.1	4.2
Alkali extraction stage (E)		
NaOH applied, % (O.D. pulp basis)	2.5	–
Initial pH	11.7	–
Final pH	9.9	–
Extraction stage		
NaOH applied, % (O.D. pulp basis)	–	3.0
H_2O_2 applied, % (O.D. pulp basis)	–	0.5
O_2 pressure, kg/cm^2	–	5.0
$MgSO_4$ applied, % (O.D. pulp basis)	–	0.1
Final pH	–	11.6
Chlorine dioxide stage (D_2)		
ClO_2 applied as available Cl_2, % (O.D. pulp basis)	0.6	0.6
ClO_2 consumed as available Cl_2, % (O.D. pulp basis)	0.55	0.549
ClO_2 consumed, %	91.6	91.5
Final pH	4.1	4.0
Peroxide stage (P)		
H_2O_2 applied, % (O.D. pulp basis)	–	0.5
EDTA applied, % (O.D. pulp basis)		0.5
$MgSO_4$ applied, % (O.D. pulp basis)		0.1
Final pH		10.9
Total ClO_2 applied, % (O.D. pulp basis)	1.6	1.6
Total ClO_2 consumed, % (O.D. pulp basis)	1.45	1.409
Total ClO_2 consumed, %	90.6	88.06
Total residual ClO_2, %	9.4	11.94
Bleaching losses, %	7.6	7.7
Bleached pulp yield, %	41.44±2.6	41.40±1.4
Pulp brightness % (ISO)	84.6±0.55	90.2±1.3
Pulp viscosity, cps	9.40±0.016	9.43±0.021

Bleaching conditions	X	O	D_1	E	E_{OP}	D_2	P
Consistency, %	10	10	10-12	10	11.5	10-12	10
Temperature, ^{0}C	55±2	90±2	70±2	60±2	75±2	70±2	90±2
Time, min	120	90	180	60	70	180	60

± refers standard deviation

Table 5.12: Comparison of mechanical strength properties and combined effluent generated during ECF bleaching

Sl. No.	Particulars	ODED	XODED	% difference	OD(E_{OP})DP	XOD(E_{OP})DP	% difference
1	Pulp brightness, % (ISO)	79.2±0.47	86.5±0.55	+7.3	85.5±1.0	90.2±1.3	+4.7
2	Beating level, ^{0}SR	35	35	–	35	35	–
3	Tear index, mNm^2/g	5.06±0.22	5.4±0.1	+6.72	5.01±0.13	5.26±0.2	+4.99
4	Burst index, kPam^2/g	3.7±0.12	3.45±0.2	–6.76	3.6±0.1	3.09±0.2	–14.17
5	Tensile index, Nm/g	53.14±3.1	48.3±1.25	–9.1	51.47±1.4	45.16±2.27	–12.26
6	Double fold, number	58±3.2	52±5.3	–10.34	44±4.7	27±2.2	–38.64
7	Pulp viscosity, cps	9.36±0.009	9.40±0.005	+0.43	9.38±0.004	9.43±0.011	+0.53
8	Copper number	0.13±0.01	0.08±0.005	–38.46	0.08±0.005	0.06±0.001	–25
9	COD, mg/L	551	623	+13.07	570	655	+14.91
10	Color, PTU	1505	1772	+17.74	1750	1884	+7.66
11	AOX, kg/t	0.42	0.246	–41.43	0.35	0.21	–40

± refers standard deviation

Table 5.13: Effect of TCF bleaching on pulp shrinkage, brightness and viscosity of sugarcane bagasse soda-AQ pulp

Particular				O(E_{OP})P
Unbleached pulp kappa number				24.26
Unbleached pulp brightness, % (ISO)				34.3
Unbleached pulp viscosity, cps				26.5
Oxygen stage (O)				
O_2 pressure, kg/cm^2				5
$MgSO_4$ applied, % (O.D. pulp basis)				0.1
Final pH				11.8
Extraction stage				
NaOH applied, % (O.D. pulp basis)				3.0
H_2O_2 applied, % (O.D. pulp basis)				0.5
O_2 pressure, kg/cm^2				5.0
$MgSO_4$ applied, % (O.D. pulp basis)				0.1
Final pH				11.6
Peroxide stage (P)				
H_2O_2 applied, % (O.D. pulp basis)				2.0
EDTA applied, % (O.D. pulp basis)				0.5
$MgSO_4$ applied, % (O.D. pulp basis)				0.1
Final pH				11.7
Total H_2O_2 applied, % (O.D. pulp basis)				2.5
Bleaching losses, %				7.1
Bleached pulp yield, %				41.67±0.6
Pulp brightness ,% (ISO)				71.3±1.05
Pulp viscosity, cps				9.8±0.011
Bleaching conditions	O	(E_{OP})	P	
Consistency, %	10	11.5	10	
Temperature, ^{0}C	90±2	75±2	90±2	
Time, min	90	70	90	

± refers standard deviation

Table 5.14: Effect of xylanase pretreatment on pulp shrinkage, brightness and viscosity of sugarcane bagasse soda-AQ pulp during TCF bleaching

Particular	OX(E_{OP})P	XO(E_{OP})P
Unbleached pulp kappa number	24.26	24.26
Unbleached pulp brightness, % (ISO)	34.3	34.3
Unbleached pulp viscosity, cps	26.5	26.5
Xylanase stage (X)		
Amount of xylanase added (on O.D. pulp basis), IU/g	–	8
Final pH	–	6.4
Oxygen stage (O)		
O_2 pressure, kg/cm^2	5	5
$MgSO_4$ applied, % (O.D. pulp basis)	0.1	0.1
NaOH applied, % (O.D. pulp basis)	1.5	1.5
Final pH	11.3	11.2
Xylanase stage (X)		
Amount of xylanase added on pulp, IU/g	8	–
Final pH	6.4	–
Extraction stage		
NaOH applied, % (O.D. pulp basis)	3.0	3.0
H_2O_2 applied, % (O.D. pulp basis)	0.5	0.5
O_2 pressure, kg/cm^2	5.0	5.0
$MgSO_4$ applied, % (O.D. pulp basis)	0.1	0.1
Final pH	11.6	11.4
Peroxide stage (P)		
H_2O_2 applied, % (O.D. pulp basis)	2.0	2.0
EDTA applied, % (O.D. pulp basis)	0.5	0.5
$MgSO_4$ applied, % (O.D. pulp basis)	0.1	0.1
Final pH	11.2	11.1
Total H_2O_2 applied, % (O.D. pulp basis)	2.5	2.5
Bleaching losses, %	7.5	7.8
Bleached pulp yield, %	41.49±1.5	41.35±1.2
Pulp brightness % (ISO)	77.4±1.01	79.5±0.5
Pulp viscosity, cps	10.13±0.013	10.05±0.023

Bleaching conditions	X	O	(E_{OP})	P
Consistency, %	10	10	11.5	10
Temperature, ^{0}C	55±2	90±2	75±2	90±2
Time, min	120	90	70	90

± refers standard deviation

Table 5.15: Comparison of mechanical strength properties and combined effluent generated during TCF bleaching

Sl. No.	Particulars	O(E_{OP})P	OX(E_{OP})P	% difference	XO(E_{OP})P	% difference
1	Pulp brightness, % (ISO)	71.3±1.05	77.4±1.01	+6.1	79.5±0.5	+8.2
2	Beating level, ^{0}SR	35	35	–	35	–
3	Tear index, mNm^2/g	5.2±0.1	5.51±0.12	+5.96	5.4±0.24	+3.85
4	Burst index, kPam^2/g	3.08±0.09	2.57±0.07	–16.56	2.45±0.13	–20.45
5	Tensile index, Nm/g	51.38±1.0	47.12±1.02	–8.29	42.09±2.6	–18.08
6	Double fold, number	27±3.1	22±1.6	–18.51	24±2.5	–11.11
7	Pulp viscosity, cps	9.8±0.011	10.13±0.013	+3.37	10.05±0.008	+2.55
8	Copper number	0.09±0.001	0.06±0.003	–33.3	0.06±0.001	–33.3
9	COD, mg/L	710	745	+4.92	776	+9.3
10	Color, PTU	2692	3250	+20.72	3580	+32.98

