STUDIES ON ENZYMATIC ASPECTS OF MICROBIAL ORIGIN AND BIO-BLEACHING OF HARDWOODS

Ph.D. THESIS

by

POONAM

DEPARTMENT OF PAPER TECHNOLOGY
INDIAN INSTITUTE OF TECHNOLOGY ROORKEE
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A THESIS

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ABSTRACT

In view of wood-fiber crises and preservation of forests, the pulp and paper industry is moving towards fast growing wood species that can grow on waste land. The pulp and paper industry is under constant pressure for reducing the pollution load due to stringent rules of the governments; hence it is adopting the eco-friendly technologies. Therefore, the present investigation aims to produce crude enzyme preparations having high xylanase activity from the isolated fungal strain using cheap agro-residues as the substrate, and to develop an eco-friendly technology by using crude xylanase as prebleaching agent in different bleaching sequences of kraft pulps produced from two fast growing hardwood trees growing on waste land i.e. *L. leucocephala* and *C. equisetifolia*. The ultimate objective of this research work is to evaluate the feasibility of reducing the chemical consumption and pollution load while preserving the strength properties of *L. leucocephala* and *C. equisetifolia* kraft pulps through enzymatic treatment.

To achieve this, an investigation is undertaken for isolating, screening and identifying a potent fungal strain comprising the potential ability of secreting xylanase. A thorough investigation of a variety of natural sites has led to isolation of xylanase producing fungal strain RM-1. This strain has notably higher xylanase activity along with moderate cellulase activity and is identified as white rot basidiomycete *Coprinopsis cinerea* from National Fungal Culture Collection of India, Agharkar Research Institute, Pune (India). Enzyme production from fungal strain RM-1 is evaluated under SSF and SmF conditions, and SSF results into higher levels of xylanase as well as cellulase production. Various agro-residues are evaluated for their xylanase and cellulase producing ability under SSF and the combination of wheat bran and corn cob (7:3) is selected as the substrate for crude xylanase production based on its privilege of providing high xylanase along with minor cellulase contamination. On the other hand, sugarcane bagasse is selected for crude cellulase production as the highest cellulase activity by *C. cinerea* RM-1 is obtained on this substrate under SSF. The different components of NSS are also optimized for crude xylanase and crude cellulase production.

The eight operating parameters are screened under SSF through Plackett–Burman experimental design for achieving the optimum level of xylanase secretion. Out of eight variables studied through Plackett–Burman experimental design, three variables namely, initial pH, incubation temperature and incubation time show significant influence on xylanase and cellulase

production, and are selected for further optimization by central composite experimental design. Under optimal conditions, the predicted responses agree very well with the experimental data, and this confirms the fitness and applicability of the models obtained for crude xylanase and crude cellulase production. Statistical analysis confirms the significance of all the data. The biochemical characterization of crude xylanase and crude cellulase produced by fungal strain RM-1 confirms that the enzymes produced by *C. cinerea* RM-1 are thermo-alkali-tolerant. Therefore, this test strain *C. cinerea* RM-1 is chosen for the further bio-bleaching studies of *L. leucocephala* and *C. equisetifolia* kraft pulps.

The detailed anatomical and proximate chemical analysis of *L. leucocephala* and *C. equisetifolia* are determined in order to assess their suitability for pulp and paper making. The studies indicate that these two plants can satisfactorily be used as the raw materials for pulp and paper production. Kraft pulping process is used for pulping of *L. leucocephala* and *C. equisetifolia* wood chips. The three operating cooking variables for kraft pulping i.e. active alkali (as Na_2O), maximum cooking temperature and cooking time are optimized using CCD. Statistical analysis confirms the significance of all the models. Under optimal conditions, the predicted responses agree very well with the experimental data. The mechanical strength properties are also optimized at different beating levels and beating level of 40±1 °SR is found optimum for *L. leucocephala* and 45±1 °SR for *C. equisetifolia*. The strength properties of *L. leucocephala* kraft pulp are found superior than that of *C. equisetifolia*. Bauer-McNett fiber classifier is used for fiber length distribution of kraft pulps. It confirms that kraft pulp of *L. leucocephala* has more percentage of long sclerenchymatous fiber. It indicates that the kraft pulp of *L. leucocephala* is stronger than that of *C. equisetifolia*.

The crude xylanase enzyme produced by *C. cinerea* RM-1 is analyzed for its application in bio-bleaching of *L. leucocephala* and *C. equisetifolia* kraft pulps and reduction of toxicity in effluents generated, in terms of AOX while preserving mechanical strength properties during various bleaching sequences. Xylanase prebleaching stage (X) is optimized in terms of xylanase dose, retention time and pulp consistency, and the pulp filtrates are analyzed for the release of reducing sugars and chromophores from the kraft pulps of *L. leucocephala* and *C. equisetifolia*. The effect of crude xylanase preparation is observed on different bleaching sequences i.e. conventional (CEHH and CEHHP), ECF (ODED and ODEP) and TCF ($O(E_{OP})P$). Xylanase pretreatment results in small gain in viscosity over controls for conventional, ECF and TCF bleaching sequences of kraft pulps of *L. leucocephala* and *C. equisetiolia*. This indicates that the

cellulase contamination in the crude enzyme extract has no adverse effect on the pulps of two raw materials. A gain in COD and color values of bleach effluents from all bleaching sequences is noticed by xylanase pretreatment. This indicates the solubilization of residual lignin carbohydrate complexes. Xylanase pretreatment of *L. leucocephala* and *C. equisetifolia* kraft pulps also shows the reduction in refining energy in terms of PFI revolutions for achieving the fixed beating level as compared to their respective controls during all bleaching sequences. The xylanase pretreatment of *L. leucocephala* and *C. equisetifolia* kraft pulps reduces the total chlorine demand and the AOX formation in bleach effluents while still attaining the high brightness and slightly improving the mechanical strength properties as compared to their respective controls.

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CHAPTER 1

INTRODUCTION

1.1. STATUS OF PULP AND PAPER INDUSTRY

The pulp and paper industry is one of the most important industries in the world, and it has a direct impact on the socio-economic development of the country. The pulp and paper industry consists of about 5000 pulp and paper mills worldwide. It supplies an essential product- paper to over 5 billion people across the world. World demand for paper and paperboard is likely to grow by 2.1% annually in the long term, reaching an estimated 490 million tonnes by the year 2020 [35]. China and India are estimated to be the most rapidly growing production countries within Asia accounting for 39% and 8% production of the world by 2020 [71]. Indian paper industry is more than a century old, consisting of about 715 paper mills, and accounts for 1.6% of the world's production of paper and paperboard [70]. The Indian paper industry utilizes wood, agro-residues and recycled papers as the major raw materials [75]. In early seventies, wood was the chief fibrous raw material for the pulp and paper industry as the share of woody raw materials was 84%. Subsequently, due to scarcity of forest based raw materials, the share of wood has decreased remarkably, and falls down to 39% [39]. The production of paper and paperboard products from different cellulosic raw materials i.e. wood based, agricultural residues and waste paper is shown in Table 1.1. At present, majority of Indian pulp and paper mills are based on recycled fiber (551.0), then agro-based (139.0) and wood-based (25.0) [70]. Only 3-4% of total wood is utilized by the pulp and paper industry. Due to forest policy, the paper industry is constrained not to take wood from any of the national forest reserves [33].

With the increasing world population and paper demand, the paper industry is facing the severe shortage of forest based and conventional pulpable raw materials globally. Figure 1.1A indicates the projected population and literacy rate of India till 2020. India consists of 20.6% forest cover of the country's surface area and only 0.8 ha/person per capita forest area. Total fiber consumption for the production of paper and paperboard in India is estimated to be growing from 7.4 to 13.7 million tonnes per year in between 2006 and 2016 [17]. The growth of Indian paper industry depends mainly on the sustained availability of cellulosic raw materials which include forests, agricultural residues and the recycled fibers [75]. India's total

wood fiber scarcity is expected to enhance at a rate of 11.3% per year by 2016 [17]. The wood demand of pulp and paper industry is anticipated to grow to 9 million tonnes by the year 2010 and 13 million tonnes by the year 2020 [55] (Figure 1.1B).

1.1.1. Raw materials

In view of wood-fiber crisis, increasing attention is currently being directed towards fast growing wood species that can grow on waste land to make a sustainable supply of wood fibers. Fast growing tree farming may be a sustainable and economically viable land-use activity to get rid of wood-fiber-crisis [30]. Many high yielding annual and perennial plants as well as agro-based cellulosic raw materials have been identified and assessed for their suitability of pulp production [26, 27, 28, 42, 69]. Moreover, many fast growing hardwood plants such as *Leucaena leucocephala* [44], *Casuarina equisetifolia* [3], *T. orientalis* [31] and *Eucalyptus* sp. [43, 51], non-woody plants such as lemon and sofia grasses [36], kenaf [53, 54] and agricultural residues [15, 16] such as wheat straw, rice straw and bagasse [12, 59, 60, 61] have been cultivated and studied for their potentiality as alternative source of raw materials for pulp and paper industry. In the past, few studies have been done on *L. leucocephala* and *C. equisetifolia* to test their suitability for pulp and paper making by using the kraft process of pulping [8, 19, 20]. *L. leucocephala* and *C. equisetifolia* (Photographs 1.1A and B) are fast growing trees; belong to the family Fabaceae and Casuarinaceae, respectively. These plants can grow vigorously on barren and polluted sites, increase soil fertility and prevent erosion with facilitating the establishment and growth of other plant species [40]. These plants not only help to conserve environment but also give higher yield of pulpable biomass with a high calorific value. Moreover, these plants have an additional importance because of their nitrogen-fixing ability which is due to their symbiotic association with the bacterial species. In view of shortage of wood fibers and the usability of *L. leucocephala* and *C. equisetifolia*; these two fast growing hardwoods are selected as the raw materials for pulping and bio-bleaching experiments.

1.2. ENZYME INDUSTRY

The estimated value of enzyme industry was 3.3 billion US$ in 2010 in the global market, and expected to grow by a compound annual growth rate of 6% over the period of 5 years, reaching an estimated 4.4 billion US$ by the year 2015. Technical enzymes were worth over 1 billion US$ in the year 2010. This sector is estimated to grow by an annual growth rate

of 6.6%, reaching an anticipated 1.5 billion US$ by the year 2015; the highest demand of technical enzymes are in leather followed by bioethanol industry [65]. India contributes to about 2% of the global biotechnology industry. India ranks third after Japan and Korea, in the Asia-Pacific region, and is anticipated to reach 25 billion US$ by 2015 [29]. Novozymes and Biocon are the largest players in this sector having a global market of about 24 million US$ and 23 million US$, respectively. These two companies account for about 50% of the total sector's value. Including manufacturing and marketing sector, about fifteen companies are involved in enzyme business in India. Most of the enzyme companies are focused on textile, chemicals, food processing, dairy, aquaculture, breweries and animal nutrition for the application of their products. On the other hand, the consumption of enzymes in pulp and paper industry is very less as compared to other sectors. To the total market of industrial enzymes, hydrolytic enzymes account for about 75% and glycosidases (cellulases, amylases and hemicellulases) comprise second major group after protease [7]. The government of India has approved many projects related to industrial enzymes for the growth of industry.

1.2.1. Enzymes used in pulp and paper industry

Enzymes are the fundamental elements for biochemical processes and catalyze all aspects of cell metabolism, and also have valuable industrial and medical applications [24]. Enzymes are distinct biological molecules that catalyze (i.e. increase the rate of) chemical reactions, and convert substrates to particular products. They are usually very specific in function (as to which reactions they catalyze) and the substrate, and increase the rate of reactions by providing alternative pathways of lower activation energy without being consumed. Complementary shape, charge and hydrophilic/hydrophobic characteristics of enzymes and substrates are responsible for their specificity. Enzymes are the best alternative to polluting chemical technologies due to their eco-friendly usage.

Pulp and paper industry is forced to adopt more cost effective and environmental friendly alternative technologies due to increasing environmental pressures. The main policies of pulp and paper industry are high productivity, improved quality of products and energy savings with lesser environmental impacts. Biotechnological processes are consistent with these polices and are getting interest to imply in pulp and paper industry [5]. The driving force of biotechnology is the ability of microorganisms of utilizing the various carbon sources, occurring as pollutants [2]. Fungi and their enzymes have different biotechnological potential [63]. The enzymes were utilized in the pulp and paper industry, for the first time in the year

1986. However until now, the use of enzymes in pulp and paper industry is not feasible due to lack of suitable enzymes [4]. Still, several scientific institutions and enzymatic companies are involved in the research regarding the application of biotechnology in diverse areas of pulp and paper industry [13]. However, most of the prospective enzymes are still in the research and development stage, and many have been commercialized. The use of enzymes in pulp and paper industry has continued to gain an interest of researchers [13] as demonstrated by the increasing numbers of scientific papers and patents related to this topic every year since 1995 (Figures 1.2 and 1.3). Table 1.2 shows the different types of enzymes used in pulp and paper industry and their applications. The most commonly used enzymes in pulp and paper industry are cellulases, xylanases, laccases and lipases. Cellulases are primarly applied in pulp refining, deinking and vessel picking reduction experiments. Whereas xylanase and laccase are most commonly used in bleaching and delignification processes. Lipases are primarily applied in pitch control and processing of paper.

Today, the pulp and paper industry is adopting the ecofriendly technologies to reduce the pollution and to meet the challenges of globalization. The focus is on the alternative bleaching technologies which can reduce the AOX and TOCL in bleach plant effluents. From an industrial point of view, the thermo-tolerant and alkali-tolerant characteristics of enzyme are desirable because the pulps obtained after kraft cooking are warm and alkaline, and it is difficult and expensive to control the temperature due to the cost of cooling. Moreover, the degradation products in the kraft pulp and wash water are in abundant amount, hence, the enzymes used in these processes should resist inhibition creating by kraft degradation products. Cellulase free thermo-tolerant and alkali-stable xylanases are gaining importance in pulp and paper technology because of their use as a bleach-boosting agent [45, 46, 50, 67] in bio-bleaching where the high temperature (55-70 °C) and alkaline pH are essential. Currently, the xylanase prebleaching technology is the most important application of enzymes, and is being used at several mills worldwide [4, 37, 52]. Hence, the focus of future developments is to identify the xylanase with higher temperature stability at alkaline pH and developing process technologies for commercial application of such enzymes. Many studies have been carried out for producing the xylanase with thermo-tolerant and alkali-tolerant characteristics for their application in pulp and paper industry [72, 1, 34]. Xylanases are used as catabolic agent for delignification in the bleaching process. They react with xylans and eliminate them [73, 22] from the pulp; thereby breaking the linkage between cellulose and lignin. The lignin is set free

and washed out rapidly in subsequent bleaching stages [74, 56]. Cellulase has been used for reduction of vessel picking and refining or beating time of pulp [4, 38, 11]. However, for commercial application, enzymes should ideally be produced in high titers with a rapid rate using cheap agro-industrial wastes such as wheat straw, rice straw, wheat bran, corn cob and sugarcane bagasse etc. as the substrate for making the process more economic at an industrial level [32, 21, 58]. Moreover, lignocellulosic wastes or agro-residues are generated every year over the globe in large quantities; if these are not properly discharged or used, cause the environmental problems [18]. Agro-residues consist of cellulose, hemicellulose and lignin which may act as the substrate for the production of different enzymes like xylanase, cellulase and laccase etc.

1.3. OPTIMIZATION AND RESPONSE SURFACE METHODOLOGY

Optimization is a complex heterogeneous process, involving the interaction effect of many variables. In general, the conventional, OVAT method of optimization is used which may be effective in some experiments but fails to consider the interaction effects of all involved variables [6]. This approach is insufficient for optimization of multivariable system [48]. This method is determined by single dimensional search by changing one variable while keeping the other variables at a constant level; this gives unreliable results and often requires a considerable amount of work and time. RSM has been very popular for optimization studies in recent past years as it can overcome the limitations of OVAT approach, and illustrates the effect of the independent variables, individually or in combinations [6]. The application of RSM involving the statistical experimental designs including PBD and CCD in optimization experiments can result in improved product yields, reduced process variability, development time and overall costs [62, 25]. PBD [57] is a powerful tool to screen 'n' variables in just 'n+1' experiments, which may reduce the total number of experiments and has been widely used in optimization of fermentation processes [64, 76, 68, 41]. This technique cannot determine the interaction effect but it is very useful for the initial step of an optimization procedure, and provides indications and tendency regarding the necessity of each factor in relatively few experiments. RSM is a collection of statistical techniques for designing experiments, building models, evaluating the effects of several variables and searching for the optimum conditions [10], and have successfully been used in the optimization of bioprocesses [23]. RSM may evaluate multiple parameters and their interactions meanwhile reducing the number of experimental trials. Many researchers have been used RSM successfully in fermentation processes for the production of

enzymes from different microorganisms [66, 9, 49, 14]. The basic theoretical aspects, fundamental assumptions and experimental implications of RSM have been discussed elsewhere [47]. Therefore, keeping in view the advantages of RSM, the majority of the optimization work is being done using response surface methodology.

This Thesis is interdisciplinary in nature. The major objectives of the Thesis are:

1. To design the experiments using RSM for optimization of various operating physicochemical parameters and culture conditions for the production of thermo-tolerant and alkali-stable xylanase and cellulase from the isolated fungal strain.
2. To determine the anatomical and chemical properties of the two hardwood raw materials for evaluating their potential utilization in pulp and paper production.
3. To design the experiments using RSM for optimization of variables associated with kraft pulping process for *L. leucocephala* and *C. equisetifolia.*
4. To use crude xylanase enzyme for developing an environmental friendly technology for bleaching of kraft pulps of *L. leucocephala* and *C. equisetifolia*. The ultimate objective of this study is to evaluate the feasibility regarding reduction in the chlorine demand and toxicity of effluents generated during various bleaching sequences, in terms of AOX while preserving mechanical strength properties.

1.4. ORGANIZATION OF THE THESIS

Keeping in view the above discussed objectives, the Thesis has been organized as follows:

Chapter 1: It is introductory in nature. It states the relevant definitions and furnishes the objectives of carrying out this work.

Chapter 2: In this chapter an attempt has been made for isolation, screening and identification of potent fungal strains producing thermo-alkali tolerant xylanase and cellulase by using cheap agro-residues as the substrate. The experiments are designed using OVAT, PBD and CCD for optimization of xylanase and cellulase production by the screened fungal isolate. MINITAB-16 software is used for generating the PBD and CCD, and the experiments are performed for data collection. The data are modelled using method of least squares. The results are validated by experimenting again at the obtained input.

Chapter 3: It represents the analysis of the anatomical characteristics of the two raw materials; *L. leucocephala* and *C. equisetifolia* followed by their proximate chemical analysis to confirm the suitability of these hard wood fibers for production of kraft pulps.

Chapter 4: In this chapter an attempt has been made for optimization of variables associated with kraft pulping process for *L. leucocephala* and *C. equisetifolia*. This optimization process involves three major steps: (i) The effect of sulphidity, additive (AQ) and surfactant (Tween 20) is studied by using OVAT approach (ii) the three pulping variables i.e. alkali charge (active alkali as Na_2O), maximum temperature and cooking time (at temperature) are optimized using CCD and their effects are investigated with respect to screened pulp yield, pulp kappa number and pulp viscosity (iii) Modeling of data for each of the response variable. The unbleached pulps of *L. leucocephala* and *C. equisetifolia* are beaten to different beating levels and laboratory hand sheets are prepared. These sheets are evaluated for paper properties.

Chapter 5: This chapter illustrates the effect of bio-bleaching (enzyme aided bleaching) of kraft pulps of *L. leucocephala* and *C. equisetifolia* in reducing the chlorine demand and toxicity of effluents generated during various bleaching sequences, in terms of AOX. The effect of enzyme treatment is also observed on brightness, viscosity and mechanical strength properties like tear, tensile, burst indexes and double fold.

Chapter 6: This chapter depicts the conclusions of the work presented in earlier chapters. Moreover, a brief discussion for future aspects of this work is described.

Table 1.1: The estimated production of paper from different cellulosic raw materials in million tonnes

Year	Wood resources	Agro based resources	Recycled paper	Total production	Baseline production
2010-2011	3.2	2.2	4.7	10.1	10.1
2011-2012	3.4	2.3	5.1	10.9	10.9
2012-2013	3.7	2.5	5.7	11.8	11.7
2013-2014	4.0	2.7	6.2	12.9	12.5
2014-2015	4.3	2.9	6.8	14.1	13.3
2015-2016	4.6	3.2	7.5	15.3	14.1
2016-2017	5.0	3.4	8.3	16.7	14.8
2022-2023	8.0	5.4	14.7	28.0	19.6
2024-2025	9.3	6.3	17.8	33.4	22.0
2026-2027	10.8	7.4	21.5	39.7	23.5

(**Source:** CPPRI, Sub-committee on raw material (Group-I) for wood based paper industry)

Table 1.2: The key enzymes used in pulp and paper industry and their applications [13]

Enzymes	Applications
Cellulase	Deinking, refining, vessel picking reduction, drainage enhancement
Xylanase	Bleach boosting, Beating
Laccase	Delignification
Lipase	Pitch control
Manganese peroxidase	Bleaching
Lignin peroxidase	Bleaching
Amylase	Drainage enhancement, Slime control
Esterase	Stickies control
Protease	Paper conservation

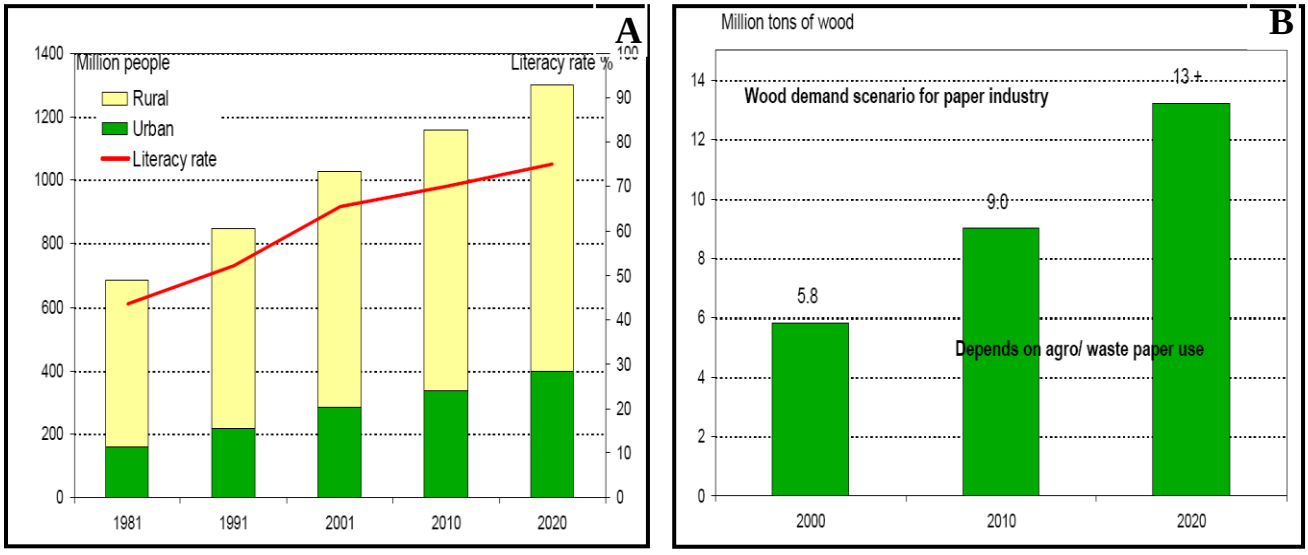

Figure 1.1: Projected Indian population and literacy rate (A) and the wood demand scenario for pulp and paper industry in India (B) [55].

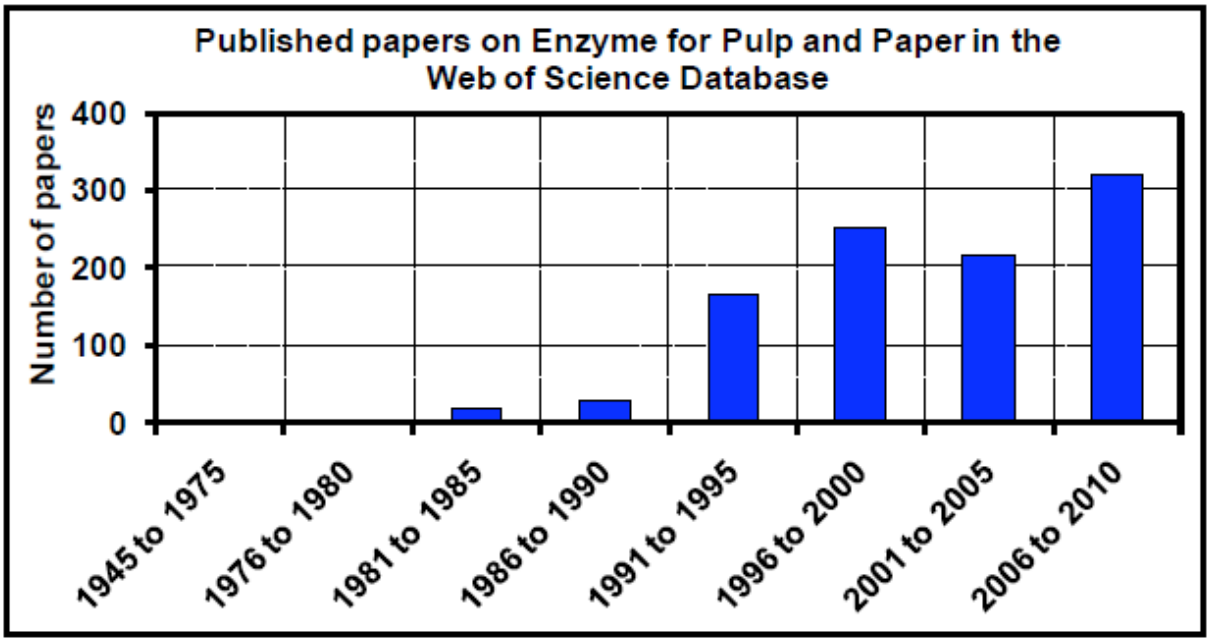

Figure 1.2: Published documents on the application of enzymes for pulp and paper industry from 1945 to July 2010 [13].

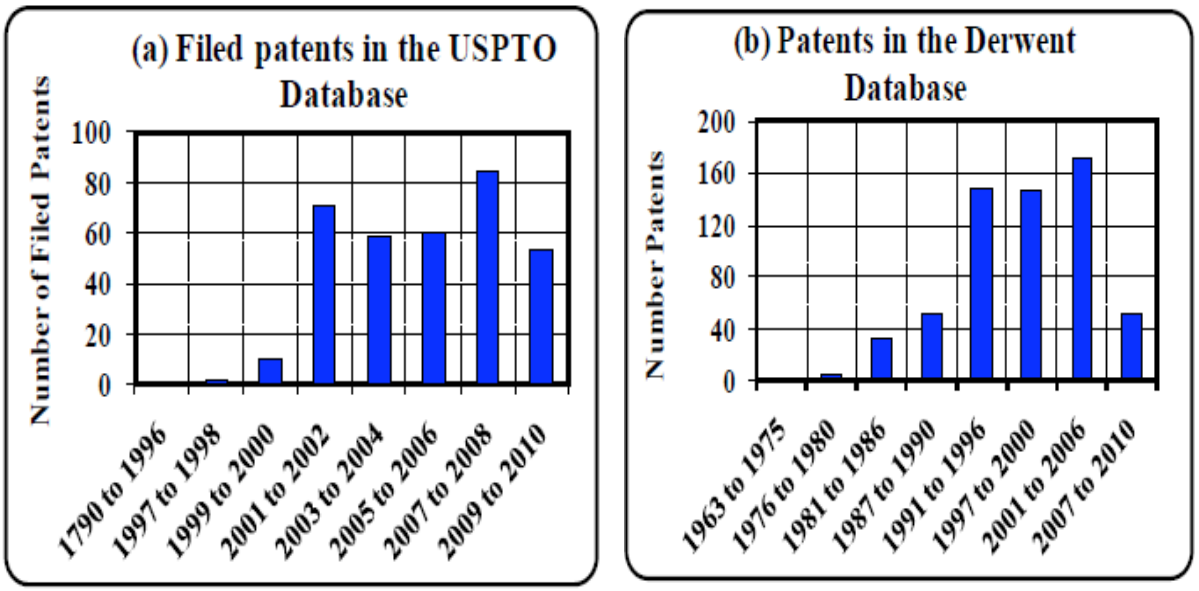

Figure 1.3: Patents filed for the application of enzymes in pulp and paper industry; in USPTO database from 1970 to July 2010 (a) and in Derwent database from 1963 to 2010 (b) [13].

Photograph 1.1: *L. leucocephala* plant (A) and *C. equisetifolia* plant (B).

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ANNEXURE 1

ABBREVIATIONS

%	Percent
AOX	Adsorable organic halides
AQ	Anthraquinone
CCD	Central composite design
etc.	et cetera
i.e.	That is
OVAT	One-variable-at-a-time
PBD	Plackett-Burman design
RSM	Response surface methodology
sp.	Species
TOCL	Total organic chlorides

CHAPTER 2

STUDIES ON XYLANASE AND CELLULASE PRODUCTION

2.1. INTRODUCTION

Xylanase and cellulase are the most important enzymes attracting the attention because of their several applications in different industries [136, 15, 79, 11, 12, 135]. Currently, the major applications of xylanase and cellulase are in pulp and paper, feed, food, textile and baking industries [22, 141, 147, 9, 152, 114]. Xylanase and cellulase are wide spread in nature and produced by a wide variety of bacteria, yeast, actinomycetes, fungi, marine algae, protozoa, snails, insects and seeds of terrestrial plants [32, 10, 79, 27, 21]. Among them, fungal enzymes are commonly used in industries due to various technical reasons, including the feasibility of obtaining enzymes in high concentration by solid state fermentation [96]. From the foregoing research, it is apparent that a number of microorganisms including the strains of *Coprinopsis cinerea* [72], *Coprinellus disseminates* [1, 132], *Aspergillus* sp. [13], *Aspergillus carneus* [38], *Penicillium oxalicum* [99], *Aspergillus kawachii* [62], *Trichosporon cutaneum* [85], *Aspergillus nidulans* [41] and *Streptomyces* [69] have been isolated and exploited for xylanase and cellulase production, and this list is still growing continuously. The white-rot fungi belonging to basidiomycetes are the most efficient producers of xylanase, cellulase as well as other extracellular lignocellulolytic enzymes [3, 4, 51, 132]. White-rot basidiomycetes and the component of their lignocellulolytic system are therefore, at the moment of great interest for biological pulping and bleaching [132, 72]. The potential use of white-rot fungi for bio-pulping, bio-bleaching and treatment of pulp mill waste effluents has been reviewed. Keeping in view, the huge potential of white rot fungi as xylanase and cellulase producers, extensive studies are need to be done to tap this promising source of enzymes.

Various efforts have been made to mitigate or eliminate the use of chlorine in pulp bleaching processes due to stringent regulations and soaring environmental concern. This has compelled industry to research and develop cleaner processes, and shifted their main focus to the use of less polluting pulping and bleaching techniques [87]. Over the years, the application of xylanase and cellulase has been increased significantly in pulp and industry. These enzymes are ubiquitous, and their judicious use in industry might result in lower pollution load and consumption of energy [111]. Use of xylanase in the treatment of pulp improves the liberation

of lignin by hydrolyzing residual xylan and represents a successful and newer technology that may remarkably reduce the demand of molecular chlorine or chlorine-based bleaching chemicals. It results in an increase in pulp brightness and paper properties, and also reduces pollution load [146, 139, 148, 12, 26]. Cellulase is used to treat the hardwood pulp for reducing the vessel picking, and thus improves the paper and print quality [28]. Cellulase is also used to reduce refining or beating time of pulp [155]. In the past two decades, the oxidative and hydrolytic enzymes mainly produced from lignocellulose degrading microorganisms have been used in pulp and paper industry [12].

Lignocelluloses comprise cellulose, hemicellulose and lignin. Cellulose, the most abundant organic polymer, is a linear chain of β-1,4-D-glucopyranose units [48]. Hemicellulose is a branched polymer of several different sugars such as xylose, glucose, mannose, rhamnose, galactose and arabinose. Lignin is an organic polymer of aromatic alcohols. Lignocelluloses are commonly degraded by combined action of lignocellulolytic enzymes such as cellulase, hemicellulase and Laccase etc. Cellulase consists of many enzymes with various isozymes which act synergistically for breaking cellulose into glucose [25]. The cellulolytic enzyme system in fungi is considered to comprise three major components: (i) endoglucanase or CMCase (endo-1,4- β-D-glucanase; EC 3.2.1.4) randomly cleaves the cellulose backbone at internal amorphous parts and generating new chain ends (ii) exoglucanase or cellobiohydrolase (exo-1,4-β-D-glucanase; EC 3.2.1.91) cleaves at reducing or non-reducing ends of cellulose chain and release cellobiose units (iii) β-glucosidase or cellobiase (EC 3.2.1.21) hydrolyzes the short-chain cellooligosaccharides and cellobiose to glucose.

Xylanase is glycosidase (O-glycoside hydrolase, EC 3.2.1.x) which catalyzes the endohydrolysis of 1,4-β-D-xylosidic linkages in xylan. Xylan (mainly heteroxylan) constitutes the major component of hemicellulose, and is composed of xylopyranose units linked by β-1,4-xylosidic bonds with different substitute groups in the side chains [138]. The structure of xylan, found in cell wall of plants, can differ greatly depending on their origin and different structures attached to the xylan backbone [79, 82]. They are highly branched and in firm association with other polymers. Wood xylan exists as O-acetyl-4-O-methylglucuronoxylan in hardwoods and as arabino-4-O-methylglucuronoxylan in softwoods while xylan in cereals and annual plants is typically arabinoxylan [79]. The complete enzymatic hydrolysis of xylan requires the synergistic action of a group of "xylanolytic enzymes". This is due to structural heterogeneity of xylan from different sources [115, 49, 24, 17]. The xylanolytic system represents the

repertoire of hydrolytic enzymes mainly composed of endo-β-1,4-xylanases (endo-1,4-β-D-xylan xylanohydrolases; EC 3.2.1.8) that randomly cleave the xylan backbone and β-1,4-xylosidases (1,4-β-D-xyloside xylohydrolases; EC 3.2.1.37) that cleave xylose monomers from the non-reducing end of xylo-oligosaccharides and xylobiose along with debranching enzymes (L-arabinofuranosidases and glucuronidases) and esterases (acetylxylan esterases, ferulic acid esterases and coumaric acid esterases) that allow the complete degradation of the xylooligosaccharides to their constituent monosaccharides [27, 49, 138]. There are an abundance of diverse xylanases with varying specificities, primary sequences and folds due to heterogeneity and complexity of xylan but the main enzyme needed to enhance the delignification of pulp is endo-β-1,4-xylanase. However enrichment with other hemicellulolytic components has been shown to improve the effect of enzymatic treatment. Xylanase can be classified into different groups mainly by taking into account the different criteria. Wong and co-workers [153] suggested that endoxylanase could be grouped into two, on the basis of their molecular weight and pI: (i) having low molecular weight (<30 kDa) basic protein with high pI value and (ii) having high molecular weight (>30 kDa) acidic protein with low pI value. This classification pattern also takes into consideration of the sequence analysis and can predict the crystal structure. However, this pattern has several exceptions [138, 93]. On the basis of kinetic parameters, xylanase can be classified into three types [33, 10]: (i) Endo-β-(1-4)-D-xylan xylanohydrolase, [EC 3.2.1.8] acts randomly on xylan to produce large amount of xylo-oligosaccharides of various chain lengths (ii) Exo-β-(1-4)-D-xylanase, [β-(1-4)-D-xylan xylanohydrolase] removes single xylose units from non-reducing terminal of xylan chain and (iii) β-xylosidase or xylobiase [EC 3.2.1.37] hydrolyzes disaccharides like xylobiose and the higher xylooligosaccharides with decreasing specific affinity. Furthermore, these patterns are not applied on many xylanase, and the divergent research have evolved that many xylanase families have related three dimensional structures and can be grouped into hierarchical levels (clans) [20]. Hence, a more complete classification system has been introduced on the basis of crystal structure which grouped xylanase into two families:

Family 11/family G: members of this family result into oligosaccharides with lower degree of polymerization [64, 150].

