STUDIES ON DEVELOPMENT OF BIO-BLEACH PROCESSES FOR CYMBOPOGON MARTINI AND CYMBOPOGON CITRATUS

A THESIS

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

DOCTOR OF PHILOSOPHY

by

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CANDIDATE'S DECLARATION

I hereby certify that the work which is being presented in the thesis entitled **"STUDIES ON DEVELOPMENT OF BIO-BLEACH PROCESSES FOR CYMBOPOGON MARTINI AND CYMBOPOGON CITRATUS"** 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 August, 2006 to May, 2011 under the supervision of Dr. Dharm Dutt, Associate Professor and Dr. C.H. Tyagi, Associate Professor, 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.

Harjeet Kaur

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

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The Ph.D. Viva-Voce examination of **Mrs. Harjeet Kaur**, Research Scholar has been held on....11.10.2011.....

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ABSTRACT

The path of progress of paper industry has ever since been associated with pollution; a fact that often mars the reputation of the industry. The industry is hence looked upon as a notorious pollution maker and unfortunately the truth can't be denied. The global concerns about energy, preservation of forests and elimination of pollution from pulping and bleaching processes have led us towards exploration of alternate fibrous resources other than wood and new pulping and bleaching processes that are compassionate with the environment 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 aimed at developing an environmental benign technology for production of pulp and paper using the left over waste (bagasse) of two aromatic, fibrous raw materials; lemon grass and sofia grass (after extraction of their essential oils by steam distillation). The prior objective of the study was to evaluate and estimate the feasibility regarding reductions in chemical consumption, while preserving mechanical strength properties of the lemon and sofia grass soda-AQ pulps through enzymatic treatment.

The detailed morphological and anatomical features of lemon and sofia grasses were determined and thermo-chemical characterization of the two raw materials was carried out in order to assess their suitability for pulp and papermaking. The studies indicated that the steam distilled, left over lignocellulosic biomass of lemon and sofia grasses could satisfactorily be used for pulp and paper production thereby reducing the environmental load and also seizing the tree logging.

Soda pulping process was used to pulp the dried bagasse of steam distilled lemon and sofia grasses. Owing to their open and loosened anatomy, they required milder cooking conditions. The various operating process variables for soda pulping like, maximum cooking temperature, cooking time and active alkali dose were optimized for soda process and effect of anthraquinone (AQ) was then observed. An active alkali dose of 14% (as Na_2O), and cooking time of 90 min at a maximum cooking temperature 150 and 160 ^0C respectively for lemon and sofia grasses were found optimum for soda pulping. AQ (0.1%) as a pulping additive accelerated the delignification rates along with improvement in screened pulp yields. The mechanical strength properties were also

optimized at different beating levels and beating level of 40±1 ^{0}SR. Fiber length distribution of soda-AQ pulps using Bauer-McNett fiber classifier confirmed that sofia grass soda-AQ pulp had maximum percentage of fibers in middle and bottom fractions, while lemon grass in top fraction. The soda-AQ pulps thus obtained were of good quality for paper making. The scanning electron microscopic (SEM) study was carried out to understand the procedure of alkaline pulping and its effect on the fibers of lemon and sofia grasses.

An investigation was undertaken to isolate, screen and identify a potent microorganism having the potential ability to secrete xylanase. A thorough survey of various sites led to isolation of two credible xylanase producing fungal strains. The strains had notably higher xylanase activity along with a minor cellulase contamination and were identified as two different strains of white rot basidiomycete *Coprinopsis cinerea* from Forest Research Institute, Dehradun (India). The test fungi were also tested as per Bavendamm plate assay for their ability to produce phenoloxidases, which hence confirmed that the two strains were white rotters. The two strains were designated as HK-1 and HK-2 respectively. Further the strain HK-1, with a higher xylanase activity was sent to Agharkar Research Institute, Pune (India) for confirmation of its identity on the basis of internally transcribed sequences (ITS). The detailed morphological study of both of the test strains was carried out using SEM which showed that the hyphae of fungal strain HK-1 were thick and compact whereas, the hyphae of the fungal strain HK-2 were thin and ribbon like. Occurrence of clamp connections and club shaped basidiospores further confirmed their identity under basidiomycetes. Xylanase production from HK-1 and HK-2 was evaluated under submerged and solid-state fermentation conditions; of these, the level of xylanase production observed was higher under solid-state fermentation (SSF) conditions. Various operating parameters, such as, incubation period, temperature, pH, carbon source, nitrogen source and moisture content were optimized under SSF for the hyper-xylanase producing *C. cinerea* strains HK-1 and HK-2 to achieve their maximum levels of xylanase secretion. The biochemical characterization of crude xylanases produced by the two fungal isolates confirmed that the xylanase produced by *C. cinerea* HK-1 was more thermo-alkali-tolerant in comparison to that produced by HK-2. Therefore the test strain *C. cinerea* HK-1 was chosen for the further biobleaching studies of lemon and sofia grass soda-AQ pulps.

The xylanase produced by *C. cinerea* HK-1 was analyzed and evaluated for its application in biobleaching of lemon and sofia grass soda-AQ pulps and reduction of toxicity in effluents generated during various bleaching sequences, in terms of AOX. Various operating parameters for xylanase prebleaching i.e. xylanase dose, retention time and pulp consistency were optimized and the pulp filtrates were checked for the release of reducing sugars and chromophores from the pulp samples. The xylanase preparation was used in biobleaching of the soda-AQ pulps of lemon and sofia grasses during the conventional (CEHH and OCEHH), ECF (ODED and ODEDP) and TCF ($O(E_{OP})P$) bleaching sequences.

The xylanase pretreatment of lemon and sofia grass soda AQ pulps depreciated the total chlorine demand by 22.67 and 29.73% for CEHH and 11.60 and 16.92% for OCEHH bleaching sequence, while still achieving high degree of brightness and preserving mechanical strength properties of the two soda-AQ pulp samples in comparison to their respective controls. Xylanase pretreatment also reduced the AOX formation in lemon and sofia grasses bleach effluents by 26.64 and 29.96% for CEHH while 35.11 and 30.73% for OCEHH bleaching sequences. For ECF (ODED and ODEDP) and TCF ($O(E_{OP})P$) bleaching sequences; xylanase pretreatment increased the overall brightness ceiling of the pulps at the same chemical charge with decrease in all the mechanical strength properties except tear index. Xylanase pretreatment resulted in small gains in viscosity over controls for all the bleach sequences. AOX formation reduced by 84.84 and 82.67% respectively, from lemon and sofia grass combined bleach effluents following investigated ECF bleaching sequences when compared to CEHH bleaching sequence. For lemon grass, after ODED and ODEDP, COD decreased by 14.71 and 18.15% respectively while colour decreased by 28.08 and 43.46% when compared to CEHH sequence, whereas for sofia grass, the respective decreases in COD were 15.06 and 19.71% and decrease in colour were 33.02 and 17.85%. The introduction of xylanase stage before ODED and ODEDP sequences reduced the AOX formation by 35.68 and 46.88% in lemon and sofia grasses respectively. The reduction in copper number after xylanase pretreatment for all bleaching sequences showed that there was no brightness reversion with time; whereas an increase was noticed in effluent COD and color values after xylanase pretreatment. SEM studies were carried out in order to attain a better understanding regarding the effect of xylanase pretreatment on lemon and sofia grass pulp bleachabilities which revealed that xylanase

pretreatment brought about fiber surface modifications and rendered 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 improved the mechanical as well as optical properties of paper along with reduction in chlorine consumption which in turn mitigated the pollution load, thereby, signifying it as an ecofriendly and environmentally benign bleaching technology.

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Harjeet Kaur

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INTRODUCTION

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INTRODUCTION

The dynamic dimensions of science and technology ensure that the frontiers of knowledge are continually expanding. In the modern era, paper has taken the most important role in the human life. The pulp and paper industry marks uniqueness with respect to the noble product coming out of it in the form of paper. Paper promotes literacy. World demand for paper has increased at an average annual rate of 4.7% over the past 40 years (1). The world consumption of paper and paper board in 2006 was 380.28 million tonnes, of which 36% was consumed in Asia and 64% in rest of the world (58) (Figure 1). The world demand for paper and paper board as per forecast is likely to grow by 2.1% annually in the long term and has been estimated to reach 490 million tonnes by the year 2020, according to a recent research paper based on demand and supply study (30). China and India will be the most rapidly growing production areas within Asia accounting for 39% and 8% of the world's incremental production by 2020 (62).

Paper industry is regarded as one of the 35 high priority industries, by the Government of India. Indian paper industry is the 15th largest in the world and provides employment to 1.3 million people in the country, contributing rupees 25 billion to the government. As per CRIS-INFAC reports, the annual demand in India is projected to grow from 6.8 million tonnes in 2006 to 8.2-8.5 million tonnes per annum in 2010 and to 11 million tonnes per annum in 2015, with an overall industrial segment growth of 7-8% (26). The consumption of paper goes hand in hand with population and literacy rates. Indian population, with an augmentation rate of 1.2% per year shall strike the figure of 1.3 billion by 2020. Literacy rate is expected to grow over 70% by 2020 (45). Table 1 infers that the import of writing grade paper is quite high in Indian paper market (Figure 2) (45).

The per capita consumption of paper is considered as a bench mark of modernization of any country, 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 per capita consumption of paper and paper board, which was 11 kgs/ person for five selected countries of Asia while, 49 kgs/ person for China alone in 2006 is

expected to increase by 14 and 66 kgs/ person respectively (Figure 3). The consumption of paper in India is one of the lowest i.e. 7 kgs/person compared to average world consumption i.e. 50 kgs/person (58) and will be expected to increase to 9 kgs/person by 2010. This low per capita consumption infers that an enormous scope exists for the consumption to rise in India in the near future. With no major paper mills in neighbouring countries like Srilanka, Nepal and Bangladesh, India can become the prime regional player of this area. During 2005-06, paper prices increased by 17-20% and demand grew by 6.3% (26).

There are at present about 710 units engaged in the manufacture of paper and paper boards and newsprint in India, while numerous small mills are not working at their full capacity. According to a report on market published by the British consultancy firm, Hawkins Wright, the average capacity of a paper machine is about 14,000 tonnes per year against a global average of 42,000 tonnes per year. Likewise, the average paper machine speed at Indian mills is 200-260 m/min which is below the global average of 600 to 700 m/min. Most of the Indian mills are small, only 34 Indian mills have a capacity of over 33,000 tonnes/annum. The number of Indian mills capacity wise is shown in Figure 4 (45). The major paper industries of India have been shown in the map of Indian paper industry (Figure 8) (39).

Wood is the main raw material for the pulp and paper industry in developed countries while non-wood cellulosic fibers occupy small niche markets providing special properties to a range of high value added products. However where wood based fibers are not available, as in the developing world, non wood fibers are a major source for the pulp and paper industry (19). The three major raw materials on which Indian paper industry relies are: forests, agricultural residues and secondary fibers (70). Pulp and paper industry uses 39% of forest based fiber, 31% agro based and 30% secondary fiber obtained from waste paper (35). Proportionally, the percentage of non-wood raw materials and waste paper is increasing over the years, and at present about 60.8% of non wood raw materials and 39.2% of wood based raw materials are in use (55). The production capacity of Indian paper industry, based on their raw material usage has been divided into three categories as shown in Figure 5.

Total forest area in 2005 was estimated to be around 30% of the planet's land area, just under 40 million km^2, corresponding to an average of 0.62 ha (6200 m^2) per capita

(72). Total land area of India is 328.8 million ha. Agricultural land occupies 47% of the total land area, while uncultivated, non-agricultural and barren land accounts for 30% (41). Total tree cover of India as reported in 2005 by Forest Survey of India (FSI) was 23.41%, including a forest cover of 20.60% and the rest being trees outside forests (TOF) (21). This 20.6% (Figure 6) of the country's forest cover translates to just 0.8 ha/person, one of the lowest in the world (41). This forest and tree cover of 23.39% of geographical area in 2005 touched 25% in 2008 and is likely to touch 30% in 2012. The goal is to bring 33% under forest and tree cover by 2020 (2).

The paper industry's wood demand is expected to grow from 5.8 million tonnes to 9 million tonnes by 2010 and 13 million tonnes by 2020 (45) (Figure 7). Total fiber consumption for the production of paper and paperboard in India will nearly double between 2006 and 2016, while growing from 7.4 million tonnes/year to 13.7 million tonnes/year. The pace of economic growth including infrastructure and industrial growth is sure to influence the demand for paper and paper products and it is certain that the business-as-usual scenario is unable to keep pace with demands. The total wood fiber deficit in India will increase at an annual rate of 11.3% by 2016, as per forecast (41). The pulp and paper industry uses only 3-4% of total wood. As per the existing forest policy, the paper industry cannot use wood from any of the national forest reserves (28). The emerging trends of challenges and opportunities are sending signals for change which might be even ahead of our knowledge and experience. Serious doubts about sustained supplies of wood fibers from natural forests and forest plantation arise in view of following fact:

- Intense biotic pressure on forests and plantations leading to illicit removal of firewood, excessive and uncontrolled grazing, fires, poor productivity, etc. (35).
- With the recognition of climate change as a consequence of anthropogenic carbon emission, every effort is being made to either mitigate impact by reducing emission or working on adaptations to the changing situations. Forests can be used as sinks for immobilizing large quantity of carbon dioxide (the most prevalent green house gas) for long periods. Thus the role of forests in climate change is determined by the state and management (27).

The above facts leave their impact on industry as:-

1. The import of timber from other countries is on rise which is highly uneconomical.
2. The industries have also started obtaining a part of their raw materials from farmers, for short rotation tree species, agro-residues etc.
3. Waste paper based industries accounts for about $1/3^{rd}$ of Indian paper capacity. The recovery of waste paper has increased from 65,000 tonnes in 1995 to 850,000 tonnes in 2000. Most of the paper is recovered but due to alternative uses, the recovery rate for paper industry is still only about 20%. This is low by international standards: Thailand (42%), China (33%) and Germany (71%) (18) and therefore, requires more attention in India.
4. Trees outside the forests in the form of farm forestry, agro forestry, home stead forestry, etc will bridge part of the gap between demand and supply scenario. TOF's have immense potential for socio-economic and cultural development (2). Also, the current trends in forestry sector favour the promotion of agro forestry. This is marked by the enhancement of the area under agro forestry from about 10 million hectares (about 5% of the total agricultural area) at present to at least 20 million hectares in near future (28).
5. Selection of the best fibrous raw material which will be available on sustained basis appears to be of prime importance and further, the quality of end product will largely depend upon the type of fiber to processing and its blends in making the stock (56). The paper industry has turned over to the use of fast growing wood species, alternative non-wood fibers and secondary fibers for production due to fast depletion of forests resources and its impact on ecological balance (35).

Since 1970, the wood/ forest based segments of paper industry has considerably shrunk from 84% to just 31% in 2000 (60). The share of agro based segments of the paper industry has been evidenced by the escalating growth from a meager share of 9% in 1970 to nearly 31% in 2000 (60). 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 fiber in India is given in Table 2 (45).

Looking over to global scenario, non-wood fibered pulp represents only 7% of the total world pulp production (45) but at over 10 million tonnes per annum; it still represents a substantial quantity. About 70% of this non-wood pulp production occurs in India and China where domestic wood fiber fails to sustain a continuous supply of fibers to pulp and paper industry. The estimated total availability of non-woody fibrous plant is 2300 million tonnes out of which about 50% are straws.

From the foregoing, it is apparent that the grass family presently provides the greatest source of non-woody raw materials (10). Two factors may account for this;-

1. Technologically, monocotyledons have a less complex system of fibers and associated botanical components than the dicotyledons (10). The chemistry of grass lignocelluloses varies considerably from that of wood. There is less lignin in grasses than in woody plants (66, 68).
2. Many species become available as residues or by-product of agricultural or industrial operations.

The first factor leads to greater simplicity in processing and the second one makes it possible to charge off substantial portion of the expense of harvesting, collection and cleaning to the primary product.

The economically sound agronomic aspects for a new crop, favour its prospects in competing with pulp wood. It is hence necessary to develop the proper condition and techniques for exploiting new fibrous crops in the best possible manner. What continuous research has done for pulp woods in the paper making industries should also be possible for prospective non woody fibrous crop, since both necessity and special properties account for their use (10).

The fibrous residues of many aromatic grasses are available as industrial by-product after extraction of their essential oils (10). With this view, efforts have been made in the present study to discover the paper making potential of two such non-woody fibers: *Cymbopogon citratus* (lemon grass) and *Cymbopogon martini* (sofia grass). The two raw materials belong to grass family, *Poaceae* (20). The genus *Cymbopogon* includes about 80 species, distributed in the world tropics. Most of them are aromatic and yield essential oils of commercial importance (71). Among the most important Indian species are *C. martini, C. citratus, C. nardus, C. coloratus, C. jwarancusa, C. nervatus, C. schoenanthus,*

C. caesius and *C. polyneuros*, etc (71). The essential oils are chiefly obtained by the process of steam distillation (36, 51).

The two raw materials grow as tall, tufted perennial grasses often with aromatic leaves. Their inflorescences are densely clustered in large panicles, hidden within the spathe. Lemon grass is often cultivated as a border to roads and paths (20). On account of the production of essential oil from its leaves, it has acclaimed significant global demand. Table 3 enlists the miraculous range of applications of lemon grass oil in different industries. Medicinal use of lemon grass is known to mankind since antiquity. It is used to cure various ailments like cough, cold, rheumatism, digestive problems, as a mouthwash for toothache and swollen gums (9), as analgesic, antipyretic, oral antitumor drug (43, 73). A review of literature also establishes lemon grass as a potent antibacterial (6, 42) and antifungal agent (8, 13, 53), based on the presence of citral, the most important component of the oil of lemon grass. Similarly sofia grass contains geranial as the chief component of its essential aromatic oil (50).

More than 250 types of essential oils (1, 20,000 t world annual production) worth US$ 1.2 billion per annum are traded in the world market. India ranks second in the world trade of essential oils (50). According to an estimate, the world wide availability of solid waste of *Cymbopogon* per year is about 200,000 tonnes (51). After recuperation of the essential geranium oil from sofia grass and citronella oil from lemon grass, the solid waste (bagasse) of these grasses is mainly used for land filling and a fraction is burnt to generate steam for stripping; the rest is left in the fields for a natural biodegradation, thus it creates environmental problems (52). The aim of the study hence would be the production of chemical grade pulp from the hitherto unexploited sources of fibers of lignocellulosic residues of lemon and sofia grasses. It would surely be a step forward in green chemistry by mitigating the environmental pressure of logging trees for paper making. Besides, the process of steam distillation makes the anatomy of their lignocellulosic bagasse more open and looser (67), which would facilitate the further process of pulping.

The pulp and paper industry is an environmental sensitive sector and falls under red category of industries (49). Due to stringent rules of the government, it faces a constant pressure to reduce and modify environmental emissions to air and water. Like any other large scale industry, the pulp and paper industry exerts its own impact on the environment. In the process of paper making, chemical pulping is the first step in which

fibers are broken apart and most of the lignin is removed (24). The residual lignin is then removed by a multistep bleaching process (3, 14).

Delignification after pulping, takes place in the chlorination stage of bleaching process. By increasing the total chlorine demand during pulp bleaching, the brightness of the pulp can be improved (18) but, there is no good without evil and no light without darkness. The increase in total chlorine demand adversely affects the pulp strength, brightness stability and pollution load. It has been found that C- stage is the first point where 2,3,7,8-TCDD, 2,3,7,8-TCDF and 1,2,7,8-TCDF congeners were always present (54, 32, 34, 59). The organochlorine compounds formed during the bleaching of chemical pulp have attracted most attention in the recent years. While not all absorbable organic halides (AOX) are harmful to health or the environment, a number of these have been determined to be so (4). The E-stage filtrate releases the highest concentrations of dioxins (48), known for changing the blood chemistry and causing liver damage, skin disorders, lung lesions and tumor types at numerous sites within the body, including liver and thyroid (47, 25, 32). The industry is hence looked upon as a notorious pollution maker and unfortunately the truth can't be denied.

The environmental regulations have been set across the world, to limit the amount of effluent discharges based on AOX or dioxin discharge values (57). Such regulations have forced the pulp paper industry to reduce the AOX discharges by combining new developments in their pulping and bleaching processes (preventive strategy) and/or effluent treatment technologies (curative strategy. Due to the fact that it is better to avoid the formation of AOX than trying to perform an effluent cleanup, the driving force of present pulping technologies has turned on to remove as much lignin as possible, so that less chemicals must be required during bleaching (38, 44). This approach will permit to lower the amount of chlorine or increase its substitution for chlorine di-oxide to bleach pulp at equivalent quality standards. Alternatives to achieve this goal can be; extended delignification, oxygen delignification, increased chlorine di-oxide substitution, peroxide reinforced oxidative extraction, optimization of chlorine-stage parameters, enzymatic pretreatments and/or non chlorine bleaching sequences (38, 44, 61). To reduce the use of chlorine and chlorine compounds in bleaching which are the most polluting, industries/researchers have developed alternative bleaching methods (40) by the replacement of elemental chlorine free (ECF) bleaching sequences (7), and without elemental chlorine or chlorine containing compounds, termed as totally chlorine free

(TCF) bleaching process. These bleach techniques make use of oxidants such as oxygen, ozone and peroxide, etc. which oxidatively degrade lignin, thereby decreasing the molecular size and increasing its water and alkali solubility (16). These alternatives are however quite expensive to adopt since, they require a lot of changes in the infrastructure and hence are only viable to large paper mills (40). For agro residue pulp mills, with production capacity less than 100 tonnes per day (TPD), these alternate bleaching technologies may not be feasible on techno-economical grounds in developing countries like India (64). Moreover a risk in loss of pulp viscosity and strength is always there (29). So switching over to biobleaching has proven to be the most promising alternative for eliminating chlorine based chemicals in the pulp bleaching (4).

A hypothesis was laid down by the researchers, a few decades ago, for the use of microbial enzymes in paper industry, based on the fact that paper is composed of natural polymers viz. cellulose, hemicelluloses and lignin materials. The last century has witnessed the turning point of that hypothesis into reality in the form of lignin degrading enzymes and hemicellulases, which have been successfully used in paper processing such as bleaching with xylanases, lacasses, etc., pitch removal with lipases and increasing freeness of the pulp fibers (37, 63).

Enzymes can carry out myriad of biochemical reactions under ambient conditions, which makes their use eco-friendly and often the best alternative to polluting chemical technologies. The usage of enzymes at various industrial levels has gained momentum (17). Xylanases have occupied the center stage of all the bio-chemical processes involved in pulp and paper industry. Vikarii et al. was the first to demonstrate that xylanases are applicable for delignification in the bleaching process (69). Over the years usage of xylanases at industrial level has increased significantly. The positive effect of xylanases is observed in their act of catabolism which eliminates the xylans (63, 22), thereby breaking the existing link between cellulose and lignin. The lignin is hence set free and is more rapidly eliminated in subsequent bleaching stages (66, 46) with minimal damage to the pulp.

Xylanases are ubiquitous and diverse by nature (11), and the judicious use of xylanases in industries could result in cleaner reactions, higher yields and lower consumption of energy (11, 22). The interest in cellulase free, thermo-alkali stable xylanases has been of more recent origin in the pulp and paper industry. Any cellulase activity will have serious economic implications in terms of cellulose loss, decrease in

degree of polymerization, degraded pulp quality and increased effluent treatment cost (33, 22, 31).

By presenting an overview over the current status of paper industry and the future prospects of the use of xylanase enzyme as an effective bio-agent at various levels of processing in paper industry, the present study aimed at developing an environmentally benign technology for the production of pulp and paper using *C. citratus* (lemon grass) and *C. martini* (sofia grass). The focus of the study was evaluation and estimation of the feasible reduction in chemical consumption and energy, while preserving the mechanical strength properties of pulp of both raw materials via enzymatic treatments. Keeping the above discussed problems in view, the outline of the work was prepared as:

1. Morphological and anatomical studies of the two raw materials, followed by their proximate chemical and thermo analysis to check the suitability and potential of these non-woody fibers for production of chemical grade pulps.
2. Optimization of soda-AQ pulping process and mechanical strength properties of the paper produced from pulps in order to reduce the Kappa number prior to bleaching and, escalate the pulp yield.
3. Isolation of potent fungal strains producing thermo-alkali tolerant xylanase, from lignocellulosic wastes and their screening.
4. Characterization of the screened fungal isolates for production of different extra-cellular enzymes, mainly for higher xylanases and lower cellulase activity.
5. To analyze the xylanase production under submerged and solid state fermentation systems. Selection of fermentation system in order to achieve high xylanase levels and optimization of various enzyme production parameters.
6. To study the effect of enzyme aided bleaching on brightness, viscosity; PFI revolutions during beating and mechanical strength properties like tear index, tensile index, burst index and double fold.
7. To analyze and evaluate the xylanase preparation for its application in bio-delignification of lemon and sofia grass soda- AQ pulps and to reduce the toxicity of effluents generated during conventional, ECF and TCF bleaching sequences of the respective pulps to make the process eco-friendly and cost effective.

Table 1: Paper Market in India in 2000 (Each figure is given in 1000 tonnes) (46)

Particulars	Production	Imports	Exports	Consumption
News print	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 2: Annual potential of agro based fibers in India in 2001 (46)

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 3: Uses of essential oil from lemon grass

S. No.	Industries	Uses
1	Perfumery and cosmetics	For its strong lemon like flavour with a deodorizing effect (12).
2	Food and beverage	As a flavouring and preservative agent, as an herb in dried and powered form, in teas, soups & curries (20).
3	In agriculture	As an insect repellant and as a bio-fungicide (20).
4	In soaps, detergents and pharmaceuticals	For its antiseptic, antimicrobial properties (5). As a starting material for manufacture of synthetic vitamin-A & ionene (15)
5	Aromatherapy	For its aromatic healing properties in treatment of serious skin diseases, acne, superficial mycoses (23). Excellent for tired and aching feet (20).

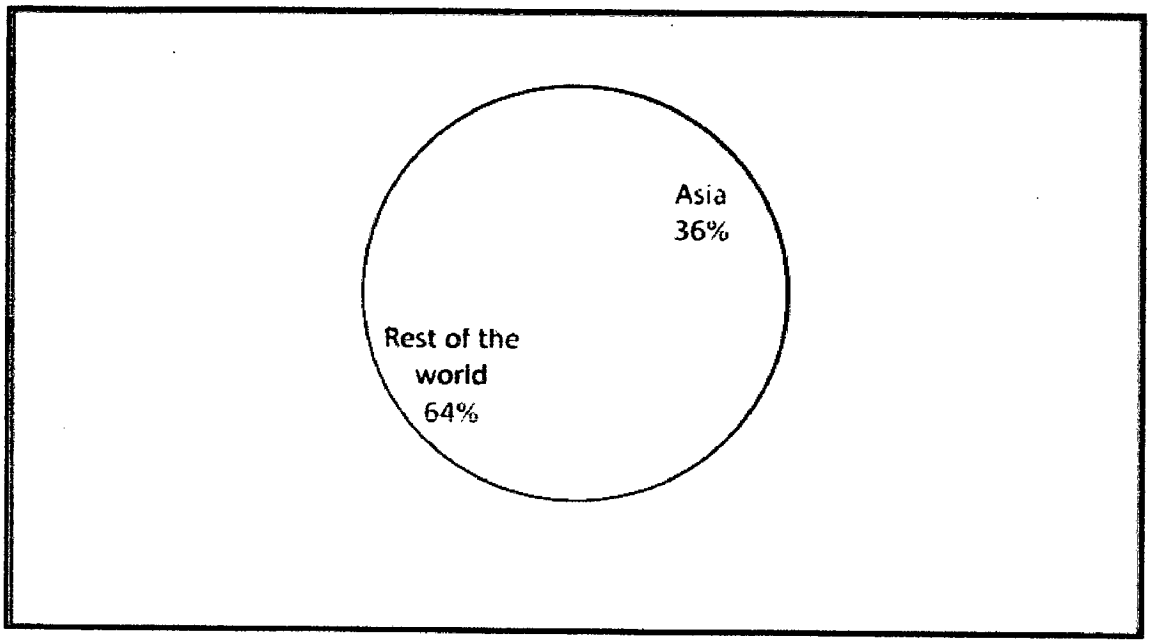

Figure 1.1: Total world paper and paperboard consumption in 2006 (58)

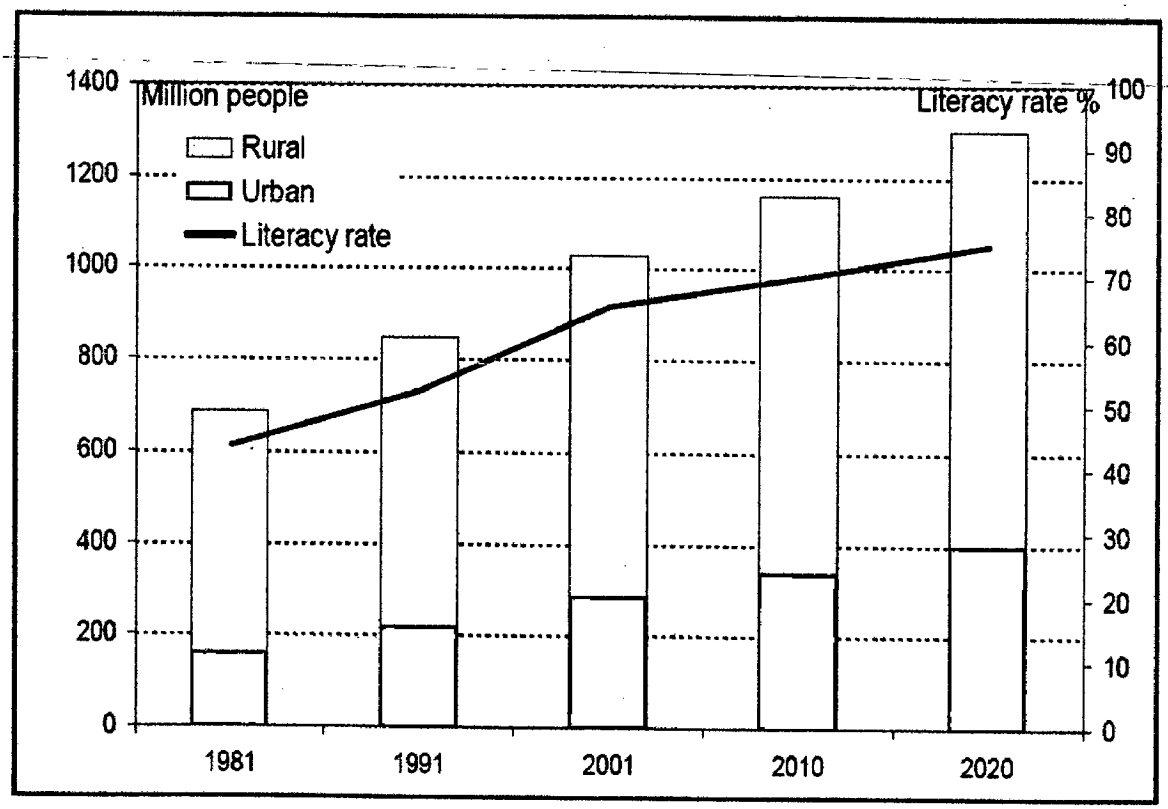

Figure 1.2: Indian population and literacy rate (46)

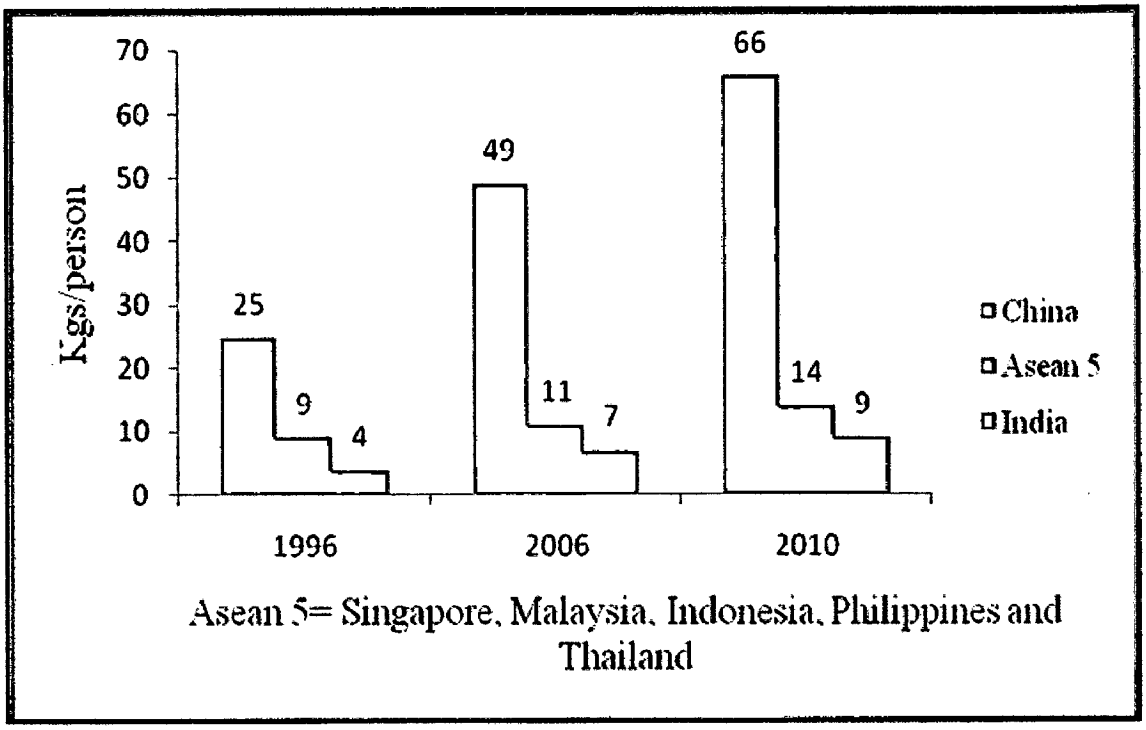

Figure 1.3: Per capita consumption of paper and paper board in Asia (46)

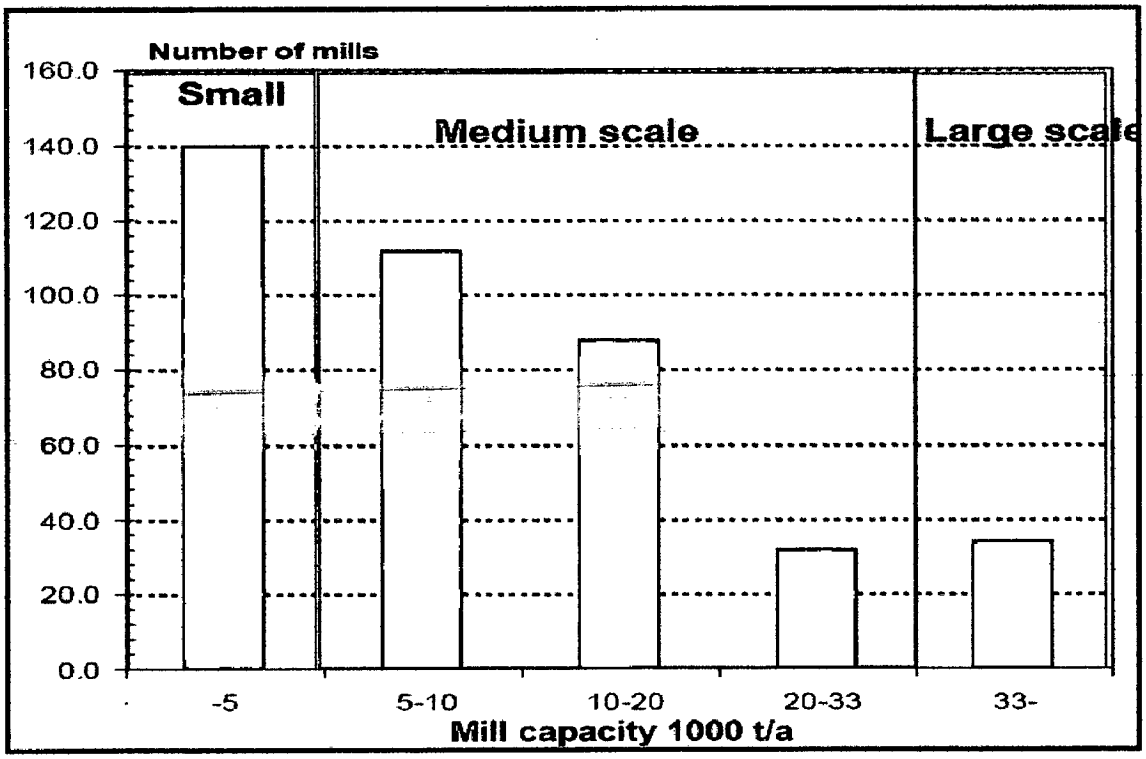

Figure 4: Indian paper mills structure in 2001 (46)

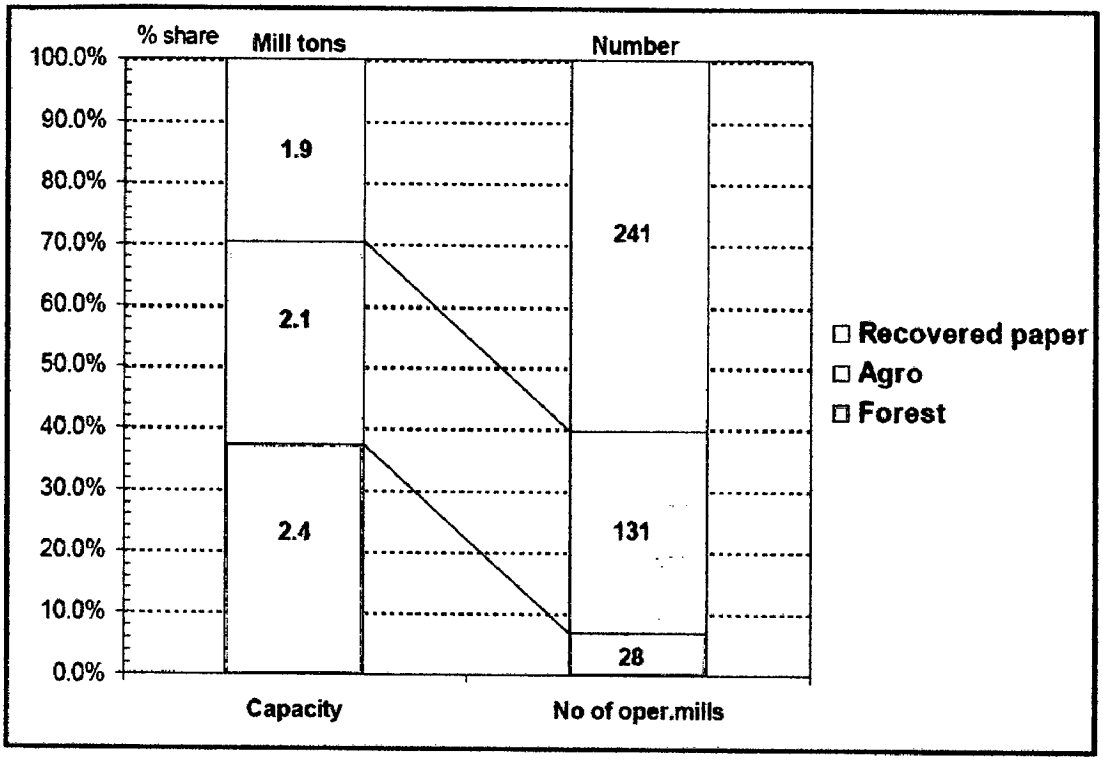

Figure 5: Structure of Indian paper industry based on fibrous raw materials in 2001 (46)

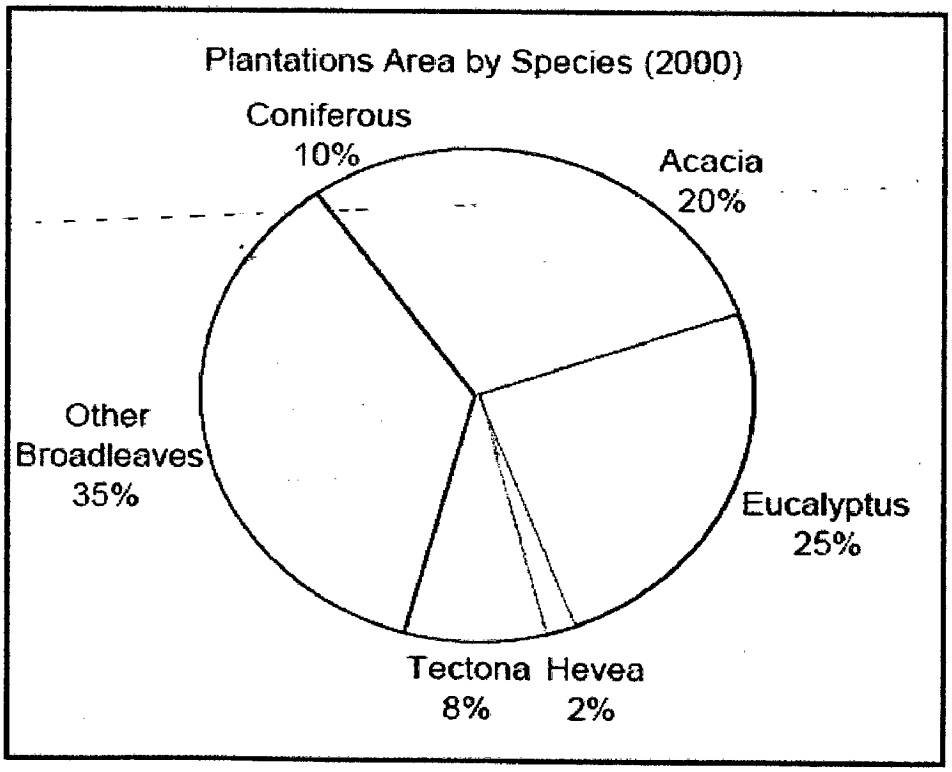

Figure 6: Forest plantation in India (46)

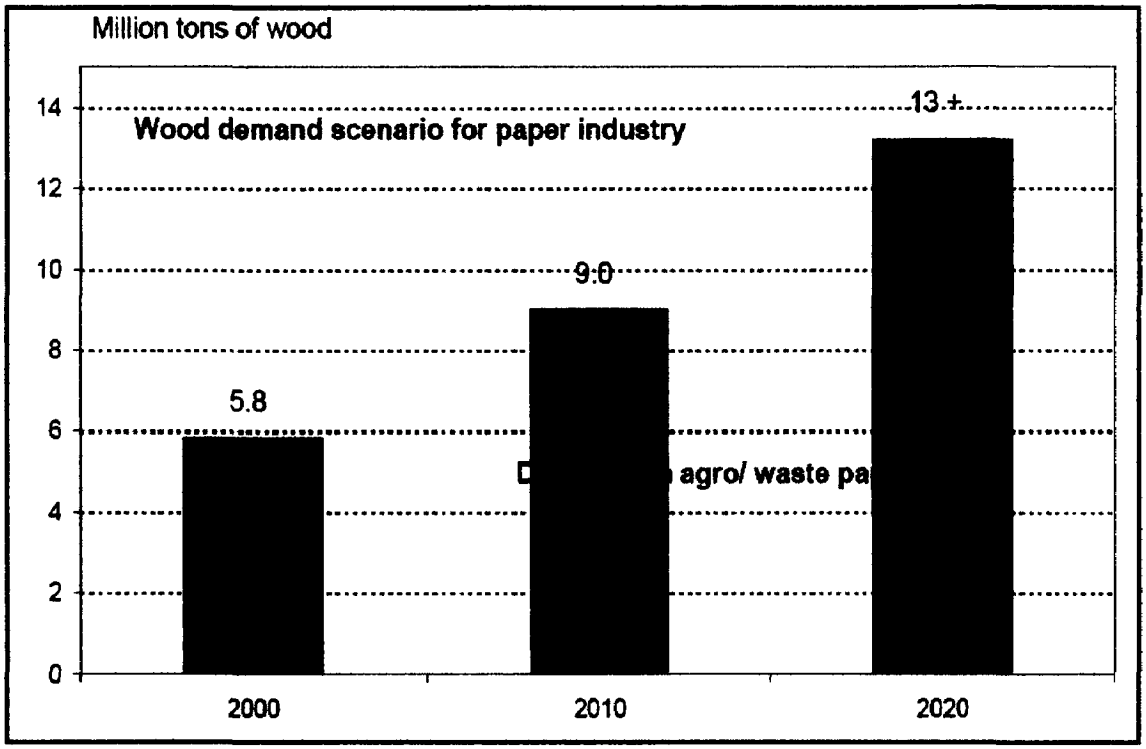

Figure 7: Industrial wood demand scenario for India (46)

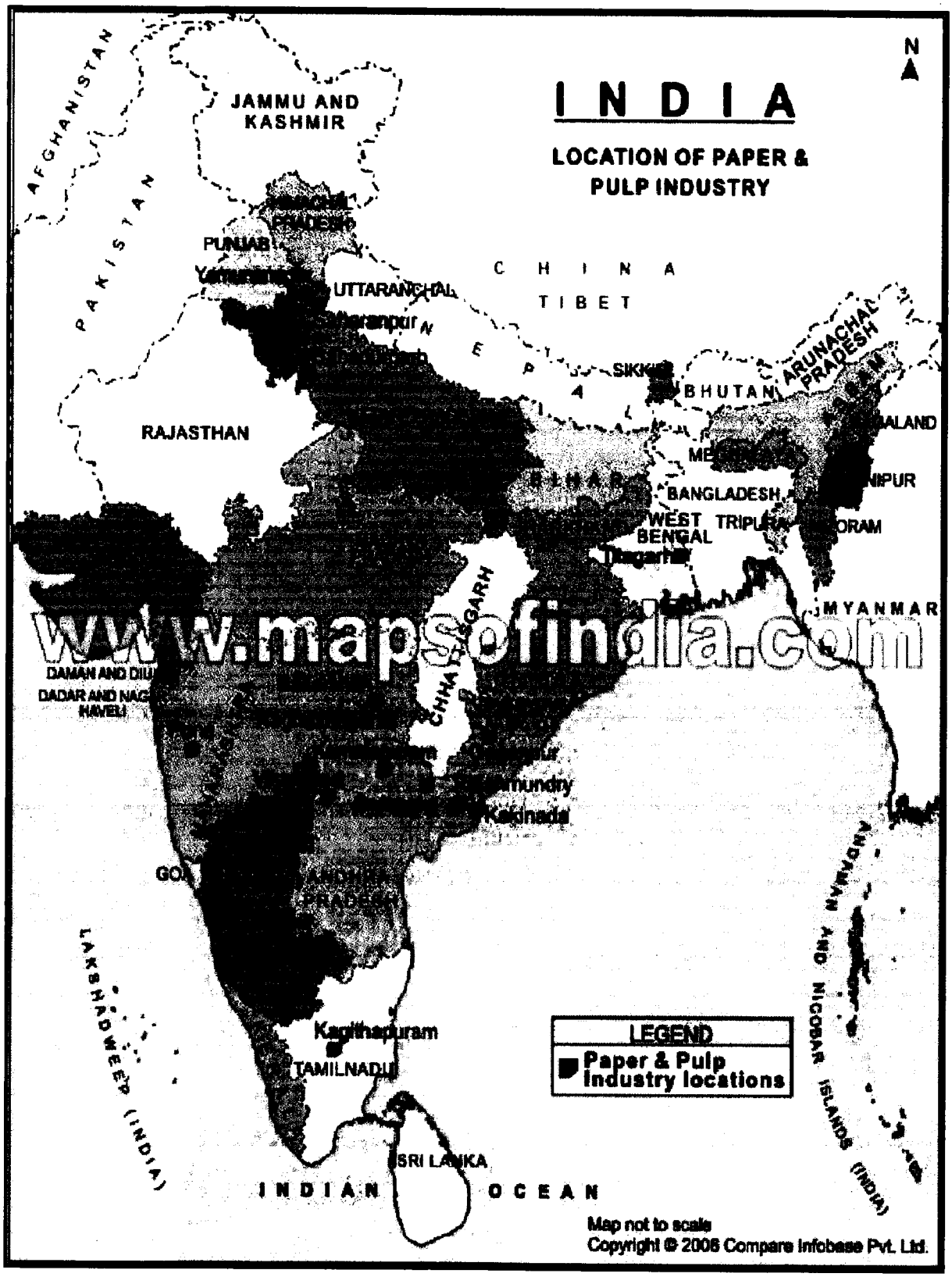

Figure 8: Paper industry map of India (40)

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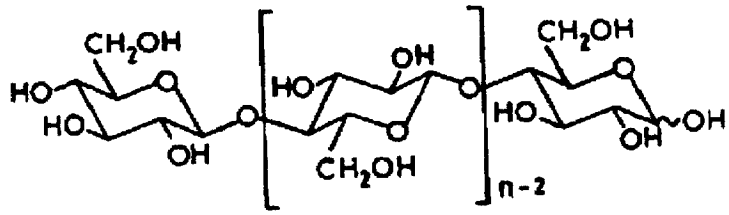
CHAPTER 2

ANATOMICAL, MORPHOLOGICAL AND CHEMICAL COMPOSITION OF LEMON AND SOFIA GRASSES



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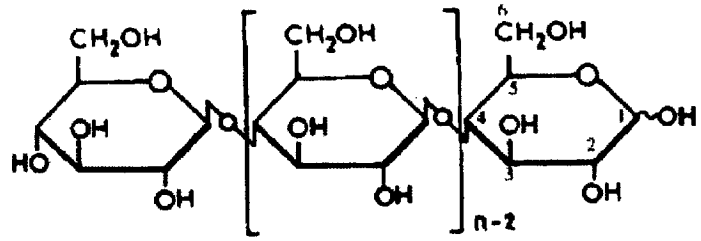

Non-reducing end Reducing end

Figure 2.1: Molecular structure of cellulose (28)

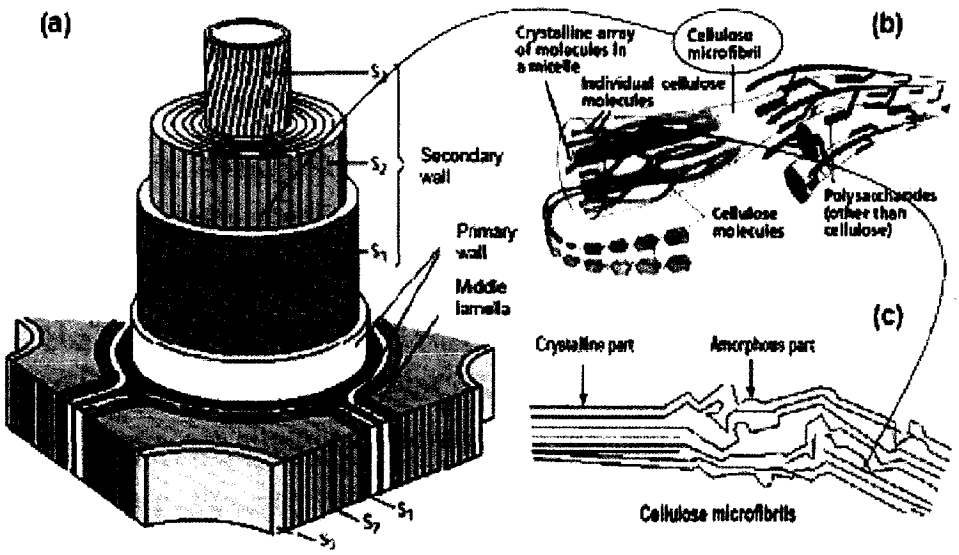

Figure 2.2: Simplified composition of the wood cell wall (a) The cell wall is divided into primary (P) and secondary (S_1, S_2 and S_3 layers) cell wall. The lines in the secondary cell w represent the microfibrillar alignment (100) **(b) A schematic representation of the cellulo microfibrils. Amorphous hemicellulose and some lignin are located between the crystalli cellulose microfibrils (c) Schematics of a cellulose microfibril** (13).

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) (Table 2.3). 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 "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} (32). 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 (86, 54, 26). Table 2.4 shows the general cell wall chemical composition of coniferous fibers (58).

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 (44, 45). 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, colouring matters and starches in wood and pulp. Water solubles affect the pulp yield to some extent. Therefore, water solubles in a plant should be as low as possible (5). 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 (52). 1% NaOH solution extracts low-molecular-weight carbohydrates consisting mainly of hemicellulose and degraded cellulose in wood and pulp. The solubility of wood indicates the degree of fungal decay or of degradation by heat, light, oxidation, etc. As the wood decays or degrades, the percentage of alkali-soluble material increases (65, 82); which indicates that wood is more susceptible to deterioration during storage (65). 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 (3).

Cellulose is the main component in chemical pulping; its amount in wood hence directly reflects the variation in pulp yield and economy in pulp production. Cellulose is long chain polymer while 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 behaviour of the fiber (2). Paper strength also depends on cellulose content of the pulp and, mechanical strength of the pulp (especially tensile strength) is directly proportional to the cellulose content (59).

Lignin represents what is called the "incrusting material" forming a part of the cell wall and middle lamella in wood. Lignin, together with cellulose is one of the main components of the natural organic material from which coal and some other type of solid fossil fuels had been formed. After cellulose, lignins are the most widely occurring compounds of biological origin; their content being about 25–30% in softwood and 19–23% in hardwood. It is estimated that currently, planet earth contains 300 billion metric

tons of lignin, with an annual biosynthetic rate of production of 20 billion metric tonnes (7). Thus, with a perceived shortage of petroleum-based materials as well as a desire to utilize 'green materials', the chemistry and technology of lignins is seeing renewed interest. Lignin is a multifunctional natural polymer that has the potential to be developed into a major industrial raw material for a multitude of applications (36, 107). In contrast to cellulose, which is a polymeric compound with a specific set of characteristics, lignin is a macromolecular organic compound of indeterminate chemical and physical structure (91). It is an aromatic, amorphous substance containing phenolic, methoxyl, hydroxyl and other constituent groups; its chemical structure has not been fully elucidated. 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 colour, are also associated with the lignin content (92). The residual lignin present in the pulp influences the paper properties and causes 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.

Pulping, for paper is one of the most important applications from agricultural resources. Removal of lignin is a main objective of pulping and bleaching processes. A matter of great concern here is that millions of tonnes of lignins produced annually as by-products of pulping processes are predominantly burnt or disposed off with a consideration that they have low energy value (64, 110). Recently, lignin, as the most abundant natural aromatic material, has been considered for new economical applications such as bio-fuel, binder, dispersant or emulsifier, phenolic resins, carbon fibers (14, 49), automotive brakes, wood panel products (104), polyurethane foams (37), epoxy resins for printed circuit boards (56) and so on. The large scale utilization of lignin requires the thorough understanding of its structural features. Direct structural characterization is complicated by the fact that lignin is cross-linked, amorphous and practically insoluble in its native state. Various thermal decomposition techniques have proven to be useful for a rapid structural characterization of lignins in the microanalytical scale (39). Taking into consideration the above fact, thermal studies of the two raw materials, lemon and sofia grasses and their isolated Klason lignins were carried out via thermogravimetric analysis (TGA). Lignocellulosics burn because the cell wall polymers undergo pyrolysis reactions

with increasing temperature to give off volatile, flammable gases. The hemicellulose and cellulose polymers are degraded by heat much before the lignin. The lignin component contributes to char formation, and the charred layer helps insulate the composite from further thermal degradation (88). Hence, the knowledge from this basic study can be beneficial to the food technologist, material scientist, and polymer chemist for future applied research studies.

2.2 EXPERIMENTAL METHODOLOGY

Lemon and sofia grasses were collected from Punjab Agriculture University Ludhiana (India) at the start of the rainy season. The freshly cut lemon and sofia grasses were hand-chopped manually into 15-25 mm long pieces and sun-dried for 20 days and then essential oils were extracted by steam distillation in crude iron direct-fired stills having false bottom, over which the grasses were charged. The lignocellulosic residues (LCR) after extraction were air dried and kept in ventilated polythene bags.

2.2.1 Anatomical and morphological studies

The detailed anatomical study of both the raw materials was carried out using surface electron microscope (SEM, Leo 435 VP, England). The cross sections were subjected for fixation using 3% (v/v) glutaraldehyde - 2% (v/v) formaldehyde (4:1) for 24 h. Following the primary fixation, cross sections were washed thrice with double distilled water and then treated with alcohol gradients of 30%, 50%, 70%, 80%, 90% and absolute for dehydration and kept for 15 min each up to 70% alcohol gradient, thereafter treated for 30 min each for subsequent alcohol gradients. After treating with absolute alcohol, cross sections were air dried and examined under SEM using gold shadowing technique (29). Electron photomicrographs were taken at 15 kV using detector SE1 at desired magnifications. The anatomical structures of lemon and sofia grass are shown in Plates 2.1 and 2.2. The morphological characteristics of both lemon and sofia grasses were compared to those of *Populous deltoids*, wheat straw and sugarcane bagasse and are reported in Table 2.6.

For fiber length determination, small slivers of both the grasses were macerated with 10 mL of 67% HNO_3 and boiled in a water bath at 100 °C for 10 min (70). 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. About 0.5 mL of macerated fiber suspension was finally placed on a slide (standard, 7.5 cm × 2.5 cm) by means of a medicine dropper of about 10 cm length and 8 mm internal diameter with one end fitted with a rubber bulb and the other carefully smoothened but not tapering (35). All fiber samples were viewed under a calibrated microscope; a total of 100 randomly chosen fibers were measured. For fiber diameter, lumen diameter and cell wall thickness determinations, cross-sections of 25 μm thickness were cut on Leitz base sladge microtome 1300 and were stained with 1:1 aniline sulphate–glycerine mixture to enhance cell wall visibility (cell walls retain a characteristic yellowish colour). Using fiber dimensions, the derived wood properties like, Runkel ratio (2 × fiber cell wall thickness/ lumen diameter) (89), Luce's shape factor [(fiber diameter^2 – fiber lumen diameter^2)/ (fiber diameter^2 + fiber lumen diameter^2)] (57), slenderness ratio (fiber length/fiber diameter) (103), solids factor [(fiber diameter^2 – fiber lumen diameter^2) × fiber length)] (12) and flexibility coefficient (90, 70) were then determined.