± refers standard deviation

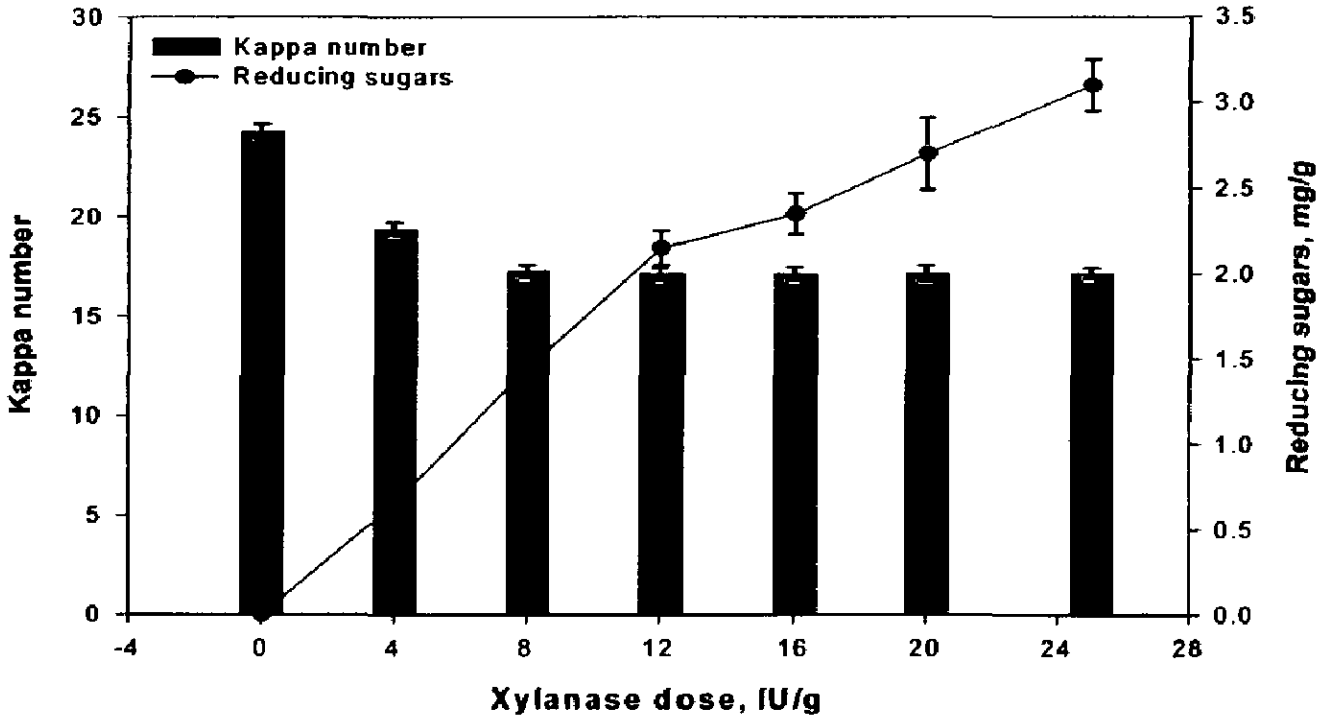

Figure 5.1: Effect of xylanase doses on reducing sugars released and kappa number of soda-AQ pulp of sugarcane bagasse

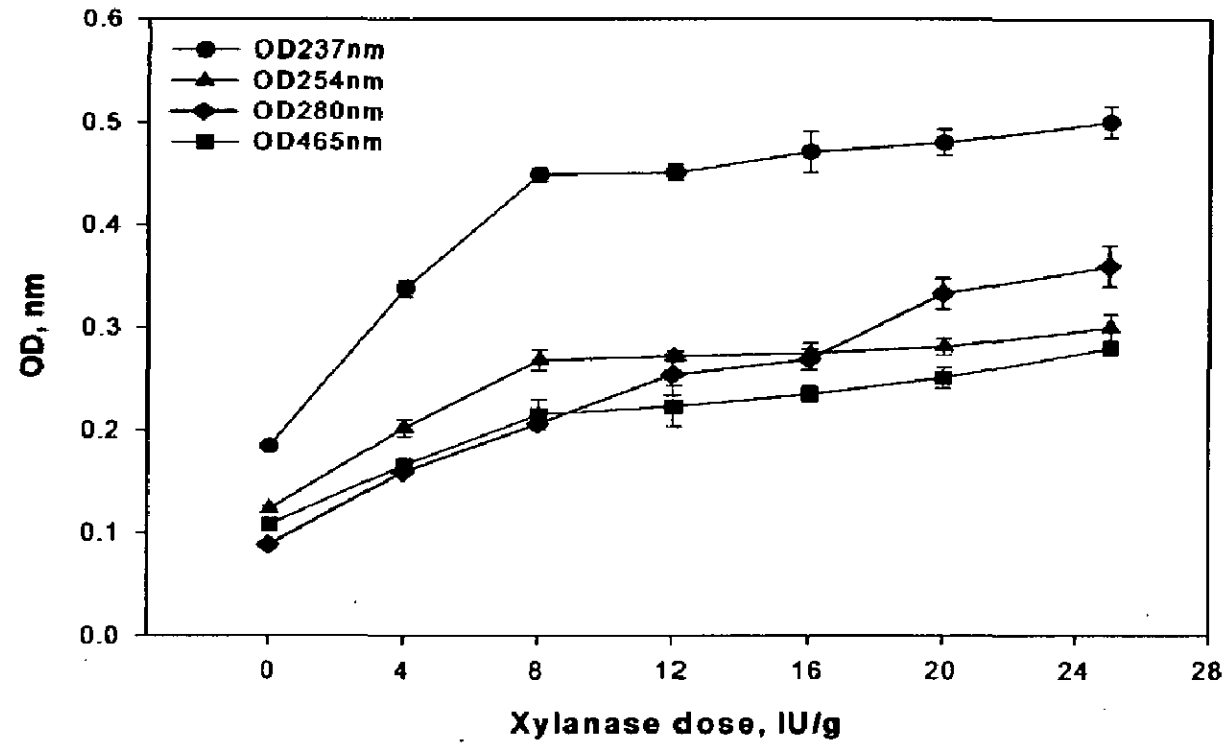

Figure 5.2: Spectrophotometric analysis of pulp filtrate generated during xylanase treatment of soda-AQ pulp of sugarcane bagasse at different xylanase doses

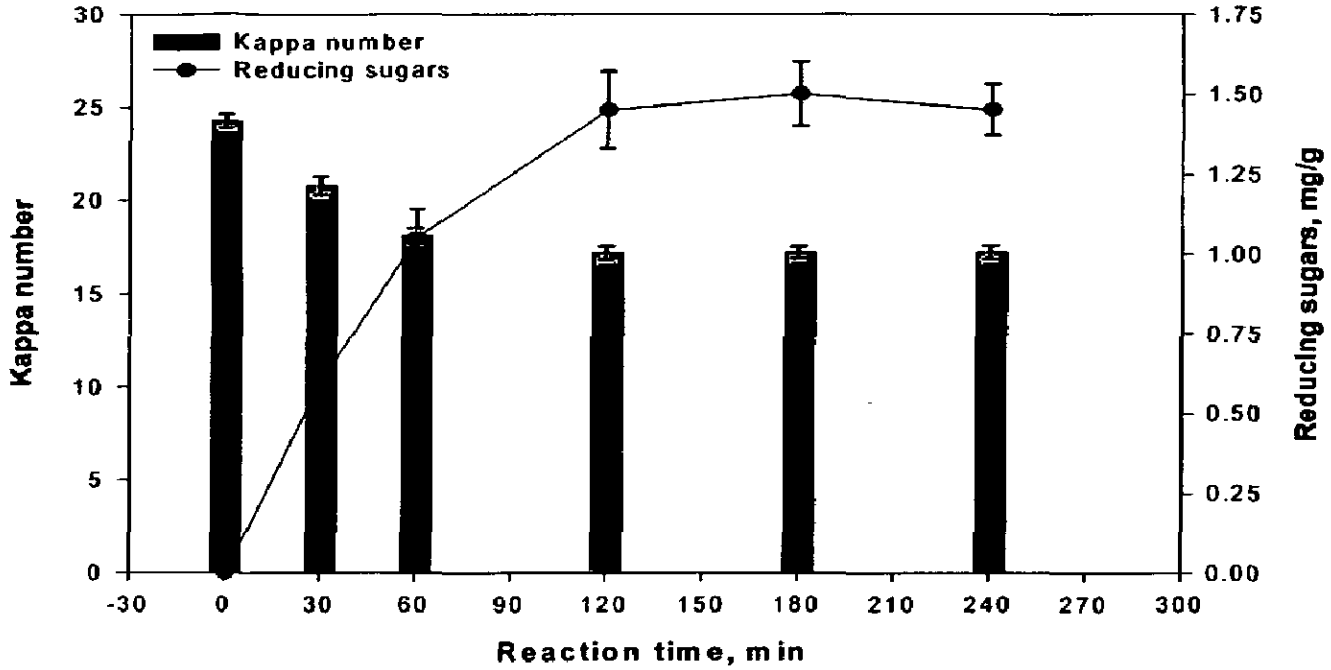

Figure 5.3: Effect of reaction time on reducing sugars released and kappa number of soda-AQ pulp of sugarcane bagasse during xylanase treatment