Family 10/family F: these are true xylanase, lacking the cellulase activity. Catalytic domain of this family consists of the β-pleated sheets, formed into a bilayered trough that surrounds the catalytic site [151, 64]. Moreover, it has been established that like the families 10 and 11, a

distinct catalytic domains with a demonstrated endo-1,4-β-xylanase is found in some families like 5, 7, 8 and 43. Thus, according to current view, the enzymes with xylanase activity should include families 10, 11, 5, 7, 8 and 43 [27].

The various biotechnological techniques like submerged fermentation and solid state fermentation have been employed for xylanase and cellulase biosynthesis [46, 69]. SSF is gaining interest instead of SmF because it presents many advantages such as the higher yields, better product characteristics, lower operation and capital cost [106, 116]. SSF is defined as the fermentation process allowing the growth of microorganisms on moist solid materials in the absence of free-flowing water [105, 108]. Filamentous fungi are ideally suited to SSF due to their hyphal mode of growth and also due to their physiological capabilities. They are well adapted to spread over and penetrate into solid substrate because on this media, the fungi grow under conditions close to their natural habitats due to which they may be more capable of producing certain enzymes and metabolites [7]. In SSF, the colonies arising from the adjacent spores soon merge and then the hyphal density within the mycelium increases. Hyphal growth also gives the fungus a much higher penetration than unicellular microorganism. Furthermore, SSF offers numerous opportunities in processing of agro-industrial residues [107].

The industrial and other practical suitability of the enzymes depends mostly on their alkali and thermal stability, and also on other kinetic and thermodynamic properties. Most of the xylanases which have been used in industries belong to the mesophilic and/or neutrophilic origin [55]. However, the thermo-tolerant enzymes could be used in the processes where a cooling step would be uneconomical or high temperature is essential to enhance the solubility of substrates, and to reduce the risk of contamination [89]. Most of the xylan-degrading enzymes from thermophilic fungi belong to endoxylanase [90]. The efficient production of xylanolytic and cellulolytic enzymes depends on the nature of inducing substrate and optimum medium composition [78]. The smaller molecules such as xylose, xylobiose, xylooligosaccharides, glucose, lactose, cellulose and their positional isomers act as a potent inducer for production of xylanase and cellulase [119, 31]. The nature of the solid substrate is one of the most important factors affecting the fermentation process and its selection depends on several factors mainly related with cost and availability. The use of purified substrate for inducing the enzyme production makes the process expensive. Therefore, many studies have been conducted involving the use of several cheap lignocellulosic wastes such as wheat straw, wheat bran and rice straw etc. as the substrate for the production of xylanase and cellulase [53,

113, 19, 72]. There are many factors that have their importance in enzyme production. It has been reported that several factors including incubation time, pH, carbon and nitrogen source, temperature and substrate concentration etc. can influence the production of fungal enzymes in various fermentation processes [55, 154, 18]. Therefore, screening of important nutritional factors and optimization of fermentation conditions are of essential importance to determine the optimal parameters for efficient production.

Therefore, the aim of the present study is to optimize the various operating physico-chemical parameters and growth conditions for maximum production of xylanase (preferable) and cellulase enzyme by the isolated fungal strains through suitable fermentation system by using agro-residues as the substrates. RSM based on CCD is adopted in this study for evaluating the significant factors, building models and for determining the optimum conditions of variables for maximum production of xylanase and cellulase enzyme. Statistical software Minitab-16 is used for generating the PB and CCD tables, facilitating the analysis, generating the contour plots and for optimizing the independent variables. The xylanase and cellulase enzyme produced under optimized conditions are characterized biochemically in terms of alkali and thermo-tolerant nature for their successful utilization in pulp and paper industry.

2.2. MATERIALS AND METHODS

2.2.1. Materials

Birchwood xylan and yeast extract were purchased from Sigma Chemical Co. (USA); BSA and DNS were purchased from Loba Chemie, India. All other reagents were of laboratory grade and procured from standard manufacturers. Wheat bran, rice straw, corn cob, sugarcane bagasse and coconut coir were purchased from the local market (District Saharanpur, U.P., India).

2.2.2. Methods

2.2.2.1. Isolation of xylanase and cellulase producing fungal strains

A total 51 samples (21 from wood sources+30 from soil) were collected from the decaying wood, decomposing manure, forest soil and paper industry waste sites of Western U.P. (Saharanpur District) and Uttarakhand (Dehradun District), India. Out of 21 wood samples, 11 strains of wood rotting fungi were isolated by enrichment technique, in which the decaying wood samples were buried in the wet rice straw and incubated at 37 °C in a BOD incubator. The growth was observed every day, and the moisture level was carefully controlled

with 0.85% NaCl for isolating the alkali stable enzyme producing strains. Upcoming fungal cultures were isolated on rice straw agar plates (4% w/v rice straw, 2% w/v agar-agar, pH 7), and the plates were incubated at 37 °C. Serial dilution technique was used for isolation of fungal strains from 30 soil samples.

Briefly, soil samples from different sites were collected and mixed separately, and 1.0 g soil from it was suspended in 100 mL saline (0.85% NaCl). These suspensions were incubated in an orbital shaker incubator (Sanyo, Orbi-safe, UK) at room temperature with shaking at 140 rpm for 30 min. Mixtures were allowed to settle and then suitable dilutions were spread on rice straw agar media and plates were incubated at 37 °C. Morphological observation of the colonies indicated that a total 21 different strains were isolated from soil samples, and these strains were then further purified and stabilized on same medium by routinely sub culturing. All 32 purified fungal cultures were maintained on PDA slants, incubated at 37 °C for 4 days and then kept at 4 °C. The fungal cultures were also preserved as a suspension of hyphae and spores in sterile glycerol, 15% (v/v), at -20 °C.

2.2.2.2. Preliminary screening of fungal isolates by plate assay technique

Primary screening of all the isolated strains for xylanase and cellulase production was carried out on xylan agar (XA) and CMC agar (CMCA) plates, respectively [143]. The XA medium contained 1% w/v birch wood xylan and 2% w/v agar-agar while CMCA medium contained 1% w/v CMC and 2% w/v agar-agar. These plates were inoculated with four days old cultures grown on rice straw agar medium and incubated for 4 days at 37 °C. Thereafter, the plates were stained with Congo red solution (0.5% Congo red and 5% ethanol in distilled water) for 15 min followed by washing with 1M NaCl. The xylanase and cellulase producing strains were selected by observing clear zones surrounding the colonies against the red background. Primary screening was done for selecting the fungal strains exhibiting higher xylanase and cellulase activities.

2.2.2.3. Secondary screening of xylanase and cellulase producing isolates

Out of 32, secondary screening of 5 selected fungal strains exhibiting good area of clear zones on XA medium as well as on CMCA medium was carried out on the basis of higher xylanase [8] and cellulase [92] activities of crude sample produced under SSF conditions as shown in Table 2.2. Finally, four fungal strains i.e. RM-l, RM-2l, RM-l4 and RM-4 exhibiting higher activities of xylanase as well as cellulase were selected for morphological identification.

2.2.2.4. Identification of selected isolates

Four selected fungal strains (RM-l, RM-2l, RM-l4 and RM-4) were sent to ITCC, Division of Plant Pathology, IARI, New Delhi, India, for their morphological identification. Furthermore, the white rot fungal strain RM-1 showing the highest activity of xylanase and moderate activity of cellulase was sent to ARI, Pune, India for its molecular identification. Molecular identification was done up to species level by isolating the genomic DNA. About 500 bp r-DNA fragments were amplified using universal primers with sequencing PCR which was set up with ABI-BigDye® Terminator v3.1 Cycle Sequencing Kit. By aligning the sequenced data with publically available sequences, the fungal isolate was identified as *Coprinopsis cinerea* RM-1 with accession number NFCCI-3086.

2.2.2.5. Scanning electron microscopy

SEM was carried out, using a FEI Quanta 200 F microscope for morphological studies of selected fungal strain RM-1. Fungal mat and spores were subjected for fixation using 3% (v/v) glutaraldehyde and 2% (v/v) formaldehyde in a ratio of 4:1 for 24 h. After primary fixation, samples were washed thrice with double distilled water and then treated with the alcohol gradients of 30, 50, 70, 80, 90 and 100% for dehydration. These samples were kept for 15 min in each alcohol gradient upto 70% and subsequently treated for 30 min under each gradient. Fungal samples were then examined under SEM at desired magnifications using gold shadowing technique [43].

2.2.2.6. Fermentation medium

2.2.2.6.1. Nutrient salt solution

NSS, as prepared by Vishniac and Santer [149] and standardized by Singh and Garg [131] was used as a fermentation medium. It contained, as g/L, 1.5 KH_2PO_4, 0.5 $MgSO_4.7H_2O$, 4.0 NH_4Cl, 0.5 KCl and 1.0 yeast extract in distilled water with trace elements solution (0.04 mL/L) comprising; $ZnSO_4.7H_2O$ (180 µg/L), $FeSO_4.7H_2O$ (200 µg/L) and $MnSO_4.7H_2O$ (20 µg/L). The pH of the medium, as desired, was adjusted with the help of $NaOH/H_2SO_4$ by pH meter (Knick, Germany, Model-761 Calimatic).

2.2.2.6.2. Preparation of substrates

The agro-residues, namely, wheat bran, rice straw, corn cob, sugarcane bagasse and coconut coir were washed 3-4 times in hot and cold distilled water for removing starchy and

other undesirable materials. The water was then decanted, and substrates were immediately dried in sunlight. Following this, the above agro-residues were subjected to high pressure steam treatment in which all the substrates were steam heated at 121±2 °C temperature and high pressure for 20 min [67, 84], and powdered using laboratory grinder. The powders were then exposed to sunlight again and sieved by using screens of different mesh size, and the fractions so obtained were stored in polythene begs for further use in fermentation processes.

2.2.2.7. Enzyme production

2.2.2.7.1. Solid state fermentation

Solid state fermentation was performed in Erlenmeyer flasks (250 mL) initially containing 5 g of rice straw (pretreated and 100 µm mesh size fraction) and 15 mL of NSS (no free water available) to maintain a solid to liquid ratio of 1:3 [74]. Other substrates in place of rice straw also have been used as where indicated. Initial pH of NSS was adjusted as desired. Culture medium was autoclaved at high pressure (15 psi) for 15 min, and inoculated with 2 discs (5 mm each) of actively growing fungal isolate RM-1 (4 days old culture) followed by incubation at desired temperature. After incubation, the flasks were harvested as per requirement of the experiments.

2.2.2.7.2. Submerged fermentation

As in above process, enzyme production under SmF was also carried out in 250 mL flasks containing 40 mL of NSS and 0.8 g rice straw at pH 6.5 [131]. Medium was autoclaved and inoculated with 2 discs (5 mm each) of fungal isolate RM-1. The inoculated flasks were incubated at 37 °C in an orbital shaker incubator with constant shaking (120 rpm) for 7 days.

2.2.2.7.3. Harvesting and storage of enzymes

The enzyme produced under SmF was harvested by filtering the content of flask through four-layered cheese cloth on 7^{th} day. In SSF, the content of flask was crushed by using a glass rod in distilled water (15 mL) and shaken on laboratory shaker at 100 rpm for 30 min at 25 °C for harvesting of the enzymes. The content of the flask was then filtered and the filtrate was centrifuged (Sigma laboratory centrifuge, 2K-15) at 5000x at 4 °C temperature for 10 min [99]. The clear supernatant was used as crude enzyme sample and stored at -20 °C for future usage.

2.2.2.8. Analytical methods

2.2.2.8.1. Estimation of xylanase activity

Xylanase activity was estimated by measuring the amount of reducing sugar released after incubation of diluted culture supernatant (crude enzyme) with 1% birch wood xylan solution (in 0.05 M sodium phosphate buffer) in a ratio of 1:9 at 55 °C [8]. After 15 min, 3 mL of DNS reagent was added to stop the reaction [95]. The reaction mixture was then boiled for 5 min and cooled under tap water. Blanks were repeated in the similar manner using reagents and enzyme, separately. Xylanase activity was reported in terms of IU. One IU per mL of xylanase was defined as 1 µmol of reducing sugar (xylose) produced in 1 min by 1 mL of enzyme under the assay conditions. The Xylose units released in this reaction were estimated at 540 nm using UV-Vis spectrophotometer (Cary 100 Bio Varian-Australia) at 25 °C.

2.2.2.8.2. Estimation of cellulase (CMCase) activity

The cellulase activity (CMCase) was determined using CMC as the substrate [92]. The reaction mixture, in a total volume of 4 mL, contained 2 mL of 2% (w/v) CMC prepared in 0.05M citrate buffer and 2 mL of the enzyme preparation in appropriate dilution. The mixture was incubated at 55 °C for 30 min. Thereafter, the tubes were kept in an ice bath and 3 mL of DNS reagent [95] was added to 1 mL of reaction assay followed by mixing and boiling of reaction mixture on boiling water bath for 5 min. The tubes were immediately cooled under tap water. The controls having reagent and enzyme separately were also treated similarly. The reducing sugars released were measured at 575 nm in a UV-Vis spectrophotometer. The enzyme activity was expressed as IU equivalent to µ moles of glucose units released in 1 min by 1 mL of enzyme at 55 °C.

2.2.2.8.3. Estimation of supernatant protein concentration

Protein concentration in culture filtrates produced under SSF conditions was quantified by using Lowry method with BSA as a standard [86].

2.2.2.9. Optimization methodology for xylanase and cellulase production

The methodology of this work for optimizing xylanase and cellulase production under SSF involved three major steps: (i) Initial studies were carried out in order to improve the nutrient medium (solid substrate+NSS) for xylanase and cellulase production using conventional method based on the OVAT technique, in which, one independent variable was

studied while fixing all others at a specific level, (ii) Screening of important variables responsible for xylanase and cellulase production was done through PB design and (iii) RSM was used to optimize the screened components for enhanced xylanase and cellulase production using CCD. All experiments were carried out independently in triplicate, and the results were the average of three replicate experiments.

(i) Optimization through OVAT

SSF was carried out to study the effect of different components of the production medium on xylanase and cellulase activities. The conventional method (change-one-variable-at-a-time) was used to optimize the different components (solid substrate and NSS) of the production medium for achieving enhanced levels of xylanase and cellulase produced by the fungal isolate RM-1. The fermentation was carried out in 250 mL flasks each containing 5 g of various substrates and moistened with 15 mL of NSS medium. The flasks containing SSF media were inoculated with 2 discs (5 mm each) of actively growing fungal isolate RM-1 and the initial culture conditions were set at 37 °C and pH 6.5 for 7 days of incubation period as recommended by Kaur and co-workers [71]. The enzyme was then harvested by filtering the content of the flasks through the four-layered cheese cloth, and enzymatic assays for xylanase [8] and cellulase [92] were carried out. The nutrient variables optimized through OVAT are described as under:

(A) Selection of solid substrates for xylanase and cellulase production

Various natural agro-residues namely, wheat bran, rice straw, corn cob, sugarcane bagasse and coconut coir, singly and in combinations (1:1), were used as the substrate to assess their suitability in promoting xylanase and cellulase production from fungal isolate RM-1.

(B) Effect of wheat bran-corn cob (WB+CC) ratio on xylanase production

To evaluate the effect of substrate ratio on xylanase synthesis, different ratio of wheat bran and corn cob (100:0, 90:10, 70:30, 50:50, 30:70, 10:90 and 0:100) were tested. A total 5 g of each ratio was taken in flask, to which 15 mL of NSS was added.

(C) Standardization of nutrient salt solution

The NSS medium was also standardized using sequential optimization procedure in which one component of NSS was changed while fixing others at a fixed level. This was done for achieving enhanced levels of xylanase and cellulase production by the test isolate RM-1.

The fermentation was carried out in 250 mL flasks each containing substrate and NSS medium in a ratio of 1:3 (5 g substrate and 15 mL NSS). The combination of wheat bran and corn cob (7:3) was used as the solid substrate for xylanase production while sugarcane bagasse was used for cellulase production under SSF in further optimization process, describing below:

(C_1) Effect of additional carbon sources on xylanase and cellulase production

Various carbohydrates such as glucose, lactose, xylose, galactose, maltose and CMC in NSS medium were tested as an additional supplement of carbon source for enzyme production in 1.0 g/L concentration along with control (having no additional carbon).

(C_2) Effect of organic nitrogen sources on xylanase and cellulase production

To determine the effect of various organic nitrogen sources (peptone, beef extract, malt extract and urea) on enzyme production, 1.0 g/L of each was added separately to the NSS medium in place of yeast extract.

(C_3) Effect of inorganic nitrogen sources on xylanase and cellulase production

In order to investigate the effect of different inorganic nitrogen sources, NH_4Cl in the NSS medium was replaced with NH_4NO_3, $NaNO_3$, $(NH_4)_2SO_4$ and KNO_3 at a concentration of 4 g/L, individually.

(C_4) Effect of surfactants on xylanase and cellulase production

The influence of surfactants on enzyme production was examined by adding Tween 20, Tween 80 and SDS, individually, in the NSS medium at an initial concentration of 0.1 g/L. The flasks containing different surfactants along with control were inoculated and incubated at previously described conditions of solid state fermentation as in section (i).

(C_5) Effect of different concentrations of organic nitrogen, $(NH_4)_2SO_4$ and Tween 80 on xylanase and cellulase production

The optimized nutrients from the above experiments were tested, sequentially, at different concentrations in the NSS medium such as yeast extract for xylanase enzyme and peptone for cellulase enzyme production in a concentration of 0 to 3 g/L. Thereafter, $(NH_4)_2SO_4$ (0 to 5 g/L) and tween-80 (0 to 0.4 g/L) were tested in the SSF culture medium (NSS) to optimize both the enzyme samples.

This standardized medium as optimized above was used for further work, describing below:

(ii) Screening of factors through PBD

The Plackett-Burman experimental design was employed to investigate the significance of various culture conditions on xylanase and cellulase production. This was a fractional factorial design with certain combinations of the eight factors i.e. initial pH, incubation time, substrate concentration, temperature, particle size, moisture ratio, inoculums size and inoculums age, studied in the experimental plan. In this study, eight assigned variables selected from literature as possible factors affecting xylanase and cellulase production, were tested at two distinct levels i.e. high and low and denoted by "+" and "–" respectively. The boundary limits of each variable were chosen as per literature. Table 2.13 illustrated the maximum and minimum levels of eight variables chosen for trials in PBD. The design of 12 trials with two levels for each variable with the resultant enzyme activities was shown in Table 2.14. The SSF was carried out according to the design table in 250 mL flasks and enzymatic assays for xylanase [8] and cellulase [92] were carried out as per standard protocols. This design did not describe the interaction among the factors. It was used to screen and evaluate the important factors that influenced the response (xylanase or cellulase activity), and to rank the factors according to their importance. The software Minitab-16 was used for generating the design table, and for analyzing the data. Variables with the highest t-value and confidence level over 95% were considered to be highly significant for xylanase and cellulase production.

(iii) Response surface methodology

Based on the above results, three factors that significantly affected the xylanase and cellulase production were identified and further optimized by using RSM. A 2^3-factorial CCD, with six axial points ($\alpha = 1.682$) and six replications at the centre points ($n_0 = 6$) leading to a total number of 20 experimental runs (Table 2.17) was employed. The selected variables were initial pH (X_1), incubation time (X_2) and temperature (X_3), and were tested at 5 different levels (Table 2.16). The boundary limits of each variable were chosen as prior experience about the process, and the independent variables were coded for statistical calculation according to the following equations:

$$X_i = \frac{x_i - x_0}{\Delta x} \qquad (1)$$

Where, X_i is the dimensionless coded value of i^{th} independent variable; x_i is the actual value of that independent variable; x_0 is the actual value of the same variable at the centre point and Δx

is the step change of the variable. The experiments were done according to the design table under the SSF conditions as optimized above and the results for xylanase and cellulase production were shown in Table 2.17. The SSF was carried out in 250 mL flasks containing substrate and NSS in a ratio of 1:3, and enzymatic assays for xylanase [8] and cellulase [92] were carried out. The experimental data were fitted with a second order polynomial/quadratic equation:

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_i X_j \quad (2)$$

Where, Y is the predicted response (xylanase or cellulase, IU/mL); β_0 is the interception coefficient; β_i is the linear coefficient; β_{ii} is the quadratic coefficient and β_{ij} is the coefficient of interaction effect. X_i and X_j denote the coded levels of variable x_i and x_j in experiments. The Minitab-16 software was used for creating the CCD table, and for regression and graphical analysis (contour plots) of the data obtained. The optimum values of the selected variables were obtained by solving the regression equation 2 [29]. ANOVA was used to determine the significance of each term in the equations fitted and to estimate the goodness of fit in each case.

2.2.2.10. Characterization of crude xylanase and crude cellulase

2.2.2.10.1. Optimum pH and pH stability

The pH stability of xylanase and cellulase enzyme was determined in the pH range of 3.8-10.3 at 55 °C. Three different buffers: citrate buffer for pH 3.8-5.8, potassium-phosphate for pH 5.8-7.8 and Tris-HCl buffer for pH 7.8-10.3 were used. After incubation for 1 h at different pH, the residual xylanase [8] and cellulase activities [92] of the crude enzyme samples were measured under standard assay conditions.

2.2.2.10.2. Optimum temperature and thermo-stability

In order to assess the thermo-stability of the xylanase and cellulase enzyme, the crude enzyme samples were incubated at different temperatures (45-85 °C) for 1 h. After that, the samples were analyzed for residual xylanase [8] and cellulase [92] activities following the standard assay conditions.

2.2.2.11. Mass production of crude xylanase

For the use in bio-bleaching experiments, mass production of crude xylanase from *C. cinerea* RM-1 was carried out using optimized conditions of SSF as described in Table 2.26.

The mass production was carried out in 2 L flasks using 30 g of wheat bran and corn cob in a ratio of 7:3, impregnated with 90 mL of NSS. The flasks were autoclaved after maintaining the pH of media as described previously. Thereafter, the flasks were inoculated with 12 discs (5 mm diameter each) of actively growing fungal strain *C. cinerea* RM-1 and incubated under optimum conditions. These were then harvested for the extraction of enzymes. The xylanase and cellulase activities and supernatant protein concentration were determined as per standard protocols.

2.3. RESULTS AND DISCUSSION

2.3.1. Isolation and screening of fungal strains for enzyme production

A total of 32 fungal strains are isolated from a variety of natural sites in the present study. Out of these 32 fungal strains, 11 strains are isolated from decomposing wood samples and 21 are isolated from soil samples of different sites i.e. forest (12 samples) and paper industry waste sites (9 samples). These strains are isolated initially on the basis of spore color and colony appearance at 37 °C following four days of incubation. These isolated strains are purified by regular sub-culturing on rice straw agar media, as it has already been shown to be a cheap and promising carbon source for xylanase production [110]. Table 2.1 shows the morphological characteristics of 32 fungal strains. These fungal strains are primarily screened for xylanase and cellulase producing ability by the XA and CMCA plate assay, respectively. These plates are stained with Congo red for enhancing the visibility of hydrolyzed area. Out of the 32 strains, 20 are resulted into zone formation onto XA as well as on CMCA plates. Five, among these 20 isolates that have shown good zone formation on both the plates i.e. XA and CMCA, are selected for secondary screening for their xylanase and cellulase producing ability under SSF conditions as described in Table 2.2. Fungal strain RM-1 exhibits maximum clear zone diameter on XA and moderate clear zone diameter on CMCA media plates (Photographs 2.1E and F). Secondary screening of these 5 isolates indicates that fungal strain RM-1 produces highest xylanase activity (345.2 IU/mL) followed by RM-21 (111.6 IU/mL), RM-14 (75.8 IU/mL), RM-4 (61.5 IU/mL) and RM-26 (25.4 IU/mL). These strains produce respectively, 1.65, 2.92, 1.89, 1.30 and 2.75 IU/mL cellulase activity (Figure 2.1). The four strains (RM-l, RM-2l, RM-l4 and RM-4) producing higher xylanase as well as cellulase activity have been sent for morphological identification.

2.3.2. Identification of screened isolates

The four fungal isolates RM-l, RM-2l, RM-l4 and RM-4 are identified as *basidiomycete* Sp., *Trichoderma* Sp., *Aspergillus niger* and *basidiomycete* Sp. from ITCC, Division of Plant Pathology, IARI, New Delhi, India (Photographs 2.1A, B, C and D). A lot of work has been done on production of xylanase and cellulase from fungi of the genera *Trichoderma* and *Aspergillus*. On the other hand, white rot basidiomycete fungi have effective hemicellulase and cellulase systems and represent a good source for these enzymes [145, 144, 61] but relatively little is known about their patterns of decay. Hence, white rot fungal strain RM-1 is selected for further optimization process of xylanase and cellulase production, based on the privilege of producing the highest xylanase activity, among the four selected isolates, and is sent to ARI, Pune, for molecular identification. RM-1 is identified as white-rot basidiomycete *Coprinopsis cinerea* (Schaeff.) Redhead, Vilgayls & Moncalvo. This strain has been deposited in NFCCI, ARI, Pune, India, and allotted the NFCCI Culture No.-3086 (Photograph 2.1A). *C. cinerea* is a multicellular basidiomycete mushroom belongs to the family Psathyrellaceae [94]. It is a higher fungus (Agaricales) comprised of many cell types and, therefore, provides a window on the development of multicellularity within a single kingdom. *C. cinerea* has become important model organism to study multicellularity of fungi because it grows on define media, has life cycle of two weeks [97], and can be manipulated easily in any phase of development [76].

2.3.3. Morphological characteristics of *C. cinerea* RM-1

The SEM microphotographs of mycelia and spores of selected strain RM-1 reveal the presence of highly tapered, tubular and filamentous hyphae, basidium, basidiospores, hymenial layers and chlamydospores (mitotic submerged spores) (Photographs 2.3A, B and C). The jointly long hyphae can explore and utilize food substrates very proficiently and secrete enzymes, and absorb the food particles at their tips [5]. Clamp connections are also observed when test strain is directly inoculated on PDA medium (Photograph 2.3A). After 5 to 6 days of inoculation, small etiolated streaks are raised and they grow eventually into the complex structures called fruiting bodies (Photograph 2.2A) which later on undergo rapid autolysis, a specific feature of coprini. Fruiting bodies produce the black spores (club-shaped) on the 10^{th} day of growth (Photograph 2.2B). It is easily recognized as a Coprinoid mushroom by its grayish black gills and cap which is initially white but soon begins to turn light grayish brown, and it quickly crumbles when handled [80].

2.3.4. Analysis of critical parameters for enhancement of xylanase and cellulase production by white rot fungal strain *C. cinerea* RM-1

2.3.4.1. Comparative analysis of fermentation systems

Table 2.3 represents the effect of two fermentation systems (SSF and SmF) on xylanase and cellulase production by *C. cinerea* strain RM-1, with its graphical illustration in Figure 2.2. The xylanase activity of *C. cinerea* RM-1 under SSF conditions is 68.2% higher than SmF while cellulase activity is also found to be 53.5% higher in SSF than SmF. The high xylanase and cellulase production by the fungal strain RM-1 under SSF as compared to SmF may be because SSF provides the white rot fungus an environment close to its natural habitat. This might have stimulated this strain RM-1 for the production of higher levels of hemicellulolytic enzymes [130].

The aim of SSF is to strengthen the contact between microorganism and solid substrate [63] for achieving the higher concentration of substrate during fermentation [58] which is not feasible during SmF. In SSF, the growth of fungus is restricted to the solid surface only while in SmF, the fungus is exposed to hydrodynamic forces that lead to negative effects on its growth and enzyme production also [130]. Moreover, the heterogeneous nature of the solid matrix [133], the restricted drop in substrate concentration [120], the concentration gradients of mineral salts and soluble sugars [129] during SSF process, are responsible for minimizing catabolic repression [133, 120], and for better metabolic activity of microorganisms. Kaur and co-workers observed 54 and 35% increment in xylanase and cellulase production under SSF as compared to SmF by the fermentation of wheat bran for *C. cinerea* HK-1 [72]. Malarvizhi and co-workers observed 30-fold improvement in xylanase production under SSF as compared to SmF using wheat bran for the fungal strain *Ganoderma lucidum* [91].

Thus, in order to achieve further higher levels of xylanase and cellulase production, and to economize the process, solid state fermentation is optimized using a range of cheap solid substrates of agricultural origin.

2.3.4.2. Selection of solid substrate for xylanase and cellulase production under SSF

Cost-effective production of enzymes from low-cost agricultural residues is vital for their extensive and economic industrial applications. The utilization of agricultural wastes by microorganisms depends upon several physical and chemical factors such as nature of cellulose and hemicellulose, surface and pore size of the particles, sugar composition of substrate,

pretreatment of substrate, the presence of activator or inhibitor and diffusion of catabolites in the media [130]. In the present study, five different agro-residues i.e. wheat bran, rice straw, corn cob, sugarcane bagasse and coconut coir, singly and in combinations (initially in 1:1 ratio and impregnated with the fermentation medium, NSS), are used as the solid substrate during SSF for enzyme production by the strain *C. cinerea* RM-1 (Table 2.4). All the agro-residues promote the growth and enzyme production by RM-1 but maximum xylanase production (696.4 IU/mL) is observed for combination of wheat bran and corn cob. While sugarcane bagasse produces the highest cellulase (2.33 IU/mL) among all the tested agro-residues on 7^{th} day of incubation at 37 °C. The descending order of xylanase activity for different agro-residues is as follows: wheat bran+corn cob >wheat bran >corn cob >corn cob+rice straw >wheat bran+rice straw >xylan agar >rice straw >corn cob+sugarcane bagasse >wheat bran+sugarcane bagasse >sugarcane bagasse >coconut coir. While the descending order of cellulase activity for different agro-residues is as follows: sugarcane bagasse >wheat bran+sugarcane bagasse >rice straw >corn cob+sugarcane bagasse >wheat bran+rice straw >corn cob+rice straw >coconut coir >wheat bran >wheat bran+corn cob >corn cob >xylan agar (Table 2.4 and Figure 2.3). The difference in production levels is probably due to the structural difference of the solid matrices used, that may cause difference in nutrient absorption and fungal entrapment; therefore, resulting into differential levels of growth and the enzyme secretion.

It is evident from Figure 2.3 that corn cob and wheat bran show good induction potency for xylanase production as rice straw and sugarcane bagasse individually do not support xylanase activity to that level as they do in combination with corn cob or wheat bran. However, cellulase production seems to be negatively affected by both corn cob and wheat bran. This suggests that xylanase and cellulase are inducible enzymes and their induction is substrate-dependent, and they may act under different regulatory mechanism or the same, depending upon the enzyme producer [55]. Many microorganisms show the independent regulatory mechanism for the synthesis of xylanase and cellulase [55]. Kaur and co-workers reported that *C. cinerea* HK-1 showed highest activity of xylanase and average activity of cellulase on wheat bran containing media [72]. They also reported that rice straw supported the xylanase activity but suppressed the cellulase activity for the strain HK-1, as the second highest activity of xylanase with negligible activity of cellulase were obtained on rice straw containing media.

The suitability of wheat bran and corn cob as the substrate for xylanase production may be related to the nutritional content and biochemical composition of these substrates. The biochemical composition of wheat bran [81] indicates that wheat bran contains considerable amount of soluble sugars, as % dry wt., 42.5 glucose, 15.4 xylose, 3.1 arabinose and 2.7 galactose required for the instigation of growth and replication of fungi. Corn cob contains, as % dry wt., 23 xylose and 28 xylan [54]. Corn cob xylan is a rich carbon source and more favourable to induce enzyme production [54]. Wheat bran and corn cob containing media exhibit higher levels of enzyme production; this may be because of the formation of bran and the corn cob xylan which is more conducive to induce enzyme production. The findings of the present study are in line with the results of Ninawe and Kuhad [101]. They reported that use of wheat bran and corn cob as a carbon source was the best for the production of xylanase by *Streptomyces cyaneus* SN32. The findings are also in close conformity with the findings of Jin and co-workers. They reported the combination of wheat bran and corn cob as the best substrate for xylanase production by *A. niger* [66]. Many other researchers also found maximum activity of xylanase by using the combination of wheat bran and corn cob [36]. Wheat bran had been described as a potent substrate and an inducer for the production of xylanase in several studies [72, 73, 77, 23, 53, 142]. Many researchers also observed that corn cob gave maximum activity of xylanase as compared to other substrates [52, 59]. In concerns with cellulase, the high production of cellulase by the fungal strain RM-1 on sugarcane bagasse containing media may be due to the altered nature and amount of cellulose, low hemicellulose and lignin content or due to the presence of other activators in sugarcane bagasse. The pretreated sugarcane bagasse contains 84.7% cellulose, 9.5% lignin, 3.3% hemicellulose and 1.1% ash [124]. Alves and Rocha reported good CMCase activity (27,017 U x L–1) on sugarcane bagasse by fungal strain *Trichoderma harzianum* [125].

Corn cob and wheat bran cause a selective induction for xylanase with repressive action on cellulase production while sugarcane bagasse acts as an inducer for cellulase production. Thus, the combination of wheat bran and corn cob (1:1) is selected in further optimization process for xylanase production based on its privilege of providing high xylanase and negligible cellulase activity. The crude enzyme sample having high xylanase and negligible cellulase activities produced by using wheat bran and corn cob under SSF is represented as “crude xylanase” for further description. On the other hand, sugarcane bagasse is selected for cellulase production as highest cellulase activity by *C. cinerea* RM-1, is obtained on this

substrate under SSF; the crude enzyme sample produced by using sugarcane bagasse is represented as “crude cellulase”.

2.3.4.3. Effect of wheat bran and corn cob ratio on xylanase production

Generally, the induction of enzymes by microorganisms is a complex phenomenon, and highly affected by the nature and amount of substrate used in the fermentation medium [46]. In order to determine the best ratio of solid substrates for crude xylanase production, different ratio of wheat bran and corn cob as described in Table 2.5, are tested. The results show that the xylanase activity is increased initially with decreasing the ratio of wheat bran and corn cob from 100:0 to 70:30 (Figure 2.4). Thereafter, a decline in xylanase activity is observed with further decreasing the wheat bran and corn cob ratio. Maximum xylanase activity (708.9 IU/mL) is obtained at 70:30, wheat bran and corn cob ratio. The xylanase activity is increased by 16.4 and 18.8% on wheat bran and corn cob ratio of 70:30 as compared to its respective activities at wheat bran and corn cob as a sole carbon source. Hence, based on the results, wheat bran and corn cob in a ratio of 70:30 are selected and used as the solid substrate for further optimization studies of crude xylanase production by RM-1 under SSF conditions.

2.3.4.4. Effect of different sugars on enzyme production

The carbon source used in the production medium is one of the major nutritional factors influencing the xylanase and cellulase activities. The production of xylanase and cellulase has been shown to be inducible, and is dependent upon the carbon source present in the fermentation medium [65]. Keeping this in view, different carbon supplements such as glucose, lactose, xylose, galactose, maltose and CMC at fixed concentration (1 g/L) in NSS medium are used to observe their effects on the production of crude xylanase and crude cellulase by fungal strain RM-1 under SSF conditions (Table 2.6). Figures 2.5A and B show the effects of these various carbon substitutes on xylanase and cellulase activities, respectively. The activity of xylanase on wheat bran and corn cob containing media is found to be repressed by addition of different sugars and the maximum activity is being attained in the absence of these sugars (control). Lower concentration of sugar substrates is mainly utilized for growth of the fungus while comparatively high amount of sugars acts as an inducer for the synthesis of enzymes. Further increase in concentration represses the enzyme production due to osmotic problems faced by the fungus in the combination of the inhibition due to catabolites repression. Catabolites repression refers to the repression of enzyme synthesis by glucose or other easily

metabolized sugars. In the present case, we find the repression in xylanase activity by the addition of easily metabolized sugars which may be attributed to the fact that wheat bran and corn cob are a rich source of xylan, xylose and other sugars themselves, and are capable for stimulating the xylanase synthesis and xylose production. Hence, further addition of sugars may cause catabolites repression due to which the xylanase activity is decreased. Many researchers observed the repression of xylanase in different microorganisms in presence of easily metabolized sugars [47].