2.2.2 Proximate chemical analysis

Dried lemon and sofia grasses were pulverized in a laboratory Wiley mill (Weverk, A-47054, Sweden) and their fractions passing through −48 mesh size but retained on +80 mesh size were used for analysis of water solubility (TAPPI T 207 cm-99 "Water solubility of wood"), 1% caustic soda solubility (TAPPI T 212 om−98 "One percent caustic soda solubility of wood") and alcohol-benzene solubility (TAPPI T 204 cm−97 "Alcohol-benzene solubility of wood"). Moisture content was calculated based on oven dry weight of the sample. Dust samples of the two raw materials were next extracted in a Soxhlet apparatus with ethanol–toluene (1:2, v/v) for 6 h (TAPPI T 264 cm-97 "Preparation of wood for chemical analysis"). After air drying, the extractive-free samples were subjected to further chemical analysis like holocellulose (TAPPI T 249 cm−00 "Holocellulose in wood"), lignin (TAPPI T 222 om−02 "Lignin in wood"), ash (TAPPI T 211 om−93 "Ash in wood"), pentosan (TAPPI T 223−cm-01 "Pentosans in wood") as per Tappi Standard Test methods: 2007 (6). The results of proximate chemical analysis were compared with sugarcane bagasse, sunflower stalks and *Arundo donax* as reported in Table 2.7.

The determination of carbon was done in a Leco SC-144DR instrument using direct combustion and infrared detection. In nitrogen determination, the sample is dropped into a hot furnace and flushed with pure oxygen for very rapid combustion, and by-

products of combustion are formed (CO_2, H_2O, NO_x, and N_2). The material then is passed through the furnace filter and thermoelectric cooler for subsequent collection in a ballast apparatus. These collected gases in the ballast are mixed, and a small aliquot dose is then used for further conversion of the gases. The remaining aliquot that has been reduced is measured by the thermal conductivity cell for nitrogen, in a Leco FP-528. Two determinations per sample were performed according to CEN/TS 15104 (3) to determine the carbon and nitrogen contents.

A Leco TruSpec TRSCHNC was used to determine hydrogen. The system is based on the Dumas method of combustion. There are three phases during an analysis cycle: purge, burn, and analyze. In the sample-drop purge phase, the encapsulated sample is placed in the loading head, sealed, and purged of any atmospheric gases that have entered during sample loading. The ballast volume (zero volume at this point) and gas lines are also purged. During the burn phase, the sample is dropped into the primary furnace (950°C), and flushed with pure oxygen for very rapid combustion. The products of combustion are passed through the after-burner furnace, furnace filter, pre-cooler, and thermoelectric cooler before being collected in the ballast volume. In the analysis phase, the combustion gases in the ballast become homogeneous by means of passive mixing. A series of infrared detectors measure the evolved gases for hydrogen. In addition, a 3 cm^3 aliquot captured in a loop before the ballast piston is forced down to evacuate the ballast. An optimized detector was used for hydrogen. The final result was displayed as weight percentage, according to CEN/TS 15104 (3).

2.2.3 Thermo-gravimetric analysis

The extractive free aliquots of both the homogenized non-woody dust samples (passed through −48 and retained on +80 mesh size) after being subjected to moisture determination (drying at 105 °C to constant weight) and their Klason lignin isolated thereby (TAPPI T 222 om−02 "Lignin in wood") (6) were used as samples (in a mass range of 7 to 10 mg) for the TGA studies. This analysis consisted of recording the loss in weight of samples due to drying, volatilization, and gasification under dynamic conditions between 29 °C (room temperature) and 900 °C at a constant heating rate 10 °C/min under an inert atmosphere of N_2 using an EXSTAR TG/DTA 6300. The samples were placed in a little cup made of aluminium hanging from a microbalance (47). The variation of mass of the samples allowed drawing the TG (variation of the mass in function of the

temperature) and TGD (derivative of loss of mass versus the time) thermograms. The combination of these two thermograms gave a clear indication of number of stages of the thermal degradation.

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

2.3.1 Anatomical and morphological studies of lemon and sofia grasses

Plate 2.1 shows T.S. of leaf of lemon grass, demonstrating its anatomical features under various magnifications. For lemon grass, the outermost layer i.e. epidermis was wavy and uniseriate, made of living parenchyma cells varying in shape and size (Plate 2.1A). A large amount of silica remains endorsed in the epidermis, which makes this layer completely undesirable for pulp and papermaking because the epidermal cells dissolve very slowly and incompletely during chemical pulping. The characters of diagnostic importance in the identification of lemon grass were prickle hairs (trichomes), the unicellular outgrowths of epidermis, seen frequently with pointed tips and elongated swollen bases (Plate 2.1A). Trichomes were absent in sofia grass. The epidermis was followed by layers of collenchyma cells with an unevenly thickened cell wall. They were mainly supportive in function. Cells were tightly packed with no intercellular spaces (Plate 2.3B). The collenchyma cells having depositions of cellulose and pectin compounds in their primary walls, became lignified and thickened, to form sclerenchymatous cells at maturity (Plate 2.1B). They hence served to provide rigidity to the plant part. Besides these sclerenchyma cells, the bundles of sclerenchymatous cells, found in the phloem region were called bast fibers or phloem fibers (Plate 2.1B and 2.3A). A cap of bast fibers on the phloem side of the vascular bundles represented the most valuable, fibrous material in the lemon grass, encrushing the epidermis. The ground tissue was composed of parenchyma cells which were large, barrel shaped, isodiametric and thin walled when moving away from the vascular bundles, while parenchyma cells were smaller in the vicinity of vascular bundles (Plate 2.1B and 2.3C). The parenchymas primarily function to store solutes and foodstuffs for the plant (38).

The conductive tissues, xylem and the phloem fibers were surrounded by a strong sheath of sclerenchyma cells (Plate 2.1B). Plate 2.1D shows a vessel element of lemon grass, having dense, reticulate and lignified thickenings; thus they were capable of stretching. The conductive tissues or vascular bundles in lemon grass, were oval shaped (Plate 2.1 B), conjoint and collateral i.e. xylem and phloem both occurred in contact with each other, with xylem lying towards the inner side while phloem on the outer side. The xylem was peculiarly demarcated by the presence of very large reticulate tapering vessels arranged in a Y-shape in lemon grass. Phloem near the periphery (protophloem) was highly crushed and appeared to be fused with a strong sheath of sclerenchyma cells or bast fibers. The vascular bundles were embedded in a ground tissue of parenchymatous cells. The essential oil secreted by lemon grass was essentially stored in modified, large parenchymatous cells which took up very dark stain on staining with Schiff's reagent (used to stain aldehydes). Lemon grass oil essentially being rich in citral, and that of sofia grass in geranial, both being aldehydes, took up dark pink colour with this stain and retained it even after washing (53). SEM microphotograph of lemon grass in Plate 2.3D shows that oil glands bursted as a result of steam distillation, thereby releasing oil. The rupturing of cell walls loosened the anatomy, thereby abating the problem of mass transfer and facilitating faster penetration of cooking liquor during pulping which further reduces the overall cooking time.

Plate 2.4A shows the T.S. of stem of sofia grass. The outermost part of a sofia grass stem was cuticle, that covered the single layered epidermis which was led by 5 to 6 layers of highly lignified collenchyma cells (dark stain on staining with Schiff's reagent , Plate 2.2A) with unevenly thickened cell wall, that function supportively. The cortex region contained circular, conjoint and collateral vascular bundles. The xylem vessels arranged in a V-shape were peculiarly demarcated by the presence of annular thickenings (Plate 2.4B), and blunt ends (Plate 2.2B). Phloem near the periphery (protophloem) was highly crushed and appeared to be fused with a strong sheath of sclerenchyma cells or bast fibers, also known as phloem fibers (Plate 2.4B). The vascular bundles were embedded in a large ground tissue made up of thin walled, modified parenchymatous cells, barrel shaped without intercellular spaces (Plate 2.4C). Plate 2.2D shows circular oil glands in sofia grass with condensed oil clouds (Plate 2.4D).

The minor vascular bundles in both the grasses, concentrated close to the epidermis formed an almost continuous ring of fibrous tissue. This peripheral part contained the most

valuable sclerenchyma fiber bundles which form the main fibrous raw material for producing pulp. The epidermis layer and ground tissue form a major part of fines fraction in the pulp and are perhaps the most undesirable elements of the stalk so far as pulping is concerned. Moreover, their cells dissolve very slowly and incompletely during the pulping process, masquerading the problem of fluff at dryer or printing machine due to larger surface area of the non-fibrous cells (20). The majority of non-fibrous cells was short in length, and might cause poor drainage in pulp (46). The parenchyma cells are easily deformed, to generate the fines (15), which result into reduced freeness and increased water retention by the pulps; yet they are of importance because as the thin-walled parenchymas collapse, they aid in bonding, and contribute to the tensile strength of the unbeaten pulps (106).

The dimensions of various non-fibrous cells of lemon and sofia grass fibers have been compared with wheat straw (T259OM-93) and rice straw fibers (T259OM-93) in Table 2.5. The parenchyma cells of lemon and sofia grass were 368.9 and 332.3 μm long while 84.7 and 64.1 μm wide; vessels were 198.2 and 147.1 μm long while 35.6 and 28.5 μm wide. The dimensions of parenchymatous cells of lemon grass lie between wheat straw and rice straw, while that of sofia grass were lowest among them. Parenchymatous cells appear in the form of primary fines (Plates 2.5 C,D) during pulping (40). Marton and Marton reported that the surface area of fine fractions is 46.8 m^2/g compared to surface area of fibers which is 9.9 m^2/g (61). Fines have a major influence on paper machine drainage performance due to their high water sorption capacity (2-3 times that of fibers) (43). These non-fibrous cells act as fillers and affect mechanical strength and surface properties like porosity, smoothness and Denninson wax pick strength (43, 62, 63, 61) etc. Higher the surface area of fines more will be the consumption of rosin size. Accordingly, more paper maker's alum [$Al_2(SO_4)_3$.14-18 H_2O] will be required for anchoring the soap size to cellulose surface. This complexity of the ground tissue is a great problem to the paper makers. Processing and screening systems must be developed for each raw material exclusively taking into consideration its specific morphology.

Table 2.6 shows the morphological characteristics of lemon and sofia grass fibers, and their comparison with those of *Populous deltoids* (2), wheat straw (96) and sugarcane bagasse (1). Fiber morphological characteristics play a key role to find out the suitability of any wood species or other raw materials for pulp and paper manufacturing. The fiber length was quite varying in both the raw materials (Plates 2.5 A,B: lemon grass and Plates

2.5C-F : sofia grass); however the average fiber length of lemon and sofia grass were 1.09 and 0.87 mm respectively as compared to *P. deltoids* (0.984 mm), sugarcane bagasse (1.18 mm) and wheat straw (1.51 mm). Fiber length generally influences the tearing strength of paper. Greater the fiber length, higher will be the tearing resistance of paper. On the other hand, longer fibers tend to give a more open and less uniform sheet structure. The fiber length is of secondary importance in determining the breaking length with other properties. The effect of fiber length has been ascribed to stress dissipation; the longer the fiber, the greater the area over which the stress is dissipated (30). The average fiber width of lemon (16.3 μm) and sofia grasses (14.7 μm) were higher when compared to wheat straw fiber (13.60 μm) but on much lower side when compared to *P. deltoides* (25.60 μm) and sugarcane bagasse (21.4 μm). The cell wall thickness of sofia grass fibers (3.86 μm) was in close proximity with wheat straw fibers (3.96 μm), and that of lemon grass (4.62 μm) exceeded *P. deltoides* (4.10 μm) whereas sugarcane bagasse held the highest cell wall thickness of 7.74 μm. Fiber diameter and wall thickness govern the fiber flexibility. Thick walled fibers adversely affect the bursting strength, tensile strength and folding endurance of paper. The paper manufactured from thick walled fibers will be bulky, coarse surfaced and containing a large amount of void volume; whereas, paper from thin walled fibers will be dense and well formed. The lumen width of sofia grass fibers (5.41 μm) resembled with wheat straw (5.68 μm), while that of lemon grass fibers (6.07 μm) resembled with bagasse fibers (6.27 μm). Fiber lumen width affects the beating of pulp. Larger the fiber lumen width, better would be the beating of pulp because of the penetration of liquids into empty spaces of the fibers.

Arithmetic ratios calculated from the dimensional measurements of fibers also helped to assess various properties of paper. The fibers of lemon grass (66.9) were more slender compared to sofia grass (59.2). The slenderness ratio (L/D), also termed as felting power, was inversely proportional to the fiber diameter and related to pulp yield (positively) and to digestibility (negatively) (72). Slenderness ratio of lemon and sofia grass was calculated as 66.9 and 59.2. They were less slender, when compared to wheat straw and sugarcane bagasse. Fibers having high slenderness ratio had a low degree of collapseness and conformability, and such type of papers gave more tear, porosity, bulk, and opacity (85). When used for applications such as paper, the slenderness ratio of individual cells in a fiber affected the flexibility and resistance to rupture of the fibers

(60). The fibers of lemon (37.24) and sofia grasses (36.8) were quite flexible and hence, showed less plasticity. Wheat straw (41.76) and *P. Deltoids* (68.75) fibers were comparatively more flexible, however, sugarcane bagasse fibers, with a flexibility coefficient of 29.29, were least flexible. Lemon and sofia grass fibers, hence offered a higher degree of collapseness and conformability within the sheet and tend to produce less opaque sheet having lower bulk and air permeability compared to wheat straw and sugarcane bagasse.

The most important and primary observation in order to find suitability of any raw material for pulp and paper manufacturing is the Runkel ratio. Standard value of this ratio is 1. The fibers with Runkel ratio above one are thick walled fibers which are stiffer, less flexible and form bulky paper sheet of lower bonded area (21). Runkel ratio is also related to paper conformability (19) and pulp yield (72). The Runkel ratio of both lemon (1.45) and sofia grass (1.52) fibers was higher than *P. deltoids* (0.465) but found place somewhere near that of wheat straw fibers with a Runkel ratio of 1.39. Yet the highest runkel ratio was found for fibers of sugarcane bagasse (2.46). Runkel ratio is directly affected by cell wall thickness but not by lumen diameter, and is related to fiber density (80). The breaking length, bursting strength, and double fold are determined by fiber density. In comparison to bagasse fibers, lemon and sofia grasses shared a lesser Runkel ratio, fiber diameter and rigidity coefficient which are measures of the flexibility and wet plasticity of fibers, and would result in a greater degree of fiber collapse and higher degree of conformability within the sheet, which would further give rise to a sheet of higher density or lower bulk (21, 20). As such the size and number of inter-fiber bonds, was improved in both lemon and sofia grass fibers. The mechanical properties along with other properties of paper related to wet plasticity might be increased by fibrillation, and by the presence of high hemicelluloses. Large amount of hemicelluloses might result in decreased tensile and bursting strength not because of the bonding effect, but possibly because the individual fiber strength might be reduced as a result of the decrease in the average molecular weight of the polymer system (102).

The Luce's shape factor of lemon grass (0.71) was lower than that of sofia grass (0.79), comparable to wheat straw and about 50% more than *P. deltoids*. On the other hand, both the raw materials stood on a much better place when compared to bagasse. It means, the mechanical and structural properties of lemon grass were well in the range of wheat straw but both the raw materials should have better properties than sugarcane

bagasse. Luce's shape factor and solids factor were found to be related to paper sheet density and could significantly be correlated to breaking length of paper in Eucalyptus by Ona et al. (72). Similar to Runkel ratio, the trend of variation of Luce's shape factor might be associated with that of wall thickness, because both the fiber diameter and the fiber lumen diameter were used to obtain the cross-sectional fiber wall area in the equation for Luce's shape factor (57). Thick walled and narrow lumen fibers with long fiber length gave maximum solid factor. Breaking length and burst index depend upon collapsibility of fibers to ribbons on pressing. Initially, the strength of paper depends upon fiber length and then extent of hydrogen bonding. Sofia grass gave lower solids factor (165.63) compared to lemon grass (240.24), wheat straw (180.19), sugarcane bagasse (632.16) and *P. deltoids* (340.07). While solid factor of lemon grass was more than sofia grass and wheat straw but less than sugarcane bagasse and *P. deltoids*. *Picca abies* and *Pinus kesiya* fibers (23) having long fiber length produced a high solids factor of 1067.2 and 1024.5 respectively. However, their thin walls and wide lumen enabled them to be converted into thin ribbon like structures which mitigated the negative side of solid factor.

The total fibers in lemon and sofia grasses were about 37.8 and 34.61% compared to 39.2% in wheat straw while 50% in *P. deltoides*. Parenchyma and epidermal cells accounted for about 35.33 and 21.64% of the total cells in lemon grass and 37.14 and 23.83% respectively in sofia grass. The parenchyma and epidermal cells have large surface area (63) and may act as fillers. They may also adversely affect the mechanical strength properties of paper (43). The vessels accounted for about 5.23% of the total cells in lemon grass which were quite near to the vessel count of wheat straw (5.14%) while sofia grass had a lower amount of vessel elements making just 4.42% of the total cells. Vessels had blunt ends, perforated end walls, and pits of various shapes.

2.3.2 Chemical characterization of lemon and sofia grasses

Both cold and hot water extractives were much higher for lemon and sofia grasses than the bagasse (Table 2.7). It means both the raw materials would require slightly higher alkali dose to neutralize acidic extractives and pulp yield would also be negatively affected. 1% alkali solubility was distinct between lemon (30.64%) and sofia grasses (28.21%), and higher than sugarcane bagasse and *Arundo donax* but it was lesser than that of the sunflower stalks indicating compositional dissimilarities between the two species. It indicated that both the grasses could not be stored for a longer period after harvesting

compared to sugarcane bagasse and *Arundo donax*. The high NaOH solubility of wheat straw was possibly due to the presence of low molar mass carbohydrates and other alkali soluble materials. The alcohol-benzene solubles in sofia grass (5.86%) were higher than lemon grass (4.33%) but, lower than that of *Arundo donax*; while *P. deltoids* had the least amount. This indicated that rice straw and wheat straw contained more of substances like waxes, fats, resins, phytosterols, and non-volatile hydrocarbons, low-molecular-weight carbohydrates, salts, and other water-soluble substances. A higher content of extractives would be converted into pitch which would adversely affect the runnability of process equipment due to choking of Fourdrinier wire and; quality of paper, due to shadow marking. Papers made from such type of fibers showed reduced water absorbency (52). Holocellulose, as a whole, adds to the overall strength of the paper. Lemon and sofia grasses had a total carbohydrate fraction (holocellulose) approximately equal to that of hardwoods. This was due to the high hemicellulose (mainly pentosans), and low lignin content compared to wood, which is a characteristic feature of agro-residues. This characteristic directly influences the fibrillation of fibers during refining operations. α–cellulose content was satisfactory for lemon (44.16%) and sofia (45.55%) grass. According to the rating system designated by Nieschlag et al. (68), plant materials with 34% and over α –cellulose content were characterized as promising for pulp and paper manufacture from a chemical composition point of view. Lemon and sofia grasses had higher cellulose content compared to sugarcane baggase, sun flower stalks and *Arundo donax*. Hemicelluloses in lemon and sofia grasses were comparable to sugarcane bagasse, sun flower stalks and *Arundo donax*. The quantity, chemical structure, distribution and degree of polymerization of hemicelluloses influenced final paper strength (24). It was shown that the higher the hemicellulose content; the better the swelling behaviour of the pulp, which lead to an increase in mechanical strength properties, including tensile, burst indices and double folds (67) and reduction in beating/refining energy (94). Klason lignin contents in lemon (17.39%) and sofia (17.04%) grasses were much lower than sugarcane baggase and *Arundo donax*. This, in practice, means that these materials would need milder pulping conditions (lower temperatures and chemical charges) than those of softwoods and hardwoods in order to reach a satisfactory kappa number. It also indicates the potential of these materials to undergo bleaching more easily and with the utilization of fewer chemicals. Also, the higher the lignin content, greater would be the stiffness of fibers (22). Examples of milder pulping conditions leading to satisfactory delignification

levels are abundant in the literature. Kaur et al. (50) reported such conditions for lemon and sofia grasses, Singh et al. (96) for wheat straw, Agnihotri et al. (1) for sugarcane bagasse, Dutt et al. (23) for *Ipomea carnea* and *Cannabis sativa* and Dutt et al. (22) for *Hibiscus cannabinus* and *Hibiscus sabdariffa*. Ash content (carbonates, Ca, K and some trace elements) was higher in lemon grass (7.05%) followed by sofia grass (5.11%); although high ash contents were undesirable, as they would pass into the pulp; ash contents in this study were in the typical range for non-wood plants and are not expected to have any significant effect on pulp mechanical strength properties. The silica content in lemon grass (3.12%) was on higher side than that of sofia grass (2.10%) and was relatively high as compared to wood. Silica caused rather serious difficulties during recovery and poor drainage of straw pulp during papermaking (101). At the same time, silica could play a role of inhibitor for O_2 delignification and bleaching with H_2O_2, thereby eliminating the need for additional inhibitors to mask transition metals ions during pulping/ bleaching (79). The major elemental constituents of lemon and sofia grasses respectively were carbon (27.3758 and 30.1568%), oxygen (22.6307 and 20.0247%), and hydrogen (48.8130 and 49.1204%). The elemental contents partially determine energetic properties of agro-residues. Some previous researchers found that calorific value of biomass increased with a higher proportion C and H contents (18). The concentration of nitrogen in lemon and sofia grasses were 0.4018 and 0.3561% respectively. The main environmental impact would be the generation of NOx in the chemical recovery furnace (69). Ultimate analysis was very important in order to determine the theoretical air-fuel ratio in thermo-conversion systems, to evaluate the heating values, and also to have knowledge of the pollution potential.

2.3.3 TGA studies of whole lemon and sofia grasses and their Klason lignins

The plant cell wall is principally made of the polymer cellulose in the form of microfibrils, hemicellulose and lignin. Biomass pyrolysis generally proceeds through a series of complex reaction pathways. The composition of the three constituents varies from one species to another and presents the different physical structures. Among the lignocellulosic materials, lignin is the most thermostable component mainly due to the inherent structure of aromatic rings with various branches (105). The information regarding thermal stability of a material is necessary to determine its thermo-mechanical properties. The thermal decomposition of biomass from the lemon and sofia grasses occurred in four distinct phases: (i) moisture evolution, (ii) hemicellulose decomposition, (iii) cellulose decomposition and (iv) lignin decomposition (108, 84). The thermal (TG)

curves of whole lemon and sofia grasses (Figure 2.3a and 2.4a) predicted two major mass loss steps. The TG curves showed that first stage of degradation (~5-10% weight loss), in the range of 100–200 °C, was due to the gradual evaporation of residual moisture. The second mass loss, in the range of 200 to 500 °C was due to decomposition of the three major constituents of raw materials i.e. cellulose, hemicellulose and lignin. The weight loss between 200-300 °C could be approximated to degradation of hemicelluloses while that between 300-350 °C corresponded to weight loss particularly due to celluloses and further from 350-500 °C region could be assigned mainly to lignin degradation. The lignin thermograms (Figure 2.3a and 2.4a) obtained for the two grasses also depicted that thermal range of lignin degradation actually overlapped in the thermal range of cellulose. The DTG curves (Figure 2.3b and 2.4b) further made it clear that lignin degradation also overlapped in the region of hemicellulose degradation. Some researchers believed that the mechanism of wood pyrolysis is a superposition of the mechanisms of the three components (84, 98). For the temperatures above 300 °C, the deterioration of the lignin overlapped with the decomposition of cellulose and stabilized around 500 °C (95). The T_{onset} for both lemon and sofia grass lignin samples was somewhere near 300 °C while T_{maxima} was recorded at 425 and 390 °C for lemon and sofia grass respectively, which hence proved greater thermostability of lignin from lemon grass which could be seen as a result of greater extent of cross linking (95). The lignin has a tridimensional structure. Sharp peaks were not associated with lignin owing to its heterogeneous nature since, it is a polyphenolic polymer formed with three elementary motifs, the coumaryl, sinapyl, and coniferyl alcohols (105). Nearly 40 and 43.3% weight loss could be recorded up till 500 °C for lignin samples from lemon and sofia grasses respectively with a slight loss in weight thereby continuing up till 900 °C. Lignin degradation was very slow with a high rate of non volatile products. It might be attributed to the slow carbonization of lignin, and carbon could be the main product; lignin was the main component responsible for the production of char (74, 109). At 700 °C, % weight loss recorded for the lignin samples from lemon and sofia grasses were 49.6 and 51.6% respectively, whereas at 900 °C, 52.6 and 55% weight loss were recorded. Further pyrolysis was not achieved for lignin samples because in an inert atmosphere (N_2), lignin does not completely decompose and undergoes char formation. As a matter of fact, the many functional groups present in lignins and their derivatives led to complex cross linked structures that formed a char and did not decompose even at such high temperatures (95). In order to completely combust

all of the lignin in a woody biomass sample, an oxidizing environment must be used (99). It can further be concluded that lemon grass showed greater extent of charring. Orfao et al. also stated that these thermal events were different depending upon the chemical environment in the pyrolysis chamber, either inert (nitrogen) or oxidizing (air) (74). Thus the thermal analysis of lignins from the two raw materials provided an evidence of their heterogeneity and extent of cross linking. Also it could be stated that among the lignocellulosic materials, lignin was the most thermostable component mainly due to the inherent structure of aromatic rings with various branches and more thermostable a component is, more is its molecular mass (105). Because the reactivity range of lignin is quite wide, the degradation of lignin occurred in a wide temperature range. The thermogravimetric studies on lignin had shown that the combustion of lignin took place in the following steps: (i) cleavage of α- and β-aryl-alkyl-ether linkages occured between 150 °C and 300 °C; (ii) aliphatic side chains started splitting off from the aromatic ring around 300 °C; (iii) the carbon-carbon linkage between lignin structural units were cleaved at 370-400 °C (10); decomposition or condensation of aromatic rings was believed to take place at 400-600 °C (73). Most lignins showed their maximum rate of weight loss between 300 °C and 400 °C (31, 27). Our results were in good agreement with the theoretical stoichiometric values, based on the fact that lignocellulose materials were chemically active and decomposed thermo-chemically between 150 and 500 °C; hemicellulose mainly between 225-325 °C, cellulose between 305-375 °C and lignin between 250 and 500 °C (81, 108). The variation of constituent fractions in biomass, types of species and plant origin gave different thermal behaviour and products namely non-condensable gases, heavy volatiles (tar) and char (83).

From the DTG curves of whole lemon and sofia grass samples, the maximum degradation rates were found to be 1.02 and 0.65 mg/min respectively which were attained at 350 °C (Figure 2.3b and 2.4b). Owing to homogeneous nature of the carbohydrate polymers, it was noticed that the two peaks were sharp, which confirmed the faster rate of carbohydrate degradation as compared to lignin which degraded slowly. Normally, DTG curves from biomass exhibited a peak at high temperatures that was mainly due to the pyrolysis of the cellulose and a shoulder at lower temperatures that could be attributed to the pyrolysis of the hemicelluloses (66).

Table 2.1: Comparison of fiber dimensions of softwood, hardwood and non-wood cellulosic raw materials (8)

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 (97)

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 (33).

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° (latewood) and 20-30° (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 (58)

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: Morphological characteristics of parenchyma cells and vessels of lemon and sofia grasses and their comparison with wheat and rice straws

Particulars	Lemon grass	Sofia grass	Wheat straw (T259OM-93)*	Rice straw (T259OM-93)*
Parenchyma				
Length, μm	368.9±3.6	332.3±2.9	450	350
Width, μm	84.7±3.1	64.1±3.3	130	82
Vessels				
Length, μm	198.2±2.5	147.1±1.1	100	650
Width, μm	35.6±1.3	28.5±1.4	60	40

± refers standard deviation

* Tappi Standard Test Methods: 2007 (6)

Table 2.6: Morphological characteristics of Lemon and Sofia grasses

Sl. No.	Particulars	Lemon grass	Sofia grass	*P. deltoids* (2)	Wheat straw (96)	Sugarcane bagasse (1)
1	Fiber length (L), mm	1.09±0.43	0.87±0.30	0.984	1.18±0.08	1.51±0.08
2	Fiber width (D), μm	16.3±1.6	14.7±1.3	25.60	13.60±1.7	21.4±1.6
3	Lumen diameter (d), μm	6.73±0.4	5.07±0.5	17.60	5.68±1.09	6.27±0.4
4	Cell wall thickness (w), μm	4.62±0.2	3.86±0.4	4.10	3.96±0.08	7.74±0.2
5	Slenderness ratio (L/D)	66.9	59.2	38.43	86.76	70.56
6	Flexibility coefficient (d/DX100)	31.1	30.0	68.75	41.76	29.29
7	Runkel ratio (2w/d)	1.45	1.52	0.465	1.39	2.46
8	Luce's shape factor $[(D^2- d^2)/(D^2 + d^2)]$	0.71	0.79	0.36	0.70	0.84
9	Solids factor $[(D^2- d^2)L]$	240.24	165.63	340.07	180.19	632.16
10	Rigidity coefficient (2w/D)	0.57	0.53	0.32	0.49	0.72
11	Fiber, %	37.80±.05	34.61±.03	50.00	39.20±0.06	—
12	Parenchyma, %	35.33±.06	37.14±.05	—	32.10±0.05	—
13	Vessels, %	5.23±.03	4.42±.04	32.40	5.14±0.05	—
14	Epidermis, %	21.64±.05	23.83±.06	—	23.56±0.04	—

± refers standard deviation

Table 2.7: Proximate chemical analysis of lemon and sofia grasses

Sl. No	Particulars	Lemon grass	Sofia grass	Sugarcane bagasse (1)	Sunflower stalks (55)	*Arundo donax* (48)
1	Cold water solubility, %	10.95±0.04	8.61±0.05	3.02	–	–
2	Hot water solubility, %	12.08±0.02	7.44±0.03	7.42	21.1	4.73
3	1% NaOH solubility, %	30.64±0.08	28.21±0.1	32.29	50.4	26.80
4	Alcohol -benzene solubility, %*	4.33±0.01	5.86±0.02	1.85	4.07	7.30
5	Holocellulose, %*	72.13±0.5	72.21±0.63	71.03±0.5	66.9	70.20
6	α-cellulose, %*	44.16±0.32	45.55±0.2	42.34	37.6	40.46
7	Pentosans, %*	25.61±0.18	21.92±0.24	23.9	–	–
8	Hemicellulose, %*	29.07±0.31	28.12±0.45	28.60	29.3	29.74
9	Lignin (acid insoluble), %*	17.39±0.34	17.04±0.3	21.7	10.8	22.34
10	Ash content, %*	7.05±0.03	5.11±0.05	2.10	7.90	–
11	Silica content, %*	3.12±0.007	2.10±0.003	0.98	–	–
12	Hydrogen, %	48.8130	49.1204	–	–	–
13	**Carbon, %	27.3758	30.1568	–	–	–
14	**Oxygen, %	22.6307	20.0247	–	–	–
15	**Nitrogen,%	0.4018	0.3561	–	–	–

± refers standard deviation

* Values on extractive free basis

** Elemental mol %

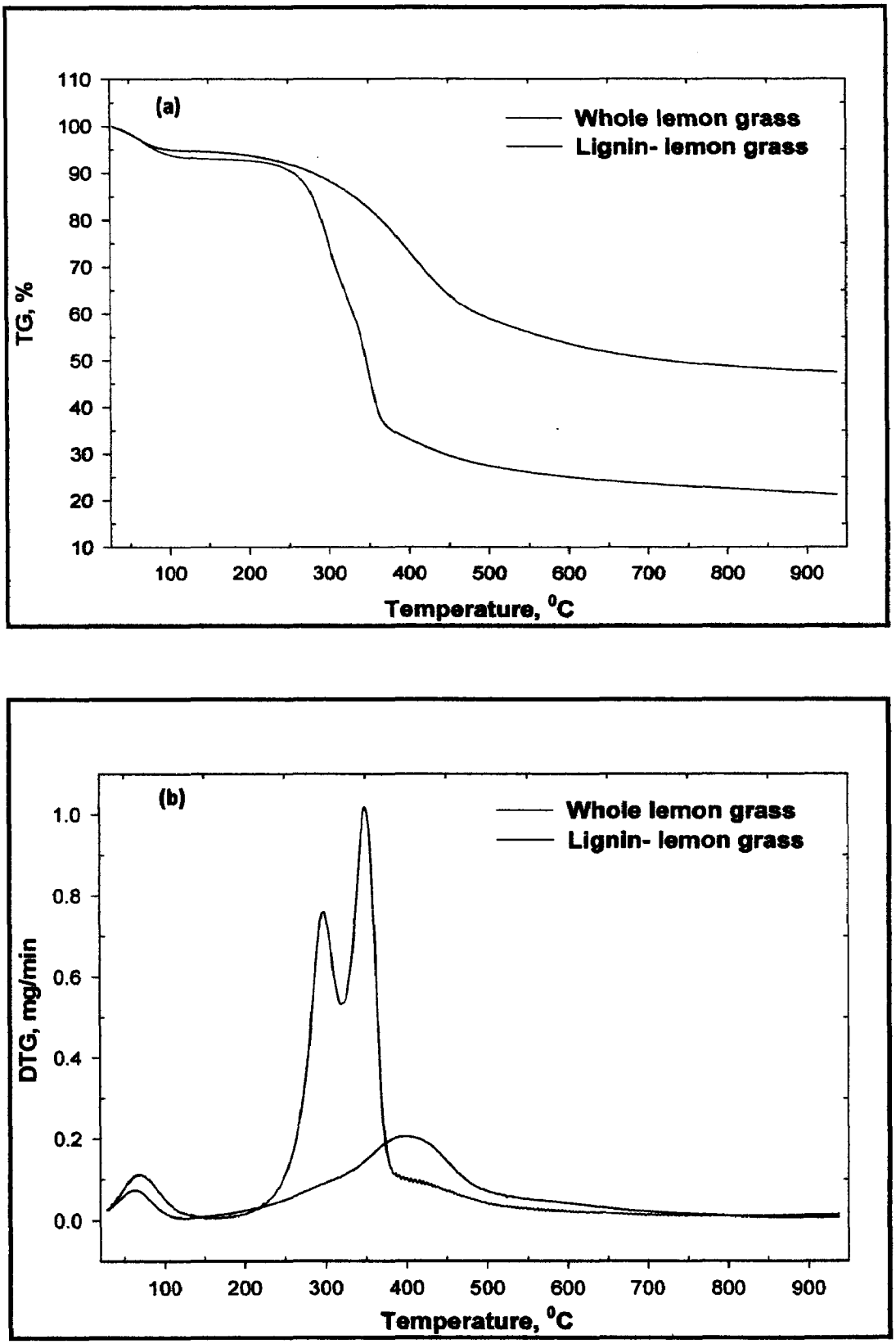

Figure 2.3: (a) TG curves and (b) DTG curves of whole lemon grass and its lignin (Klason).

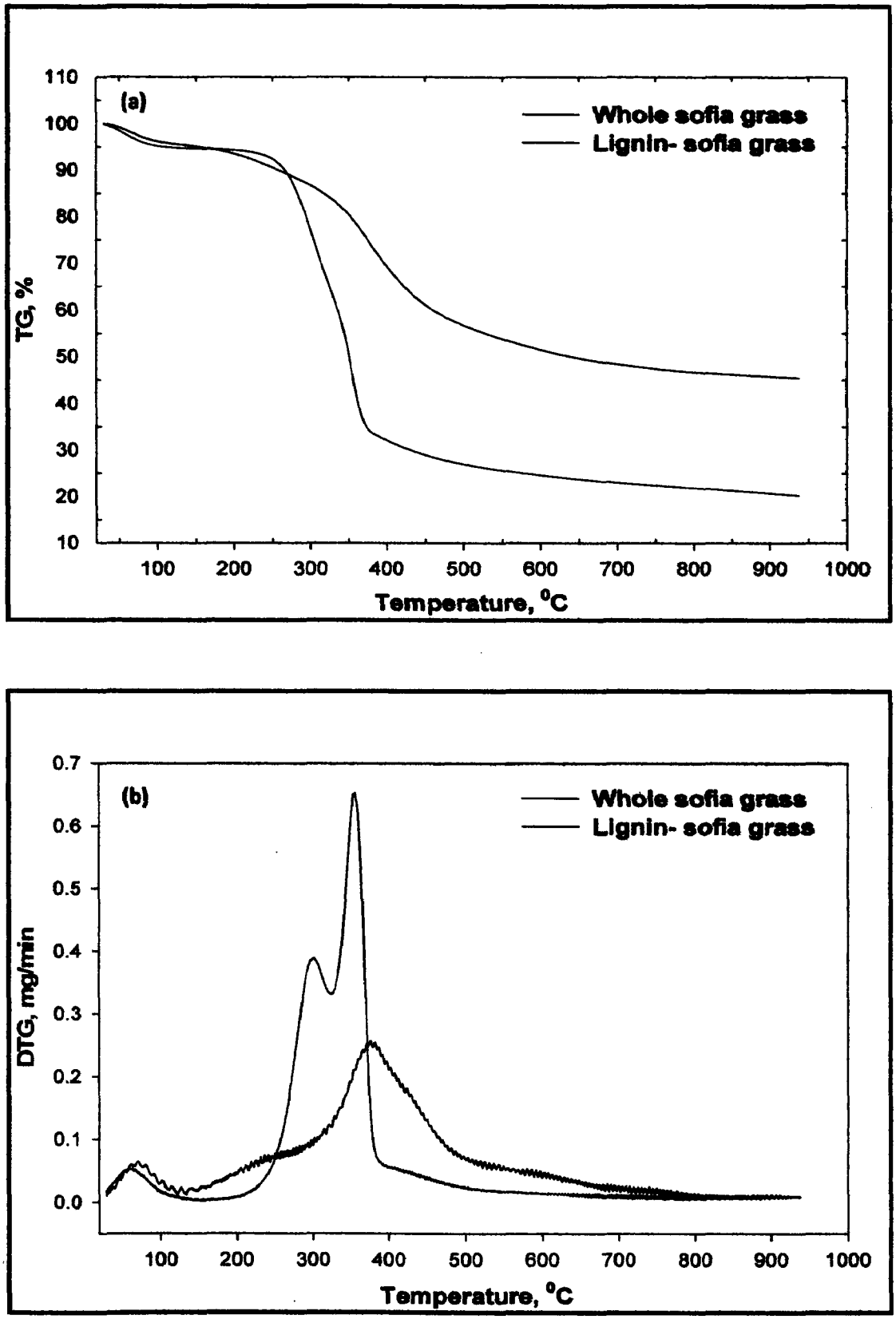

Figure 2.4: (a) TG curves and (b) DTG curves of whole sofia grass and its lignin (Klason)

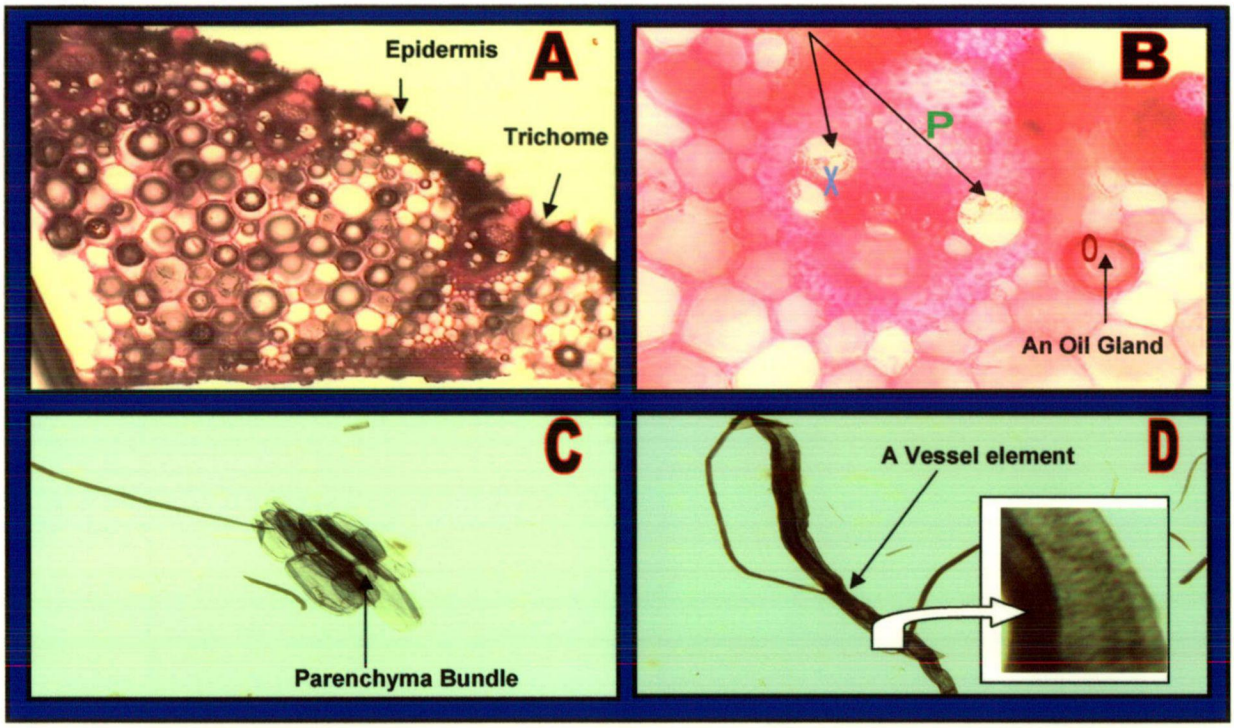

Plate 2.1: Light photomicrographs (A) T.S. of lemon grass leaf at 10X showing epidermis, sclerenchyma fibers, scattered vascular bundles and parenchyma (ground tissue);(B) Enlarged view of a vascular bundle (40X), also showing a prominent oil gland- O, xylem vessel-X and phloem fibers-P;(C) A bundle of parenchyma cells at 10X;(D) A lemon grass vessel showing perforation at 10X

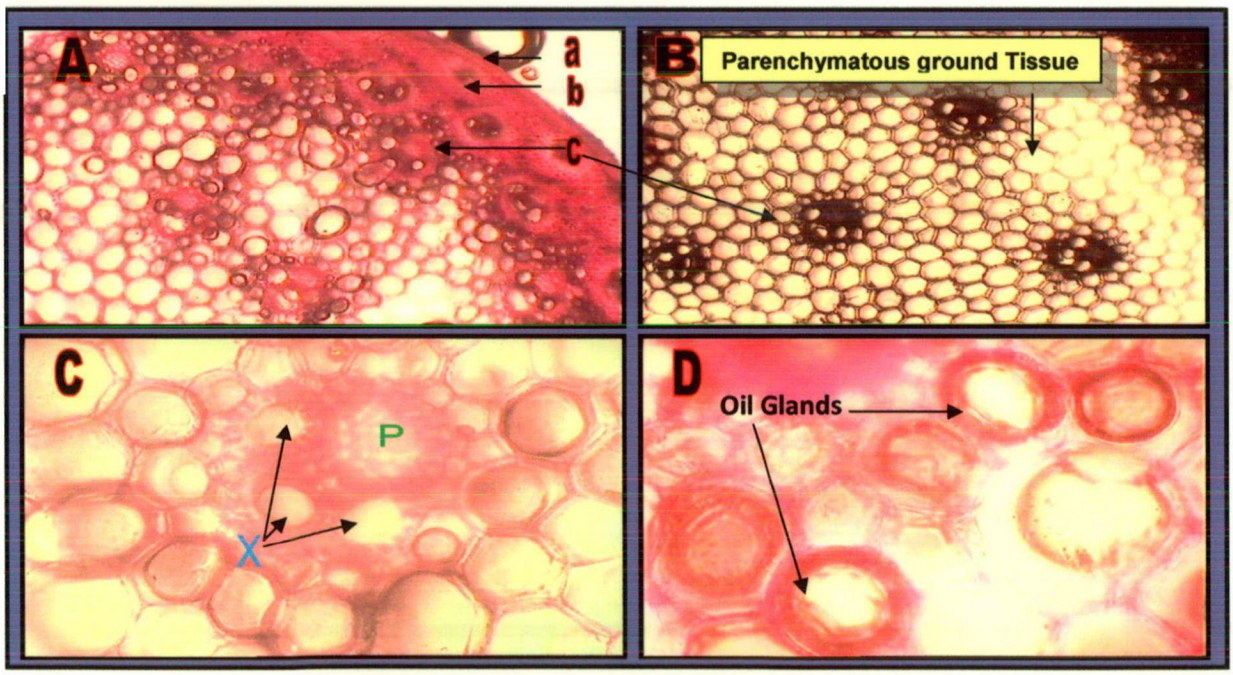

Plate 2.2: Light photomicrographs (A) T.S. of sofia grass before steam distillation, a: epidermis,b: sclerenchyma fibers, c: vascular bundles;(B) T.S. of sofia grass (after steam distillation) at 10X showing parenchymatous ground tissue, sclerenchyma and scattered vascular bundles;(C) An enlarged view of a vascular bundle (40 X) showing xylem (X) and phloem (P);(D) Dark stained oil glands of sofia grass at 40 X

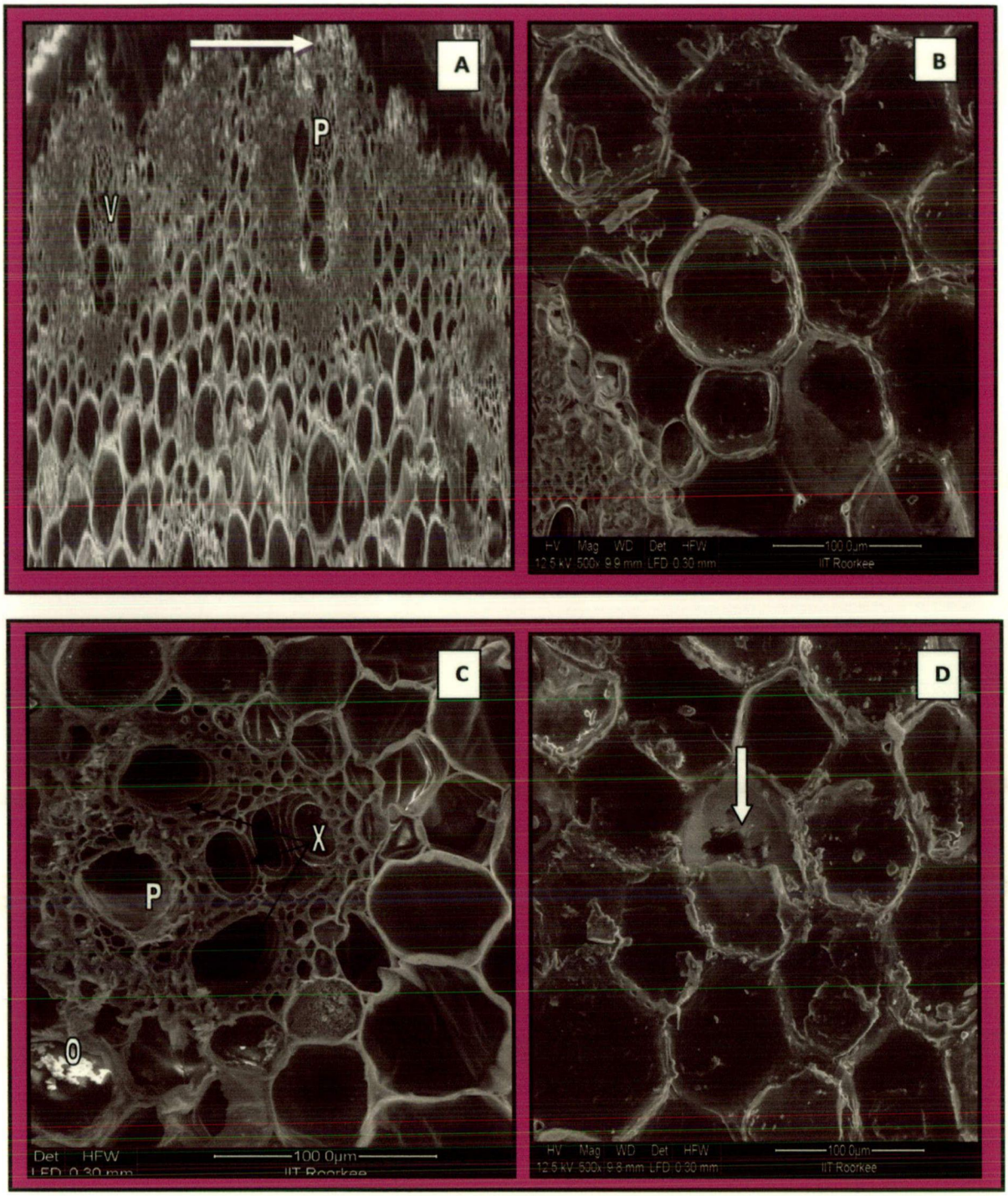

Plate 2.3: SEM photomicrographs, (A) Gross anatomy of lemon grass (100 X), Arrows showing bundles of sclerenchyma fibers, Vascular bundles –V, phloem fibers- P; (B) Collenchyma cells in an enlarged view at 500 X; (C) A prominent vascular bundle at 500 X showing, xylem-X, phloem-P and an oil gland-O, and surrounding parenchymas; (D) Parenchyma cells after steam distillation showing ruptured cell walls and loose anatomy, arrow shows a burst oil gland (500 X)

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CHAPTER 3

SODA AND SODA-AQ PULPING OF LEMON AND SOFIA GRASSES

Chapter 3

SODA AND SODA- AQ PULPING OF SOFIA GRASS AND LEMON GRASS

3.1 INTRODUCTION

In the last 30 years, world's non-wood pulp production has increased continuously and the current share of non-wood fibers on the global pulp capacity is almost 12% (7). The principal interest of pulping the non-woody raw materials is that they provide pulp of excellent quality for making speciality paper or constitute the sole affordable source of fibrous raw materials in some geographical areas. In addition, non-wood plants are an alternative to the increasingly scant forest wood as a source of pulp fibre. To bridge over the extensive gap between demand and supply, many fast-growing annual and perennial plants of higher biomass have been recognized, and investigated to appraise their suitability for pulp production. These include non-woody plants such as *Cannabis sativa*, *Ipomea carnea* (17), *Hibiscus cannabinus*, *Hibiscus sabdariffa* (20), *Sesbania aculeata*, *Sesbania sesban* (18, 19), as well as agricultural residues including sugarcane bagasse (4) and wheat straw (50); fast growing hardwoods via. social forestry such as *Anthocephalus cadamba* (42), *Casurina equisetafolia* and *Leucaena leucocephala* (46) and waste papers. With short growth cycles, low lignin content, and high pentosan or hemicellulose content, non-woody plants offer several advantages over wood, resulting in reduced energy and chemical consumption during pulping (35, 36). An inherent problem is however associated with grass and straw pulping i.e. the high content of small parenchyma cells, leading to a high level of so-called primary fines in the pulp (38).

Pulping involves the systematic rupturing of bonds within the wood structure, which ultimately reduces the wood (or other fibrous raw material) to a fibrous mass (52). On the basis of energy and chemical requirements for fiber separation; four main pulping processes have been described: chemical, semi-chemical, chemi-mechanical and mechanical pulping. Whereas, the chemical pulping methods rely on the effect of chemicals to separate fibers, the mechanical pulping methods show a complete reliance on giving mechanical (physical) action to the fibers (11). The chemical cooking of

lignocellulosics may involve the use of an alkali (kraft and soda pulping), an acid or an organic solvent (organosolv pulping).

Lignin (around 30% of wood composition), a reticulated polymer composed of phenyl propane units linked by ether and covalent carbon–carbon bonds, plays two main functions in the tree; binding the fibres to each other and providing rigidity to their cell walls. In chemical pulping, enough lignin from the (lignin-rich) middle lamella needs to be dissolved in order to separate the undamaged wood fiber cells from each other (8). It involves the application of several alkaline, neutral or acidic components at an elevated temperature and pressure, resulting in dissolution of lignin and some carbohydrates from wood chips. These chemicals penetrate from the lumen through the cell wall towards the middle lamella (29). In general, it has been established (5) that during alkaline pulping of lignocellulosics, two types of degradation of carbohydrates (cellulose and hemicelluloses) take place in carbohydrate chains:

1. Peeling of different end units.
2. Alkaline hydrolysis of glycosidic bonds.

The former (primary peeling) of alkali-catalyzed reactions is mainly responsible for the loss of cooking yield. The peeling reaction involves a stepwise elimination of monosaccharide moieties from carbohydrates starting at their reducing ends and continuing along the polymeric chain until an alkali-stable end group is formed by a competing reaction (the stopping reaction). In contrast to peeling reaction, alkaline hydrolysis is non-terminal including a random cleavage of glycosidic bonds and leading to a harmful formation of new reducing end groups which are then subjected to further degradation (secondary peeling). The alkaline hydrolysis of glycosidic linkages proceeds slowly and only limited amounts of linkages is broken before reaching the maximum temperature of the cook (5). It is also known that hemicelluloses are hydrolyzed much more readily than cellulose due to the highly crystalline nature and high degree of polymerization of cellulose. The rapid hydrolysis of acetyl groups of (galacto) glucomannans in softwoods and xylan in hardwoods (51, 28) occurs by the action of alkali. Substantial amounts of xylan and around 75% of glucomannan are lost from softwoods during cooking.

By and large, it is known (15) that non-wood feed stocks, compared with wood have higher hemicellulose content and lower lignin content. In addition, alkaline pulping

of wood has to be carried out at higher temperatures (165–175 °C), where the degradation of cellulose during delignification, especially at the later **bulk** and the **residual phases**, cannot be avoided. This also means that the viscosity generally decreases as delignification proceeds. In contrast, non-wood straws and grasses can be pulped more easily, and delignification proceeds at lower temperatures (145–165 °C). It has been reported (15) that the **bulk phase** of delignification of straw and grass (by soda, soda-AQ, and kraft processes) occurs before 100 °C with a 50–70% removal of total lignin. Simultaneously, a large amount of hemicelluloses is also dissolved into the cooking liquor during this bulk phase. The high solubility of lignin and hemicelluloses in straw and grass is probably ascribed to their relatively low molecular masses; high content of phenolic structure; high content of phenolic acids, mainly *p*-coumaric and ferulic acids; and high amount of lignin–carbohydrate complexes (24, 14, 53). In addition, most of the alkali is consumed during the **heating-up stage**. Thus, such cooking conditions do not cause much cellulose depolymerization but may vary the amount of dissolved hemicelluloses at different cooks, resulting in characteristic variations in pulp viscosity (*23*).

The pulp and paper industry has turned to modified pulping and bleaching technologies due to increasing global market demands for improved pulp quality, economic constraints imposed on energy use, elevated concerns on environmentally friendly technologies. Kraft process, the dominating pulping process today, suffers from a great disadvantage, owing to the release of malodorous reduced sulphur compounds to the atmosphere (54). Although the possibility of using sulphur-free organic solvents has been known for long time, the process has so far been implemented on a pilot plant scale only (39, 33, 47). This, together with the scarcity of alternative processes, has refocused attention on the oldest known chemical pulping process, which uses soda to cook raw materials (30).

Soda pulping has been the dominant chemical pulping process for non-wood plant materials ever since, where the advantages of sulphidity are not of much importance. But it is faced with severe drawbacks. Strongly alkaline cooking liquors dissolve carbohydrates to a great extent with negative impact on pulp yield (31, 22). Moreover, the pulp produced is highly coloured, leading to an increased consumption of bleaching chemicals (26). But on the other hand, paper manufactured from soda pulps bears high bulk, opacity, absorbency and printability. Soda pulps are therefore best suited to paper

grades where pulp strength requirements are not much demanding. Blending of longer and stronger fibers can strengthen the soda pulps, which are otherwise too soft to be used alone. They better serve as "filler fibers", added to the paper (17, 20). Some drawbacks of soda pulping could be overcome by the use of suitable pulping additives such as anthraquinone (AQ) or anthraquinone-2-sulfonate (AQ-S) (9, 10). With the presence of anthraquinone (AQ) as a chemical additive in the alkaline pulping process, the delignification rate and the preserved pulp yield can be improved (8, 48).

Anthraquinone (AQ), when added to soda cooks protects the reducing aldehyde groups of carbohydrates against alkaline peeling and improves lignin solubility. Compared to soda pulps the soda-AQ pulps have slightly better bleachability, higher yield and sometimes better strength (31). Soda-AQ process is sulphur-free, which simplifies the recovery of chemicals and eliminates emissions of odorous sulphur compounds to the air (25). As a redox catalyst, at moderate temperatures around 50 °C or higher, the AQ stabilises the carbohydrates by oxidation of reducing end groups to alkali stable aldonic acid groups and, accelerates delignification by reducing lignin or some solubilised lignin fragments, hence preserving the pulp yield (45, 12). In this process of catalysis, AQ is reduced to anthrahydroquinone (AHQ) by the polysaccharide end groups. AHQ then effectively cleaves the β- aryl ether linkages in free phenolic phenylpropane units of lignin (hence accelerating delignification) and simultaneously gets oxidised to AQ, hence completing a redox cycle (56). Although the retention of the hemicelluloses and lower degradation of cellulose contribute to the superior strength, easy beating, denser and more transparent paper from the soda-AQ pulps, compared to soda pulps, the brittleness of the pulp increases and thus the tear resistance is affected to some extent (40). Since AQ is insoluble in caustic solutions, the effectiveness of AQ during the alkaline pulping process could be limited, mainly by mass transfer.

3.2 EXPEREIMENTAL METHODOLOGY

3.2.1 Materials

Lemon and sofia grasses were collected from Punjab Agriculture University Ludhiana (India) at the start of the rainy season. After steam distillation, the lignocellulosic residues (LCR) of the two grasses were stored in polythene bags after drying in sun-light. Both the grasses were used as raw materials for preparation of soda-

AQ pulps. The reagents used were of laboratory grade from Qualigens Fine Chemicals, Mumbai.

3.2.2 Methods

3.2.2.1 Pulping studies

The chopped chips of lemon and sofia grasses were delignified in WEVERK electrically heated rotary digester of 0.02 m^3 capacity having four bombs, each of 1 L capacity. The two raw materials were cooked under different cooking conditions like, maximum temperature ranging from 140 to 170 °C for sofia grass and 130 to 160 °C for lemon grass, cooking time from 30 to 120 min, active alkali from 11 to 16% for sofia grass and 10 to 16% for lemon grass (as Na_2O) respectively, holding a fixed liquor to raw material ratio of 4.5:1. Based on experimental results at optimum cooking conditions, 0.1% anthraquinone (AQ), a carbohydrate stabilizer, was added (based on o.d. raw material) to investigate its impact on pulp yield, screening rejects and kappa number, while keeping the other operating parameters constant. Sofia and lemon grasses were thereby also delignified at varying active alkali doses ranging from 10 to 12 % (as Na_2O) in the presence of 0.1% AQ while keeping other operating parameters constant, following which the effect of varying AQ doses (0.05 to 2%) was analysed. After termination of cooking, the pulps were washed on a laboratory flat stationary screen having 300 mesh wire bottom for the removal of residual cooking chemicals. The pulps were disintegrated and screened through WEVERK vibratory flat screen with slot size of 0.15 mm and the screened pulps were washed, pressed and crumbled. The pulps were then analyzed for kappa number (TAPPI T 236 cm-85 "Kappa number of pulp"), screened pulp yield, lignin (TAPPI T 222 om-02 "Acid-insoluble lignin in wood and pulp") and screening rejects (6). The results are shown in Tables 3.1 to 3.5 and Figures 3.1 to 3.8.