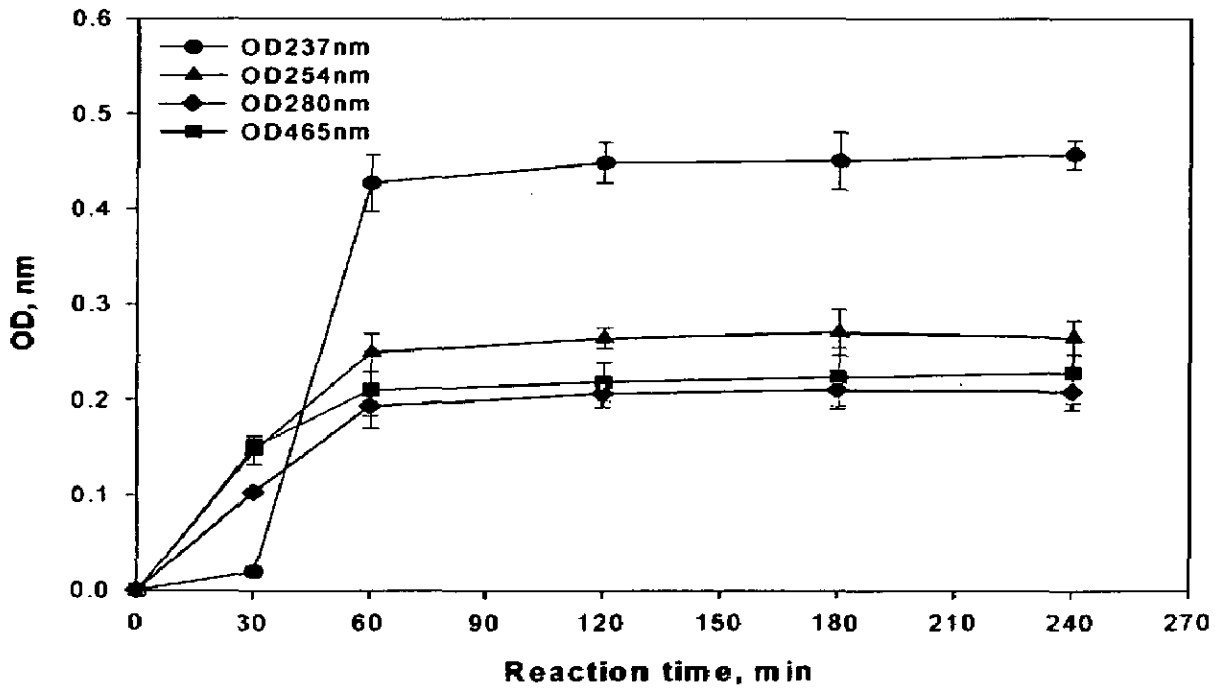

Figure 5.4: Spectrophotometric analysis of pulp filtrates generated during xylanase treatment of soda-AQ pulp of sugarcane bagasse at different reaction times

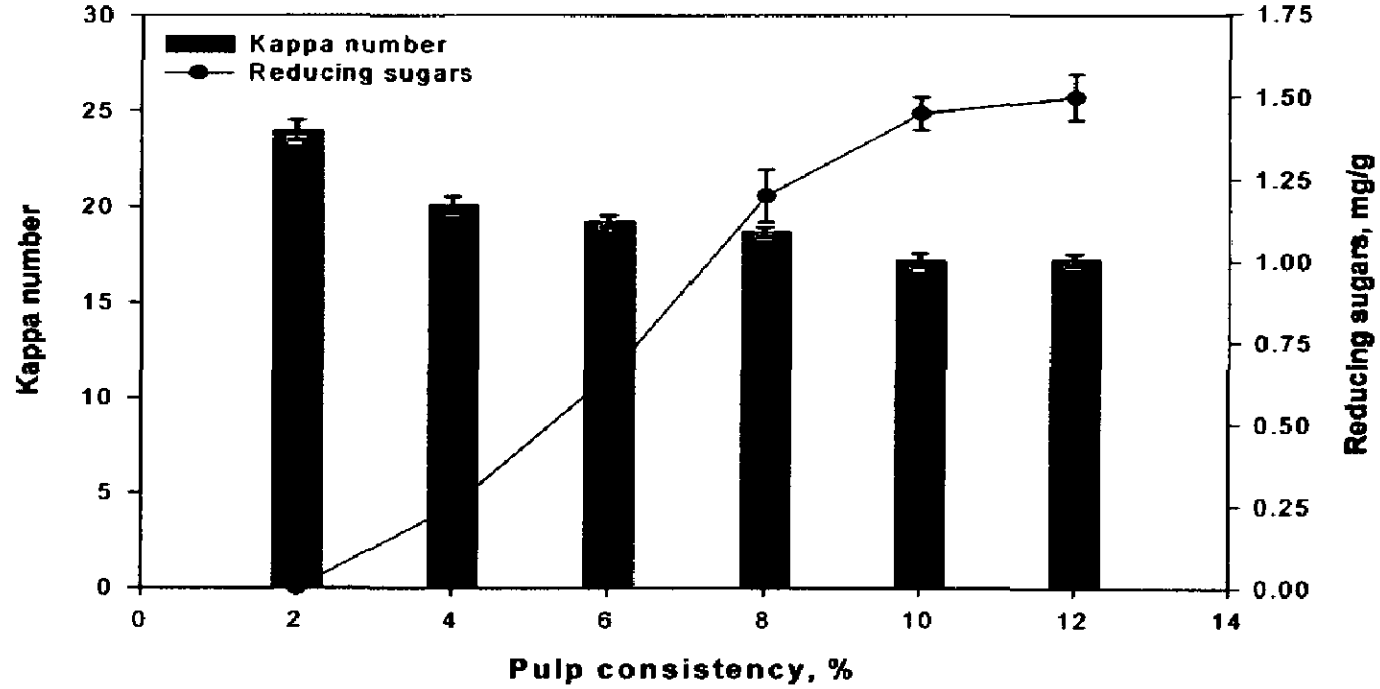

Figure 5.5: Effect of pulp consistency on reducing sugars released and kappa number during xylanase treatment of soda-AQ pulp of sugarcane bagasse