On the other hand, addition of different sugars has positive or negative effect on cellulase activity (Figure 2.5B). Maximum cellulase activity (2.9 IU/mL) occurs when sugarcane bagasse medium is supplemented with lactose, followed by CMC, maltose and galactose while glucose and xylose have negative effect on cellulase activity. The results indicate that cellulase production is strongly induced by lactose and suppressed by glucose and xylose. The mechanism of lactose induction is supposed to be due to a series of enzymatic reactions of Leloir pathway which controls the signalling [127]. Many researchers described lactose, cellobiose and sophorose as the most commonly known inducers of cellulase production [14, 88]. Fang and co-workers observed that lactose acted as an inducer for cellulase production by filamentous fungus *Acremonium cellulolyticus* [39, 71]. The inhibitory effect of xylose and glucose on cellulase production may be due to the catabolites repression. Many researchers reported the repressive effect of glucose on cellulase production [60, 154].

Since, the highest cellulase activity is obtained with the addition of lactose (1 g/L) in NSS media; lactose (1 g/L) is added as carbon source for further optimization studies of crude cellulase produced by *C. cinerea* RM-1 under SSF using sugarcane bagasse as the substrate. On the other hand, no additional sugar is used for production of crude xylanase enzyme as highest xylanase activity is obtained without addition of any sugar source.

2.3.4.5. Effect of organic nitrogen sources on enzyme production

Organic nitrogen sources contain some nutrients and activators which contribute to the enhanced production of enzymes [128]. In an attempt to optimize various media components, different organic nitrogen sources are used separately in place of yeast extract in NSS media to find out the suitability of organic nitrogen sources on production of crude xylanase and crude cellulase by fungal isolate RM-1 under SSF conditions (Table 2.7). The highest xylanase activity (707 IU/mL) is obtained in yeast extract while the highest cellulase activity (3.2 IU/mL) is obtained in peptone, as the most effective organic nitrogen source. The order of

suitability of organic nitrogen sources for xylanase production in the present study is: yeast extract >beef extract >urea >peptone >malt extract (Figure 2.6A), and for cellulase production is: peptone >beef extract >yeast extract >urea >malt extract (Figure 2.6B). It has been proved in many studies that both the source and concentration of nitrogen substance are important parameters in regulating the enzyme production by white rot fungi [137, 156]. These results show that the choice of nitrogen source for enzyme synthesis depends not only on the fungal species but on the lignocellulosic substrate also [30, 70, 68]. The highest production of xylanase with yeast extract may be because of direct absorption of yeast extract's amino acids through the mycelia of the test isolate RM-1 on wheat bran and corn cob containing medium. These findings agree with earlier studies in which yeast extract were found to be the best nitrogen source for xylanase production [132, 16]. About 11.7% increase in cellulase activity is found by using peptone (3.17 IU/mL) as the nitrogen source in place of yeast extract (2.80 IU/mL). Agnihotri and co-workers found peptone as the best nitrogen source for induction of cellulase enzyme from white-rot strain *Coprinellus disseminatus* SW-2 NTCC1165 [1]. Many other researchers also reported peptone as the best nitrogen source for cellulase production [44].

Yeast extract and peptone are chosen as organic nitrogen sources in NSS medium for further optimization studies of crude xylanase and crude cellulase, respectively, produced by *C. cinerea* RM-1 under SSF.

2.3.4.6. Effect of inorganic nitrogen sources on enzyme production

Different inorganic nitrogen sources such as NH_4NO_3, $NaNO_3$, $(NH_4)_2SO_4$ and KNO_3 are also used by replacing NH_4Cl in NSS medium for observing their effects on crude xylanase and crude cellulase production by strain RM-1 under SSF (Table 2.8). All the inorganic nitrogen sources show good improvement in xylanase and cellulase activities except $NaNO_3$. The maximum xylanase (785.1 IU/mL) and cellulase (3.59 IU/mL) activities are achieved with $(NH_4)_2SO_4$ followed by NH_4NO_3 (Figures 2.7A and B). Xylanase and cellulase activities are increased about 9.3 and 12.8%, respectively, by using $(NH_4)_2SO_4$ in place of NH_4Cl in NSS medium. The results are in accordance with the work of Kachlishvili and co-workers who reported the peptone and $(NH_4)_2SO_4$ as the best nitrogen sources for stimulating cellulase and xylanase production by the white rot fungal strains, *L. edodes* IBB 363 and *P. dryinus* IBB 903 [68]. $(NH_4)_2SO_4$ was the best nitrogen source for *M. plumbeus* and *A. terreus* **[**103].

As shown by the results, $(NH_4)_2SO_4$ is found to be the best in stimulating xylanase as well as cellulase production; NH_4Cl is replaced by $(NH_4)_2SO_4$ as the inorganic nitrogen source in NSS medium for further studies of crude xylanase and crude cellulase production by *C. cinerea* RM-1 under SSF.

2.3.4.7. Effect of surfactants on enzyme production

Many reports have been shown the stimulatory effects of surfactants on enzyme production by microorganisms in SmF and SSF [121, 109, 50, 2, 122]. Most of the surfactants used for enzyme production are chemically synthesized surfactants such as Tween 80, Tween 20, SDS and Triton X-100 etc. Keeping this in view, different surfactants such as Tween 20, Tween 80 and SDS at a fixed concentration (0.1%) in NSS medium have been used to observe their effects on crude xylanase and crude cellulase production by fungal strain RM-1 under SSF conditions (Table 2.9). It is pragmatic from Figures 2.8A and B that the xylanase and cellulase production by fungal strain RM-1 increase considerably in Tweens containing media and reduce in SDS containing media. The highest activities of xylanase (826.9 IU/mL) and cellulase (3.9 IU/mL) are found on Tween 80 containing media; which is at a hike of 4.36% (for xylanase) and 6.2% (for cellulase) as compared to their respective controls (without surfactant). The results are in close association with other researchers who reported the Tweens as the most effective surfactants tested for the stimulation of enzyme production [77, 102]. Non-ionic surfactants are known to increase the permeability of cell membranes, and cause a more rapid secretion of enzymes [121]. Surfactants contain both hydrophilic and hydrophobic heads by which they can attach to lignocellulosic substrates and alter their surface properties to make them more accessible for enzymatic hydrolysis. Helle and co-workers revealed that surfactants improved the cellulase stability and prevented the denaturation of enzymes during hydrolysis [57].

Tween 80 is chosen as best surfactant in NSS medium for further studies of crude xylanase and crude cellulase production by *C. cinerea* RM-1 under SSF.

2.3.4.8. Effect of concentrations of yeast extract, peptone, $(NH_4)_2SO_4$ and Tween 80 on enzyme production

The concentration of medium components plays an important role on the enzyme production and their activities. Based on the above experiments, yeast extract has been chosen as optimum nitrogen source for crude xylanase while peptone has been chosen for crude

cellulase production in present investigation. Xylanase and cellulase activities increase with increasing concentration of yeast extract and peptone, respectively, from 0-1g/L and decrease thereafter with further increasing the concentration (Table 2.10). Xylanase production improves by about 16.8% in presence of 1 g/L yeast extract while cellulase production improves by about 19.7% in presence of 1 g/L peptone as compared to the cultivation in absence of any nitrogen sources (Figures 2.9A and B). Our results agree with earlier studies in which 1 g/L yeast extract concentration was found optimum for xylanase production [132].

Table 2.11 represents the effect of different concentrations of $(NH_4)_2SO_4$ from 0-5 g/L on crude xylanase and crude cellulase production by fungal strain RM-1 under SSF conditions. Xylanase and cellulase activities increase with increasing concentration of $(NH_4)_2SO_4$ from 0-3 g/L in NSS medium and follow a decreasing trend thereafter with further increasing the concentration. A hike of 8.2 and 11.0% is observed in xylanase and cellulase activities, respectively, in presence of 3 g/L $(NH_4)_2SO_4$ as compared to the cultivation in absence of any inorganic nitrogen source (Figures 2.10A and B).

Table 2.12 represents the effect of different concentrations of Tween 80 from 0-0.4 on xylanase and cellulase production by fungal strain RM-1. Xylanase activity increases with increasing concentration of Tween 80 from 0-0.1 g/L in NSS medium, and follows a declining trend thereafter (Figure 2.11A). While cellulase activity increases with increasing concentration of Tween 80 from 0-0.2 g/L in NSS medium and then it starts to decrease (Figure 2.11B). Xylanase activity increases 6.28% in presence of 0.1 g/L Tween 80 while cellulase activity increases 10.5% in presence of 0.2 g/L Tween 80 as compared to the cultivation in absence of surfactant. Ding and co-workers observed an about two fold increment in xylanase activity produced by *S. olivaceoviridis* E-86 with increasing the concentration of Tween 80 from 0-1.5% [35]. Based on the above results, the NSS medium is modified for further xylanase and cellulase production as under:

NH_4Cl (4 g/L) is replaced by $(NH_4)_2SO_4$ (3 g/L) and Tween 80 (0.1 g/L) is also added for crude xylanase production using wheat bran+corn cob as the substrate. For cellulase production, NH_4Cl (4 g/L) and yeast extract (1 g/L) are replaced by $(NH_4)_2SO_4$ (3 g/L) and peptone (1 g/L), respectively, with the addition of lactose (1 g/L) and Tween 80 (0.2 g/L) using sugarcane bagasse as the substrate. The fungal strain RM-1 produces high xylanase (864.8 IU/mL) and cellulase (4.2 IU/mL) activities, representing 18.0 and 44.5% improvement in

xylanase and cellulase activities, respectively, compared to that obtained under unmodified NSS medium (708.9 IU/mL for xylanase and 2.33 IU/mL for cellulase).

From this point, the above respective modified NSS media have been used for further optimization process of crude xylanase and crude cellulase production by *C. cinerea* RM-1 under SSF.

2.3.5. Analysis of PBD

Variations in initial pH, incubation period, substrate concentration, cultivation temperature, particle size, moisture ratio, inoculum size and inoculum age can affect enzyme production [36, 56]. The relative importance of these eight variables for xylanase and cellulase production by *C. cinerea* RM-1 is investigated at two levels, high (+) and low (–) using PBD. Table 2.13 reveals the high and low levels of eight variables. Table 2.14 illustrates the 12 trials of PBD with two levels for each variable with the resultant enzyme activities for xylanase and cellulase. Plackett-Burman experiments show a wide variation in xylanase (from 302.1 IU/mL to 705.6 IU/mL) as well as in cellulase activity (from 0.96 IU/mL to 2.25 IU/mL). This variation indicates towards the importance of optimization for attaining higher productivity. The significance of each variable is determined by student's t-test and p-value, which are listed in Table 2.15. The variable showing larger the magnitude of t-test and smaller the p-value, is considered as more significant [75].

Out of the eight variables studied, three variables namely, pH (A), incubation period (B), and temperature (D) have significant influence on xylanase and cellulase production as evidences by their p-value ($p < 0.05$, significant at 95% confidence level). The main effects of all variables are also calculated. When the main effect value of a tested variable is positive, its influence on response variables is greater at high level; when it is negative, its influence is greater at low level. For xylanase production, the variables, initial pH (A) and moisture ratio (F) display negative effects while all other variables have positive effects. For cellulase production, initial pH (A), particle size (E) and moisture ratio (F) display negative effects while all other variables have positive effects. pH is found to be significant although with a negative coefficient as most of the fungi grow well on slightly acidic media [123]. pH is one of the regulatory parameter in production of enzymes as it causes inactivation of the enzyme system if fermentation is carried out at above or below the optimum range of pH [144, 37]. Many white-rot fungi had been reported to grow the best on slightly acidic pH [72, 123, 1].

The effect of moisture level on enzyme production may be due to the alteration in physical properties of solid substrate. The free excess liquid causes reduction in porosity and oxygen transfer, and creates gummy texture with modification in solid substrate particle structure while low moisture level reduces the swelling of solid substrate and decreases the solubility of nutrients [117, 40]. A higher moisture level than optimum imposes an additional diffusion barrier in the media which results into a decrease in growth and enzyme production [98]. Many researchers obtained maximum xylanase production by different white rot fungi at substrate to moisture ratio of 1:3 under SSF using wheat bran as the substrate [132, 72, 1].

The particle size of the solid substrates has an influence on surface/volume ratio and accessibility of the nutrients for the microorganism [6]. Hence, an appropriate size of substrate particles is required for optimum growth of microorganism and production of enzymes. Sugarcane bagasse with 300 µm particle size possibly provides sufficient surface area and good aeration for growth of *C. cinerea* RM-1 under SSF resulting in increased cellulase production. Lower xylanase production obtained on smaller particle size of wheat bran and corn cob may be due to increased thickness of fungal mycelia around the solid substrate particles which reduces the porosity of the substrate and diffusivity of oxygen in the bed [118]. Jiang and co-workers obtained maximum xylanase activity for strain *Thermomyces lanuginosus* CAU44 at corn cob particle size of 0.300 µm-450 µm [65]. Moreover, the Pareto graph is used to rank the variables according to their importance, and to show the effect of all variables on xylanase and cellulase production (Figures 2.12A and B). The Pareto graph represents the magnitude of each variable, and is an easy way to view the results of a PB design [134]. The Pareto graphs confirm the significance of variables; initial pH (A), incubation period (B) and temperature (D) for xylanase and cellulase production, as stated earlier. The normal effect plot can also be a useful resource for visualizing the significance of variables. The significant effect strays farther from the line, on the contrary, the insignificant effect drops along a line. As shown by the plots (Figures 2.13A and B), the effects of variables incubation period (B) and temperature (D) are placed farther from the line on right side which illustrate that their higher levels have a significant effect while the effect of variable initial pH (A) lies on left side of the line, indicating that its lower level contributes to higher value of response variables.

Based on above statistical analysis, the significant variables namely, temperature, pH and incubation period are selected for further optimization by CCD to attain a maximum production of crude xylanase and crude cellulase on the WB+CC and sugarcane bagasse

containing media, respectively. Other variables showing positive value of main effect i.e. substrate concentration, particle size, inoculum size and inoculum age are kept at their higher level i.e. 5 g/flask, 600 µm, 3 disks/flask and 5 days, respectively, and showing negative value i.e. moisture ratio is kept at its lower level i.e. 66.67% for xylanase production. For cellulase production, the three variables i.e. substrate concentration, inoculum size and inoculums age are fixed at their higher level i.e. 5 g/flask, 3 disks/flask and 5 days while particle size and moisture level are kept at their lower level i.e. 300 µm and 66.67%, respectively.

2.3.6. Analysis of CCD

Based on the results from PBD experiment three variables i.e. initial pH (X_1), incubation period (X_2) and temperature (X_3), are selected for the further analysis of their effects on xylanase and cellulase production by CCD. For RSM analysis based on the CCD, a set of 20 experiments is carried out and their response values are demonstrated in Table 2.17, for xylanase and cellulase production. Response surface methodology allows the modeling of a second-order polynomial/quadratic equation 2 that describes the process. Xylanase and cellulase production are analyzed by the least squares method of multiple regressions, and the following models are obtained by considering the significant terms:

$$Y = 1002.31 - 95.76X_1 + 35.37X_2 - 89.34X_3 - 192.58X_1^2 - 52.16X_2^2 - 178.71X_3^2 + 19.63X_1X_2 + 30.64X_1X_3 - 12.31X_2X_3 \quad (3)$$

$$Y^* = 4.4987 - 0.7311X_1 - 0.2970X_2 - 0.50646X_3 - 0.2720X_1^2 - 0.5779X_2^2 - 1.0410X_3^2 + 0.3025X_1X_2 + 0.7475X_1X_3 + 0.5725X_2X_3 \quad (4)$$

Y and Y* denote the response variables; xylanase and cellulase activities, respectively. The significance of each coefficient (main effects, quadratic and interaction between factors) of the independent variables (initial pH, incubation period and temperature) is determined by student's t-test and p-value, which are listed in Table 2.18. The goodness of fit of equations 3 and 4 are observed by determination coefficient (R^2). In this case, the R^2 value of 99.88% for equation 3 and 99.60% for equation 4 indicates that the sample variations of 99.88 and 99.60% for xylanase and cellulase production, respectively, are attributed to the independent variables and their interactions, and only about 0.12 and 0.40% of the total variation cannot be explained by the obtained models i.e. equation 3 and equation 4, respectively. The values of the adjusted determination coefficient (R^2_{adj}) for above two models are also high enough (99.77 and

99.25%) which indicate the significance of the models. The values of predicted R^2 for xylanase and cellulase (99.26 and 97.59%) are in reasonable agreement with the values of $R^2_{adj,}$ indicating a good adjustment between the experimental and predicted values of the responses (xylanase and cellulase activities).

The significance of the two models for xylanase and cellulase production is also estimated by the F-test for ANOVA which shows that both models are statistically significant at 95% ($p < 0.05$) confidence level (Table 2.19). The linear terms, quadratic terms and cross terms are all statistically significant based on the F-value. Furthermore, the p-values of lack of fit for both the models are more than 0.05 (nonsignificant) which further indicates that both the models are significant. Thus, it can be concluded that the regression models obtained could be used for analyzing the trends of response variables. The interaction effects of variables on xylanase and cellulase production are studied by plotting contour plots against any two independent variables while keeping other variable at its central (0) level.

2.3.6.1. Effect of initial pH and incubation period on xylanase and cellulase production

Figures 2.14A and B illustrate the interaction between initial pH and incubation time with respect to xylanase and cellulase activities, at a constant temperature at its middle level, 0 (40 °C). Figures 2.14A shows that there are many combinations of pH and incubation time that produce the desired response (average xylanase activity of >1000 IU/mL). Maximum activity of xylanase (1018 IU/mL) is obtained at pH level of -0.19 (5.8) and incubation time level of 0.28 (7.2 days). These conditions may be considered as optimum conditions for production of xylanase enzyme. Moreover, at the pH levels of <-0.5 (<5.5) and >0.5 (>6.5), the contour lines are almost parallel to the incubation time axis, indicating that variation in incubation time at a fixed initial pH essentially does not affect xylanase production in that region.

For cellulase, maximum activity (5.20 IU/mL) is obtained at pH level of -1.5 (4.5) and incubation time level of -0.77 (6.2 days) (Figure 2.14B). The contour plots for cellulase enzyme indicate towards the falling ridge of cellulase activity with respect to pH and incubation time. It means that the cellulase activity decreases with increasing pH or incubation time. The cellulase activity decreases sharply (from 4.7 to 0.53 IU/mL) with increasing pH (from level -1.68 to +1.68) while the decrease in cellulase activity is not to that great extent (from 4.7 to 2.0 IU/mL) with increasing incubation time (from level -1.68 to +1.68). Hence, initial pH plays more important role as compared to incubation time in that region for cellulase production. pH is an important parameter in fermentation system, and it influences the charge and ionic components

of the substrate as well as the transport of enzymes across the cell membrane [113]; thereby affecting the catalytic properties of the enzymes. Each microorganism exhibits optimal growth and enzyme production under certain pH range. The reduction in xylanase production after optimum period may be due to the proteolysis of enzyme or due to the depletion of nutrients in the media [42]. Xylanase and cellulase are primary metabolites expressed at end of the exponential phase maximally. This indicates the harvesting time of the fungus [79]. Many microorganisms exhibited the optimum pH for xylanase production towards the acidic side at pH 2.0 to 6.0 [140, 79]. White rot fungi exhibit a slow rate of growth, and produce maximum xylanase as on 7^{th} day in *C. cinerea* HK-1 [72], 7^{th} day in *C. disseminates* SH-1 [132] and 8^{th} day in *Volvariella diplasia* [112]. The optimum pH was 6.5 for xylanase production by *T. lanuginosus* wild type and M7 by using corncob and corn steep liquor under shake flask fermentation [19].

2.3.6.2. Effect of initial pH and incubation temperature on xylanase and cellulase production

Figures 2.15A and B illustrate the interaction between initial pH and incubation temperature with respect to xylanase and cellulase activities, at a constant incubation time at its middle level, 0 (7 days). Maximum activity of xylanase (1027 IU/mL) is obtained at pH level of -0.24 (5.7) and incubation temperature level of -0.26 (38.5 °C) (Figure 2.15A). Starting from the above mentioned maximum points, the xylanase activity decreases by stepping in any direction. As shown by the plots, the xylanase activity is found optimum in the vicinity of central levels of both the variables; pH and incubation temperature. These plots also indicate that the xylanase activity decreases sharply with increasing initial pH or incubation temperature from their central levels as compared to that at their respective lower levels.

Figure 2.15B shows that maximum activity of cellulase (5.6 IU/mL) is obtained at pH level of -1.6 (4.4) and incubation temperature level of -0.69 (36.5 °C). In the region of lower levels of temperature (<0), with increasing pH from level -1.68 to +1.68, the cellulase activity decreases sharply as compared to the decrease at higher levels of temperature (>0). Moreover, at low levels of pH (<0), cellulase activity decreases sharply with increasing temperature from middle level (>0). Each fungus shows evidence of maximum growth and enzyme production under an optimum range of pH and temperature. Many researchers found 37 °C temperature as an optimum for xylanase production under SSF [1, 11]. Gawande and Kamat suggest that the production of proteins by the microorganisms is slow down at high temperatures, and only

essential proteins required for growth and other physiological processes are produced [45]. The kinetic energy of reacting molecules is increased while proceeding towards optimum temperature; this increases the reaction rate for enzyme production. On further elevation in temperature, the denaturation of protein takes place which leads to a loss of enzyme activity [104].

2.3.6.3. Effect of incubation time and temperature on xylanase and cellulase production

Figures 2.16A and B show the interaction between incubation time and incubation temperature with respect to xylanase and cellulase activities at a constant initial pH at its middle level, 0 (6). Maximum activity of xylanase (1019 IU/mL) is obtained at incubation time level of 0.2 (7.2 days) and incubation temperature level of -0.2 (38.5 °C) (Figure 2.16A). At high levels of temperature >0 (>40 °C), contour lines are approximately parallel to the incubation time axis, thus variation in time at a fixed temperature does not affect xylanase activity very much in this region.

Maximum activity of cellulase (4.65 IU/mL) is obtained at incubation time level of -0.47 (6.53 days) and incubation temperature level of -0.36 (38.2 °C) (Figure 2.16A). It is also indicated that at higher levels of temperature and incubation time, the cellulase activity is decreased sharply as compared to their respective lower levels.

2.3.6.4. Optimum culture conditions for xylanase and cellulase production

The response optimizer module in Minitab helps to identify the combinations of independent variable settings that jointly optimize a single response or a set of responses. The details about the numerical optimization have been discussed elsewhere [100]. Minitab can draw an optimization plot which can be used to interactively change the settings of independent variables to search for more desirable solutions. Table 2.20 shows the goals and selected ranges of values of responses that are used to obtain the optimum culture conditions. However, other goals and ranges of responses can also be selected depending upon the desired properties of responses. Tables 2.21 and 2.22 illustrate some of the optimum culture conditions for variables; initial pH, incubation temperature and incubation time during SSF process for crude xylanase and crude cellulase production, respectively. Minitab calculates the predicted values of their associated responses i.e. xylanase and cellulase activities. The experiments are performed in triplicate under the best predicted conditions (in bold) and the experimental results agree quite

closely with the predicted values, proving the validity of the models (Table 2.23). Hence, the predicted values appear to be reliable and verify the estimation models.

2.3.7. Biochemical characterization of xylanase and cellulase

2.3.7.1. Effect of pH on the activity and stability of xylanase and cellulase

The alkali-tolerant characteristic of enzymes is crucial while considering their application in broad range of industrial processes including bleaching of pulps. All enzymes (except ribozymes) are proteinaceous entities, and are likely to denature under harsh conditions like change in pH and high temperature etc. Enzymes lose the basic structure of their active sites in presence of harsh conditions of pH and temperature change, and this might result into the reduction in enzyme activity. The pH stability of the crude xylanase and crude cellulase from *C. cinerea* RM-1 is examined under a pH range of 3.8-10.3 at temperature 55 °C for 30 min of reaction time (Table 2.24). The optimum pH for maximum xylanase and cellulase activities is found to be 5.8 and 4.3 respectively (Figure 2.17A). The crude enzymes xylanase and cellulase are active in the pH range of 3.8 to 9.3. The xylanase enzyme retains 90, 70 and 48% of activity at pH 7.3, 8.3 and 9.3 respectively, in comparison to its activity obtained at optimum pH of 5.8. The cellulase enzyme retains 93, 72 and 30% of activity at pH 5.3, 6.8 and 9.3, respectively, in comparison to its activity obtained at optimum pH of 4.3. Thus, the enzymes show the good pH stability which proves their alkali-tolerant nature. This quality makes these enzymes potentially valuable in pulp and paper industry. These findings confirm the results that most xylanases produced by fungi are stable over a broad range of pH [83]. These findings are in accord with those demonstrating that most fungal origin xylanases are active under acidic conditions [123, 72]. The pH tolerance of xylanase produced by *Pleurotus ostreatus* was found in the range of 3-7 [115]. The xylanase produced by *C. disseminatus* SW-1 NTCC1165 was found active over a pH range of 6.0-9.0 [1].

2.3.7.2. Effect of temperature on the activity and stability of xylanase and cellulase

Table 2.25 illustrates the effect of different temperatures (45-85 °C) on the activities of xylanase and cellulase produced by *C. cinerea* RM-1 at the conditions described. The crude xylanase and cellulase exhibit optimal activity at 55 °C (Figure 2.17B). The xylanase and cellulase enzymes retain 88 and 87% of their optimum activities, respectively, at temperature 65 °C. At temperature 75 °C, the xylanase retains 65% while cellulase retains 70% of the maximal activity. On further elevation of temperature upto 85 °C, the xylanase retains just 35%

of its total activity while cellulase retains 37% of its maximal activity. These results are in consistence with other study in which the xylanase is optimally active in the temperature range of 50-60 °C [72]. The optimal temperature for xylanase production by most of the fungi lies in the range of 40-60 °C [79]. The optimum temperature for xylanase produced by *Aspergillus nidulans* KK-99 was found at 55 °C [142]. The thermo-stable characteristic of enzymes is attained by the exchange of some amino acids due to which the active site of enzyme is subjected to many structural modifications. The alteration of ion-pair interactions, hydrophobic and hydrogen bonds are responsible for providing the thermo-stability to the enzymes [126]. In pulp bleaching process, the alkali, thermo-tolerant cellulase free xylanases are highly desirable. *C. cinerea* RM-1 produced alkali, thermo-tolerant xylanase with negligible cellulase contamination (0.55 IU/mL) by fermentation of wheat bran and corn cob. Hence, this crude xylanase may be used directly in pulp and paper industry without further purification.

Table 2.1: Morphological analysis and screening of fungal isolates for xylanase and cellulase production

SI. No.	Isolated strain	Site of isolation	Mycelial appearance	Spore color	Zone diameter *(mm)	Zone diameter **(mm)
1	RM-1	Decomposing wood	Cottony, white	Black	21.0	7.2
2	RM-2	Decomposing wood	Granular, dull green	Green	2.3	6.2
3	RM-3	Decomposing wood	Fluffy, pale yellow	Black	-	4.1
4	RM-4	Decomposing wood	Velvety, white	Black	6.0	6.1
5	RM-5	Decomposing wood	Fine, dull white	Greenish	3.1	1.0
6	RM-6	Decomposing wood	Cottony, white	-	3.2	2.4
7	RM-7	Decomposing wood	Granular, brown	-	2.8	-
8	RM-8	Decomposing wood	Granular, white	White	4.1	3.1
9	RM-9	Decomposing wood	Powdery, white	Black	2.2	3.0
10	RM-10	Decomposing wood	Fine, white	Black	2.5	2.1
11	RM-11	Decomposing wood	Wooly, white	Black	3.2	1.2
12	RM-12	Forest soil	Cottony, yellow	Dark brown	-	1.2
13	RM-13	Forest soil	Fluffy, brown	-	-	-
14	RM-14	Forest soil	White	Black	6.1	9.7
15	RM-15	Forest soil	Dense, white	Black	2.0	4.1
16	RM-16	Forest soil	Greenish	Green	1.6	5.3
17	RM-17	Forest soil	Velvety, white	Black	2.2	7.0
18	RM-18	Forest soil	Fine, white	Black brown	-	2.1
19	RM-19	Forest soil	Velvety, grey	Yellow	-	-
20	RM-20	Forest soil	Floccose, grey green	Grey	1.0	-
21	RM-21	Forest soil	Irregular, white	Green	7.3	14.7
22	RM-22	Forest soil	Fluffy, white	Yellowish brown	1.2	1.5
23	RM-23	Forest soil	Granular, bluish	Smoky blue green	2.2	2.0
24	RM-24	Paper industry waste site	Granular, pale yellow	Black	-	2.4
25	RM-25	Paper industry waste site	Loose, dull green	Green	3.1	7.0
26	RM-26	Paper industry waste site	Blackish	Black	2.1	13.0
27	RM-27	Paper industry waste site	-	Black	-	3.6
28	RM-28	Paper industry waste site	Dense, off white	Black	-	4.7
29	RM-29	Paper industry waste site	Granular, greenish	Smoky blue green	2.4	2.5
30	RM-30	Paper industry waste site	Floccose, white	-	1.0	-
31	RM-31	Paper industry waste site	Cottony, dirty white	Powdery, grey	2.9	2.0
32	RM-32	Paper industry waste site	Granular, whitish brown	-	-	1.4

*** On xylan agar (XA) medium**

**** On CMC agar (CMCA) medium**

Table 2.2: Xylanase and cellulase production from the selected fungal isolates

SI. No.	Fungal strain	Zone diameter* (mm)	Zone diameter** (mm)	Xylanase activity (IU/mL)	Cellulase activity (IU/mL)
1	RM-1	21.0	7.2	345.15±8.45	1.65±0.04
2	RM-21	7.3	14.7	111.6±2.25	2.92±0.09
3	RM-14	6.1	9.7	75.8±2.10	1.89±0.03
4	RM-4	6.0	6.1	61.5±1.34	1.30±0.08
5	RM-26	2.1	13.0	25.4±0.82	2.75±0.12

Fermentation conditions: Solid substrate = rice straw Substrate:NSS = 1:3 pH = 7 Temperature = 37 °C Incubation time = 7 days	**Assay conditions:** **Xylanase:** Temperature = 55 °C, pH = 7.5, incubation time = 15 min **Cellulase:** Temperature = 55 °C, pH = 7.5, incubation time = 30 min

***On XA medium, ** On CMCA medium, ± refers standard deviation**

Table 2.3: Effect of different fermentation systems on xylanase and cellulase production by *C. cinerea* RM-1

SI. No.	Fermentation system	Xylanase activity (IU/mL)	Cellulase activity (IU/mL)
1	Solid state fermentation	397.22±7.12	1.79±0.04
2	Submerged fermentation	126.25±2.14	0.832±0.01

Fermentation conditions: **Solid state fermentation** Rice straw = 5 g, NSS = 15 mL, pH = 6.5 Temperature = 37 °C, incubation time = 7 days **Liquid state fermentation** Rice straw = 0.8 g, NSS = 40 mL, pH = 6.5 Temperature = 37 °C, incubation time = 7 days	**Assay conditions:** **Xylanase:** Temperature = 55 °C, pH = 6.4, incubation time = 15 min **Cellulase:** Temperature = 55 °C, pH = 4.8, incubation time = 30 min

± refers standard deviation

Table 2.4: Effect of different agro-residues as the substrate on xylanase and cellulase production by *C. cinerea* RM-1

SI. No.	Solid substrate	Xylanase activity (IU/mL)	Cellulase activity (IU/mL)	Protein concentration (mg/mL)
1	Wheat bran (WB)	590.43±10.25	0.59±0.02	5.09±0.19
2	Corn cob (CC)	581.30±9.06	0.31±0.01	4.97±0.15
3	Rice straw (RS)	402.54±7.24	1.75±0.05	3.92±0.07
4	Sugarcane bagasse (SB)	118.93±5.59	2.33±0.12	2.73±0.06
5	Coconut coir (CK)	95.60±4.24	0.74±0.20	1.29±0.02
6	Wheat bran+corn cob (WB+CC)	696.37±9.07	0.46±0.01	6.10±0.21
7	Wheat bran+rice straw (WB+RS)	492.14±9.05	1.16±0.04	4.26±0.12
8	Wheat bran+sugarcane bagasse (WB+SB)	162.92±5.65	2.07±0.14	3.20±0.07
9	Corn cob+rice straw (CC+RS)	514.79±8.03	0.98±0.03	4.45±0.09
10	Corn cob+sugarcane bagasse (CC+SB)	205.52±5.29	1.42±0.04	3.48±0.08
11	Xylan agar (XA)	430.35±9.39	0.09±0.003	4.17±0.10

Fermentation conditions:
Substrate:NSS = 1:3
pH = 6.5
Temperature = 37 °C
Incubation time = 7 days
Substrate combination ratio = 1:1

Assay conditions:
Xylanase:
Temperature = 55 °C, pH = 6.4, incubation time = 15 min
Cellulase:
Temperature = 55 °C, pH = 4.8, incubation time = 30 min

± refers standard deviation

Table 2.5: Effect of wheat bran and corn cob ratio on xylanase production by *C. cinerea* RM-1.