3.2.2.2 Preparation of laboratory hand sheets and testing

The unbleached pulps of sofia and lemon grasses were beaten in PFI mill (TAPPI T 248 sp-00 "Laboratory beating of pulp [PFI mill method]") at different beating levels. Laboratory handsheets of 60 g/m^2 were prepared on a British sheet former (TAPPI T 221 cm-99 "Drainage time of pulp"), pressed, air-dried under atmospheric conditions, and evaluated for various physical strength properties, such as tear index (TAPPI T 414 om-

98 "Internal tearing resistance of paper [Elmendorf-type method]"), tensile index (TAPPI 494 om-01 "Tensile properties of paper and paperboard [using constant rate of elongation apparatus]"), burst index (TAPPI T 403 om-97 "Bursting strength of paper"), double fold (TAPPI T 423 cm-98 "Folding endurance of paper [Schopper type tester]") as per Tappi Standard Test Methods: 2007. Both temperature and relative humidity have significant effects on the physical properties of paper and board. Therefore, laboratory handsheets were preconditioned at 27±2 °C at a relative humidity of 65±2% (6). The results are shown in Table 3.6 and Figures 3.9 to 3.12.

3.2.2.3 Bauer-McNett fiber classification

This method has been designed to measure the weighted average fibre length of a pulp. If a fibre is 1 mm in length and weighs w mg, then for a given pulp, the weighted average length (L) is $\Sigma(wl)/\Sigma w$, or the sum of the products of the weight times the length of each fibre divided by the total weight of the fibres in the specimen. The fiber fractionation of soda-AQ pulps of sofia and lemon grasses were carried out with the help of Bauer-McNett type four screen fiber classifier (T 233 cm-06) using different mesh screen numbers 20, 48, 100, and 200 (6). The results are reported in Table 3.7.

3.2.2.4 Scanning electron microscopy

The detailed morphological studies of soda-AQ pulps of sofia and lemon grasses were conducted using a scanning electron microscope, (SEM, Leo 435 VP, England). Pulp samples were taken and subjected to fixation using 3% (v/v) glutaraldehyde-2% (v/v) formaldehyde (4:1) for 24 h. Following the primary fixation, samples were washed thrice with double distilled water. The samples were then treated with the 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 (27). Electron photomicrographs were taken at 15.00 kV using detector SE1 and at desired magnifications and are shown in Plates 1 and 2.

3.2.2.5 Statistical analysis

For determination of kappa number, pulp yield, lignin, burst, tear and tensile indices and double fold number, three experimental values were taken in each case and the results are the mean ± standard (SD) of the values.

3.3 RESULTS AND DISCUSSION

3.3.1 Influence of temperature and time

Figure 1 reveals the effect of cooking time at temperatures varying from 140 to 170 °C (sofia grass) and 130 to 160 °C (lemon grass) on residual lignin. The curves could be approximated by two straight lines at each temperature investigated. The curves with steeper slopes pertained to rapid solubilisation of lignin (bulk delignification), whereas part of curves with gentler slopes were related to the slow solubilisation of the residual lignin (residual delignification). Bulk delignification corresponded to the removal of easily accessible lignin present in the middle lamella and residual delignification corresponded to the removal of lignin present in the primary wall, secondary wall layers (S_1, S_2, and S_3) and the central interconnection cavities. The individual fibers are bonded together by a lignin-rich region known as middle lamella. Cellulose contains its highest concentration in the S_2 layer (~50%), and lignin is most concentrated in the middle lamella (~90%), which, in principle, is free of cellulose (1, 2, 3). Although the reaction patterns were not fully understood, most kinetic models described delignification as the consecutive or simultaneous dissolution of three types of lignin present in wood from the beginning: initial, bulk, and residual lignin (44). Three distinct phases of delignification were hence observed in most systems: an initial phase that involved the rapid removal of about 20% of the lignin, a slower stage of bulk delignification, and finally, an even slower residual delignification (44). Larocque and Maass also mentioned that in alkaline pulping, two sets of delignification reactions, termed as bulk and residual delignifications having different velocity constants were involved (43). The fast process (bulk delignification) solubilised an average of 80% of the whole lignin dissolved, and this process occurred more rapidly at 100 °C (37).

The delignification is also associated with the solubilisation of significant amounts of hemicelluloses (41). Also it is indicated that with the decrease in temperature from 170-140 °C for sofia grass, and 160 to 130 °C for lemon grass, the time to reach transition from bulk to residual delignification, and the lignin content of the pulp corresponding to this transition point both increased. Figure 3.1 also reveals that at lower temperatures, the residual lignin decreased sharply while at higher temperatures, the magnitude of decrease in lignin was insignificant. Moreover at higher temperature, the degradation of carbohydrates also increases, thereby reducing the pulp yield (41). In other words, at the

transition point, lower pulp lignin content was obtained at 160±2 °C for sofia grass and 150±2 °C for lemon grass. Beyond these transition points, alkaline hydrolysis (depolymerization) of the polysaccharide chains occured in addition to the peeling reaction; thereby subjecting them to further degradation reactions (secondary peeling) (34). The curves after transition points were almost horizontal lines, indicating the termination of bulk delignification; therefore, it was not economical to continue delignification beyond 160 °C for sofia and 150 °C for lemon grasses. The effect of maximum cooking time on kappa number and pulp yield, as shown in Tables 3.1 and 3.2, reveals that there was hardly any drop in kappa number beyond a cooking time of 1.5 h, while the pulp yield reduced sharply (Figure 3.2). Therefore, a cooking time of 1.5 h and cooking temperature of 160 °C for sofia grass and 150 °C for lemon grass were considered optimum. Application of a shorter cooking time (30 min) raised the residual lignin content of the pulp and the amount of rejects; while a longer cooking time (2 h) exceeding the optimal value, decreased the selectivity of the cook and led to lower yields. Similar findings have been reported by Hedjazi et al. (31) for AS/AQ pulping of wheat straw.

3.3.2 Influence of alkali charge

Effective alkali charge (EA) has a significant impact on pulp yield, screening reject and kappa number (23). Table 3.3 shows the effect of different active alkali doses with or without the presence of AQ (0.1%) on screened pulp yield, kappa number and screening rejects during soda pulping of sofia and lemon grasses. As per Figure 3.3 the screened pulp yield increased with an increase in active alkali from 11 to 14% (as Na_2O) for sofia grass and 10 to 14% for lemon grass and then declined sharply, where as both kappa number and screening rejects decreased sharply, and then both the parameters remained almost constant. The active alkali charge of 14% (as Na_2O) was thereby considered optimal for both the raw materials, providing a screened pulp yield of 43.7 and 41.4% for sofia and lemon grass respectively, at kappa numbers 20 and 12.5. A fairly high-silica content, associated with grasses got dissolved to a high extent in strongly alkaline cooking liquors and thereby created precipitation problems in evaporators, recovery boilers and in the causticising plant. Some of the mentioned deficiencies of soda pulping were overcome by addition of anthraquinone (31, 48). The addition of 0.1% AQ at different alkali doses brought about a major reduction in kappa numbers, and screening rejects with a slight increase in pulp yield of the two grassy raw materials (Figure 3.4). Here, the kappa

number and screening rejects for sofia grass, showed a decrease by 26% (5.2 units) and 1%, with a 0.2% increase in screened pulp yield, while for lemon grass the respective decreases in kappa number, and screening rejects were 8% (1 unit) and 0.6% along with an associated increase of 0.4% in pulp yield.

The increase in pulp yield and reduction in kappa number could be explained on the basis of redox catalytic activity of AQ. In the very first step of reaction, AQ reacts with the reducing group of a carbohydrate, thus stabilizing the carbohydrate against alkaline peeling and producing the reduced form of AQ, i.e. anthrahydroquinone (AHQ), which is soluble in alkali. The AHQ reacts with quinone-methide segment of lignin polymer increasing the rate of delignification. At the same time, AHQ is converted back to AQ, which can then participate again in the redox cycle. Because AQ goes through a cyclic process, it is typically used at about 0.1% on an oven dry raw material basis, and results in an increase in pulp yield (13, 55, 21).

3.3.3 Influence of time

Table 3.4 shows the effect of maximum cooking time on screened pulp yield, screening rejects, and kappa number during soda pulping of sofia and lemon grasses, while keeping all other variables constant, such as alkali dose 14% (as Na_2O), liquor to raw material ratio of 4.5:1, and maximum cooking temperature 160 °C for sofia grass and 150 °C for lemon grass. Elevation of cooking time from 30 to 90 min improved the screened pulp yield from 37.4 to 43.7%, while kappa number dropped from 32.5 to 20 units for sofia grass. Likewise, for lemon grass, pulp yield increased from 37.6 to 41.4%, and kappa number descended from 26.4 to 12.5 units. Beyond that, a sharp decrease in screened pulp yield was noticed, while kappa number remained almost constant. Hence, optimum cooking time for soda pulping of both the raw materials was optimized as 90 min (Figure 3.5). The addition of 0.1% AQ (o.d. pulp basis) under different time at temperature while keeping other parameters constant improved pulp yields along with reductions in kappa number, and screening rejects for both the raw materials compared to their respective controls. The addition of AQ at a reaction time of 90 min improved pulp yield by 0.8% and reduced kappa number and screening rejects by 5.2 units, and 0.1% for sofia grass, whereas there was a 0.5% increase in pulp yield with a reduction in kappa number by one unit and screening rejects by 0.65% respectively for lemon grass (Figure 3.6).

3.3.4 Influence of AQ dose

Table 3.5 shows the effect of varying AQ doses on pulp yield, kappa number, and screening rejects under optimum cooking conditions for soda pulping of sofia grass and lemon grasses. Different doses of AQ (ranging from 0.05 to 0.2% on an oven dry raw material basis) at 14% alkali dose (as Na_2O) were used to observe the effect on kappa number. The addition of 0.1% AQ brought the kappa number down from 20.0 to14.8 units for sofia grass (Figure 3.7), and from 12.5 to 11.5 for lemon grass (Figure 3.8). A higher dose of AQ (0.2%) reduced the kappa number further, but to a lesser extent. The addition of AQ marginally improved the pulp yield in both the cases. The screening rejects in the pulp were also found to be lowered with increasing AQ doses. A similar trend was observed for *E. tereticornis* (16) as well as a binary mixture of *E. tereticornis* and *P. roxburghii* (49) in the ratio of 70:30, and for sugarcane bagasse (4).

3.3.5 Optimization of mechanical strength properties

Table 3.6 reveals the mechanical strength properties of sofia and lemon grasses at 14% active alkali charge. All the mechanical strength properties increased with increasing beating level up to 40±1 °SR (degree Schopper Reigler) except tear strength. Removal of primary wall exposed the secondary wall layers. However, the primary wall is permeable to water but does not participate in bond formation. Therefore, tearing energy required to pull the fibres from the mesh would 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 declined, whereas all other properties depending upon hydrogen bonding improved with pulp beating. A beating level of 40±1 °SR imparted tear, tensile and burst indices and double fold number as 3.74 mNm^2/g, 53.89 Nm/g, 3.5 $kPam^2/g$ and 50 for sofia grass, while 4.54 mNm^2/g, 68.52 Nm/g, 5.4 $kPam^2/g$ and 59 respectively for lemon grass. The strength properties of soda-AQ pulp of lemon grass were much superior to those of sofia grass at a beating level of 40±1 °SR (Figure 3.9).

3.3.6 Fiber length distribution of sofia and lemon grasses

Table 3.7 shows the fiber length distribution of soda-AQ pulps of sofia and lemon grass at 15 °SR. Important quantitative information about the fiber length distribution of sofia and lemon grass pulps could be best achieved by fiber fractionation. Furthermore, the fractionation process using the Bauer-McNett fiber classifier with screens of 20, 48, 100,

and 200 mesh sizes not only separated fibers according to the fiber length, but also to a great extent, it separated the fractions of sclerenchyma fibers and parenchyma cells. Lemon grass showed +20 fractions of 62.63% as compared to 41.72% for sofia grass, which consisted of mostly long sclerenchymatous fiber cells. Sofia grass retained 27.94% fibers in +48 fractions compared to 17.74% for lemon grass. This holds that sofia grass soda-AQ pulp contained maximum middle and bottom portions, while lemon grass had a maximum top fraction. The -200 fractions were just doubled in the case of sofia grass than that of lemon grass, which included shortened fibers, parenchyma cells, large vessel fragments, cell debris, and single epidermal cells (32). This implies that sofia grass might cause fluff generation during paper making and printing operation.

3.3.7 SEM studies of sofia and lemon grass fibers

SEM studies revealed that fibers in the soda-AQ pulp of sofia grass were non-uniform; the cell wall was distinguished by longitudinal striations and transverse fractures with somewhat swollen fissures, which were conspicuous. The fibers were rectangular in shape; they were moderately thin to thick walled (Plate 1A). The fibers of lemon grass were uniform, straight, and intact with a smooth, silky surface, and bore an appearance of compactness (Plate 2A). Plates 1D and 2D show the lumen of the sofia grass and lemon grass soda-AQ pulp fibers. As pulping proceeded the connection between the fibers was loosened, firstly along radial planes, and eventually the fibers remained stuck together only along those edges where several cells meet, and delignification was still incomplete. In addition, fibers showed no sign of external fibrillation or formation of fibrils. During alkaline pulping some part of hemicelluloses (mainly xylan) was solubilised, and reprecipitated on to the fiber surface as shown in plates 1A and 2B. Finally after removal of most of the lignin, the fibers lost their rigidity and collapsed readily (Plate 1B). Plates 1C and 2C show external fibrillation or formation of fibrils as an outcome of beating of the sofia and lemon grass pulp fibers, which resulted into an increased area of contact for bonding. The pith parenchymas were easily broken down (Plates 1D and 2C) to flexible flakes with some bonding effect.

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

Temperature, °C	Time at temperature, h	Sofia grass		
		Yield, %	Kappa number	Lignin, %
140	0.50	65.3±3.5	–	12.22±0.40
	1.00	57.5±2.2	–	9.35±0.60
	1.50	50.5±2.4	32.7±1.7	7.05±0.28
	2.00	45.6±1.9	–	6.10±0.50
	2.50	42.4±1.1	–	5.65±0.35
	3.00	41.7±1.7	–	5.10±0.30
150	0.50	62.4±2.3	–	10.55±0.65
	1.00	54.3±1.8	–	7.45±0.80
	1.50	47.4±1.2	25.6±0.5	4.75±0.72
	2.00	43.3±1.7	–	4.41±0.28
	2.50	40.5±1.2	–	3.65±0.30
	3.00	39.5±1.5	–	3.15±0.34
160	0.50	57.5±1.9	–	9.10±1.0
	1.00	50.5±1.7	–	6.35±0.40
	1.50	44.1±1.5	20±0.3	4.10±0.25
	2.00	40.4±1.2	–	3.45±0.28
	2.50	37.6±1.6	–	2.92±0.22
	3.00	35.5±1.8	–	2.65±0.17
170	0.50	55.5±2.1	–	8.15±0.70
	1.00	47.8±1.8	–	5.75±0.35
	1.50	42.5±1.1	13.5±0.3	3.62±0.30
	2.00	38.5±1.5	–	2.95±0.23
	2.50	35.4±1.0	–	2.41±0.18
	3.00	34.2±1.2	–	2.22±0.10

± refers standard deviation

Cooking conditions:

Liquor to wood ratio : 4.5:1
Active alkali : 14% (as Na_2O)
Digester pressure : 5.0 kg/cm^2
Time from room temperature to 105±2 °C : 45 min
Time from 105 to 160±2 °C : 55 min

Table 3.2: Effect of maximum cooking temperature on pulp yield, lignin and kappa number of lemon grass

Temperature, °C	Time at temperature, h	Lemon grass		
		Yield, %	Kappa number	Lignin, %
130	0.50	53.5±1.9	–	12.25±0.34
	1.00	50.2±2.0	–	9.52±0.30
	1.50	47.6±1.8	22.5±0.4	6.80±0.50
	2.00	44.3±0.9	–	5.85±0.35
	2.50	42.8±1.3	–	5.35±0.50
140	0.50	50.6±1.5	–	10.45±0.65
	1.00	47.5±1.1	–	7.42±0.80
	1.50	43.8±1.2	14.8±0.5	5.40±0.35
	2.00	41.5±1.5	–	4.52±0.62
	2.50	39.7±1.0	–	3.90±0.30
150	0.50	47.8±1.8	–	9.35±1.0
	1.00	45.2±1.3	–	6.80±0.71
	1.50	42.2±0.8	12.5±0.3	4.55±0.28
	2.00	38.7±1.1	–	3.50±0.34
	2.50	37.6±1.6	–	2.80±0.25
160	0.50	45.2±1.8	–	8.78±0.80
	1.00	42.4±1.2	–	6.10±0.40
	1.50	38.8±1.5	11.6±0.3	3.82±0.29
	2.00	35.5±1.3	–	2.75±0.20
	2.50	34.6±0.9	–	1.85±0.15

± refers standard deviation

Cooking conditions:

Liquor to wood ratio	:	4.5:1
Active alkali	:	14% (as Na_2O)
Digester pressure	:	5.0 kg/cm^2
Time from room temperature to 105±2 °C	:	45 min
Time from 105 to 160±2 °C	:	45 min

Table 3.3: Effect of active alkali (as Na_2O) on screened pulp yield, screening rejects and kappa number of sofia and lemon grasses

Sl. No.	Active alkali, % (as Na_2O)	Total yield, %	Screened pulp yield, %	Screening rejects, %	Kappa number
Sofia grass					
1	11	48.8±3.1	38.2±1.8	10.6±0.8	36.2±0.5
2	11*	47.5±3.0	42.0±2.6	5.5±0.3	26.8±0.5
3	12	46.1±1.7	41.3±2.9	4.8±0.1	31.5±1.1
4	12*	46.2±2.8	43.6±1.6	2.6±0.3	21.0±0.6
5	13	46.9±1.9	43.6±2.6	3.3±0.25	26.2±0.8
6	13*	45.7±2.4	44.2±1.7	1.5±0.11	17.7±0.6
7	14	44.8±2.6	43.7±1.5	1.1±0.08	20.0±0.4
8	14*	44.0±1.8	43.9±2.8	0.1±0.02	14.8±0.9
9	15	39.8±1.3	39.7±1.8	0.1±0.01	14.4±0.7
10	15*	39.5±1.0	39.2±1.6	0.3±0.05	12.3±0.3
11	16	34.5±1.5	34.4±1.1	0.1±0.02	13.7±0.3
12	16*	32.4±1.2	32.3±1.4	0.1±0.01	12.0±0.1
Lemon grass					
13	10	46.7±2.5	39.5±1.3	7.2±0.70	29.2±0.8
14	10*	45.8±3.0	40.7±1.2	5.1±0.61	26.2±0.8
15	12	45.6±2.1	40.8±1.5	3.8±0.35	20.8±0.5
16	12*	44.2±2.7	42.4±1.9	1.8±0.20	16.5±0.5
17	14	42.2±1.9	41.4±2.1	0.8±0.10	12.5±0.1
18	14*	42.0±2.1	41.8±1.5	0.2±0.04	11.5±0.3
19	16	38.8±1.5	38.6±1.7	0.2±0.05	11.2±0.1
20	16*	37.4±1.1	37.3±2.3	0.1±0.02	10.6±0.2

± refers standard deviation, * = 0.1% AQ on o.d. raw material basis, **lemon grass

Cooking conditions:

Liquor to raw material ratio	:	4.5:1
Digester pressure	:	5.0 kg/cm^2
Time from room temperature to 105±2 °C	:	45 min
Time from 105 to maximum temperature 160±2 °C	:	55 min
**Time from 105 to maximum temperature 150±2 °C	:	45 min
Time at maximum temperature, 160±2 °C	:	90 min
**Time at maximum temperature, 150±2 °C	:	90 min
Active alkali	:	14% (as Na_2O)

Table 3.4: Effect of maximum cooking time on screened pulp yield screening rejects and kappa number of sofia and lemon grasses

Sl. No.	Maximum cooking time, min	Total yield, %	Screened pulp yield, %	Screening rejects, %	Kappa number
Sofia grass					
1	30	47.6±2.3	37.4±1.3	10.2±1.3	32.5±0.75
2	30*	46.4±3.1	40.2±1.1	6.2±0.8	24±0.68
3	60	46.3±2.9	39.5±1.9	6.8±0.8	24.4±0.32
4	60*	45.5±1.8	42.8±2.1	2.7±0.9	16.4±0.33
5	90	44.1±2.5	43.7±2.4	0.4±0.08	20.0±0.62
6	90*	44.8±1.4	44.5±1.4	0.3±0.041	14.8±0.10
7	120	37.4±0.9	37.2±1.5	0.2±0.025	17±0.25
8	120*	37.8±0.8	37.7±0.8	0.1±0.01	14.4±0.22
Lemon grass					
9	30	47.8±3.1	37.6±2.5	10.2±1.5	26.4±1.4
10	30*	46.2±2.5	42.0±1.6	4.2±0.9	20.0±0.54
11	60	45.2±2.0	39.1±3.0	6.1±0.6	19.3±0.36
12	60*	43.8±3.1	43.3±2.8	0.5±0.05	16.2±0.28
13	90	42.2±1.6	41.4±3.2	0.8±0.11	12.5±0.15
14	90*	42.05±1.2	41.9±1.6	0.15±0.05	11.5±0.21
15	120	38.7±1.3	38.6±2.2	0.1±0.01	11.2±0.11
16	120*	38.5±1.8	38.4±1.5	0.1±0.02	10.8±0.09

± refers standard deviation, * = 0.1% AQ on o.d. raw material basis, **lemon grass

Cooking conditions:

Liquor to raw material ratio	:	4.5:1
Alkali dose	:	14% (as Na_2O)
Digester pressure	:	5.0 kg/cm^2
Time from room temperature to 105±2°C	:	45 min
Time from 105 to maximum temperature 160±2°C	:	55 min
**Time from 105 to maximum temperature 150±2°C	:	45 min

Table 3.5: Effect of AQ on pulp yield, kappa number and screening rejects during soda pulping of sofia and lemon grasses at optimum cooking conditions

AQ doses, %	Total pulp yield, %	Screened pulp yield, %	Screening rejects, %	Kappa number
Sofia grass				
0.0	44.3±2.0	43.2±1.8	1.1±0.05	20.0±0.40
0.05	44.4±2.5	43.8±1.3	0.6±0.03	17.3±0.25
0.1	44.8±1.8	44.5±0.8.	0.3±0.02	14.8±0.37
0.2	44.7±1.3	44.6±1.5	0.1±0.01	12.8±0.20
Lemon grass				
0.0	42.2±2.3	41.4±1.5	0.8±0.05	12.5±0.23
0.05	42.0±2.9	41.8±2.1	0.2±0.02	11.8±0.09
0.1	42.0±1.5	41.8±2.3	0.2±0.02	11.5±0.11
0.2	41.6±1.0	41.5±1.2	0.1±0.006	10.6±0.13

± refers standard deviation, * = 0.1% AQ on o.d. raw material basis, **lemon grass

Cooking conditions:

Liquor to raw material ratio	:	4.5:1
Digester pressure	:	5.0 kg/cm^2
Time from room temperature to 105±2 °C	:	45 min
Time from 105 to maximum temperature 160±2 °C	:	55 min
**Time from 105 to maximum temperature 150±2 °C	:	45 min
Time at maximum temperature, 160±2 °C	:	90 min
**Time at maximum temperature, 150±2 °C	:	90 min
Active alkali	:	14% (as Na_2O)

Table 3.6: Mechanical strength properties of unbleached sofia and lemon grasses soda-AQ pulp at optimum pulping conditions

Active alkali, % (as Na_2O)	Beating level, °SR	Tensile index, Nm/g	Tear index, mNm^2/g	Burst index, $kPam^2/g$	Double fold, number
Sofia grass					
14%	15	20.02±0.56	2.11±0.09	0.7±0.08	4±1.0
	30	41.39±1.2	4.36±0.16	2.0±0.11	18±1.0
	35	47.53±2.10	4.04±0.22	2.6±0.15	34±2.1
	40	53.89±2.91	3.74±0.25	3.5±0.06	50±2.5
	45	51.04±3.03	3.02±0.15	2.8±0.21	49±2.2
Lemon grass					
14%	15	23.66±0.72	3.02±0.33	1.2±0.09	10±1.1
	30	48.92±2.20	4.36±0.14	3.3±0.20	28±1.6
	35	61.53±1.93	5.23±0.11	4.6±0.17	46±3.0
	40	68.52±1.80	4.54±0.24	5.4±0.15	59±4.6
	45	67.80±2.50	4.02±0.20	5.1±0.21	57±3.3

± refers standard deviation

Table 3.7: Bauer-McNett fiber classification of soda-AQ pulps under optimum pulping conditions of sofia grass and lemon grass

Sl. No.	Mesh size	Fibers retained, %	
		Sofia grass	Lemon grass
1	+20	41.72	62.63
2	-20 to +48	27.94	17.74
3	-48 to +100	8.57	1.6
4	-100 to +200	1.12	0.11
5	-200	20.65	10.02

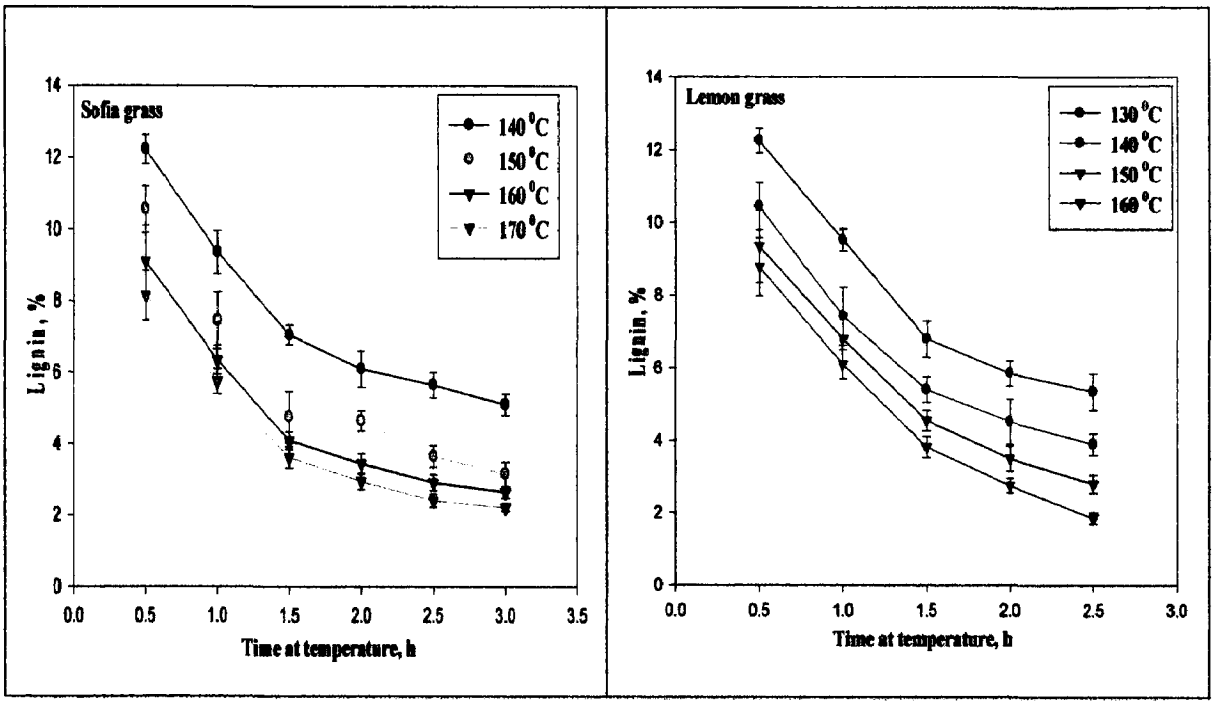


Figure 3.1: Curves of lignin vs different reaction times at maximum cooking temperature during soda pulping of sofia and lemon grasses

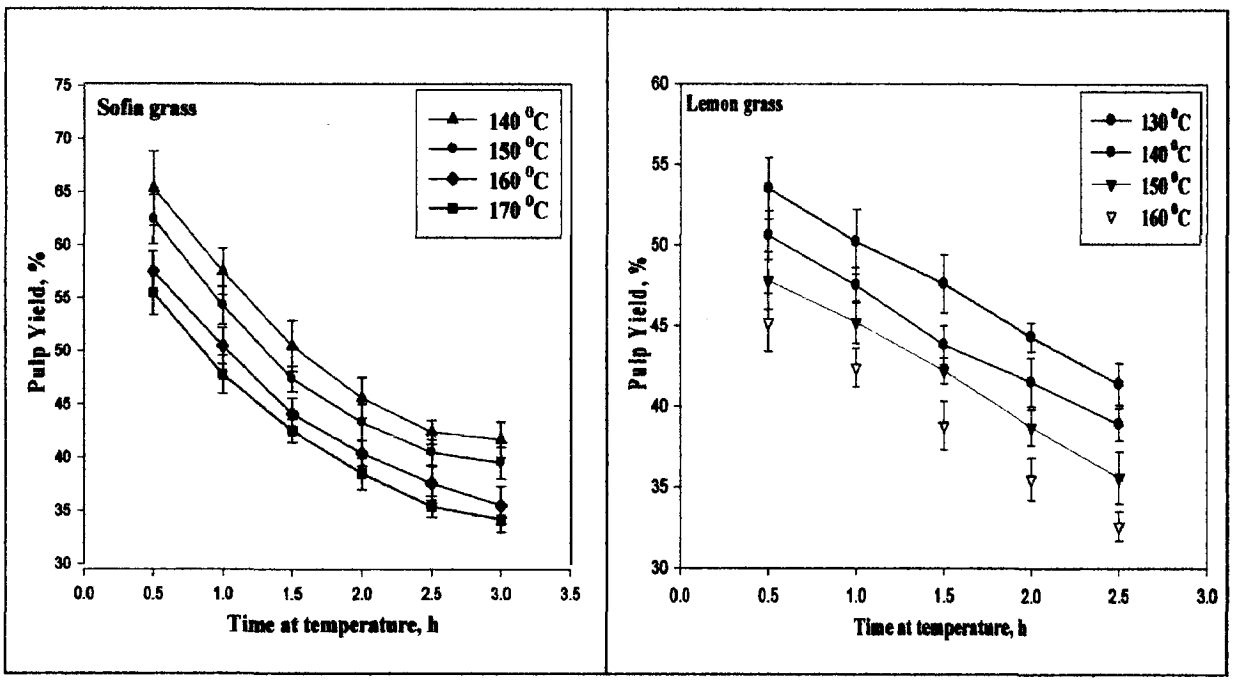


Figure 3.2: Curves of pulp yield vs different reaction times at maximum cooking temperature during soda pulping of sofia and lemon grasses

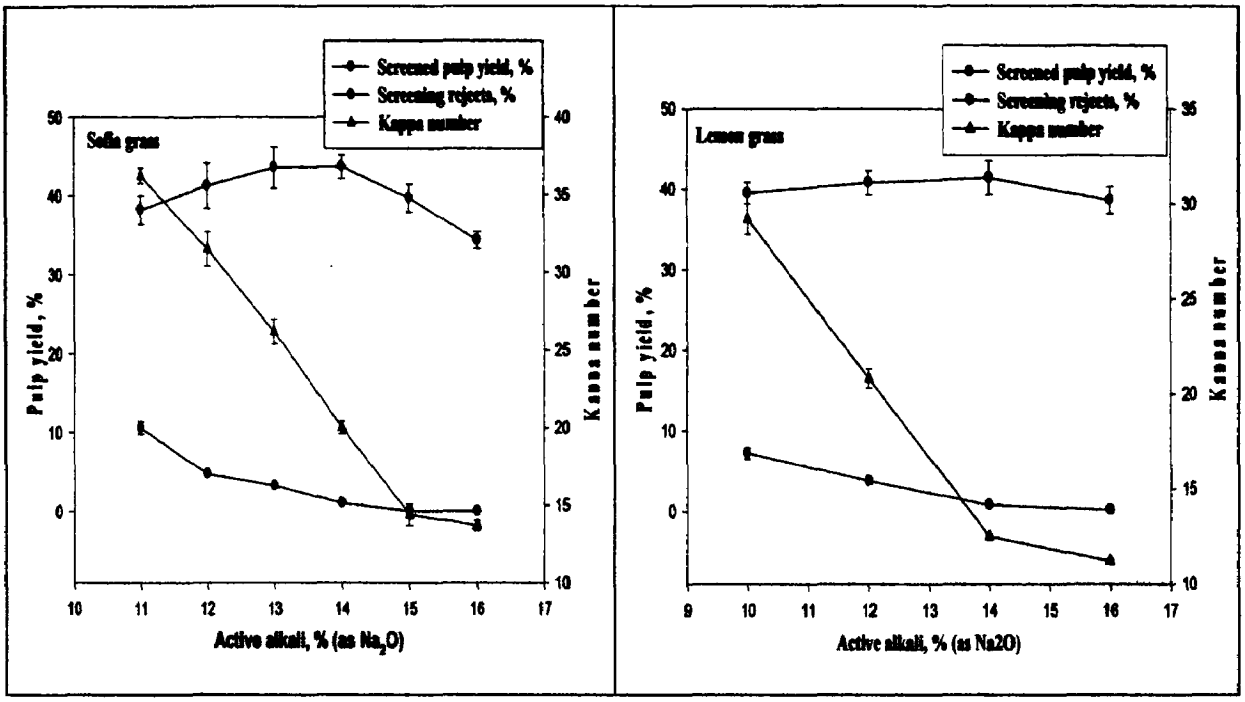
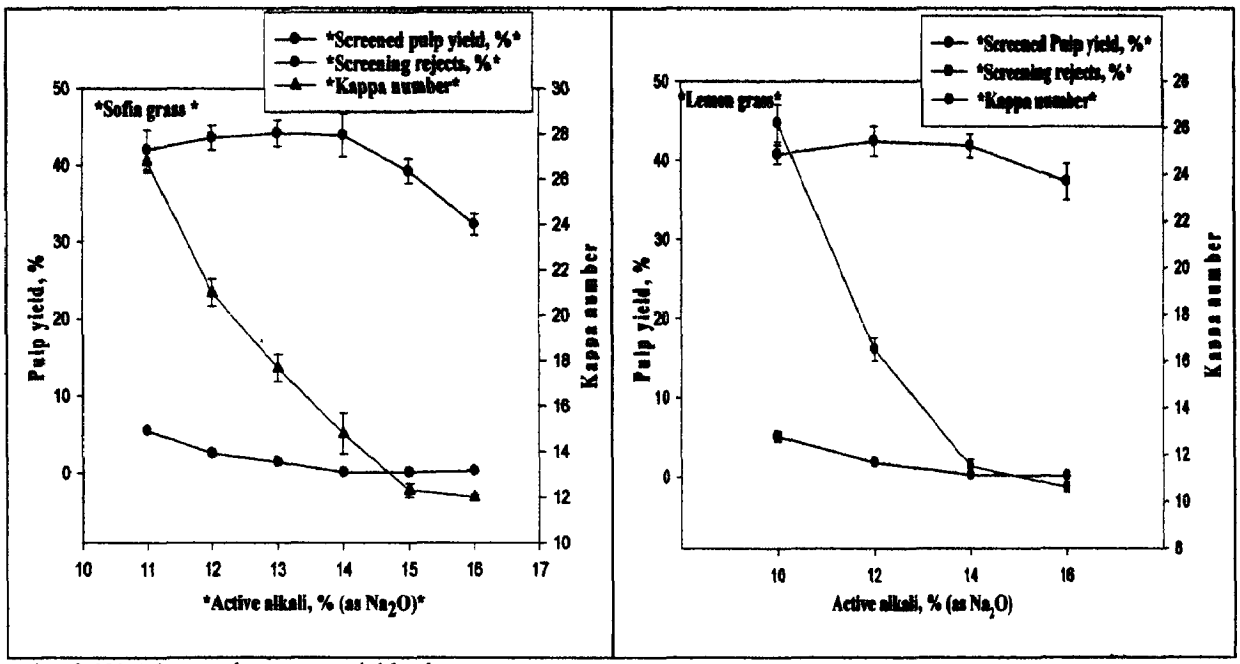


Figure 3.3: Effect of active alkali dose on screened pulp yield, screening rejects and kappa number during soda pulping (without AQ) of sofia and lemon grasses



* = 0.1% AQ on o.d. raw material basis

Figure 3.4: Effect of active alkali dose on screened pulp yield, screening rejects and kappa number during soda-AQ pulping of sofia and lemon grasses

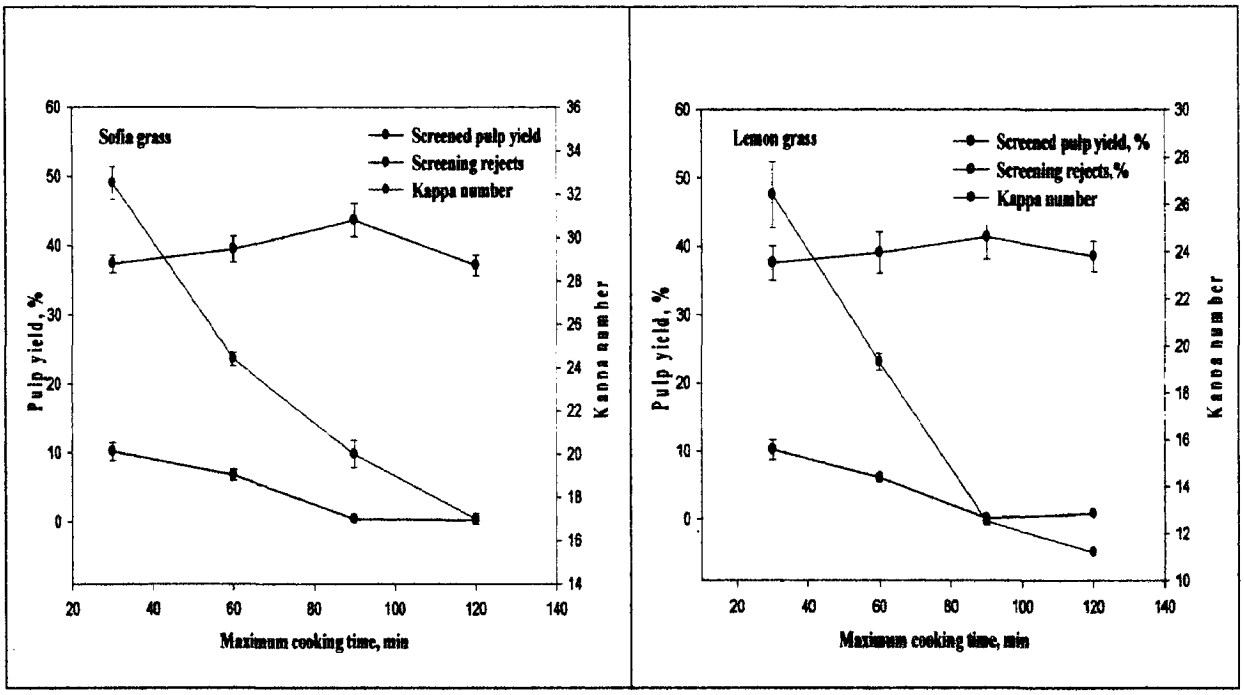
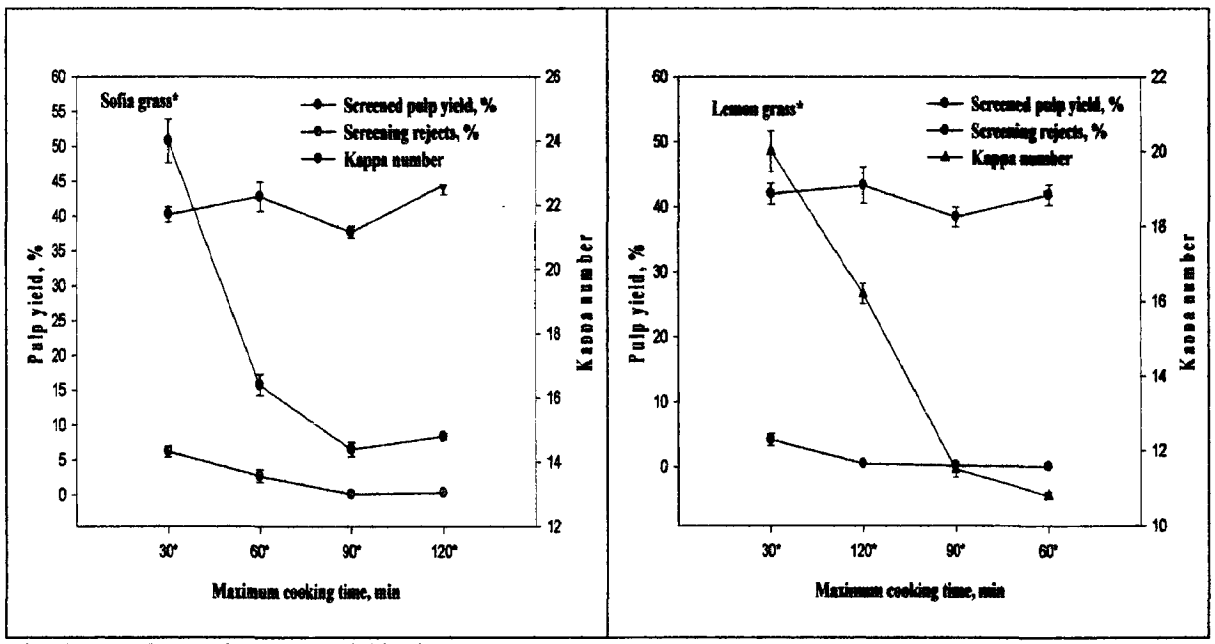


Figure 3.5: Effect of maximum cooking time on screened pulp yield, screening rejects and kappa number during soda pulping (without AQ) of sofia and lemon grasses



* = 0.1% AQ on o.d. raw material basis

Figure 3.6: Effect of maximum cooking time on screened pulp yield, screening rejects and kappa number during soda-AQ pulping of sofia and lemon grasses

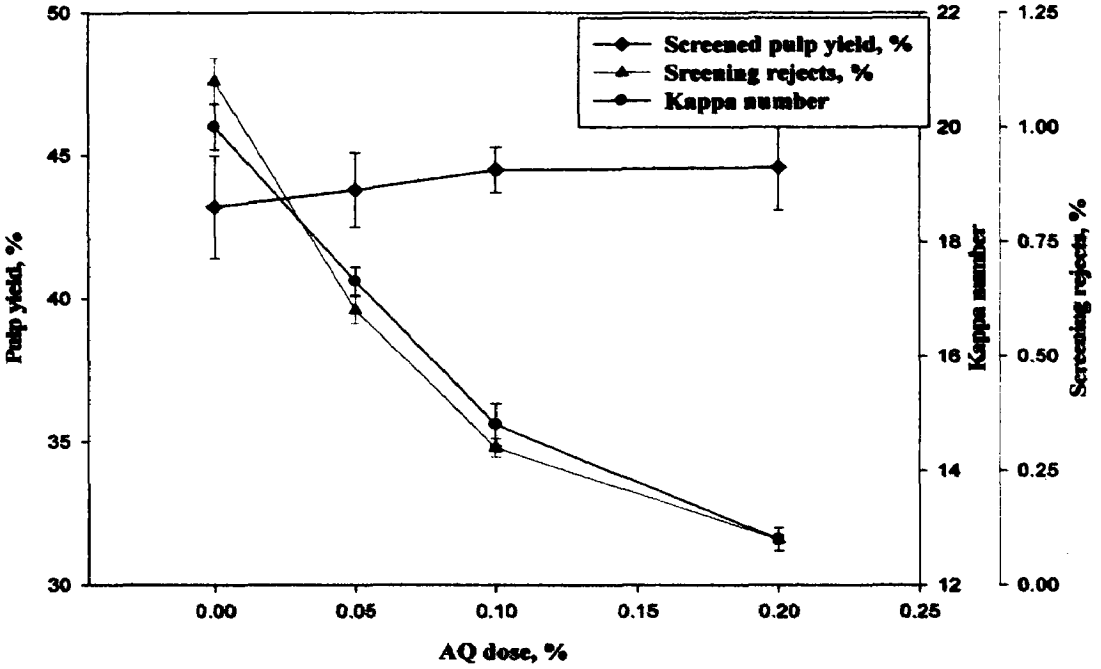


Figure 3.7: Effect of anthraquinone (AQ) dose on screened pulp yield, screening rejects and kappa number at optimum pulping conditions during soda pulping of sofia grass

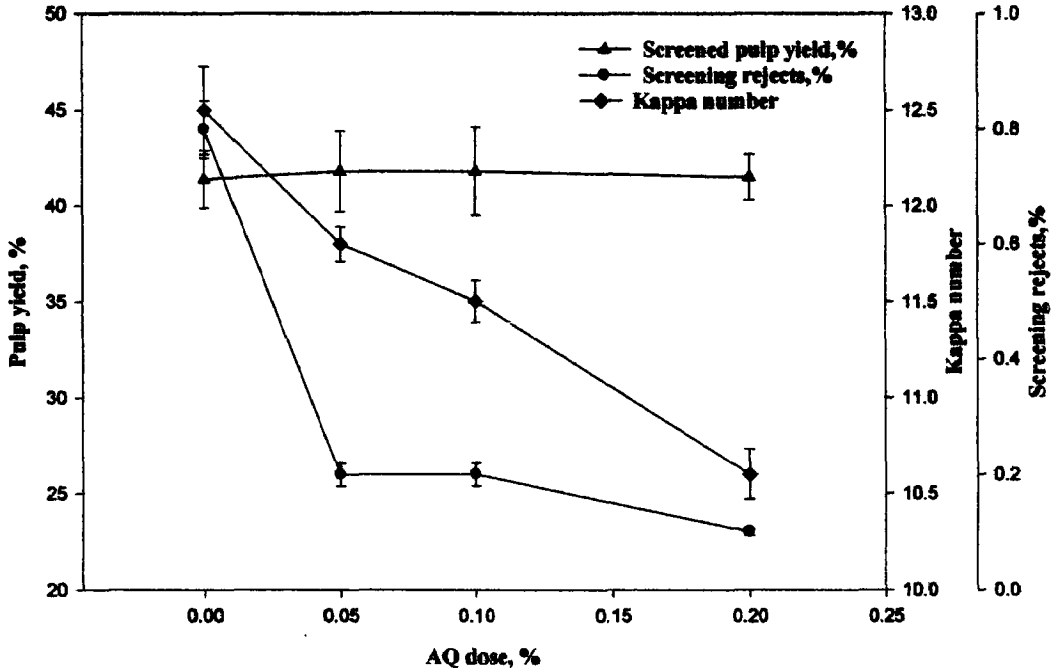


Figure 3.8: Effect of anthraquinone (AQ) dose on screened pulp yield, screening rejects and kappa number at optimum pulping conditions during soda pulping of lemon grass

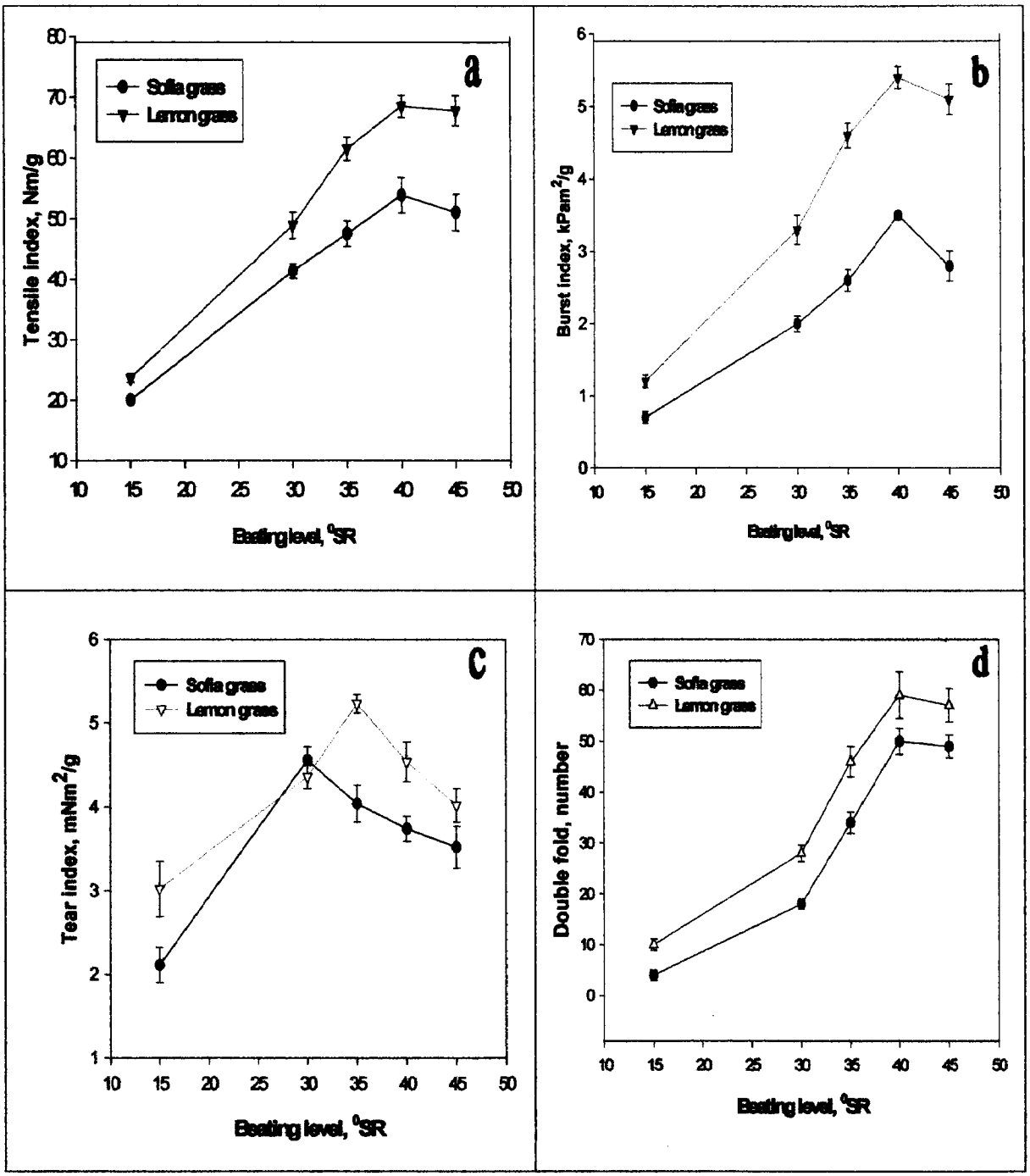


Figure 3.9: Effect of beating level (° SR) on (a) tensile index (b) burst index (c) tear index (d) double fold number of soda-AQ pulps of sofia and lemon grasses

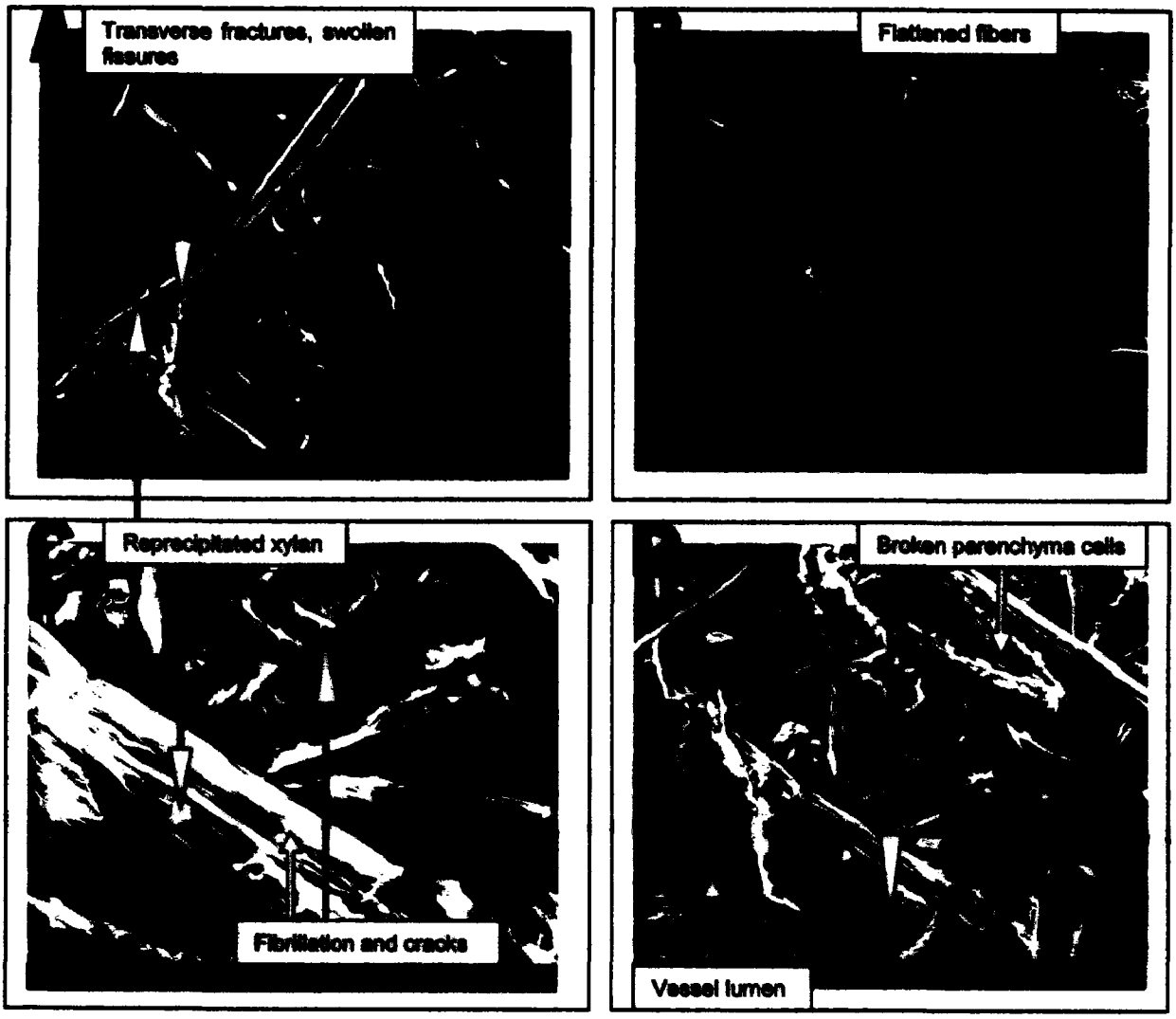


Plate1. Microphotographs of (A) complete fibers of unbeaten soda-AQ pulp of sofia grass with primary wall (2000 X)(B) flattened fibers after pulping (500 X)(C) beaten pulp (40 °SR) fibers of sofia grass showing reprecipitated xylan on their surface along with fibrillation and cracks (2000 X)(D) beaten soda-AQ fibers (40 °SR) of sofia grass showing vessel lumen and broken parenchymatous cells (1000X).

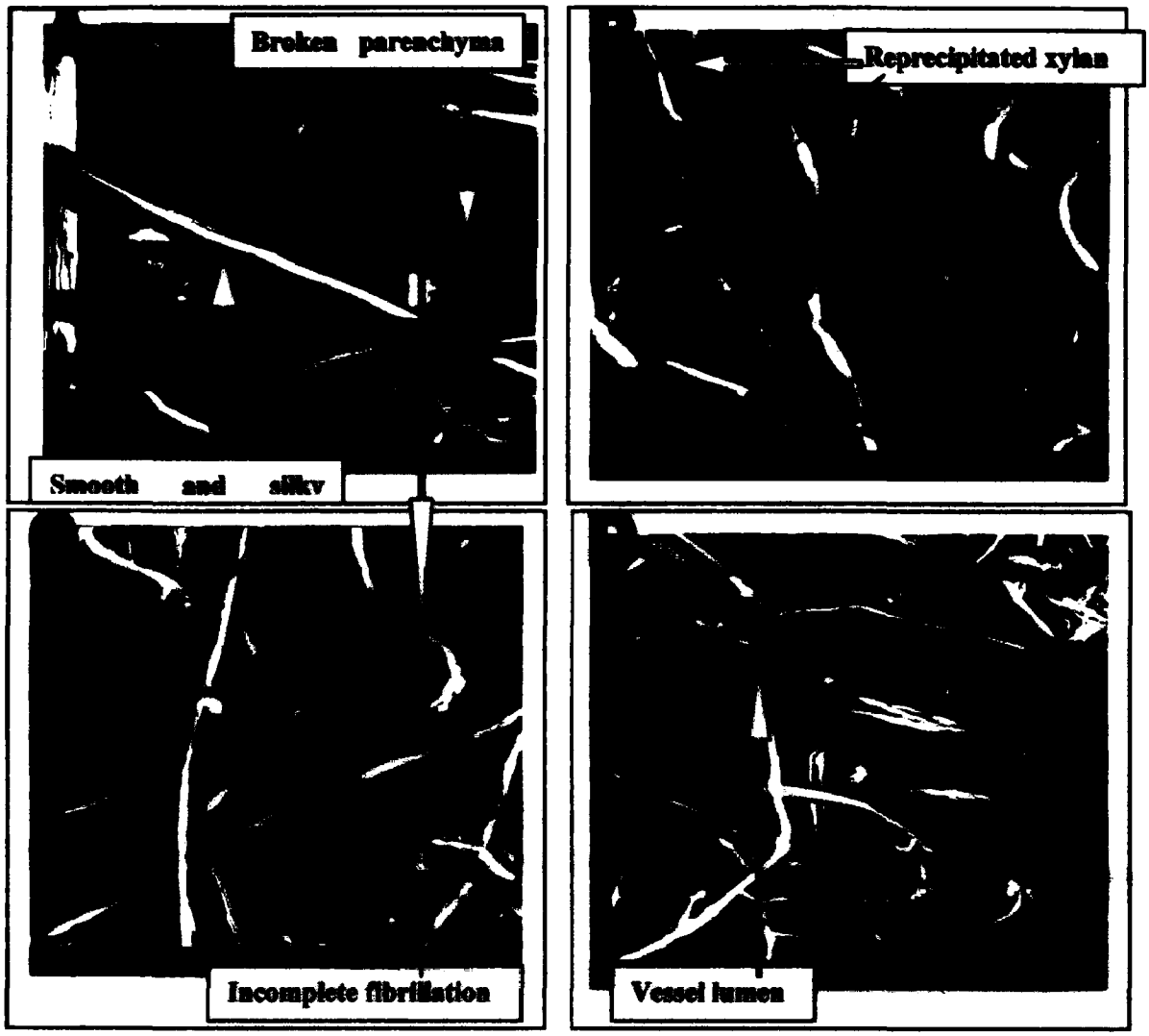


Plate2. Microphotographs of (a) complete smooth fibers of unbeaten soda-AQ pulp of lemon grass with primary wall (2000 X) (b) fibers showing reprecipitated xylan on their surface (2000 X) (c) beaten pulp (40 °SR) of lemon grass showing incomplete fibrillation and broken parenchyma cells (2000 X) (d) unbeaten soda-AQ fibers of lemon grass showing lumen (1000x).