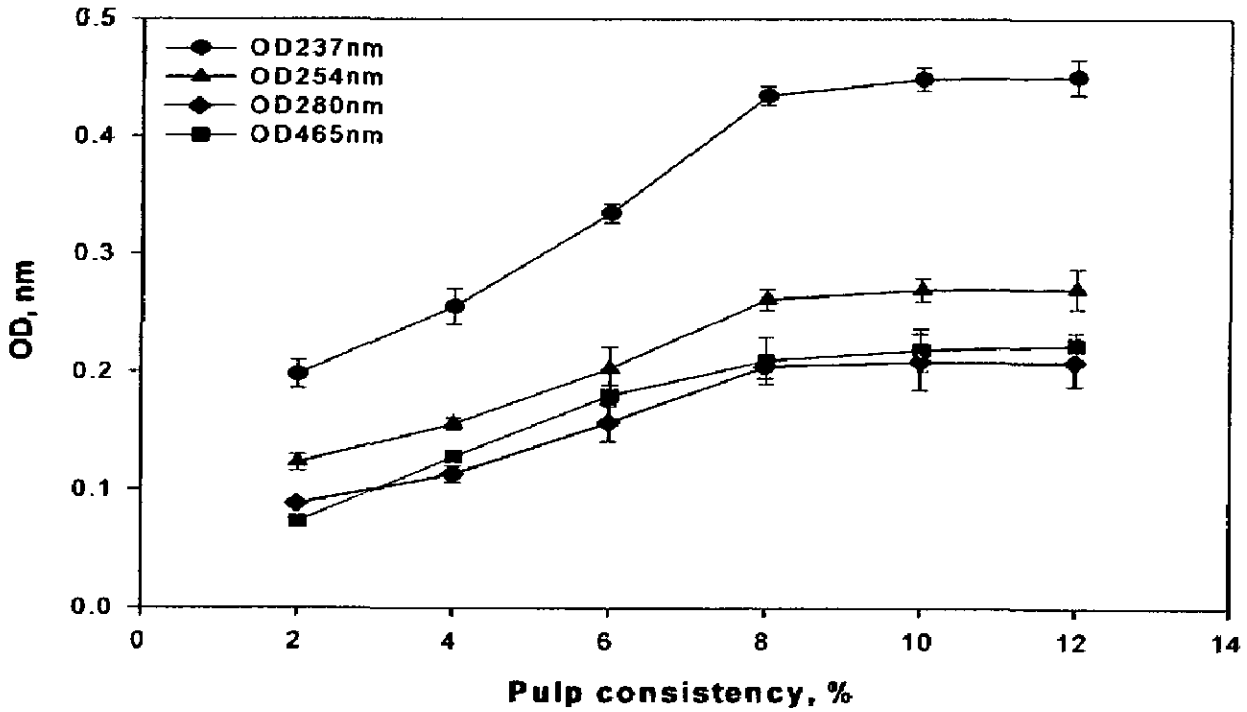

Figure 5.6: Spectrophotometric analysis of pulp filtrates generated during xylanase treatment of sugarcane bagasse soda-AQ pulp at different pulp consistency

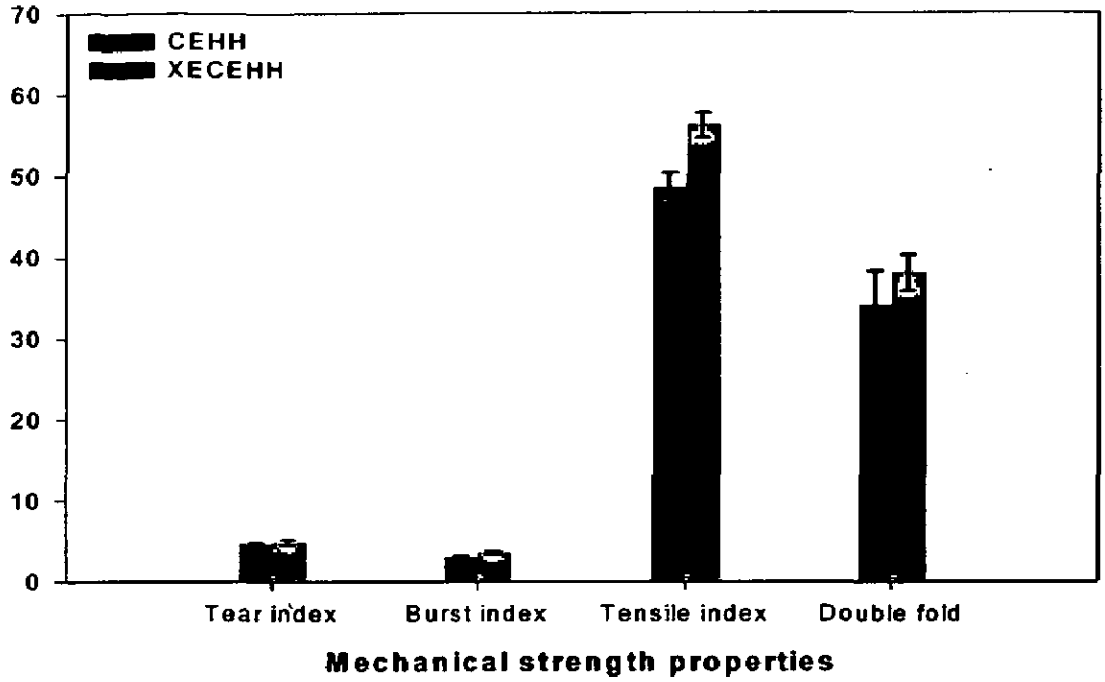

Figure 5.7: Comparison of mechanical strength properties of soda-AQ pulp of sugarcane bagasse during CEHH and XECEHH beaching sequences

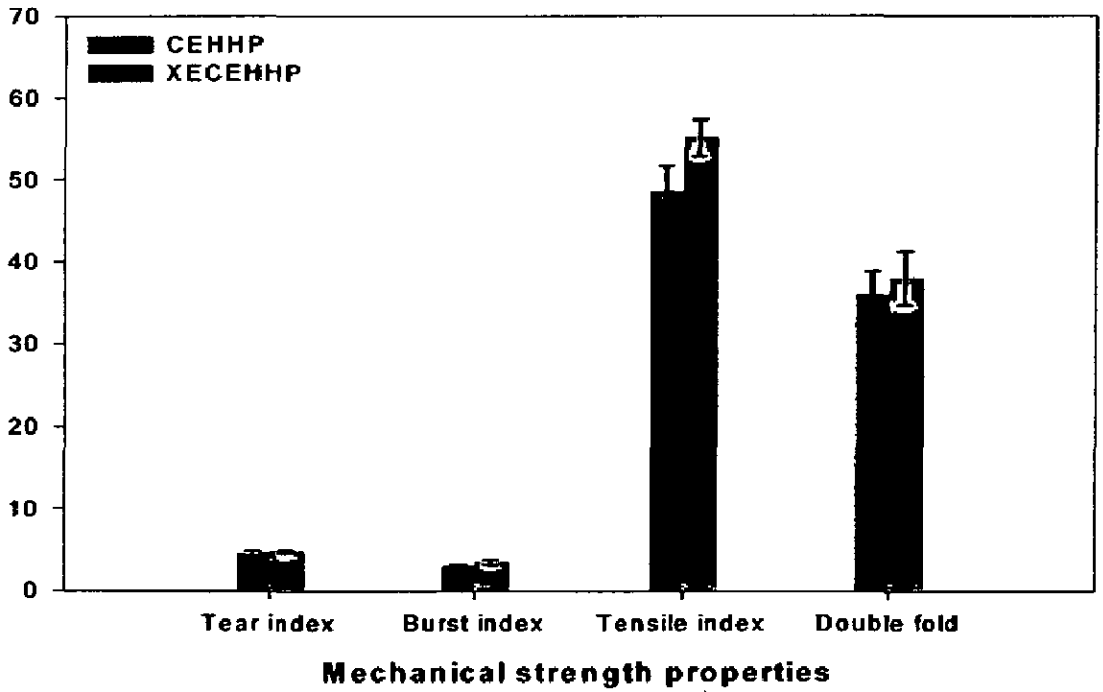

Figure 5.8: Comparison of mechanical strength properties of soda-AQ pulp of sugarcane bagasse during CEHHP and XECEHHP bleaching sequences