SI. No.	Solid substrate ratio (WB+CC)	Xylanase activity (IU/mL)
1	100:0	592.37±9.13
2	90:10	630.28±10.47
3	70:30	708.86±11.47
4	50:50	699.54±11.78
5	30:70	665.19±10.07
6	10:90	613.87±9.67
7	0:100	575.58±10.47

Fermentation conditions:
Substrate:NSS = 1:3
pH = 6.5
Temperature = 37 °C
Incubation time = 7 days

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

± refers standard deviation

Table 2.6: Effect of different sugars on xylanase and cellulase production by *C. cinerea* RM-1

SI. No.	Sugar concentration (1 g/L)	Xylanase activity (IU/mL)	Cellulase activity (IU/mL)
1	Glucose	590.00±8.08	1.11±0.04
2	Lactose	645.99±9.01	2.91±0.11
3	Xylose	655.99±9.37	1.25±±0.04
4	Galactose	692.65±9.37	2.59±0.09
5	Maltose	700.73±12.39	2.63±0.09
6	CMC	609.13±9.06	2.80±0.12
7	Control	712.48±13.87	2.31±0.07
Fermentation conditions: Solid substrate = WB+CC, 7:3 (for xylanase), SB (for cellulase) Substrate:NSS = 1:3, pH = 6.5, temperature = 37 °C, incubation time = 7 days		**Assay conditions:** **Xylanase:** Temperature = 55 °C, pH = 6.4, incubation time = 15 min **Cellulase:** Temperature = 55 °C, pH = 4.8, incubation time = 30 min	

± refers standard deviation

Table 2.7: Effect of different organic nitrogen sources on xylanase and cellulase production by *C. cinerea* RM-1

SI. No.	Organic nitrogen source (1 g/L)	Xylanase activity (IU/mL)	Cellulase activity (IU/mL)
1	Peptone	670.12±9.28	3.17±0.16
2	Beef extract	702.04±14.31	2.89±0.11
3	Yeast extract	707.04±13.85	2.80±0.09
4	Malt extract	625.89±10.75	2.18±0.12
5	Urea	694.46±10.83	2.24±0.16
Fermentation conditions: Solid substrate = WB+CC, 7:3 (for xylanase), SB (for cellulase) Substrate:NSS = 1:3, pH = 6.5, temperature = 37 °C, incubation time = 7 days		**Assay conditions:** **Xylanase:** Temperature = 55 °C, pH = 6.4, incubation time = 15 min **Cellulase:** Temperature = 55 °C, pH = 4.8, incubation time = 30 min	

Table 2.8: Effect of different inorganic nitrogen sources on xylanase and cellulase production by *C. cinerea* RM-1

SI. No.	Inorganic nitrogen source (4 g/L)	Xylanase activity (IU/mL)	Cellulase activity (IU/mL)
1	NH_4NO_3	735.74±12.95	3.33±0.15
2	$NaNO_3$	698.84±9.42	2.81±0.11
3	$(NH_4)_2SO_4$	785.10±11.52	3.59±0.14
4	KNO_3	727.37±12.50	3.17±0.13
5	NH_4Cl	711.83±11.11	3.13±0.16
Fermentation conditions: Solid substrate = WB+CC, 7:3 (for xylanase), SB (for cellulase) Substrate:NSS = 1:3, pH = 6.5, temperature = 37 °C, incubation time = 7 days		**Assay conditions:** **Xylanase:** Temperature = 55 °C, pH = 6.4, incubation time = 15 min **Cellulase:** Temperature = 55 °C, pH = 4.8, incubation time = 30 min	

Table 2.9: Effect of different surfactants on xylanase and cellulase production by *C. cinerea* RM-1

SI. No.	Surfactant (0.1%)	Xylanase activity (IU/mL)	Cellulase activity (IU/mL)
1	Tween 20	815.46±13.14	3.76±0.18
2	Tween 80	826.85±14.31	3.87±0.17
3	SDS	765.28±12.04	3.52±0.16
4	Control	790.79±12.16	3.63±0.14
Fermentation conditions: Solid substrate = WB+CC, 7:3 (for xylanase), SB (for cellulase) Substrate:NSS = 1:3, pH = 6.5, temperature = 37 °C, incubation time = 7 days		**Assay conditions:** **Xylanase:** Temperature = 55 °C, pH = 6.4, incubation time = 15 min **Cellulase:** Temperature = 55 °C, pH = 4.8, incubation time = 30 min	

Table 2.10: Effect of different concentrations of yeast extract on xylanase and peptone on cellulase production by *C. cinerea* RM-1

SI. No.	Organic nitrogen concentration (g/L)	Xylanase activity (IU/mL)	Cellulase activity (IU/mL)
1	0	690.89±8.94	3.10±0.12
2	1	830.51±13.69	3.86±0.17
3	2	816.22±13.2	3.79±0.17
4	3	795.19±12.53	3.72±0.16
Fermentation conditions: Solid substrate = WB+CC, 7:3 (for xylanase), SB (for cellulase) Substrate:NSS = 1:3, pH = 6.5, temperature = 37 °C, incubation time = 7 days		**Assay conditions:** **Xylanase:** Temperature = 55 °C, pH = 6.4, incubation time = 15 min **Cellulase:** Temperature = 55 °C, pH = 4.8, incubation time = 30 min	

Table 2.11: Effect of different concentrations of ammonium sulphate on xylanase and cellulase production by *C. cinerea* RM-1

SI. No.	$(NH_4)_2SO_4$ concentration (g/L)	Xylanase activity (IU/mL)	Cellulase activity (IU/mL)
1	0	785.68±13.10	3.56±0.11
2	1	810.98±13.54	3.72±0.16
3	2	832.13±14.15	3.89±0.2
4	3	855.81±15.40	4.0±0.21
5	4	835.04±14.74	3.87±0.21
6	5	820.91±14.19	3.83±0.18
Fermentation conditions: Solid substrate = WB+CC, 7:3 (for xylanase), SB (for cellulase) Substrate:NSS = 1:3, pH = 6.5, temperature = 37 °C, incubation time = 7 days		**Assay conditions:** **Xylanase:** Temperature = 55 °C, pH = 6.4, incubation time = 15 min **Cellulase:** Temperature = 55 °C, pH = 4.8, incubation time = 30 min	

Table 2.12: Effect of different concentrations of Tween 80 on xylanase and cellulase production by *C. cinerea* RM-1

SI. No.	Tween 80 concentration (g/L)	Xylanase activity (IU/mL)	Cellulase activity (IU/mL)
1	0.0	810.45±12.09	3.77±0.13
2	0.1	864.77±13.89	4.10±0.21
3	0.2	860.18±16.24	4.21±0.23
4	0.3	847.05±15.01	4.11±0.22
5	0.4	832.29±14.11	3.94±0.18
Fermentation conditions: Solid substrate = WB+CC, 7:3 (for xylanase), SB (for cellulase) Substrate:NSS = 1:3, pH = 6.5, temperature = 37 °C, incubation time = 7 days		**Assay conditions:** **Xylanase:** Temperature = 55 °C, pH = 6.4, incubation time = 15 min **Cellulase:** Temperature = 55 °C, pH = 4.8, incubation time = 30 min	

Table 2.13: Variables and their levels employed in PBD for screening of culture conditions affecting xylanase and cellulase production by *C. cinerea* RM-1

SI. No.	Variable		Level	
	Variable code	Variable name	-1	+1
1	A	Initial pH	6	8
2	B	Incubation time (days)	4	7
3	C	Substrate concentration (g/flask)	3	5
4	D	Incubation temperature (°C)	30	40
5	E	Particle size (µm)	300	600
6	F	Moisture ratio (%)	66.67	80
7	G	Inoculum size (No. of disc, mm)	1	3
8	H	Inoculum age (days)	3	5

Table 2.14: PBD for xylanase and cellulase production by *C. cinerea* RM-1

Run No.	A	B	C	D	E	F	G	H	Xylanase activity (IU/mL)	Cellulase activity (IU/mL)
1	-1	-1	1	1	1	-1	1	1	705.61	2.04
2	-1	1	1	1	-1	1	1	-1	670.36	2.25
3	-1	1	-1	-1	-1	1	1	1	579.34	1.65
4	1	-1	1	-1	-1	-1	1	1	401.21	1.34
5	1	-1	1	1	-1	1	-1	-1	385.56	1.17
6	1	1	-1	1	-1	-1	-1	1	582.19	1.90
7	1	1	1	-1	1	1	-1	1	595.35	1.45
8	1	1	-1	1	1	-1	1	-1	634.28	1.96
9	-1	-1	-1	-1	-1	-1	-1	-1	396.35	1.40
10	-1	1	1	-1	1	-1	-1	-1	652.21	1.75
11	1	-1	-1	-1	1	1	1	-1	302.12	0.96
12	-1	-1	-1	1	1	1	-1	1	479.61	1.52

Solid substrate:
For xylanase = WB+CC, 7:3
For cellulase = SB

Assay conditions:
Xylanase:
Temperature = 55 °C, pH = 6.4, incubation time = 15 min
Cellulase:
Temperature = 55 °C, pH = 4.8, incubation time = 30 min

Table 2.15: Analysis of PBD for xylanase and cellulase production by *C. cinerea* RM-1

SI. No.	Variable		Xylanase			Cellulase		
	Variable code	Variable name	Effect	t-ratio	p-value	Effect	t-ratio	p-value
1	A	Initial pH	-97.13	-4.16	0.025	-0.3050	-4.17	0.025
2	B	Incubation time (days)	173.8	7.45	0.005	0.4217	5.77	0.010
3	C	Substrate concentration (g/flask)	72.73	3.12	0.063	0.1017	1.39	0.258
4	D	Incubation temperature (°C)	88.51	3.79	0.032	0.3817	5.22	0.014
5	E	Particle size (µm)	59.03	2.53	0.085	-0.0050	-0.07	0.950
6	F	Moisture ratio (%)	-59.92	-2.57	0.083	-0.2317	–3.17	0.054
7	G	Inoculum size (No. of disc, mm)	33.61	1.44	0.245	0.1683	2.30	0.105
8	H	Inoculum age (days)	50.40	2.16	0.120	0.0683	0.93	0.419

Table 2.16: Variables and their levels employed in CCD for xylanase and cellulase production by *C. cinerea* RM-1

Independent variable	Coded level of variable				
	-α	**-1**	**0**	**+1**	**+α**
Initial pH, X_1	4.32	5	6	7	7.68
Incubation time, X_2 (days)	5.32	6	7	8	8.68
Temperature, X_3 (°C)	31.59	35	40	45	48.41

Table 2.17: CCD for xylanase and cellulase production by *C. cinerea* RM-1

SI. No.	Independent variable						Response variable	
	Coded			Real				
	X_1	X_2	X_3	Initial pH	Incubation time (days)	Temperature (°C)	Xylanase activity (IU/mL)	Cellulase activity (IU/mL)
1	0	-1.68	0	6.0	5.3	40	796.08	3.41
2	1	-1	-1	7.0	6.0	35	469.09	2.30
3	1.68	0	0	7.7	7.0	40	300.52	2.42
4	1	-1	1	7.0	6.0	45	383.35	1.48
5	-1.68	0	0	4.3	7.0	40	616.53	5.11
6	1	1	-1	7.0	8.0	35	596.81	1.12
7	0	0	1.68	6.0	7.0	48.4	331.03	0.86
8	-1	-1	1	5.0	6.0	45	562.21	2.01
9	-1	1	-1	5.0	8.0	35	819.72	3.43
10	1	1	1	7.0	8.0	45	475.47	2.70
11	0	0	0	6.0	7.0	40	1005.37	4.60
12	0	0	0	6.0	7.0	40	1012.31	4.61
13	-1	1	1	5.0	8.0	45	562.16	1.91
14	0	0	-1.68	6.0	7.0	31.6	664.49	2.32
15	0	1.68	0	6.0	8.7	40	915.27	2.39
16	0	0	0	6.0	7.0	40	989.34	4.41
17	0	0	0	6.0	7.0	40	996.27	4.44
18	-1	-1	-1	5.0	6.0	35	756.88	5.71
19	0	0	0	6.0	7.0	40	1003.61	4.50
20	0	0	0	6.0	7.0	40	1006.62	4.42

Solid substrate:
For xylanase = WB+CC, 7:3
For cellulase = SB

Assay conditions:
Xylanase:
Temperature = 55 °C, pH = 6.4, incubation time = 15 min
Cellulase:
Temperature = 55 °C, pH = 4.8, incubation time = 30 min

Table 2.18: Regression analysis of CCD for xylanase and cellulase production by *C. cinerea* RM-1

Parameter	Xylanase			Cellulase		
	CE	t-ratio	p-value	CE	t-ratio	p-value
Constant	1002.31	208.14	0.000	4.4987	88.481	0.000
X_1	-95.76	-29.97	0.000	-0.7311	-21.672	0.001
X_2	35.37	11.07	0.002	-0.2970	-8.803	0.000
X_3	-89.34	-27.96	0.000	-0.5064	-15.011	0.000
X_1^2	-192.58	-61.92	0.000	-0.2720	-8.284	0.002
X_2^2	-52.16	-16.77	0.001	-0.5779	-17.597	0.000
X_3^2	-178.71	-57.46	0.000	-1.0410	-31.701	0.001
X_1*X_2	19.63	4.70	0.001	0.3025	6.863	0.003
X_1*X_3	30.64	7.34	0.000	0.7475	16.960	0.000
X_2*X_3	-12.31	-2.95	0.015	0.5725	12.989	0.002
R^2	99.88			99.60		
R^2 (pred.)	99.26			97.59		
R^2 (adj.)	99.77			99.25		

Table 2.19: ANOVA for xylanase and cellulase production by *C. cinerea* RM-1

Source	DF	Xylanase				Cellulase			
		SS	MS	F-value	p-value	SS	MS	F-value	p-value
Linear	3	251315	83772	600.90	0.000	12.0050	4.0017	257.49	0.000
Square	3	910224	303408	2176.35	0.000	19.1272	6.3757	410.25	0.000
Interaction	3	11808	3936	28.23	0.000	7.8242	2.6080	167.82	0.000
Lack of fit	5	1060	212	3.17	0.116	0.1153	0.0231	2.87	0.136
Pure error	5	334	67			0.0401	0.0080		
Total	19	1174741				39.1118			

Table 2.20: Criteria for optimization of xylanase and cellulase production by *C. cinerea* RM-1

Response	Goal	Lower	Target	Upper	Importance
Xylanase activity (IU/mL)	Maximize	900	1000	-	1
Cellulase activity (IU/mL)	Maximize	3.0	5.0	-	1

Table 2.21: Some optimized conditions calculated by Response optimizer for xylanase production by *C. cinerea* RM-1 using WB+CC (7:3) as the substrate

Initial pH	Incubation time (days)	Temperature (°C)	Xylanase activity (IU/mL)	Composite desirability
5.8	**7.3**	**38.5**	1032.9	1
5.8	7	38.5	1027.7	1
6	7	38.5	1012.6	1
6	7	37.4	1000.0	1
5.8	7	37.0	1004.3	1
5.8	7.4	36.5	1002.7	1
5. 7	7.1	36.5	1004.6	1
5. 7	6.8	36.5	988.2	0.882

Table 2.22: Some optimized conditions calculated by Response optimizer for cellulase production by *C. cinerea* RM-1 using SB as the substrate

Initial pH	Incubation time (days)	Temperature (°C)	Cellulase activity (IU/mL)	Composite desirability
4.3	**5.7**	**34.0**	6.5	1
4.7	5.9	34.0	6.1	1
4.7	5.9	36.1	6.1	1
4.9	6.1	37.5	5.7	1
4.9	6. 6	37.5	5.6	1
5.5	6. 6	37.5	5.2	1
5.8	6.7	37.5	4.8	0.918
5.8	6.4	36.3	4.8	0.899

Table 2.23: Experimental values and calculated values of xylanase and cellulase produced by *C. cinerea* RM-1 using above selected optimum conditions (in bold)

Response	Mean experimental value	Calculated value	Diff. (%)
Xylanase activity (IU/mL)	1020.5±25.2	1032.9	1.20
Cellulase activity (IU/mL)	6.54±0.31	6.5	0.61

Fermentation conditions: **For xylanase:** Solid substrate = WB+CC (7:3), pH = 5.8, temperature = 38.5 °C, incubation time = 7.3 days **For cellulase:** Solid substrate = SB, pH = 4.3, temperature = 34 °C, incubation time = 5.7 days	**Assay conditions:** **Xylanase:** Temperature = 55 °C, pH = 6.4, incubation time = 15 min **Cellulase:** Temperature = 55 °C, pH = 4.8, incubation time = 30 min

Table 2.24: pH stability of crude xylanase and crude cellulase produced by *C. cinerea* RM-1

SI. No.	Buffer pH	Xylanase activity (IU/mL)	Relative xylanase activity (%)	Cellulase activity (IU/mL)	Relative cellulase activity (%)
1	3.8	626.7±6.35	60	6.43±.16	97
2	4.3	730.92±9.52	70	6.64±0.15	100
3	4.8	825.5±10.15	79	6.52±0.21	98
4	5.3	971.27±9.24	93	6.18±0.12	93
5	5.8	1044.56±13.45	100	5.65±0.20	85
6	6.3	1023.45±12.45	98	5.30±0.14	80
7	6.8	993.34±12.35	95	4.78±0.16	72
8	7.3	940.26±11.27	90	4.26±0.11	64
9	7.8	867.5±10.5	83	3.79±0.10	57
10	8.3	731.15±9.10	70	3.40±0.09	51
11	8.8	605.01±7.20	58	2.79±0.08	42
12	9.3	500.35±6.10	48	1.98±0.06	30
13	9.8	355.0±4.35	34	1.15±0.03	17
14	10.3	198.15±3.45	19	0.59±0.01	9

Fermentation conditions:
For xylanase: Solid substrate = WB+CC (7:3), initial pH = 5.8, temperature = 38.5 °C, incubation time = 7.3 days
For cellulase: Solid substrate = SB, initial pH = 4.3, temperature = 34 °C, incubation time = 5.7 days
Assay conditions:
Temperature = 55 °C, time = 1 h

Table 2.25: Temperature stability of crude xylanase and crude cellulase produced by *C. cinerea* RM-1

SI. No.	Temperature (°C)	Xylanase activity (IU/mL)	Relative xylanase activity (%)	Cellulase activity (IU/mL)	Relative cellulase activity (%)
1	45	949.70±9.12	92	6.37±0.20	95
2	55	1035.35±14.25	100	6.68±0.17	100
3	65	928.4±9.10	90	5.82±0.16	87
4	75	685.50±7.51	66	4.68±0.12	70
5	85	370.91±5.25	36	2.47±0.08	37

Fermentation conditions:
For xylanase: Solid substrate = WB+CC (7:3), initial pH = 5.8, temperature = 38.5 °C, incubation time = 7.3 days
For cellulase: Solid substrate = SB, initial pH = 4.3, temperature = 34 °C, incubation time = 5.7 days
Assay conditions:
pH = 5.8 (for xylanase), 4.3 (for cellulase), time = 1 h

Table 2.26: Mass production of crude enzyme using wheat bran and corn cob (7:3) as the substrate by *C. cinerea* RM-1

Xylanase activity (IU/mL)	Cellulase activity (IU/mL)	Protein concentration (mg/mL)
1038.5±18.25	**0.465±0.03**	**7.24±0.40**
Fermentation conditions: Solid substrate = WB+CC (7:3), initial pH = 5.8, temperature = 38.5 °C, incubation time = 7.3 days	**Assay conditions:** **Xylanase:** Temperature = 55 °C, pH = 6.4, incubation time = 15 min **Cellulase:** Temperature = 55 °C, pH = 4.8, incubation time = 30 min	

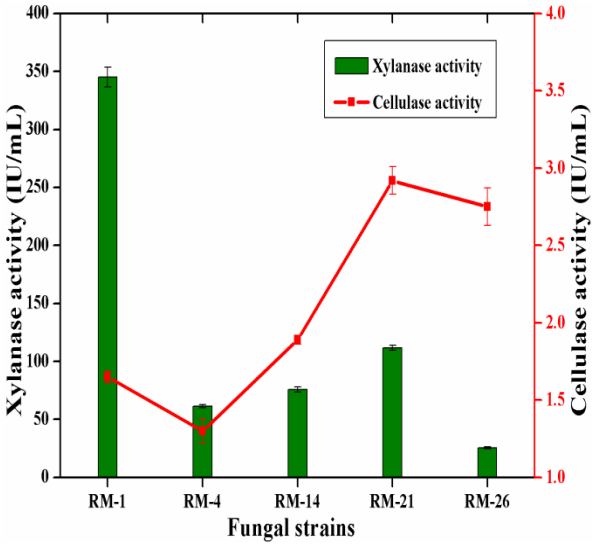


Figure 2.1: Xylanase and cellulase production from the isolated fungal strains under SSF conditions.

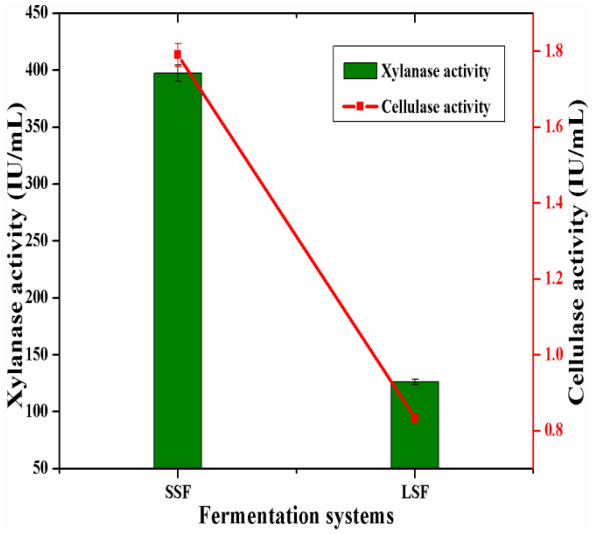


Figure 2.2: Comparison of fermentation systems for xylanase and cellulase production by *C. cinerea* RM-1.

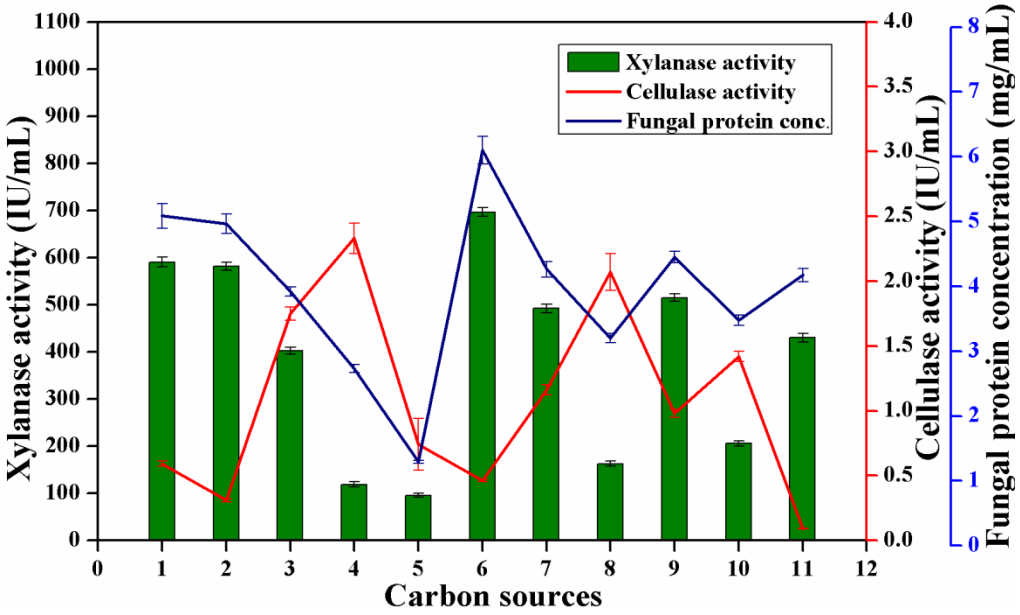


Figure 2.3: Effect of lignocelluloses as the substrate on enzyme production and fungal protein concentration by *C. cinerea* RM-1 (1: wheat bran, 2: corn cob, 3: rice straw, 4: sugarcane bagasse, 5: coconut coir, 6: wheat bran+corn cob, 7: wheat bran+rice straw, 8: wheat bran+sugarcane bagasse, 9: corn cob+rice straw, 10: corn cob+sugarcane bagasse and 11: xylan agar).

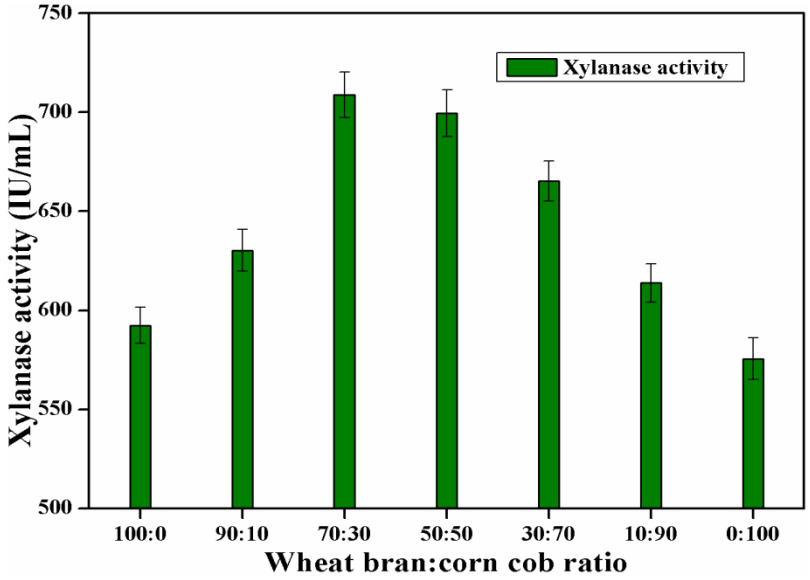


Figure 2.4: Effect of substrate ratio (wheat bran:corn cob) on xylanase production by *C. cinerea* RM-1.

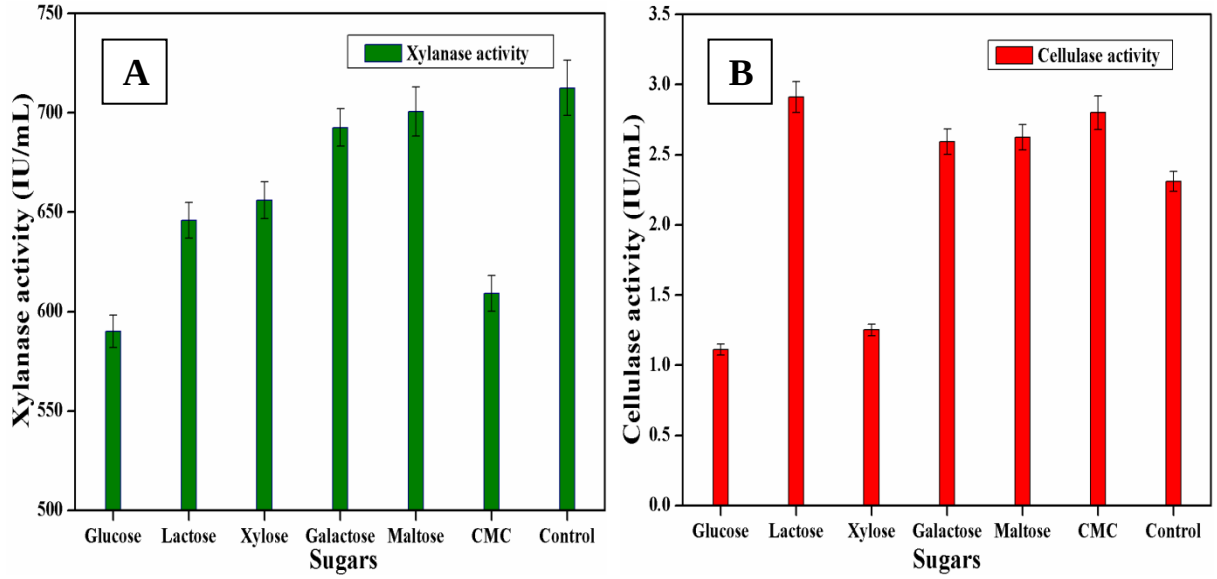


Figure 2.5: Effect of sugars on xylanase (A) and cellulase (B) production by *C. cinerea* RM-1.

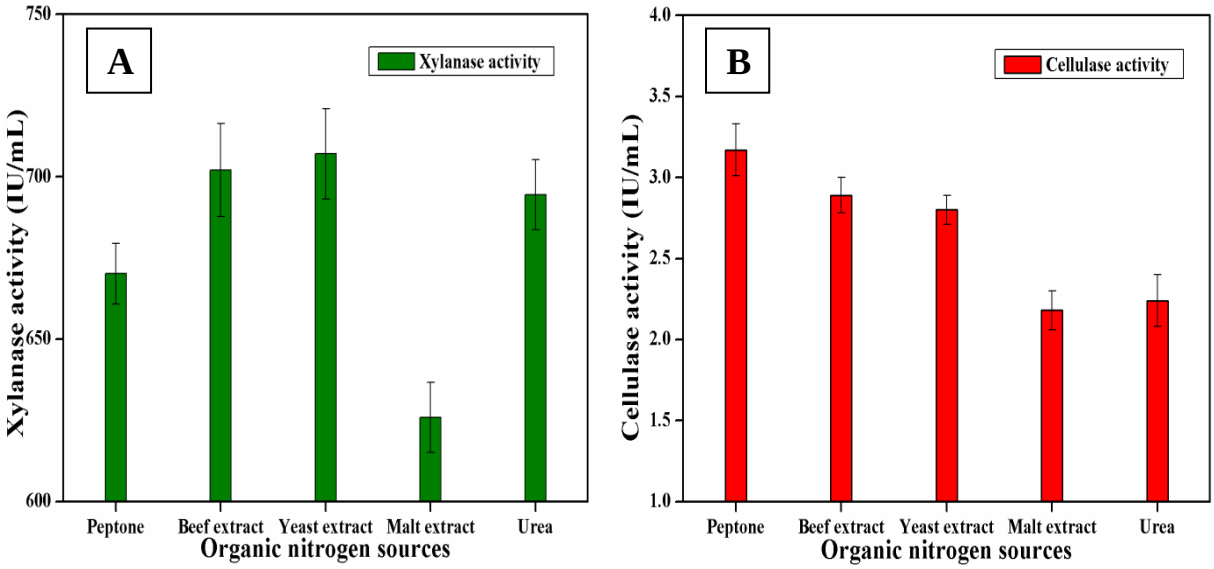


Figure 2.6: Effect of organic nitrogen sources on xylanase (A) and cellulase (B) production by *C. cinerea* RM-1.

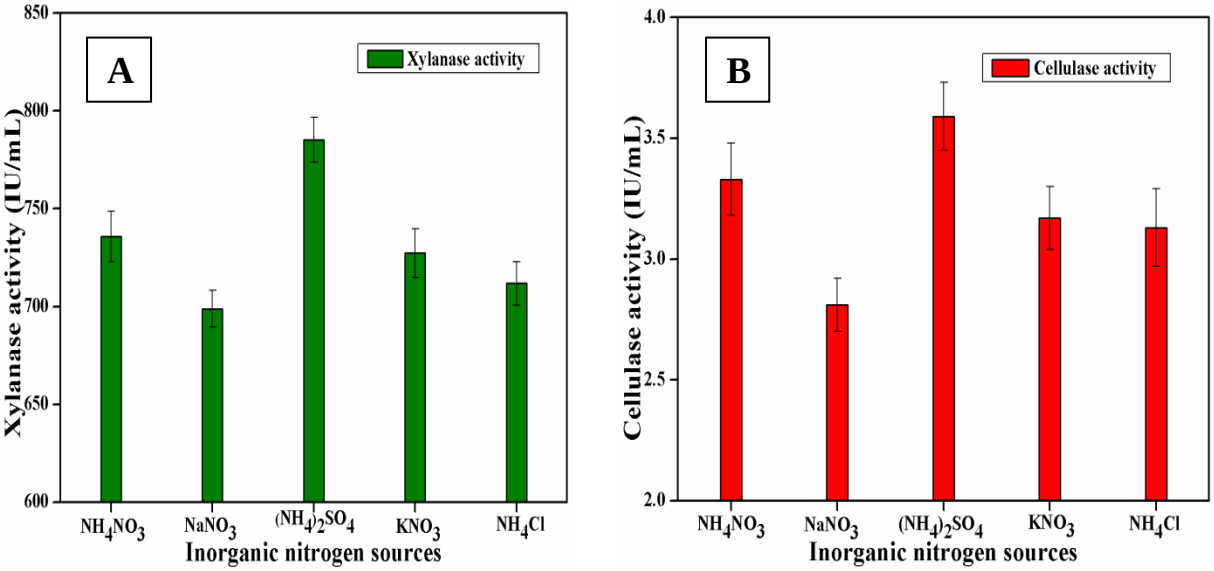


Figure 2.7: Effect of inorganic nitrogen sources on xylanase (A) and cellulase (B) production by *C. cinerea* RM-1.

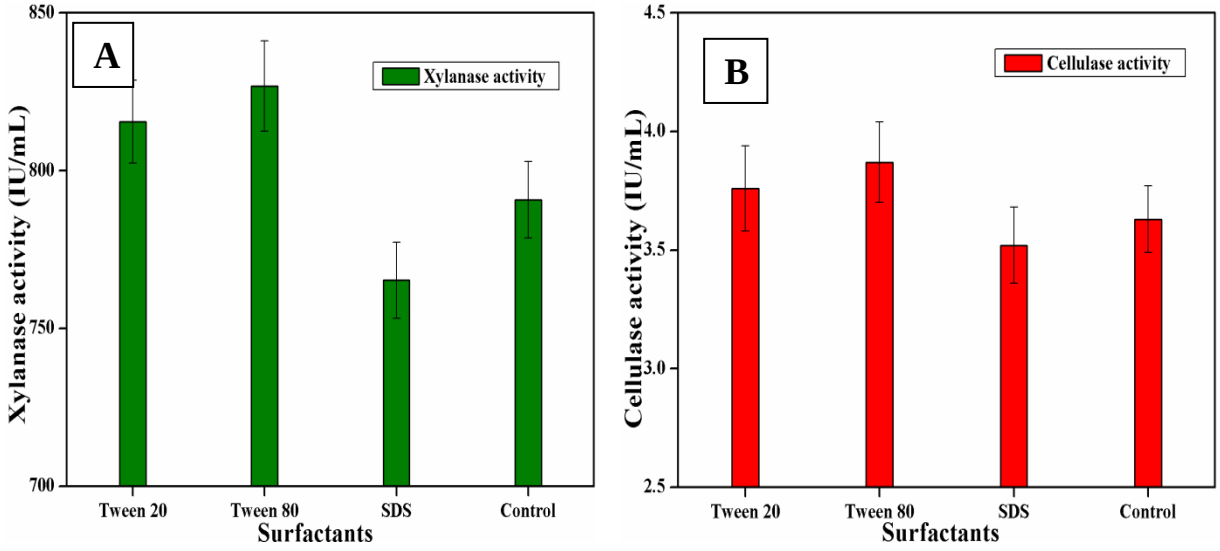


Figure 2.8: Effect of surfactants on xylanase (A) and cellulase (B) production by *C. cinerea* RM-1.

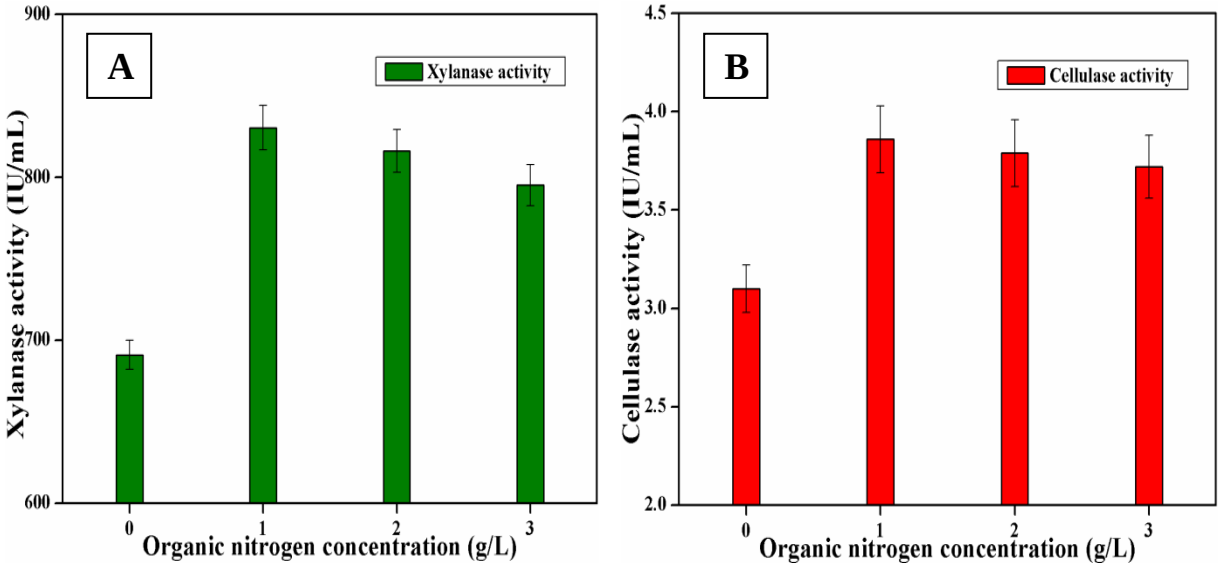


Figure 2.9: Effect of organic nitrogen concentrations on xylanase (A) and cellulase (B) production by *C. cinerea* RM-1.