References

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CHAPTER 4

STUDIES ON XYLANASE PRODUCTION

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4.1 INTRODUCTION

Lignocelluloses, the major structural components of woody plants and non-woody plants such as grass represent the most abundant source of renewable organic biomass on the Earth. Lignocelluloses consist of cellulose, hemicellulose and lignin. The chemical properties of the components of lignocellulosics make them a substrate of enormous biotechnological value (109, 93). A huge amount of residual plant biomass considered as "waste" can potentially be converted into different value-added products. Their utilization can allow self sustainable processes and products. The main processes for these materials to which the biotechnological approaches can be of help, are the improvement of the digestibility for feeding animals (6), the mushroom production, the energy area (biofuels), the retting of textile fibers, the biodegradation of toxic molecules (bioremediation) and many aspects concerning the paper pulp production (18,13, 168).

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 (15). Microbial enzymes have shown tremendous potential for different applications. Like all catalysts enzymes work by lowering the activation energy of a reaction, thus, dramatically accelerating rate of the reaction. In their catalysis process, neither are they consumed, nor do they alter the equilibrium of these reactions (8). Enzymes can carry out their myriads of biochemical reactions under ambient conditions, which make their use ecofriendly and often the best alternative to polluting chemical technologies. The usage of enzymes at various industrial levels has hence gained momentum (44). 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 the past two decades, however, microbial enzymes, particularly oxidative and hydrolytic ones produced mainly from lignocellulose degrading microorganism have been used in pulp and paper industry (15).

component are xylans, mannans, galactans, and arabinans (11). The most abundant hemicellulose present on the Earth's surface, xylan represents up to 30–35% of the total dry weight of land plants (79). It contributes over 70% of the hemicellulose structure (148). It is a major constituent of plant cell walls and the second most abundant renewable polysaccharide in nature after cellulose (138). It is typically located in the secondary cell wall of plants, but is also found in the primary cell wall, particularly in monocots (187, 56).

Within the cell wall structure, all three constituents (cellulose, hemicellulose and lignin) interact via covalent and non-covalent linkages, with the xylan being found at the interface between lignin and cellulose where it is believed to be important for fiber cohesion and plant cell wall integrity (15). 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%) (163).

Xylan molecules mainly consist of D-xylose as the monomeric unit, and traces of L-arabinose are also present (11). Xylan is a heteropolysaccharide and is made up of a homopolymeric backbone chain of 1,4-linked β-D-xylopyranosyl units, which can be substituted to varying degrees with glucuronopyranosyl, 4-O-methyl-D-glucuronopyranosyl, α-L-arabinofuranosyl, acetyl, feruloyl, and/or p-coumaroyl side chain groups (95).

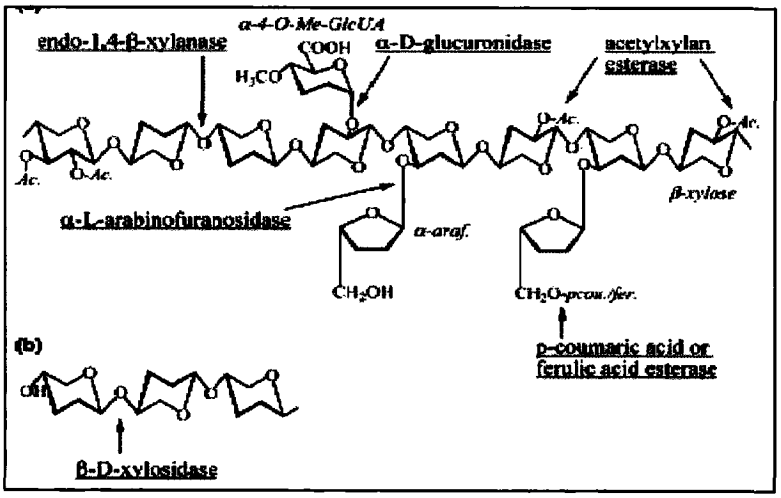

Figure 4.1: (a) Structure of xylan showing sites of xylan attack by xylanolytic enzymes. The backbone of the substrate is composed of 1, 4-β-linked xylose residues. Ac., Acetyl group; α-araf., α-arabinofuranosidases; α-4-O-Me-GlcUA, α-4-O-methylglucuronic acid; pcou., p-coumaric acid; fer., ferulic acid. (b) Hydrolysis of xylo-oligosaccharide by β-xylosidase (169)

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Because of heterogeneity and the complex chemical nature of plant xylan, its complete breakdown requires the action of a complex of several hydrolytic enzymes with diverse specificity and modes of action. Endo-1,4-β-D-xylanases (EC 3.2.1.8) randomly cleave xylan backbone, and β-D-xylosidases (EC 3.2.1.37) cleave xylose monomers from the nonreducing end of xylo-oligosaccharides and xylobiose, while the removal of side groups is catalysed by α-L-arabinofuranosidases (EC 3.2.1.55), α-D-glucuronidases (EC 3.2.1.139), acetyl xylan esterases (EC 3.2.1.72), ferulic acid esterases (EC 3.1.1.73), and p-coumaric acid esterases (EC 3.1.1.–) (35). The hypothetical structure of xylan attacked by xylanolytic enzymes is given in Figure 4.1 (169).

It is plausible that xylanases can be found in all organisms living in habitats where the substrates for these enzymes are abundant (72). The main sources of xylanases are microorganisms, which produce extracellular xylanases in presence of suitable inducer such as, xylan. Production of xylanolytic enzymes by various bacteria and fungi is found to be inducible. Smaller molecules (low molecular mass), which are the degradation products of the complex polysaccharide xylan, act as inducers. A basal level of these hydrolytic enzymes is always present in the cell. As a result of the activity of these enzymes, small soluble signal molecules are generated owing to degradation of polysaccharide substrate (95). Facilitated by transferases, these signal molecules are translocated into the cell cytoplasm where they induce synthesis of the corresponding enzyme (136). The potent inducer molecules are xylose, xylobiose, xylooligosaccharides, heterosaccharides of xylose and glucose and their positional isomers. In some cases xylan itself acts as inducer, but weakly, due to its complex structure and high molecular mass it can't penetrate the cell wall (95). The most prominent signalling molecule is probably xylobiose, which has been found to be an effective inducer in a range of microorganisms, together with other xylooligosaccharides (136).

The efficient production of xylanolytic enzymes essentially relies on the choice of an appropriate inducing substrate and optimum medium composition (94). The induction

of enzyme production using purified substrate makes the process expensive. The use of agricultural residues rich in hemicellulose increases xylanase production, lowering the cost of biobleaching of pulp (94). A variety of inducers have been used for induction of xylanases such as, sawdust, corn cob, wheat bran, and sugar beet pulp and sugarcane bagasse. Wheat bran is found to be the best substrate for xylanase production by a thermophile *Bacillus licheniformis* grown on solid substrate (5). Xylanases are often co-induced with cellulases by pure cellulose, as in *T. aurantiacus*, *Chaetomium thermophile* var. *coprophile*, and *Humicola insolens*. However, cellulase-free xylanases are sought more importantly for their potential industrial applications (126).

Fungi, actinomycetes and bacteria are the most important xylanolytic enzyme producers (72, 100). Some of the most important one's include the *Aspergillus, Trichoderma, Streptomycetes, Phanerochaetes, Chytridiomycetes, Ruminococci, Fibrobacteres, Clostridia and Bacilli* (167, 169, 188). Many of these organisms have been found to produce multiple forms of xylanases. The white-rot fungus *Phanerochaete chrysosporium* has been shown to produce multiple endoxylanases (87). These may have diverse physico-chemical properties, structures, specific activities, and yields, as well as overlapping but dissimilar specificities, thereby increasing the efficiency and extent of hydrolysis and also the diversity and complexity of the enzymes (128). This multiplicity may be the result of genetic redundancy (187) or differential post translational processing (20). Filamentous fungi are particularly interesting as industrial xylanase producers because they release xylan degrading enzymes into the medium (extracellular xylanases) in much higher levels than yeasts or bacteria, and their enzymatic systems are more complete. Intracellular xylanases occur in rumen bacteria and protozoa (40).

Based on similarities in their amino-acid sequence, Henrissat and Bairoch (68, 69) proposed a classification of endoxylanases into families: (1) family 10, also called F/10, includes high-molecular-weight enzymes (above 30 kDa) with low isoelectric points, and (2) family 11, G/11, consisting of low-molecular-weight enzymes (below 30 kDa) with high isoelectric points (88, 21, 187). Family 11 xylanases are generally more thermolabile because their conformation is sensitive to sequence modifications, whereas family 10 xylanases appear to be more thermostable and present greater catalytic versatility (21, 52). However, several exceptions to this pattern have been found (169) and approximately 30% of presently identified xylanases, in particular fungal xylanases, cannot be classified by

this system (35). Endoxylanases have been identified in *Aspergillus niger* (31) *Aspergillus foetidus* (158); *Aspergillus fischeri* (156); *Bacillus pumilus* (137); *Cellulomonas NCIM 2353* (30); *Streptomyces sp.* (14); *Staphylococcus sp. SG-13* (63); *Cryptococcus albidus* (121); *Trichoderma reesei* (177); and many other microorganisms (16, 90, 103). The majority of xylan-degrading enzymes from thermophilic fungi are endoxylanases (107).

Xylanases from alkalophilic and thermophilic fungi are receiving considerable interest because of their application in pulp and paper industry for biobleaching, in which the enzymatic removal of xylan from lignin-carbohydrate complexes (LCC) facilitates the leaching of lignin from the fiber cell wall, obviating the need for chlorine for pulp bleaching. Two endoxylanases from *H. insolens* with isoelectric point 9.0 and 7.7 have been purified, and characterized to be potentially important in biobleaching process (47). Xylanases that have a great potential for commercial application in pulp and paper industry are produced by various microorganisms such as *Aspergillus niger* (17), *Aspergillus oryzae* (173), *Arthrobacter* sp. (85), *Thermomyces lanuginosus* strain M7 (26), *Thermomyces lanuginosus* (32), *Streptomyces cyaneus* SN32 (127), *Streptomyces sp.* QG-11-3 (14), *Bacillus subtilis* ASH (150), *Bacillus* NCIM 59 (94), etc.

Suitability of an enzyme in the industry and for other practical purposes depends upon the enzyme's thermal stability and other kinetic and thermodynamic properties. Many of the xylanases used in industry today appear to be of mesophilic and/or neutrophilic origin (66). However, the extremophiles producing thermophilic enzymes could be used in applications where a cooling step would be uneconomical or where high temperatures are required to increase the bioavailability and/or solubility of substrates, to reduce viscosity and/or to reduce the risk of contamination (106). The major current application of xylanases is in the pulp and paper industries as a bleach-boosting agent (163, 86), where the high temperature (55–70 °C) and alkaline pH of the pulp substrate requires thermo-alkalophilic enzymes for efficient biobleaching (15, 182).

These include *Thermus aquaticus*, *Bacillus stearothermophilus*, and other hyperthermophilic microorganisms, mainly belonging to the archaea, that thrive under extreme environmental conditions (temperatures above 60 °C) (34, 97, 42). Some of the thermophilic fungi, *Chaetomium thermophile, Humicola insolens* (syn. *Scytalidium thermophilum*), *Sporotrichum thermophile, Thermoascus aurantiacus,* are usually isolated from composting materials and are known to play an important role in amelioration of

complex carbon sources by secreting a battery of thermostable cellulose and hemicellulose degrading enzymes (160, 122). *Thermomyces lanuginosus* (synonym *Humicola lanuginosa*) is a thermophilic fungus that produces high levels of extracellular, cellulase-free xylanase (82). Production of a highly thermostable xylanase by a mutant strain of *Thermomyces lanuginosus* has been reported recently with temperature optima of 70 °C and a half life of 31.79 min at 80 °C on 2% corn-cob media under submerged fermentation conditions (25).

Though exploitation of natural sources is one of the ways to identify enzymes that are thermally stable, but stability of the enzyme can also be increased by chemical modification, cross-linking, immobilization, treatment with additives and protein engineering (62). For this reason, the study of the complete xylanolytic enzyme system from both mesophilic and thermophilic xylanolytic microorganisms is important to satisfy the need of xylanases for different industrial applications. Most of the xylanases have a modular structure consisting of two domains: a catalytic domain (CD), responsible for the hydrolysis reaction, and a carbohydrate-binding module (CBM), mediating binding of the enzymes to the substrate, and thus improving the hydrolysis of insoluble substrates (116). CBMs, originally identified as "thermostabilizing" modules in xylanases from thermophilic bacteria (29), have also been found in mesophilic bacteria such as *Cellulomonas fimi* (33) and *Bacillus* sp. (24).

The optimum temperature for the activity of endoxylanase from bacterial and fungal sources varies from 40 to 100 °C (95, 15), and they commonly have a broad optimal pH range of 3.6–10. Xylanase preparations have been obtained, at an industrial scale, mainly from fungi of the genera *Trichoderma* and *Aspergillus* (18). Among genus *Aspergillus*, it has been reported that the optimum temperature of *Aspergillus niger and Aspergillus ochraceus* xylanase activity was 65 °C, while for *A. niveus* it was approximately 55-65 °C under SSF using wheat bran as the substrate (17). *Aspergillus sydowii* SBS 45 produces two xylanases, xylanase- I and II with optimal activity at 50 °C under SSF with a combination of wheat bran and birch wood xylan as the carbon source. At 40 and 60 °C, xylanase-I retained 50.45 and 99.4% of normal activity, while xylanase-II retained 87.64 and 25.95% of normal activity (125). Kitpreechavanich et al. has reported a temperature of 65 °C for xylanase stability in *Aspergillus fumigatus* (89). A strain of

A. niger reported by John et al. produces two enzymes with a broad temperature activity and maximal activity between 65 and 80 °C (78).

Xylanases useful for facilitating the bleaching of kraft pulps ought to be free of cellulase activity, capable of penetrating the micropore structure of the fibers, and active at neutral or alkaline pH. Because the kraft pulping process is strongly alkaline and because alkali remains trapped in the fibers even after extensive washing; the pH tends to drift higher during enzyme treatment. Enzymes with substantial activity in the alkaline region can hence be very useful. Alkalophilic *Bacillus subtilis* ASH produces cellulase free xylanase using wheat bran under Submerged Fermentation (SmF) up to an alkaline pH of 11 at 60 °C, however maximum activity was obtained at pH 7, 37 °C (149). A newly isolated strain of *Bacillus pumilus* grown under SmF in a basal medium supplemented with wheat bran (2%) at pH 8 and temperature 37 °C produces a cellulase free, thermo stable xylanase which is stable in neutral to alkaline pH region at 70 °C (12). Temperature stability is desirable because the pulps are warm after they come out of the kraft cook and washing steps. The enzymes should resist inhibition by kraft degradation products because these components are abundant in the pulp and the wash waters. Most importantly, the enzymes must be effective.

Although a majority of actinomycetes express maximum endoxylanase activity at slightly acid to neutral pH (112), broad pH optima have been demonstrated for some strains including *Thermomonospora fusca* (113, 191). Endoxylanases from *Streptomyces roseiscleroticus* show thermal stability (at 60 °C) and activity of xylanases at an alkaline pH (7-9) (59), which enable them to be used under conditions more appropriate for the kraft-pulping process. Stability at the extremes of pH appears to be characterized by a spatially biased distribution of charged residues. Enzymes stable in alkaline conditions are typically characterized by a decreased number of acidic residues and an increased number of arginines. Furthermore, a recent comparative structural study of family 11 enzymes suggests a correlation between pH activity/stability and the number of salt bridges, with acidophilic xylanases having much less of these interactions than their alkalophilic homologues (65).

Most microbial xylanases are single-subunit proteins with molecular weights ranging from 8 to 145 kDa (95, 37, 187). However, a strain of *Thermotoga thermarum* produced two xylanases, one of which was monomeric with MW of 35 kDa and the other

of which was dimeric, showing two bands on SDS-PAGE. Both were catalytic subunits with MWs of 105 and 150 kDa, respectively (170). More cases of dimeric enzymes, with subunits having MWs of 151 and 350 kDa, have been reported from *Talaromyces emersoni* (181). A cell-associated xylanase from *Thermoanaerobacterium* has a MW of 234 kDA, with two heterosubunits having MWs of 180 kDa and 24 kDa, respectively, on SDS-PAGE (151). High-molecular- weight multicomponent xylanase complexes can appear as a single band on SDS (189).

Solid state fermentation (SSF) holds tremendous potential for the production of xylanase (176) and can be of special interest in those processes where the crude fermented product may be used directly as enzyme source (132). The term SSF is applied for the processes in which insoluble materials in water are used for the microbial growth (119). However the moisture level should not exceed the capacity of saturation of the solid bed in which the microorganisms grow. Water is essential for the microbial growth and in SSF it is present in thin layers and is often absorbed inside the substrates (123). The relatively low content of humidity in SSF reduces the chances of microbial contamination, but leads to the formation of gradients of temperature, nutrients and products, as well as the production of enzymes and secondary metabolites (161).

SSF is considered as the most appropriate method for filamentous fungi (156) and actinomycetes (127) cultivation and lignocellulosic enzyme production. Since they grow under conditions close to their natural habitats i.e. moist substrates with less moisture content, 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 (131). Bacteria need high moisture content to grow (137) and there are quite fewer reports related to successful utilisation of bacteria for SSF (137, 184). SSF is highlighted by the utilisation of less energy (no aeration or agitation), low wastewater output, high concentration of metabolites at low cost and is technically easier (71, 132, 133, 114). It hence offers apparently economised advantages over the classical SmF process. The selections of suitable strain, substrate and process parameters are crucial factors that affect the SSF process (132). The stability of produced enzymes at high temperature or extreme pH has also been reported to be better in SSF (41).

The degradation of lignocellulosic material is currently understood as an enzymatic process where fungi play the main role (101, 111). The fungi mostly develop highly

diffuse cellular bodies made up of a spreading network (mycelium) of very narrow, tubular, branching filaments called hyphae. These hyphae exude enzymes, and absorb food at their growing tips. Hyphae are collectively very long, and can explore and exploit food substrates very efficiently (4, 84). Wherever some moisture is available, the spores of the fungal strains, present in environment will germinate and the hyphae arising from them will attack almost any kind of organic matter including human food, fabric, paper, wooden material, above a certain water threshold (84).

Xylanases have been isolated from basidiomycetes (91, 180, 36, 117, 75), but relatively little is known about the patterns of decay. Depending on the preferential degradation of certain cell wall structures, two basic forms of wood decay are known in homobasidiomycetes: brown rot and the white rot. Brown rot fungi depolymerise cellulose and degrade all polysaccharides early in the decay process (23), hemicellulose being apparently degraded prior to cellulose (70). The lignin is not degraded, but only slightly modified. This modified lignin is responsible for the characteristic colour of brown rotted wood (60, 155). The white rot fungus exhibits 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 (190).

Geographically diverse strains vary in their characteristics and levels of enzyme produced (77). 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 strains producing the compound of interest is a crucial and an exhaustive step for accomplishing the production of a particular compound. Since any biotechnology process is likely to be based on crude enzymes (58), it is important to optimize simultaneous production of the constituent enzymes for realizing their biotechnological potential. Although, white-rot fungi have effective hemicellulase systems, only a few studies have been made on their hemicellulases. The present study aimed at isolating, screening and identifying the white rot basidiomycetes, capable of producing extracellular xylanases with little or no cellulose activity. The optimisation of various operating physico-chemical parameters was done to achieve the higher xylanase activity from the screened strains. The xylanase was biochemically characterised 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). All other chemicals were of analytical grade and purchased from standard commercial manufacturers. Wheat bran, rice bran, rice straw, sorghum, sugarcane bagasse and soyabean meal were purchased from local market of Saharanpur, U.P. (India).

4.2.2 Strain isolation

Different fungal strains were isolated from lignocellulosic wastes (dead and decaying woods), decomposing manure, sugarcane dumping site, fruiting bodies 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; Mussoorie; Forest research institute (FRI), Dehradun 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 32 °C. The moisture level was carefully controlled with sterile tap water, so as to provide a solid substrate for fungal growth, with no free water available. The plates were observed for the appearance of fungal growth for 2-10 days.

A total of 14 fungal colonies with different morphological features appeared in different Petri plates. Amongst the isolated strains, many were exhibiting fruit bodies (indicative of growth of basidiomycetes). The different fungal hyphae and the fruiting bodies from the above isolates were transferred to wheat-bran agar media plates, prepared by dissolving 4% wheat bran and 2% agar in 1 L of double distilled water and autoclaved at 15 psi for 15 min. 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 37 °C for 5 days and further stored at 4 °C for future usage. The cultures were also maintained as a suspension of spores and hyphal fragments in 15% (v/v) sterile glycerol at -20 °C for long term preservation.

4.2.3 Screening of isolates for potent xylanase producing strains

A set of highly discriminatory procedures were used to select the fungal strains, best among the isolates in terms of higher xylanase activity. The 14 fungal isolates obtained in the present work were primarily screened for their abilities to produce extracellular xylanases while growing on xylan-agar medium as described (175). To prepare the xylan agar medium, 1% xylan and 2% of agar were 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 32 °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 °C, 20 min) supernatant did not produce any clear zones. The strains were hence primarily screened for their xylanase production ability on the basis of clear zone diameter obtained on the xylan-agar (XA) plate. The screened isolates were further subjected to solid state fermentation (SSF) conditions to determine their actual xylanase activities. The enzymes from all the isolates were harvested on 6^{th} day of incubation and the xylanase and cellulase activities of enzyme samples from each isolate were determined. The supernatant protein concentrations of the enzyme samples were also determined. The aim of primary screening was to select fungal strains exhibiting higher xylanase activities.

Secondary screening of the 10 selected fungal strains was carried out on the basis of higher xylanase (115) and lower cellulase (110) activity of crude enzyme extract produced by SSF and finally two strains were selected for further studies.

4.2.3.1 Screening of the test strains HK-1 and HK-2 for phenoloxidases by plate assay technique

The test fungi were tested as per Bavendamm plate assay for their ability to produce phenoloxidases (that catalyze the oxidation of a number of aromatic substances such as diphenols, methoxy-substituted monophenols, etc.,) against tannic acid; a feature characteristic to basidiomycetes (147). Tannic acids are water-soluble phenolic

compounds that exhibit distinct properties such as the ability to precipitate alkaloids, gelatin, and other proteins (67). The characteristic that sets tannic acids apart from all other phenolics is their ability to precipitate proteins. Therefore, having an enzyme capable of degrading tannic acid or that is insensitive to the presence of tannic acid may be useful for various applications of plant tissue materials. The two strains were thereby grown on wheat bran-agar media supplemented with 0.02% tannic acid. 5 mm discs from 4 day old cultures of the test fungi were inoculated in this medium, followed by plate incubation at 32 °C for 10 days, to get sufficient growth of the cultures on the medium.

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 treatment for 30 min was given for subsequent alcohol gradients. After treating with 100% alcohol, samples were air dried and examined under SEM using gold shadowing technique (53). Electron photomicrographs were taken at desired magnifications. Results are reported in Plate 4.3 and 4.4.

4.2.5 Identification of strains

Finally, two selected fungal strains were sent to Forest Research Institute, Dehradun, Uttaranchal (India) for morphological identification and further the strain with highest xylanase activity (HK-1) was sent to Agharkar Research Institute (ARI), Pune (India) for its molecular identification up to species level by isolation of the genomic DNA in pure form, followed by amplification of nearly 3000 base pair r-DNA fragments using 4 different pairs of universal primers (SSU-NS1 and NS4, NS3 and NS8, LSU-5.8SR and LR7, LR7R and LR12). The sequencing PCR was set up with ABI-BigDye® Terminator v3.1 Cycle Sequencing Kit (Part No. 4337455) and the sequence data was manually edited using appropriate software, whereby the test isolate was identified as *Coprinopsis cinerea* HK-1 with accession no NFCCI 2032.

4.2.6 Fermentation medium

4.2.6.1 Nutrient salt solution

For production of extracellular enzymes, the nutrient salt solution (N.S.S.) was prepared according to Vishniac and Santer (185) and standardized as by Singh and Garg (162). 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

All the starchy material from wheat bran (lignocellulosic substrate) was completely removed by a subsequent thorough wash in hot and cold distilled water. It was immediately dried in sunlight, grinded up to +100 mesh size and stored in sealed polythene bags for further use.

4.2.6.3 Inoculum

From PDA slants, fungal cultures were transferred to Petri-plates (wheat bran agar medium) and incubated at 32 °C for 4 days. From this actively growing culture, 2 disks of size 5 mm, cut with the help of a borer were 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) (162). Medium was inoculated with fungal cultures following incubation at 32 °C for 6 days in an orbital incubator shaker (Sanyo, Orbi-safe, UK) with constant shaking (120 rpm). The crude enzyme was harvested and assayed for xylanase (115), and cellulase (110) activities as per standard protocols. Results are reported in Table 4.3 and Figure 4.3.

4.2.7.2 Solid-state fermentation (SSF)

Solid-state fermentation was performed as described by Beg *et al.* (14). Slurry of the fermentation medium containing 5 g of wheat bran and 15 mL of NSS was added

thereby maintaining a solid to liquid ratio of 1:3 (85) in Erlenmeyer flasks (250 mL) and inoculated with fungal culture. The culture flasks were inoculated with 2 discs of 5mm diameter from both the strains and incubated at 32 °C for 6 days. The crude enzyme was harvested and assayed for xylanase (115), and cellulase (110) activities as per standard protocols. Results are reported in Table 4.3 and Figure 4.3.

4.2.7.3 Enzyme harvesting

The enzyme was directly filtered through four layers of cheese cloth in submerged fermentation process while in solid state fermentation, the contents of the flask were crushed with the help of a glass rod and were shaken for at least 30 min using 15 mL of distilled water 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 °C. The clear brown colored supernatant was used as the crude enzyme sample and was stored at –20 °C until used.

4.2.8 Analytical methods

4.2.8.1 Estimation of xylanase activity

Xylanase activity was estimated by analysing the xylose released as per DNS method (115). 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 reagent was stored in dark at 4°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 °C for 15 min. From the incubated mixture, 0.3 mL of solution was taken in a test tube and 0.9 mL of dinitrosalicylic acid (DNS) reagent was then added, followed by heating for 5 min on boiling water bath and estimation of xylose released colorimetrically at 540 nm using UV-Vis spectrophotometer (Cary 100 Bio Varian-Australia) at 25 °C. Blank was prepared by using distilled water in place of enzymatic reaction products and 3 mL of DNS reagent. One unit of enzyme activity corresponded to one μmol of xylose released per min per mL of the reaction mixture under the assay conditions.

4.2.8.2 Estimation of cellulase (CMCase) activity

Carboxymethyl cellulase (CMCase) activity was determined as described by Mandels (110). 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 regularly included in which either enzyme or substrate were omitted and treated similarly. The mixture was incubated at 50 °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 °C using DNS reagent (115). One unit of enzyme activity was expressed as one μmol of glucose liberated per min per mL of the reaction mixture under above defined conditions.

4.2.8.3 Estimation of protein concentrations

Protein concentration was estimated according to Lowry et al., with bovine serum albumin (BSA) as a standard (104) as described below,

Reagents

Lowry-A: 2% Na_2CO_3 in 0.1M NaOH

Lowry-B: 0.5% $CuSO_4$ in 1% sodium potassium tartarate (freshly prepared)

Lowry-C: 50 mL of Lowry-A mixed with 1.0 mL of Lowry-B

Lowry-D: 1 mL of 1N Folin–Ciocalteu's phenol reagent in 3 mL of distilled water

A total volume of 200 μL of protein sample was taken and 1 mL of Lowry-C was added. The reaction mixture was put for 10 min at room temperature. After 10 min 100 μL of Lowry-D was added and incubated for 30 min at room temperature. Colour developed was measured at 750 nm using UV-visible spectrophotometer (Cary 100 Bio, Varian-Australia) at 25 °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 (39) 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 °C. Enzyme activity was expressed as the amount of enzyme which produced an increase of 1.0 absorbance unit per 30 seconds.

4.2.9 Various physicochemical parameters affecting extracellular enzyme production under SSF

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

4.2.9.1 Effect 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 psi for 15 min and inoculated aseptically with 2 discs of 5 mm diameter from 4-day old culture of each fungal strain. These were then incubated at 32 °C and were harvested after 2^{nd}, 3^{rd}, 4^{th}, 5^{th}, 6^{th}, 7^{th}, 8^{th}, 9^{th}, 10^{th} and 11^{th} day. The estimation of protein concentration, xylanase and cellulase activity was done as per standard protocols described previously. Results are reported in Tables 4.4-4.5 and Figures 4.4-4.5.

4.2.9.2 Effect of initial pH

For the optimization of initial pH, a set of 250 mL Erlenmeyer flasks was prepared as described above. pH of the flasks, varying from 4.4 to 11, was adjusted using 1 N $NaOH/H_2SO_4$ separately with the help of a pH meter. These flasks were autoclaved at 15 psi for 15 min, inoculated with fungal cultures aseptically as described above and incubated at 32 °C. Flasks were harvested after an optimum period of 7 days for strain HK-1 and 8 days for HK-2 and the supernatant protein concentration, xylanase and cellulase activities were measured as already described. Results are reported in Table 4.6 and Figure 4.6.

4.2.9.3 Effect of Incubation temperature

The effect of incubation temperature on the production of xylanases by test fungi was studied by incubating the inoculated flasks at different temperatures varying from 27 to 52 °C for an optimum period of 7 days for strain HK-1 and 8 days for HK-2. 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 °C for 7 days. The supernatant

protein concentration, xylanase and cellulase activities were determined as described earlier. Results are reported in Table 4.7 and figure 4.7.

4.2.9.4 Effect of carbon source (lignocellulosic substrates)

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) as well as the two grasses under study, sofia grass and lemon grass were used as substrates for xylanase production by both of the test strains. The substrates were moistened with NSS, autoclaved, inoculated and incubated at 37 °C. The crude enzyme was extracted on 7^{th} day and assayed for supernatant protein concentration, xylanase and cellulase activities as previously described. Results are reported in Table 4.8 and Figure 4.8.

4.2.9.5 Effect of complex organic nitrogen source

A set of 6 Erlenmeyer flasks (250 mL) was prepared as described above and each flask was supplemented with a different nitrogen source including peptone, beef extract, yeast extract, malt extract, soya bean meal and urea in the NSS at a concentration of 1.0 g/L. The pH of NSS was set at 6.4. Fermentation was carried out for 7 days and 8 days for the strains HK-1 and HK-2 respectively at 37 °C. The enzymes were then harvested and the supernatant protein concentration, xylanase and cellulase activities were estimated as per the standard protocols described earlier. Results are reported in Table 4.9 and Figure 4.9.

4.2.9.6 Effect 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 and 8 days respectively for the strain HK-1 and HK-2 at 37 °C. The supernatant protein concentration, xylanase and cellulase activities in crude enzyme extract were checked as per standard protocols mentioned earlier. Results are reported in Table 4.10 and Figure 4.10.

4.2.9.7 Effect of glucose and lactose concentration

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 4.11. Xylanase activity was determined as per standard protocol mentioned earlier. Results are reported in Table 4.13 - 4.14 and Figure 4.11(a, b) respectively for glucose and lactose.

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 (115) of the crude enzyme samples was determined, under standard assay conditions. Results are reported in Table 4.15 and Figure 4.12.

4.2.10.2 Optimum temperature and thermostability

Thermostability of the enzyme was determined by incubating the crude enzyme preparations at temperatures ranging between 45-85 °C for up to 15 min. Samples were withdrawn after 15 min and analyzed for residual xylanase activity (115) under standard assay conditions. Results are reported in Table 4.16 and Figure 4.13.

4.2.11 Mass production of xylanase

After optimization of fermentation conditions, mass production of xylanases from both the fungal strains was carried out under optimised conditions of SSF for their use in biobleaching experiments. The mass production was carried out in flasks of 2 L capacity. 30 g of wheat bran and 90 mL of NSS were added to each flask. The pH of each flask was adjusted as described earlier. The flasks were autoclaved and inoculated with 12 discs, each of 5 mm diameters from 4-day old culture of each test fungi. These were then incubated under optimum conditions of temperature, pH and incubation period, and harvested for the extraction of enzymes. The xylanase, cellulase, laccase activities and supernatant protein concentration were determined as per standard protocols. Results are reported in Table 4.17.

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 4.1 reveals the morphological characteristics of 14 fungal strains collected from different lignocellulosic sources. The fungal strains were isolated on wheat bran agar plates, incubated at 32 °C and colour of mycelia, spores and colony appearance was observed (Plates 4.2 I-L). The formation of aerial fruiting bodies and white thread like mycelial network on the decaying wood, after successive degradation was an indication of the growth of basidiomycetes (Plate 4.1 D) (Plate 4.1D, G and 4.2D, E). During primary screening of the fungal isolates, their xylanase production ability was tested by xylan-agar (XA) plate assay. XA-plates were stained with Congo red dye to enhance the visibility of hydrolyzed area observed as a clear zone. Out of 14, ten isolates were able to form a clear zone on XA-plate plates, of which HK-1 showed maximum clear zone diameter (Plate 4.1E) followed by HK-2 (Plate 4.2G). These isolates were chosen as potent xylanase producers. The above 10 strains were next subjected to secondary screening for their xylanase production ability under conditions of solid-state fermentation (Table 4.2). The highest xylanase activities were detected in the culture filtrates of three fungal strains namely, HK-1, HK-2, and HK-6. These strains produced respectively 456.12, 164.22, and 132.07 IU/mL xylanase activity with the least cellulase activity i.e. 0.744, 0.815 and 0.700 IU/mL respectively on wheat bran at 32 °C after 6 days of incubation. Two fungal isolates namely HK-1 and HK-2 were selected for further studies based on their privilege of providing high xylanase and minor cellulase activity.

4.3.1.1 Screening of test strains for production of phenoloxidases

As per Bavendamms plate assay technique, the two test strains were screened for the production of phenoloxidases by supplementing the wheat bran-agar media with tannic acid. After a period of 10 days, the oxidation of tannic acid was manifested as a dark browning around the colonies, which was measured to correlate with enzyme activity. Both strains showed a good phenoloxidase activity (Plate 4.1F and 4.2H). Many edible mushrooms such as *Lentinus edodes, Pholiota aurivella* also showed positive reaction on such agar plates (120).

4.3.1.2 Identification of selected isolates

The two fungal isolates HK-1 and HK-2 were identified as different strains of white-rot basidiomycete *Coprinopsis cinerea* (Schaeff.) (Pers.: Fr.) Redhead, Vilgayls &

Moncalvo, 2001 (144) *(=Coprinus cinereus* (Per.:Fr.) Gray), from Forest Research Institute (FRI), Dehradun, Uttarakhand (India). Both of these strains were deposited in the National Type Culture Collection (NTCC), Forest Pathology Division, FRI, Dehradun and were allotted the NTCC Culture No. 1870 and 1871 respectively. *Coprinopsis cinerea* has been scientifically classified as Fungi, *Basidiomycota, Agaricomycotina, Agaricomycetes, Agaricomycetidae, Agaricales, Coprinaceae, Coprinopsis.* The basidiomycete *Coprinopsis cinerea* has been a classic experimental model for multicellular development in fungi because it grows on defined media, completes its life cycle in 2 weeks, produces some 108 synchronized meiocytes, and can be manipulated at all the developmental stages by mutation and transformation (92). It is commonly isolated from horse dung (92), degraded forestry products or composting materials. Their occurrence could closely be associated with the ability to produce a variety of cell wall degrading enzymes.

The identification of fungal strain HK-1 was further confirmed by sequencing the SSU (SSU- NS1, NS4, NS3, NS8) and LSU region (LSU- LR7, LR7R, LR12) of rDNA for *C .cinerea* HK-1.

Primer NS1 (721 bases)

>NS1-HK1 sequence exported from NS1-HK1_B03-edited.ab1

5'AAACTGCGAATGGCTCATTAAATCAGTTATAGTTTATTTGAAGAGTATCTTA
CTACATGGATAACTGTGGTAATTCTAGAGCTAATACATGCAATCAAGCCCCGA
CTTCCGGAAGGGGTGTATTTATTAGATAAAAAACCAACGCGGCTCGCCGCTCC
CTTGGTGATTCATAATAACTTCTCGAATCGCATGGCCTTGTGCCGGCGATGCTT
CATTCAAATATCTGCCCTATCAACTTTCGATGGTAGGATAGTGGCCTACCATG
GTTTCAACGGGTAACGGGGAATAAGGGTTCGATTCCGGAGAGGGAGCCTGAG
AAACGGCTACCACATCCAAGGAAGGCAGCAGGCGCGCAAATTACCCAATCCC
GACACGGGGAGGTAGTGACAATAAATAACAATATAGGGCTCTTTTGGGTCTTA
TAATTGGAATGAGTACAATTTAAATCCCTTAACGAGGAACAATTGGAGGGCA
AGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCCAATAGCGTATATTAAAGT
TGTTGCAGTTAAAAAGCTCGTAGTTGAACTTCAGACCTGGCTGGGCGGTCCGC
CTAACGGCGTGTACTGTCTGGCTGGGCCTTACCTCTTGGTGAGCCGGCGTGCC
CTTTATTGGTGTGCGTCGGGGAACCAGGACTTTTACCTTGAGAAAATTAGAGT
GTTCAAAGCAGGCCTTTGCCCGAATACATTAGCAT3'

Primer NS4 (725 bases)

>NS4-HK1 sequence exported from NS4-HK1_A04-edited.ab1

5'TTTTGATTTCTCGTAAGGTGCCGAGCGACACATCAAAATTGAGGTAGCACCG
ATCCCTAGTCGGCATAGTTTACTGTTAAGACTACAACGGTATCTAATCGTTTTC
GATCCCCTAACCTTCGTTCTTGATTAATGAAAACATCCTTGGCAAATGCTTTCG

CAGTAGTTGGTCTTGAGTCAATCCAAGAATTTCACCTCTAGCGACTCAATACC
AATGCCCCCAACTATCCCTATTAATCATTACGGCGACTCTAGAAACCAACAAA
ATAGAACCGCACGTCCTATTTTATTATTCCATGCTAATGTATTCGGGCAAAGG
CCTGCTTTGAACACTCTAATTTTCTCAAGGTAAAAGTCCTGGTTCCCCGACGCA
CACCAATAAAGGGCACGCCGGCTCACCAAGAGGTAAGGCCCAGCCAGACAGT
ACACGCCGTTAGGCGGACCGCCCAGCCAGGTCTGAAGTTCAACTACGAGCTTT
TTAACTGCAACAACTTTAATATACGCTATTGGAGCTGGAATTACCGCGGCTGC
TGGCACCAGACTTGCCCTCCAATTGTTCCTCGTTAAGGGATTTAAATTGTACTC
ATTCCAATTATAAGACCCAAAAGAGCCCTATATTGTTATTTATTGTCACTACCT
CCCCGTGTCGGGATTGGGTAATTTGCGCGCCTGCTGCCTTCCTTGGATGTGGTA
GCCGTTTCTCAGGCTCCCTCTCCGGAATCGAA3'

Primer NS3 (306 bases)

>NS3-HK1 sequence exported from NS3-HK1_H04-edited.ab1

5'GTTGTTGCAGTTAAAAGCTCGTAGTTGAACTTCAGACCTGGCTGGGCGGTCC
GCCTAACGGCGTGTACTGTCTGGCTGGGCCTTACCTCTTGGTGAGCCGGCGTG
CCCTTTATTGGTGTGCGTCGGGGAACCAGGACTTTTACCTTGAGAAAATTAGA
GTGTTCAAAGCAGGCCTTTGCCCGAATACATTAGCATGGAATAATAAAATAGG
ACGTGCGGTTCTATTTTGTTGGTTTCTAGAGTCGCCGTAATGATTAATAGGGAT
AGTTGGGGGCTTTGGTTCTTCGAGTCGCTAGTGGTGGAATTATGAGCCCCCCG
GTAATCTCATATTCCCTCGACCCGCCCCCCCTTCGCCCTGGGATCGGGGAATAT
GTTGTACCTCTTACCTCCCTCCCCCTCTCCCCTGTCCTTGGTG3'

Primer NS8 (616 bases)

>NS8-HK1 sequence exported from NS8-HK1_G05-edited.ab1

5'GAGACCTCACTAATGCCATTCAATCGGTAGTACTTTGTACGGGCGGTGTGTA
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TCACAAGATTACCCAGACCTTCCGGCCAAGGAAAAGAACTCGCTGGCTCTGTC
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GCCTCAAACTTCCGTCAGCTAGACGCTGACAGTCCCTCTAAGAAGCCGGCGAC
CATCCAAAGACGGCCTGGCTATTTAGCAGGTTAAGGTCTCGTTCGTTATCGGA
ATTAACCAGACAAATCACTCCACCAACTAAGAACGGCCATGCACCACCACCC
ATAAAATCATGAAAGAGCTATCAATCTGTCAATCCTAGTTATGTCTGGACCTG
GTGAGTTTCCCCGTGTTGAGTCAAATTTAAAGCCGCAGGCTCCACACCCTTGG
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Primer LR7 (770 bases)

>HK1LR7 sequence exported from SAT_250510_D12_HK1 **LR7**_4-edited.ab1

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CCAAGCGGTCCAATTAAGAGACGGCTTGTTCTTACATATTTAAAGTTTGAGAA
TAGGTTAAGGTTGTTTCAACCCCAAGGCCTCTAATCATTCGCTTTACCACATAA
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AGGCATAGTTCACCATCTTTCGGGTCCTAGCATACATGCTTTACCTCACATCCG
TCCGTAAACTTCAGGTGCGGGCGCCAGTGCTCCCCCATGGAAGGGGATCCTGG
ACTTTCACTTTCATTACGCGTCCGGGGTTTTCCACCCAACACTCGCAGGTATGT
TAACTCCTTGGTCCGTGTTTCAAGACGGGTCGATTAAAGCCATTATGCCGCTCC
TTAAGCACGAACGGGTTCGAAACCGGCCTGCCGGGTGGCTGAATTCCTCAGTC
CCACCGATGTTTACGACCAAGGGTTTAACCTTCCGGAAAAGCCCATTCCCTGG
GATTTTTCCCCCGGCCAAATGA3'

Primer LR7R (800bp bases)

>HK1-LSU2-LR7R sequence exported from SAT_200410_A08_HK1-1.ab1-

5'GGTCATGGCGTCATGTCTGATCCTAAGAGATAGGGAAGCTCCGTTTCAAAGT
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AGCCCCGGGAAGAGTTATCTTTTCTCCTTAACAGTCTATCACCCTGAAATCAAT
TTGTTTGGAGCTAGGGTTCAATGACTGGTAGAGCACAACACCTCTGTTGTGTC
CGGTGCGCTCTCGACAATCCTTGAAAATCCAAGGGAATGAATAATTTTCACAC
CTGGTCGTACTCATAACCGCAGCAGGTCTCCAAGGTGAACAGCCTCTAGTTGA
TAGAACAATGTAGATAAGGGAAGTCGGCAAAATAGATCCGTAACTTCGGGAA
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GGGTGTGGGCGACCTTGGCTGCTCTTCGGGGCGTCCGGTGTACGCTTAACAAC
CAACTTAGAACTGGTACGGACAAGGGGAATCTGACTGTCTAATTAAAACATA
GCATTGCGATGGCCAGAAAGTGGTGTTGACGCAATGTGATTTCTGCCCAGTGC
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AATGGATACG3'

Primer LR12 (833 bases)

>HK1-LSU2-LR12 sequence exported from SAT_200410_B08_HK1-edited.ab1-

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AAATTCCGAGGGACTAAAGGATCGATAGGCCACACTTTCATGGTTTGTATTCA
CACTGAAAATCAAAATCAAGGGGACTTTTACCCTTTTGTTCTACGCGAGATTT
CTGTTCTCGCTGAGTCCCCCTTAGGACACCTGCGTTATCTTTTAACAGATGTGC
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CGTTTACCCCGCTGGGTGATTCTTCCTTTGACATTCAAGCATGGGCAGAATCAC
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CTTGGCGTACAGTTTAATTGGTGTAAGTAACCGA3'

4.3.1.3 Morphological characteristics of *Coprinopsis cinerea*

Morphological studies of the fungal isolates *C. cinerea* HK-1 and HK-2 with SEM revealed a spreading, white coloured mycelial network of highly tapered, tubular, branched filamentous hyphae (Plates 4.4 and 4.5). Small etiolated stipes arising after 4-5 days of inoculation, eventually grew into unique and complex structures called fruiting bodies which subsequently suffered rapid autolysis, a feature that is specific for many species of the *coprini*. The fruiting bodies grew as immature primordia (stipes) (Plates 4.1B and 4.2D), which on 8^{th} or 9^{th} day produced black club-shaped spores (Plates 4.1 G,I and 4.2E) which was a characteristic feature of all the members of basidiomycotina group. Filamentous fungi have the tendency to grow adhered to surfaces and the influence of this type of growth on fungal physiology has not yet been thoroughly studied, particularly when related to productivity (183). These filaments exuded enzymes, and absorbed food at their growing tips. Hyphae were collectively very long, and could explore and exploit food substrates very efficiently (4). The finer structural details of the mycelia and spores as observed through the scanning electron microscopy (SEM) are shown in Plates 4.3 and 4.4. Clamp connections were easily visualized as in Plates 4.3 A, C; which was a feature characteristic for identification of the fungal strains as basidiomycetes. The hyphae of fungal strain HK-1 were thick and compact (Plate 4.3A-C) whereas, the hyphae of the fungal strain HK-2 were thin, elongated (Plates 4.4 A,B) and ribbon like (Plate 4.4C). The basidiospores produced by both the fungal strains were club shaped (Plates 4.3D,E and Plate 4.4D,E) which was a characteristic feature of all the members of basidiomycotina group.

Since, fungal morphology influences the productivity of fungal fermentations; it was of major importance to know the fungal behaviour during culture for enzyme production. Fermentation medium components as well as physico-chemical factors could be responsible (50) for variation in morphology of the 2 isolated *coprinoid* strains (HK-1 and HK-2) with a clear transition from cottony growth in fungal culture HK-1 to pelleted forms in HK-2 (Plates 4.1A-C, 4.2A-C). Plates 4.1 I and 4.2 F shows the growth of two strains under SSF held in flasks.

Xylanase production has been described for many fungal species, including the thermo-tolerant and thermophilic fungi (57), but until now, no study on the xylanase

production from any *Coprinopsis cinerea* strain using SSF, ever appeared in the literature. Present study investigated the xylanase producing potential of indigenously isolated wild strains of *Coprinopsis cinerea* HK-1 and HK-2 in context with its alkali-thermo-tolerant nature. The study examined a detailed optimization of SSF culture including pH, temperature, cheap and readily available carbon and nitrogen substrates, substrate to NSS ratio for high production of xylanase. The biochemical characterization of enzyme was further made to mark the effect of pH and temperature on the activity and stability of xylanase.

4.3.2 An analysis of critical parameters for betterment of the xylanase production by the strains HK-1 and HK-2 of *Coprinopsis cinerea*

The optimization of composition of medium and cultural conditions was carried out based on stepwise modification of the governing parameters for xylanase production.

4.3.2.1 Fermentation system

Table 4.3 and Figure 4.3 reveal that a high level of extracellular xylanase production (465.25 IU/mL and 214.1 IU/mL) with wheat bran as the lignocellulosic substrate for fermentation at 32 °C (pH 6.0) was observed for *C. cinerea* HK-1 on the sixth day of incubation under SSF and submerged fermentation (SmF) conditions respectively; however, accessory enzyme levels (cellulase) were low. Similarly, 156.92 and 97.40 IU/mL xylanase activities were achieved for strain HK-2 under the respective fermentation modes. The enzyme production by both the strains under SSF conditions was impeccably well as compared to SmF. SSF marked a 53.7 and 37.9% hike over SmF regarding xylanase producing potential of strain HK-1 and HK-2, along with an associated improvement in cellulase activity of the two strains by 35.4 and 55.9% respectively.

Filamentous fungi could grow to significant extent in the absence of free water (54). Considering that submerged free floating fungal growth is not natural, growth on and within solid substrates was fundamentally related to cell adhesion (183). The heterogeneous nature of the substrates used in SSF process (166), the concentration gradients of sugars and mineral salts (159) and the localised drop in substrate concentration (143) allowed enhanced metabolic activity in microorganisms growing in SSF and significantly minimized catabolic repression (166, 143).

SSF aimed at strengthening the contact between fungal mycelia and the insoluble substrate thereby achieving the highest substrate concentrations (71), which was not possible during SmF. Thereby, SSF was chosen for further optimization studies for crude xylanase production by the *C. cinerea* HK-1 and HK-2. A hike in xylanase activity of *Coprinellus disseminatus* SW-1 NTCC1165 under SSF using wheat bran was reported by Agnihotri et al., (2) in comparison to SmF, while a 30-fold enhancement in xylanase production under SSF was observed by Malarvizhi et al., (108) in contrast to liquid culture using wheat bran as the substrate for a culture of *Ganoderma lucidum.*

4.3.2.2 Effect of incubation period

Xylanase activities of the two basidiomycetous strains under SSF were determined after every 24 h of incubation in order to determine the optimum incubation period for obtaining maximum xylanase titers. The enzyme production however started after 24 h of inoculation which steadily attained its maxima (537.83 IU/mL) with a 13.5% increase on 7^{th} day of incubation for HK-1, whereas for strain HK-2, xylanase production hiked by 26.9% on 8^{th} day (230.56 IU/mL) at 32 °C when compared to their respective activities of 6^{th} day (Tables 4.4 and 4.5). Basidiomycetes are slow growing fungi. so maximum xylanase production was achieved after 7 days in *Pleurotus ostreatus* (141), 6 days in *Phanerchaete chrysosporium* (172) and 8 days in *Volvariella diplasia* (135). A decrease in enzyme activity (491.4 and 213.97 IU/mL) for strains HK-1 and HK-2 was recorded respectively with further increase in incubation period. The productivity of xylanase in SSF for *C. cinerea* strains HK-1 and HK-2 was compared with those reported for other xylanase-producing microorganisms. In some fungi, high xylanase production was shown to be linked strictly to cellulase production (66, 83), but *C. cinerea* did not produce much cellulase despite of the use of cellulose-rich substrate. Maximum cellulase production was 0.690 IU/mL for HK-1 and 0.965 IU/mL for HK-2 which was achieved on the 6^{th} and 7^{th} day respectively. The results depicted in Figures 4.4 and 4.5 indicate that the enzyme production corresponded closely to the growth of the fungus in terms of increased fungal biomass; as the maximum xylanase activity and protein biomass (3.92 and 4.03 mg/mL) achieved for the strains HK-1 and HK-2 almost corresponded to the optimised period of incubation. The protein biomass continued to increase from day-2 to day-8 for both the

strains, and became nearly stationary thereafter for the strain HK-1, while a decrease of 46% was noticed on day-11 for the strain HK-2.

The reduction in xylanase yield after optimum period was probably due to the depletion of nutrient available to microorganism or due to proteolysis (51). Being primary metabolites, xylanases were optimally expressed at end of the exponential phase, which correlated the harvesting time of the fungus to the production of enzymes (95). The metabolic enzymes of the xylanase producers such as proteases (134) and transglycosidases had also been shown to affect the actual yield of the enzymes (73). The xylanase production and supernatant protein concentration increased up to seven days of incubation for two strains SH-1 and SH-2 of *C. disseminatus* on a wheat bran based media at 37 °C under SSF (164).

4.3.2.3 Effect of initial pH of the medium

pH of the medium is one of the regulatory parameters during fermentation. Effect of media pH on enzyme production is shown in Figure 4.6. pH 6.4 conferred to be optimal for growth of *C. cinerea* HK-1 and HK-2 with 578.27 and 315.50 IU/mL of respective xylanase activities. For fungal strain HK-1, when NSS with pH between 6.0 and 7.4 were used, no significant differences were found in the enzyme yield (Table 4.6 and Figure 4.6). The pH range 6.4-8.4 provided similar observations for strain HK-2. This could be explained by the fact that wheat bran possesses excellent buffering capacity (133). Similar observations were made by Pal and Khanum (130) for xylanase from *Aspergillus niger* DFR-5 in wheat bran-based medium. At a pH of 8.0, 70 and 81.3% of the maximum xylanase activity was retained by strains HK-1 and HK-2 respectively, which was quite remarkable. The enzyme production in strain HK-1 was severely hampered at an alkaline pH of 10.0 (212.0 IU/ml). However, with increase or decrease in pH, by adjusting to values other than the optimal value, the production of xylanase was found to decrease gradually. The preferred pH range for attaining optimal cellulase titers was 4.4-6.0 for both the *C. cinerea* strains. While moving towards the acidic end of the pH range, the maxima were obtained with 0.923 and 1.092 IU/mL of cellulase activities for strain HK-1 and HK-2 respectively, whereas a sharp decrease in cellulase activities was noticed above a pH of 6.0.

In view of the fact that enzymes are proteins, the ionic character of the amino and carboxylic acid groups on the protein surface were liable to be influenced by pH changes and the catalytic properties of the enzymes were strikingly affected. Fermentation at lower and higher pH proved to be detrimental perhaps because of the inactivation of the enzyme system (48, 178, 76). Each microorganism thereby, holds certain pH range for its optimal growth and enzyme production. Initial pH influences many enzymatic systems and the transport of several species of enzymes across the cell membrane (137). Most of the white-rot fungi had been reported to grow best at slightly acidic pH (145). Xylanase from different organisms showed an optimum pH within a range of 4.0 to 7.0. However, certain xylanases from *Aspergillus* sp. RSP-6, *Penicillium herque* and some other fungi including *Fusarium oxysporum* exhibited an optimum pH more towards the acidic side (pH 2.0-6.0) (171, 95). The optimum initial medium pH was 6.5 for xylanase production under shake flask fermentation condition using corncob and corn steep liquor as the carbon and nitrogen sources for both *T. lanuginosus* wild type and M7 (26). The optimum pH for xylanase activity from 7·0 to 9·0 was found in many bacterial strains (46). However, several alkali-tolerant xylanases have also been characterized recently (179, 22, 43, 45).

4.3.2.4 Effect of incubation temperature

Temperature is one of the important parameters that determine the success of optimization system. Therefore, the effect of temperature on xylanase production by *Coprinopsis cinerea* HK-1 and HK-2 was examined between temperature ranges of 27 to 52 °C at a pH of 6.4 for 7 and 8 days respectively for the two *Coprini* and the results obtained are shown in Figure 4.7 and Table 4.7. The maximal production of xylanase was achieved at temperature 37 °C for both strains with an activity of 668.45 IU/mL for HK-1 and 356.42 IU/mL for HK-2. In comparison to activities at 32 °C, a net increase of 14.2 and 10.6% was noticed with strains HK-1 and HK-2 respectively at a growth temperature of 37 °C. A decrease in xylanase titer was obtained with cultivation temperatures either lower or above the optimum temperature of 37 °C. Strain HK-1 retained 50% of its maximal activity at 47 °C, whereas, strain HK-2 could retain just 24% at this temperature. Even at a temperature of 52 °C, growth was visible for strain HK-1 with a xylanase activity of 34.80 IU/mL, whereas no growth was observed in case of HK-2. Hence, the thermo-tolerance of strain HK-1 was found to be much better than HK-2. Production of

cellulase by the two strains HK-1 and HK-2 were maximum at 32 °C, which decreased by 29.18 and 10.23% on incubation under temperature 37 °C. The associated cellulase contamination of the crude enzyme was thereby reduced by optimising the culture conditions.

Temperature was a cardinal factor affecting the amount and rate of growth of an organism and the increasing temperature had the general effect of increasing enzyme activity (118) but the enzyme began to suffer thermal inactivation at higher temperatures. The decreased yield at low temperatures was possibly due to lower transport of substrate across the cells. On proceeding towards optimum temperature for enzyme production, increased kinetic energy of reacting molecules increased the reaction rate. At higher temperatures, thermal denaturation of enzymes of the metabolic pathway occured which increased the maintenance energy requirement of cellular growth, thereby resulting in poorer production of the metabolites (3) and even loss of enzyme activity (130) signifying that the end-point of fermentation ought to be controlled carefully. A citation also stated that microorganisms synthesized only a reduced number of proteins essential for growth and other physiological processes under conditions of high temperatures (55). Agnihotri et al. (2) reported 37 °C as the optimal temperature for growth of a thermotolerant fungus *Coprinellus disseminatus* SW-1 NTCC 1165 under SSF, using wheat bran as substrate. An identical optimum temperature for xylanase production in SSF using a wheat bran based media was reported by Beg et al. (14) for *Streptomyces* sp. QG-11-3 and Battan et al. for *Bacillus pumilus* ASH 7411 (12). *S. cuspidosporus* also grew rapidly and produced maximum xylanase at a temperature of 37 °C (105). In contrast, the highest xylanase production by *B. licheniformis* A99 was observed at 50 °C (5).

4.3.2.5 Effect of carbon sources

Requirements for efficient xylanase production differed from one fungal strain to another as far as carbon source was concerned. It is pragmatic from Table 4.8 that *C. cinerea* was maximally supported to produce these enzymes on wheat bran as the sole lignocellulosic carbon substrate with the xylanase activities as 668.1 and 362.5 IU/mL respectively for the strains HK-1 and HK-2. Wheat bran and wheat straw have been

known for being ideally suitable for xylanase production in *C. disseminatus* strains SH-1 and SH-2 (164), *Penicillium citrinum and Fusarium solani* cultures (125, 64).

Just providing nutrient to the microorganisms couldn't be sufficient criteria for choosing a substrate; oxygen transfer and heat dispersion were equally important. An insight into the biochemical composition of wheat bran unveiled that it was a nutrient base that contained a considerable amount of soluble sugars: 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 (102). The cell wall polysaccharides of wheat bran contained about 40% xylans and 28% proteins, which could serve as the source of carbon and nitrogen for the microorganisms. The lignin and silica contents of wheat bran have also been reported to be quite low (149).