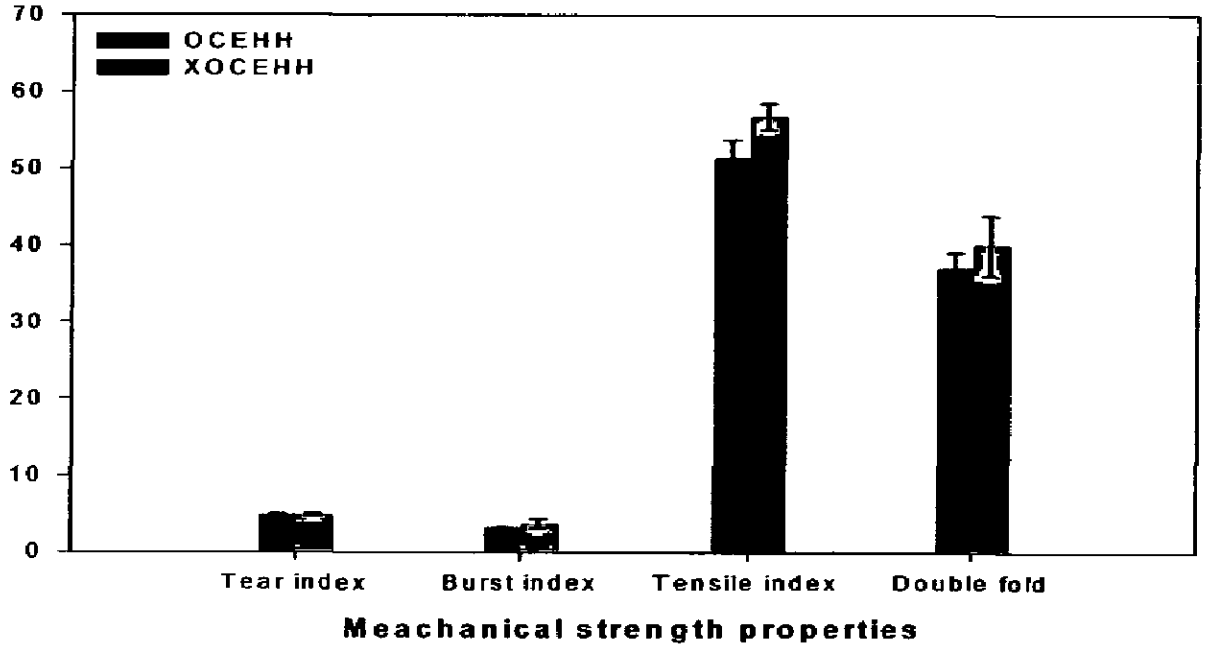

Figure 5.9: Comparison of mechanical strength properties of soda-AQ pulp of sugarcane bagasse during OCEHH and XOCEHH bleaching sequences

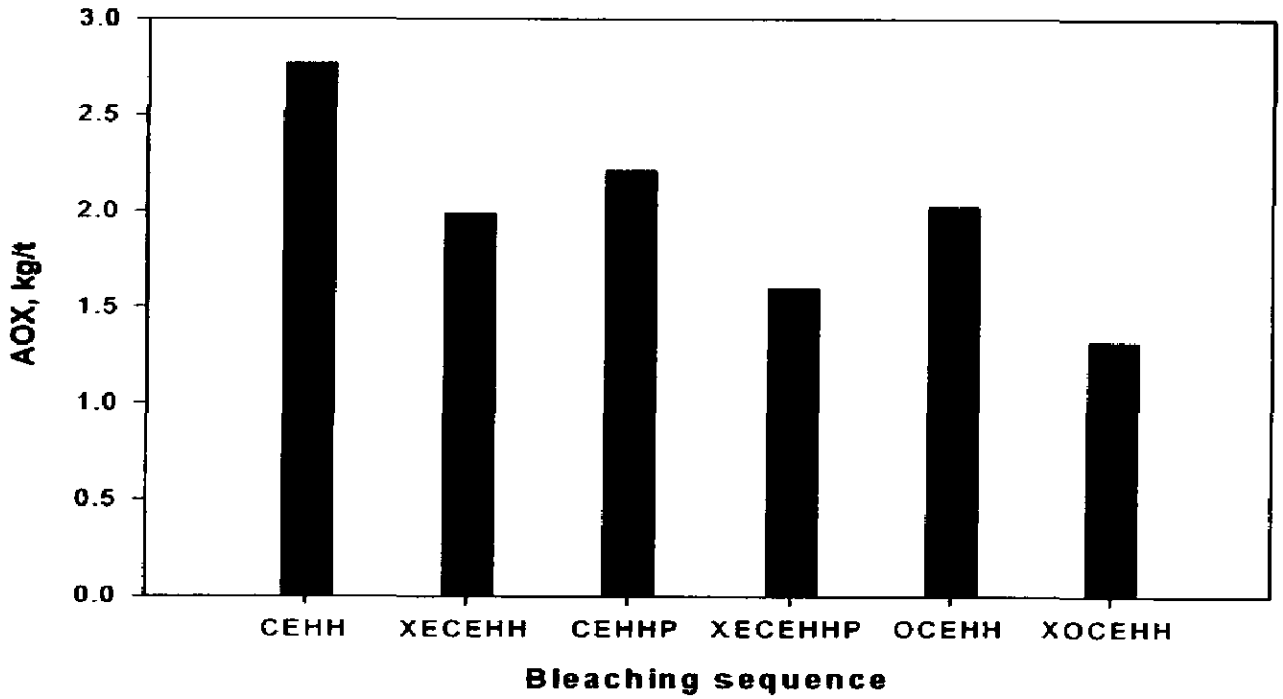

Figure 5.10: Comparison of AOX formation during conventional bleaching sequences of soda-AQ pulp of sugarcane bagasse

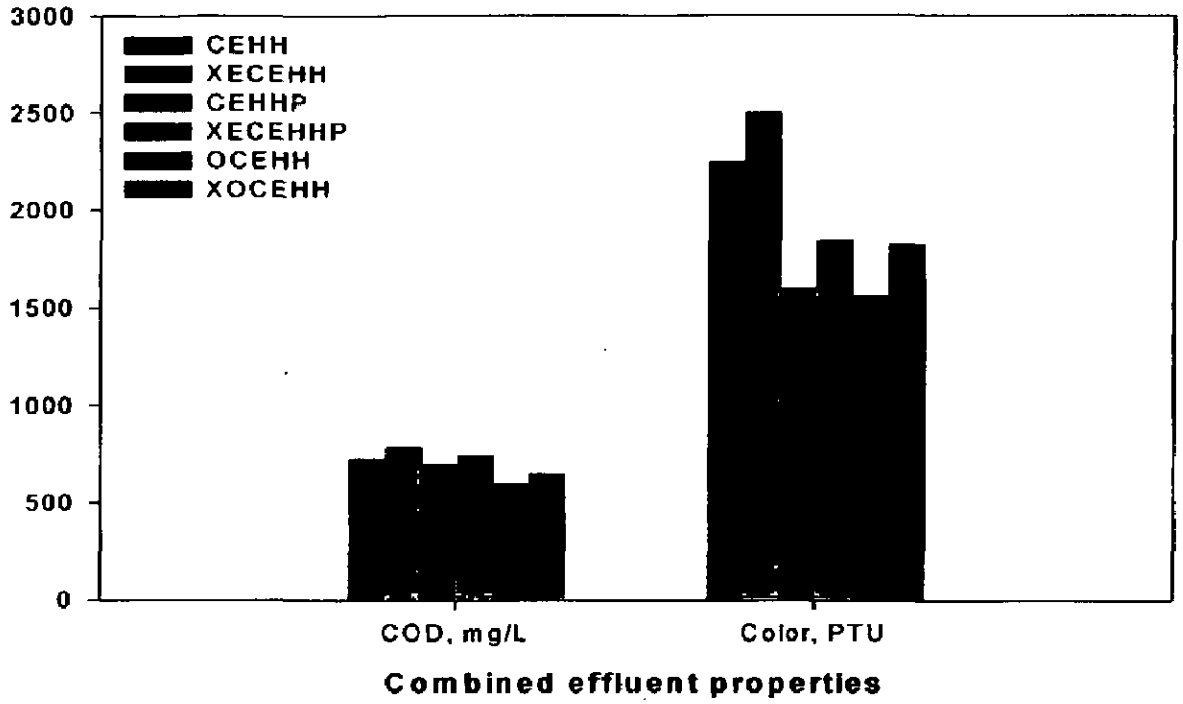

Figure 5.11: Comparison of COD and color of combined bleach effluents generated during conventional bleaching of soda-AQ pulp of sugarcane bagasse