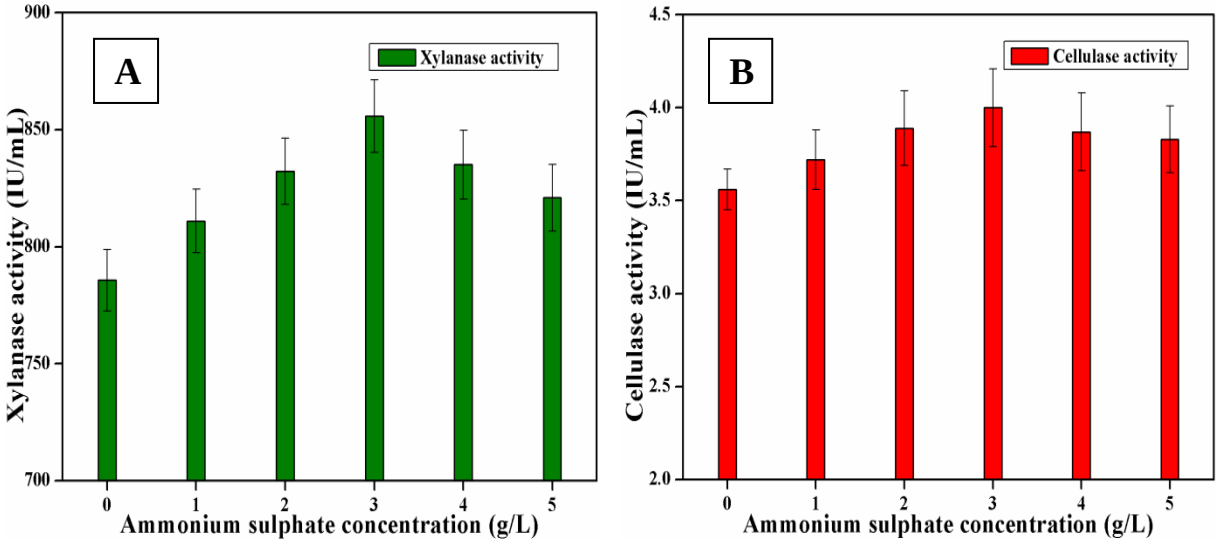


Figure 2.10: Effect of ammonium sulphate concentrations on xylanase (A) and cellulase (B) production by *C. cinerea* RM-1.

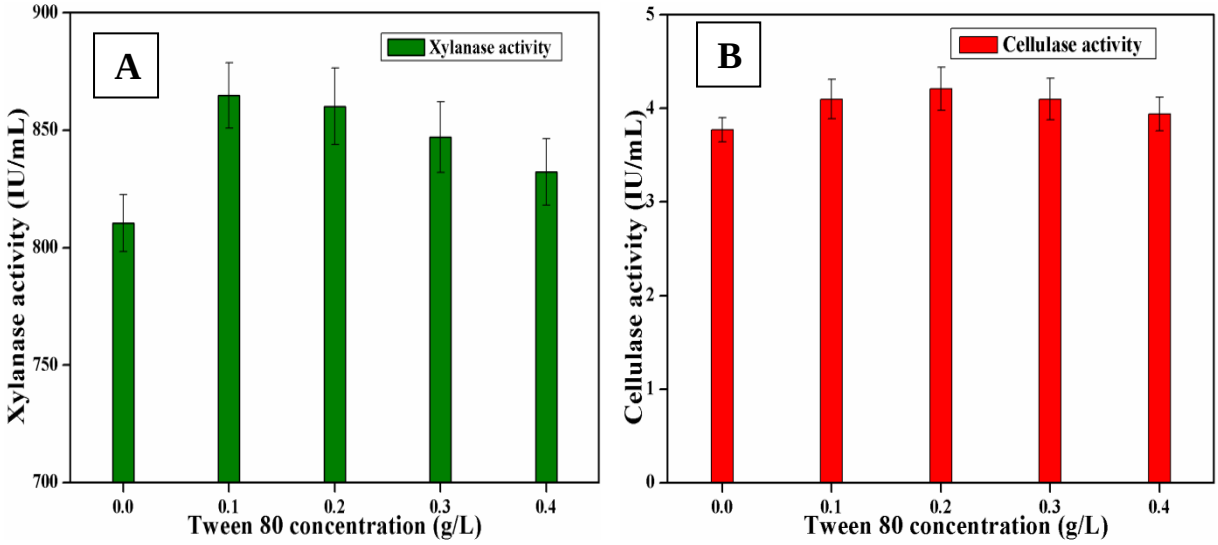


Figure 2.11: Effect of Tween 80 concentrations on xylanase (A) and cellulase (B) production by *C. cinerea* RM-1.

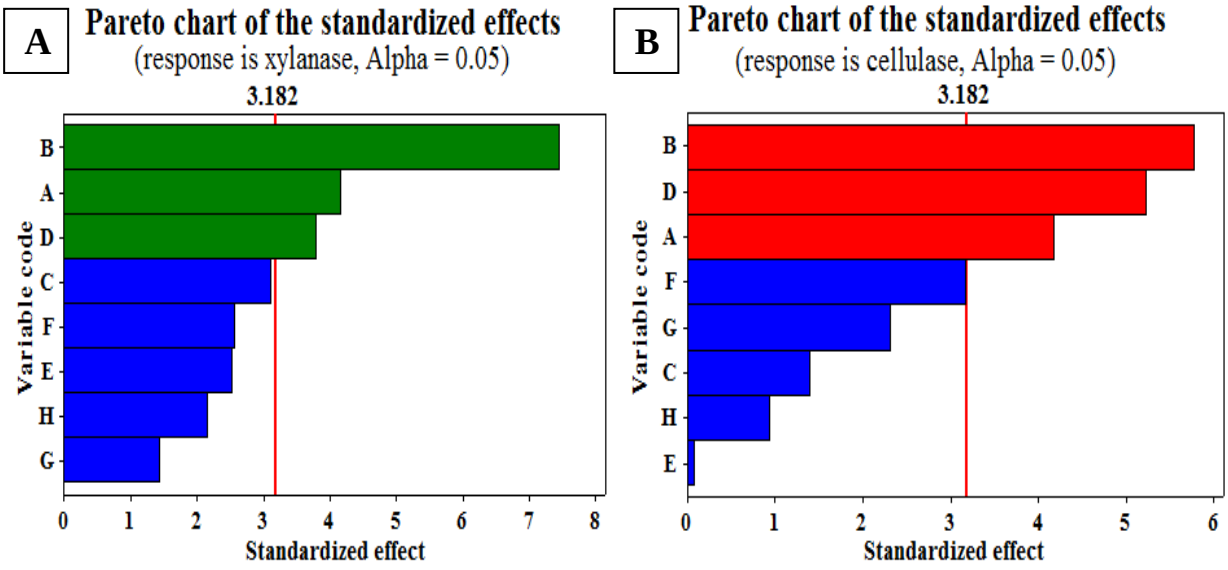


Figure 2.12: Effects Pareto plots for xylanase (A) and cellulase (B) production by *C. cinerea* RM-1.

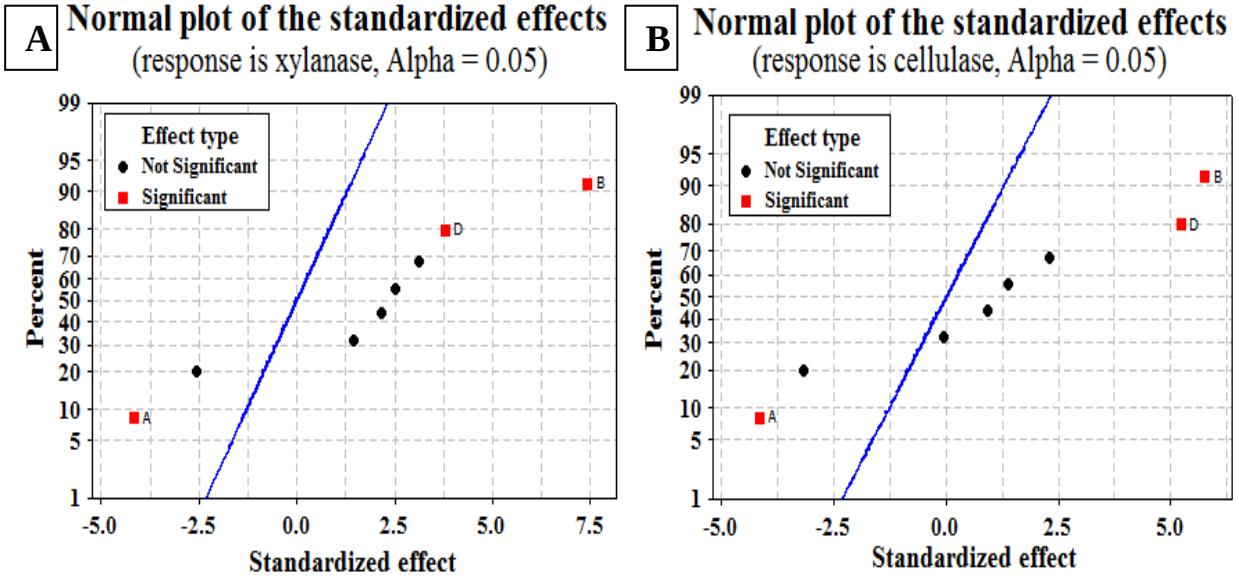


Figure 2.13: Normal Effect plots for xylanase (A) and cellulase (B) production by *C. cinerea* RM-1.

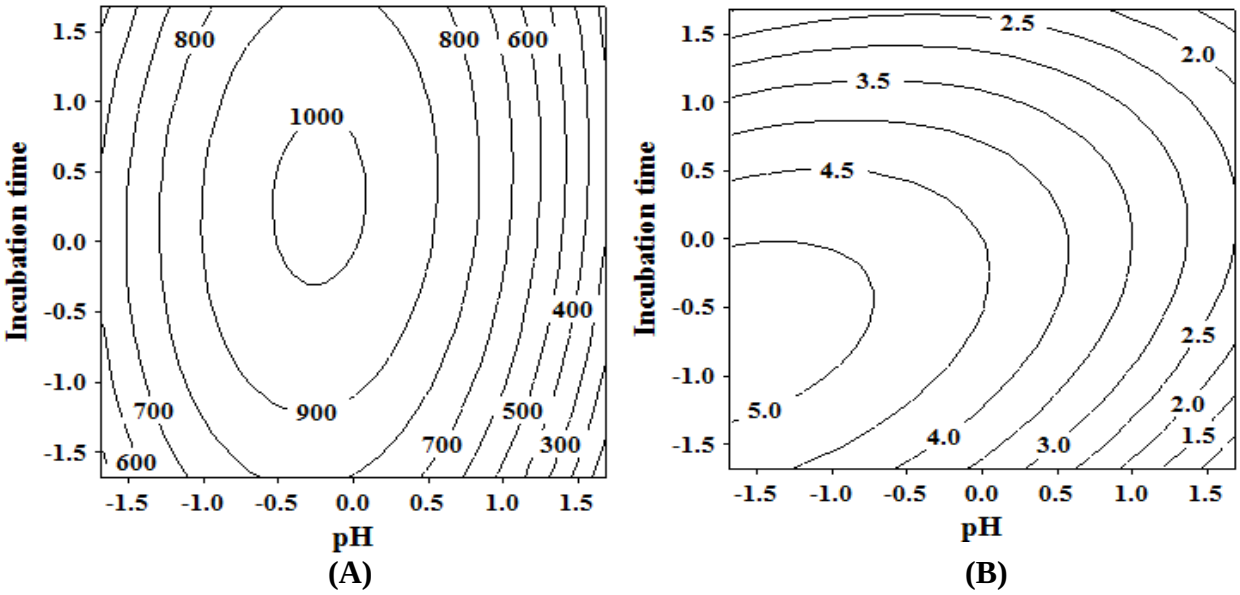


Figure 2.14: Effect of initial pH and incubation time on xylanase (A) and cellulase (B) production by *C. cinerea* RM-1.

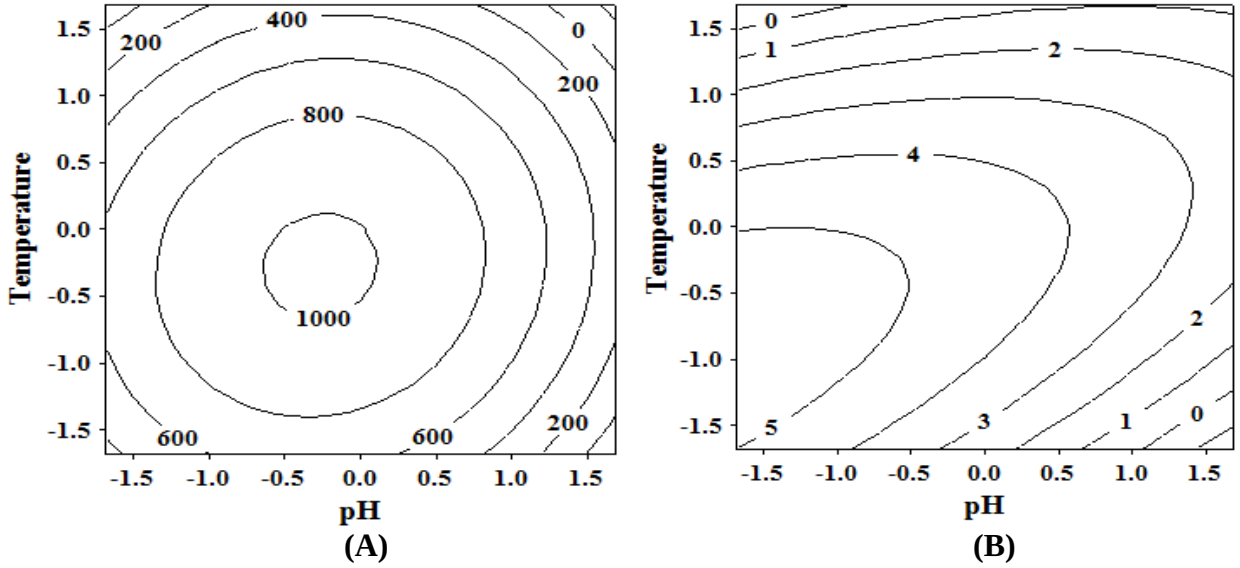


Figure 2.15: Effect of initial pH and incubation temperature on xylanase (A) and cellulase (B) production by *C. cinerea* RM-1.

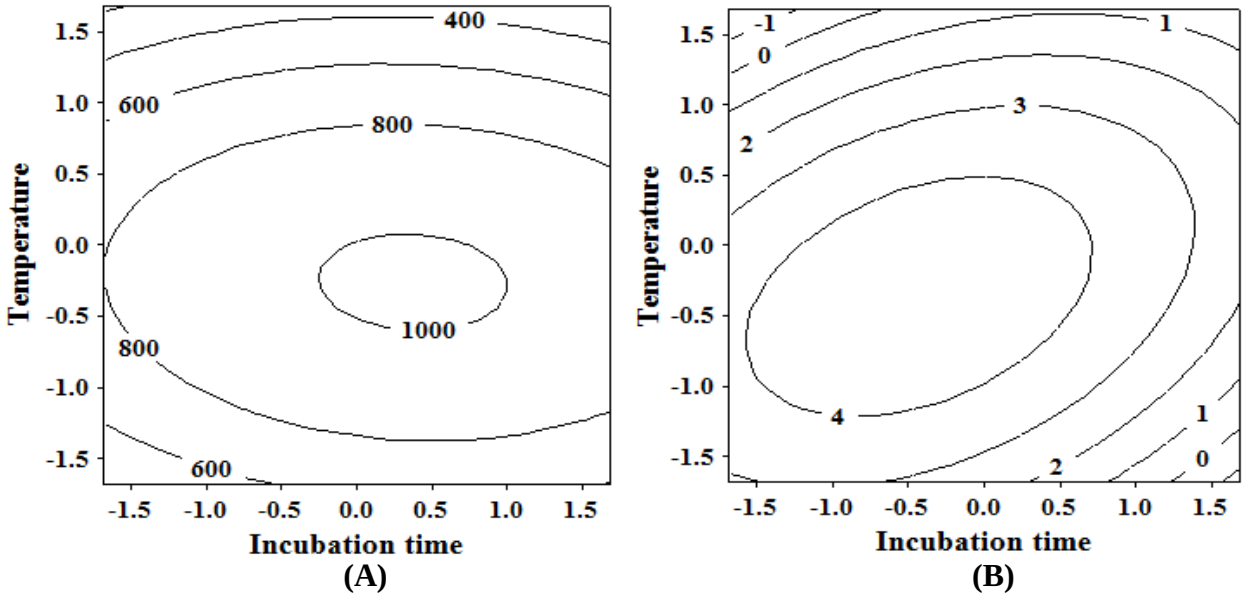


Figure 2.16: Effect of incubation time and temperature on xylanase (A) and cellulase (B) production by *C. cinerea* RM-1.

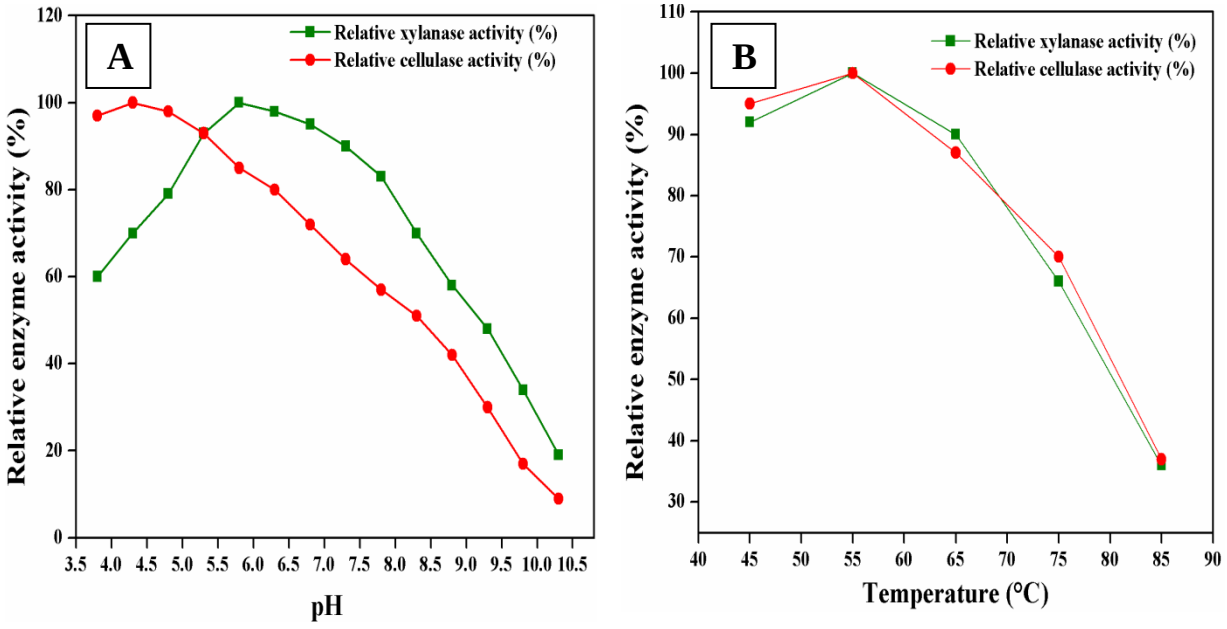
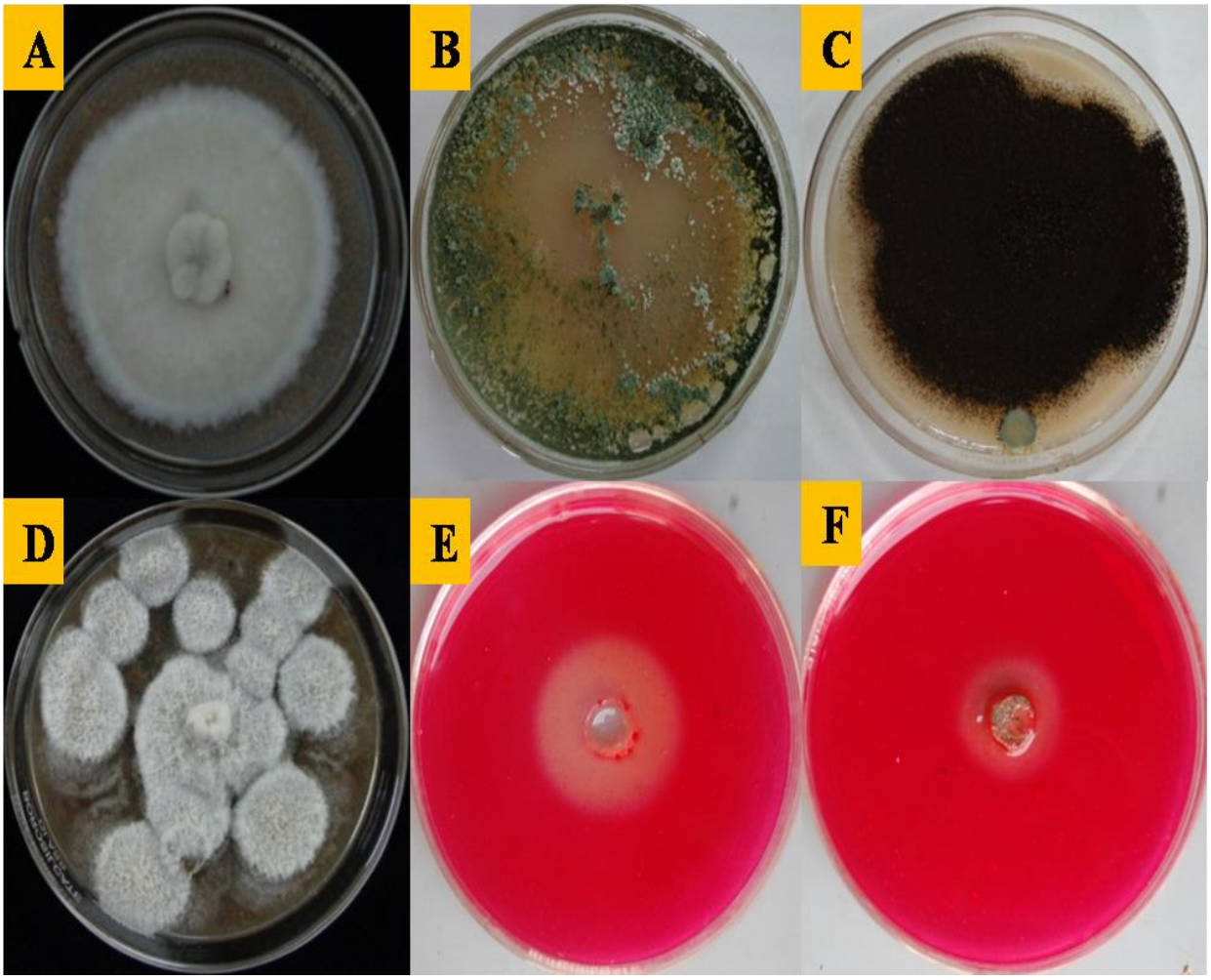
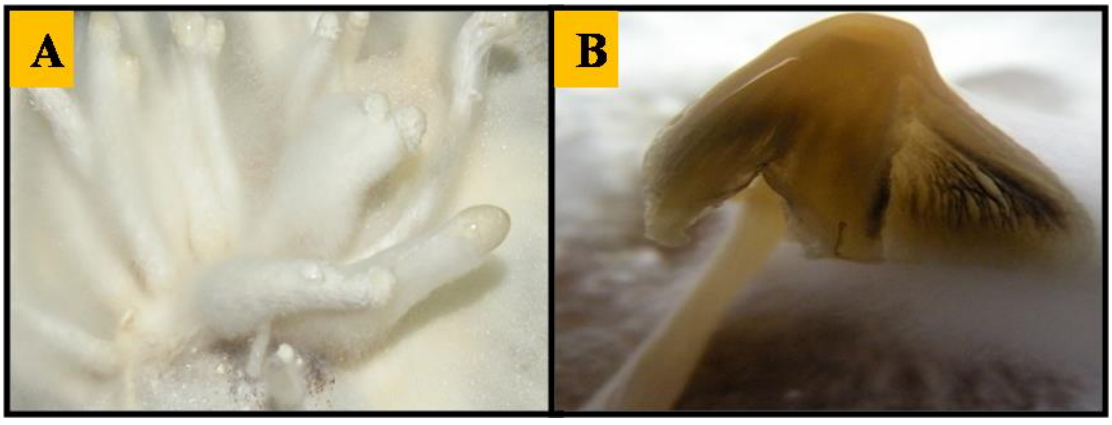


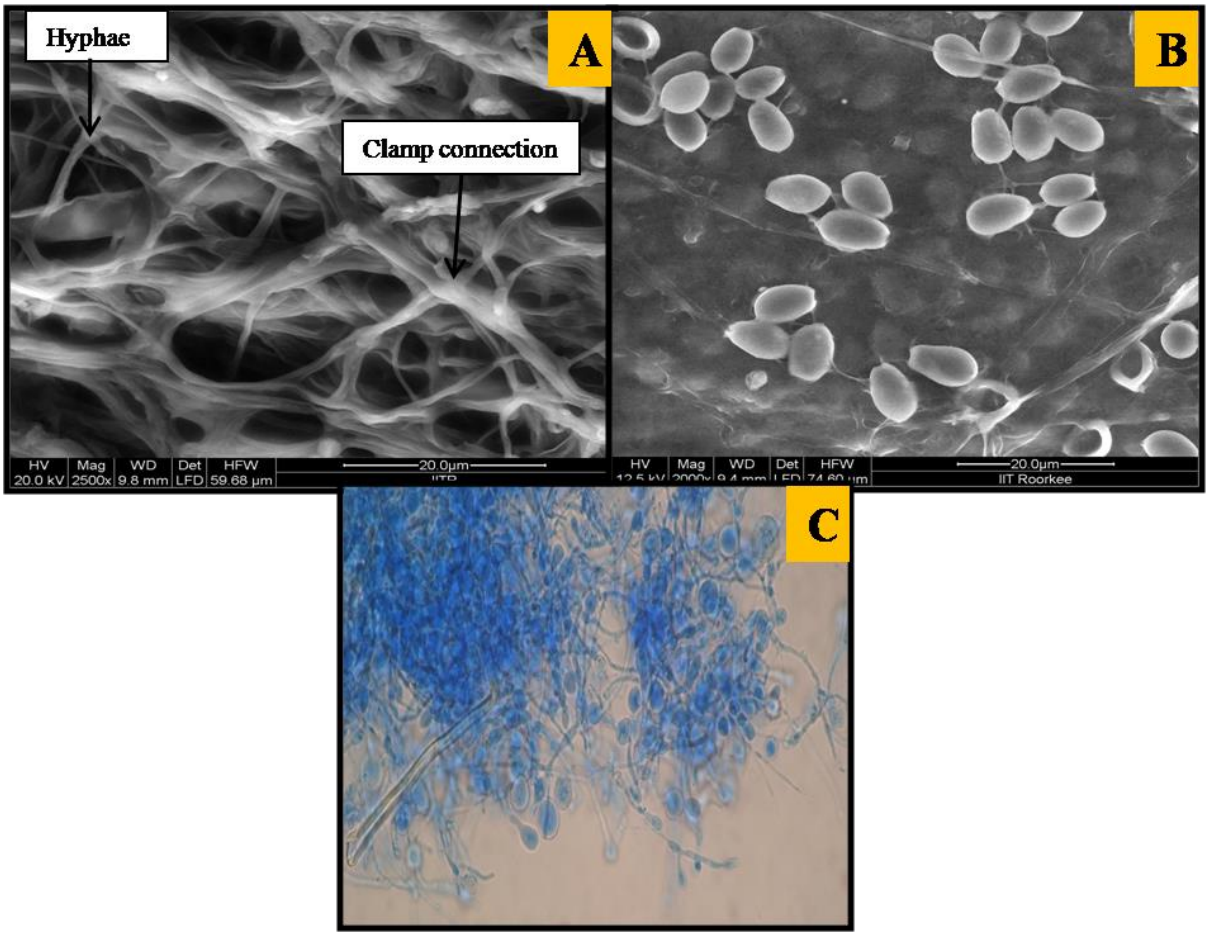
Figure 2.17: pH (A) and temperature stability (B) curves of xylanase and cellulase produced by *C. cinerea* RM-1.



Photograph 2.1: Morphological features of different fungal isolates i.e. RM-l, RM-2l, RM-l4 and RM-4 (A-D); xylan-agar plate assay for strain RM-1 (E); CMC-agar plate assay for strain RM-1 (F).



Photograph 2.2: Fungal strain *C. cinerea* RM-1 showing fruiting body primordia in 8 days old culture (A); sporulation stage in 10 days old culture (B).



Photomicrograph 2.3: Fungal strain *C. cinerea* RM-1 showing highly branched fungal mycelial mat and solid hyphae with clamp connection (A); club shaped basidiospores (B and C).

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ANNEXURE 2

ABBREVIATIONS

%	Percent
<	Less than
>	More than
°C	Degree celcius
µg	Micrograms
µg/L	Micrograms per Litre
µm	Micrometers
µmol	Micromoles
ARI	Agharkar Research Institute
bp	Base pairs
BSA	Bovine serum albumin
CCD	Central composite design
CE	Coefficient
CMC	Carboxymethyl cellulose
CMCA	Carboxymethyl cellulose agar
DNS	3, 5-dinitrosalicylic acid
etc.	et cetera
g	Grams
g/L	Grams per litre
h	Hours
i.e.	That is
IARI	Indian Agricultural Research Institute
ITCC	Indian Type Culture Collection
IU	International units
IU/mL	International units per millilitres
kDa	Kilo Dalton
L	Litres
min	Minutes
mL	Millilitres
mm	Millimeters
NFCCI	National Fungal Culture Collection of India
nm	Nanometer
NSS	Nutrient salt solution
OVAT	One-variable-at-time
PBD	Plackett-Burman design
PDA	Potato dextrose agar
pI	Isoelectric point
R^2	Determination coefficient
R^2adj	Adjusted determination coefficient
r-DNA	Ribosomal DNA
rpm	Revolutions per minute
RSM	Response surface methodology

SB	Sugarcane bagasse
SDS	Sodium dodecyl sulphate
SEM	Scanning electrone microscopy
SmF	Submerged fermentation
sp.	Species
SSF	Solid state fermentation
UV-Vis	Ultravilot-Visible
v/v	Volume per volume
w/v	Weight per volume
WB+CC	Wheat bran+corn cob
wt.	Weight
XA	Xylan agar

CHAPTER 3

PROXIMATE ANALYSIS OF *L. LEUCOCEPHALA* AND *C. EQUISETIFOLIA*

3.1. INTRODUCTION

The main component of the biosphere is trees, and their wood has a major role as a sustainable and renewable raw material for industries. Wood formation in plants can be explained by vascular development which can be divided into two distinct phases: primary and secondary development [55]. During primary development, the apical meristem in plants develops the procambial tissue made up of only one layer of juvenile cells, called initials. The cambial initials produce phloem and xylem mother cells by further cell divisions. The phloem and xylem mother cells form primary conductive tissues (xylem and phloem). Secondary vascular development occurs through a complex series of steps involving cell division, differentiation phase and finally programmed cell death to form hollow tube-like wood cells. Procambial tissue undergoes further asymmetric periclinal cell divisions and forms a lateral meristem, the vascular cambium (secondary meristem). By the process of cell division, vascular cambial cells proliferate and form a few layers of narrow, elongated, thin-walled cell types, called “fusiform initials” and “ray cell initials”, and comprise two types of highly vacuolated cells. Fusiform initials (spindle shape) are elongated fiber like cells which divide through periclinal division and produce wood elements (mostly tracheids in gymnosperms but also vessel elements, vessel-associated cells, axial parenchyma and fibers in angiosperms) inwards and secondary phloem cells (sieve tubes in gymnosperms and companion cells, axial parenchyma and fibers in angiosperms) outwards [36]. Ray initials give rise to rays that play an important role in translocation of nutrients between phloem and xylem. Tracheids and vessels are involved in water transport and mechanical support of the entire tree, and act as a good source for paper making fibers with which the paper industry is concerned [5].

The differentiation phase of the wood fiber development involves four major steps: cell expansion, deposition of a thick multilayered secondary cell wall, followed by lignification and cell death [36]. At first step of differentiation, the formation of a new highly elastic layer, primary wall (P), of 0.1 μm thickness takes place. Primary wall is made up of several layers of cellulosic microfibrils exhibiting no definite orientation. This layer has a high amount of lignin, hemicellulose and pectic substances which may be found between the MFs. Primary wall of

one fiber is attached to the primary wall of another fiber through intercellular layer (I) or middle lamella. The middle lamella is made up of lignin and pectic substances, and is only 0.5 to 1.5 µm thick. The wood cells expend longitudinally and radially during the formation of the primary wall and many enzymes like xyloglucan endotransglycosylases, endoglucanases, expansins, pectin methylesterases and pectinases play a determinant role in this process [36]. Once expansion is completed, the formation of the secondary cell wall begins inside the primary cell wall [6].

According to the generally accepted model, secondary cell wall is divided into three different layers; S_1, S_2 and S_3 [52, 15]. S_1 is the thinnest outermost layer of secondary cell wall. The thickness of the S_1 layer varies between 0.1 to 0.35 µm and accounts for 5 to 10% of the total thickness of the cell wall. This layer functions as an intermediate between the primary cell wall and the S_2 and S_3 layers. The S_2 layer is the thickest middle layer in the secondary cell wall, being 1 to 10 µm thick, and representing 75 to 80% of the total thickness of the cell wall. The S_3 layer is the innermost layer of the secondary cell wall, being only 0.5 to 1.10 µm thick. The three layers of secondary cell wall are composed of cellulose MFs, and are organized in a plywood type of construction [20, 54]. The cellulose MFs in each layer are associated in an ordered and parallel arrangement which differs from one S layer to another S layer. MFA, the angle between MFs and the longitudinal cell axis, is the most important characteristics of fibers [19], and exhibits the orientation of cellulose MFs in wood cells. Each layer of secondary cell wall differs in MFA [41, 3]. The MFA in S_1 and S_3 layers is large, varying from 40 to 120° [9, 36] while the MFA in S_2 layer is small, varying from 5 to 30° [20, 36]. The MFA in S_2 layer can be even higher, depending on external mechanical stress [36]. The highest amount of cellulose is present in S_2 layer as compared to S_1 and S_3 layers, on the other hand, hemicellulose is higher in S_3 layer while lignin is the dominant compound in the middle lamella [14].

Many properties of paper are reliant on the chemical composition of wood [45]. The softwoods (gymnosperms) consist of mainly thin-walled, long tapering cells called tracheids, storage parenchyma, epithelial cells and ray tracheids. Tracheid cells (softwood fibers) are long, generally 3-7 mm in length, and function in conducting water and providing mechanical support to the tree. The parenchyma tissue of softwoods often contains resin-filled ducts which are part of the tree's defense system against insects and fungal diseases. Ray tracheids may be absent in some species. The pulps of softwoods provide more surface area for bonding during

pressing of sheet due to their thin walled fibers that collapse readily to double walled ribbon structure. Hardwoods contain mainly long, narrow cells called fiber cells, storage parenchyma, vessels elements and ray parenchyma. The hardwood fibers can be divided into two broad categories: “tracheids” and “true fibers”. Tracheids include vascular tracheids and vesicentric tracheids, both of which are found in only a few woods. On the other hand, the true fibers include fiber tracheids and/or libriform fibers which are found in all species, and are the major component of hard wood pulps [24]. The average length of hardwood fibers is 1-2 mm [50]. 90-96% of softwood cells may be considered as fibers while only about 60-65% of cells in hardwoods are fibers. Vessel elements are large diameter cells which function as water pipes in transporting water throughout the tree. Vessel elements create serious problems of linting, fuzzing and picking during offset printing of paper. The large size vessels are poorly bonded with fibers. Moreover, they are easily picked out from the paper surface by the ink tackiness of the printing press and resulting [32] in deteriorate quality of print with repeating white spot in solid and halftone areas. Over a period of time, different methods including high intensity refining, hydrocyclone refining, enzymatic refining, wet-end strength additives and surface sizing have been used to reduce the vessel picking tendency of hardwood pulps [51, 37, 7].