Moreover, it provided a large surface area and efficient aeration by remaining loose even under moist conditions during the SSF mode of culturing (7, 19). Wheat bran was hence a suitable supporter and carrier pertaining to its porosity and cheapness. Solid substrate used in SSF should be insoluble in water, since water gets absorbed onto substrate particles which could be used by the microbe for growth and metabolic activity (5). Wheat bran was described as a potent substrate and an enhancer for xylanase production in SSF by *Arthrobacter sp.*, and *Streptomyces cyaneus* SN 32 (85, 127).

Followed by wheat bran it was rice straw which supported the fungal growth and xylanase production at the second highest level, with xylanase activities of 255.64 and 113.82 IU/mL respectively for strains HK-1 and HK-2. The cellulase producing ability of the two strains however seemed to be negatively affected by rice straw. Both the strains showed a cellulase activity of 0.423 and 0.591 IU/mL on wheat bran media but a negligible activity on rice straw as 0.091 and 0.062 IU/mL respectively. This suggested that xylanase and cellulase induction were substrate-dependent and may or may not be under a common regulatory mechanism depending on the particular enzyme producer (66). Rice straw was followed sequentially by sorghum, wheat straw and bagasse for inducing the xylanase activity in strain HK-1. In HK-2, however, wheat straw had better induction potency as compared to sorghum. A combination of carbon sources with wheat bran in a ratio of 1:1 was also tested. Wheat bran and rice straw together worked out to be the best among all combinations, with xylanase activities as 370.82 and 189.50 IU/mL for

the strains HK-1 and HK-2 respectively, though, individually rice straw did not support xylanase activity to the level as it did in combination with wheat bran. A recent research established a synergy between various inducers present in rice straw and wheat bran, which resulted in high production of cellulases and hemicellulases (77). Both the strains reacted in an unexpectedly better manner under these combo carbon sources with their increasing xylanase activities being in order of wheat bran + rice straw, wheat bran + wheat straw, wheat bran + bagasse and wheat bran + sorghum. In concern with cellulase activities of the two strains, maxima were achieved with wheat bran as the sole carbon source (HK-1: 0.423 and HK-2: 0.591 IU/mL), followed by wheat straw. While going through combination sources, wheat bran and rice straw when together, gave highest cellulase activity for the fungal strain HK-1 while HK-2 produced its maximum cellulase activity with a combination of wheat bran and wheat straw. It is evident from Table 4.8 that the requirements for efficient xylanase production differed from one fungal strain to another as far as carbon source was concerned. Based on the above results, wheat bran was chosen as the lignocellulosic substrate for further optimisation studies of HK-1 and HK-2 under SSF conditions.

Due to the fact that the use of pure xylan is uneconomical for commercial xylanase production, the current impetus lies on the utilization of xylan-rich, low-cost agricultural by-products (96) to carry out the mass production of xylanases which would offer cost effective and better substrate for xylanase production (93, 61). From this point of view, various lignocelluloses, singly as well as in combinations were used. Also the two grasses under investigation in the present study- sofia and lemon grass were taken in combination with wheat bran, to find out their potential in supporting the xylanase production by the two test isolates HK-1 and HK-2. Both the grasses however seemed to suppress the fungal growth and their xylanase producing potentials. On sofia grass with wheat bran, the respective xylanase activities of the two strains were, 11.68 and 8.328 IU/mL while no activity was found with the combination of lemon grass and wheat bran. Possibly this occurred due to presence of certain antifungal compounds in the two grasses. Both grasses were aromatic, oil producing plants; sofia grass containing geranial while lemon grass having citral as main constituents of their oils respectively. A review of literature established lemon grass as a potent antibacterial (27, 129) and antifungal agent

(38, 28) based on the presence of citral, the most important component of the oil of lemon grass.

4.3.2.6 Effect of nitrogen sources

The mechanisms that preside over the mycelial growth and formation of extra cellular enzymes are influenced by the availability of precursors for protein synthesis (95). The optimal nitrogen source varied from microorganism to another and was equally important in determining optimized conditions for enzyme production (74). Using wheat bran as the carbon source, different organic nitrogen sources (at a concentration of 1g/L to NSS) were evaluated for xylanase production. From Figure 4.9 it is evident that all the tested N- sources were able to stimulate xylanase production in both the fungal strains under study.

Among the various complex organic nitrogen sources used (Table 4.9), the order of suitability for production of xylanase in the present study was: beef extract > yeast extract > urea > malt extract > peptone > soya bean meal for the *Coprinopsis cinerea* strain HK-1 and for the strain HK-2, yeast extract > peptone > urea > malt extract > beef extract > soya bean meal. Beef extract favoured maximum xylanase productivity for the strain HK-1 of *Coprinopsis cinerea.* This might be attributed to the better absorption of amino acids present in beef extract by the mycelia of the strain HK-1 (141). About 3% increase in xylanase activity was found to occur in HK-1 by using beef extract (690.71 IU/mL) as the nitrogen source in place of yeast extract (672.50 IU/mL). The strain HK-2 attained maximal cellulase activity as 0.761 IU/mL using beef extract whereas, for strain HK-1, cellulase maxima (0.797 IU/mL) was attained by using soyabean meal as the nitrogen source, which was at hike of 33.5% as compared to its activity on beef extract (0.530 IU/mL). These results indicated that quality of proteins for enzyme production varied from species to species (157). Peptone was the best nitrogen source with white-rot basidiomycetes, to achieve optimal xylanase activity from *Pleurotus ostreatus* (141) and *Trichoderma harzianum* (1), under SmF conditions and *Lentinus edodes* IBB 363 (81) under SSF. Soya bean meal as a source of nitrogen was mentioned for yet another white-rot *Coprinellus disseminatus* SW-1 NTCC1165 (2) under SSF, however, in the present study, xylanase activity obtained with beef extract (690.71IU/mL) increased by 16% for

strain HK-1 in comparison to soya bean meal (583.34IU/mL) whereas, for HK-2 the activity was nearly 22% better with yeast extract (364.28 IU/mL) which was quite significant when compared to soya bean meal (286 IU/mL). Similar results were shown for *Aspergillus* sp. RSP-6, in which, soya bean meal and beef extract were found to be the least and best preferred sources for xylanase production, respectively (171). These results were also in agreement with those reported in the literature where fungi were found to produce higher xylanase activities on organic nitrogen sources (140, 10).

4.3.2.7 Effect of moisture content

Table 4.10 shows the effect of moisture content on xylanase production by *C. cinerea* strains HK-1 and HK-2. The moisture level is a crucial factor in SSF that determines the success of the process. The optimal xylanase titers for strain HK-1 (695.8 IU/mL) and HK-2 (360.28 IU/mL) were obtained at a substrate to moisture (NSS) ratio of 1:3 (Figure 4.10). Cellulase activity for both of the fungal strains too was maximum i.e. 0.541 IU/mL for HK-1 and 0.596 IU/mL for HK-2 at solid substrate to moisture content ratio of 1:3 and decreased further on increasing substrate to moisture content ratio. By decreasing the ratio to 1:2, nearly half of the production could be obtained for HK-1 while HK-2 retained 59.1% of the maximal enzyme activity. Nearly, 45 and 51% of the maximal titers were retained by strain HK-1 at a ratio of 1:4. Increase in the moisture content was thereby found to be more detrimental as compared to the decrease. The free excess liquid presented an additional diffusion barrier together with that imposed by the solid nature of the substrate and led to a decrease in growth and enzyme production (124).

The importance of moisture level in SSF media and its influence on microbial growth and product biosynthesis might be attributed to the impact of moisture on the physical properties of solid substrate. A higher than optimum moisture level caused decrease in porosity, lower oxygen transfer, gummy texture and alteration in wheat bran particle structure whereas a lower than optimum moisture level caused reduction in solubility of nutrients in the solid substrate and decreased swelling of solid substrate (49, 142). In SSF using wheat bran and eucalyptus kraft pulp as the primary solid substrates, *Streptomyces sp.* QG-11-3 (14) produced maximum xylanase yield at substrate-to-moisture ratio of 1:2.5 and 1:3, respectively. Singh et al., obtained maximum xylanase

production and supernatant protein concentration for strains SH-1 and SH-2 of a white rot fungus *C. disseminatus* at substrate to moisture (N.S.S.) ratio of 1: 3 on 7th day of incubation under SSF using wheat bran (164).

Based on above discussions, the various optimized physico-chemical parameters for crude xylanase production by the two *Coprinopsis* strains were inferred as: incubation period of 7 days for HK-1 and 8 days for HK-2; incubation temperature-37 °C; pH-6.4; solid substrate (wheat bran) to moisture (NSS) ratio-1:3; carbon source-wheat bran; and nitrogen source-beef extract for strain HK-1 and yeast extract for HK-2 (Table 4.11). Table 4.12 shows the enzyme production by both of the fungal strains under optimized conditions. The xylanase activities of HK-1 and HK-2 were 693.6 and 368.5 IU/mL while cellulase activities 0.550 and 0.615 IU/mL respectively. The laccase activity was 26.5 IU/mL for HK-1 and 14.8 IU/mL for HK-2 while the respective protein biomass was 4.8 and 4.4 mg/mL.

4.3.2.8 Effect of glucose and lactose on xylanase production

The production of xylanase by *C. cinerea* HK-1 and HK-2 were found to be repressed by increasing concentrations of both glucose and lactose from 1 to 5 g/L; the maximum activity being attained in the absence of these simple sugars (Figure 4.11 a,b). The production of extra cellular xylanase by both strains was thereby found to be inducible and controlled by catabolite repression. Glucose in fermentation medium generally repressed enzyme production by moulds and only after exhaustion of glucose, the fungus started the production of xylanase. Such xylanase repression seemed to be an indicative of the fact that enzyme synthesis was controlled by transition state regulators and catabolite repression, as was also observed by Sanghi et al. (150) in *Bacillus subtilis* ASH, using wheat bran under SSF at temperature 37 °C and pH 7.0 and Smith and Wood (165) in *Aspergillus awamori* using ball-milled oat straw under SmF at temperature 30 °C and pH 4.0. On contrary to this, resistance to catabolite repression by glucose had been cited from a mutant strain, M7 of *Thermomyces lanuginosus* (26).

4.3.3 Biochemical characterization of xylanase

4.3.3.1 Effect of pH on the activity and stability of xylanase

The tolerance to alkaline condition is crucial, while considering the potentiality of xylanases in bleaching of cellulose pulps at high pH. Table 4.15 shows the effect of pH on

the xylanase activity of *C. cinerea* HK-1 and HK-2 at temperature 55 °C for 15 min of reaction time. Figure 4.12 infers that the crude xylanase produced by fungal strains HK-1 and HK-2 were active in the pH range of 6.0 to 7.2 with the maximum xylanase activity obtained at pH 6.4. At pH 6.0, 92 and 85% of the respective xylanase activities were maintained by strains HK-1 and HK-2 in comparison to xylanase activity obtained at optimum pH of 6.4. At pH 8, 50 and 35% of the maximum activity were retained by HK-1 and HK-2 respectively, thereby proving their alkali-tolerant nature. Strain HK-1 xylanase hence possessed better pH stability than HK-2. Enzymes, proteinaceous entities are liable to denaturation under harsh conditions posed by pH change, high temperature, or presence of high concentration of metal ions, with adverse effects on their active sites resulting in a subsequent loss of enzyme activity. The pH activity profiles of enzymes also depend on pKa (ionization constant) of catalytic residues (80). Lower the pKa value, higher is the pH stability. Amino acid residues contributing positive charges and hydrogen bonds lower the pKa values with shorter bonds having a more definite effect (35). The optimal pH range for some strains of *T. lanuginosus* was found to be 6.0–7.0 (163). The activity of extracellular xylanase from *C. disseminatus* SW-1 NTCC1165 was found within a pH range of 6.0-9.0 with optima at pH 6.4 (2). 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 (139). Ninawe et al. (128) worked with purified xylanase from *Streptomyces cyaneus* SN at a pH 6.0.

4.3.3.2 Effect of temperature on the activity and stability of xylanase

The thermal stability of the crude xylanase from *C. cinerea* HK-1 and HK-2 was examined under a temperature range of 45-85 °C at pH 6.4 for 15 min, and the results (Figure 4.13) were indicative of good thermal-tolerance; the temperature of 55 °C conferred to be optimal for the crude xylanase activity of both the strains (HK-1: 691.86 IU/mL and HK-2: 367.27 IU/mL) and beyond that xylanase activity decreased. At temperature 65 °C, xylanase produced from strains HK-1 and HK-2 retained 78 and 31% of their optimum xylanase activities. On being assayed at further temperature elevation (75 °C); the xylanase from strain HK-2 lost most of its xylanase activity, retaining just 17% of the total; while xylanase from HK-1 yet retained 46% of the maximal activity. At

85 °C, strain HK-2 xylanase couldn't manage to hold an activity more than 4.5% while HK-1xylanase could retain 23% of optimum xylanase activity. HK-1 hence stood more thermo-tolerant in comparison to HK-2 on the basis of its resistance to temperature elevations. Same as the xylanase in this study, most of the other known xylanases were also optimally active in the range of 50–65 °C (95).

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 (152). The optimal temperature for the crude xylanase activity of both the strains was 55 °C, which substantiates with the finding that the optimum temperature for the xylanase produced by most of the fungi is in the range of 40-60 °C (95). 55 °C was considered as the optimum temperature for xylanase activity of *Aspergillus nidulans* KK-99 (174) while xylanase from *Aspergillus fumigatus* AR1 exhibited maximum xylanase activity at temperature 60-65 °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 °C) conditions. *C. cinerea* HK-1 hence surpassed HK-2 by holding characteristic features of alkali-thermo-tolerance and negligible cellulase contamination (0.55 IU/mL) associated with the crude enzyme which empowered its direct use in pulp and paper industry without any further purification step, making the entire process economical. Thus, it was selected for further biobleaching experiments. The mass production of crude xylanase by HK-1 under optimized conditions is summarized in Table 4.17. HK-1 exhibited xylanase activity 721.28 IU/mL, laccase activity 22.86 U/mL and supernatant protein concentration 5.31 mg/mL which explains that crude enzyme obtained from strain HK-1was concentrated solution of a variety of proteins. The associated cellulase contamination in the crude enzyme extract was determined as 0.576 IU/mL. Although an overestimation of soluble proteins in crude extracts because of interferences with other compounds was possible, its profile agreed with increase in enzyme.

Table 4.1: Morphological analysis and screening of fungal isolates for xylanase

SI No.	Isolates	Sources	Appearance of mycelia	Spore colour	Xylanase activity
1	A	Fruiting Body	White, lateral	No spore formation	++
2	B	Dead and decaying wood	White	Light brown	++++
3	C	Mango tree bark	Off white	No spore formation	++
4	D	Dead and decaying wood	Cottony white aerial, in rings	Black	++++
5	E	Decomposing manure	White	Black	+++
6	F	Decomposing manure	Dull white	Black	++
7	G	Paper industry waste	White, aerial	Brown	+++
8	H	Paper industry waste	Black	Black	+
9	I	Paper industry waste	Yellowish white	No spore formation	+
10	J	Dead and decaying wood	White	Brown	+++
11	K	Dead and decaying wood	Green	Green	+
12	L	Dead and decaying wood	Dense white, irregular	No spore formation	+++
13	M	Sugarcane dumping site	White, lateral	No spore formation	++
14	N	Sugarcane dumping site	Green	Green	+

\+ Very poor activity
++ Average activity
+++ Good activity
++++ Very good activity

Table 4.2: Screening of isolated potent xylanase producers under SSF conditions

Sl. No.	Screened Isolates	Fungal strains	Xylanase activity, IU/mL	Cellulase activity, IU/mL	Protein concentration, mg/mL
1	B	HK-1	456.12±12.14	0.744±0.08	3.565±0.15
2	D	HK-2	164.22±8.01	0.815±0.13	2.288±0.40
3	E	HK-3	131.42±9.33	2.12±0.18	1.892±0.24
4	G	HK-4	59.96±4.40	2.62±0.09	1.090±0.20
5	J	HK-5	80.14±4.18	0.940±0.18	1.890±0.40
6	L	HK-6	132.07±6.87	0.700±0.25	1.600±0.08
7	A	HK-7	53.94±4.14	1.324±0.31	0.940±0.05
8	C	HK-8	11.20±3.02	2.960±0.09	0.382±0.03
9	F	HK-9	61.55±4.19	0.141±0.12	1.324±0.21
10	M	HK-10	9.10±0.40	0.185±0.01	0.235±0.23

Fermentation conditions:

Wheat bran, g	= 5
Nutrient salt solution, mL	= 15
pH	= 6.0
Temperature, °C	= 32
Incubation period, days	= 6

Assay conditions:

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

± Standard deviation from the mean

Table 4.3: Comparison of solid-state and submerged fermentation conditions for xylanase production by *Coprinopsis cinerea* strains HK-1 and HK-2

SI. No.	Fermentation condition	*Coprinopsis cinerea* strains			
		HK-1		HK-2	
		Xylanase activity, IU/mL	Cellulase activity, IU/mL	Xylanase activity, IU/mL	Cellulase activity, IU/mL
1.	Submerged fermentation	214.1 ±7.3	0.481±0.14	97.40±8.6	0.345±0.04
2.	Solid-state fermentation	462.25±10.41	0.743±0.05	156.92±8.3	0.783±0.09

Fermentation conditions:

Submerged fermentation		**Solid-state fermentation**	
Wheat bran, g	= 0.8	Wheat bran, g	= 5
Nutrient salt solution, mL	= 40	Nutrient salt solution, mL	= 15
pH	= 6.0	pH	= 6.0
Temperature, °C	= 32	Temperature, °C	= 32
Incubation period, days	= 6	Incubation period, days	= 6

± Standard deviation from the mean

Table 4.4: Effect of incubation period on xylanase production by *Coprinopsis cinerea* HK-1 and HK-2

SI. No	Day	*Coprinopsis cinerea* strain HK-1		
		Xylanase activity, IU/mL	Cellulase activity, IU/mL	Protein concentration, mg/mL
1	2	8.4±2.1	0.063±0.002	0.08±0.004
2	3	27.8±2.5	0.187±0.012	0.40±0.042
3	4	82.41±5.7	0.354±0.025	0.85±0.050
4	5	196.82±8.4	0.492±0.010	1.45±0.075
5	6	454.5±12.3	0.690±0.030	2.64±0.140
6	7	537.83±10.3	0.640±0.020	3.60±0.200
7	8	491.4±9.2	0.565±0.013	3.88±0.300
8	9	466.8±9.8	0.423±0.010	3.92±0.150
9	10	415.2±10.5	0.368±0.012	3.81±0.180
10	11	384.4±12.7	0.311±0.035	3.75±0.100

SI.No	Day	*Coprinopsis cinerea* strain HK-2		
		Xylanase activity, IU/mL	Cellulase activity, IU/mL	Protein concentration, mg/mL
1	2	7.29±1.30	0.091±0.005	0.131±0.02
2	3	20.53±2.20	0.224±0.020	0.380±0.07
3	4	42.19±3.12	0.400±0.018	0.791±0.06
4	5	80.35±2.50	0.682±0.040	1.532±0.19
5	6	168.49±6.31	0.795±0.032	2.174±0.21
6	7	186.70±4.89	0.965±0.075	2.383±0.20
7	8	230.56±5.02	0.880±0.032	3.102±0.34
8	9	213.97±6.08	0.734±0.050	2.862±0.35
9	10	178.60±4.14	0.526±0.032	2.230±0.13
10	11	128.45±7.04	0.455±0.017	1.785±0.09

Fermentation conditions:

Wheat bran, g	=	5
Nutrient salt solution, mL	=	15
pH	=	6.0
Temperature, °C	=	32

Assay conditions:

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

± Standard deviation from the mean

Table 4.5: Effect of initial pH on xylanase production by *Coprinopsis cinerea* HK-1 and HK-2

Sl. No.	pH	*Coprinopsis cinerea* strain HK-1		
		Xylanase activity, IU/mL	Cellulase activity, IU/mL	Protein concentration, mg/mL
1	4.4	186.8±7.3	0.923±0.08	3.13±0.06
2	5.0	292.53±11.2	0.850±0.08	3.30±0.11
3	5.4	415.01±16.4	0.720±0.04	3.57±0.08
4	6.0	535.63±9.5	0.643±0.02	3.65±0.19
5	6.4	578.27±12.2	0.625±0.06	3.96±0.25
6	7.0	541.5±10.1	0.605±0.04	3.85±0.24
7	7.4	513.70±9.7	0.554± 0.04	3.72±0.18
8	8.0	403.01±13.7	0.512±0.03	3.38±0.16
9	8.4	387.20±8.3	0.480±0.01	2.91±0.13
10	9.0	345.63±9.5	0.442±0.03	2.70±0.10
11	9.4	235.80±12.6	0.311±0.01	2.59±0.09
12	10.0	212.0±7.6	0.265±0.015	1.42±0.12
13	10.4	191.21±11.03	0.088±0.005	1.16±0.08
14	11.0	103.77±5.41	0.053±0.001	0.97±0.04

Sl. No.	pH	*Coprinopsis cinerea* strain HK-2		
		Xylanase activity, IU/mL	Cellulase activity, IU/mL	Protein concentration, mg/mL
1	4.4	132±8.3	1.092±0.08	2.54±0.10
2	5.0	168.6±11.2	1.013±0.05	2.72±0.05
3	5.4	176.5±9.3	0.922±0.08	3.02±0.08
4	6.0	227.60±6.8	0.855±0.04	3.11±0.15
5	6.4	315.50±9.5	0.694±0.05	3.26±0.20
6	7.0	276.93±13.0	0.610±0.04	3.07±0.22
7	7.4	245.62±7.2	0.562±0.06	2.86±0.18
8	8.0	256.46±12.1	0.55±0.03	2.63±0.24
9	8.4	220.50±8.8	0.48±0.02	2.41±0.11
10	9.0	180.7±10.4	0.436±0.04	2.34±0.17
11	9.4	211.5±7.2	0.382±0.04	2.30±0.20
12	10.0	195.7±9.7	0.332±0.02	2.02±0.12
13	10.4	168.7±5.3	0.210±0.02	1.84±0.10
14	11.0	142.84±8.9	0.008±0.002	1.56±0.06

Fermentation conditions:

Wheat bran, g	=	5
Nutrient salt solution, mL	=	15
Incubation period, min	=	15
Temperature, °C	=	32
Incubation period (HK-1), days	=	7
Incubation period (HK-2), days	=	8

Assay conditions:

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

± Standard deviation from the mean

Table 4.6: Effect of Incubation temperature on xylanase production by *Coprinopsis cinerea* HK-1 and HK-2

Sl. No.	Temperature, °C	*Coprinopsis cinerea* strain HK-1		
		Xylanase activity, IU/mL	Cellulase activity, IU/mL	Protein concentration, mg/mL
1	27	25.78±3.50	0.391±0.020	0.19±0.03
2	32	573.82±11.09	0.610±0.080	3.93±0.17
3	37	668.45±12.08	0.432±0.040	4.41±0.20
4	42	342.10±10.15	0.250±0.020	2.45±0.04
5	47	205.50±7.62	0.156±0.018	1.63±0.07
6	52	34.80±6.55	0.063±0.003	0.30±0.04

Sl. No.	Temperature, °C	*Coprinopsis cinerea* strain HK-2		
		Xylanase activity, IU/mL	Cellulase activity, IU/mL	Protein concentration, mg/mL
1	27	18.50±3.60	0.50±0.043	0.23±0.04
2	32	318.75±10.80	0.665±0.072	3.31±0.10
3	37	356.42±9.70	0.597±0.040	3.87±0.18
4	42	135.21±8.91	0.236±0.035	1.78±0.07
5	47	85.60±4.12	0.045±0.002	1.20±0.04
6	52	No growth	-	-

Fermentation conditions:
Wheat bran, g = 5
Nutrient salt solution, mL = 15
pH = 6.4
Incubation period, days = 7 (HK-1), 8 (HK-2)

Assay conditions:
Temperature, °C = 55
Incubation period, min = 15
pH = 6.4
± Standard deviation from the mean

Table 4.7: Effect of various lignocellulosic carbon sources on xylanase production by *Coprinopsis cinerea* HK-1 and HK-2

Sl. No.	Carbon sources	*Coprinopsis cinerea* strain HK-1		
		Xylanase activity, IU/mL	Cellulase activity, IU/mL	Protein concentration, mg/mL
1	Rice straw (RS)	255.64±5.01	0.091±0.004	1.94±0.11
2	Wheat bran (WB)	668.1±13.08	0.423±0.030	4.42±0.27
3	Wheat-straw (WS)	30.65±2.51	0.250±0.020	0.26±0.05
4	Sorghum (S)	39.68±4.30	0.165±0.003	0.47±0.02
5	Bagasse (B)	22.71±3.94	0.086±0.001	0.21±0.03
6	Wheat bran + Rice straw	370.82±12.1	0.624±0.050	2.86±0.15
7	Wheat bran + Wheat straw	190.72±4.98	0.471±0.020	1.33±0.10
8	Wheat bran + Sorghum	114.50±8.72	0.330±0.010	0.92±0.08
9	Wheat bran + Bagasse	143.56±11.0	0.540±0.030	1.24±0.09
10	Wheat bran + Sofia grass (SF)	11.68±2.1	0.155±0.005	0.14±0.02
11	Wheat bran +Lemongrass (LG)	No growth	-	-

SI. No.	Carbon sources	*Coprinopsis cinerea* strain HK-2		
		Xylanase activity, IU/mL	Cellulase activity, IU/mL	Protein concentration, mg/mL
1	Rice straw (RS)	113.82±2.1	0.062±0.002	1.35±0.14
2	Wheat bran (WB)	362.50±10.2	0.591±0.050	3.96±0.22
3	Wheat-straw (WS)	27.76±7.3	0.333±0.020	0.38±0.03
4	Sorghum (S)	25.54±1.86	0.109±0.001	0.31±0.02
5	Bagasse (B)	12.04±2.1	0.140±0.006	0.22±0.02
6	Wheat bran + Rice straw	189.50±14.3	0.438±0.030	2.65±0.08
7	Wheat bran +Wheat straw	145.70±9.2	0.592±0.040	2.03±0.15
8	Wheat bran +Sorghum	78.56±6.4	0.386±0.021	1.34±0.10
9	Wheat bran + Bagasse	77.63±4.4	0.341±0.060	1.10±0.08
10	Wheat bran + Sofia grass (SF)	8.328±1.3	0.070±0.001	0.06±0.03
11	Wheat bran +Lemongrass (LG)	No growth	-	-

Fermentation conditions:
Nutrient salt solution, mL = 15
pH = 6.4
Temperature, °C = 37
Incubation period, days = 7 (HK-1), 8 (HK-2)

Assay conditions:
Temperature, °C = 55
Incubation period, min = 15
pH = 6.4
± Standard deviation from the mean

Table 4.8: Effect of various nitrogen sources on xylanase production by *Coprinopsis cinerea* HK-1 and HK-2

SI. No.	Nitrogen sources	*Coprinopsis cinerea* strain HK-1		
		Xylanase activity, IU/mL	Cellulase activity, IU/mL	Protein concentration, mg/mL
1	Peptone	589.32±15.5	0.506±0.030	3.98±0.11
2	Beef extract	690.71±12.5	0.530±0.020	4.70±0.24
3	Yeast extract	672.50±10.5	0.400±0.032	4.45±0.22
4	Malt extract	634.68±18.8	0.682±0.025	4.14±0.15
5	Soyabean meal	583.34±20.4	0.797±0.050	4.05±0.20
6	Urea	668.10±11.3	0.723±0.041	4.25±0.12
SI. No.	Nitrogen sources	*Coprinopsis cinerea* strain HK-2		
		Xylanase activity, IU/mL	Cellulase activity, IU/mL	Protein concentration, mg/mL
1	Peptone	341.12±10.2	0.543±0.020	3.87±0.17
2	Beef extract	304.40±8.90	0.761±0.011	3.12±0.18
3	Yeast extract	364.28±14.5	0.587±0.020	4.05±0.10
4	Malt extract	318.61±12.0	0.680±0.040	3.24±0.23
5	Soyabean meal	286.0±9.70	0.612±0.020	3.83±0.08
6	Urea	331.72±13.3	0.560±0.030	3.75±0.11

Fermentation conditions:
Wheat bran, g = 5
Nutrient salt solution, mL = 15
pH = 6.4
Temperature, °C = 37
Incubation period days = 7 (HK-1), 8 (HK-2)

Assay conditions:
Temperature, °C = 55
Incubation period, min = 15
pH = 6.4
± Standard deviation from the mean

Table 4.9: Effect of moisture level on xylanase production by *Coprinopsis cinerea* HK-1 and HK-2

Sl. No.	Solid substrate : moisture content	*Coprinopsis cinerea* strain HK-1		
		Xylanase activity, IU/mL	Cellulase activity, IU/mL	Protein concentration, mg/mL
1	1:2.0	351±11.5	0.402±0.015	2.40±0.15
2	1:2.5	525.01±14.1	0.515±0.039	3.26±0.16
3	1:3.0	695.8±13.8	0.541±0.021	4.74±0.24
4	1:3.5	486±10.4	0.492±0.010	3.11±0.07
5	1:4.0	311.2±9.5	0.410±0.020	2.28±0.11

Sl. No.	Solid substrate : moisture content	*Coprinopsis cinerea* strain HK-2		
		Xylanase activity, IU/mL	Cellulase activity, IU/mL	Protein concentration, mg/mL
1	1:2.0	212.75±8.9	0.450±0.020	2.06±0.15
2	1:2.5	281.50±8.4	0.470±0.030	3.54±0.12
3	1:3.0	360.28±14.5	0.596±0.029	4.08±0.10
4	1:3.5	246.7±10.11	0.580±0.020	2.98±0.17
5	1:4.0	185.21±7.6	0.435±0.032	2.04±0.20

Fermentation conditions:

Wheat bran, g = 5
Nutrient salt solution, mL = 15
pH = 6.4
Temperature, °C = 37
Incubation period, days = 7 (HK-1), 8 (HK-2)

Assay conditions:

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

± Standard deviation from the mean

Table 4.10: Various physico-chemical parameters derived for production of xylanase by *Coprinopsis cinerea* HK-1 and HK-2

Sl. No	Parameters	*Coprinopsis cinerea* strains	
		HK-1	HK-2
1	Incubation period, days	7	8
2	Incubation temperature, °C	37	37
3	Lignocellulosic carbon source	Wheat bran	Wheat bran
4	Nitrogen source	Beef extract	Yeast extract
5	pH	6.4	6.4
6	Moisture content	1:3	1:3

Table 4.11: Extracellular production of various enzymes under above listed conditions by *Coprinopsis cinerea* HK-1 and HK-2

Fungal strain	Xylanase activity, IU/mL	Cellulase activity, IU/mL	Laccase activity, IU/mL	Protein concentration, mg/mL
HK-1	693.6±12.2	0.550±0.020	26.5±3.8	4.8±1.60
HK-2	368.5±8.10	0.615±0.030	14.8±4.2	4.1±0.75

± Standard deviation from the mean

Table 4.12: Effect of glucose on xylanase production by *Coprinopsis cinerea* HK-1 and HK-2

Sl. No.	Glucose concentration, g/L	*Coprinopsis cinerea* strains	
		HK-1	HK-2
		Xylanase activity, IU/mL	Xylanase activity, IU/mL
1	0	694.21±14.8	368.28±11.61
2	1	525.66±10.41	243.09±9.50
3	2	470.21±7.45	198.77±7.10
4	3	458.20±8.12	164.62±3.65
5	4	414.06±7.90	158.30±4.28
6	5	336.50±5.88	113.73±3.80

Fermentation conditions:
Wheat bran, g = 5
Nutrient salt solution, mL = 15
pH = 6.4
Temperature, °C = 37
Incubation period days = 7 (HK-1), 8 (HK-2)

Assay conditions:
Temperature, °C = 55
Incubation period, min = 15
pH = 6.4

± Standard deviation from the mean

Table 4.13: Effect of lactose on xylanase production by *Coprinopsis cinerea* HK-1 and HK-2

Sl. No.	Lactose concentration, g/L	*Coprinopsis cinerea* strains	
		HK-1	HK-2
		Xylanase activity, IU/mL	Xylanase activity, IU/mL
1	0	692.21±11.11	365.28±8.61
2	1	616.30±9.09	283.57±6.82
3	2	537.51±13.40	231.77±10.10
4	3	510.27±9.14	212.90±12.3
5	4	448.0±7.30	178.01±5.81
6	5	371.80±6.12	162.33±4.19

Fermentation conditions:
Wheat bran, g = 5
Nutrient salt solution, mL = 15
pH = 6.4
Temperature, °C = 37
Incubation period days = 7 (HK-1), 8 (HK-2)

Assay conditions:
Temperature, °C = 55
Incubation period, min = 15
pH = 6.4

± Standard deviation from the mean

Table 4.14: pH stabilization of crude xylanase produced by *Coprinopsis cinerea* HK-1 and HK-2

Sl. No	Buffer pH	*Coprinopsis cinerea* strains			
		HK-1		HK-2	
		Xylanase activity, IU/mL	Relative xylanase activity, %	Xylanase activity, IU/mL	Relative xylanase activity, %
1	6.0	642.16±9.0	92	316.39±6.23	85
2	6.4	698.21±12.50	100	370.74±7.65	100
3	6.8	558.49±7.49	80	273.80±8.16	74
4	7.2	530.48±8.60	76	208.25±4.28	56
5	7.6	439.74±11.12	63	177.76±4.61	48
6	8.0	349.00±10.06	50	129.50±6.08	35
7	8.4	251.28±9.50	36	99.90±3.10	27
8	8.7	153.56±3.20	22	27.39±4.33	7
9	9.0	114.66±4.33	16	10.43±3.93	3

± Standard deviation from the mean
Assay conditions:
Temperature of incubation, °C : 55
Substrate concentration : 10 mg xylan/mL potassium phosphate buffer

Table 4.15: Temperature stabilization of crude xylanase produced by *Coprinopsis cinerea* HK-1 and HK-2

Sl. No.	Temperature, °C	*Coprinopsis cinerea* strains			
		HK-1		HK-2	
		Xylanase activity, IU/mL	Relative xylanase activity, %	Xylanase activity, IU/mL	Relative xylanase activity, %
1	45	594.83±8.11	86	302.05±7.04	82
2	55	691.86±13.72	100	367.27±8.86	100
3	65	538.98±9.80	78	152.67±4.28	31
4	75	319.70±11.40	46	62.39±5.02	17
5	85	159.16±7.43	23	16.40±2.60	4.5

Table 4.16: Mass production of extracellular enzymes by *Coprinopsis cinerea* HK-1

Fungal strain	Xylanase activity, IU/mL	Cellulase activity, IU/mL	Laccase activity, IU/mL	Protein concentration, mg/mL
HK-1	721.28±13.91	0.576±9.038	22.86±5.04	5.31±2.16

± Standard deviation from the mean

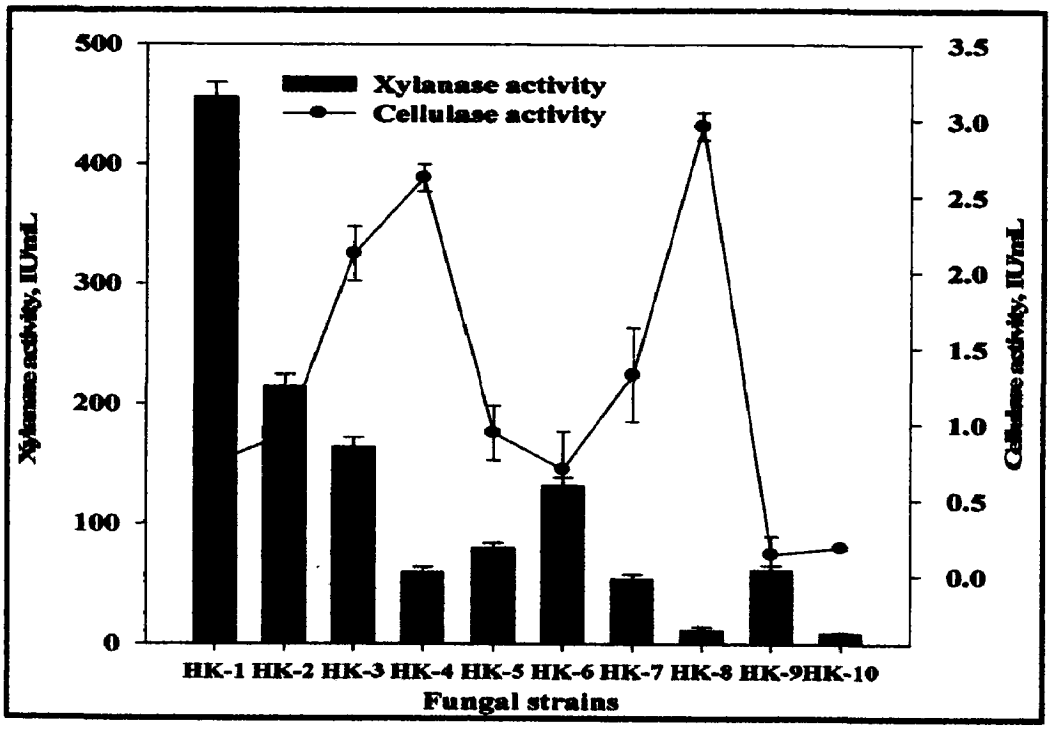


Figure 4.2: Enzyme production from the fungal isolates under SSF conditions

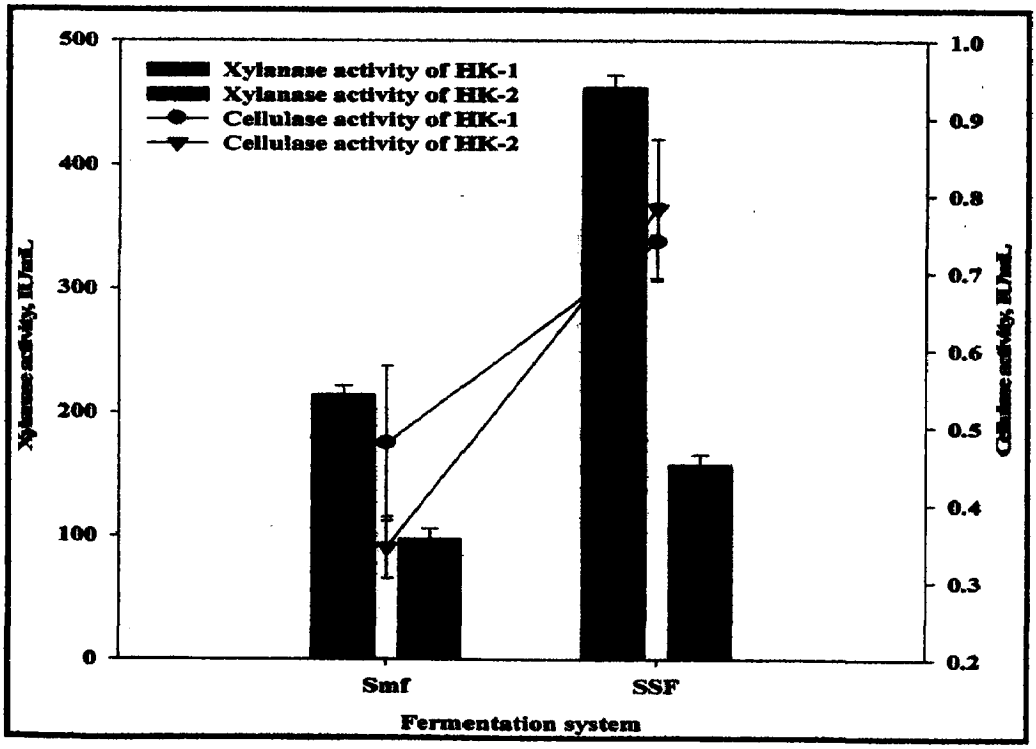


Figure 4.3: Comparison of fermentation systems for the enzyme production by *Coprinopsis cinerea* strain HK-1 and HK-2

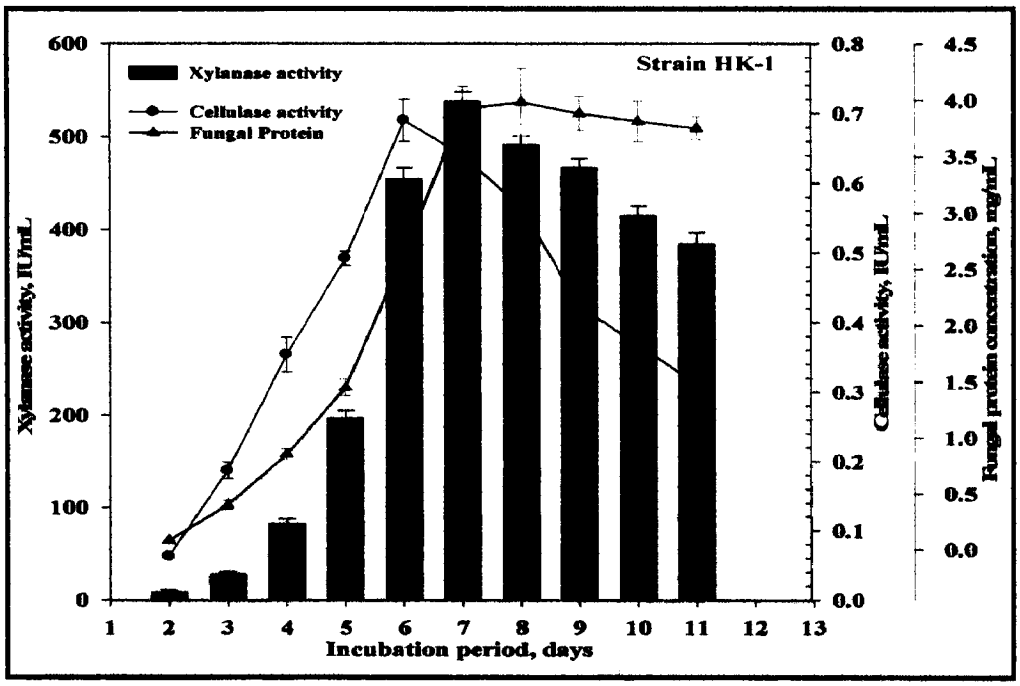


Figure 4.4: Effect of incubation period on xylanase and cellulase production by *Coprinopsis cinerea* strain HK-1 and associated fungal protein concentration

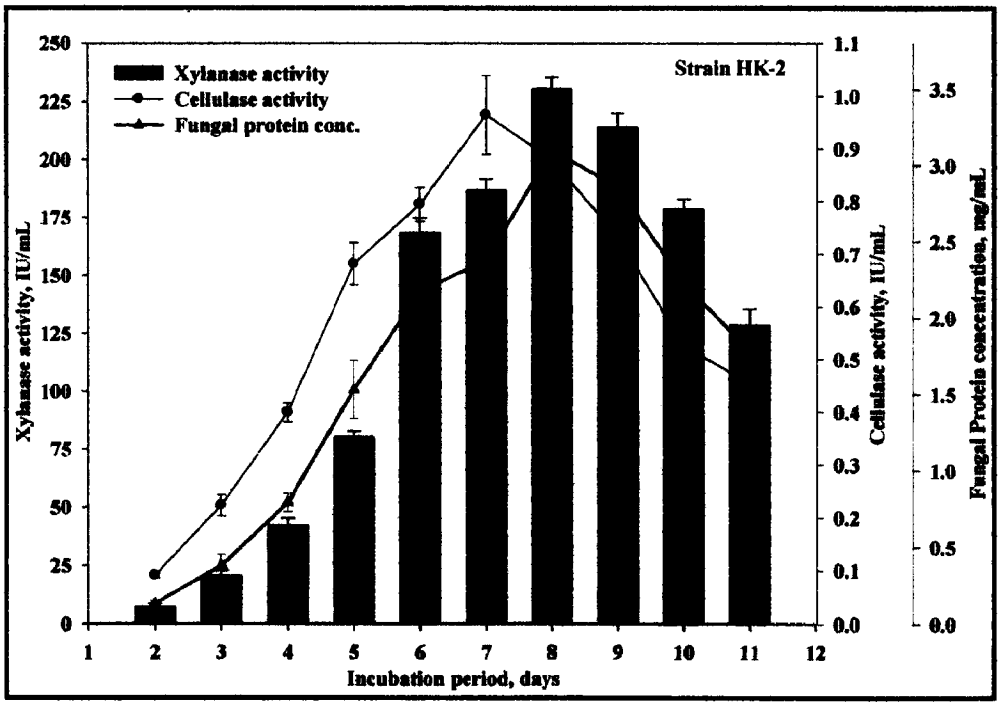


Figure 4.5: Effect of incubation period on xylanase and cellulase production by *Coprinopsis cinerea* strain HK-2 and associated fungal protein concentration

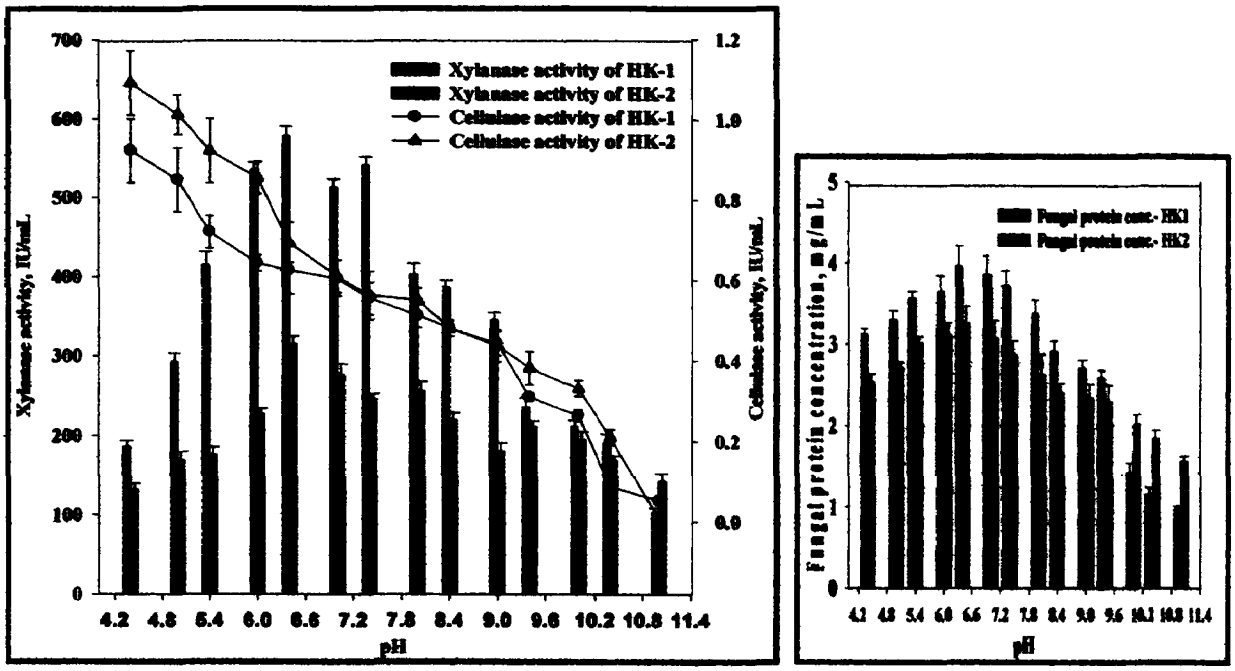


Figure 4.6: Effect of initial pH on xylanase and cellulase production by *Coprinopsis cinerea* strains HK-1 and HK-2 and associated fungal protein concentrations

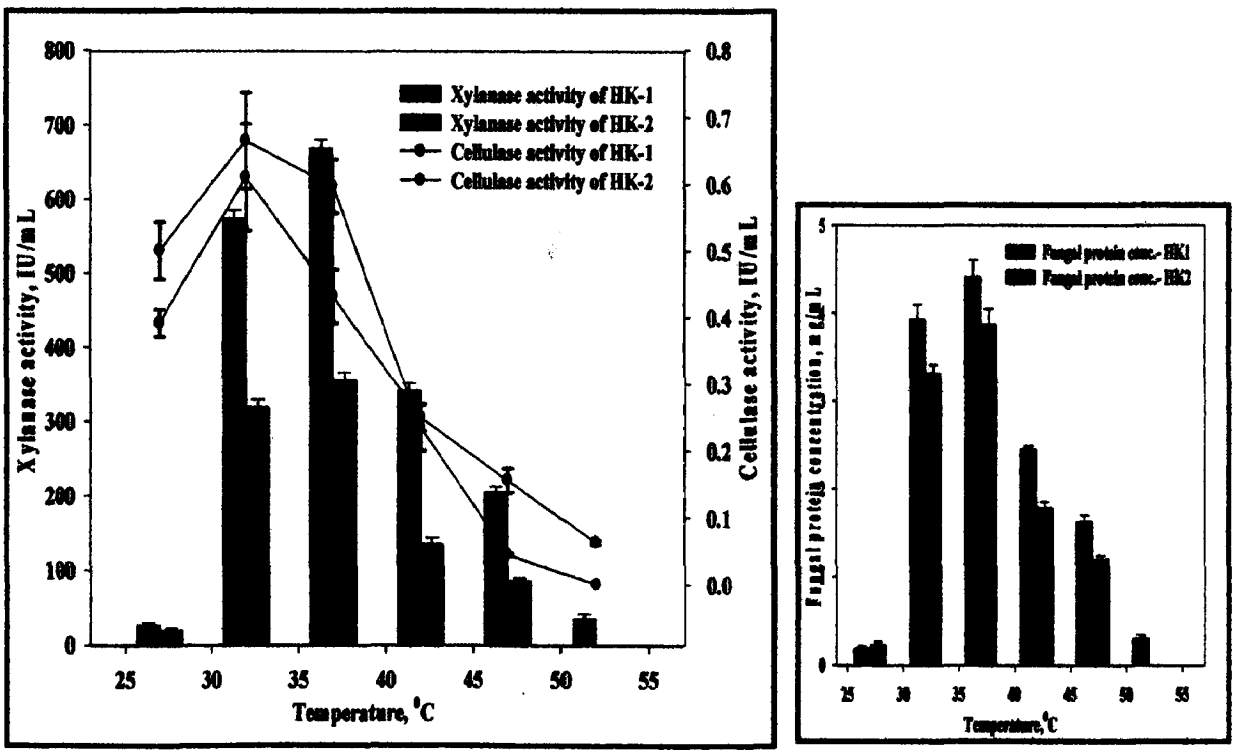


Figure 4.7: Effect of incubation temperature on xylanase and cellulase production by *Coprinopsis cinerea* strains HK-1 and HK-2 and associated fungal protein concentrations

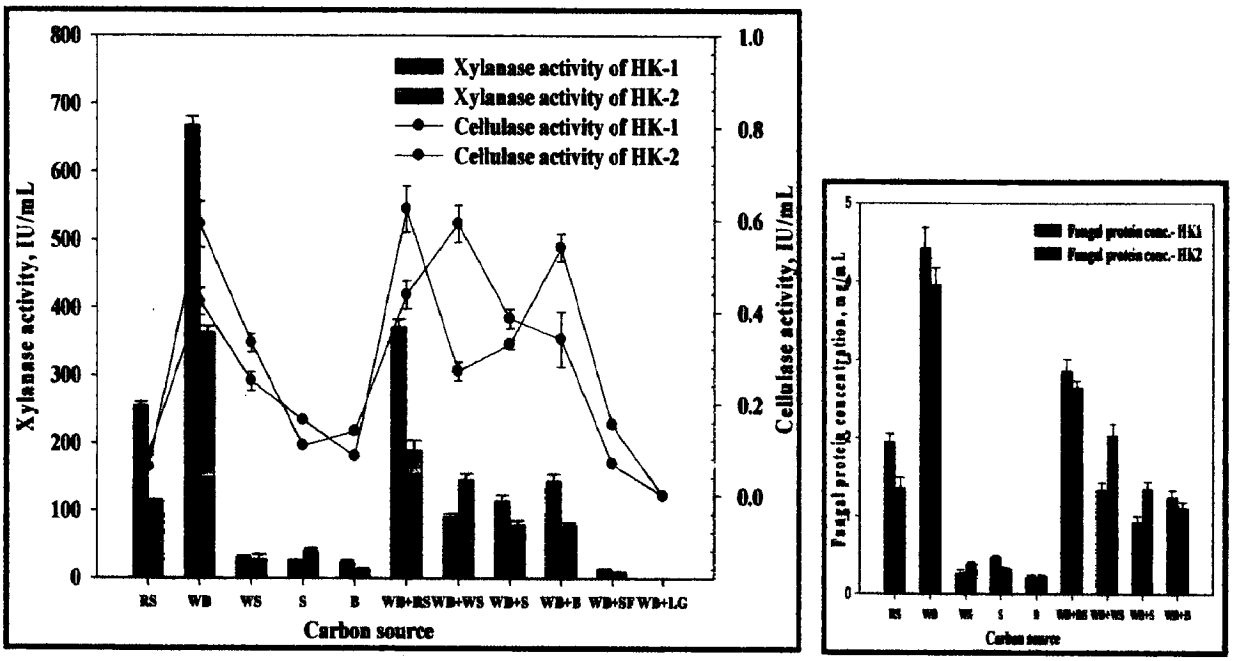


Figure 4.8: Effect of lignocellulosic carbon source on xylanase and cellulase production by *Coprinopsis cinerea* strains HK-1 and HK-2 and associated fungal protein concentrations (RS- Rice straw, WB- Wheat bran, WS- Wheat straw, S- Sorghum, B- Bagasse, SG- Sofia grass, LG- Lemon grass)

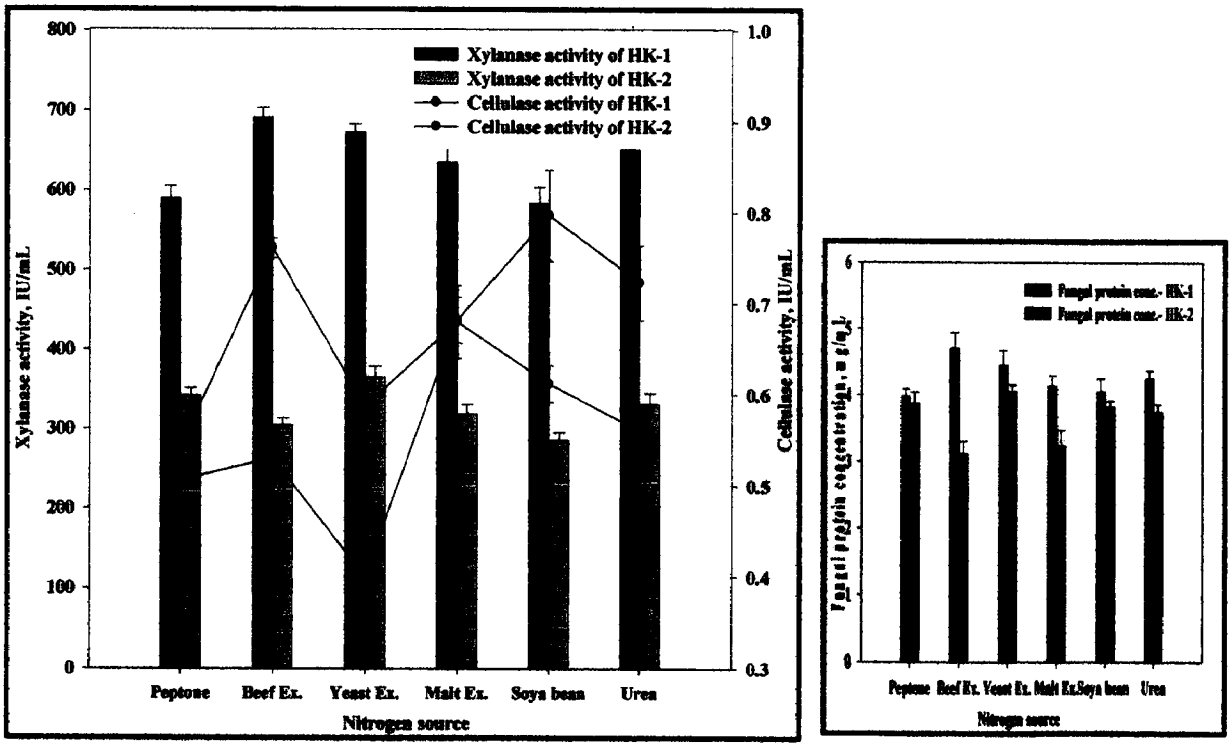
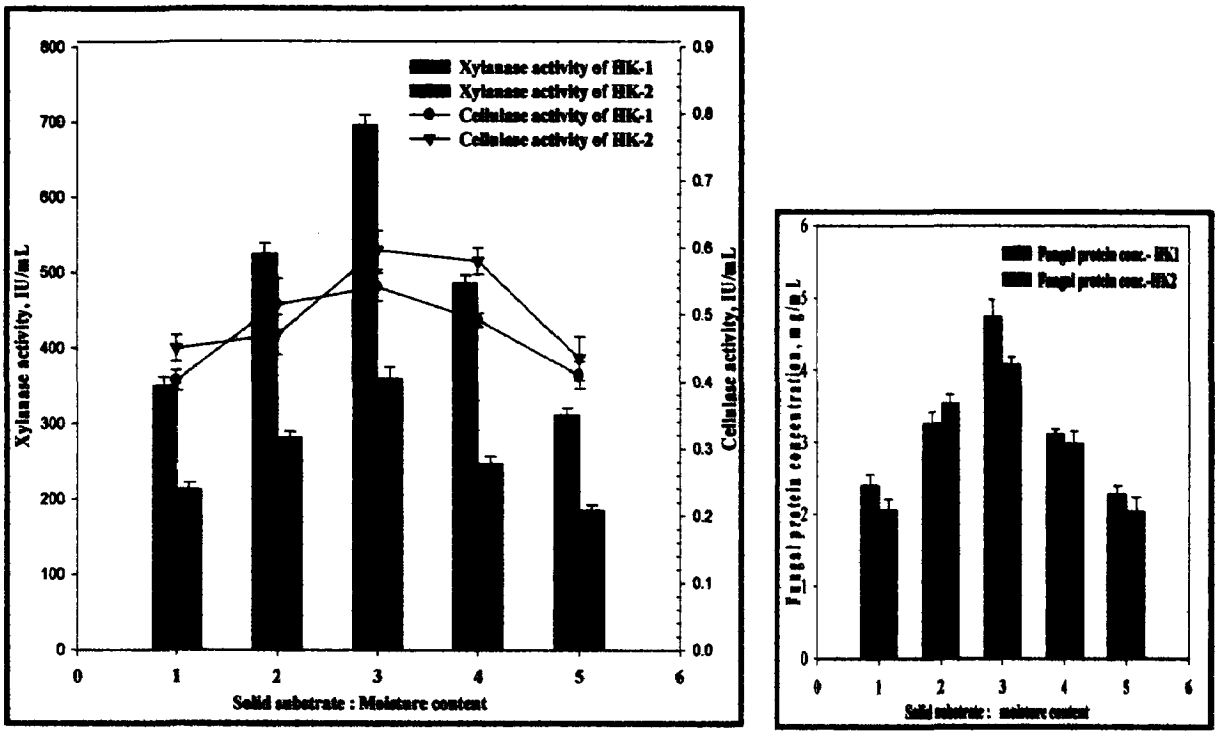