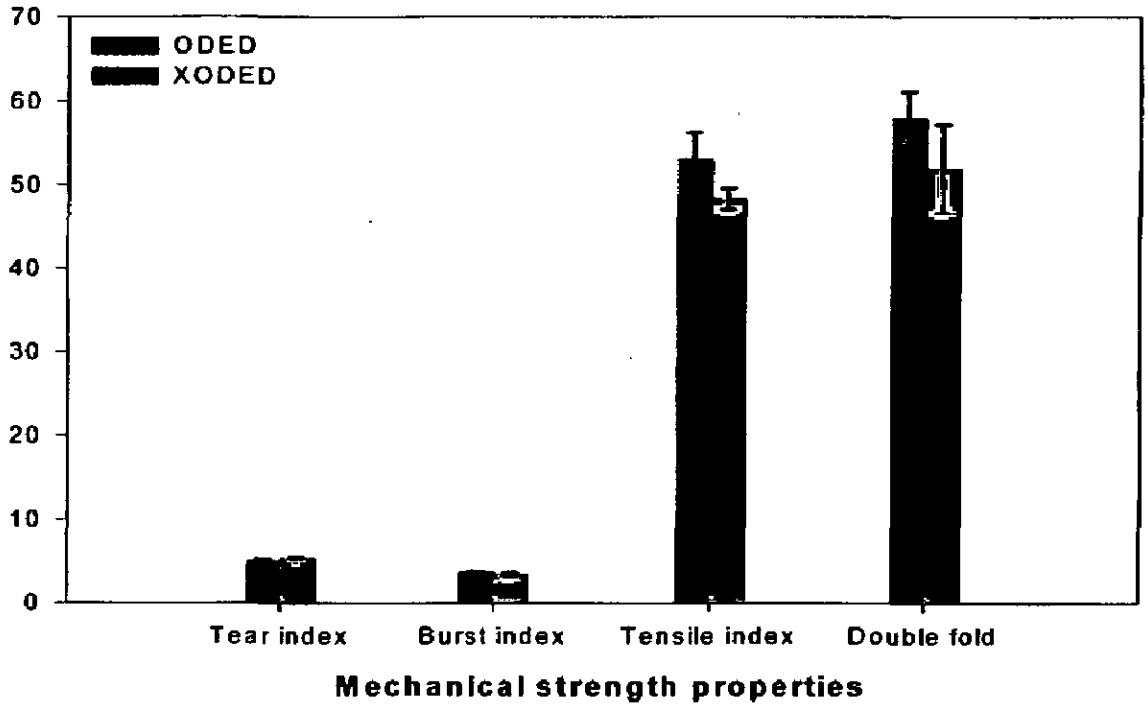

Figure 5.12: Comparison of mechanical strength properties of soda-AQ pulp of sugarcane bagasse during ODED and XODED bleaching sequences

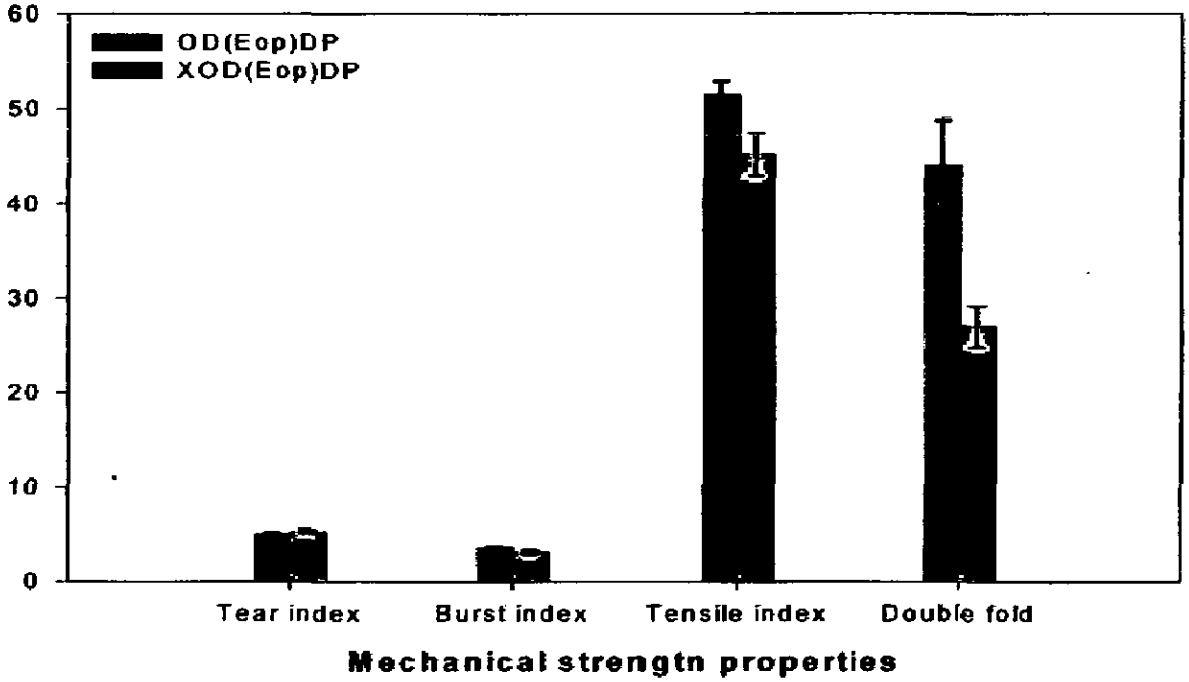

Figure 5.13: Comparison of mechanical strength properties of soda-AQ pulp of sugarcane bagasse during OD(E_{OP})DP and XOD(E_{OP})DP bleaching sequences

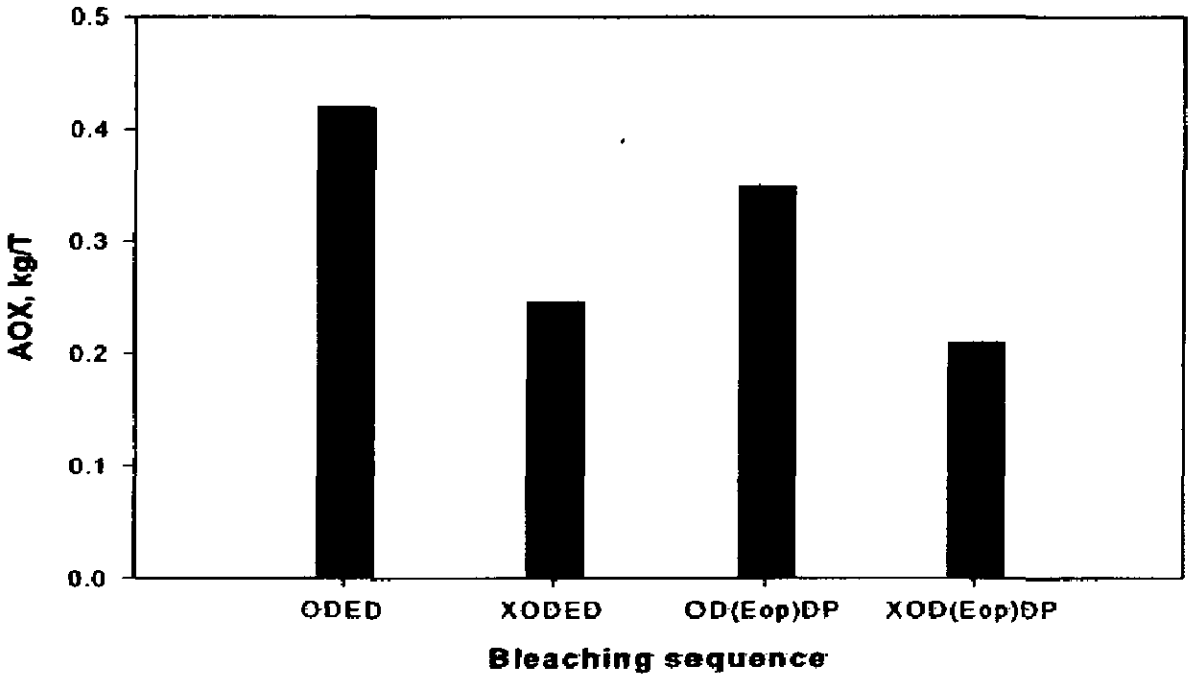

Figure 5.14: Comparison of AOX formation during ECF bleaching sequences of soda-AQ pulp of sugarcane bagasse