The papermaking behavior of pulp depends very much on anatomical and chemical properties of the raw material. Fiber morphological parameters, such as fiber length, diameter and wall thickness are fundamental properties of pulpwood that directly or indirectly affect many properties of paper. Fiber length can be an important factor in determining the strength and quality of the final product. There have been indications that under certain conditions, it strongly affects tensile strength, tearing index, burst index and fold properties of a sheet made from relatively unbeaten pulp [47]. The fiber morphology with high length, high length to width ratio (>33) and/or low Runkel ratio (<1) are desirable properties for papermaking process. The importance of the derived wood properties like Runkel ratio, Slenderness ratio and Luce’s shape factor, particularly for determining the suitability of a material for pulping and papermaking is well documented [8, 22, 29].

Wood is mainly composed of cellulose, hemicellulose and lignin. In addition, wood contains extractives such as oils, fats, gums and coloring matters. The content of cellulose, hemicellulose, lignin and extractives varies among different kinds of wood [45, 26]. Hemicellulose forms a coat around the underlying cellulose fibers, bounded by hydrogen bonding. The overlaying sheath of lignin is interspersed, intertwined and covalently linked to

hemicellulose [4]. The proximate chemical analysis helps in evaluating the paper making potential of raw material. The important tests include water solubility, 1% NaOH solubility, alcohol-benzene solubility, lignin, holocelluloses, pentosan, extractives and ash etc. The cold water removes a part of extraneous materials such as inorganic compounds, tannins, gums and sugars present in wood. The hot water treatment removes starches additionally. The degree of fungal decay of wood or degradation by heat, light and oxidation is estimated by 1% NaOH solubility. The alcohol-benzene solubility of wood is a measure of extractives like waxes, fats, resins and phytosterols etc. that are not soluble in water. These resins create pitch problems and affect the homogeneity in the paper. Lignin content of hardwoods is a complex make up of different units i.e. syringyl (S), guaiacyl (G) and *p*-hydroxyphenyl (H) units [42]. The wood having high S/G ratio is easier to delignify and provides higher pulp yield. The holocellulose content of wood is the total of carbohydrate fraction present in it. Cellulose content of the pulp determines the strength properties of paper. Tensile strength of paper is directly proportional to the cellulose content [27].

The ash content of wood provides an estimation of mineral salts and other inorganic matters. Silica and calcium carbonate are present in large amount in wood ash. Large quantity of silica in wood affects paper quality adversely and exhibits damaging effect during processing of wood. Wood properties and wood quality influence the properties of pulp to a great extent. Wood is an anisotropic material; its properties vary between different species, between different trees of same species and also within the same tree [49, 18, 34, 39]. Moreover, wood raw material supplied to the pulp mill is mostly non-uniform. The non-uniformity is due to the presence of chips from trees with different ages [38], differently aged tissue within a same tree, genetic factors (trees based on seedlings from seeds) and environmental factors (soil, climate, pests, diseases, declivity, hydrological deficit and sun-shining etc.). In addition, non-uniformity may be due to the presence of fungal decay, knots, bark attached to the chips and the poor wood storage management [16].

Therefore, the anatomical and chemical characterization of raw material is necessary before the pulping process. Keeping this in view, an attempt has been made to determine the anatomical and chemical properties of *L. leucocephala* and *C. equisetifolia* wood, and to evaluate the potential utilization of their fibers in pulp and paper production.

3.2. MATERIALS AND METHODS

3.2.1. Materials

Wood chips of *L. leucocephala* and *C. equisetifolia* were collected from ITC limited, Bhadrachalam, Andhra Pradesh, India. The chemicals were of laboratory grade and purchased from standard commercial manufacturers.

3.2.2. Methods

3.2.2.1. Anatomical characterization of *L. leucocephala* and *C. equisetifolia*

For anatomical studies, cross and tangential sections (25 µm thickness) of *L. leucocephala* and *C. equisetifolia* were cut on Leitz base sladge microtome-1300 and studied for SEM using a FEI Quanta 200 F microscope. The sections were primarily fixed with 3% glutaraldehyde and 2% formaldehyde in a ratio of 4:1 (v/v) for 24 h. Following the primary fixation, the sections were washed thrice with double distilled water and then treated with the alcohol gradients of 30, 50, 70, 80, 90 and 100% for dehydration. These sections were kept for 15 min in each alcohol gradient up to 70%, and then treated for 30 min under each alcohol gradient. Wood sections were then air-dried and studied under SEM at desired magnifications using gold shadowing technique [17].

The fiber length of *L. leucocephala* and *C. equisetifolia* was determined by macerating the small wood slices of each with 10 ml of 67% HNO_3 and boiled at 100±2 °C for 10 min [31]. The soften wood slices were placed in a small flask containing 50 ml distilled water after washing, and fiber bundles were disintegrated into individual fibers using a small motorized mixer with a plastic end. The macerated fiber suspension of each sample was finally placed on a standard microscope glass slide (25 mm × 75 mm) by means of a dropper. The graduated tube was used to deliver 0.5 mL suspension. All fiber samples were viewed under a calibrated microscope; a total of 100 randomly chosen fibers were measured. The kraft pulp samples of *L. leucocephala* and *C. equisetifolia* were used for other anatomical studies. The fiber suspensions (0.1% consistency) of *L. leucocephala* and *C. equisetifolia* each were used for detailed anatomical features including fiber width, fiber coarseness, fines, number of kinks, kink angle and kink index by using Fiber Tester (912/L&W). The fiber suspensions of 0.05% consistency were used to make slide for studying anatomical features including fiber lumen diameter, wall thickness, vessel length and diameter, parenchyma cell length and diameter. The slides were observed under Motorized Research Microscope (OLYMPUS BX 61). A total of 100 randomly

selected fibers, 25 randomly selected vessels and 25 randomly selected parenchyma cells were considered for measurement. The derived wood properties including slenderness ratio (fiber length/fiber diameter) [53], flexibility coefficient (100 × lumen diameter/fiber diameter) and Runkel ratio (2 × fiber wall thickness/lumen diameter) [43] were also calculated. The results were compared with *E. tereticornis* [1].

3.2.2.2. Chemical characterization of *L. leucocephala* and *C. equisetifolia*

100 g of wood chips of *L. leucocephala* and *C. equisetifolia* each were powdered in a laboratory Wiley mill, and the fractions passing through -48 mesh size but retained on +80 mesh size were selected for chemical analysis. The chemical characterization of *C. equisetifolia* and *L. leucocephala* was carried out [2]. The powder samples of wood chips were analyzed for alcohol-benzene solubility (TAPPI T 204 cm-97), cold and hot water solubility (TAPPI T 207 om-99) and 1% NaOH solubility (TAPPI T 212 om-98). Powdered samples of *C. equisetifolia* and *L. leucocephala* were then extracted with ethanol-toluene (1:2, v/v) for 6 h in a Soxhlet apparatus for removing extractives (TAPPI T 264 cm-97). The extractive-free samples were used for further chemical analysis like lignin (TAPPI T 222 om-02), pentosan (TAPPI T 223 cm-01), holocellulose (TAPPI T 249 cm-00), α-cellulose (TAPPI T 203 om-88), β-cellulose, γ-cellulose and ash content (TAPPI T 211 om-93). The holocellulose extraction was carried out by modified chlorite method developed by Erickson (1962) [13]. The results of proximate chemical analysis were compared with E. *tereticornis* [1] and *T. orientalis* [21].

3.3. RESULTS AND DISCUSSION

3.3.1. Anatomical characterization of *L. leucocephala* and *C. equisetifolia*

Photomicrograph 3.1 shows the transverse and tangential sections of wood chips of *L. leucocephala*, indicating its anatomical features under scanning electron microscope. The wood of *L. leucocephala* is diffuse and porous (Photomicrographs 3.1A and B). The parenchyma cells contain extractives like, crystals, silica, gum, resins, tannins, oils, latex and coloring materials etc. These cells are present in cortical region of the stem and contain starch grains. This is confirmed by iodine test; a deep red color is developed when iodine solution is applied on cross-sections of *L. leucocephala*, indicating the presence of abundant starch granules in storage cells, i.e. wood parenchyma and ray cells. The parenchyma cells are known for storing the solutes and foodstuffs for the growth of the plant [28]. Fiber cells have distinct middle lamella in between the secondary walls of adjacent cells (Photomicrograph 3.1A). Vessels are

round, mostly solitary and may be present in radial multiples. The vessel cells have dense lignified thickening in the secondary wall which is arranged as annular rings with simple and oblique perforation plate (Photomicrographs 3.1C and D). The transverse and tangential sections show uniseriate rays which are heterocellular with upright cells (Photomicrographs 3.1C and D). Fiber cells are septate. The tangential view (Photomicrograph 3.1D) shows the presence of crystalliferous parenchyma cells in the form of ray cells. Calcium and potassium are the predominating metals in *L. leucocephala*. Photomicrograph 3.2 shows the fiber, parenchyma cell and vessel cell present in kraft pulp of *L. leucocephala*. Photomicrograph 3.3 shows transverse and tangential sections of wood chips of *C. equisetifolia*. Vessel cells are round or oval in shape. The vessel cells of *C. equisetifolia* have longer tapering end and large diameter (Photomicrograph 3.4). The ray cells are uniseriate to biseriate with upright cells.

Table 3.1 represents the anatomical characteristics of *L. leucocephala* and *C. equisetifolia* and their comparison with *E. tereticornis*. The fiber length of *L. leucocephala* and *C. equisetifolia* are 0.78 and 0.74 mm, respectively, as compared to 0.65 mm for *E. tereticornis*. Primarily, all the strength properties depend upon fiber length and it is one of the major factors that influence the tearing strength of the paper which increases with the higher fiber length. The average width of fibers of *L. leucocephala* and *C. equisetifolia* is 18.3 and 16.9 µm, respectively, as compared to 14.2 µm for *E. tereticornis*. Fiber width provides the surface contact area for bonding and directly affects tear, tensile and burst indexes of paper. The cell cavity (lumen) of *C. equisetifolia* (4.70 µm) fibers is narrow as compared to *L. leucocephala* (7.98 µm) but wider than that of *E. tereticornis* (3.40 µm). Fiber lumen influences the beating, collapsibility and conformability of the fiber. The fiber with larger lumen diameter shows better beating and collapsibility and provides increased surface area for bonding. The cell wall thickness of *L. leucocephala* (5.2 µm) and *E. tereticornis* (5.4 µm) fibers is almost similar but *C. equisetifolia* (6.10 µm) fibers have comparatively thicker cell wall.

The slenderness ratio (L/D) is 42.62 for *L. leucocephala* and 43.79 for *C. equisetifolia* as compared to 45.77 for *E. tereticornis*. The slenderness ratio is also known as felting power and is directly proportional to the length of the fiber. Fiber with a low slenderness ratio has a high degree of collapsibility and conformability. The sheet made from such fibers is denser with low tear, high tensile, burst and double fold numbers [31, 40]. The flexibility coefficient is 43.6 for *L. leucocephala* and 27.8 for *C. equisetifolia* as compared to 23.9 for *E. tereticornis*.

Thin walled fibers are more flexible and improve the tensile strength, bursting strength and folding endurance of paper. The fibers having low flexibility coefficient are rigid and known to retain their tubular structure during pressing and do not collapse to double-walled ribbon structure, and provide lesser contact area for bonding. The handsheet made from such type of fibers is bulky and more opaque with high tear strength [44]. The Runkel ratio values of *L. leucocephala*, *C. equisetifolia* and *E. tereticornis* fibers are found in ascending order. The fibers with a Runkel ratio of more than 1.0 are lignified and thick-walled. The paper conformability and pulp yield are also affected by the Runkel ratio [10, 33]. *L. leucocephala* has large sized vessel cells with narrow diameter. The vessel cells of *C. equisetifolia* are comparatively smaller but wider than that of *L. leucocephala* while *E. tereticornis* contains smaller but wider vessel cells as compared to both of the raw materials. The length and width of parenchyma cells for *L. leucocephala* (128.98 and 27.50 µm) are more than those of *C. equisetifolia* (116.22 and 18.11 µm). Parenchyma and vessels cells appear as primary fines during pulping. The average vessel cells per lakh of fibers in *L. leucocephala* are 102.5 as compared to 154.0 in *C. equisetifolia*. Vessels are very much wider than fibers and their presence in pulp causes many problems. Due to their large dimensions, these cells act as a filler and form loose bond with the fibers, and picked up easily during printing and drying from the surface of the paper. Therefore, they generate fluff during papermaking and printing. Vessels picking problem is caused by vessel width, length and number per unit weight. Moreover, due to their large surface area, they increase the demand of wet-end additives, dry strength additives, sizing chemicals (alum-rosin, alkyl ketene dimer and alkyl succinic anhydride) and wet strength additives (melamine formaldehyde, urea formaldehyde and resins etc.).

Mean kink indexex for *L. leucocephala* and *C. equisetifolia* are 1.71 and 1.95, respectively, which are on lower side as compared to 2.15 for *E. tereticornis*. Kibblewhite defines the kink index as a sharp fiber curvature which forms an angle [23]. The high kink in fibers results into high tensile index, burst index and double fold number but low tear index [48]. The kink angle is found 47.72° for *L. leucocephala* and 48.11° for *C. equisetifolia* as compared to 29.93° for *E. tereticornis*. The values of kink per mm for *L. leucocephala* and *C. equisetifolia* are almost similar but this value is smaller than that of *E. tereticornis*. However, laboratory beaters are very effective in removing kink from the pulp [46].

Based on morphological characteristics both the raw materials (*L. leucocephala* and *C. equisetifolia*) are quite comparable to *E. tereticornis* and *T. orientalis*. *L. leucocephala* contains

longer, wider, thin walled and more flexible fiber cells as compared to *C. equisetifolia*. This indicates towards high tensile, tear and burst indexes of final product.

3.3.2. Chemical characterization of *L. leucocephala* and *C. equisetifolia*

Table 3.2 represents the results of chemical characterization for *L. leucocephala* and *C. equisetifolia*, and their comparison with *E. tereticornis* and *T. orientalis*. The cold and hot water soluble fractions in *L. leucocephala* are on higher side as compared to *C. equisetifolia* but both these raw materials contain lower amount of water soluble fractions as compared to *E. tereticornis* and *T. orientalis.* This may be due to the presence of lower amount of inorganic compounds, tannin, gums and sugars in both the raw materials. The low water solubility indicates the lesser requirement of pulping chemicals and production of higher pulp yield. The alkali solubility test (1% NaOH) estimates the dissolution of low molecular weight carbohydrates. The 1% NaOH solubility of wood indicates the degradation of carbohydrate in wood by light, solar radiation, oxidation and fungal attack when stacked in log yard. *C. equisetifolia* has lesser amount of alkali solubles as compared to *L. leucocephala. E. tereticornis* has alkali solubles almost in the same amount to *L. leucocephala* while *T. orientalis* shows maximum alkali solubles. It indicates that *C. equisetifolia* may be stacked for a longer period in wood yard, and *L. leucocephala* and *E. tereticornis* can be stored for the same length.

Alcohol benzene soluble substances are converted into pitch and block the openings of endless wire (Fourdrinier wire) and thereby retard the drainage. These substances deposit on press rolls and cause shadow marking and cockling in paper sheet. The alcohol benzene soluble substances in *C. equisetifolia* are lower as compared to *E. tereticornis* which contains lower alcohol benzene soluble substances than that of *L. leucocephala.* It indicates that *L. leucocephala* contains more of extractives such as aliphatic and aromatic hydrocarbons, waxes, terpenes and their derivatives, fatty acids, resins, alcohols, aldehydes, phenols and salts etc. These compounds have an adverse effect on pulping process [25]. The holocellulose (carbohydrate contents) is higher in *L. leucocephala* (75.31%) as compared to *C. equisetifolia* (74.73%) and *E. tereticornis* (70.3%). The total yield of pulp and overall strength of the paper depend on holocellulose content which includes straight chained high DP cellulose as well as low molecular weight carbohydrates.

α-cellulose is the high molecular weight and alkali (17.5% NaOH) insoluble part of cellulose. *L. leucocephala* and *C. equisetifolia* contain α-cellulose at about 42.9 and 43.9%, respectively;

both of values are higher than *E. tereticornis* (42.6%) but lower than *T. orientalis* (49.7%). Plant material with 34% and over α-cellulose content might be considered as a good source for pulp and paper manufacture, as directed by Nieschlag and co-workers in their rating system [30]. High α-cellulose content is related to high fiber and paper strength and longitivity of paper. β-cellulose is the alkali (17.5% NaOH) soluble part of cellulose. It can be precipitated with dilute acid (3% CH_3COOH) solution and represents the degraded cellulose with a lower degree of polymerization between 15-90. In *L. leucocephala* and *C. equisetifolia*, the amount of β-cellulose is almost similar which is higher than that *E. tereticornis* β-cellulose content. γ-cellulose indicates the hemicellulose contents of wood. γ-cellulose is higher in *L. leucocephala* (14.59%) as compared to *C. equisetifolia* (13.3%) and *E. tereticornis* (11.48%). Consequently, *L. leucocephala* fibers should require less beating for fibrillation due to more fiber swelling which may be resulted into better physical strength properties of paper. Other than cellulose, hardwoods contain pentosan (hemicellulose) which contributes to the strength in final product. High pentosan content is a desirable property of wood. The pentosan content of *C. equisetifolia* is higher than that of *L. leucocephala* which contains higher pentosan as compared to *E. tereticornis* while *T. orientalis* contains the highest amount of pentosan content. The pentosan content affects the fibrillation content of fibers during beating. The estimation of lignin content of wood provides information regarding the consumption of cooking liquor and time required for completion of cooking cycle. The stiffness of cellulosic fibers depends upon the lignin content. The fibers with high lignin content are stiffer [12]. The bleachability, hardness and color of the pulp are also related to the lignin content [11, 12]. The lignin content of *L. leucocephala* (21.65%) is lower than the lignin content of *C. equisetifolia* (23.05%) which is almost similar to that of *T. orientalis* (22.9%) but lower than that of *E. tereticornis* (28.5%). Therefore, the fibers of *C. equisetifolia* are stiffer than those of *L. leucocephala* and should require more pulping chemicals and time for the completion of cooking process. Moreover, the pulp of *C. equisetifolia* may be darker and require more chemicals for bleaching. The lignin present in the middle lamella is insoluble in 72% sulfuric acid and known as acid insoluble lignin or Klason lignin while the lignin present in primary and secondary wall layers is soluble in acid. The Klason lignin of *L. leucocephala* (20.83%) is lower than that of *C. equisetifolia* (22.41%) which has lower amount of Klason lignin than that of *E. tereticornis* (27.9%). The acid soluble lignin is found less than 1% in both the raw materials studied. The ash content of *C. equisetifolia* is lower than that of *T. orientalis* but it is higher than that *E. tereticornis* ash

content. On the other hand, the ash content of *L. leucocephala* is on higher side than that of *E. tereticornis* and *T. orientalis*. The ash content of wood provides an estimation of mineral salts and other inorganic matters. Wood ash contains silica and calcium carbonate as its major components.

Table 3.1: Anatomical characterization of *L. leucocephala* and *C. equisetifolia*

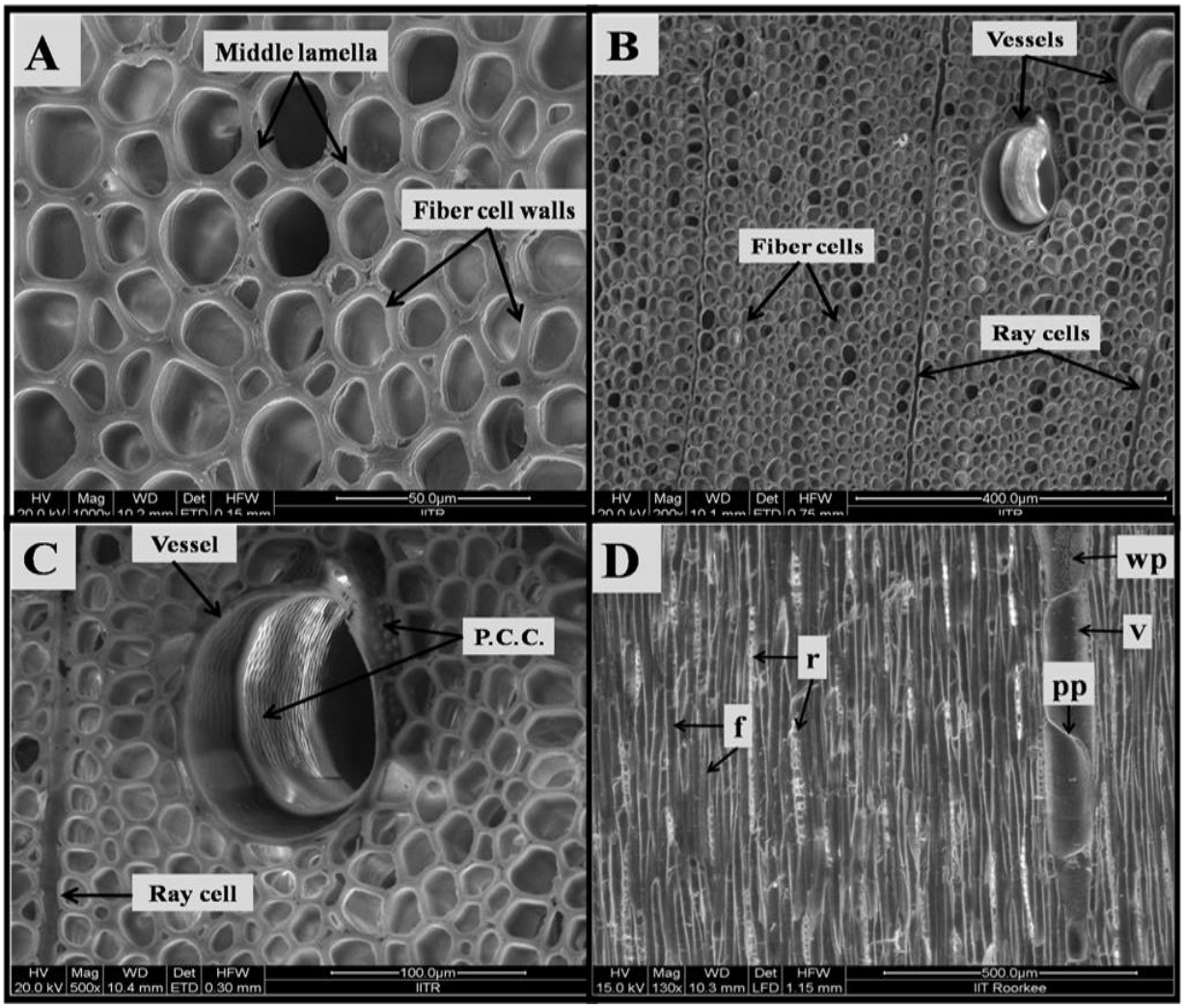
SI. No.	Parameter	*L. leucocephala*	*C. equisetifolia*	*E. tereticornis* [1]
1	Fibre length, L (mm)	0.78±0.00	0.74±0.00	0.65
2	Fibre diameter, D (µm)	18.30±0.14	16.90±0.00	14.2
3	Lumen diameter, d (µm)	7.98	4.70	3.40
4	Wall thickness, w (µm)	5.16±0.10	6.10±0.11	5.4
5	Slenderness ratio (L/D)	42.62	43.79	45.77
6	Flexibility coefficient (d/DX100)	43.61	27.81	23.94
7	Runkel ratio (2w/d)	1.29	2.59	3.18
8	Vessel length (µm)	480.55±26.93	431.24±19.7	360
9	Vessel diameter (µm)	48.31±1.98	109.76±4.36	140
10	Parenchyma cell length (µm)	128.98±10.93	116.22±7.60	69.0
11	Parenchyma cell width (µm)	27.50±2.13	18.11±1.10	23.0
12	Vessels per lakh fibres	102.50±3.54	154±1.41	-
13	Fines mass basis (%)	3.90±0.00	3.15±0.07	-
14	Number of kinks per mm	0.73±0.00	0.83±0.00	0.98
15	Mean kink angle (Degree)	47.72±0.16	48.11±0.18	29.93
16	Mean kink index	1.71±0.00	1.95±0.01	2.15
17	Number of fibers/mg	15000	11000	-

± refers standard deviation from the mean

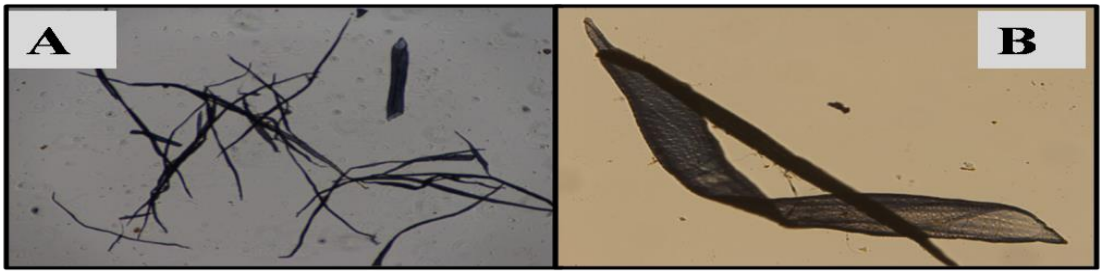
Table 3.2: Chemical characterization of *L. leucocephala* and *C. equisetifolia*

SI. No.	Particular	*L. leucocephala*	*C. equisetifolia*	E. *tereticornis* [1]	*T. orientalis* # [21]
1	Cold water solubility (%)	1.75±0.08	0.57±0.03	2.38	3.5
2	Hot water solubility (%)	3.70±0.18	2.1±0.10	4.31	5.1
3	1/10N NaOH solubility (%)	17.72±0.23	14.27±0.08	16.83	21.9
4	Alcohol-benzene solubility (%)	4.02±0.21	2.53±0.17	3.00	-
5	Holocellulose (%)	75.31±0.83	74.73±0.60	70.3	-
6	α-cellulose (%)	42.88±0.21	43.9±0.60	42.6	49.7
7	β-cellulose (%)	17.68±0.79	17.23±0.32	16.22	-
8	γ-cellulose (%)	14.59±0.52	13.3±0.49	11.48	-
9	Pentosans (%)	14.72±0.22	15.88±0.32	11.07	23.4
10	Total lignin (%)	21.65	23.05	28.5	22.9
11	Klason lignin (acid insoluble) (%)	20.83±0.16	22.41±0.41	27.9	-
12	Acid soluble lignin (%)	0.82±0.01	0.64±0.03	0.58	-
13	Ash (%)	1.20±0.06	0.87±0.03	0.66	1.0

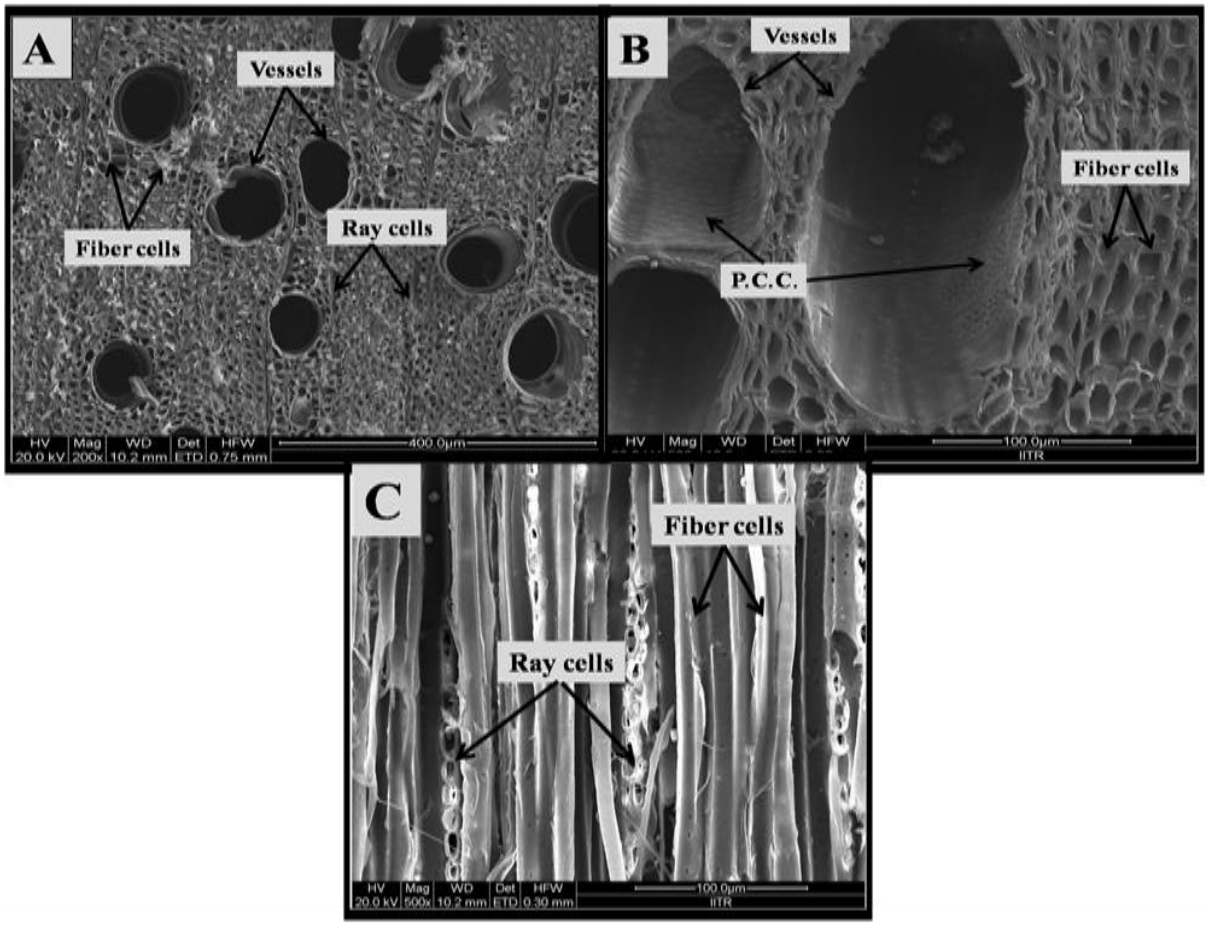
± refers standard deviation from the mean; # refers 3 years old plant



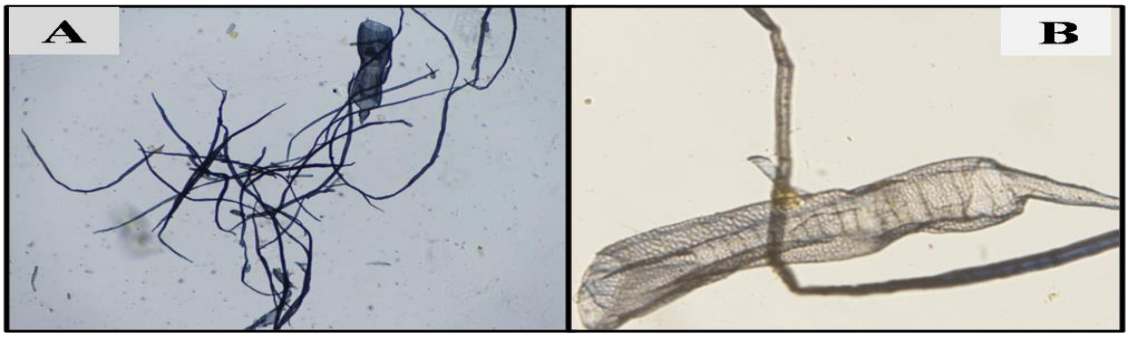
Photomicrograph 3.1: *L. leucocephala*: T.S. showing middle lamella (A) and vessels (B); magnified view of vessel showing cross-field pitting (P.C.C.) (C); tangential section showing on the right, vessel elements (v), perforation plate (pp) and wall pits (wp); rays from 4 to 26 cells high, uniseriate (r); fibers clearly septate (f) (D).



Photomicrograph 3.2: *L. leucocephala*: showing fibers, parenchyma cells and vessel (4X) (A); fiber and vessel showing bordered pits (10X) (B).



Photomicrograph 3.3: *C. equisetifolia*: T.S. showing fibers and vessels (A); magnified view of vessel showing cross-field pitting (P.C.C.) (B); tangential section showing uniseriate or biseriate rays, septate fibers with very small pits (C).



Photomicrograph 3.4: *C. equisetifolia*: showing fibers, parenchyma cells and vessel (4X) (A); fiber and vessel (10X) (B).

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ANNEXURE 3

ABBREVIATIONS

%	Percent
<	Less than
>	More than
°C	Degree celcius
µm	Micrometers
DP	Degree of polymerization
etc.	et cetera
H	Hours
MFA	Microfibrill angle
MFs	Microfibrills
Min	Minutes
Mm	Millimeters
SEM	Scanning electron microscopy
v/v	Volume per volume

CHAPTER 4

PREPARATION OF KRAFT PULPS OF *L. LEUCOCEPHALA* AND *C. EQUISETIFOLIA*

4.1. INTRODUCTION

Pulping refers to any mechanical or chemical process by which woody or non-woody biomass is reduced to a fibrous mass known as pulp. Pulp and paper are made from raw materials containing cellulosic fibers, generally wood, agricultural residues and recycled papers. The cellulosic raw materials contain cellulosic fibers together with lignin, a natural binder which cements the cellulosic fibers together in wood [38]. The pulping of wood or agricultural residues aims at dissolving the enough lignin from the middle lamella (lignin-rich) or separating the undamaged fiber cells from each other [8]. On contrary to this, recycled paper (waste paper) contains cellulose-cellulose, cellulose-hemicellulose, cellulose-starch and cellulose-wet end chemicals bonding, and other non-fibrous additives like wet strength resins to develop cross linking and surface sizing agents to reduce porosity of paper. Therefore, waste paper pulping aims at separating the cellulosic fibers by breaking these bonds, and diminishing the effect of wet strength resins and size chemicals. In this way, separation of cellulosic fibers in wood is entirely different from waste paper recycling.

Various pulping processes are available for converting woody raw materials into fibrous mass. These processes are broadly classified as mechanical, chemical and semi-chemical. Mechanical pulping (MP) involves the mechanical attrition of wood to separate the cellulosic fibers, and when mechanical pulp is produced with the use of refiners; the process is known as refiner mechanical pulping (RMP). The mechanical process has undergone extensive research in the past two decades. Most new installations include hybrid methods like thermo-mechanical pulping (TMP) and chemithermo-mechanical pulping (CTMP); these processes involve pre-softening of wood by means of thermal and/or chemical treatment. Mechanical pulping requires large amount of energy, and is used mainly for soft wood pulp and news print grade pulp.

The choice of pulping process is determined by the nature of the cellulosic raw materials to be pulped, the grade of the desired products (paper or paper products) and by economic considerations. Today, chemical pulping is the most commonly used process as it produces better quality of pulp than that of mechanical pulping, mainly with respect to paper strength [9]. The chemical pulping process can generally be classified into three types: acid

based (sulphite pulping), alkali based (soda and kraft pulping) and solvent based (organosolv pulping). These chemical pulping processes rely on different chemicals such as sulfurous acid (H_2SO_3) and bisulphate ions (HSO_3^-) in sulphite process, sodium hydroxide (NaOH) in soda process, sodium hydroxide (NaOH) and sodium sulphide (Na_2S) in kraft process, and organic solvents in organosolv process, are the principal pulping chemicals used to dissolve lignin. Sulphite pulping can be performed over a broad range of pH. Acid suphite pulping process is carried out with Ca^{+2}, Mg^{+2} and Na^+ at pH range of 1.0–2.0, and active reagents in liquor are SO_2*H_2O, H^+ and HSO_3^-. In case of bisulphite pulping process, delignification is carried out with Mg^{+2} and Na^+ at pH range of 3.0–5.0, and active reagents are HSO_3^- and H^+. Neutral sulphite semi-chemical (NSSC) pulping process is carried out with Na^+ and NH_4^+ at pH range of 5.0-7.0, and active reagents are HSO_3^- and SO_3^{-2}. Sulphite pulp is lighter in color and greater in yield than kraft pulp but the production of sulphite pulp is on smaller scale than kraft pulp due to some limitations of the sulphite process such as raw material sensitivity, lower strength properties of pulp, difficulty in chemical recovery and environmental problems.