Figure 4.9: Effect of nitrogen source on xylanase and cellulase production by *Coprinopsis cinerea* strains HK-1 and HK-2 and associated fungal protein concentrations



(1) 1:2 (2) 1:2.5 (3) 1:3 (4) 1:3.5 (5) 1:4

Figure 4.10: Effect of moisture content on xylanase and cellulase production by *Coprinopsis cinerea* strains HK-1 and HK-2 and associated fungal protein concentrations

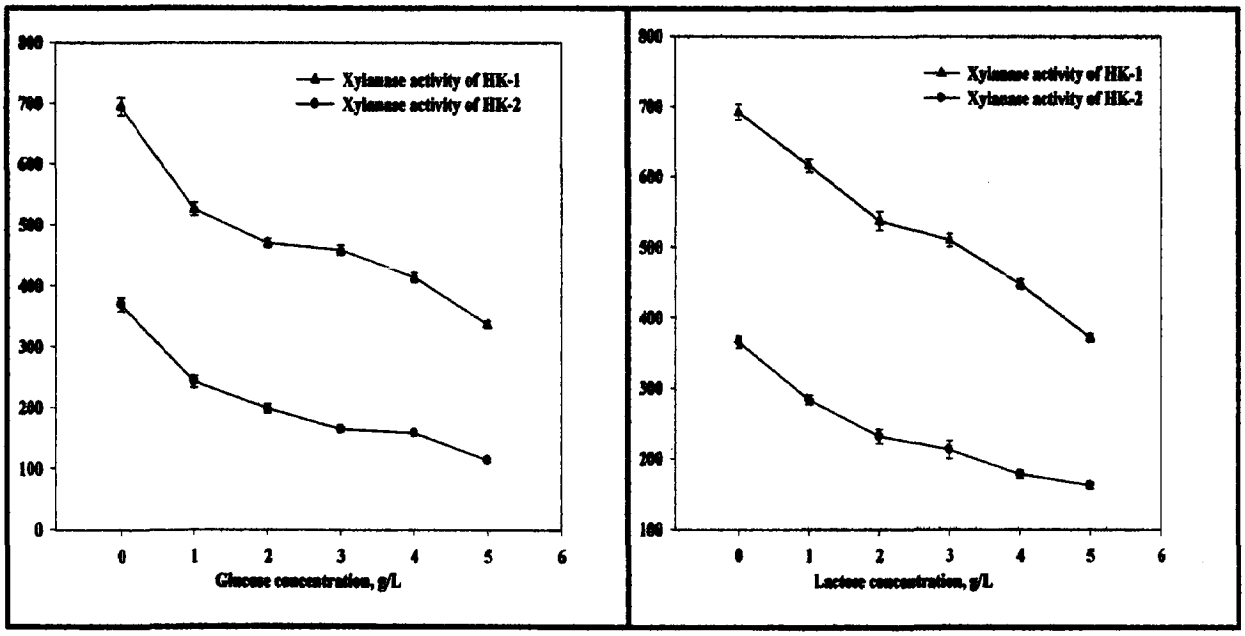


Figure 4.11: Effect of glucose (a) and lactose (b) concentration on xylanase production by *Coprinopsis cinerea* strains HK-1 and HK-2

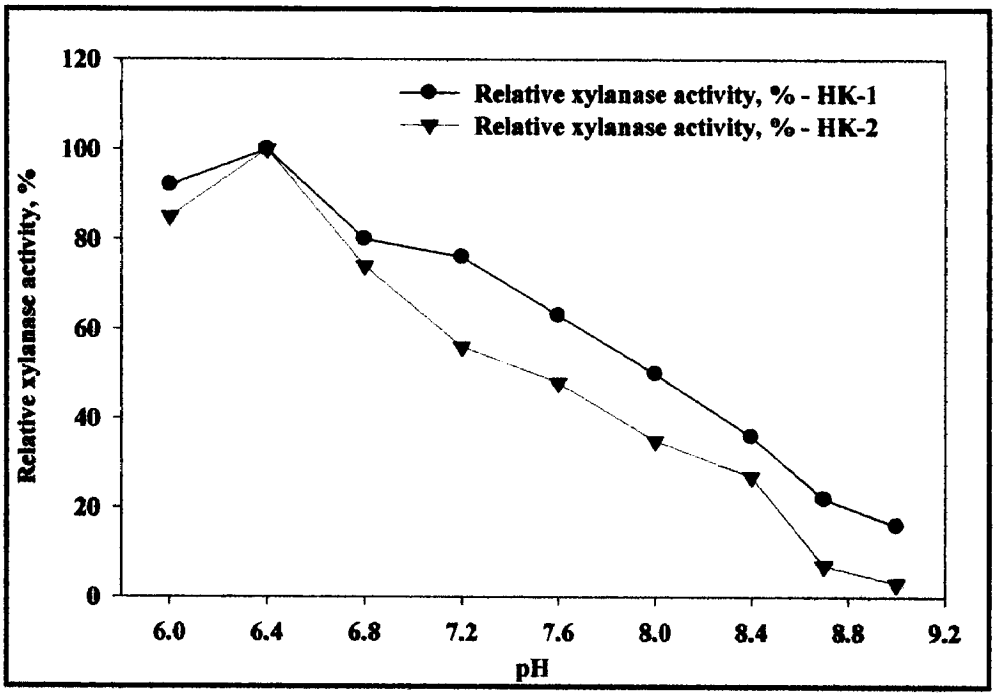


Figure 4.12: pH stability curves of crude xylanase produced by *Coprinopsis cinerea* HK-1 and HK-2

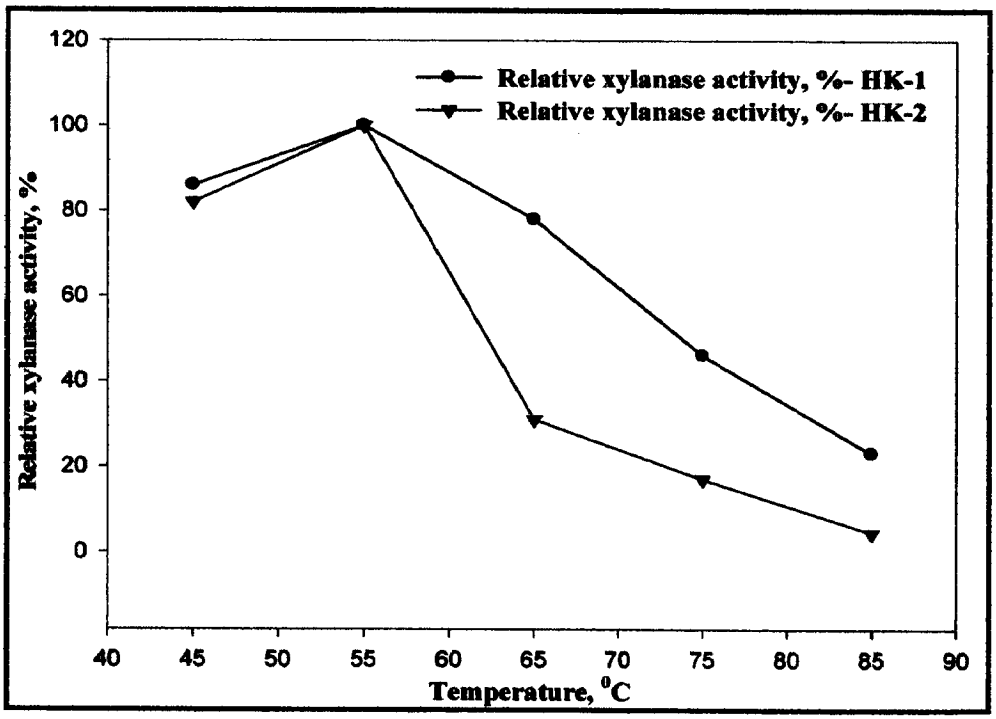


Figure 4.13: Temperature stability curves of xylanase produced by *Coprinopsis cinerea* HK-1 and HK-2

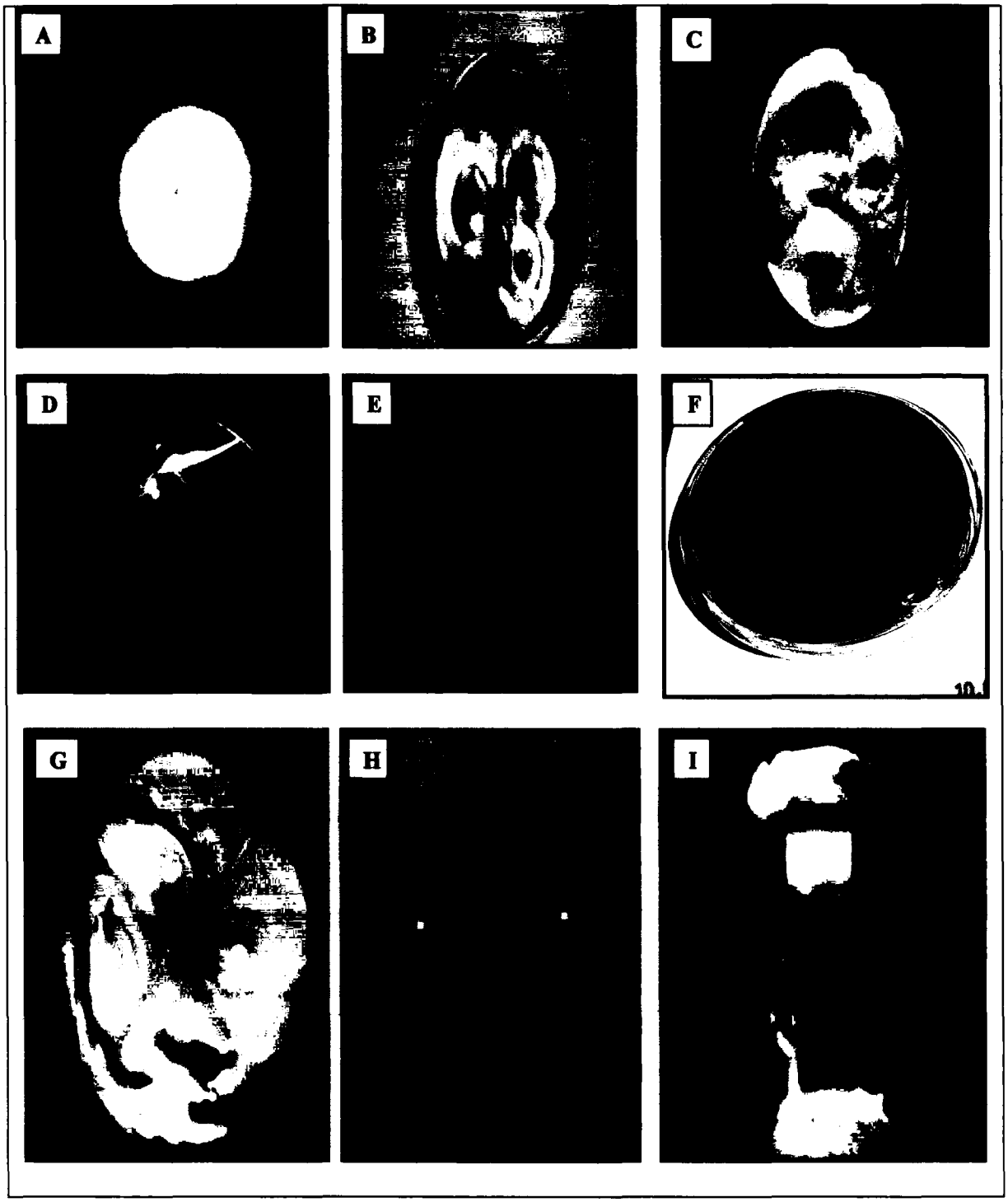


Plate 4.1: Photomicrographs showing morphological features of *Coprinopsis cinerea* HK-1 (Plate A-C); (D) Plate showing the dead and decaying wood buried in moist wheat bran for isolation of fungal cultures; (E-F) xylan-agar and phenol oxidase plate assay tests for Strain HK-1; (G) 9 days old culture of HK-1 showing sporulating stage at centre and few stipes towards the margins; (H-I) Strain HK-1 growing in PDA slants and flask with a fruiting body

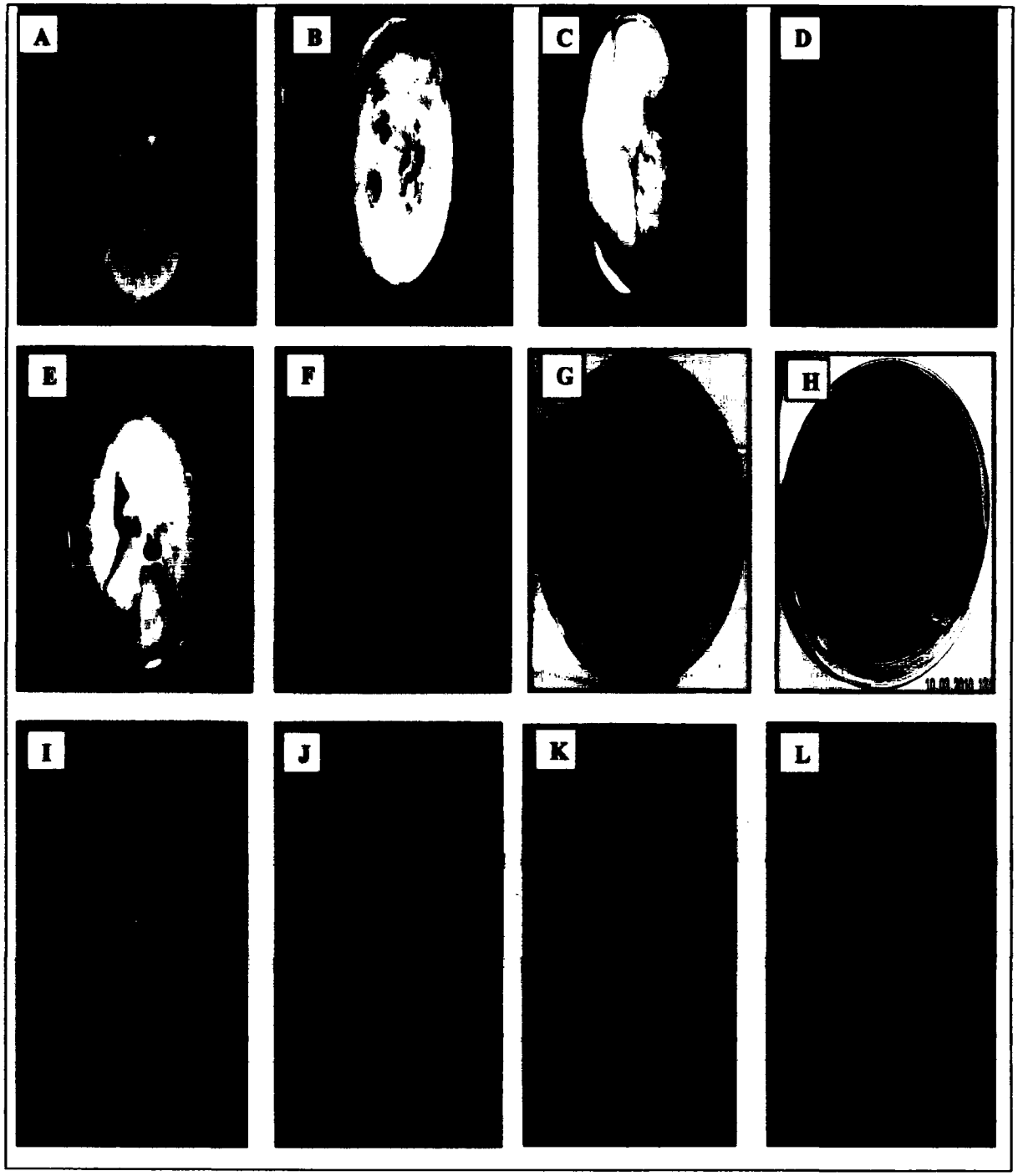


Plate 4.2: Photomicrographs showing morphological features of *Coprinopsis cinerea* HK-2 (Plates A-C); (D) plate showing immature stipes formed on a 6- day old plate of strain HK-2; (E) fruiting body formation in an eight day old culture of HK-2; (F) a 7-day old culture of strain HK-2 under solid state fermentation in a flask; (G-H) xylan-agar and phenol oxidase plate assay tests for Strain HK-2; (I-L) Morphological features of different fungal strains isolated (I) 5 days old culture of HK-6; (J) 5 days old culture of HK-7; (K) 9 days old culture of HK-3; (L) 6 days old culture of HK-9

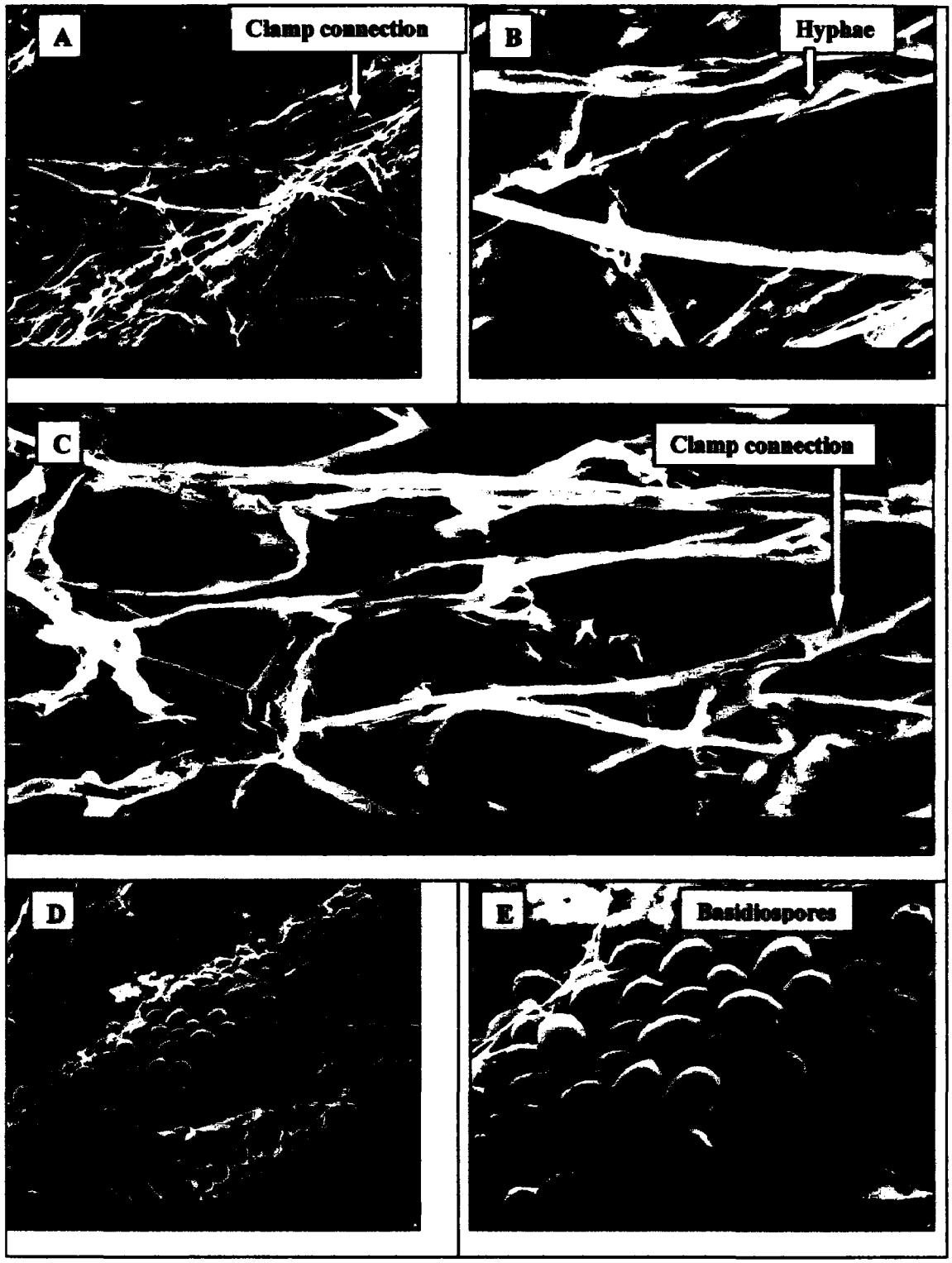


Plate 4.3: Photomicrographs of *C. cinerea* HK-1: (A-B) highly branched fungal mycelial mat with solid hyphae under Scanning electron microscope at a magnification of 500X and 2.00 K X; (C) A major clamp connection at magnification 1.00 K X; (D-E) Club shaped basidiospores at 1.00 and 2.50 K X

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CHAPTER 5

BLEACHING STUDIES ON LEMON AND SOFIA GRASS SODA-AQ PULPS

CHAPTER 5

BLEACHING STUDIES ON LEMON GRASS AND SOFIA GRASS SODA-AQ PULPS

5.1 INTRODUCTION

Removal of lignin from the lignocellulosics is the first step in the manufacturing of chemical paper pulps, kraft alkaline pulping being the most common process for woods and soda pulping for the nonwoods. Although most lignin is removed during cooking, some residual lignin remains in pulp that must be removed in subsequent oxidative bleaching reactions (57). Bleaching is a chemical process applied to cellulosic materials to increase their brightness. 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 decolourization. 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) (36). All these chemical treatments are ascertained at certain prefixed conditions of pH, temperature, concentration and time. For measuring the effectiveness of a reaction, parameters such as pulp brightness, residual lignin content and residual chemicals are often taken into account. Achieving sufficient removal or decolourisation of lignin by the application of any one chemical in a single stage is not feasible. The bleach chemicals are hence applied consecutively with intermediate washing in between the stages, with the primary objective of achieving high brightness. Depending on the end use, the secondary objectives may be high brightness stability, pulp cleanliness along with high cellulose content. These objectives must be met without compromising the strength of the final product; cellulose degradation during bleaching can lead to significant loss in strength (36).

In India, CEH, CEHH and CHH are among the most commonly used bleaching sequences. Being a major source of polluting discharges, the bleach plants account for 60-70% of BOD and 80-90% colour load of the entire mill (with chemical recovery) (121).

Various toxic chlorinated compounds including the chlorinated phenolics, dioxins, furans, chlorinated resin and fatty acids are generated in the effluents followed by these sequences (22, 60). 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 colour imparting substances and 2-4 kg of organochlorines to bleach plant effluents (96). The range for absorbable organic halides (AOX) in final discharge of agro-based mills with and without chemical recovery process is 7-11 and 14.2-21.5 mg/L, whereas, AOX in final discharge of wood based mills is 0.60-9 mg/L. It was found that the C stage was generally the point in which 2,3,7,8-TCDD, 2,3,7,8- TCDF congeners were always present (148, 151, 73). The extraction (E) stage filtrate was found to have the highest concentration of dioxins (135) well known for changing the blood chemistry and causing liver damage, skin disorders, lung lesions and tumour types at numerous sites within the body, liver and thyroid included (110, 111, 71).

The use of such chemically treated papers for the manufacture of direct body contact consumables like baby diapers, food packaging e.g. tea bag paper (39), bread and biscuits wrappers and crimped and curd cups (40) used in railway catering services and confectioners to hold the better class sweetmeats, and crystallized fruits and agriculture research like seed germination paper (38) is of major concern, since it is associated with chlorinated compounds including the animal carcinogen dioxins and furans (97).

Sustainability is a key principle in natural resource management, and it involves operational efficiency, minimisation of environmental impact and socio-economic considerations; all of which are interdependent. It has become increasingly obvious that continued reliance on chlorine and chlorine based chemicals for bleaching purpose has created hazardous environmental impact. Therefore, there are vigorous research initiatives aimed at developing potential alternates for bleaching.

The bleaching plant is among the sections in the pulp and paper industry which have undergone the greatest changes in response to environmental concerns arising from the formation of chlorinated dioxins and other chlorinated compounds during pulp production. Various methods to lower molecular chlorine multiple prior to bleaching like, oxygen (O_2) delignification (97) modified cooking processes i.e. extended modified continuous cooking (EMCC) (109), modified conventional batch cooking (MCBC) (16) and rapid displacement heating (RDH) (46) etc., use of cooking aids (117) and substitution

of chlorine with chlorine dioxide were available but first two methods required huge capital investment. Elemental chlorine free (ECF) and total chlorine free (TCF) bleaching have brought about a major technological revolution in the pulp and paper industry which has led to the production of environmentally benign paper and mitigation of environmental emissions (57, 131). With the replacement of elemental chlorine (Cl_2) by chlorine dioxide (ClO_2) in bleaching sequences, the pulp and paper industry has reduced considerably the formation and discharge of chlorinated organic material into the aquatic environment (150). A decrease in 48-65% AOX in Kraft bleaching has been reported by adoption of ECF (72). Meeting the tough environmental demands of Best Available Technology (BAT), set up by the European commission seems to be quite possible through these modern bleaching sequences of ECF and TCF (119).

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 (118). By substituting chlorine dioxide at levels of 70 to 100%, an apparent decrease of 80 to 90% in the level of chlorinated organics was found in mill effluent along with the reduction in 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 (118). ClO_2 substitution decreases effluent colour in proportion to the percent substitution; one estimate is that a 1% decrease in colour occurs for each 2% increase in percent substitution (88, 28, 87,). 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 (88, 87, 81). 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 (7). The other benefits of ECF bleaching include reduction in chloroform formation and chlorinated organic compound (AOX) by 90%; efficient utilization of forest resources; contribution to eco-system recovery; and compatibility with emerging minimum-impact mill technologies (115). 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 any bleaching method, environmental and economic considerations demand that the lignin content of the pulp should be reduced as much as possible before bleaching. Nowadays peroxide, oxygen and ozone constitute environmentally friendly alternatives to develop totally chlorine-free (TCF) sequences (57).

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 ($MgSO_4$) (126, 54, 95). 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 (107, 14, 29), 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 (17). 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 (156).

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 (62, 165, 116, 37), 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 (36). Use of this chemical agent in the bleaching of non-woody chemical pulps via TCF bleaching sequences has been reported to be quite effective (144, 68, 127, 10, 34, 84). A final peroxide bleaching stage is quite useful to improve pulp brightness stability (102, 155). 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 (51). 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 (11). However, most TCF chemical reagents, due to their lower delignification power, are less efficient than chlorine reagents in attaining high and stable pulp brightness degrees (57).

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 (122). The available options of oxygen delignification, extended delignification, substitution of ClO_2 for Cl_2, H_2O_2 and ozone (13) are highly capital intensive in terms of process change.

Nowadays, the enzymatic bleaching could be a simple and economic alternative for cleaner pulp production (47, 147). Xylanases (X) are hydrolytic enzymes that catalyse xylan degradation. Their use appears to be quite promising for chlorine-free pulp bleaching processes. Their favourable effect has been ascribed to partial removal of xylans from fibre surfaces which make fibres more accessible to the reagents in subsequent bleaching stages (160, 129). Treatment with xylanases boosts overall bleaching process by improving subsequent stages (164, 128) and has even been incorporated into bleaching sequences in some pulp mills (3, 113). The enzymatic treatment with oxido reductases such as laccases is another promising alternative under intensive research (93).

Currently, the most promising applications of xylanases in the prebleaching of pulps (13, 136, 2), include improved pulp fibrillation and water retention, reduction of beating times in virgin pulps, restoration of bonding and increased freeness in recycled fibers, and selective removal of xylans from dissolving pulps (13). Many of the fungi used for xylanase production also form abundant cellulases. This application however requires that xylanases should be completely free of any cellulase activity if one is to avoid losses in cellulose viscosity resulting from enzyme treatment (108). The treatment of kraft pulp with cellulase free xylanases increases pulp viscosity due to partial hydrolysis of xylan in the pulp (106, 28). Furthermore, fungal xylanases commonly have pH optima in the acidic range (pH 4.5-5.5). Because the kraft and soda pulping processes are strongly alkaline and

because alkali remains trapped in the fibers even after extensive washing; the pH tends to drift higher during enzyme treatment. Enzymes with substantial activity in alkaline region can be very useful (108). Temperature stability is also desirable because the pulps are warm after they come out of the cooking and washing steps (13, 149). Most importantly, the enzymes must be effective. The enzymes must be fine-tuned before their final industrial implementation. Thus, screening criteria for xylanases with better thermostability and possibly higher pH optima have received greater attention (30). The strains reported for the commercial production of xylanases include *Trichoderma reesei*, *Thermomyces lanuginosus*, *Aureobasidium pullulans*, *Bacillus subtilis*, and *Streptomyces lividans* (13). Their application conditions must also be optimized in order to reduce enzyme consumption, and also reaction times. The effect of enzyme treatment on pulp kappa number, viscosity and chemical demand are amongst the most reliable factors taken into consideration while evaluating the efficacy of an enzyme (108).The efficacy of microbial xylanases in bleaching process has also been studied for *Aspergillus oryzae* (26), *A. niger* (173) and *A. fumigatus* (136). The utilization of xylanases could lead to the replacement of 5–7 kg of chlorine dioxide per ton of kraft pulp and an average fall of 2–4 units in kappa number (112).

The biobleaching process is based on the action of the microorganisms and/or enzymes. The interest for xylan degrading enzyme and its applications in the pulp and paper industries has advanced significantly over the past few years (9, 49, 26, 149). In this process, the bond between lignin and hemicelluloses, primarily between lignin and xylan, can be removed by xylanase. Once this layer of hemicellulose is removed, the lignin layer is easily available for degradative action of the ligninolytic enzymes (44). In enzyme-aided bleaching, the proposed mechanism for the action of xylanases includes:

1. The selective removal of xylan that re-precipitates into pores and surface of the cellulose fibers during cooking of the kraft pulp, and
2. The partial extraction of chromophore groups linked to residual xylan, owing to degradation of lignin-carbohydrates complexes.

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 (67, 49).

The use of xylanase as a biobleaching process allows attainment of high brightness in pulps with savings of bleaching chemicals (8, 66) and is widely used in the bleaching of non woody pulps (4, 21, 99). Therefore, xylanases are not considered direct pulp-bleaching agents, but a bleaching aid that helps to increase brightness (157, 63). Xylanases from *Staphylococcus* sp. SG-13 have 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 (55).

The properties of many commercial xylanases make them unsuitable for the real process of pulp bleaching (2), and it is noteworthy that these commercial xylanases (41, 83), are all used after being purified. For that, the application and production are seriously influenced by the higher cultivation cost for fermentation and higher risk of contamination (30). The use of abundantly available and cost-effective agricultural residues to achieve higher xylanase yields and simple, rapid purification procedures provide suitable methods to reduce the manufacturing cost of bio-bleached paper, thus facilitating the adaptation of this environmentally friendly technology in the industry (141).

Tolerance to high pH and temperature are credentials for xylanases to be effectively employed in pulp pretreatment, which improves the efficiency of conventional chemical bleaching and pollution control (20). The use of enzyme enabled the amounts of toxic compounds (chlorophenols and other forms of organically bound chlorine) in the spent bleach liquor to be reduced (58, 59, 86, 159, 5) while an increase was noted in effluent COD and colour due to the hydrolysis of hemicelluloses (129, 86, 166). At 100% ClO_2 substitution, the extent of AOX reduction in effluent of xylanase pre-treated softwood pulp was 40% (139). Effluent COD and BOD increased by 32.19% and 107.89% after treatment of eucalyptus kraft pulp with commercial xylanase while AOX reduced by 15.09% after same treatment in comparison with control (58). However, the properties of many commercial xylanases make them unsuitable for the real process of pulp bleaching (2). In the present study, we report for the first time the ~~purification~~ extraction and characterization of an alkali xylanase from a microbial fungal strain C.cinerea HKI and its application in biobleaching of 2 non-woody raw materials.

5.2 EXPERIMENTAL METHODOLOGY

5.2.1 Biobleaching of lemon and sofia grass soda-AQ pulps with xylanase produced by *C. cinerea* HK-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 soda-AQ pulps from lemon and sofia grasses were treated with varying doses of xylanase i.e. 0-25 IU/g while keeping other variables constant like, consistency 10%, reaction temperature 55 °C, reaction time 120 min and pH 6.4. and its effect was observed on, kappa number, reducing sugars released, pulp brightness and viscosity. Xylanase treated pulp samples were followed by extraction with 1.5% alkali as per conditions mentioned in Table 5.1. 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 have been reported in Table 5.1 and Figures 5.1(a) - 5.3(b).

In an another set of experiment, the soda-AQ pulp from lemon grass was treated with xylanase dose of 10 IU/g and that from sofia grass with 8 IU/g while varying the reaction time from 30 to 240 min. The soda-AQ pulps from lemon grass and sofia grass were next treated with xylanase dose of 10 and 8 IU/g respectively at different pulp consistencies i.e. 2-12%. All other operating conditions were held constant like, reaction temperature 55 °C, reaction time 120 min and pH 6.4 for both sets of experiments and the effect was evaluated on kappa number, reducing sugars released, pulp brightness and viscosity which were determined at each stage of the experiment as earlier. The filtrates after each stage were collected for further analysis. The results have been cited in Tables 5.2, 5.3 and Figures 5.4(a) - 5.9(b).

Reducing sugar concentrations in pulp filtrates were determined by dinitrosalisylic (DNS) acid method (91) and expressed as D-xylose equivalents. Enzyme mediated release of chromophoric material was monitored in filtrates by measuring absorption spectra at 237, 280 and 465 nm (108, 55). 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 (153).

5.2.2 Application of xylanase in multi-stage bleaching process

Xylanase pretreated and untreated soda-AQ pulp samples from the two raw materials were bleached by multistage conventional (CEHH, OCEHH), ECF (ODED, ODEDP) and TCF ($O(E_{OP})P$ bleaching sequences. Unbleached pulp equivalent to 50 g o.d. was taken for each lemon grass and sofia grass separately in polythene bags and bleaching experiments were performed in temperature controlled water bath except chlorination which 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 pulps of lemon grass and sofia grass were bleached by CEHH; XECEHH 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 and 'O' for oxygen bleaching stage. The bleaching conditions and results are reported in Tables 5.4 and 5.5. The xylanase prebleaching stage (X) was conducted at an enzyme dose of 10 IU/g for lemon grass and 8 IU/g for sofia grass, pH 6.4, pulp consistency 10%, reaction time 120 min and temperature 55 °C. For analysis of bleach liquor, 10 mL of bleach liquor was diluted 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 noticed as a colour change from blue to colourless (75) was recorded which 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 × 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. 70% in 'H_1' stage and 30% 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 °C, pH 11.5 and reaction time 60 min.

The soda-AQ pulps were then delignified with molecular O_2 in electrically heated WEVERK rotatory digester of capacity 2000 mL. The O_2 delignified pulps were 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) $MgSO_4$, 0.1% EDTA (o.d. pulp basis), 2% 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 °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 (75). The results of CEHH; XECEHH, and OCEHH; XOCEHH bleaching sequences are reported in Tables 5.4 - 5.7 and Figures 5.10(a) - 5.13(b).

5.2.2.2 ECF bleaching

Lemon and sofia grass soda-AQ pulp samples were bleached by ODED; XODED and ODEDP; XODEDP 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. The results and bleaching conditions are reported in Table 5.8 and 5.9. The xylanase pretreatment stage was performed under the following bleaching conditions i.e. enzyme dose of 10 and 8 IU/g respectively for the two grasses, reaction time 120 min, temperature 55 °C, pH 6.4 and pulp consistency of 10%. The xylanase pretreated and untreated (control) soda AQ pulp samples were oxygen delignified in WEVERK rotatory digester at a pressure of 5 kg/cm^2, temperature 90 °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 (75). Oxygen delignified pulp samples were treated with 2% (o.d. pulp basis) chlorine dioxide in 'D_1' and 'D_2' stages (1.34% in 'D_1' and 0.66% in 'D_2' stages respectively) at a pulp consistency of 10% at temperature 70 °C for

180 min and pH 4.0. In E-stage 2.5% NaOH was applied at 10% consistency, temperature 60 °C for 60 min and pH 11.7. In ODEDP bleaching sequence, In ODEDP, the final stage i.e. P stage was conducted at consistency 10%, temperature 90 °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 (168). The amount of $Na_2S_2O_3$ consumed was indicated by changing the colour of end point from blue to colourless. The strength of H_2O_2 was calculated by using the following formula:

$$H_2O_2, g/L = \text{Volume (mL) of 0.1 N } Na_2S_2O_3 \text{ consumed} \times 0.34$$

All the chemicals were added on o.d. pulp basis. The results of ODED; XODED and ODEDP; XODEDP bleaching sequences are reported in Tables 5.8 to 5.11 and Figures 5.14(a) to 5.17(b).

5.2.2.3 TCF bleaching

Lemon and sofia grass soda-AQ pulp samples were 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 5.12 and 5.13. Lemon and sofia grass pulp samples were treated with a xylanase dose of 10 and 8 IU/g respectively under a pulp consistency of 10% and reaction temperature 55 °C for 120 min at a pH of 6.4. After xylanase pretreatment, the pulps from the two raw materials were subjected to O_2 delignification in WEVERK rotatory digester. Pulp samples were mixed with 0.1% $MgSO_4$, 0.1% EDTA, 2% 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 °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 °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 5.14. 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 5.12 to 5.15 and Figures 5.18(a) - 5.19(b).

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 soda-AQ pulps from lemon grass and sofia grass were disintegrated in PFI mill (T 200 sp-96) at a beating level of 40±1 °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 bleach 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) (161, 172), colour (204 A) as per Standard methods for the examination of water and wastewater, American Public Health Association, 1985 and AOX by column method (163).

5.2.5 Scanning electron microscopy

The detailed morphological studies of unbleached and xylanase prebleached lemon grass and sofia grass 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 (48). SEM microphotographs were taken at desired magnifications. Results of SEM are shown in Plates 5.1 and 5.2.

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 lemon and sofia grass soda-AQ pulps

The potential of xylanases from *Coprinopsis cinerea* HK-1 to remove chromophores from the pulp has not ever been reported previously. In the present study, an effort has been made to establish this untouched applicability of the model fungus *C. cinerea* from the perspective of pulp prebleaching.

5.3.1.1 Influence of xylanase doses

Table 5.1 reveals the effect of different xylanase doses i.e. 0-25 IU/g while keeping other operating variables constant like temperature 55 °C, reaction time 120 min and pH 6.4. The reducing sugars released after applying different doses of enzyme have been shown in Figure 5.1 (a) and 5.2 (a). The curves could be approximated by two straight lines. Up to an enzyme dose of 16 IU/g for lemon grass and 12 IU/mL for sofia grass, the curves with steeper slope pertained to rapid release of sugars whereas the part of curve with gentler slope pertained to the slow release of sugars. Both parts of the curves were having different velocity constants. Reducing sugars continued to be released owing to the xylanase hydrolysis of soluble oligosaccharides. Release of chromophores was hence a better probable indicator of the kinetics of the enzyme attack on the pulp as literature indicated that oligosaccharides were released by the initial depolymerisation of the xylan coating on the fiber surface (49). Table 5.1 shows spectrophotometric analysis of filtrate generated during xylanase treatment of soda-AQ pulps of lemon grass and sofia grass at different doses of xylanase. Figures 5.3 (a, b) reveal that the absorbance at a wave length of 237 nm increased up to a xylanase dose of 10 IU/g and 8 IU/g respectively for soda-AQ pulps of lemon and sofia grass, due to release of phenolic compounds or chromophores and beyond that, no significant increase in absorbance could be noticed. The absorbance at a wave length of 465 nm increased up to an enzyme dose of 10 IU/g for lemon grass and 8 IU/g for sofia grass owing to release of hydrophobic compounds (108, 55, 67, 12) and then there was an insignificant increase in absorbance. This indicated that on further increase in enzyme dose; only small amount of additional lignocellulosic complex (LCC) was attacked. This confirmed that xylanase acted on LCC and degraded lignin hemicellulose linkages (33), thus releasing degraded chromophores into effluent (67,147). At a wave length of 280 nm, an increase in the absorbance above enzyme dosage of 8 IU/g for sofia

grass was observed (Figure 5.3 b). At higher enzyme dosage (above 8 IU/g), there was an increase in the absorbance at 280 nm supporting the observation by Ziobro that carbohydrate degradation products also attributed to the colouring matter (174, 175). The same peak for lemon grass was reached at an enzyme dosage of 10 IU/g, with a slight increase thereafter. The peak at 280 nm in the U.V. spectrum indicated the presence of lignin in the released colouring matter (74, 100). Recent studies (43, 169) have shown that the majority of the 4-*O*-methylglucuronic acid side groups in xylan are converted to hexenuronic acid (HexA) in the early phases of the kraft cooking. Xylanases have been found to further boost up the release of xylooligosaccharides branched with hexenuronic acids (HexA), giving rise to fibres with a reduced HexA and xylose content (164). These HexA contribute to the kappa number and to the yellowing of pulp (43, 169). This release could be correlated with the reduction in kappa number found after the first extraction (164). The release of reducing sugars and the release of lignin and phenolic compounds were interrelated phenomenon. When the soda-AQ pulps from lemon and sofia grass were pre-treated with xylanase, xylose and other reducing sugars released from the hemicellulose layers ultimately resulted 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 (67).

The enzymatically treated soda-AQ pulps from lemon grass and sofia grass were subjected to alkali extraction using 1.5% NaOH (as such) as per conditions mentioned in Table 5.1 in order to observe its effect on kappa number, brightness and viscosity. Figures 5.1 (a) and 5.2 (a) reveal that kappa number after XE-stage decreased on increasing enzyme dose up to a certain level and then declined. The maximum decrease in kappa number of lemon grass pulp was achieved as 20.9% at an enzyme dosage of 10 IU/g and as 27.8% for sofia grass pulp at an enzyme dosage of 8 IU/g compared to their respective controls. Further there was no significant decrease in kappa number on increasing enzyme dose. Accordingly, maximum gain in brightness by 7.8 and 6.6% (ISO) was attained at enzyme dosage of 10 IU/g and 8 IU/g for soda-AQ pulp of lemon and sofia grass respectively compared to their respective controls. No significant gain in brightness was recorded beyond the optimal dosages. The chromophore release correlates well with the

total sugar release: this activity was used throughout the experiments as a simple method to determine the efficacy of the enzyme treatment. It was observed that higher chromophore release corresponded to higher brightness. This correlation was more evident at the low brightness levels observed in early bleaching stages. Previously alkaline extraction in conjunction with enzyme treatment was shown to improve pulp characteristics (24). 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 (25). An alkaline extraction stage was henceforth carried out following xylanase treatment of lemon and sofia grass soda AQ pulps to observe the effect of xylanase on 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 (64, 108, 74, 5, 25, 143).

The maximum increase in viscosity was 8.8 and 5.6% respectively for lemon and sofia grass soda-AQ pulps compared to controls (Figure 5.1 b and 5.2 b). The increase in pulp viscosity was due to hydrolysis of low DP (degree of polymerization) xylans in the pulp by xylanase (106) which resulted in an increase in the average molecular weight of the polymer system due to change in cellulose to xylan ratio (162). In comparison to control, treatment of eucalyptus kraft pulp with xylanase from *P. corylophilum* increased viscosity and brightness by 24.85 and 9.3% respectively at an enzyme dosage of 5 IU/g for 2h (90). According to Ragauskas et al. (117), this improvement in the pulp viscosity could be caused by accumulation of high molecular polysaccharides, which occurred when xylan was selectively removed.

A xylanase and laccase concoction produced through co-cultivation of mutant *Penicillium oxalicum* SAU(E)-3.510 and *Pleurotus ostreatus* MTCC 1804 under SSF using bagasse and black gram husk (3:1) as solid substrate was evaluated for its bleach enhancing ability of mixed wood pulp (Eucalyptus/Poplar, 60:40), which resulted into a 21% decrease in kappa number, 8% increase in brightness and 5% increase in viscosity at an enzyme (xylanase/laccase, 22:1) dose of 8 IU/g, 10% pulp consistency, temperature 55 °C at pH 9.0 for 3 h. (42). A cellulase-free xylanase produced from *Bacillus stearothermophilus* SDX under SmF using wheat bran as the carbon source was used by Garg et al. for pretreatment of wheat straw pulp at 60 °C for 120 min which resulted in 7.14% decrease in kappa number and 4.75% increase in brightness at an enzyme dose of

10 U/g of o.d. pulp at pH 9 (50). Cellulase poor xylanases from two strains of *C. disseminatus* SH1-1163 (727.78 IU/ml) and SH2-1164 (227.99 IU/ml) under SSF produced at incubation time 7 days, temperature 37 °C and pH 6.4 using yeast extract as nitrogen source and cheap substrate (wheat bran) mitigated kappa number of wheat straw soda–AQ pulps by 24.38 and 27.94% and improved pulp brightness by 16.33 and 17.94% respectively after XE-stages at an enzyme dosage of 10 IU/g, reaction time of 180 min for 55°C and consistency 10% for strain SH-1 and 5% for strain SH-2 (147). Enzymatic prebleaching of a kraft pulp conducted with thermo-alkalophilic and cellulase free xylanase from *Arthrobacter* sp. MTCC 5214 produced under SSF using wheat bran as a carbon source; at an optimized enzyme dose of 20 U/g pulp, 6% consistency, 70 °C temperature for 2 h resulted in 20 % reduction in pulp kappa number along with an increase in brightness and without much change in viscosity (67).

5.3.1.2 Influence of reaction time

Table 5.2 reveals the effect of varying reaction time from 30 to 240 min while keeping other variables constant i.e. enzyme dosage 10 and 8 IU/g for lemon grass and sofia grass soda-AQ pulps respectively, at temperature 55 °C, pH 6.4 and pulp consistency of 10%. Reducing sugars released during enzymatic prebleaching at different time intervals were found to increase with the increasing reaction time (Figure 5.4-a and 5.5-a). The reducing sugars released after enzymatic prebleaching of soda-AQ pulps of lemon and sofia grasses at a reaction time of 120 min were 4.10 and 6.14 mg/g respectively and after that the increase in reducing sugars was insignificant. Table 5.2 and Figures 5.6 (a, b) depict the effect of reaction time on absorbance of filtrates of xylanase treated pulps at different wavelengths i.e. 237, 280 and 465 nm. Most of the colour and chromophores were released between a reaction time of 30 to 120 min and beyond that the release was insignificant. Removal of the reprecipitated xylan by the action of endoxylanases augmented the permeability of the fibers and elimination of lignin from pulp fiber, thus, reducing the kappa number of pulp and increasing the chromophores in filtrate (106). 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 (5). Higher enzyme dose or longer periods of incubation did not significantly enhance the extent of biobleaching in kraft pulp treated by xylanases extracted from *Arthrobacter* sp.MTCC 5214 (67).

Lemon and sofia grass soda-AQ pulps prebleached with enzyme at different reaction time while keeping other conditions constant were subjected to alkali extraction with 1.5% NaOH (as such) at 70 °C temperature and10% consistency for 90 min in order to observe its impact on kappa number, brightness and viscosity (Table 5.2). Figures 5.4-a and 5.5-a indicate that maximum kappa number mitigation of 21.6 and 27.9% were achieved at a reaction time of 120 min for lemon and sofia grass soda-AQ pulps respectively and further increase in reaction time showed insignificant change in kappa number compared to controls. Likewise, a reaction time of 120 min showed maximum increase in viscosity of lemon and sofia grass soda-AQ pulps by 7.9 and 5.5% respectively compared to controls (Figure 5.4-b and 5.5-b). Further increase in reaction time was unbearable by both the pulp samples as indicated by a nearly 3% decrease (reaction time of 240 min) in viscosity without any appreciable reduction in kappa number or gain in brightness. The pulp brightness of soda-AQ pulps after XE-stage increased by 7.8 and 6.5% (ISO) for lemon and sofia grass respectively at a reaction time of 120 min, with an insignificant increase in brightness thereon.

Xylanases from *Coprinellus disseminatus* SW-1 NTCC 1165 (enzyme activity 499.60 IU/mL) under SSF using wheat bran as a carbon source at an enzyme dosage of 8 IU/g, 10% consistency and 55 °C temperature maintained at 6.4 pH for a reaction time of 120 min produced optimal decrease in kappa number by 29.1% along with 9.42% (ISO) gain in brightness after prebleaching of sugarcane bagasse soda-AQ pulp followed by alkali extraction (1). A cellulase free *Bacillus subtilis* crude xylanase produced on wheat bran under SmF by ASH at 37 °C and neutral pH was found most effective for biobleaching of kraft pulp when used at an enzyme dose of 6 IU/g o.d. pulp under 10% consistency at 55 °C for 2h. The prebleaching effect resulted in kappa number mitigation by 4.62% and improvement in brightness by 3.3% ISO (134). Treatment of kraft pulp with the xylano-pectinolytic enzymes (in the ratio of 5:1), extracted from *B. pumilus* via SmF using wheat bran and citrus peel as carbon source at 37 °C for 48 h resulted in 8.5% reduction in kappa number and 1.92% gain in brightness after reaction time of 180 min at a xylanase-pectinase dose of 4.5 and 0.9 U/g of o.d. pulp and consistency 10% maintained at 55 °C, pH 8.4 (66). Pretreatment of wheat straw-rich soda pulp with purified xylanase from *Streptomyces cyaneus* SN-32 at a dosage of 10 IU/g of moisture free pulp under 10% pulp consistency exhibited maximum bleach boosting effect at 65 °C, pH 9.5–10.0 after

2 h of reaction time, reduced the kappa number by 8.7% and enhanced the brightness index by 3.56% (99).

5.3.1.3 Influence of pulp consistency

Figure 5.7-a and 5.8-a shows the concentration of reducing sugars released from the two pulp samples after undergoing xylanase treatment at different pulp consistencies while keeping other conditions constant like, xylanase dose 10 IU/g for lemon grass and 8 IU/g for sofia grass, temperature 55 °C, reaction time 120 min and pH 6.4. The curve shows that the increase in pulp consistency increased the amount of reducing sugars released. The maximum increase in reducing sugars was observed at a pulp consistency of 10% and beyond that a slow increase was noticed with lemon grass while the curve became relatively stable in case of sofia grass. Table 5.3 also depicts the spectrophotometric analysis of filtrates generated during xylanase prebleaching of soda-AQ pulps of lemon and sofia grasses at varying pulp consistencies. Figure 5.9 (a, b) reveals that the absorbance at different wavelengths (237, 280 and 265 nm), also increased with increasing pulp consistency of 10% and beyond that the increase in absorbance was insignificant. In case of enzymatic treatment, several factors determine the efficiency of pulp prebleaching.

Table 5.3 describes the effect of pulp consistency on kappa number, viscosity and brightness of xylanase prebleached soda-AQ pulps of lemon and sofia grasses followed by alkali extraction as per conditions mentioned earlier. Figure 5.7(a) indicates that kappa number decreased with increasing pulp consistency and maximum reduction in kappa number by 24.1% was recorded at a pulp consistency of 10% (medium consistency). Further, there was no significant decrease in kappa number. Similarly, for sofia grass, a maximum decrease in kappa number by 29.7% was observed at a consistency of 10%. The cellulosic fibers when merged in water; contain mobile and immobile layers surrounding the fibers (105). 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 (79, 123, 65). Water layer thickness now becomes the rate-determining step. However, a higher pulp consistency provides a close contact between enzymes and pulp fibers (56, 25), probably because of the reduced volume of the liquid phase, thus facilitating enzyme adsorption to pulp and the sequential hydrolysis of hemicellulose (25). High pulp consistencies helped 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 (5). Good mixing is a critical requirement for attaining maximum benefits of an enzyme during pulp prebleaching. It was difficult to get results at high consistency due to limitations in pulp mixing equipment needed to break physical barriers for good mixing. For the decrease in kappa number above pulp consistency of 10%, the pulp had to be finely shredded to separate fiber aggregates to the greatest extent possible before contacting the fiber with reactant (105, 65, 103). In the similar pattern, maximum gain in brightness by 10.9 and 7.4% (ISO) were achieved at 10% consistency for lemon and sofia grasses respectively.

The extent of product removal is largely a function of substrate characteristics, but enzyme specificity also plays an important role (70). The interaction of the enzyme with the pulp is also important, including the effective molecular weight, net ionic properties and specific action pattern (140). Thus, the response of any pulp or parameter would vary according to the characteristic features of any enzyme preparation. Chauhan et al (21) studied the prebleaching effect of a crude xylanase from *B. coagulans* on rice straw pulp at an enzyme dosage of 20 IU/g for 3h at a pulp consistency of 6% under a pH of 8.5 whereby a maximum brightness gain of 5.1 points was achieved.

Many researchers used a pulp consistency of 10% for xylanase pretreatment of nonwoods such as bagasse soda-AQ pulp (1, 82, 23), wheat straw soda-AQ pulp (147, 99), and for woods such as eucalyptus kraft pulp (90, 134, 66), oxygen delignified soda-AQ pulp of *Eucalyptus grandis* (152), as mentioned above.

A xylanase dose of 10 IU/g for lemon grass and 8 IU/g for sofia grass, a reaction time of 120 min, pulp consistency of 10%, temperature of 55 °C and pH 6.4 were considered to be optimum for enzyme prebleaching using crude xylanases obtained from *C.cinerea* strain HK-1

5.3.2 Effect of xylanase pretreatment on conventional bleaching

Table 5.4 shows the effect of CEHH bleaching sequence on brightness, viscosity and bleach losses of two non-woody soda-AQ pulps under study. The brightness and viscosity of soda-AQ pulp of lemon grass bleached by CEHH bleaching sequence were 78.4% (ISO) and 9.5 cps respectively. The same parameters when considered for sofia grass pulp were recorded as 80.6% and 8.4 cps. Table 5.5 depicts that the introduction of O_2 (pressure 5 kg/cm^2) before chlorination stage of OCEHH bleaching sequence, reduced

kappa number by 35.34% and total chlorine demand by 34.04% for lemon grass soda AQ pulp; along with 33.31 and 33.2% respective decreases for sofia grass soda AQ pulp. A 59% reduction in the kappa number of unbleached hardwood pulp and 60% for unbleached bagasse pulp after ODL was reported at an O_2 pressure 0.50 mPa, temperature 120 °C, alkali charge 2% and reaction time of 30 min (114). A drop of about 50% in kappa number of unbleached pulp of *Melocanna baccifera* (Muli bamboo) after ODL was observed at an O_2 pressure of 0.5 mPa, temperature 70 °C, alkali charge 2% and residence time 60 min (156). When compared to CEHH bleaching sequence, OCEHH bleaching sequence produced a gain in brightness and viscosity by 1.9 and 5.94% for lemon grass (brightness 80.3% (ISO) and viscosity 10.1 cps) and by 1.0 and 4.54% for sofia grass (brightness 81.6% and viscosity 8.8 cps). The bleaching losses in CEHH and OCEHH bleaching sequences were 9.6 and 9.2% respectively for lemon grass and 9.1 and 8.7% for sofia grass soda-AQ pulps.

The effect of xylanase pretreatment was studied on total chlorine demand (TCD), brightness, viscosity, strength properties and combined effluent properties of lemon and sofia grass soda-AQ pulps during CEHH and OCEHH bleaching sequences (Tables 5.4 and 5.5). A xylanase pretreatment before chlorination stage of CEHH bleaching sequence followed by alkaline extraction (XECEHH) mitigated kappa number by 24.1% and total chlorine demand by 22.67% for lemon grass while the respective decreases for sofia grass were 25.4 and 29.73%. The kappa number of lemon and sofia grass soda-AQ pulps after XO stage in XOCEHH bleaching sequence were recorded with a net decrease of 42.67 and 44.53%. While comparing the two soda AQ pulps for kappa number, XO stage of XOCEHH produced 11.3 and 16.8% extra reduction in kappa number of lemon and sofia grass in comparison to O stage of OCEHH. Enzymatic prebleaching in XOCEHH bleaching sequence mitigated TCD by 11.60 and 16.92% for lemon and sofia grass soda-AQ pulps compared to OCEHH bleaching sequence. Results from laboratory studies and mill trials showed about 35-41% reduction in active chlorine at the chlorination stage for hardwoods and 10-20% for softwoods (137, 158, 9). 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 (173). An 18% decrease in kappa number was noticed for birch wood kraft pulp after XE-stage when it was treated with cellulase free xylanases from *Streptomyces thermoviolaceus* (49).

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 (55).

The brightness of soda-AQ pulps bleached by XECEHH and XOCEHH bleaching sequences improved by 2.4 and 3.5% respectively for lemon grass while 3.0 and 2.7% for sofia grass in comparison with their respective controls. This positive gain in brightness of lemon grass and sofia grass pulps possibly resulted from the improved accessibility of bleaching chemicals due to disruption of the xylan chain as a result of xylanase action, thus facilitating the easier removal of lignin carbohydrate complex (LCC) during bleaching (137). The pre-treatment with crude xylanase produced from the cultural filtrates of *A. niger* An76 increased the final brightness of wheat straw pulp by 4-5% (ISO) in comparison with control under the identical bleaching conditions (CEH) (173). Treatment of kraft pulp with a xylano-pectinolytic enzyme concoction (xylanase–pectinase dose of 4.5 and 0.9 U/g of o.d. pulp,) from *Bacillus pumilus* resulted in 25% reduction in active chlorine consumption in subsequent bleaching stages without any decrease in brightness (66).

Viscosity of XECEHH and XOCEHH bleached soda-AQ pulp of lemon grass improved by 2.06 and 2.89% and for sofia grass by 3.37 and 2.15% respectively compared to their controls. The increase in viscosity reflects the selective hydrolysis of low DP xylan in the pulp and enrichment of high molecular weight polysaccharides (106, 132, 145, 166). The crude xylanase extract used in the study had a negligible cellulase contamination; and the increase in viscosity of pulps under study confirmed that there was no adverse effect due to the small amount of cellulases present in the crude enzyme extract. The nonspecific endoglucanases were reported to negatively affect the viscosity of softwood kraft pulp, indicating the degradation of cellulose chains (167, 146). On contrary, a few researchers have also reported that endo-glucanases might play a crucial role in the improvement of drainage (170). The bleaching losses after XECEHH and XOCEHH bleaching sequences were 8.6 and 8.5% respectively for lemon grass soda AQ pulp compared to 9.6 and 9.2% for CEHH and OCEHH bleaching sequences. Similarly, the bleaching loss for XECEHH bleached soda-AQ pulps of sofia grass was lower by 0.6% compared to CEHH bleaching sequence. However, the difference between bleaching losses of sofia grass pulps bleached by XOCEHH and OCEHH sequences were almost negligible.