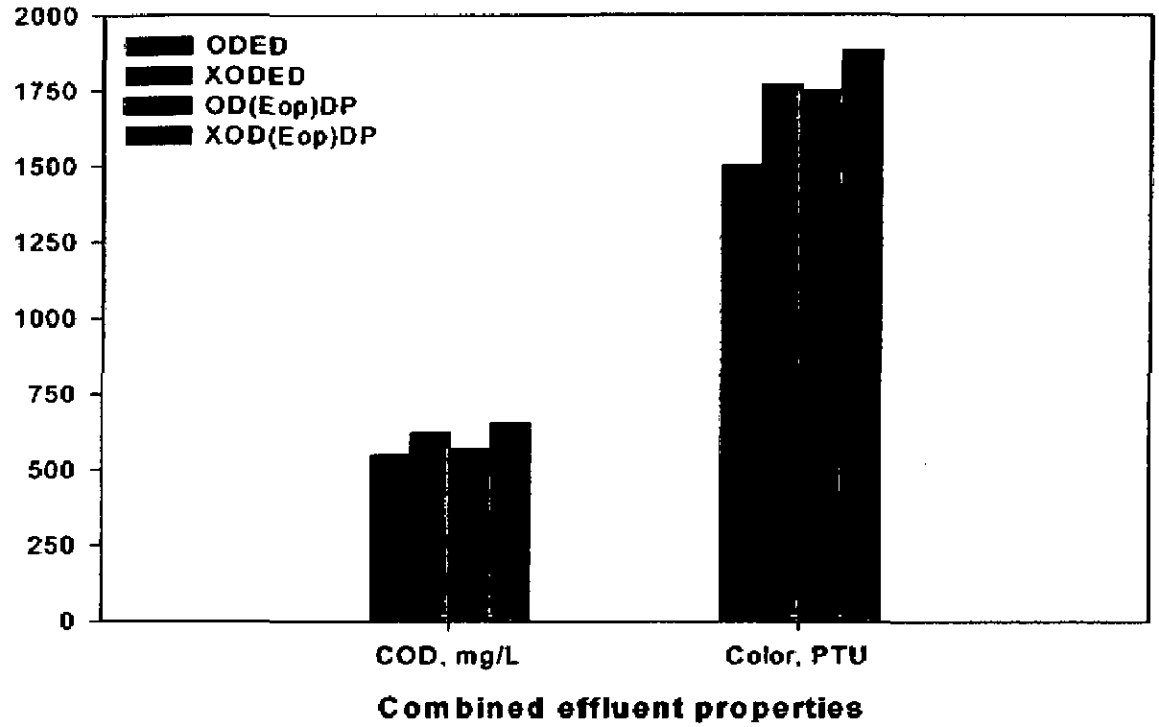

Figure 5.15: Comparison of COD and color of combined bleach effluents generated during ECF bleaching of soda-AQ pulp of sugarcane bagasse

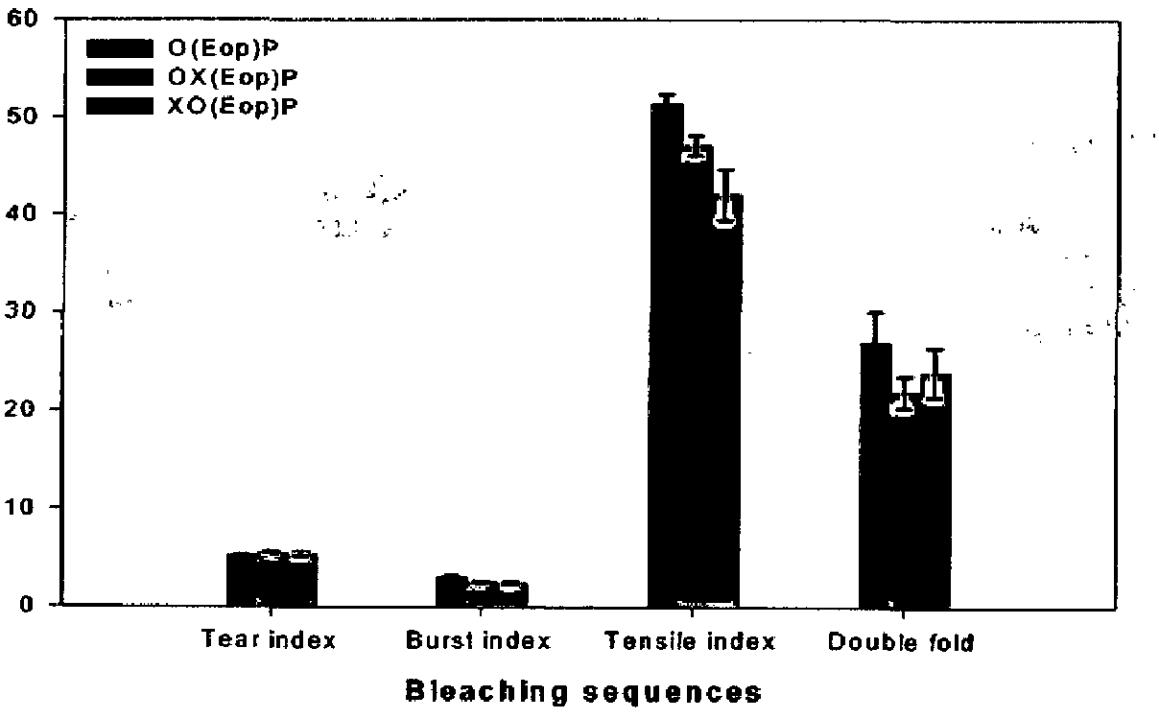

Figure 5.16: Comparison of mechanical strength properties of soda-AQ pulp of sugarcane bagasse during $O(E_{OP})P$, $OX(E_{OP})P$ and $XO(E_{OP})P$ bleaching sequences

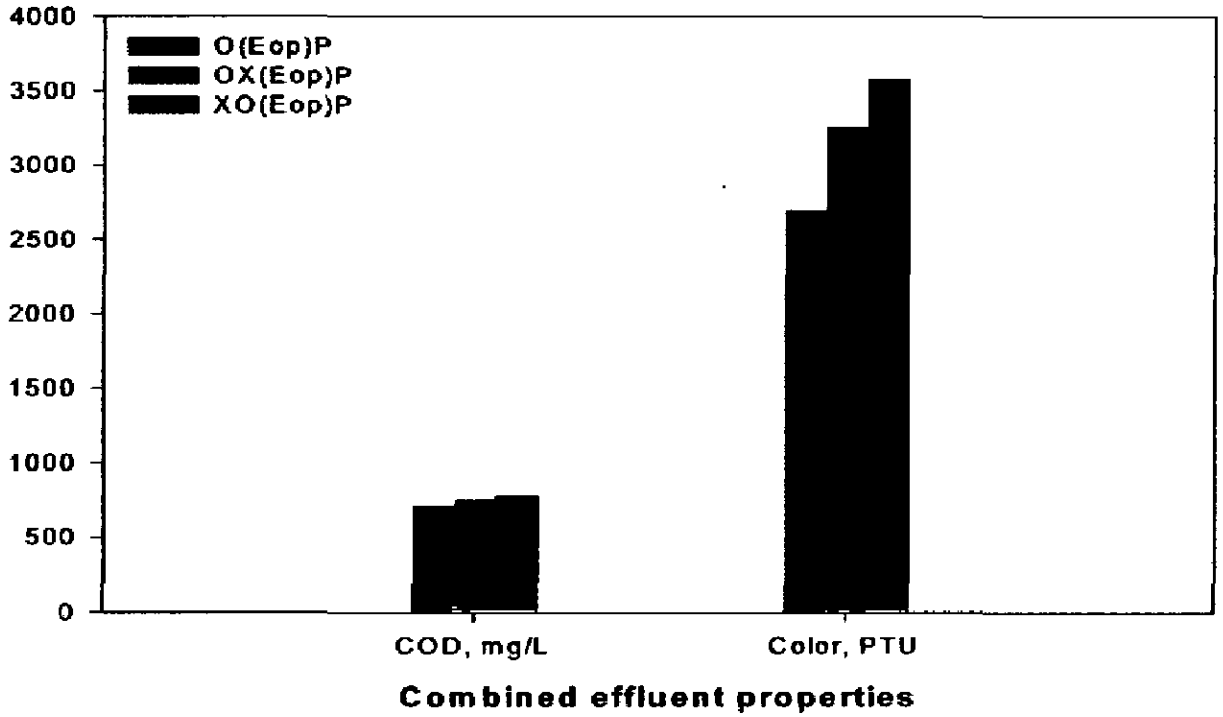

Figure 5.17: Comparison of COD and color of combined bleach effluents generated during TCF bleaching of sugarcane bagasse soda-AQ pulp