Practically, the most widely used chemical process to transform wood into pulp is the kraft pulping process [33, 19] which is accounting for the production of two-third of the world's virgin pulp and for over 90% of chemical pulp [79]. The kraft process has the advantages over other pulping processes because of the ability of process to handle almost all commercially available woods, the favorable economics due to the high chemical recovery efficiency (about 97%) and the excellent properties, mainly high strength of the pulp which it produces [79, 52, 37]. The industrial application of kraft process was first developed by C.F. Dahl in 1879 [15, 76] and patented in 1884 [18]. The world's first kraft process for unbleached pulp was started up in Sweden in 1885 [33]. The kraft wood pulping employs white liquor (NaOH and Na_2S) to dissolve lignin in order to promote cellulose fibers separation from the wood matrix. It is also known as "sulphate pulping" because of the use of sodium sulphide in its pulping liquor.

Kraft pulping has been the dominant chemical pulping process for hardwood plants ever since, but it is also faced with some drawbacks related to the release of malodorous reduced sulphur compounds to the atmosphere and discharge of hazardous bleaching effluents. The focus of pulp and paper industry has turned to the advancement of kraft pulping due to elevated concerns on environmental friendly technologies. Therefore, new concepts of kraft pulping technology like extended delignification have been developed during the past 15 years.

Extended delignification can be performed by the processes like EMCC, Isothermal cooking and Lo-Solids cooking [45, 3, 60] in continuous digesters, and Rapid displacement heating (RDH) [2], Super-Batch [69] and ENERBATCH [83] in batch digesters. Extended delignification processes require high concentration of residual alkali with prolonged cooking which dissolves carbohydrates to a great extent resulting into the lower pulp yield. These, together with the high cost of pulp production, have refocused attention on the conventional kraft pulping.

Kraft pulping is a complex process which involves various chemical reactions between the cooking chemicals (mainly OH^- and HS^-) and components of wood. These reactions are degradation reactions which take place for degradation of lignin and extractives as well as carbohydrates, and condensation reactions. The prevalent degradation reactions include the reactions of hydroxide (OH^-) and hydrosulphide (HS^-) anions, present in the pulping liquor with the lignin and cause the polymer to fragment into smaller water or alkali-soluble fragments. The fragmentation of lignin macromolecule proceeds through the cleavage reactions of α-aryl ether and β-aryl ether bonds in phenolic units as well as cleavage of β-aryl ether bonds in non-phenolic units of lignin [28, 29]. These reactions are mainly alkali promoted and sulphidolytic cleavage reactions. As a result of these cleavage reactions, the free phenolic hydroxyl groups and glycolic groups are liberated from the lignin structure [30, 29, 27]. The free phenolic hydroxyl groups increase the hydrophilicity of lignin and lignin fragments [7]; consequently, the solubility of lignin in the cooking liquor increases. The rate of delignification slows down remarkably as the concentration of dissolved lignin increases. Alkali depletion in any delignification phase of the kraft process escorts to re-condensation of lignin polymer either with themselves or with carbohydrates, and further dissolution of lignin stops. Moreover, the condensed lignin is more difficult to remove from the fibers [52].

Basically, delignification during kraft pulping proceeds in three distinct phases: [82, 68, 7, 29] the initial, the bulk and the residual delignification phase. Initial delignification takes place in the impregnation stage in a very rapid manner up to a temperature of about 150 °C [29, 73], and dissolves only 20-25% of total lignin [82]. In this phase, only small lignin (low molecular weight) fragments are extracted from the S_2 cell wall. The delignification rate during this phase is controlled by diffusion [73, 68], and independent of the hydroxide and hydrosulphide ions concentrations [29]. The bulk phase of delignification starts when the cooking temperature is elevated from 150 °C and continues up to 170 °C, and 70–80% of all

lignin dissolves during this phase. This phase is known to dissolve the lignin fractions of higher molecular weight [27]. The rate of delignification in the bulk phase is controlled by the concentrations of hydroxyl and hydrosulphide ions in the cooking liquor [51, 82]. The residual phase of delignification starts when most of the lignin (about 90%) have been removed, and it marks the end of cooking process [16]. Substantial amount of carbohydrates also dissolve during residual phase due to poor selectivity of this phase [16]; despite the fact that delignification is the prime goal of pulping.

The carbohydrate degradation during kraft pulping can be divided into three different phases: deacetylation, end-wise degradation (peeling) and alkaline hydrolysis [74, 32]. In deacetylation reactions, the acetyl group in acetylated hemicelluloses i.e. the galactoglucomannan in softwoods and 4-O-methy-glucuronoxylan in hardwoods is saponified. In peeling reactions, wood carbohydrates start to degrade at about 100 °C [29] by end-initiated de-polymerization leading to yield loss. The peeling reactions stop by a stopping reaction which may be due to a rearrangement of the reducing end groups which makes them stable for peeling further, or because the reducing end groups have become physically inaccessible to the alkaline reagents. The alkaline hydrolysis starts at about 150 °C temperature during the bulk phase, and results primarily in reduced viscosity of the pulp [29]. The alkaline hydrolysis of glycosidic bonds degrades cellulose more severely due to the high DP of cellulose, and also creates new reducing end-groups which are subjected to peeling reactions again (secondary peeling) that further results into the low yield.

During the past few decades, a large number of digester additives have been used to improve pulping productivity [43, 58, 62, 63]. These additives can be categorized into two: anthraquinone (AQ)-based and surfactant-based additives. The use of AQ with the soda or the kraft process was studied by many researchers [85, 25, 42, 48, 17, 64]. AQ creates the two fold improvement during pulping; it preserves carbohydrates and simultaneously accelerates delignification [14, 57]. It preserves carbohydrates by reacting with their reducing end groups and stabilizing them against alkaline peeling. This leads to an increase in pulp yield. The alkali soluble, reduced AHQ is produced during this process (Figure 4.1). AHQ reacts with lignin to make it more reactive and converts back to AQ which can then reacts with carbohydrates again [84]. The AQ acts as a redox catalyst which transfers electrons from carbohydrates to lignin, and is typically used in small amounts like 0.05-0.2%, and results in an improvement in pulp yield without any adverse effect on bleachability or paper strength [13, 35]. AQ is non toxic,

alkali stable and cost effective to use, and has no adverse effects on environment [12]. The surfactant-based additives allow the meticulous wetting of the wood chip's surfaces by reducing the surface tension between the liquor and chip, and facilitate rapid penetration of liquor into the inner matrix of the chip [1]. This gives rise to reduced amount of AQ residuals in black liquor, reduced amount of rejects and reduction in kappa number with overall improvement in pulp quality [20, 21]. Duggirala examined the benefits of AQ and surfactants individually and in combinations in kraft softwood pulping, and reported that the AQ/surfactant program improved the delignification by maximizing the efficiency and effectiveness of cooking chemicals during different phases of kraft pulping [21].

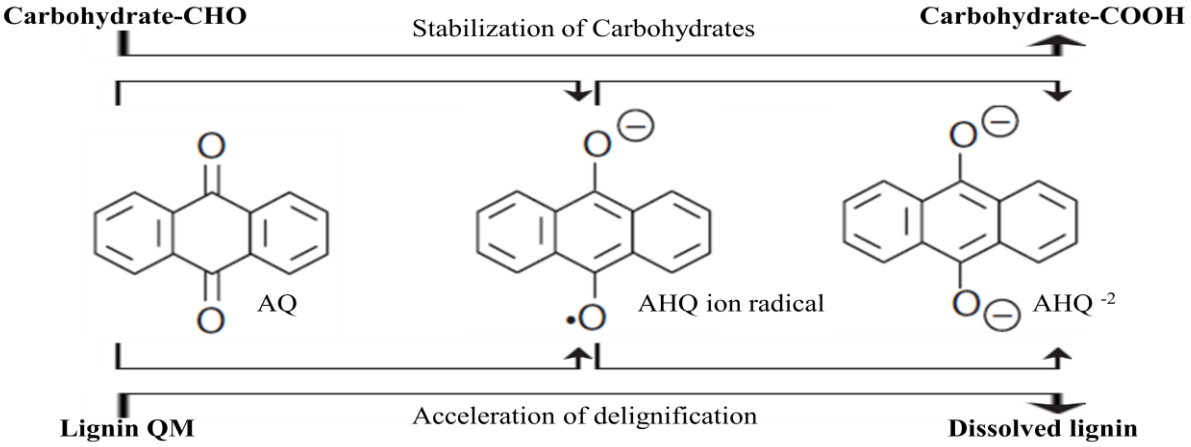


Figure 4.1: Proposed mechanism of redox cycle for AQ in alkaline pulping [4].

However, pulping is a complex heterogeneous process, involving the interaction effect of many variables. RSM has been used in many pulping processes to optimize the pulping conditions for various raw materials [86, 80, 31]. Therefore, keeping in view the above facts, the present study is done to determine the optimum cooking conditions leading to high screened pulp yield, low kappa number and high pulp viscosity, and to statistically investigate the influence of three pulping variables; alkali charge (active alkali as Na_2O), maximum cooking temperature and cooking time (at temperature), on kraft pulping of *L. leucocephala* and *C. equisetifolia*. In the present study, RSM based on CCD is adopted for evaluating the effective factors, building models, studying interaction between the variables and determining the optimum conditions of variables for the desirable responses. Statistical software Minitab-16 is used to generate CCD tables, facilitate the analysis, generate the contour plots and to optimize the above mentioned three parameters.

4.2. MATERIALS AND METHODS

4.2.1. Materials

Screened wood chips of *L. leucocephala* and *C. equisetifolia* were procured from ITC limited, Bhadrachalam, Andhra Pradesh, India. The chips were stored in separate polythene bags for attaining uniform moisture after mixing thoroughly, and were used as raw materials for preparation of kraft pulps. The chemicals were of laboratory grade and purchased from standard commercial manufacturers.

4.2.2. Methods

4.2.2.1. Kraft pulping process

The cellulose pulps from *L. leucocephala* and *C. equisetifolia* were obtained using a electrically heated rotary digester (0.02 m^3 capacity) having four bombs of 1 L capacity each. The wood chips and the white liquor were introduced into the bombs, and the bombs were sealed and placed into the digester for cooking at different conditions, as per requirement of the experiment. At the end of cooking, pressure was released quickly to obtain low pressure level, and the bombs were placed in water tank for cooling. Following cooling, the pulps were separated from the liquor by washing the pulps on a flat stationary screen (300 mesh size bottom). After washing, the total pulp yields were determined and the pulps were disintegrated for 3 min at 2500 rpm and then screened through vibratory flat screen of WEVERK (0.15 mm slot size) in order to isolate the uncooked materials (screening rejects). The screened pulps were then analyzed for screened pulp yield, kappa number (TAPPI T 236 cm-85) and viscosity (TAPPI T 230 om-04) [6].

4.2.2.2. Kraft pulping optimization methodology

The optimization of variables associated with kraft pulping process for *L. leucocephala* and *C. equisetifolia* was carried out into two major steps: (i) Preliminary experiments were carried out to study the effect of sulphidity, additive (AQ) and surfactant (Tween 20) (ii) Finally, response surface design was used to optimize the three operating variables; namely, alkali charge (active alkali as Na_2O), maximum temperature and cooking time (at temperature), and their effect was investigated on screened pulp yield, pulp kappa number and pulp viscosity. All experiments were carried out independently in triplicate, and the results were the average of three replicate experiments.

(i) Preliminary experiments

The screened wood chips of *L. leucocephala* and *C. equisetifolia* were digested at different sulphidity varying from 15 to 35%. The effect of different levels of sulphidity was studied on screened pulp yield, total pulp yield, screening rejects and pulp kappa number at different maximum temperatures varying from 160 to 180 °C. Similarly, the effect of AQ and Tween 20 varying from 0.0 to 0.2%, singly and in combinations, was also studied on screened pulp yield, screening rejects and pulp kappa number at optimum pulping conditions as mentioned in Table 4.2. The pulping variables, alkali charge and cooking time were held constant at 20% and 90 min, respectively, by taking into consideration the pulping conditions in previous research work [5]. The liquor to wood ratio and the weight of chips taken in each bomb were fixed at 3:1 and 100 g (based on O.D.), respectively, throughout the pulping process. The cooking schedule was fixed at ambient to 100 °C for 45 min and 100 °C to 170 °C for 45 min, all through the pulping experiments.

(ii) Response surface methodology

RSM was used to optimize the various variables associated with kraft pulping of *L. leucocephala* and *C. equisetifolia*, and a fully randomized factorial design (2^3 CCD) was adopted. Three operating parameters including, alkali charge (A), maximum temperature (T) and cooking time (t) were selected as the major independent variables and were tested at 5 different levels (Table 4.3). Table 4.4 and 4.5 represented the experimental designs of 20 runs each with the experimental results of this study for *L. leucocephala* and *C. equisetifolia*, respectively. The variables A, T and t were usually called 'natural variables' because they were expressed in their natural unit of measurement. Therefore, if A, T and t denoted the natural variables i.e. alkali charge, maximum temperature and cooking time, respectively, then the transformation of these natural variables to coded variables was calculated by equations 1, 2 and 3:

$$X_1 = (A\text{-}17\%)/3\% \quad (1)$$

$$X_2 = (T\text{-}170\ °C)/10\ °C \quad (2)$$

$$X_3 = (t\text{-}90\ min)/30\ min \quad (3)$$

X_1, X_2 and X_3 are the coded form of natural variables; A, T and t, respectively. The low and high levels of variables; X_1, X_2 and X_3 were denoted by "-1" and "+1". The position of 2^3 factorial points and the central points were represented by (±1, ±1, ±1) and (0, 0, 0),

respectively. The axial points were placed at (±1.68, 0, 0), (0, ±1.68, 0) and (0, 0, ±1.68). This axial distance corresponded to a rotatable design. The boundary limits of each variable were determined from literature reviews [4]. The experimental data were fitted with a quadratic/polynomial equation (second degree). The model form was as shown below:

$$Y = \beta_0 + \beta_1X_1 + \beta_2X_2 + \beta_3X_3 + \beta_{11}X_1^2 + \beta_{12}X_1X_2 + \beta_{22}X_2^2 + \beta_{13}X_1X_3 + \beta_{23}X_2X_3 + \beta_{33}X_3^2 \quad (4)$$

Where, Y is the predicted response; β_0 is constant; β_1, β_2 and β_3 are the linear coefficients; β_{11}, β_{22} and β_{33} are the quadratic coefficients; β_{12}, β_{13} and β_{23} are the two factor interaction coefficients; X_1, X_2 and X_3 are the coded form of the natural variables; A, T and t, respectively. The optimum values of the selected variables were obtained by solving the regression equation 4 [49]. ANOVA was used to determine the significance of each term in the equations fitted and to estimate the goodness of fit in each case.

4.2.2.3. Bauer-McNett fiber classification

The fiber fractionations of optimized kraft pulps of *L. leucocephala* and *C. equisetifolia* were carried out in Bauer-McNett fiber classifier (TAPPI T 233 cm-06) containing four different mesh screens i.e. 20, 48, 100 and 200 [6].

4.2.2.4. Pulp beating, hand sheets preparation and paper properties estimation

The unbleached pulps of *L. leucocephala* and *C. equisetifolia* were beaten to different beating levels in PFI mill (TAPPI T 200 sp-96). Laboratory hand sheets of 60 g/m^2 were prepared on a British hand sheet former (TAPPI T 221 cm-99), and the sheets were air dried and tested for tensile index (TAPPI 494 om-01), tear index (TAPPI T 414 om-98), burst index (TAPPI T 403 om-97) and double fold (TAPPI T 423 cm-98) [6].

4.2.2.5. SEM studies of kraft pulps

SEM of the optimized kraft pulps of *L. leucocephala* and *C. equisetifolia* was carried out using a FEI Quanta 200 F microscope. The process used to carry out SEM for the pulp samples was similar to the process which was used in chapter 3 section 3.2.2.1.

4.3. RESULTS AND DISCUSSION

4.3.1. Preliminary analysis of kraft pulping

4.3.1.1. Effect of sulphidity and maximum temperature

Table 4.1 represents the effect of different sulphidity levels (15-35%) at maximum temperature varying from 160 to 180 °C on pulp yields, reject contents and pulp kappa numbers of *L. leucocephala* and *C. equisetifolia*. The plots of sulphidity versus screened pulp yield indicate that screened pulp yield increases with increasing sulphidity levels up to 25% for *L. leucocephala* and 20% for *C. equisetifolia* at each temperature investigated, and beyond that the screened pulp yield does not show any significant increase (Figure 4.2). Also, the screened pulp yield at each sulphidity levels increases with increasing temperature from 160 to 170 °C, and beyond that there is found a sharp decrease in screened pulp yield.

The plots of total pulp yield versus sulphidity (Figure 4.3) indicate that total yield decreases continuously with an increase in sulphidity level from 15 to 35 at each temperature investigated but the decrease in pulp yield after 25% sulphidity for *L. leucocephala* and 20% for *C. equisetifolia* is more prominent. Furthermore, the decrease in total pulp yield at each sulphidity level improves up to maximum cooking temperature of 170 °C but it decreases more severely with further increasing the temperature. The plots of screening rejects versus sulphidity (Figure 4.4) at various maximum temperatures indicate that reject contents decrease continuously with an increase in sulphidity level from 15 to 35 but the decrease in screening rejects after 25% sulphidity for *L. leucocephala* and 20% for *C. equisetifolia* is not to a great extent.

The screening rejects decrease sharply with increasing temperature from 160 to 170 °C while beyond that temperature the magnitude of decrease in rejects is insignificant. Therefore, further elevation in temperature beyond 170 °C does not affect screening rejects significantly.
The graphs are also plotted between sulphidity and pulp kappa number (Figure 4.5) at different maximum temperatures. These curves can roughly be approximated by two straight lines at each temperature studied. The part of curves with steeper slope, up to a sulphidity level of 25% for *L. leucocephala* and 20% for *C. equisetifolia*, is related to rapid decrease in kappa number (bulk delignification), and the curves with gentler slope, beyond the sulphidity levels of aforementioned, indicate a slow decrease in kappa number (residual delignification). Hence, there is practically no gain (in terms of screened yield and kappa number) of using higher doses of sulphidity. It is clearly shown in Figure 4.5 that the curves plotted at 160 °C are fairly apart from the curves plotted at 170 and 180 °C which are very close to each other. This indicates that the kappa number decreases sharply in the temperature range of 160 to 170 °C and after that range; there is no profound reduction in kappa number.

From these results, it is evidenced that lower sulphidity promotes the total pulp yield and reduces the screened pulp yield; this may be due to the presence of higher amount of uncooked materials (rejects) and shives in the total pulp yield at cooking conditions of lower sulphidity. Similar results were found by Islam who reported the decrease in total pulp yield, rejects content and kappa number with increasing sulphidity while screened pulp yield was increased [40].

Also, it is indicated that screened pulp yield and total pulp yield drop sharply in the range of cooking temperature 170 to 180 °C. This may be related to the de-polymerization of the polysaccharide chains at the reducing end sites (peeling reactions) thereby decreasing the pulp yield and creating the new reducing ends which further subjected to degradation reactions (secondary peeling) [34]. The degradation of cellulose and hemicellulose also increases at higher temperature; thereby the pulp yield is reduced [50]. The kappa number is also higher at lower sulphidity which may be due to the slow rate of delignification at low concentration of HS^- ions. The rate of delignification is accelerated in presence of HS^- ions, and the high concentration of HS^- is essential for a selective delignification with limited attack on carbohydrates [65, 70]. Moreover, a low HS^- ions concentration may result in the formation of enol ether (alkali stable) structures in the early stages of conventional kraft pulping. This impairs delignification and results in the formation of more resistant residual lignin structures [78, 26, 30]. Furthermore, lignin-carbohydrate complexes (LCC) are formed during kraft pulping [28]. These complex structures are alkali stable and resistance to delignification. The hydrosulphide ions inhibit or reduce the formation of LCC linkages and facilitate lignin fragmentation via sulphidolytic cleavage of the β-aryl ether units. In addition, a high sulphidity prevents the coupling reactions of reactive intermediates (oxirane type) with carbohydrates, and thus reducing the yield loses further [55].

Three distinct phases of delignification can be observed in kraft pulping process: an initial, bulk and residual delignification phase. The bulk delignification takes place at about 150 to 170 °C and the main portion (about 70-80%) of lignin dissolves during this phase [56]. The residual delignification phase starts at 170 °C and leads to dissolution of roughly 10-15% of the lignin. The selectivity for lignin in this phase is poor, as evidenced from an increased degradation of carbohydrates relative to the rate of delignification. Substantial amount of carbohydrates also dissolves during residual phase due to poor selectivity of this phase [16]. The curves of kappa number at different sulphidity levels and 170 °C almost coincide to the

curves plotted at 180 °C, for both the raw materials. It indicates that bulk delignification is over up to 170 °C; hence, it is not economical in terms of energy and yield to continue the pulping process further.

Therefore, based on the results, a maximum cooking temperature of 170 °C and sulphidity level of 25% for *L. leucocephala* and 20% for *C. equisetifolia* are considered as optimum cooking conditions for the kraft pulping of the respective raw material. Cooking at low sulphidity and/or low temperature results into the lower screened pulp yield, higher pulp kappa number and higher content of rejects; while cooking at higher levels of sulphidity and temperature (exceeding the optimum levels), decreases the screened pulp yield but does not reduce the rejects and pulp kappa number significantly. These results are consistent with other studies in which 20% sulphidity and 165 °C temperature have been found optimum for kraft pulping of *I. carnea* and *C. sativa* [24]. Similar findings were reported in previous studies for kraft pulping of different plants [53, 23].

4.3.1.2. Effect of AQ and Tween 20

Table 4.2 reveals the effect of AQ and Tween 20 on screened pulp yield, reject contents and pulp kappa number during kraft pulping of *L. leucocephala* and *C. equisetifolia* at optimum pulping conditions. Different doses of AQ and Tween 20 (0.0 to 0.2%), based on oven dry of pulps, are used singly and in combinations. As per Figure 4.6, the screened pulp yield for both the raw materials increases with increasing AQ doses up to 0.1%, and beyond that the screened pulp yield starts to decrease slightly. On the other hand, the screening rejects and pulp kappa number decrease sharply with increasing AQ doses up to 0.1%, and beyond that the decrease in rejects content and pulp kappa number is found to be insignificant. AQ increases the screened pulp yield by 3.5% and mitigates the rejects and pulp kappa number by 1.7% and 3.4 units, respectively, at a dose of 0.1% as compared to control (without AQ), for *L. leucocephala*. For *C. equisetifolia*, the respective increase in screened pulp yield is of 3.8% along with an associated decrease of 3.4% in screening rejects and 3.9 units in kappa number in comparison to control.

AQ acts as a redox catalyst when added to pulping liquor. It oxidizes the reducing end groups of cellulose and hemicellulose in the pulp for protecting them against alkaline degradation or peeling reactions [77]; leading to an increase in pulp yield. Simultaneously, the reduced AHQ reacts with lignin and accelerates delignification [84]. In this way, the lignin becomes more water soluble, and is readily displaced from the pulp during washing. Singh and

co-workers observed an increase in pulp yield by 0.75% and reduction in pulp kappa number by 26.1% with the addition of 0.1% AQ during soda pulping of wheat straw [72]. Similar trend was observed by other researchers [47]. They observed that addition of AQ (0.1%) showed 0.2 and 0.4% improvement in pulp yield of sofia and lemon grasses with the respective reduction of 26 and 8% in kappa number during soda pulping.

In an another set of experiments, the screened pulp yield increases maximally by 1.6%, and screening rejects and pulp kappa number decrease by 1.4% and 2.8 units, respectively, with the addition of Tween 20 at a dose of 0.1% during kraft pulping of *L. leucocephala*. For *C. equisetifolia*, the respective increase in screened pulp yield is 2.1% along with an associated decrease of 2.5% in screening rejects and 2.9 units in kappa number, in comparison to control (without Tween 20). The addition of non-ionic, surfactant-based additives (like Tween 20) reduces the surface tension forces between the liquor and wood chip; therefore, they improve the wetting of wood-chip's surfaces [1, 21]. They also improve the dispersion of HS^- and OH^- ions of cooking liquor into the wood matrix during kraft pulping [81, 10]. Surfactants facilitate the penetration of cooking liquor by modifying the surface properties of solid (wood chips) and liquid (white liquor) phases. They improve the absorption of white liquor by removing the resins from wood ducts or by the combination of three different phases i.e. surface wetting, absorption and adsorption (TAPPI T558 pm-956). Blackstone observed a 5% decrease in screening rejects during kraft pulping of southern pine at the fixed kappa number with surfactant [11]. The addition of 0.5% sodium dodecyl benzene sulphonate improved the penetration of cooking liquor during soda pulping of wheat straw [54]. The addition of 0.1% surfactant (ethoxylated alcohol) improved total pulp yield by 0.5 to 0.8% at a fixed kappa number, and mitigated the screening rejects and enhanced the de-resination of wood chips during kraft pulping of different hardwood and softwood species like *Betula pendula*, *Eucalyptus globulus*, *Eucalyptus grandis*, Lodgepole pine, aspen and mountain pine beetle [22].

Finally, the combined effect of AQ and Tween 20 on screened pulp yield, rejects and pulp kappa number of *L. leucocephala* and *C. equisetifolia*, is investigated. The addition of AQ at a dose of 0.05% in combination with 0.05% Tween 20 increases the screened pulp yield by 4.3% and reduces the rejects and pulp kappa number by 1.8% and 4.2 units, respectively, for *L. leucocephala*. For *C. equisetifolia* kraft pulping, the optimum dose of AQ and Tween 20 combination is 0.05 and 0.1%, and the respective increase in screened pulp yield is 4.9% along

with an associated decrease in screening rejects and pulp kappa number by 3.7% and 5.1 units as compared to control. The results show that AQ/surfactant combinations improve the screened pulp yield and reduce the pulp kappa number better as compared to each individual treatment. This can be explained based on the fact that AQ/surfactant program accelerates selective delignification, preserves carbohydrates and facilitates faster penetration of cooking liquor [59]. These results are similar with other studies conducted by Duggirala [20]. They found 2.7 point increment in pulp yield with the addition of 0.05% surfactant in combination with 0.05% AQ.

4.3.2. Analysis of RSM

For RSM analysis, two sets of 20 pulping experiments are carried out, and their response values with different combinations of three variables are demonstrated in Tables 4.4 and 4.5 for *L. leucocephala* and *C. equisetifolia*, respectively. The experimental data are analyzed through the least squares method of multiple regressions and the following models are obtained by substituting the experimental data into equation 4:

$$Y_{SCY} = 53.65 + 1.20*X_1 + 0.13*X_2 - 1.05*X_3 - 3.27*X_1^2 - 2.44*X_2^2 - 4.88*X_3^2 + 0.84*X_1*X_2 - 0.89*X_1*X_3 + 2.39*X_2*X_3 \quad (5)$$

$$Y_{KAP} = 17.38 - 6.70*X_1 - 6.12*X_2 - 3.21*X_3 + 3.27*X_1^2 + 3.37*X_2^2 + 3.09*X_3^2 - 1.65*X_1*X_2 + 1.50*X_1*X_3 - 1.30*X_2*X_3 \quad (6)$$

$$Y_{VIS} = 969.82 - 23.37*X_1 - 21.56*X_2 + 50.77*X_3 - 81.66*X_1^2 - 114.07*X_2^2 - 47.32*X_3^2 + 25.61*X_1*X_2 + 42.01*X_1*X_3 + 63.24*X_2*X_3 \quad (7)$$

$$Y^*_{SCY} = 50.81 + 1.48*X_1 - 0.26*X_2 - 1.10*X_3 - 2.98*X_1^2 - 2.40*X_2^2 - 4.04*X_3^2 + 1.03*X_1*X_2 - 1.70*X_1*X_3 + 1.10*X_2*X_3 \quad (8)$$

$$Y^*_{KAP} = 19.03 - 7.18*X_1 - 6.76*X_2 - 4.22*X_3 + 2.05*X_1^2 + 3.45*X_2^2 + 2.40*X_3^2 - 1.41*X_1*X_2 + 2.29*X_1*X_3 - 0.09*X_2*X_3 \quad (9)$$

$$Y^*_{VIS} = 993.62 + 14.61*X_1 - 15.61*X_2 - 21.38*X_3 - 52.85*X_1^2 - 101.14*X_2^2 - 29.53*X_3^2 + 32.95*X_1*X_2 - 10.15*X_1*X_3 + 27.32*X_2*X_3 \quad (10)$$

Y_{SCY}, Y_{KAP} and Y_{VIS} denote the response variables; screened pulp yield, pulp kappa number and pulp viscosity, respectively, for *L. leucocephala* and Y^*_{SCY}, Y^*_{KAP} and Y^*_{VIS} denote the screened pulp yield, pulp kappa number and pulp viscosity, respectively, for *C. equisetifolia*.

Table 4.6 represents the regression analysis of the above six model equations. The regression analysis for all the polynomial models confirms the adequacy of the fitted models. The significance of each coefficient (main effects, quadratic and interaction between factors) of the independent variables is determined by t-test and p-value. Larger the magnitude of t-test and smaller the p-value, the coefficient is considered as more significant [49]. The goodness of fit of all six models is examined by R^2. The all six values of R^2 for equations 5, 6, 7, 8, 9 and 10 are found to be near to 1 which indicates a close agreement between the experimental results and the theoretical values predicted by the model equations. The values of the R^2_{adj} for all the six models are also high enough indicating the significance of the models. The values of predicted R^2 are in reasonable agreement with the values of R^2_{adj} indicating a good adjustment between the experimental and predicted values of the responses. Table 4.7 represents ANOVA results for model equations 5, 6, 7, 8, 9 and 10. ANOVA evaluates the significance of model terms, and all models are statistically significant at the 0.95 ($p<0.05$) confidence level. It is indicated from the F-value that all the terms are statistically significant. Moreover, the p-values of lack of fit for all the models are more than 0.05 (nonsignificant) which further indicates that all the six models are significant. Thus, it can be concluded that the regression models obtained may be used for analyzing the trends of response variables. Overall, the models are significant and able to predict the response variables. The interaction effect of variables on pulping process is studied by plotting contour plots against any two independent variables, while keeping other variable at its central (0) level.

4.3.2.1. Screening rejects

The uncooked material, retaining on the screen after screening, is known as rejects. Since, the screened pulp used for paper making process is free from rejects; the reject contents are not considered for statistical analysis.

4.3.2.2. Effect of alkali level and maximum temperature on screened pulp yield, pulp kappa number and pulp viscosity

The effect of variations in alkali charge and maximum temperature is studied with respect to three response variables; screened pulp yield, pulp kappa number and pulp viscosity

for *L. leucocephala* and *C. equisetifolia* while keeping the third variable, cooking time, at its central level 0 (90 min). Figures 4.7A and B show that there are many combinations of alkali charge and maximum temperature that produce the desired response (average screened yield of >50%). The screened pulp yield of *L. leucocephala* reaches a maximum (53.7%) at about alkali level of 0.04 (17.1%) and temperature level of -0.01 (169.9 °C), while the screened pulp yield of *C. equisetifolia* reaches a maximum (50.9%) at about alkali level of 0.04 (17.1%) and temperature level of -0.13 (168.7 °C). Starting from the above mentioned maximum points, the screened pulp yield of respective raw material is decreased by stepping out in any direction. As shown by the graphs, the screened pulp yield is found optimum in the vicinity of central levels of both the variables; alkali charge and maximum temperature. These is most likely due to the poor delignification and high reject contents which lead to a reduction in screened pulp yield at low temperature and alkali charge. On the contrary, higher temperature and OH^- concentration (alkali charge) may cause alkaline hydrolysis (de-polymerization) of the carbohydrate chains by peeling reactions; as a result, the screened pulp yield is decreased [51, 34]. These results are consistent with other study by Wanrosli and co-workers [80]. They reported a decrease in screened pulp yield in combination of very high and very low levels of alkali or temperature.

The contour plots for pulp kappa number indicate towards the falling ridge of pulp kappa number with respect to alkali charge and maximum temperature (Figures 4.8A and B). These plots also indicate that at higher temperature, with increasing the alkali charge, the kappa number decreases sharply while at lower temperature, the decrease in kappa number is not to a great extent. At a fixed central level of alkali 0 (17%), the kappa number drops faster from 37 to 17 (for *L. leucocephala*) and from 40 to 19 (for *C. equisetifolia*), with the increase in temperature from level -1.68 (153.2 °C) to 0 (170 °C). It means cooking temperature plays an important role between these two above mentioned levels. The solubilization of lignin in this region may be related to bulk delignification phase of pulping. Many researchers described that bulk delignification took place in the temperature range of 150 to 170 °C [16]. On the other hand, in the region >170 °C and at a fixed central level of alkali (17%), only slight decrease in kappa number takes place indicating the slow solubilization of residual lignin (residual delignification). The residual lignin is resistant to delignification because it is chemically linked to carbohydrates [41]. Moreover, at the high levels of alkali (>17%) and temperature (>170 °C), the contour lines tend to be almost parallel with the respective axis indicating that the solubilization of lignin from the wood chips becomes slow. It also indicates that the bulk

delignification is over upto the above mentioned transition points and it is not economical to continue the pulping process beyond these optimum levels of alkali (17%) and temperature (170 °C).

The contour plots for pulp viscosity depict that there are many combinations of active alkali and temperature in the experimental range that produce the average pulp viscosity of >900 (Figures 4.9A and B). The pulp viscosity reaches a maximum at about central level of all of the three variables (active alkali, maximum temperature and cooking time) as also shown in Tables 4.4 and 4.5. With increasing the temperature from its central level, the pulp viscosity starts to decrease which corresponds to the de-polymerization of cellulose. Similarly, the pulping at higher levels of alkali yields into lower pulp viscosity which may be due to cellulose chain scission reactions caused by alkaline hydrolysis. The decrease in cellulose viscosity is mainly dependent on the alkaline hydrolysis of the glycosidic linkages which is a reaction influenced by the OH^- concentration. Also, the xylan dissolution is down rapidly to a certain level, depending on the OH^- concentration in the cooking liquor.

4.3.2.3. Effect of alkali level and cooking time on screened pulp yield, pulp kappa number and pulp viscosity

The effect of variations in alkali charge and cooking time is studied with respect to three response variables; screened pulp yield, pulp kappa number and pulp viscosity for *L. leucocephala* and *C. equisetifolia* while keeping the maximum temperature at its central level 0 (170 °C). Figures 4.10A and B show that the screened pulp yield of *L. leucocephala* reaches a maximum (53.7%) in a combination of alkali level of 0.03 (17.1%) and cooking time level of -0.15 (85.5 min) while the screened pulp yield of *C. equisetifolia* reaches a maximum (50.9%) at about alkali level of 0.01 (17.03%) and cooking time level of -0.15 (85.5 min). These conditions may be considered as better conditions of pulping for respective raw materials. The pulping with low alkali dose (below 17%) and especially for shorter times, results into a very low screened pulp yield which may be due to the poor delignification of raw materials with high content of rejects. The high alkali dose with prolonged cooking, also results into very low screened pulp yield which may indicating towards the direct attack of pulping chemicals on cellulose. The decrease in screened pulp yield due to prolonged cooking had been discussed by many researchers [61, 44, 46, 39].