Table 5.6 shows a comparison in mechanical strength properties of CEHH, XECEHH bleached soda-AQ pulps of lemon and sofia grass beaten at a beating level of 40±1 °SR. The XECEHH bleached soda AQ pulp of lemon grass showed an improvement in tear index (5.19%), burst index (16.23%), tensile index (10.7%) and double fold (17.65%) over CEHH bleached pulp (Figure 5.10a). Alike, sofia grass XECEHH bleached soda-AQ pulp followed the similar trends but tear index of sofia grass hiked by 3.25% in contrast to lemon grass while burst index of lemon grass was better than sofia grass by 6.04% (Figure 5.11a)

Xylanase systems have been developed to ensure selective hydrolysis of the hemicellulose without loss of fiber strength (69). Enzyme treatment of pulp, modified the pulp properties such as improved fiber flexibility and fibrillation (internal and external) both (61). Enzyme helped to soften the fiber walls and increase access to cellulose fibers by breaking the primary wall, which is thin (0.05 micron thick) and relatively impermeable. It also facilitated refining and reduced fines which in turn resulted in better physical properties (29). Although environmentally driven process modifications have made over chlorination much less likely, it must be noted that degradation of cellulose might have resulted if the chlorination charge was too high (36). Reduction of chlorine demand for xylanase pretreated pulps could hence be a possible reason for improved strength properties as higher chlorine charge proved to be detrimental for paper strength (173). Pretreatment of eucalyptus kraft pulp with xylanase from *Streptomyces* sp. QG-11-3, resulted in an improvement in tensile strength and burst factor by 63 and 8% respectively prior to CEHH bleaching sequence (12). The cellulases present in the enzyme preparations could also have played an important role in improvement in mechanical strength properties of enzymatically treated pulps. Pulp fibrillation by cellulases was recognized as a means to enhance strength properties as early as 1959 by Bolaski and co-workers (19). Crude xylanase preparation contaminated with cellulase (0.54 IU/mL) might have resulted into hydrolysis of β-cellulose (degraded cellulose having DP less than 90) that envelops the surface of fibers and slightly improves other mechanical strength properties. In general, the α-cellulose indicates undegraded, higher-molecular-weight cellulose content in pulp; the β-cellulose indicates that of degraded cellulose, and the γ-cellulose consists mainly of hemicellulose (120, 171).

Tables 5.6 and 5.7 describe a comparison in mechanical strength properties of OCEHH, XOCEHH bleached soda-AQ pulps of lemon and sofia grass beaten at a beating level of 40±1 °SR. XOCEHH bleached soda-AQ pulp of lemon grass showed an increment in tear index (14.81%), burst index (13.57%), tensile index (16.03%) and double fold (14%) in comparison with OCEHH bleached pulp (Figure 5.10b). Likewise, 7.69, 9.01, 10.28 and 9.09% augmentations in the respective strength properties, attributable to xylanase action were also established for sofia grass after XOCEHH sequence (Figure 5.11b). The strength properties were comparatively better for XOCEHH bleached lemon and sofia grass soda AQ pulps as held against their XECEHH counterparts. The tear, burst and tensile indices and double fold numbers hiked by 19.8 and 13.89%, 7.01 and 2.37%, 3.26 and 4.69% and 32 and 13.64% for lemon and sofia grass respectively. Furthermore, the properties of lemon grass soda AQ pulp were better as to sofia grass in all respects, which could be attributed to the higher fiber length of lemon grass.

In oxygen bleaching, the reaction that caused yield loss in alkaline media, the peeling reaction, was usually of less importance than random chain cleavage. For peeling reactions, presence of reducing end group (carbonyl group) was essential; however the soda pulps because of their long previous exposure to strongly alkaline conditions in the digester contained very few such reducing end units that had not already been converted to the stable form by the stopping reaction. Moreover, oxygen itself converted reducing end groups to the stable oxidized forms (133), due to which yield loss was generally not a serious problem in oxygen bleaching. Oxygen bleaching was also found to be affected by traces of transition metals (Fe, Cu, Mn) that were unavoidably present in unbleached pulps. They catalyzed the formation of reactive, oxygen based radicals such as hydroxyl radicals that randomly attacked the cellulose chain, thereby decreased the average length of the cellulose chain ultimately leading to chain breakage, manifested as a drop in pulp viscosity (124). $MgSO_4$ applied at 0.05-0.1% Mg^{+2} on o.d. pulp acted as a carbohydrate protector, by precipitating magnesium hydroxide which adsorbed the metal ions, making them unavailable for catalysis of peroxide decomposition (124, 52). Addition of EDTA was also been found to be effective in retarding carbohydrate degradation. The improvement in strength properties of OCEHH bleached pulp over CEHH bleached was probably due to reduced chlorine demand.

The respective copper numbers for CEHH and OCEHH bleached pulps of lemon grass were found to be 0.22 and 0.13 while 0.25 and 0.15 for sofia grass; which got reduced to 0.17 (–22.73%) and 0.11 (–15.38%) in lemon grass while 0.19 (–24%) and 0.12 (–20%) in sofia grass after XECEHH and XOCEHH bleaching sequences respectively. The copper number might be regarded as an index of impurities in paper, such as oxycellulose, hydrocellulose, lignin, and sugars, which possess reducing properties. It was hence a useful criterion for determining changes accompanying deterioration and might therefore be considered as a factor having an indirect bearing on the permanence of paper. It thereby denoted the degree of damage to cellulose in paper (94). As a result of xylanase pretreatment of both the pulp samples under consideration, there was a reduction in TCD which thereby reduced the copper number, as validated by improvement in mechanical strength properties of XECEHH and XOCEHH bleached pulps as compared to respective controls. The results have been mentioned in Tables 5.6 and 5.7.

Figure 5.12 (a, b) shows the effect of xylanase pretreatment on the AOX generated during CEHH and OCEHH bleaching sequences. The AOX levels after CEHH and OCEHH bleaching sequences were 2.44 and 1.88 kg/t of pulp for lemon grass respectively which were markedly reduced to 1.79 (–26.64%) and 1.22 (–35.11%) kg/t of pulp respectively after XECEHH and XOCEHH bleaching sequences. AOX levels for sofia grass were recorded as 2.77 and 2.05 kg/t of pulp for CEHH and OCEHH bleaching sequences which decreased to 1.94 (–29.96%) and 1.42 (–30.73%) as a result of the xylanase treatment (Tables 5.6 and 5.7). In comparison with CEHH bleaching sequence, AOX level reduced by 23% and 26% after OCEHH as a result of oxygen delignification of lemon and sofia grass soda-AQ pulps respectively before 'C' stage, which eventually reduced TCD. AOX reduction was quite remarkable as AOX reduction of about 20–45% for xylanase treated pulps had been reported by Senior and Hamilton (137).

Organochlorine compounds are produced mainly by the reactions between residual lignin present in wood fibers and chlorine used for bleaching (7). Reduction in TCD observed after xylanase pretreatment of bleaching sequences, resulted in lowering the toxicity of the bleach plant effluents also. So the xylanase pretreatment reduced the amount of chlorophenols and other forms of organically bound chlorine (AOX) in the spent bleach liquor (5, 159). The crude xylanase proved its efficacy in making the entire process economical in terms of reduced chemical / chlorine demand and reduced

environmental burden which was being created by the generation of aromatic organic halides. 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 (138). Effluent AOX was reduced by 15.09% after eucalyptus kraft pulp was pretreated by commercial xylanase after same treatment compared to control (58).

Tables 5.6 and 5.7 reveal that the COD in combined bleach effluent of OCEHH bleaching sequence dropped by 17 and 19.44% respectively for lemon and sofia grass compared to CEHH bleaching sequence because of reduction in TCD as a result of O_2 delignification prior to bleaching. The COD load, which was 1251 mg/mL in combined bleach effluent, after CEHH bleaching of lemon grass pulp elevated to 1630 mg/mL (+23.25%) in XECEHH sequence (Figure 5.13a); while after OCEHH, the COD value recorded as 1038 mg/L increased to 1322 mg/L (+21.48%) in the combined effluent of XOCEHH bleach sequence. In case of sofia grass, the respective COD elevations after XECEHH and XOCEHH were recorded as 18.59 and 16.28% in comparison to controls (Figure 5.13b). In comparison to CEHH, a decrease in colour of combined bleach effluent by 29.72 and 30.66% was recorded for lemon and sofia grass soda-AQ pulps respectively in OCEHH bleaching sequence. Regardless, of the bleaching chemicals used, oxygen delignification decreased the BOD, COD and colour of the effluents (156). The colour of lemon grass soda-AQ pulp in combined bleach effluent generated during CEHH and OCEHH bleaching sequences were 2140 and 1504 PTU respectively and introduction of xylanases in XECEHH and XOCEHH sequences further increased the colour to 2480 (+13.71%) and 1847 (+18.57%) PTU respectively. An increase in colour (+14.6% and +19.69%) was also noticed for sofia grass in the combined bleach effluent generated during XECEHH and XOCEHH compared to respective controls.

Hydrolytic action of enzyme weakens carbohydrate bonds in the pulp and causes its dissolution, which lead to increased concentration of residual lignin carbohydrate complexes (RLCC) and hydrolyzed xylan in the effluent, leading to increase in COD and color. Similar observations were made by Roncero et al. (132, 86, 166). 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 (58, 59). These effluents were amenable to biological degradation, due to high proportion

of degraded xylan as had been confirmed by Onysko (104), Senior and Hamilton (138). An increase of 27.76% was noted in the colour of bleaching effluent when *E. globulus* pulp was pretreated with xylanase in a bleaching sequence OD_1PD_2 (166). The colour increase was highest in case of XOCEHH as xylan hydrolysis was more when oxygen delignification was carried out after xylanase stage (129).

5.3.3 Effect of xylanase pretreatment on ECF bleaching

Tables 5.8 and 5.9 show the effect of elemental chlorine free (ECF) bleaching sequences ODED and ODEDP and their enzyme associated sequences (XODED and XODEDP) on lemon grass and sofia grass soda-AQ pulps brightness, viscosity and bleaching losses. The brightness and viscosity of soda-AQ pulps bleached by ODED bleaching sequence were 80.5% (ISO) and 9.91 cps for lemon grass; 81.8% (ISO) and 8.93 cps for sofia grass respectively. In ODEDP bleaching sequence of lemon grass, the pulp brightness further improved by 2.3% with a fall in pulp viscosity by nearly 2%. Similar results were obtained for sofia grass soda-AQ pulp in which, ODEDP bleaching produced a slight increment in brightness (1.1%), along with a decrease in viscosity (1.5%) over ODED bleached pulp. A comparison of peroxide and conventional oxygen bleaching showed higher brightness and lower viscosity for a peroxide bleached pulp than for a corresponding oxygen bleached kraft pulp; that is, the selectivity for peroxide bleached pulp was lower (76, 77). The bleaching losses for ODED and ODEDP bleached pulps of lemon grass were 7.45 and 7.6% along with 8.1 and 7.8% for sofia grass respectively.

The brightness and viscosity of ODED and ODEDP bleached pulps of lemon and sofia grass showed improvement over CEHH sequence. However, introduction of oxygen prior to CEHH sequence (OCEHH) further enhanced both the properties. Also, an overall improvement in mechanical strength properties of the pulps was noticeable after ODED and ODEDP bleaching sequences in comparison with that of CEHH and OCEHH bleaching sequences because of the less damaging action of chlorine dioxide towards cellulose than chlorine (36). The combined effluent generated during ODED and ODEDP bleaching of lemon and sofia grass soda-AQ pulp showed a decrease in AOX, COD and colour compared to CEHH bleaching sequence. In combined effluent of lemon grass ODED and ODEDP bleaching sequences, COD decreased by 14.71 and 18.15% respectively while colour decreased by 28.08 and 43.46 % respectively in comparison with

CEHH bleaching sequence. For sofia grass, the respective decreases in COD were 15.06 and 19.71% whereas the decrease in colour was noticed as 33.02 and 17.85% respectively. AOX formation from lemon grass and sofia grass was reduced by 84.84 and 82.67% respectively following investigated ECF bleaching sequences when compared to CEHH bleaching sequence.

As suggested by Pryke, ECF bleaching holds potential for depreciating the chloroform and total organic halide (AOX) formation by 90% (115); use of chlorine dioxide in place of elemental chlorine reduced the amount of AOX in the effluents (89, 92). Chlorine dioxide is a stronger bleaching agent than chlorine. During bleaching the reactions with chlorine dioxide are oxidative to a large extent, which reduces the amount of AOX formed. The atomic chlorine content of chlorine dioxide is lower than chlorine, which lead to decreased AOX in effluents (142). The chlorinated phenols generated in C-stage filtrate, carried over to E-stage undergo condensation to form dioxins. Bleach filtrate received after E-stage hence was perceived as the point with largest concentration of dioxins and the 2,3,7,8-TCDD, 2,3,7,8-TCDF and 1,2,7,8-TCDF congeners (135). Between bleaching stages caustic chemicals had often been used to remove the dissolved lignin and wash the fibers before they were subjected to additional bleaching. It was apparently during this extraction that much of the dioxin created by chlorine bleaching found its way into the waste stream. An NCASI review (98) found that a 50-80% decrease in colour occured at 90-100% ClO_2 substitution, while 20-25% of the COD reduced at 100% chlorine dioxide substitution (81). While taking into consideration, the oxygen prebleaching, 63-80% decreases in colour of bleaching effluent (35) were found whereas the COD values were lowered in proportion to the reduction in kappa number (typically 40-50%) (45).

As a matter of fact, oxygen delignification could cause pulp degradation and reduce paper strength; however the problem was tackled by the addition of $MgSO_4$ which prevented degradation of cellulose fibres. Oxygen delignification and bleaching produced pulp that compared favourably with conventional bleached pulps in most ways. Strength properties of oxygen pulps were slightly less than conventional pulps, but might be acceptable for some products if delignification by oxygen did not exceed about 40 to 50% of the pretreatment lignin level (154). Experts differed as to the viscosity and strength of oxygen pulp compared to conventional pulp. The hemicellulose polymers present in the

pulp protected the cellulose from degradation by hydroxyl free radicals ($OH^{\bullet}$) present during oxygen delignification as suggested by Guay (53). The hemicellulose polymers, with a low molecular weight, competed with the cellulose for hydroxyl free radicals and thus avoided undue loss of molecular weight of the cellulose. Use of sodium salts of ethylenediamine tetraacetic acid (EDTA) and diethylenetriaminopentaacetic (acid DTPA) masked the activities of transition metals by forming polydendate ligand molecules and thereby mitigated the degradation of carbohydrates. Brightness stability for oxygen pulps was equal to or better than that of conventional pulps. Pulp viscosity increased dramatically with only 5-10% chlorine dioxide substitution and then decreased slightly as percent substitution increased further at a fixed chemical charge.

While considering the effect of xylanase pretreatment during XODED and XODEDP bleaching sequences on brightness, viscosity and bleaching losses of the two soda-AQ pulps, it was observed that brightness of lemon grass soda-AQ pulp increased by 2.6 and 2.2% (ISO) respectively in comparison with ODED and ODEDP bleaching sequences at the same chlorine dioxide charge, whereas the respective increases for sofia grass pulp were 3.3 and 4.4% (Tables 5.8 and 5.9). Xylanase treatment improved the accessibility of the pulps for the bleaching chemicals. It decreased the diffusion resistance to the outward movement of the degraded lignin fragments and allowed the removal of less degraded lignin fragments from the fiber wall. This was the reason for the higher brightness of the xylanase treated pulps at the same bleaching reagent consumption (160). 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 (18) 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 (82).

The concentration of residual chlorine dioxide in combined filtrate of XODED bleached pulp of lemon and sofia grass increased by 32.43 and 39.64% compared to ODED bleaching sequences at the same ClO_2 charge (1.34%). It means xylanase treated pulps required lesser ClO_2 charge compared to control which directly confers the savings in chemical charge attaining the same brightness of pulp. This also suggests that the treated pulps reached the chlorine saturation point sooner than the control pulp (56). An increased level of residual ClO_2 and pulp brightness obtained from the bleaching

sequences with crude xylanase pretreatment indicate that the ClO_2 charge might be reduced to a good extent to achieve a given target brightness (90). By using crude xylanase from *Bacillus licheniformis 77-2*, Damiano et al. (31) reported a 30% reduction in the requirement of ClO_2 for bleaching of eucalyptus kraft pulp.

The viscosity of XODED and XODEDP bleached lemon grass soda-AQ pulp increased by 0.43 and 0.53% in comparison with ODED and ODEDP bleaching sequences respectively. Likewise, for sofia grass, the respective increases in viscosity were 1.0 and 4.35%. The results indicate that the crude xylanase only hydrolyzed xylan (106, 28, 132, 145, 166) and not cellulose chains in pulp (167, 146). The bleaching losses after XODED and XODEDP were slightly more in comparison with ODED and ODEDP bleaching sequences as both (control and xylanase pretreated) pulps had been charged with same chlorine dioxide charge. Copper number for lemon grass pulp decreased by 36.67 and 29.71% while 33.33 and 25% for sofia grass pulp, respectively after XODED and XODEDP bleaching sequences in comparison with controls. The results depict that the xylanase pretreatment bestowed reduction in the degree of damage to cellulose of both the soda AQ-pulps after full bleaching sequences in terms of depreciation in copper number.

For lemon grass and sofia grass ECF bleached pulps, Tables 5.10 and 5.11 present a comparison of mechanical strength properties at same beating level during ODED and XODED; ODEDP and XODEDP bleaching sequences. Tear index for lemon grass increased by 6.28%; burst index, tensile index and double fold numbers deflated by 5.32, 12.42 and 9.62% respectively after XODED in comparison with ODED bleaching sequence (Figure 5.14a). Similarly, for sofia grass, tear index marked a net increase of 9.4%, while all other strength properties decreased (Figure 5.15a) as a result of xylanase pretreatment in XODED bleach sequence, when compared to the control (ODED). The results for strength properties during ODEDP and XODEDP bleach sequences as presented in Figure 5.14b, demonstrate a 4.41% increase in tear index while burst index, tensile index and double fold were reduced by 9.41, 12.98 and 34.78% respectively after XODEDP bleaching sequence of lemon grass soda-AQ pulp in comparison with control. In case of sofia grass (Figure 5.15 b), tear index marked an increase of 11.85% with burst index, tensile index and double fold number being reduced by 9.06, 8.49 and 17.39% respectively after treatment with xylanases in XODEDP sequence. In comparison to control, all the strength properties decreased with an exception of tear strength, after the xylanase pretreatment in both the ECF sequences.

Tear strength is a complicated combination of fiber strength, fiber length and fiber bonding. The necessary work needed to be done to pull the fibers loose depends on the length of the fibers as well as the bond strength (85). Enzyme treatment hydrolyses xylan (low molecular weight) from the pulp and results into an increase in the average molecular weight of the polymer system (132, 106). Inspite of the fact that molecular chains in hemicelluloses are much shorter than those in cellulose and do not make the same contribution to the strength of paper sheets, hemicelluloses are extremely important for bonding in paper sheets (36). Removal of hemicelluloses hence weakened the inter fiber bonds which caused the fibers to be pulled out intact as the fracture propagated across the sample. Therefore, tear index slightly improved but other properties like, burst index, tensile index and double fold numbers which depended upon hydrogen bonding might decrease due to depolymerization of xylan (147). The increase in tear index could hence be attributed to a greater tendency of xylanase treated pulps towards external fibrillation, owing to the elimination of xylans deposited on the surface of fiber, which would have limited this fibrillation (129, 99). The retention of hemicelluloses is considered to be favourable for the mechanical strength properties of papermaking fibers, because of their positive effect on the interfibrillar bonding during paper-sheet formation (27). Partial hemicellulose (xylan) removal during enzymatic stage could therefore have adversely affected the pulp strengths (specially tensile and burst index which depended on interfiber bonding of paper). A decline in interfiber bonding was observed after action of xylanases on pulps (101, 125). In ECF bleaching of lemon grass and sofia grass pulps, another strong reason for lower strength properties of xylanase pretreated pulps seemed to be the use of same chemical charges in both ODED and XODEDP sequences. After bleaching with the same chemical loadings, some reports indicated an improved tear index while others claimed a little change in papermaking properties (29, 132). The pretreatment of wheat straw pulp with xylanase produced by *Streptomyces cyaneus* SN32, increased the tear index and burst factor (99) while xylanase pretreatment increased the tear index of *Eucalyptus globulus* kraft pulp leaving tensile index unaffected (129). The xylanase pretreated wheat straw pulp showed a slightly lower tensile index and breaking length than the control (80) 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 (56).

Figure 5.16 (a, b) shows the effect of xylanase pretreatment on the AOX generation from lemon grass and sofia grass ODED and ODEDP bleach sequences. While considering the effect of crude xylanases from *C.cinerea* HK-1 on AOX formation after ECF bleach sequences (ODED and ODEDP), a major decrease in AOX from 0.37 Kg/t (ODEDP) to 0.238 Kg/t was recorded (-35.68%) after XODED in lemon grass. For sofia grass, the AOX values were found to be 0.44 Kg/t after ODED which decreased to 0.255 Kg/t after XODED with a net percent decrease of 46.88%. The introduction of xylanase pretreatment before ECF bleach sequences resulted into an increase in residual ClO_2 from 6.25 to 9.25% in lemon grass and 6.7 to 11.1% in sofia grass, which provided an evidence for the fact that the xylanase treated pulps used lesser chlorine dioxide than control. This suggested that the treated pulps reached the chlorine saturation point sooner than the control pulp (56). It explains the lower level of AOX obtained after xylanase pretreatment of the two pulps before ODED and ODEDP bleaching sequences.

Figure 5.17 (a, b) shows the comparison between combined effluents generated after ODED, ODEDP, XODED and XODEDP bleaching sequences. For lemon grass, the COD after ODED and ODEDP bleaching sequences were 1067 and 1024 mg/L, which increased to 1440 (+25.91%) and 1288 (20.50%) mg/L after XODED and XODEDP bleaching sequences. In sofia grass, the xylanase treatment under XODED and XODEDP increased the COD of the effluent thus generated by 16.78% and 14.06% compared to respective controls. An increase of 51.13% was noticed in the COD of bleaching effluent generated during the bleaching of *E. globulus* pulp in ODPD sequence as a result of xylanase pretreatment (166). Similar trends for increase in COD after xylanase pretreatment had been pre-established by Bajpai (6), Mathur et al. (86) and Jain et al. (59) as a result of hemicellulose removal from pulps.

The values of colour in combined bleach effluent generated after ODED and ODEDP bleaching sequences were 1539 and 1210 PTU which increased to 1915 (+19.64%) and 1702 (+28.91%) PTU after xylanase pretreatment in XODED and XODEDP bleaching sequences. For sofia grass the respective colour increments were recorded as 20.16 and 18.50% compared to respective controls. An increase in colour of bleaching effluents after xylanase pretreatment was also observed by Roncero et al. (132) and Vidal et al. (166) 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 sequence

Tables 5.12 and 5.13 reveal the effect of xylanase treatment before and after oxygen treatment ($OX(E_{OP})P$ and $XO(E_{OP})P$) on $O(E_{OP})P$ bleaching sequence on brightness, viscosity and bleaching losses of lemon grass and sofia grass soda-AQ pulps. The brightness for lemon grass and sofia grass $O(E_{OP})P$ bleached soda-AQ pulps were 74.2 and 76.4% (ISO) respectively, while the respective viscosity values were 10.8 and 9.7 cps. The bleaching losses after $O(E_{OP})P$ bleaching sequence were 6.6% for lemon grass and 6.4% for sofia grass. The pulp viscosity after $O(E_{OP})P$ bleaching sequence was quite high and bleaching losses were low for both the pulps in comparison to that of CEHH, OCEHH (conventional) and ODED & ODEDP (ECF) bleaching sequences because of the fact that no chlorine containing chemical was used during $O(E_{OP})P$ sequence.

While considering the effect of xylanase pretreatment during $O(E_{OP})P$ bleaching on brightness, viscosity and bleaching losses of the two soda-AQ pulps, the efficacy of xylanase was checked in two ways, i.e. by applying the crude xylanases before oxygen delignification ($XO(E_{OP})P$) and after oxygen delignification ($OX(E_{OP})P$). Brightness of lemon grass soda-AQ pulp increased by 2.7 and 4.5% (ISO) along with 2.2 and 4.0% ISO brightness gains for sofia grass soda-AQ pulp respectively after $OX(E_{OP})P$ and $XO(E_{OP})P$ bleaching sequences in comparison with $O(E_{OP})P$ bleaching sequence. A xylanase pretreatment before oxygen delignification ($XO(E_{OP})P$) yielded better brightness gains than xylanase pretreatment after oxygen delignification ($OX(E_{OP})P$). Xylanase pretreatment hydrolyzed the pulp xylan, hence allowing better access of bleaching chemicals to the residual lignin and easier extraction of lignin from pulp fibers, which in turn improved the brightness of pulp (32). The xylan hydrolysis was more effective when the xylanase pretreatment was applied prior to oxygen stage rather than after oxygen stage as had also been suggested by Roncero et al. (129).

The pulp viscosity for lemon grass improved by 4.4 and 1.8% respectively after $OX(E_{OP})P$ and $XO(E_{OP})P$ bleaching sequences when compared to control, while the viscosity of sofia grass pulp improved by 3.96 and 5.83% respectively due to removal of low DP xylan as a result of xylanase pretreatment (106, 28, 132, 145 166, 167, 146). The viscosity gains for both the pulps were higher with $OX(E_{OP})P$ bleach sequence than $XO(E_{OP})P$.

Bleaching losses in lemon grass increased by 7.0 and 7.3% along with 6.9 and 7.1% losses in sofia grass 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. These results also clarify the fact that a comparatively low pulp yield was obtained by applying xylanases before oxygen delignification stage because bleaching losses were 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.

Tables 5.14 and 5.15 show 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 in the two pulps under study. Copper number for lemon grass soda AQ pulp after O(E_{OP})P bleaching sequence was 0.09 which decreased by 33.3 and 27.8 % after OX(E_{OP})P and XO(E_{OP}) bleaching sequences. For sofia grass, a net decrease of 25% in copper number was recorded after both the sequences. Here, the reduction in copper number was a sign of reduction in degree of cellulose damage of the two soda-AQ pulps as a result of implication of xylanase pretreatment stage.

Figure 5.18(a, b) shows the comparison of mechanical strength properties of lemon and sofia grass soda-AQ pulps bleached by O(E_{OP})P, OX(E_{OP})P and XO(E_{OP})P bleaching sequences at 40±1 °SR. Figure 5.18a shows that for lemon grass pulp, tear index increased by 4.7 and 2.0% 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 numbers decreased by 5.4, 7.1 and 15.7% after OX(E_{OP})P bleaching sequence and 11.8, 12.5 and 11.8% respectively after XO(E_{OP})P bleaching sequence when compared with O(E_{OP})P bleaching sequence. Figure 5.18b indicates the similar trends in results for sofia grass wherein the tear index got increased by 2.4 and 1.63%, with all other strength properties showing a decrease after OX(E_{OP})P and XO(E_{OP})P bleaching sequence in comparison to the control. The probable reason for decrease in strength properties noticed could be the extra removal of xylan after xylanase pretreatment of pulp which could have adversely affected the interfibrillar bonding of paper (27). Xyloglucans were found to act as "tie molecules" in connecting cellulose microfibrils to each other. The degradation and dissolution of these tie molecules lead to a decrease in the fiber strength (78). The xylan removal also enhanced the degree of external fibrillation on fiber surface, which could have improved the tear index after xylanase pretreatment of lemon grass and sofia grass pulps (129). Results also throw light

on the inferior mechanical strength properties of the pulps after XO(E_{OP})P bleaching sequence compared to OX(E_{OP})P bleaching sequence. The reason being that xylan removal was more after XO(E_{OP})P bleaching sequence than that of OX(E_{OP})P bleaching sequence. Haixuan Zou et al. demonstrated that hemicelluloses, specifically xylan, could act as protective factors against cellulose degradation and losses of strength and viscosity (176). The mechanism proposed for the observed results stated that the hemicelluloses, low molecular weight polymers, competed with the cellulose for free radicals (generated during oxygen delignification) and, thus, prevented the undue loss of molecular weight of the cellulose (176).

Figure 5.19 (a) shows the comparison of COD and colour among combined bleach effluents generated during O(E_{OP})P, OX(E_{OP})P and XO(E_{OP})P bleaching sequences in lemon grass. COD after O(E_{OP})P bleaching sequence was 1430 mg/L which increased to 1588 (+9.9%) and 1650 (13.3%) mg/L respectively after OX(E_{OP})P and XO(E_{OP})P bleaching sequences. As per the results depicted in Figure 5.19 (b), the COD value obtained from sofia grass bleach effluent (O(E_{OP})P) was 1390 mg/L which marked a respective increase by 12.41 and 14.83% after OX(E_{OP})P and XO(E_{OP})P bleaching sequences. Colour of the combined effluents produced by O(E_{OP})P bleaching sequence in lemon and sofia grass soda AQ pulps were recorded as 2320 and 2522 PTU which increased to 3128 (+25.8%) and 3385 (+31.5%) PTU after OX(E_{OP})P and XO(E_{OP})P bleaching sequences for lemon grass while the respective increases in sofia grass were found to be 20.69 and 25.21%. The increase in COD and colour of bleach effluents was quite obvious in case of xylanase pretreatment since the hydrolytic action of xylanase led to weakening of the carbohydrate bonds in the pulp and its dissolution into the media. Thus the concentration of lignin and hydrolyzed xylan in the effluent was also increased markedly which further added to the colour and organic load in effluents (132, 166, 6, 86). The increase in COD and colour after XO(E_{OP})P bleaching sequence was more than that of OX(E_{OP})P bleaching sequence which indicated that removal of xylan and lignin after XO stage was more as compared to OX stage (129).

5.3.5 Scanning electron microscopy

Scanning electron microscopic (SEM) study was carried out in order to attain a better understanding of the effect of xylanase pretreatment on lemon and sofia grass pulp bleachabilities. The surface of untreated, control fibers was smooth and showed no signs

of external fibrillation and swelling as shown in microphotographs in Plates 5.1A (lemon grass) and 5.2A (sofia grass). Plates 5.1 (B,C) and 5.2 (B,C) represent the xylanase pre-treated fibers of lemon and sofia grasses respectively, bearing cracks, peelings, swelling and external fibrillation on their surface as a result of xylanase action. The fibers having undergone xylanase treatment had a rougher surface with striations and splits, i.e. a more open surface. These results confirmed that xylanase acted by hydrolyzing the xylans deposited on the surface of fibers during alkaline pulping, which constituted a physical barrier to penetration by bleaching agents. Their elimination facilitated the flow of bleaching agents, which explains the bleach boosting effect of the xylanases (129, 137). Xylanase treatment improved accessibility of bleaching chemicals to the pulps, decreased diffusion resistance to outward movement of the degraded lignin fragments and allowed the removal of less degraded lignin fragments from the cell wall. As a result, pulps treated with xylanase showed lower kappa number and higher brightness and viscosity than pulps not treated with the xylanase (130). Beg et al., reported that xylanase from *Streptomyces* sp. QG-11-3 introduced greater porosity, swelling and separation of pulp microfibrils in eucalyptus pulp fibers compared to the smooth surface of untreated pulp fiber (12). 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 (80). The observations were in agreement with other authors too (18, 128). Plates 5.1E and 5.2E show the xylanase treated fibers of lemon and sofia grasses respectively, beaten up to 40 ±1 °SR. These photomicrographs depict that xylanase pretreated fibers after beating were more fibrillated and showed more roughness on their surface than untreated fibers (Plates 5.1D and 5.2D). It could be concluded from these results that xylanase pretreated fibers would require lesser rotations in a PFI mill to reach a targeted °SR in comparison with untreated fibers thus saving a certain amount of electrical energy too. The results were 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 (15). 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 (132, 4).

Table 5.1: Optimization of xylanase dose for prebleaching of soda-AQ pulps of lemon and sofia grasses

	Xylanase dose, IU/g	* Kappa number	Reducing sugars released, mg/g	*Viscosity, cps	Bright-ness, % (ISO)	Chromophores released, Optical density		
						237 nm	280 nm	465 nm
Lemon grass	0	11.6±0.15	0.20±0.02	27.10±0.09	30.6 ±0.5	–	–	–
	4	10.75±0.12	1.50±0.02	27.94±0.08	35.1 ±0.2	0.275 ±0.002	0.219 ±0.005	0.156 ±0.003
	8	9.93±0.10	2.75±0.08	28.88±0.12	37.9 ±0.3	0.388 ±0.004	0.298 ±0.003	0.244 ±0.005
	10	9.18±0.18	4.05±0.16	29.72±0.10	38.4 ±0.6	0.452 ±0.003	0.321 ±0.003	0.287 ±0.002
	12	8.92±0.11	5.30±0.13	29.41±0.08	38.4 ±0.19	0.469 ±0.004	0.330 ±0.006	0.298 ±0.004
	16	8.83±0.08	7.36±0.16	27.67±0.11	39.0 ±0.7	0.475 ±0.002	0.345 ±0.005	0.310 ±0.004
	20	8.88±0.10	7.70±0.21	26.02±0.09	38.5 ±0.3	0.478 ±0.006	0.351 ±0.004	0.315 ±0.006
	25	8.90±0.07	8.34±0.11	24.65±0.14	38.4 ±0.3	0.481 ±0.005	0.350 ±0.007	0.318 ±0.003
Sofia grass	0	14.80±0.25	0.32±0.01	26.56±0.11	32.3 ±0.1	–	–	–
	4	12.14±0.14	2.15±0.14	27.13±0.08	36.2 ±0.5	0.343 ±0.003	0.235 ±0.002	0.173 ±0.001
	8	10.68±0.12	3.87±0.11	28.14±0.10	38. 9±0.2	0.462 ±0.002	0.326 ±0.001	0.312 ±0.003
	10	10.54±0.15	5.14±0.22	27.56±0.13	39.1 ±0.1	0.497 ±0.002	0.354 ±0.002	0.324 ±0.003
	12	10.48±0.20	5.98±0.19	27.31±0.09	39.1 ±0.4	0.516 ±0.004	0.403 ±0.004	0.356 ±0.002
	16	10.35±0.14	6.28±0.20	24.66±0.15	38.9 ±0.6	0.524 ±0.006	0.429 ±0.006	0.362 ±0.005
	20	10.28±0.10	6.79±0.16	23.20±0.05	38.5 ±0.2	0.538 ±0.006	0.435 ±0.003	0.380 ±0.007
	25	10.31±0.08	7.32±0.17	21.13±0.11	38.4 ±0.2	0.532 ±0.007	0.438 ±0.005	0.392 ±0.004

* refers values after XE extraction
± refers to standard deviation
Operational conditions: Extraction stage =1.2% NaOH at 70 °C temperature for 90 min,
X stage= reaction time 120 min, pH 6.4, consistency 8 %, temperature 55±2 °C

Table 5.2: Optimization of reaction time for prebleaching of soda-AQ pulps of lemon and sofia grasses

	Reaction time,min.	* Kappa number	Reducing sugars released, mg/g	*Viscosity , cps	Bright-ness, %(ISO)	Chromophores released, Optical density		
						237 nm	280 nm	465 nm
Lemon grass	30	10.80±0.10	1.21±0.01	27.91±0.10	34.5 ±0.20	0.114 ±0.001	0.145 ±0.002	0.15 ±0.001
	60	9.86±0.16	2.45±0.10	28.20±0.07	36.9 ±0.25	0.403 ±0.003	0.210 ±0.002	0.228 ±0.003
	90	9.44±0.13	3.84±0.12	28.85±0.05	37.8 ±0.18	0.455 ±0.004	0.345 ±0.003	0.363 ±0.004
	120	9.10±0.11	4.10±0.21	29.42±0.10	38.4 ±0.35	0.482 ±0.002	0.427 ±0.005	0.443 ±0.006
	180	8.86±0.07	4.45±0.16	29.36±0.08	38.9 ±0.22	0.495 ±0.005	0.430 ±0.004	0.448 ±0.004
	240	8.78±0.10	4.52±0.11	28.54±0.12	38.7 ±0.27	0.501 ±0.006	0.425 ±0.007	0.450 ±0.002
Sofia grass	30	12.70±0.08	3.43±0.14	26.71±0.06	35.3 ±0.26	0.134 ±0.001	0.117 ±0.001	0.122 ±0.002
	60	11.67±0.15	5.47±0.12	27.19±0.11	35.8 ±0.31	0.365 ±0.001	0.312 ±0.003	0.234 ±0.004
	90	11.12±0.10	5.89±0.22	27.56±0.18	36.7 ±0.25	0.487 ±0.003	0.393 ±0.002	0.279 ±0.002
	120	10.67±0.12	6.14±0.12	28.12±0.10	38.8 ±0.18	0.516 ±0.004	0.464 ±0.004	0.312 ±0.005
	180	10.65±0.11	6.36±0.18	27.48±0.14	38.5 ±0.13	0.510 ±0.006	0.468 ±0.004	0.315 ±0.003
	240	10.68±0.07	6.35±0.20	27.36±0.12	38.3 ±0.22	0.512 ±0.004	0.471 ±0.005	0.310 ±0.006

* refers values after XE extraction
± Refers to standard deviation
Operational conditions: Extraction stage =1.2% NaOH at 70 °C temperature for 90 min,
X stage= dose 10 IU/g for lemon grass and 8 IU/g for sofia grass, pH 6.4, consistency 8 %, temperature 55±2 °C

able 5.3: Optimization of consistency for prebleaching of soda-AQ pulps of lemon and sofia 'asses

	Cy %	* Kappa number	Reducing sugars released, mg/g	* Viscosity, cps	Bright-ness %,(ISO)	Chromophores released, Optical density		
						237 nm	280 nm	465 nm
Lemon grass	2	11.23±0.07	0.28±0.01	27.12±0.05	32.60 ±0.18	0.242 ±0.002	0.193 ±0.003	0.168 ±0.002
	4	10.20±0.11	2.91±0.09	27.48±0.11	35.43 ±0.23	0.298 ±0.004	0.235 ±0.004	0.212 ±0.003
	6	9.37±0.12	4.21±0.12	28.12±0.09	37.32 ±0.20	0.366 ±0.002	0.309 ±0.005	0.285 ±0.006
	8	9.04±0.08	5.10±0.18	29.41±0.08	39.60 ±0.35	0.452±0 .005	0.379 ±0.002	0.363 ±0.004
	10	8.81±0.11	5.38±0.20	29.72±0.10	41.55 ±0.27	0.517 ±0.006	0.398 ±0.003	0.421 ±0.007
	12	8.72±0.10	5.72±0.15	28.11±0.06	41.58 ±0.34	0.520 ±0.004	0.411 ±0.006	0.427 ±0.005
Sofia grass	2	14.21±0.10	0.35±0.02	26.54±0.10	33.61 ±0.15	0.203 ±0.002	0.137 ±0.001	0.150 ±0.002
	4	13.54±0.16	2.76±0.06	26.86±0.11	36.20 ±0.20	0.356 ±0.001	0.225 ±0.004	0.201 ±0.003
	6	12.87±0.07	4.58±0.10	27.20±0.18	37.94 ±0.18	0.425 ±0.004	0.258 ±0.002	0.277 ±0.002
	8	10.71±0.12	6.14±0.12	28.12±0.10	38.83 ±0.14	0.516 ±0.002	0.312 ±0.005	0.348 ±0.004
	10	10.40±0.12	6.88±0.11	28.65±0.14	39.74 ±0.30	0.632 ±0.006	0.387 ±0.003	0.415 ±0.004
	12	10.03±0.16	7.20±0.18	28.51±0.20	39.72 ±0.27	0.628 ±0.003	0.390 ±0.004	0.433 ±0.003

y % = Consistency %
refers values after XE extraction
Refers to standard deviation
perational conditions: Extraction stage =1.2% NaOH at 70 °C temperature for 90 min,
stage= dose 10 IU/g for lemon grass and 8 IU/g for sofia grass, reaction time 120 min, pH 6.4, temperature 55±2 °C

Table 5.4: Effect of conventional bleaching on pulp shrinkage, brightness and viscosity of soda-AQ pulps of lemon and sofia grasses

Particulars	Bleaching sequence			
	Lemon grass		Sofia grass	
	CEHH	XECEHH	CEHH	XECEHH
Unbleached pulp kappa number	11.60	11.60	14.80	14.80
Unbleached pulp brightness, % (ISO)	30.61	30.61	32.25	32.25
Unbleached pulp viscosity, cps	27.10	27.10	26.56	26.56
Xylanase stage (X)				
Amount of xylanase added (on o.d. pulp basis), IU/g	–	10	–	8
Final pH	–	6.4	–	6.4
Alkali extraction stage (E)				
NaOH applied, % (o.d. pulp basis)	–	1.2	–	1.2
Initial pH	–	11.5	–	11.5
Final pH	–	10.6	–	10.2
kappa number of xylanase treated pulp	–	8.81	–	11.04
Chlorination stage (C)				
Cl_2 applied, % (o.d. pulp basis)	1.45	1.101	1.85	1.300
Cl_2 consumed, % (o.d. pulp basis	1.446	1.097	1.843	1.297
Amount of Cl_2 consumed, %	99.7	99.5	99.6	99.7
Final pH	1.9	2.4	1.8	2.2
Alkali extraction stage (E)				
NaOH applied, % (o.d. pulp basis)	0.76	0.581	0.96	0.680
Initial pH	11.0	11.4	11.0	11.5
Final pH	11.2	11.2	11.6	11.4
Hypochlorite stage (H_1)				
Hypo applied as available Cl_2, % (o.d. pulp basis)	1.015	0.771	1.295	0.910
Hypo consumed as available Cl_2, % (o.d. pulp basis)	0.965	0.728	1.250	0.865
Hypo consumed, %	95.03	94.42	96.5	95.06
Final pH	11.5	11.0	11.4	11.2
Hypochlorite stage (H_2)				
Hypo applied as available Cl_2, % (o.d. pulp basis)	0.435	0.331	0.555	0.390
Hypo consumed as available Cl_2, % (o.d. pulp basis)	0.398	0.299	0.518	0.360
Hypo consumed, %	91.4	90.33	93.3	92.31
Final pH	11.2	11.2	11.2	11.2
Total Cl_2 applied, % (o.d. pulp basis)	2.85	2.204	3.70	2.60
Total Cl_2 consumed, % (o.d. pulp basis)	2.76	2.124	3.611	2.522
Total Cl_2 consumed on Cl_2 basis, %	96.8	96.37	97.6	97.0
Total residual Cl_2, %	3.2	3.63	2.4	3.0
Bleaching losses, %	9.6	8.6	9.1	8.5
Bleached pulp yield, %	37.31±1.3	38.22±2.2	40.46±2.2	40.70±3.0
Pulp brightness, % (ISO)	78.4±0.52	81.2±0.7	80.6±0.47	83.5±0.36
Pulp viscosity, cps	9.5±0.008	9.7±0.020	8.4±0.011	8.7±0.015

Bleaching conditions	X	C	E	H_1	H_2
Consistency, %	10	3	10	10	10
Temperature, °C	55±2	Ambient	60±2	45±2	45±2
Time, min	120	30	60	60	60

± refers standard deviation

Table 5.5: Effect of xylanase pretreatment on pulp shrinkage, brightness and viscosity of soda-AQ pulps of lemon and sofia grasses during conventional bleaching

Particulars			Bleaching sequence			
			Lemon grass		Sofia grass	
			OCEHH	XOCEHH	OCEHH	XOCEHH
Unbleached pulp kappa number			11.60	11.60	14.80	14.80
Unbleached pulp brightness, % (ISO)			30.61	30.61	32.25	32.25
Unbleached pulp viscosity, cps			27.10	27.10	26.56	26.56
Xylanase stage (X)						
Amount of xylanase added (on o.d. pulp basis), IU/g			–	10	–	8
Final pH			–	6.4	–	6.4
Oxygen stage (O)						
O_2 pressure, kg/cm^2			5	5	5	5
$MgSO_4$ applied, % (o.d. pulp basis)			0.1	0.1	0.1	0.1
EDTA applied, % (o.d. pulp basis)			0.1	0.1	0.1	0.1
NaOH applied, % (o.d. pulp basis)			2.0	2.0	2.0	2.0
Final pH			12	12	12	12
Kappa number of xylanase and O_2 delignified pulp			7.50	6.65	9.87	8.21
Chlorination stage (C)						
Cl_2 applied, % (o.d. pulp basis)			0.938	0.831	1.234	1.026
Cl_2 consumed, % (o.d. pulp basis)			0.925	0.828	1.224	1.020
Amount of Cl_2 consumed, %			98.7	99.64	99.2	99.42
Final pH			1.75	2.4	1.77	2.5
Alkali extraction stage (E)						
NaOH applied, % (o.d. pulp basis)			0.50	0.446	0.65	0.543
Initial pH			11.0	11.5	11.0	11.4
Final pH			11.1	11.3	11.3	11.1
Hypochlorite stage (H_1)						
Hypo applied as available Cl_2, % (o.d. pulp basis)			0.657	0.582	0.864	0.718
Hypo consumed as available Cl_2, % (o.d. pulp basis)			0.610	0.529	0.808	0.649
Hypo consumed, %			92.9	91.00	93.5	90.40
Final pH			11.2	11.5	11.2	11.4
Hypochlorite stage (H_2)						
Hypo applied as available Cl_2, % (o.d. pulp basis)			0.281	0.249	0.370	0.308
Hypo consumed as available Cl_2, % (o.d. pulp basis)			0.231	0.208	0.313	0.263
Hypo consumed, %			81.9	83.53	84.5	85.39
Final pH			11.0	11.1	11.0	11.0
Total Cl_2 applied, % (o.d. pulp basis)			1.88	1.662	2.47	2.052
Total Cl_2 consumed, % (o.d. pulp basis)			1.77	1.565	2.35	1.932
Total Cl_2 consumed, %			94.2	94.16	95.1	94.15
Total residual Cl_2, %			5.8	5.84	4.9	5.85
Bleaching losses, %			9.2	8.5	8.7	8.65
Bleached pulp yield, %			37.94±1.5	38.25±2.5	40.61±2.3	40.65±1.8
Pulp brightness, % (ISO)			80.3±0.38	82.4±0.71	81.6±0.25	84.7±0.36
Pulp viscosity, cps			10.1±0.020	10.4±0.015	8.8±0.013	9.2±0.018
Bleaching conditions	X	O	C	E	H_1	H_2
Consistency, %	10	10	3	10	10	10
Temperature, °C	55±2	90±2	Ambient	60±2	45±2	90±2
Time, min	120	90	30	60	60	60

± refers standard deviation

Table 5.6: Comparison of mechanical strength properties and combined effluent characteristics generated during conventional bleaching of lemon grass

Sl. No	Particulars	CEHH	XECEHH	% difference	OCEHH	XOCEHH	% difference
1	Total chlorine demand	2.85	2.204	–22.67	1.88	1.66	–11.60
2	Pulp brightness,(% ISO)	78.4±0.52	81.2±0.7	+2.80	80.3±0.38	82.4±0.71	+2.10
3	Pulp viscosity, cps	9.5±0.008	9.7±0.020	+2.06	10.1±0.02	10.4±0.05	+2.89
4	Copper number	0.22±0.003	0.17±0.005	–22.73	0.13±0.004	0.11±0.002	–15.38
5	Beating level, °SR	40±1	40±1	–	40±1	40±1	–
6	Tear index, mNm^2/g	4.57±0.22	4.82±0.35	+5.19	5.12±0.27	6.01±0.12	+14.81
7	Burst index, $kPam^2/g$	5.11±0.25	6.10±0.20	+16.23	5.67±0.42	6.56±0.11	+13.57
8	Tensile index, Nm/g	60.9±1.9	68.2±1.5	+10.7	59.2±1.4	70.5±2.0	+16.03
9	Double fold, number	28±4.2	34±2.2	+17.65	43±1.8	50±3.2	+14.0
10	COD, mg/L	1251	1630	+23.25	1038	1322	+21.48
11	Color, PTU	2140	2480	+13.71	1504	1847	+18.57
12	AOX, kg/t	2.44	1.79	–26.64	1.88	1.22	–35.11

± refers standard deviation

Table 5.7: Comparison of mechanical strength properties and combined effluent characteristics generated during conventional bleaching of sofia grass

Sl. No.	Particulars	CEHH	XECEHH	% difference	OCEHH	XOCEHH	% difference
1	Total chlorine demand	3.70	2.60	–29.73	2.47	2.05	–16.92
2	Pulp brightness,(% ISO)	80.6±0.35	83.5±0.42	+2.90	81.6±0.61	84.7±0.45	+3.10
3	Pulp viscosity, cps	8.4±0.011	8.7±0.015	+3.37	8.8±0.013	9.2±0.018	+2.15
4	Copper number	0.25±0.005	0.19±0.002	–24.0	0.15±0.004	0.12±0.002	–20.0
5	Beating level, °SR	40±1	40±1	–	40±1	40±1	–
6	Tear index, mNm^2/g	3.69±0.15	4.03±0.20	+8.44	4.32±0.17	4.68±0.35	+7.69
7	Burst index, $kPam^2/g$	3.70±0.25	4.12±0.18	+10.19	3.84±0. 1	4.22±0. 24	+9.01
8	Tensile index, Nm/g	42.02±1.9	47.37±1.5	+11.29	44.59±2.7	49.70±1.3	+10.28
9	Double fold, number	16±4.2	19±2.2	+15.79	20±2.1	22±3.9	+9.09
10	COD, mg/L	1507	1851	+18.59	1214	1450	+16.28
11	Color, PTU	2241	2624	+14.60	1554	1935	+19.69
12	AOX, kg/t	2.77	1.94	–29.96	2.05	1.42	–30.73

± refers standard deviation

Table 5.8: Effect of ECF bleaching on pulp shrinkage, brightness and viscosity of soda-AQ pulps of lemon and sofia grasses

Particulars	Bleaching sequence			
	Lemon grass		Sofia grass	
	ODED	XODED	ODED	XODED
Unbleached pulp kappa number	11.60	11.60	14.80	14.80
Unbleached pulp brightness, % (ISO)	30.61	30.61	32.25	32.25
Unbleached pulp viscosity, cps	27.10	27.10	26.56	26.56
Xylanase stage (X)				
Amount of xylanase added (on o.d. pulp basis), IU/g	–	10	–	8
Final pH	–	6.4	–	6.5
Oxygen stage (O)				
O_2 pressure, kg/cm^2	5.0	5.0	5.0	5.0
$MgSO_4$ applied, % (o.d. pulp basis)	0.2	0.2	0.2	0.2
EDTA applied, % o.d. pulp basis)	0.1	0.1	0.1	0.1
NaOH applied, % (o.d. pulp basis)	2.0	2.0	2.0	2.0
Final pH	12.0	12.0	12.0	12.0
Chlorine dioxide stage (D_1)				
ClO_2 applied as available Cl_2,% (o.d. pulp basis)	1.34	1.34	1.34	1.34
ClO_2 consumed as available Cl_2,% (o.d. pulp basis)	1.26	1.212	1.24	1.181
ClO_2 consumed on Cl_2 basis, %	94.02	90.45	92.54	88.13
Final pH	4.0	4.0	4.1	4.1
Alkali extraction stage (E)				
NaOH applied, % (o.d. pulp basis)	2.5	2.5	2.5	2.5
Initial pH	11.0	11.0	11.0	11.0
Final pH	11.2	11.2	11.6	11.2
Chlorine dioxide stage (D_2)				
ClO_2 applied as available Cl_2, % (o.d. pulp basis)	0.660	0.660	0.660	0.660
ClO_2 consumed as available Cl_2, % (o.d. pulp basis)	0.611	0.603	0.626	.597
ClO_2 consumed, %	92.58	91.36	94.85	90.45
Final pH	4.1	4.1	4.0	4.0
Total ClO_2 applied, % (o.d. pulp basis)	2.0	2.0	2.0	2.0
Total ClO_2 consumed, % (o.d. pulp basis)	1.871	1.815	1.866	1.778
Total ClO_2consumed on Cl_2 basis, %	93.75	90.75	93.30	88.90
Total residual ClO_2, %	6.25	9.25	6.70	11.10
Bleaching losses, %	7.45	7.5	8.1	8.04
Bleached pulp yield, %	38.62±1.3	38.65±2.12	40.90±2.11	40.92±2.05
Pulp brightness, % (ISO)	80.5±0.5	83.1±0.5	81.8±0.3	85.1±0.2
Pulp viscosity, cps	9.91±0.015	10.10±0.020	8.93±0.011	9.02±0.009

Bleaching conditions	X	O	D_1	E	D_2
Consistency, %	10	10	10-12	10	10-12
Temperature, °C	55±2	90±2	70±2	60±2	70±2
Time, min	120	90	180	60	180

± refers standard deviation

Table 5.9: Effect of xylanase pretreatment on pulp shrinkage, brightness and viscosity of soda-AQ pulps of lemon and sofia grasses during ECF bleaching

Particulars	Bleaching sequence			
	Lemon grass		Sofia grass	
	ODEDP	XODEDP	ODEDP	XODEDP
Unbleached pulp kappa number	11.60	11.60	14.80	14.80
Unbleached pulp brightness, % (ISO)	30.61	30.61	32.25	32.25
Unbleached pulp viscosity, cps	27.10	27.10	26.56	26.56
Xylanase stage (X)				
Amount of xylanase added (on o.d. pulp basis), IU/g	–	10	–	8
Final pH	–	6.4	–	6.5
Oxygen stage (O)				
O_2 pressure, kg/cm^2	5.0	5.0	5.0	5.0
$MgSO_4$ applied, % (o.d. pulp basis)	0.2	0.2	0.2	0.2
EDTA applied, % (o.d. pulp basis)	0.1	0.1	0.1	0.1
NaOH applied, % (o.d. pulp basis)	2.0	2.0	2.0	2.0
Final pH	12.0	12.0	12.0	12.0
Chlorine dioxide stage (D_1)				
ClO_2 applied as available Cl_2, % o.d. pulp basis)	1.34	1.34	1.34	1.34
ClO_2 consumed as available Cl_2, % (o.d. pulp basis)	1.212	1.212	1.181	1.181
ClO_2 consumed on Cl_2 basis, %	90.45	90.45	88.13	88.13
Final pH	4.0	4.1	4.1	4.1
Alkali extraction stage (E)				
NaOH applied, % (o.d. pulp basis)	2.5	2.5	2.5	2.5
Initial pH	11.0	11.0	11.0	11.0
Final pH	10.9	10.9	10.3	10.3
Chlorine dioxide stage (D_2)				
ClO_2 applied as available Cl_2, % (o.d. pulp basis)	0.660	0.660	0.660	0.660
ClO_2 consumed as available Cl_2, % (o.d. pulp basis)	0.611	0.603	0.626	0.597
ClO_2 consumed, %	92.58	91.36	94.85	90.45
Final pH	4.1	4.1	4.0	4.0
Peroxide stage (P)				
H_2O_2 applied, % (o.d. pulp basis)	0.5	0.5	0.5	0.5
EDTA applied, % (o.d. pulp basis)	0.5	0.5	0.5	0.5
$MgSO_4$ applied, % (o.d. pulp basis)	0.1	0.1	0.1	0.1
Final pH	10.4	10.9	10.5	10.3
Total ClO_2 applied, % (o.d. pulp basis)	2.0	2.0	2.0	2.0
Total ClO_2consumed, % (o.d. pulp basis)	1.871	1.815	1.866	1.778
Total ClO_2consumed on Cl_2 basis, %	93.75	90.75	93.30	88.90
Total residual ClO_2, %	6.25	9.25	6.70	11.10
Bleaching losses, %	7.6	7.55	7.8	7.3
Bleached pulp yield, %	38.68±2.2	38.72±1.7	41.05±1.8	41.26±2.8
Pulp brightness, % (ISO)	82.8±0.3	85.0±0.3	82.9±0.2	87.3±0.2
Pulp viscosity, cps	9.72±0.008	10.03±0.016	8.80±0.010	9.20±0.012

Bleaching conditions	X	O	D_1	E	D_2	P
Consistency, %	10	10	10-12	10	10-12	10
Temperature, °C	55±2	90±2	70±2	60±2	70±2	90±2
Time, min	120	90	180	60	180	60

± refers standard deviation

Table 5.10: Comparison of mechanical strength properties and combined effluent generated during ECF bleaching of lemon grass

Sl. No.	Particulars	ODED	XODED	% difference	ODEDP	XODEDP	% difference
1	Pulp brightness, (%ISO)	80.5±0.5	83.1±0.5	+2.6	82.8±0.3	85.0±0.3	+2.2
2	Pulp viscosity, cps	9.91	10.10	+1.88	9.72	10.03	+3.09
3	Copper number	0.15±0.04	0.095±0.002	–36.67	0.10±0.005	0.06±0.001	–29.71
4	Beating level, °SR	40±1	40±1	–	40±1	40±1	–
5	Tear index, mNm^2/g	5.82±0.14	6.21±0.17	+6.28	5.64±0.15	5.90±0.12	+4.41
6	Burst index, $kPam^2/g$	5.26±0.12	4.98±0.2	–5.32	5.10±0.24	4.62±0.18	–9.41
7	Tensile index, Nm/g	66.37±2.23	58.13±1.25	–12.42	64.54±1.82	56.16±1.50	–12.98
8	Double fold, number	52±3.5	47±4.2	–9.62	46±3.5	30±2.41	–34.78
9	COD, mg/L	1067	1440	+25.91	1024	1288	+20.50
10	Color, PTU	1539	1915	+19.64	1210	1702	+28.91
11	AOX, kg/t	0.37	0.238	–35.68	0.37	0.238	–35.68

± refers standard deviation

Table 5.11: Comparison of mechanical strength properties and combined effluent generated during ECF bleaching of sofia grass

Sl. No.	Particulars	ODED	XODED	% difference	ODEDP	XODEDP	% difference
1	Pulp brightness, % (ISO)	81.8±0.3	85.1±0.2	+3.3	82.9±0.41	87.3±0.66	+4.4
2	Pulp viscosity, cps	8.93	9.02	+1.0	8.80	9.20	+4.35
3	Copper number	0.12±0.01	0.08±0.003	–33.33	0.08±0.005	0.06±0.002	–25
4	Beating level, °SR	40±1	40±1	–	40±1	40±1	–
5	Tear index, mNm^2/g	3.76±0.22	4.15±0.15	+9.40	3.72±0.13	4.22±0.16	+11.85
6	Burst index, $kPam^2/g$	3.52±0.10	3.21±0.15	–8.81	3.42±0.11	3.11±0.2	–9.06
7	Tensile index, Nm/g	54.11±1.4	46.39±2.27	–14.27	52.87±1.76	48.38±2.25	–8.49
8	Double fold, number	28±3.2	25±2.8	–10.71	23±2.7	19±2.2	–17.39
9	COD, mg/L	1280	1538	+16.78	1210	1408	+14.06
10	Color, PTU	1841	2291	+19.64	1608	1965	+18.17
11	AOX, kg/t	0.48	0.255	–46.88	0.48	0.255	–46.88