CHAPTER 6

CONCLUSIONS AND SUGGESTIONS

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6.1 CONCLUSIONS

Based on the present study carried out and the investigations made; the following are the major conclusion drawn:

1. Sugarcane bagasse contains 71.36% useful fibers and 15.48% pith after dry and wet depithing. The average fiber length of depithed sugarcane bagasse is 1.51 mm with a fiber diameter of 21.4 μm. The length and width of an average parenchyma cell are 326.9 and 53.4 μm respectively while length and width of vessel cell are 152.3 and 28.1 μm respectively. The dimensions of parenchyma and vessel cells are on higher side than that of fiber dimensions. Therefore, if they are present in excess, may affect the strength of paper and runnability of paper machine adversely and lead to fluff formation.
2. Proximate chemical analysis of depithed sugarcane bagasse depicts that it requires milder cooking conditions due to less lignin content and more open and looser anatomy.
3. Sugarcane bagasse produces the screened pulp yield of 42.18% and kappa number 28.16 by soda pulping at optimum cooking conditions like: active alkali dose 12% (as Na_2O), maximum cooking temperature 150 ^0C, maximum cooking time 60 min, digester pressure 5 kg/cm^2 and liquor to wood ratio 4:1.

4. The addition of 0.1% anthraquinone (AQ) accelerates delignification during soda pulping. At optimum cooking conditions it improves the pulp yield by 2.62% with kappa number reduction of 3.9 units.

5. The sugarcane bagasse soda-AQ pulp has been beaten at different freeness levels and it is observed that at a beating level of 45 ^{0}SR and active alkali dose 12% (as Na_2O); the mechanical strength properties are optimum for sugarcane bagasse soda-AQ pulp.

6. The whole sugarcane bagasse spent liquor contains higher total solid concentration, BOD, COD and color values than that of depithed sugarcane bagasse. However, the spent liquor of whole sugarcane bagasse is important from energy conservation point of view because the presence of larger portion of pith in it.

7. The Baur-McNett fiber classification of soda-AQ pulp of sugarcane bagasse shows that it contains 75.30% medium sized pulp fractions retained on mesh sizes –20 to +48 and –48 to +100. Therefore, sugarcane bagasse soda-AQ pulp is found to produce good mechanical strength properties.

8. Scanning electron microscopy of sugarcane bagasse fiber after soda-AQ pulping depicts that fibers are smooth and flat without any external fibrillation and a part of the xylan is reprecipatated on their surface.

9. Among twelve fungal strains isolated from different lignocellulosic sources, two stains of *Coprinellus disseminatus* i.e. SW-1 and SW-2 have been selected as the potent xylanase producers along with minimum cellulase contamination.

10. Solid-state fermentation (SSF) results into higher xylanase yield than the submerged fermentation (SmF) from both of the strains of *C. disseminatus*.

11. The various optimized physico-chemical parameters under SSF conditions for crude xylanase production by fungal strains SW-1 and SW-2 are: incubation period 7 day, incubation temperature 37 ^{0}C, pH 6.4, solid substrate: moisture content 1:3 and carbon source wheat bran except nitrogen source which is soya bean meal for strain SW-1 and peptone for SW-2.

12. It is concluded from the biochemical characterization that the crude xylanase produced by *C. disseminatus* SW-1 is more thermo and alkali-tolerant in comparison with that produced by *C disseminatus* SW-2. Hence white rot strain *C. disseminatus* SW-1 has been chosen for further biobleaching studies.

13. The xylanase produced by strain SW-1 maintains its high stability over a pH range of 6.0 to 7.6 and temperature range of 55 to 65 ^{0}C which is a critical requisite for paper industry.

14. The molecular weight of xylanase produced from *C. disseminatus* SW-1 is found to be 43 kDa following biochemical characterization.

15. The biobleaching effect of crude xylanase produced from *C. disseminatus* SW-1 on sugarcane bagasse soda-AQ pulp is maximum at a xylanase dose of 8 IU/g, reaction time 120 min, pulp consistency 10%, temperature 55 ^{0}C and pH 6.4. Xylanase also causes the release of reducing sugars and chromophores from sugarcane bagasse soda-AQ pulp indicating its biobleaching potential.

16. Xylanase pretreatment reduces the total chlorine demand by 29.70% for CEHH and CEHHP bleaching sequences and 36.53% for OCEHH bleaching sequence along with a significant gain in brightness.

17. The AOX in combined bleach effluent of CEHH, CEHHP and OCEHH bleaching sequences gets reduced by 28.16, 27.6 and 34.65% respectively as a result of xylanase pretreatment in comparison with control.

18. A gain in all mechanical strength properties is noticed as a result of xylanase pretreatment in CEHH, CEHHP and OCEHH bleaching sequences.

19. The xylanase pretreatment in ODED and OD(E_{OP})DP bleaching sequences improves the pulp brightness by 7.3 and 4.3% respectively at the same chemical dose.

20. The AOX in combined bleach effluent of ODED and OD(E_{OP})DP bleaching sequences reduces by 84.84 and 87.3%, COD by 23.8 and 21.16% and color by 33.11 and 22.2% respectively compared to that of CEHH bleaching sequence.

21. A decrease of 41.43 and 40% is noticed in AOX of combined bleach effluent generated during ODED and OD(E_{OP})DP bleaching sequences as a result of xylanase pretreatment.

22. TCF bleaching sequence O(E_{OP})P produces the pulp of higher viscosity along with reduced bleaching losses compared to conventional (CEHH, CEHHP and OCEHH) and ECF (ODED and OD(E_{OP})DP) bleaching sequences.

23. It is concluded that keeping xylanase stage before oxygen delignification is more beneficial to attain a higher brightness ceiling in TCF bleaching as brightness increases by 8.2% after XO(E_{OP})P and 6.1% after OX(E_{OP})P compared to O(E_{OP})P bleaching sequence.

24. Xylanase pretreatment causes a slight increase in pulp viscosity for conventional (CEHH, CEHHP and OCEHH), ECF (ODED and OD(E_{OP})DP) and TCF (O(E_{OP})P) bleaching sequences while a decrease is noticed in copper number which suggests that there is no brightness reversion with time.

25. A small decrease is noticed in all mechanical strength properties except tear index as a result of xylanase pretreatment of ODED, OD(E_{OP})DP and O(E_{OP})P bleaching sequences at same chemical dose.

26. COD and color of combined bleach effluents increase slightly as a result of xylanase pretreatment of all bleaching sequences.

27. It is clear from the SEM studies that xylanase pretreatment modifies the fiber surface by introducing cracks, peelings, swelling and external fibrillation compared to smooth surface of untreated fibers.

Therefore, it is concluded that the sugarcane bagasse can be successfully used as a raw material for paper making process and crude xylanase produced by *C. disseminatus* SW-1 has tremendous potential not only for reducing the bleach chemical demand and toxicity of various bleaching effluents in terms of AOX but also for improving various paper properties. The cost of the biobleaching is satisfactorily low as xylanase is produced using wheat bran as carbon source which is an inexpensive agro residue and used in its

crude form as it contains a negligible cellulase contamination and so does not require any purification step.

6.2 SUGGESTIONS FOR FUTURE WORK

With reference to the present work done and targets achieved; the following suggestions are made for the future work:

- *C. disseminatus* SW-1 may be checked for ligninolytic enzyme production and various parameters may be optimized to get the increased level of enzyme which can be further used for pulp processing.
- Biobleaching process may be carried out at higher temperature and pH values in order to check the viability of crude xylanase produced by *C. disseminatus* SW-1 in extreme conditions.
- *C. disseminatus* SW-1 and SW-2 may be genetically modified for making their enzyme preparations more thermo and alkali stable to be used in various pulp and paper making operations.
- It is recommended to take plant trials with xylanase produced by *C. disseminatus* SW-1 in agro-based industry using sugarcane bagasse to validate laboratory results and cost reduction studies must be carried out to calculate the economic viability of enzyme.

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