± refers standard deviation

Table 5.12: Effect of TCF bleaching on pulp shrinkage, brightness and viscosity of lemon grass soda-AQ pulp

Particulars		Lemon grass		
		O(E_{OP})P	OX(E_{OP})P	XO(E_{OP})P
Unbleached pulp kappa number		11.60	11.60	11.60
Unbleached pulp brightness, % (ISO)		30.61	30.61	30.61
Unbleached pulp viscosity, cps		27.10	27.10	27.10
Xylanase stage (X)				
Amount of xylanase added (on o.d. pulp basis), IU/g		–	–	10
Final pH		–	–	6.5
Oxygen stage (O)				
O_2 pressure, kg/cm^2		5.0	5.0	5.0
$MgSO_4$ applied, % (o.d. pulp basis)		0.2	0.2	0.2
EDTA applied, % (o.d. pulp basis)		0.1	0.1	0.1
NaOH applied, % (o.d. pulp basis)		2.0	2.0	2.0
Final pH		12.0	12.0	12.0
Xylanase stage (X)				
Amount of xylanase added (on o.d. pulp basis), IU/g		–	10	–
Final pH		–	6.5	–
Extraction stage				
NaOH applied, % (o.d. pulp basis)		3.0	3.0	3.0
H_2O_2 applied, % (o.d. pulp basis)		0.5	0.5	0.5
O_2 pressure, kg/cm^2		5.0	5.0	5.0
$MgSO_4$ applied, % (o.d. pulp basis)		0.1	0.1	0.1
Final pH		11.6	11.6	11.5
Peroxide stage (P)				
H_2O_2 applied, % (o.d. pulp basis)		2.0	2.0	2.0
EDTA applied, % (o.d. pulp basis)		0.5	0.5	0.5
$MgSO_4$ applied, % (o.d. pulp basis)		0.1	0.1	0.1
Final pH		11.7	11.7	11.7
Total H_2O_2 applied, % (o.d. pulp basis)		2.5	2.5	2.5
Bleaching losses, %		6.6	7.0	7.3
Bleached pulp yield, %		39.05±0.45	38.87±0.84	38.75 ±0.53
Pulp brightness ,% (ISO)		74.2±1.05	76.9±0.28	78.7±0.51
Pulp viscosity, cps		10.8±0.011	11.3±0.013	10.8±0.023
Bleaching conditions	X	O	(E_{OP})	P
Consistency, %	10	10	11.5	10
Temperature, °C	55±2	90±2	75±2	90±2
Time, min	120	90	70	90

± refers standard deviation

Table 5.13: Effect of xylanase pretreatment on pulp shrinkage, brightness and viscosity of sofia grass soda-AQ pulp during TCF bleaching

Particulars		Sofia grass		
		$O(E_{OP})P$	$OX(E_{OP})P$	$XO(E_{OP})P$
Unbleached pulp kappa number		14.80	14.80	14.80
Unbleached pulp brightness, % (ISO)		32.25	32.25	32.25
Unbleached pulp viscosity, cps		26.56	26.56	26.56
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		5.0	5.0	5.0
$MgSO_4$ applied, % (o.d. pulp basis)		0.2	0.2	0.2
EDTA applied, % (o.d. pulp basis)		0.1	0.1	0.1
NaOH applied, % (o.d. pulp basis)		2.0	2.0	2.0
Final pH		12.0	12.0	12.0
Xylanase stage (X)				
Amount of xylanase added (on o.d. pulp basis), IU/g		–	8	–
Final pH		–	6.5	–
Extraction stage				
NaOH applied, % (o.d. pulp basis)		3.0	3.0	3.0
H_2O_2 applied, % (o.d. pulp basis)		0.5	0.5	0.5
O_2 pressure, kg/cm^2		5.0	5.0	5.0
$MgSO_4$ applied, % (o.d. pulp basis)		0.1	0.1	0.1
Final pH		11.4	11.5	11.5
Peroxide stage (P)				
H_2O_2 applied, % (o.d. pulp basis)		2.0	2.0	2.0
EDTA applied, % (o.d. pulp basis)		0.5	0.5	0.5
$MgSO_4$ applied, % (o.d. pulp basis)		0.1	0.1	0.1
Final pH		11.7	11.7	11.7
Total H_2O_2 applied, % (o.d. pulp basis)		2.5	2.5	2.5
Bleaching losses, %		6.4	6.9	7.1
Bleached pulp yield, %		41.74±0.63	41.42±1.50	41.31±1.20
Pulp brightness ,% (ISO)		76.5±0.33	78.6±0.20	80.4±0.23
Pulp viscosity, cps		9.7±.020	10.1±0.015	10.3±0.020
Bleaching conditions	X	O	(E_{OP})	P
Consistency, %	10	10	11.5	10
Temperature, °C	55±2	90±2	75±2	90±2
Time, min	120	90	70	90

± refers standard deviation

Table 5.14: Comparison of mechanical strength properties and combined effluent generated during TCF bleaching of lemon grass

Sl. No.	Particulars	$O(E_{OP})P$	$OX(E_{OP})P$	% difference	$XO(E_{OP})P$	% difference
1	Pulp brightness, % (ISO)	74.2±1.05	76.9±0.28	+2.7	78.7±0.51	+4.5
2	Pulp viscosity, cps	10.8±0.011	11.3±0.013	+4.4	11.0±0.023	+1.8
3	Copper number	0.09±0.001	0.06±0.003	–33.3	0.065±0.001	–27.8
4	Beating level, °SR	40±1	40±1	–	40±1	–
5	Tear index, mNm^2/g	4.42±0.24	4.64±0.15	+4.7	4.51±0.18	+2.0
6	Burst index, $kPam^2/g$	5.35±0.2	5.06±0.15	–5.4	4.72±0.12	–11.8
7	Tensile index, Nm/g	66.67±1.5	61.93±2.3	–7.1	58.35±2.6	–12.5
8	Double fold, number	51±2.7	43±3.3	–15.7	45±2.5	–11.8
9	COD, mg/L	1430	1588	+9.9	1650	+13.3
10	Color, PTU	2320	3128	+25.8	3385	+31.5

± refers standard deviation

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

Sl. No.	Particulars	$O(E_{OP})P$	$OX(E_{OP})P$	% difference	$XO(E_{OP})P$	% difference
1	Pulp brightness,%(ISO)	76.4±0.33	78.6±0.20	+2.2	80.4±0.23	+4.0
2	Pulp viscosity, cps	9.7±.020	10.1±0.015	+3.96	10.3±0.020	+5.83
3	Copper number	0.12±0.002	0.09±0.001	–25.0	0.09±0.001	–25.0
4	Beating level, °SR	40±1	40±1	–	40±1	–
5	Tear index, mNm^2/g	3.61±0.1	3.70±0.12	+2.4	3.67±0.15	+1.63
6	Burst index, $kPam^2/g$	3.47±0.09	3.08±0.07	–11.2	2.92±0.13	–15.85
7	Tensile index, Nm/g	51.50±1.0	47.12±1.02	–8.5	43.39±2.6	–15.50
8	Double fold, number	35±2.1	30±1.2	–14.3	32±1.1	–8.57
9	COD, mg/L	1390	1587	+12.41	1632	+14.83
10	Color, PTU	2522	3180	+20.69	3372	+25.21

± refers standard deviation

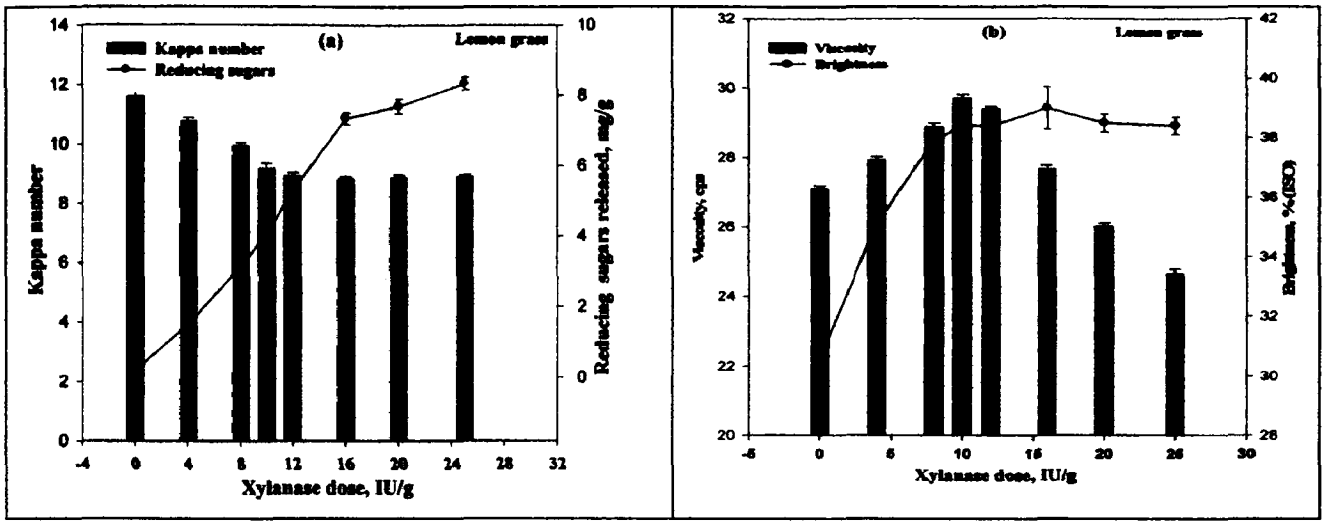

Figure 5.1: Effect of enzyme dose on (a) reducing sugars released and kappa number (b) viscosity and brightness of soda-AQ pulp of lemon grass during xylanase treatment

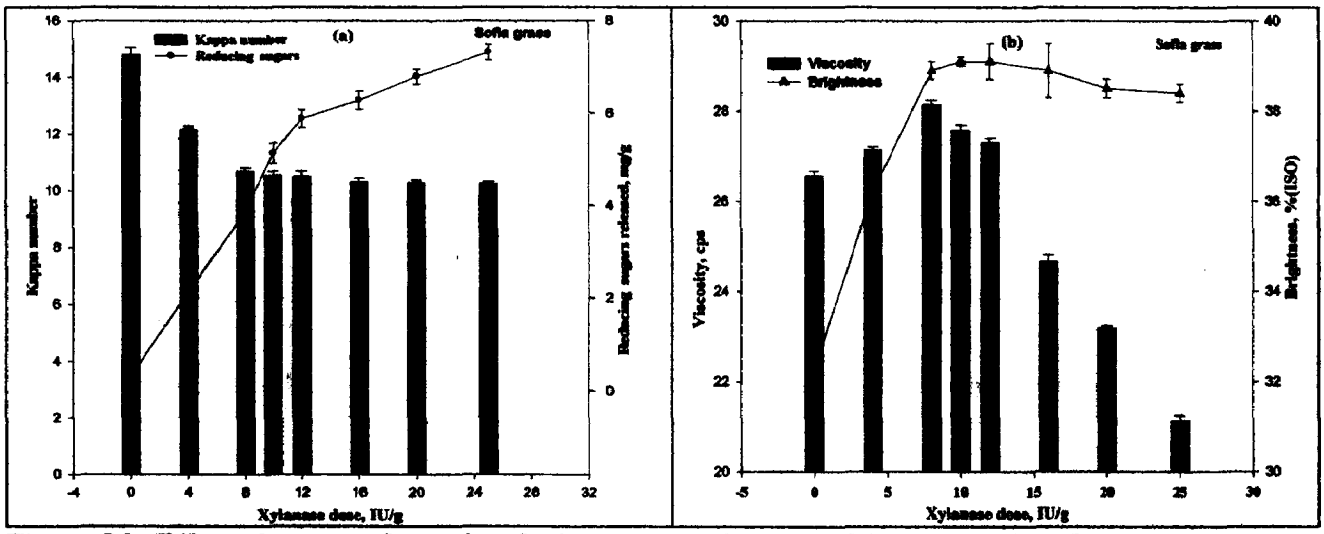

Figure 5.2: Effect of enzyme dose (a) reducing sugars released and kappa number (b) viscosity and brightness of soda-AQ pulp of sofia grass during xylanase treatment

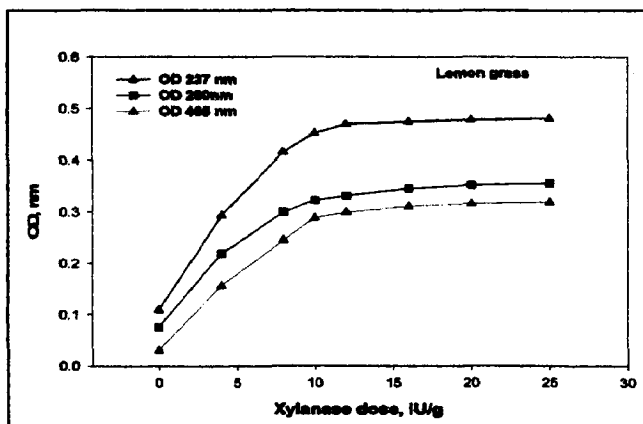

Figure 5.3 (a): Spectrophotometric analysis of pulp filtrate generated during xylanase treatment of soda-AQ pulp of lemon grass at different xylanase doses

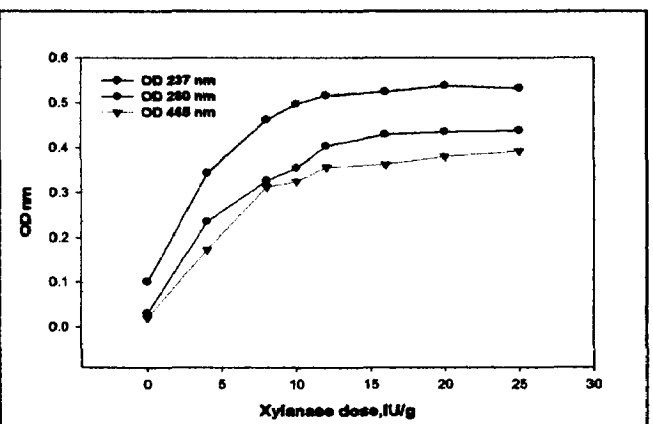

Figure 5.3 (b): Spectrophotometric analysis of pulp filtrate generated during xylanase treatment of soda-AQ pulp of sofia grass at different xyl doses

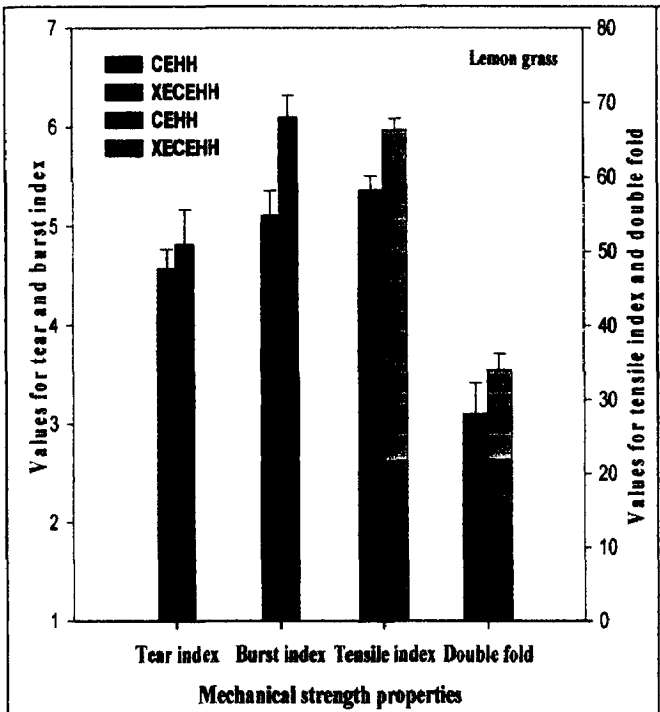

Figure 5.10 (a): Comparison of mechanical strength properties of soda-AQ pulp of lemon grass during CEHH and XECEHH beaching sequences

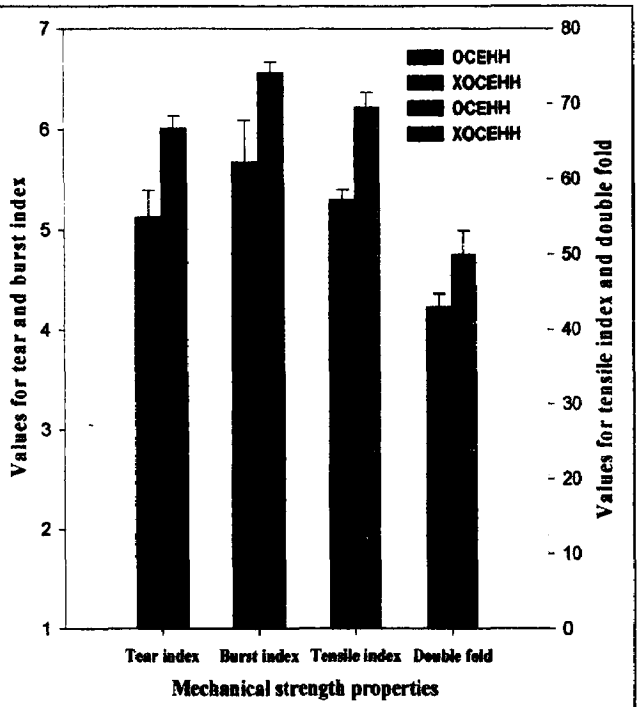

Figure 5.10 (b): Comparison of mechanical strength properties of soda-AQ pulp of lemon grass during OCEHH and XOCEHH beaching sequences

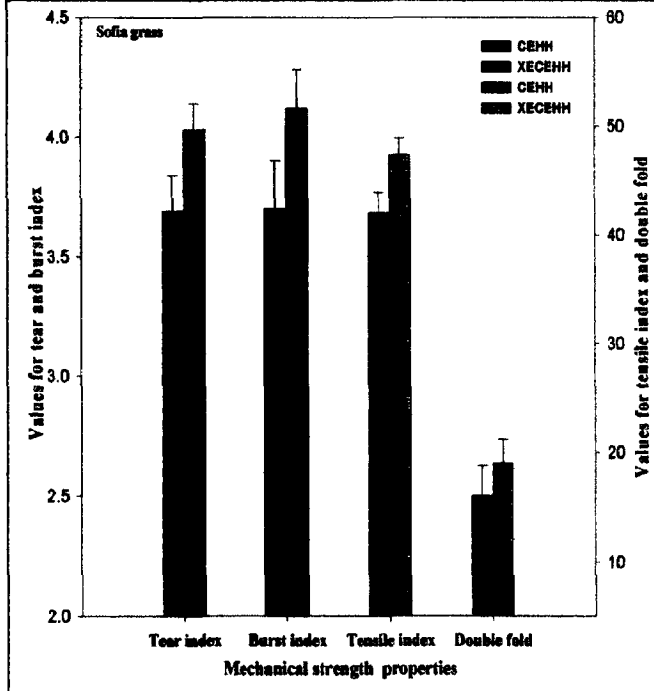

Figure 5.11 (a): Comparison of mechanical strength properties of soda-AQ pulp of sofia grass during CEHH and XECEHH beaching sequences

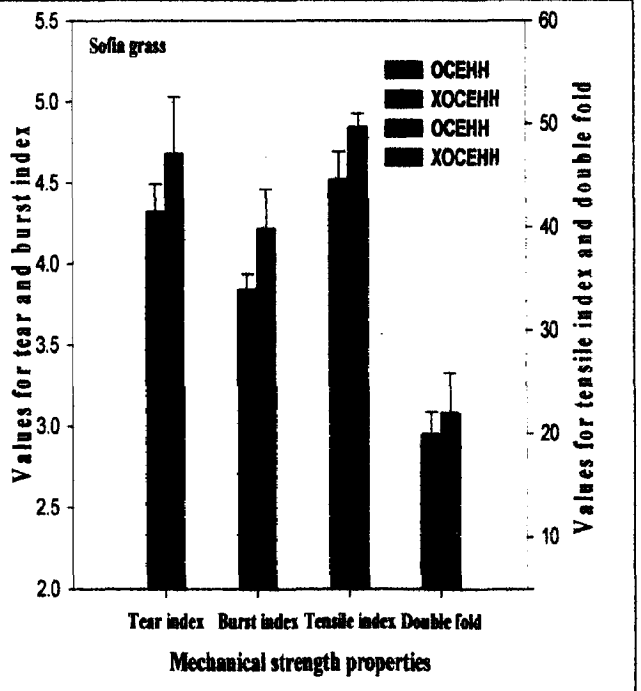

Figure 5.11 (b): Comparison of mechanical strength properties of soda-AQ pulp of sofia grass during OCEHH and XOCEHH beaching sequences

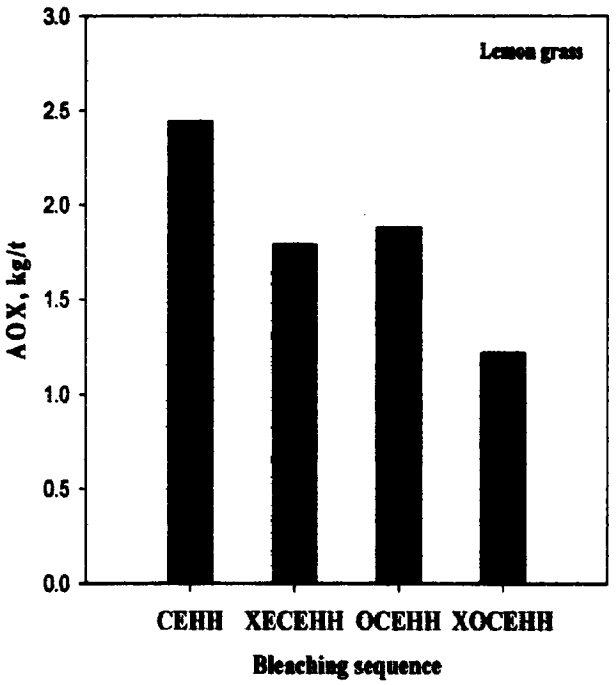

Figure 5.12 (a): Comparison of AOX formation during conventional bleaching sequences of soda-AQ pulp of lemon grass

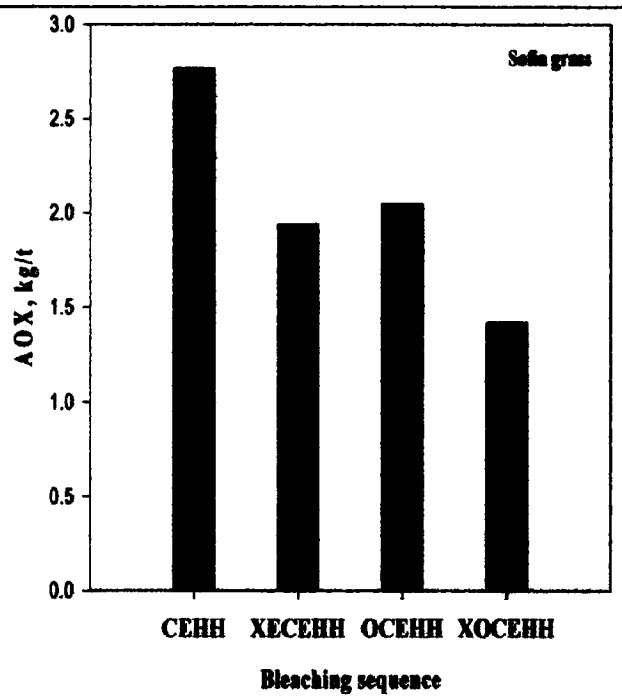

Figure 5.12 (b): Comparison of AOX formation during conventional bleaching sequences of soda-AQ pulp of sofia grass

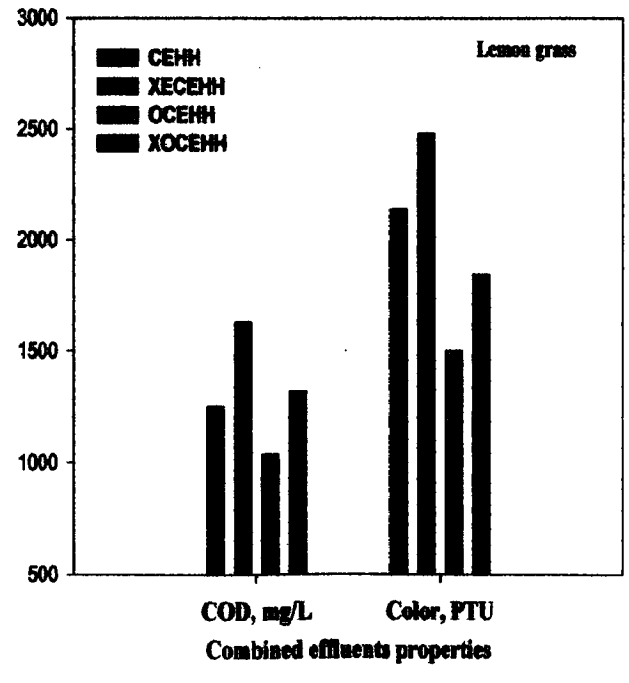

Figure 5.13 (a): Comparison of COD and color of combined bleach effluents generated during conventional bleaching of soda-AQ pulp of lemon grass

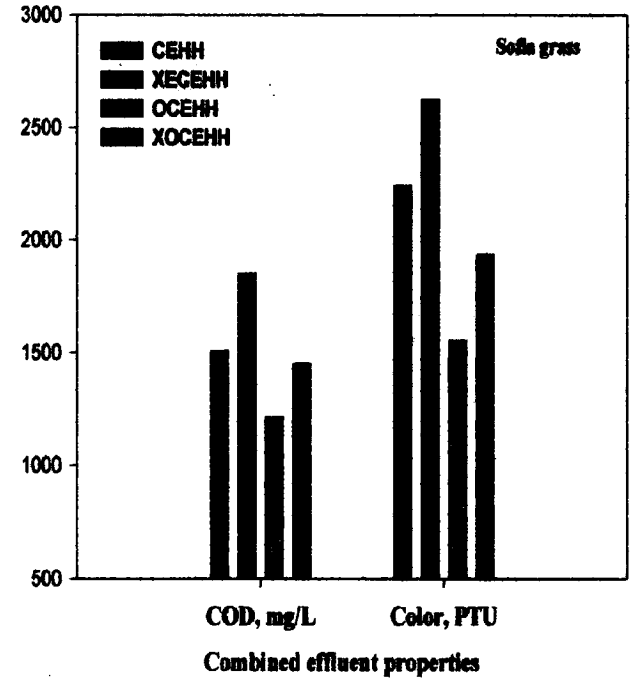

Figure 5.13 (b): Comparison of COD and color of combined bleach effluents generated during conventional bleaching of soda-AQ pulp of sofia grass

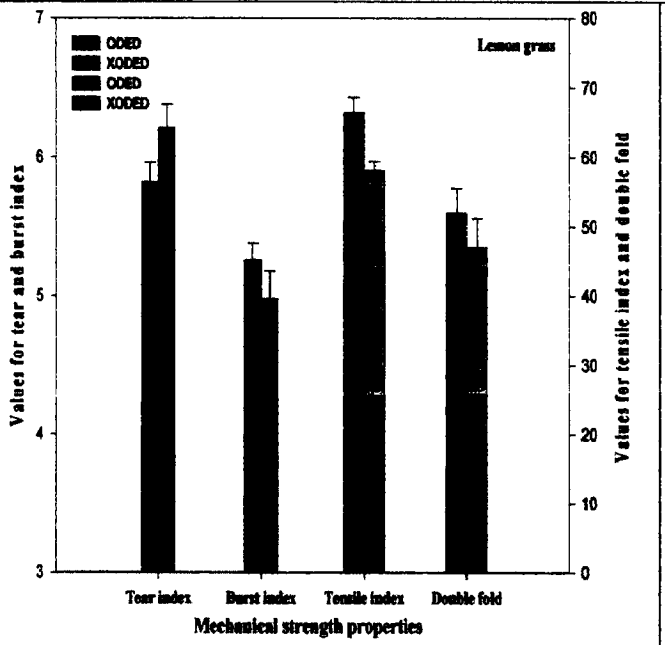

Figure 5.14 (a): Comparison of mechanical strength properties of soda-AQ pulp of lemon grass during ODED and XODED bleaching sequences

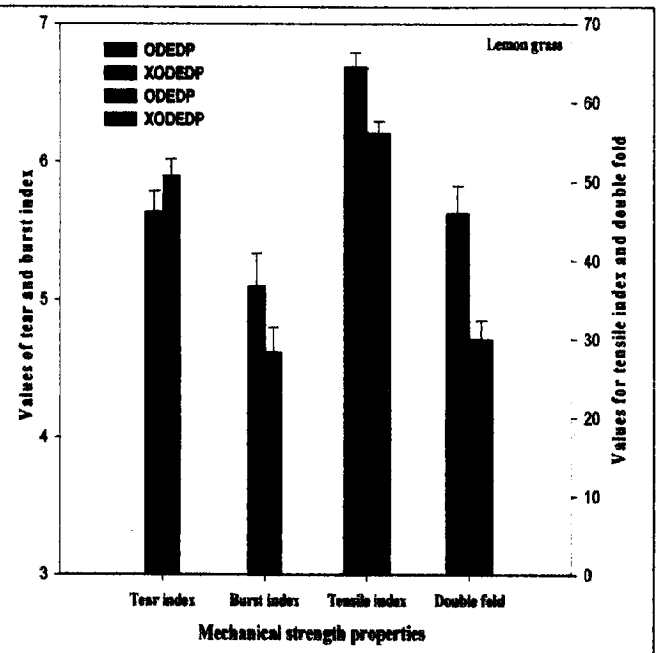

Figure 5.14 (b): Comparison of mechanical strength properties of soda-AQ pulp of lemon grass during ODEDP and XODEDP bleaching sequences

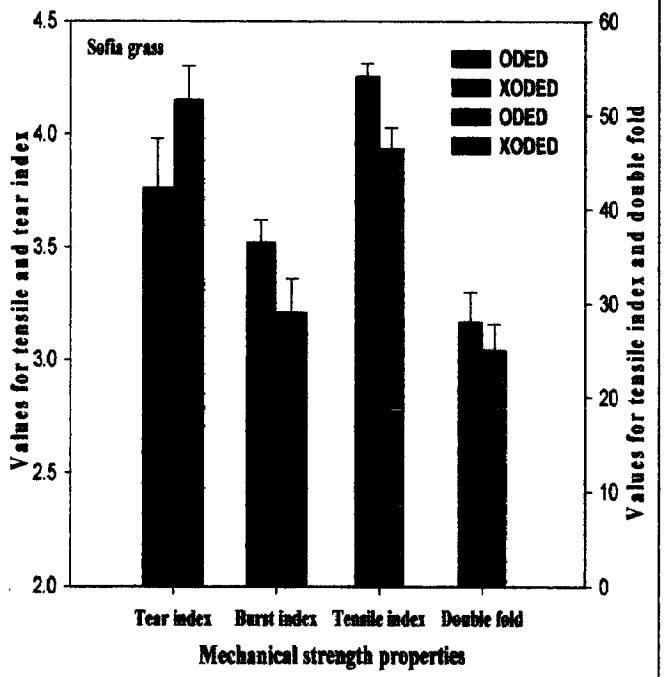

Figure 5.15 (a): Comparison of mechanical strength properties of soda-AQ pulp of sofia grass during ODED and XODED bleaching sequences

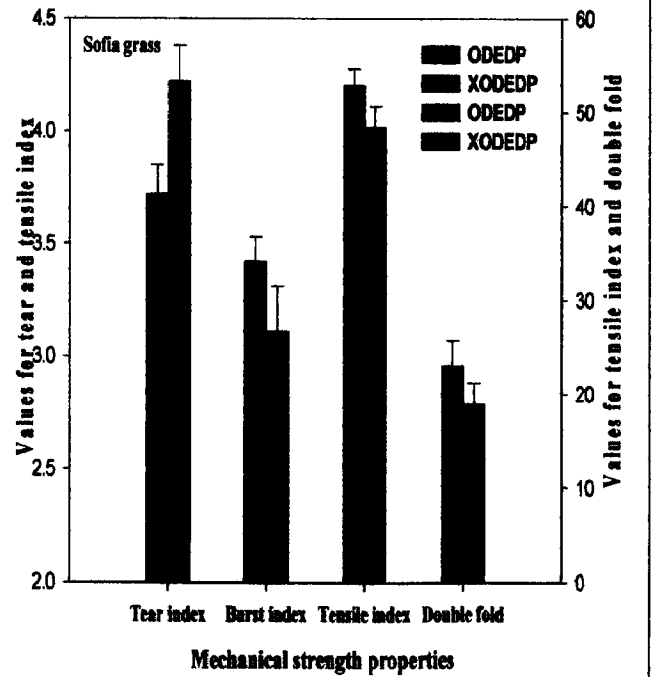

Figure 5.15 (b): Comparison of mechanical strength properties of soda-AQ pulp of sofia grass during ODEDP and XODEDP bleaching sequences

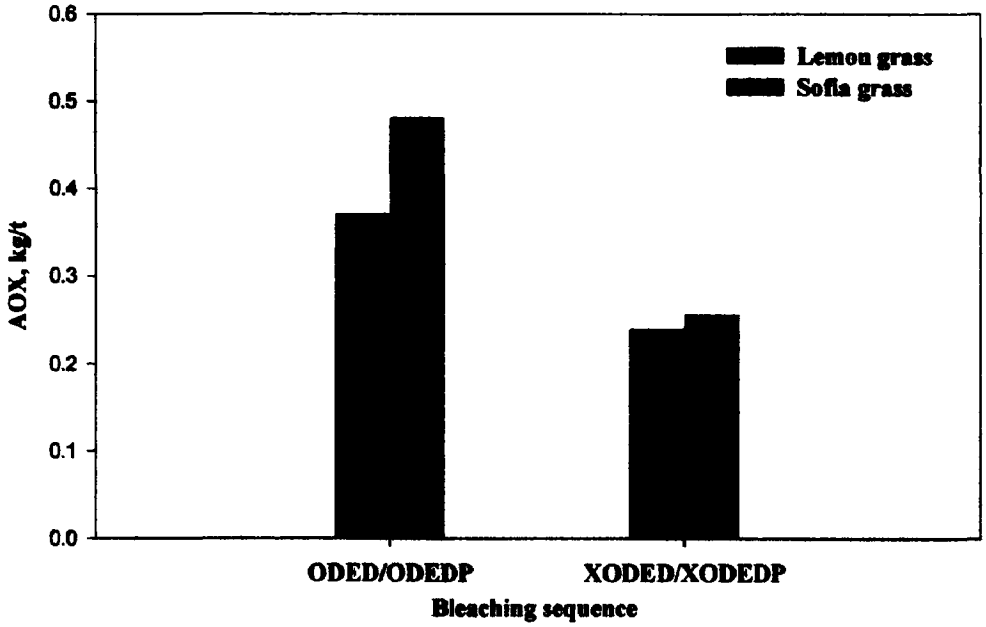

Figure 5.16: Comparison of AOX formation during ECF bleaching sequences of soda-AQ pulps of lemon and sofia grasses

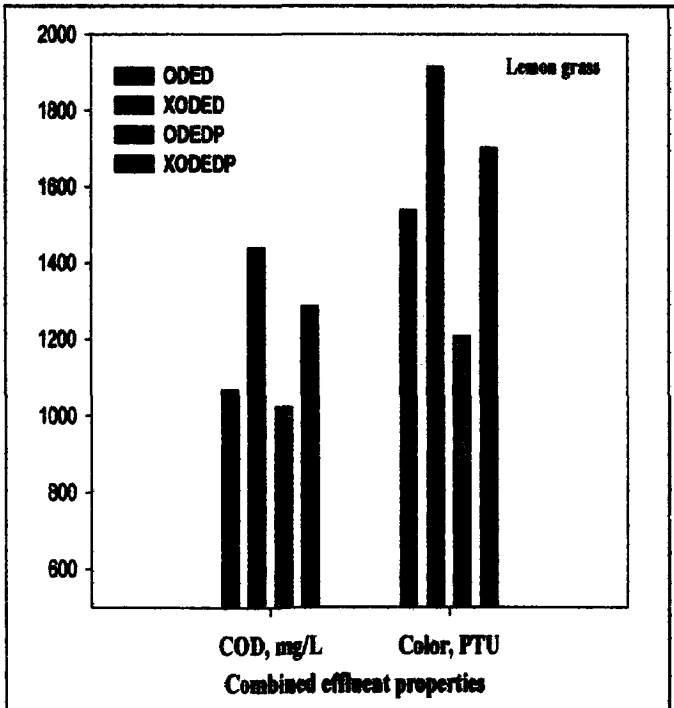

Figure 5.17 (a): Comparison of COD and color of combined bleach effluents generated during ECF bleaching of soda-AQ pulp of lemon grass

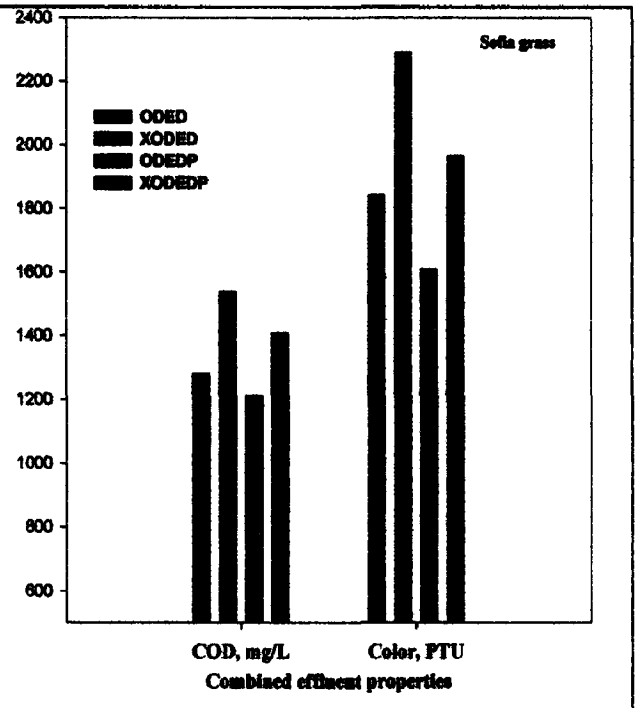

Figure 5.17 (b): Comparison of COD and color of combined bleach effluents generated during ECF bleaching of soda-AQ pulp of sofia grass

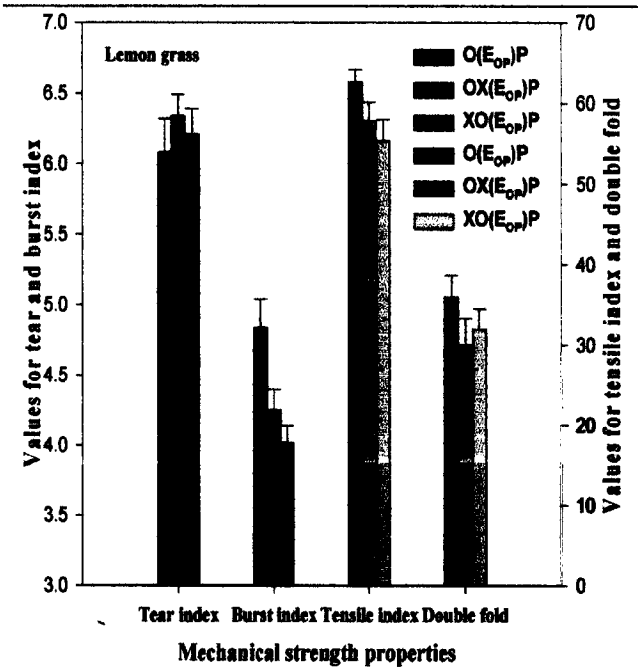

Figure 5.18 (a): Comparison of mechanical strength properties of soda-AQ pulp of lemon grass during $O(E_{OP})P$, $OX(E_{OP})P$ and $XO(E_{OP})P$ bleaching sequences

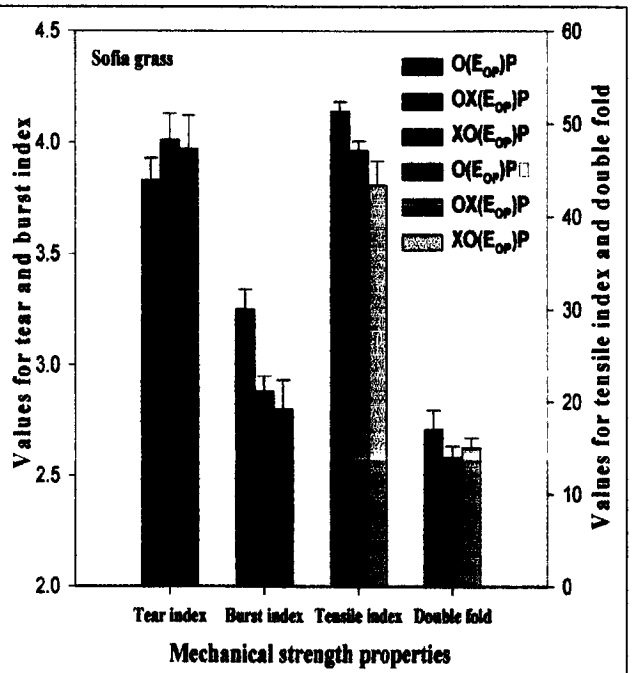

Figure 5.18 (b): Comparison of mechanical strength properties of soda-AQ pulp of sofia grass during $O(E_{OP})P$, $OX(E_{OP})P$ and $XO(E_{OP})P$ bleaching sequences

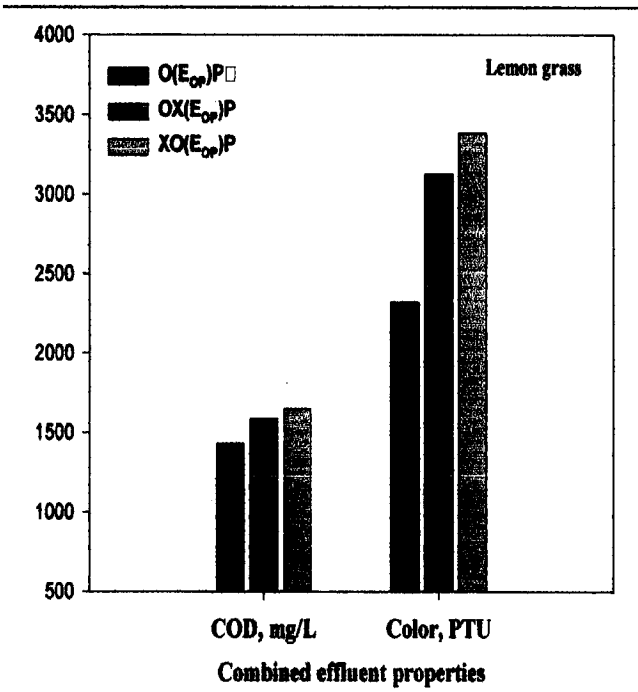

Figure 5.19 (a): Comparison of COD and color of combined bleach effluents generated during TCF bleaching of soda-AQ pulp of lemon grass

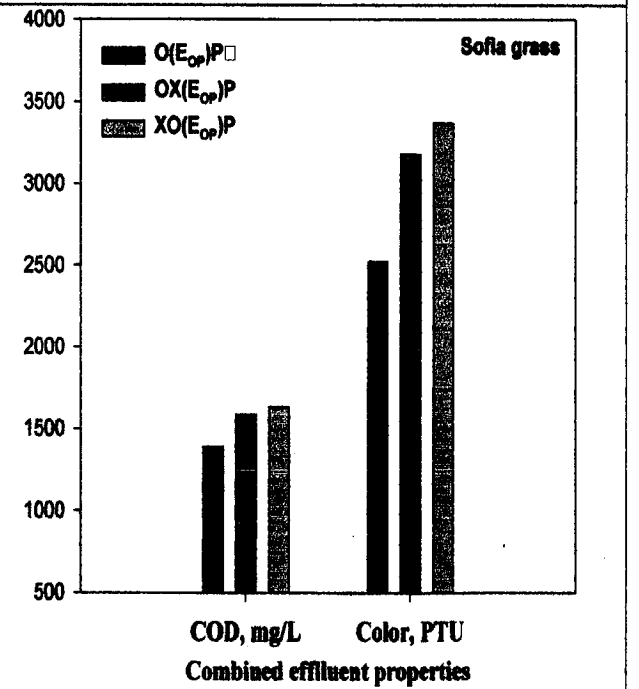

Figure 5.19 (b): Comparison of COD and color of combined bleach effluents generated during TCF bleaching of soda-AQ pulp of sofia grass

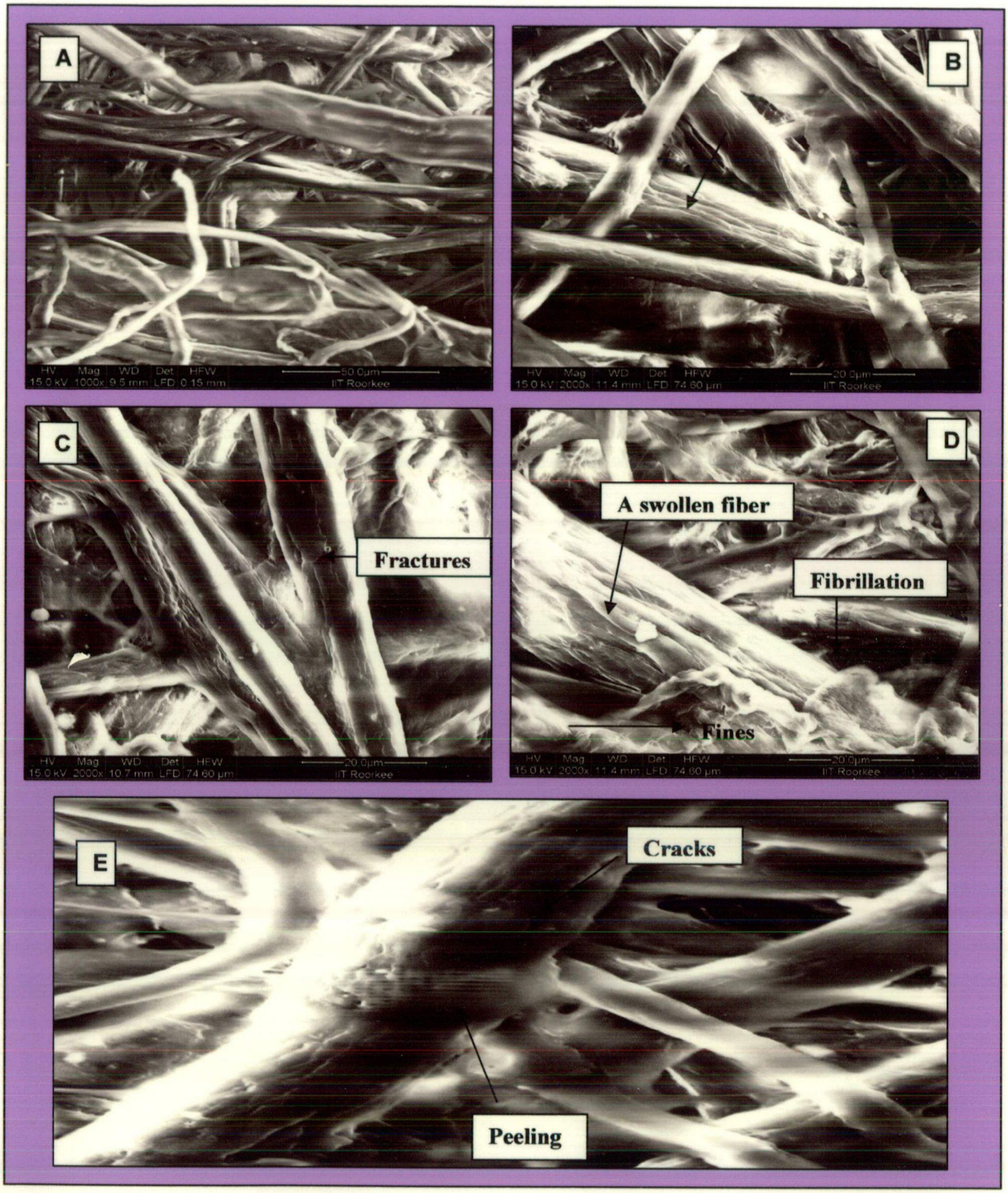

Plate 5.2: SEM photomicrographs, (A) Unbleached, control, ribbon-like pulp fibers with fibers of sofia grass with a smooth surface (1.0 KX); (B) Enzyme treated sofia grass fibers with a rough surface (2.0 KX); (C) Enzyme treated swollen sofia grass pulp fibers showing flakes, peeling, fractures and fibrillation (2.5 KX); (D) Untreated sofia grass fibers beaten to a level of 40 °SR, showing fibrillation, also showing fines generated during beating (2.0 KX); (E) Enzyme treated sofia grass fibers after beating at a level of 40 °SR(4.5 KX)

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CHAPTER 6

CONCLUSIONS AND SUGGESTIONS

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6.1 CONCLUSIONS

From the present study, based on the characterization and usage of two non-woody raw materials *C. martini* (sofia grass) and *C. citratus* (lemon grass) with regard to their unexploited potential in the pulp and paper industry and applying environmentally benign bleaching technologies following the use of crude xylanase from white-rot test fungus *Coprinopsis cinerea* HK-1, following are the major conclusions drawn:

1. SEM studies of lemon and sofia grasses showed that as a result of steam distillation, oil glands bursted, releasing oil and rupturing the cell walls which loosened the anatomy. Steam distillation process removed extractives along with essential oils, thus mitigating the problem of mass transfer, facilitating the faster penetration of cooking liquor, and solving various problems associated with soluble during pulping, and papermaking process.

2. Lemon and sofia grasses had an average fiber length of 1.09 and 0.87 mm with a fiber diameter of 16.3 and 14.7 μm. The length of an average parenchyma cell in lemon and sofia grass was 368.9 and 332.3 μm while they were 84.7 and 64.1 μm wide; vessels were 198.2 and 147.1 μm long while 35.6 and 28.5 μm wide respectively. The dimensions of parenchyma and vessel cells were on higher side than that of fiber dimensions. These non-fibrous cells appear in the form of fines and their presence in an excess adds to an adverse impact on paper machine drainage performance (runnability). They also act as fillers, affecting the mechanical strength and surface properties

3. Proximate chemical analysis of lemon and sofia grasses depicted that they required milder cooking conditions owing to lesser lignin content and more open and looser anatomy. Both the grasses however could not be stored for a longer period after harvesting due to a higher 1% alkali solubility.

4. The Lignocellulosic residues (LCR) left after extracting oils by steam distillation method from sofia and lemon grasses was successfully used for the production of

chemical grade pulp. Sofia grass produced a screened pulp yield of 43.2% with kappa number 20 at an active alkali dose of 14% (as Na_2O), maximum cooking time and temperature of 90 min and 160 °C, and liquor to wood ratio of 4.5:1. On contrary to this, lemon grass produced a screened pulp yield of 41.4% with kappa number 12.5 under the same pulping conditions except maximum cooking temperature of 150 °C.

5. The introduction of 0.1% AQ descended the kappa number by 26 and 8% with marginal increase in screened pulp yield by 0.2 and 0.4% respectively for sofia and lemon grasses.
6. The optimal mechanical strength properties were obtained at a beating level of 40±1 °SR. The strength properties of soda-AQ pulp of sofia grass were inferior to lemon grass.
7. The +20 fraction of lemon grass pulp fibers separated by a Bauer-McNett fiber classifier was 62.63% as compared to 41.72% for sofia grass, which mainly consisted of long sclerenchymatous fiber. While sofia grass retained 27.94% fibers in +48 fractions compared to 17.74% for lemon grass. Sofia grass soda-AQ pulp therefore had maximum percentage in middle and bottom fractions, while lemon grass in top fraction. The -200 fractions were just doubled in case of sofia grass compared to lemon grass, highlighting the possibility of fluff generation at the dryer section of paper machine or the printing blanket part of offset printing which could be mitigated either by using wet end bonding additives or by increasing pulp refining.
8. SEM studies showed that the fibers of soda-AQ pulps of sofia grass were non-uniform, the cell wall was distinguished by longitudinal striations, and transverse fractures with somewhat swollen fissures. On the other hand, the fibers of lemon grass were uniform, straight, and intact with a smooth, silky surface, bearing an appearance of compactness.
9. Among fourteen fungal strains isolated from different lignocellulosic sources, two strains of *Coprinopsis cinerea* i.e. HK-1 and HK-2 were selected as the potent xylanase producers along with minimum cellulase contamination.
10. Bavendamms plate assay screening also confirmed the production of a good phenol-oxidase activity by the two test strains.

11. Solid-state fermentation (SSF) resulted into higher xylanase yield than the submerged fermentation (SmF) from both of the strains of *C. cinerea*.
12. The various optimized physico-chemical parameters under SSF conditions for crude xylanase production by fungal strains HK-1 and HK-2 were: incubation period 7 (HK-1) and 8 days (HK-2), incubation temperature 37 °C, pH 6.4, carbon source wheat bran, nitrogen source beef extract (HK-1) and yeast extract (HK-2) and solid substrate: moisture content 1:3.
13. It is concluded from the biochemical characterization that the crude xylanase produced by *C. cinerea* HK-1 was more thermo and alkali-tolerant in comparison with that produced by *C. cinerea* HK-2. Hence white rot strain *C. cinerea* HK-1 was chosen for further biobleaching studies.
14. The xylanase produced by strain HK-1 could maintain a high stability over a pH range of 6.0 to 7.2 along with a 50% alkali tolerance even at a pH of 8.0; and a temperature range of 55-65 °C which is a critical requisite for paper industry.
15. The biobleaching effect of crude xylanase produced from *C. cinerea HK*-1 on lemon and sofia grass soda-AQ pulps was maximum at a xylanase dose of 10 and 8 IU/g, reaction time 120 min, pulp consistency 10%, temperature 55 °C and pH 6.4. Xylanase also caused the release of reducing sugars and chromophores from two soda-AQ pulps depicting its biobleaching potential.
16. Xylanase pretreatment in lemon and sofia grass soda-AQ pulps reduced the total chlorine demand by 22.67 and 29.73% for CEHH bleaching sequence while 11.60 and 16.92% for OCEHH bleaching sequence along with a significant gain in brightness.
17. The AOX in combined bleach effluent of CEHH and OCEHH bleaching sequences got reduced by 26.64 and 35.11% in lemon grass whereas in sofia grass the respective reductions were 29.96 and 30.73% as a result of xylanase pretreatment in comparison with control.
18. A gain in all mechanical strength properties was noticed as a result of xylanase pretreatment in CEHH and OCEHH bleaching sequences.
19. The xylanase pretreatment in ODED and ODEDP bleach sequences improved the pulp brightness by 2.6 and 2.2% respectively for lemon grass pulp along with 3.3 and 4.4% increments for sofia grass at the same chemical dose.

20. The AOX in combined bleach effluents from lemon and sofia grasses generated during ODED and ODEDP bleaching sequences reduced by 84.84 and 82.67% respectively compared to that of CEHH bleaching sequence. COD and colour also marked a major decrease.
21. Following the implementation of xylanase in the ECF sequences (ODED and ODEDP), a net percent decrease in AOX for lemon and sofia grass came out as 35.68 and 46.88%, in comparison to their respective controls.
22. TCF bleaching sequence $O(E_{OP})P$ marked its impact over conventional (CEHH and OCEHH) and ECF (ODED and ODEDP) bleaching sequences by producing the pulps of higher viscosity along with reduced bleaching losses.
23. It is concluded that keeping xylanase stage before oxygen delignification was more beneficial in attaining a higher brightness ceiling in TCF bleaching as brightness increased by 4.5 and 4.0% after $XO(E_{OP})P$ while 2.7 and 2.2% after $OX(E_{OP})P$ for lemon and sofia grasses respectively, compared to $O(E_{OP})P$ bleaching sequence.
24. Xylanase pretreatment led to a slight increase in pulp viscosity for conventional (CEHH and OCEHH), ECF (ODED and ODEDP) and TCF ($O(E_{OP})P$) bleaching sequences while a decrease was noticed in copper number which hence leads to a fact that there would be no brightness reversion with time.
25. A small decrease was noticed in all mechanical strength properties except tear index as a result of xylanase pretreatment of ODED, ODEDP and $O(E_{OP})P$ bleaching sequences at same chemical dose.
26. Xylanase pretreatment however also led to an increase in COD and colour of combined bleach effluents from all bleaching sequences.
27. It became clear from the SEM studies that xylanase pretreatment modified the fiber surface by introducing cracks, peelings, swelling and external fibrillation compared to smooth surface of untreated fibers.

Therefore, it is concluded that both the raw materials used in the study can successfully be used as raw materials for paper making process and crude xylanase produced by *C. cinerea* HK-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 study hence brings about development of sequences that would ensure environmental compliances with respect to AOX generation

for soda-AQ pulps of lemon and sofia grass, indicating the effectiveness of xylanase biobleaching for improving environmental performance of the bleach plant.

Realistic cost estimates and improvement in process economics are the key factors for the commercial success of any technology. The enzyme production was designed in a way so as to keep the process as cost effective as possible, like, cheap lignocellulosic substrates were used for the enzyme production under SSF and crude xylanases were used for enzyme bio-bleaching as it contained only a negligible cellulase contamination and thereby did not required any purification step. Still, it must be clearly understood that no enzyme-based process for bleaching can be as inexpensive as using chlorine or even organic chlorine compounds. The added expenses incurred by the use of enzymes must be viewed in terms of their accrued indirect benefits like prevention of environmental derangement and reduced health hazards to mankind. At the same time, xylanase treatment can easily be applied to any traditional or major process changes.

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. cinerea* HK-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. cinerea* HK-1 in extreme conditions.
- *C. cinerea* HK-1 and HK-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.
- Further work on purification and characterization of the xylanases obtained from the test strains is suggested for a better understanding of their enzyme system.
- It is recommended to take plant trials with xylanase produced by *C. cinerea* HK-1 in nonwoody fiber-based industry using lemon and sofia grasses to validate laboratory results and cost reduction studies must be carried out to

calculate the economic viability of enzyme. Also, studies should be carried out to see the effect of xylanase from the two test strains on different raw materials and on pulps produced by different methods.

- Chromatographic analysis to study the change in molecular weight profiles of lignin and carbohydrates in the pulp should be carried out for a better understanding of the attack of xylanase on lignin-carbohydrate complexes (LCC).

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