The contour plots of pulp kappa number (Figures 4.11A and B) represent that there are many combinations of alkali charge and cooking time close to their central levels that produce

the desirable value of response (kappa number <20). The pulp kappa number reduces sharply with increasing the alkali charge and cooking time from lower to central level, and may be corresponded to bulk delignification. Further increasing the dose of alkali beyond the central level, with or without prolonged cooking, reduces the pulp kappa number slightly which may corresponded to the residual delignification phase of pulping. Moreover, the contour lines are spaced more widely in the region of higher alkali and higher cooking time indicating the slight decrease in pulp kappa number with a slower rate.

It is indicated from Figures 4.12A and B that the increase in alkali charge beyond the central level, decreases the pulp viscosity sharply while increase in cooking time does not affect pulp viscosity significantly in that region. Hence, alkali charge is more important parameter for affecting the pulp viscosity during kraft pulping process. It is also clear from the graphs that the optimum pulp viscosity occurs when alkali charge and cooking time are at intermediate levels.

4.3.2.4. Effect of maximum temperature and cooking time on screened pulp yield, pulp kappa number and pulp viscosity

The effect of variations in maximum temperature and cooking time is studied with respect to screened pulp yield, pulp kappa number and pulp viscosity for *L. leucocephala* and *C. equisetifolia* while keeping the third variable, alkali charge, at its central level 0 (17%). In Figures 4.13A and B, the screened pulp yield follows the similar trend as in Figures 4.10A and B, which show the decrease in screened pulp yield at very high and very low levels of alkali charge and cooking time. The optimum values of screened pulp yield for both the raw materials are obtained in the close vicinity of the central levels of both the variables; maximum temperature and cooking time. Moreover, at the time levels of >0.24 (>97.2 min) for *L. leucocephala* and >0.5 (>105 min) for *C. equisetifolia*, the contour lines are almost parallel to the temperature axis, indicating that variation in temperature at a fixed time essentially does not affect screened pulp yield in that region.

The contour plots of pulp kappa number (Figures 4.14A and B) indicate that both variables; maximum temperature and cooking time are equally important in promoting delignification. In the lower left region of the contour plots (low temperature and low cooking time), the pulp kappa number is reduced sharply with increasing the either of these two variables. However, in the higher levels of cooking time, the contour lines are approximately parallel to the time axis indicating that variation in time at a fixed temperature does not affect

kappa number dramatically. Similar to Figures 4.11A and B, the pulp kappa number is found optimum (<20) close to central levels of both the variables for *L. leucocephala* and *C. equisetifolia*.

It is clearly indicated in Figures 4.15A and B that temperature is more effective in affecting pulp viscosity than cooking time as at very low <-1 (<160 °C) and very high >0.5 (>175 °C) levels of temperature, contour lines are approximately parallel to the time axis, thus variation in time at a fixed temperature does not affect pulp viscosity very much in this region. The contours indicate that viscosity of pulps initially increases with increasing temperature, and then follows a decline trend. In this case, the high temperature may cause degradation of cellulose resulting into the low viscosity of pulps obtained. Like the above results, the optimum pulp viscosity occurs when both the variables are at medium levels. It is evidenced from the results that the optimum values for the response variables i.e. screened pulp yield, pulp kappa number and pulp viscosity are obtained in the close vicinity of the central levels of all three independent variables; alkali charge (17%), maximum temperature (170 °C) and cooking time (90 min). This corresponds to the better conditions for delignification and the dissolution of polysaccharides is not excessive. It is therefore advisable to operate under intermediate operating conditions in order to obtain higher pulp yield, low pulp kappa number and higher pulp viscosity. These results are in close agreement with another study carried out by Lal and co-workers [53]. They reported 16% alkali charge, 165 °C temperature, 90 min time and 20% sulphidity as the optimum conditions for the kraft pulping of *A. Cadamba*.

4.3.2.5. Some optimum pulping conditions for *L. leucocephala* and *C. equisetifolia*

Table 4.8 shows the goals and selected range of values of responses that are used in the response optimizer module to obtain the optimum pulping conditions. Table 4.9 illustrates some of the optimum pulping conditions for independent variables; alkali charge, maximum temperature and cooking time during kraft pulping process of *L. leucocephala* and *C. equisetifolia*. Minitab calculates the predicted values of their associated responses i.e. screened pulp yield, pulp kappa number and pulp viscosity. The experiments are performed in triplicate under the best predicted conditions (in bold) and the experimental results agree quite closely with the predicted values, proving the validity of the models (Table 4.10). Hence, the predicted values appear to be reliable and verify the estimation models.

4.3.3. Fiber classification studies of *L. leucocephala* and *C. equisetifolia* kraft pulps

Table 4.11 represents the weighted fiber length distribution of optimized kraft pulps of *L. leucocephala* and *C. equisetifolia* at initial 15 °SR. The Bauer-McNett fiber classifier separates the fibers according to fiber length along with it separates the fractions of parenchyma cells. The top fractions (+20 fractions) of the *L. leucocephala* kraft pulp (16%) are found more than double value of *C. equisetifolia* (7.5%) indicating that the kraft pulp of *L. leucocephala* is stronger than that of *C. equisetifolia*. Further, the middle portion (+48 fractions) of the *L. leucocephala* kraft pulp is about 9.8% less compared to *C. equisetifolia* pulp. These fractions of pulp mainly contain medium sized fibers. The +100 fractions constitute 18% for *L. leucocephala* and 20% for *C. equisetifolia*, and are made up of short fibers, parenchyma cells and vessel elements. On the other hand, +200 fractions contain mainly shortened parenchyma cells and other broken cellular elements and it is 2.4% more in case of *L. leucocephala* than that of *C. equisetifolia*. This indicates that *L. leucocephala* may cause more fluff generation during paper making as compared to *C. equisetifolia*. Fluff are short fibers, ray cells, broken vessels, parenchyma cells and shives etc. –200 fractions which are washed away contain mainly cell debris, shortened fibers and broken vessel elements. The weight loss in these fractions is about 0.96% for *L. leucocephala* and 0.11% for *C. equisetifolia*. If we keep all other paper making variables constant for both the raw materials, we can say that *L. leucocephala* may cause more fluff generation as it has little more +200 and -200 fractions as compared to *C. equisetifolia*.

4.3.4. Mechanical strength properties of *L. leucocephala* and *C. equisetifolia* kraft pulps

Table 4.12 shows the mechanical strength properties of optimized kraft pulps of *L. leucocephala* and *C. equisetifolia,* at different beating levels varying from 15 to 50 °SR. All mechanical strength properties show improvement with respect to beating level upto 40 °SR for *L. leucocephala* and upto 45 °SR for *C. equisetifolia*, except tear index (Figure 4.16). The tear index increases with increasing beating level upto 30 °SR, and then declines. The °SR of pulp fibers shows the bonding ability and increases gradually with pulp beating/refining. It creates hydrogen bonds between fibers due to increased specific surface area and volume as a result of gradual beating [36, 66]. As a result of beating process, the primary wall and S_1 layer start to loosen up and ultimately broken down into layers. This creates a larger surface area for bonding. Also, the S_2 layer is opened up and fibrillated due to further increase in beating level

(°SR) [75]. Moreover, beating produces many tiny fibers which can fill in between the gap among the long fibers and increase the hydrogen bonding.

Therefore, strength properties like tensile and burst indexes and also double fold number governed by hydrogen bonding will be improved by increasing the °SR. On the other hand, average fiber length and coarseness decrease continuously whereas fiber diameter first increases and then decreases with increasing beating level (°SR). Hence, the tensile index, burst index and double fold number start to decrease due to fiber cutting after a beating level of 40 °SR for *L. leucocephala* and 45 °SR for *C. equisetifolia*. The tear index depends on many fiber properties such as fiber length, strength and coarseness [71]. In a weakly bonded sheet (lower °SR), the fibers are comparatively longer and coarser. In this case, the tearing resistance is controlled more by the number of bonds that break along the length of fibers. The single-fiber strength is more at low beating level but only a little friction is present among the fibers, so more fibers are pulled out than break in the tear zone. Therefore, tearing resistance depends strongly on the fiber length. In a well bonded sheet (higher °SR), the friction among the fibers increases while the coarseness of fibers reduces, since more fibers break than pull out in tear zone; thus this increases the tear index [71]. However, when coarseness of fiber is very low due to increasing °SR, the single-fiber strength is also reduced. This leads to a decrease in tear index.

4.3.5. SEM studies of *L. leucocephala* and *C. equisetifolia* kraft pulps

Photomicrographs 4.1A and C show the unbeaten fibers with large vessels in kraft pulps of *L. leucocephala* and *C. equisetifolia*, respectively, under optimized pulping conditions. The unbeaten fibers do not show fibrillation and the vessel cells have large surface area. Vessel cells are one of the mechanisms by which liquids flow through the tree in hardwoods. Their presence in paper poses the vessel's picking problem which is greatly influenced by vessel width, length and number per unit area. The vessel picking problem is a phenomenon that some of the hardwood vessel elements in the paper surface tend to be picked off by an ink-tackiness of the printing press [67]. It is clearly shown in Photomicrographs 4.1B and D that fibers exhibit fibrillation after beating, and vessel cells are entangled with beaten fibers of *L. leucocephala* and *C. equisetifolia*. Well bonded reinforcing fiber gives Z-direction strength and helps holding the vessels in place. Better bonding reduces vessel picking.

Table 4.1: Effect of maximum temperature and sulphidity on pulp yields, screening rejects and pulp kappa numbers of *L. leucocephala* and *C. equisetifolia*

Maximum temperature (°C)	Sulphidity (%)	Screened pulp yield (%)	Total pulp yield (%)	Screening rejects (%)	Pulp kappa number
L. leucocephala					
160	15	38.6±0.8	50.7±1.5	12.1±0.84	39.9±2.0
	20	40.8±1.2	50.3±2.1	9.5±0.68	33.7±1.7
	25	42.3±1.6	49.8±1.9	7.5±0.40	28.6±1.7
	30	42.5±1.7	49.1±1.9	6.6±0.25	28.2±1.6
	35	42.4±1.4	47.7±1.4	5.3±0.35	28.0±1.2
170	15	42.8±1.8	48.2±1.6	5.4±0.31	30.1±1.9
	20	44.2±1.2	47.9±1.5	3.7±0.21	25.0±1.7
	25	45.6±1.5	47.6±1.7	2.0±0.15	22.0±1.6
	30	45.5±1.9	47.0±1.9	1.5±0.06	21.8±1.2
	35	45.3±1.6	46.2±1.6	0.9±0.01	21.8±1.1
180	15	40.5±1.3	45.1±1.5	4.6±0.15	28.5±1.5
	20	42.1±1.8	44.6±2.0	2.5±0.11	23.7±1.1
	25	43.4±1.4	44.2±1.4	0.8±0.06	20.6±0.8
	30	43.3±1.2	43.9±1.2	0.6±0.02	20.6±0.7
	35	43.1±1.8	43.5±1.8	0.4±0.01	20.4±0.7
C. equisetifolia					
160	15	40.0±1.3	50.1±3.0	10.1±0.31	44.6±2.1
	20	42.1±1.5	49.6±2.2	7.5±0.33	37.6±1.6
	25	42.2±1.3	49.0±2.1	6.8±0.26	37.1±1.5
	30	42.1±1.5	48.2±1.2	6.1±0.31	37.0±1.2
	35	41.9±1.9	46.9±1.6	5.0±0.21	36.8±1.3
170	15	42.9±1.3	48.8±2.0	5.9±0.14	33.3±1.3
	20	44.5±1.6	48.2±1.5	3.7±0.15	26.0±1.1
	25	44.4±1.7	47.2±1.4	2.8±0.10	25.9±1.1
	30	44.2±1.5	46.0±1.3	1.8±0.10	25.9±1.0
	35	43.8±1.3	45.0±1.3	1.2±0.03	25.8±1.1
180	15	41.1±1.8	46.1±1.9	5.0±0.36	31.6±1.6
	20	42.6±1.6	45.4±1.7	2.8±0.11	24.9±1.0
	25	42.6±1.5	44.3±1.8	1.7±0.07	24.8±1.1
	30	42.4±1.2	43.4±1.3	1.0±0.02	24.7±1.2
	35	42.2±1.5	42.8±1.4	0.6±0.01	24.7±1.1

± refers standard deviation

Cooking conditions: liquor to wood ratio = 3:1, active alkali = 20% (as Na_2O), time from room temperature to 105±2 °C = 45 min, time from 105 °C to maximum temperature = 45 min, time at maximum temperature = 90 min.

Table 4.2: Effect of AQ and Tween 20 on screened pulp yields, screening rejects and pulp kappa numbers of *L. leucocephala* and *C. equisetifolia*

AQ (%)	Tween 20 (%)	*L. leucocephala*			*C. equisetifolia*		
		Screened pulp yield (%)	Screening rejects (%)	Pulp kappa number	Screened pulp yield (%)	Screening rejects (%)	Pulp kappa number
0.0	0.0	45.9±1.1	1.9±0.04	22.3±0.7	44.3±1.4	3.8±0.1	25.5±1.1
0.05	0.0	48.8±1.3	1.1±0.02	20.3±0.8	46.8±1.5	2.0±0.1	23.2±1.2
0.1	0.0	49.4±1.1	0.2±0.006	18.9±0.6	48.1±1.4	0.4±0.01	21.6±1.2
0.2	0.0	48.8±1.0	0.15±.005	18.7±0.6	47.9±1.7	0.3±.01	21.5±1.0
0.0	0.05	47.4±1.4	1.4±0.05	20.8±0.9	45.7±1.6	2.7±0.1	23.8±1.1
0.0	0.1	47.5±1.4	0.5±0.006	19.5±0.6	46.4±1.5	1.3±0.07	22.6±1.0
0.0	0.2	47.3±1.3	0.3±0.005	19.4±0.7	46.1±1.6	1.0±0.05	22.4±0.8
0.05	0.05	50.2±1.7	0.09±0.007	18.1±0.8	48.2±1.5	0.3±0.01	21.0±0.8
0.05	0.1	50.2±1.7	0.09±0.004	18.1±0.7	49.2±1.2	0.1±0.006	20.4±0.6
0.1	0.05	50.0±1.6	0.08±0.003	17.9±0.6	48.4±1.7	0.1±0.004	20.3±0.5
0.1	0.1	49.7±1.5	0.08±0.002	17.9±0.5	48.2±1.6	0.09±0.00	20.2±0.7

± refers standard deviation

Cooking conditions: liquor to wood ratio = 3:1, active alkali = 20% (as Na_2O), sulphidity = 25% (for *L. leucocephala*) and 20% (for *C. equisetifolia*), maximum temperature = 170±2 °C, time from room temperature to 105±2 °C = 45 min, time from 105 to 170 °C = 45 min, time at 170 °C = 90 min.

Table 4.3: Variables and their levels of CCD for *L. leucocephala* and *C. equisetifolia*

Independent variable	Coded level of variable				
	-α	**-1**	**0**	**+1**	**+α**
Active alkali charge, A (%)	11.96	14	17	20	22.04
Maximum temperature, T (°C)	153.2	160	170	180	186.8
Time, t (min)	39.6	60	90	120	140.4

Table 4.4: CCD and the corresponding responses for kraft pulping of *L. leucocephala*

SI. No.	Pulping variable						Response variable			
	Coded			Real						
	X_1	X_2	X_3	A (%)	T (°C)	t (min)	Screen pulp yield (%)	Rejects (%)	Pulp kappa number	Pulp viscosity (cm^3/g)
1	1	1	1	20	180.0	120.0	46.5	0.10	9.6	868.2
2	-1	-1	1	14	160.0	120.0	40.2	8.60	34.2	762.2
3	0	0	-1.68	17	170.0	39.6	42.6	7.20	30.8	792.8
4	1	-1	-1	20	160.0	60.0	48.0	8.50	28.2	642.8
5	0	0	0	17	170.0	90.0	52.6	0.60	18.5	964.2
6	0	0	0	17	170.0	90.0	54.1	0.10	17.1	994.1
7	1	-1	1	20	160.0	120.0	38.9	3.90	28.8	712.5
8	0	-1.68	0	17	153.2	90.0	46.8	8.60	37.1	702.4
9	0	1.68	0	17	186.8	90.0	47.4	3.10	15.5	615.4
10	1	1	-1	20	180.0	60.0	44.1	2.50	17.2	515.5
11	0	0	0	17	170.0	90.0	53.0	0.40	16.4	970.8
12	0	0	0	17	170.0	90.0	54.5	0.20	17.0	985.4
13	-1	-1	-1	14	160.0	60.0	43.8	10.00	42.6	830.5
14	0	0	1.68	17	170.0	140.4	37.8	2.40	20.2	902.6
15	1.68	0	0	22	170.0	90.0	45.9	1.30	14.5	735.9
16	-1	1	1	14	180.0	120.0	42.5	8.30	24.6	785.4
17	0	0	0	17	170.0	90.0	53.6	0.30	18.0	945.5
18	-1	1	-1	14	180.0	60.0	38.5	8.20	35.2	630.8
19	0	0	0	17	170.0	90.0	54.0	0.28	17.5	954.9
20	-1.68	0	0	12	170.0	90.0	43.6	11.40	37.5	765.2

Cooking conditions: liquor to wood ratio = 3:1, sulphidity = 25%, AQ = 0.05%, Tween 20 = 0.05%, time from room temperature to 105±2 °C = 45 min, time from 105 °C to maximum temperature = 45 min.

Table 4.5: CCD and the corresponding responses for kraft pulping of *C. equisetifolia*

SI. No.	Pulping variable						Response variable			
	Coded			Real						
	X_1	X_2	X_3	A (%)	T (°C)	t (min)	Screen pulp yield (%)	Rejects (%)	Pulp kappa number	Pulp viscosity (cm^3/g)
1	1	1	1	20.0	180.0	120.0	41.7	1.10	10.8	835.5
2	-1	-1	1	14.0	160.0	120.0	41.2	3.31	35.2	795.8
3	0	0	-1.68	17.0	170.0	39.6	40.5	1.80	34.1	945.5
4	1	-1	-1	20.0	160.0	60.0	46.8	2.80	29.5	870.4
5	0	0	0	17.0	170.0	90.0	52.2	0.10	17.9	1002.4
6	0	0	0	17.0	170.0	90.0	51.1	0.20	20.4	985.6
7	1	-1	1	20.0	160.0	120.0	38.7	1.60	25.4	745.5
8	0	-1.68	0	17.0	153.2	90.0	43.2	3.50	40.1	738.4
9	0	1.68	0	17.0	186.8	90.0	44.1	1.10	16.8	688.5
10	1	1	-1	20.0	180.0	60.0	45.4	0.52	11.9	847.6
11	0	0	0	17.0	170.0	90.0	49.8	0.09	18.2	978.6
12	0	0	0	17.0	170.0	90.0	52.5	0.08	18.0	995.5
13	-1	-1	-1	14.0	160.0	60.0	42.5	4.10	45.1	876.6
14	0	0	1.68	17.0	170.0	140.4	37.5	1.60	16.9	886.5
15	1.68	0	0	22.1	170.0	90.0	44.5	0.91	13.8	864.6
16	-1	1	1	14.0	180.0	120.0	40.1	2.50	22.9	750.5
17	0	0	0	17.0	170.0	90.0	48.8	0.15	20.1	987.6
18	-1	1	-1	14.0	180.0	60.0	37.0	1.50	36.5	725.5
19	0	0	0	17.0	170.0	90.0	50.6	0.18	19.7	1010.0
20	-1.68	0	0	12.0	170.0	90.0	39.5	3.40	35.2	835.5

Cooking conditions: liquor to wood ratio = 3:1, sulphidity = 20%, AQ = 0.05%, Tween 20 = 0.1%, time from room temperature to 105±2 °C = 45 min, time from 105 °C to maximum temperature = 45 min.

Table 4.6: Regression analysis for screened pulp yield, pulp kappa number and pulp viscosity of *L. leucocephala* and *C. equisetifolia*

Parameter	Screened pulp yield			Pulp kappa number			Pulp viscosity		
	CE	t-ratio	p-value	CE	t-ratio	p-value	CE	t-ratio	p-value
L. leucocephala									
Constant	53.65			17.38			969.82		
X_1	1.20	4.51	0.001	-6.70	-22.31	0.000	-23.37	-2.89	0.016
X_2	0.13	0.47	0.648	-6.12	-20.37	0.000	-21.56	-2.67	0.024
X_3	-1.05	-3.96	0.003	-3.21	-10.69	0.000	50.77	6.28	0.001
X_1^2	-3.27	-12.65	0.000	3.27	11.18	0.000	-81.66	-10.37	0.000
X_2^2	-2.44	-9.43	0.000	3.37	11.54	0.000	-114.07	-14.49	0.000
X_3^2	-4.88	-18.86	0.000	3.09	10.57	0.000	-47.32	-6.01	0.000
X_1*X_2	0.84	2.41	0.037	-1.65	-4.21	0.002	25.61	2.42	0.036
X_1*X_3	-0.89	-2.56	0.029	1.50	3.82	0.003	42.01	3.98	0.003
X_2*X_3	2.39	6.88	0.000	-1.30	-3.31	0.008	63.24	5.99	0.000
R^2	0.9840	-	-	0.9928	-	-	0.9766	-	-
R^2 (pred.)	0.8996	-	-	0.9514	-	-	0.8476	-	-
R^2 (adj.)	0.9695	-	-	0.9863	-	-	0.9556	-	-
C. equisetifolia									
Constant	50.81			19.03			993.62		
X_1	1.48	4.53	0.001	-7.18	-15.95	0.000	14.61	4.38	0.001
X_2	-0.26	-0.78	0.452	-6.76	-15.01	0.000	-15.61	-4.68	0.001
X_3	-1.10	-3.38	0.007	-4.22	-9.37	0.000	-21.38	-6.41	0.000
X_1^2	-2.98	-9.37	0.000	2.05	4.68	0.001	-52.85	-16.27	0.000
X_2^2	-2.40	-7.54	0.000	3.45	7.86	0.000	-101.14	-31.13	0.000
X_3^2	-4.04	-12.71	0.000	2.40	5.48	0.000	-29.53	-9.09	0.000
X_1*X_2	1.03	2.40	0.037	-1.41	-2.40	0.037	32.95	7.56	0.000
X_1*X_3	-1.70	-3.99	0.003	2.29	3.89	0.003	-10.15	-2.33	0.042
X_2*X_3	1.10	2.58	0.027	-0.09	-0.15	0.885	27.32	6.27	0.000
R^2	0.9697	-	-	0.9856	-	-	0.9927	-	-
R^2 (pred.)	0.8979	-	-	0.9069	-	-	0.9643	-	-
R^2 (adj.)	0.9424	-	-	0.9727	-	-	0.9860	-	-

Table 4.7: ANOVA for screened pulp yield, pulp kappa number and pulp viscosity of *L. leucocephala* and *C. equisetifolia*

Source	DF	Screened pulp yield			Pulp kappa number			Pulp viscosity		
		SS	F-value	p-value	SS	F-value	p-value	SS	F-value	p-value
L. leucocephala										
Linear	3	34.96	12.08	0.001	1264.30	342.22	0.000	49009	18.29	0.000
Square	3	498.95	172.41	0.000	380.06	102.87	0.000	272412	101.69	0.000
Interaction	3	57.51	19.87	0.000	53.30	14.43	0.001	51360	19.17	0.000
Lack of fit	5	7.07	2.75	0.146	9.49	3.35	0.105	7254	4.33	0.067
Pure error	5	2.57	-	-	2.83	-	-	1676	-	-
Total	19	601.07	-	-	1709.98	-	-	381711	-	-
C. equisetifolia										
Linear	3	47.37	10.85	0.002	1571.32	189.2	0.000	12486	27.36	0.000
Square	3	377.18	86.40	0.000	266.94	32.15	0.000	177675	389.36	0.000
Interaction	3	41.21	9.44	0.003	57.88	6.97	0.008	15483	33.93	0.000
Lack of fit	5	4.58	0.46	0.794	21.18	3.26	0.110	847	1.26	0.404
Pure error	5	9.97	-	-	6.49	-	-	674	-	-
Total	19	480.31	-	-	1923.82	-	-	207164	-	-

Table 4.8: Criteria for optimization of screened pulp yield, pulp kappa number and pulp viscosity of *L. leucocephala* and *C. equisetifolia*

Response	Goal	Lower	Target	Upper	Importance
Screened pulp yield (%)	Maximize	50	55	-	1
Pulp kappa number	Minimize	-	18	20	1
Pulp viscosity (cm^3/g)	Maximize	900	1000	-	1

Table 4.9: Some kraft pulping optimum conditions calculated by Response optimizer for *L. leucocephala* and *C. equisetifolia*

Pulping variable			Response variable			Composite desirability
*Active alkali charge (%)	Maximum temperature (°C)	Time (min)	Screened pulp yield (%)	Pulp kappa number	Pulp viscosity (cm^3/g)	
L. leucocephala						
17.1	169.8	92.6	53.54	17.13	973.67	0.805
17.5	**169.8**	**90.6**	53.69	16.30	966.0	0.787
18.3	170.9	82.8	51.09	16.70	993.48	0.974
17.9	169.8	82.8	51.14	18.34	997.94	0.976
18.3	170.2	71.2	50.45	19.26	994.63	0.955
16.8	170.2	93.3	50.52	19.01	989.24	0.957
17.3	171.6	88.1	50.89	17.60	991.11	0.968
17.3	171.2	77.5	50.65	19.60	994.31	0.961
C. equisetifolia						
17.9	**170.2**	**82.1**	51.13	17.89	997.06	0.906
17.3	171.6	88.1	50.89	17.60	991.11	0.968
17.3	171.2	77.5	50.65	19.60	994.31	0.961
17.9	169.8	82.8	51.14	18.34	997.94	0.976
18.3	169.5	81.7	51.10	17.88	995.58	0.900
18.3	170.9	82.8	51.09	16.70	993.48	0.974
18.4	168.8	77.5	50.95	18.91	995.11	0.970
16.8	170.2	86.7	50.52	19.01	989.24	0.957

*** refers active alkali charge as Na_2O**

Table 4.10: The experimental values and calculated values of responses of pulps prepared by above selected optimum kraft pulping condition (in bold) for *L. leucocephala* and *C. equisetifolia*

Response	*L. leucocephala*			*C. equisetifolia*		
	Mean experimental value	Calculated value	Diff. (%)	Mean experimental value	Calculated value	Diff. (%)
Screened pulp yield (%)	53.54±1.4	53.69	2.4	52.42±1.4	51.13	2.46
Pulp kappa number	16.70±0.6	16.30	2.39	18.12±0.7	17.89	1.27
Pulp viscosity (cm^3/g)	950.53±20.2	966.0	1.6	974.51±23.45	997.06	2.26

Table 4.11: Bauer–McNett fiber classification of optimized unbleached kraft pulps of *L. leucocephala* and *C. equisetifolia*

SI. No.	Mesh size	Fibers retained (%)	
		L. leucocephala	***C. equisetifolia***
1	+20	16.08±0.42	7.54±0.26
2	-20 to +48	57.25±1.89	67.08±2.56
3	-48 to +100	18.06±0.92	20.05±1.26
4	-100 to +200	7.65±0.35	5.22±0.24
5	-200	0.96±0.04	0.11±0.01

Table 4.12: Mechanical strength properties of optimized unbleached *L. leucocephala* and *C. equisetifolia* kraft pulps at different beating levels

Pulp	Beating level (°SR)	PFI revolution (number)	Tensile index (Nm/g)	Tear index (mNm^2/g)	Burst index ($kPam^2/g$)	Double fold (number)
L. leucocephala	15	0	31.04	5.27	1.17	11.03
	20	1555	50.26	8.70	2.85	55.20
	30	7000	62.07	9.53	4.20	98.98
	40	10000	75.91	8.38	6.29	135.79
	45	10475	73.95	7.14	5.91	125.28
	50	10975	70.71	5.25	4.82	96.61
C. equisetifolia	15	0	20.48	3.74	1.13	7.45
	20	2070	33.15	6.33	2.56	47.85
	30	8110	50.06	8.47	3.78	80.10
	40	10500	61.55	8.16	5.10	104.32
	45	11000	67.76	6.43	5.53	111.43
	50	11515	65.12	4.72	5.11	97.38

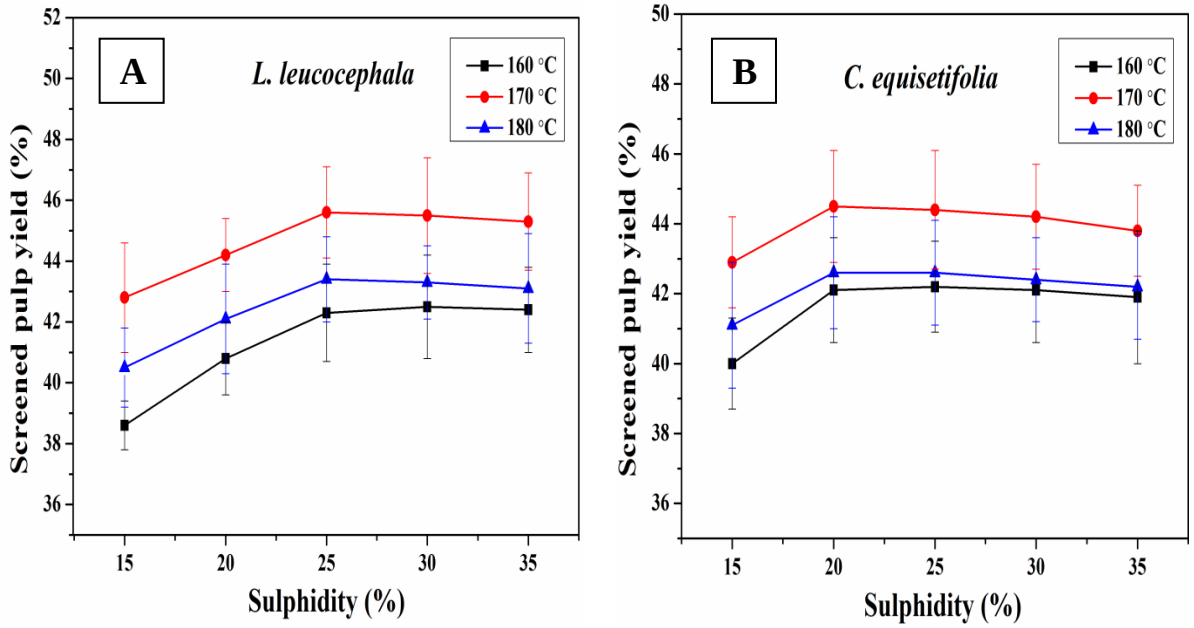


Figure 4.2: Curves of screened pua3alp yield *vs.* different sulphidity levels at maximum cooking temperatures during kraft pulping of *L. leucocephala* (A) and *C. equisetifolia* (B).

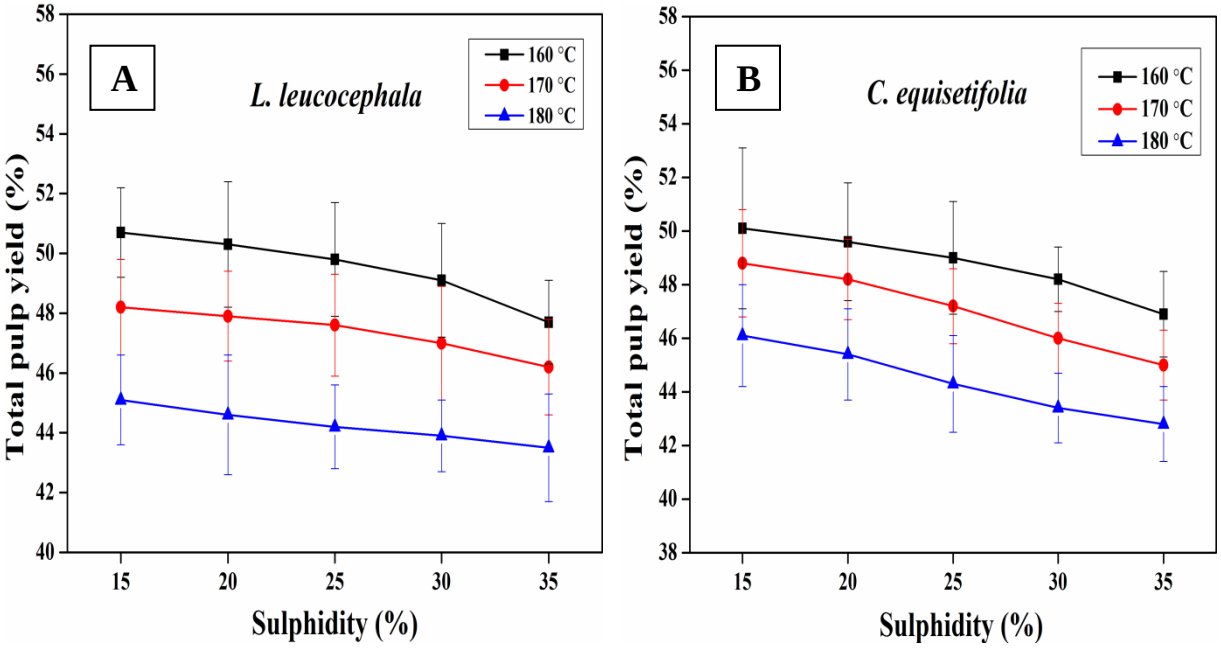


Figure 4.3: Curves of total pulp yield *vs.* different sulphidity levels at maximum cooking temperatures during kraft pulping of *L. leucocephala* (A) and *C. equisetifolia* (B).

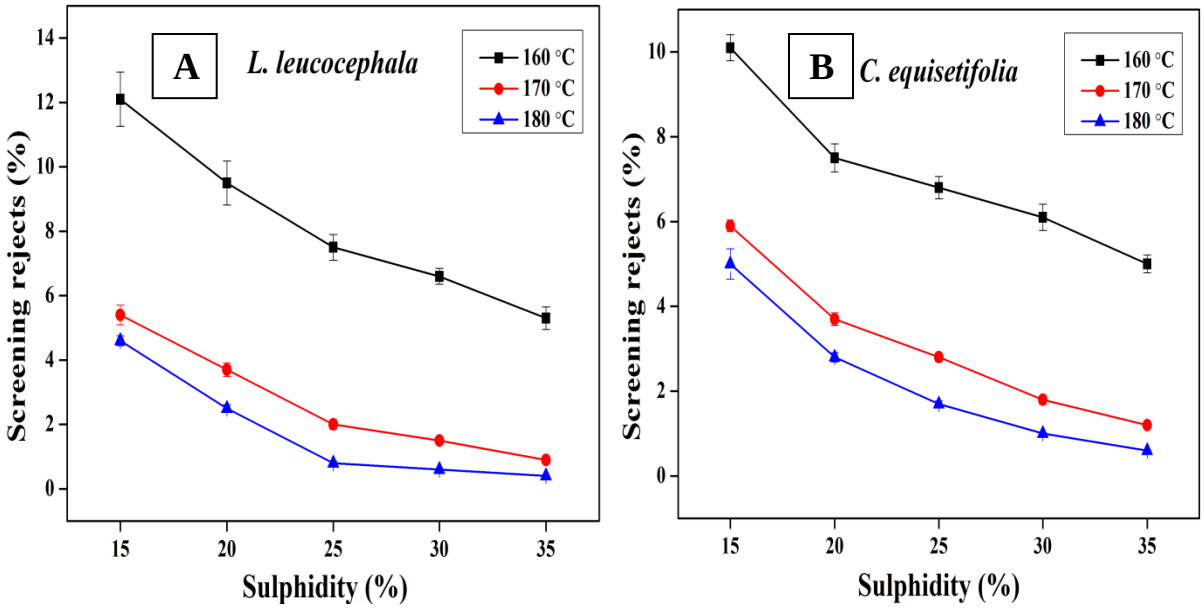


Figure 4.4: Curves of screening rejects *vs*. different sulphidity levels at maximum cooking temperatures during kraft pulping of *L. leucocephala* (A) and *C. equisetifolia* (B).

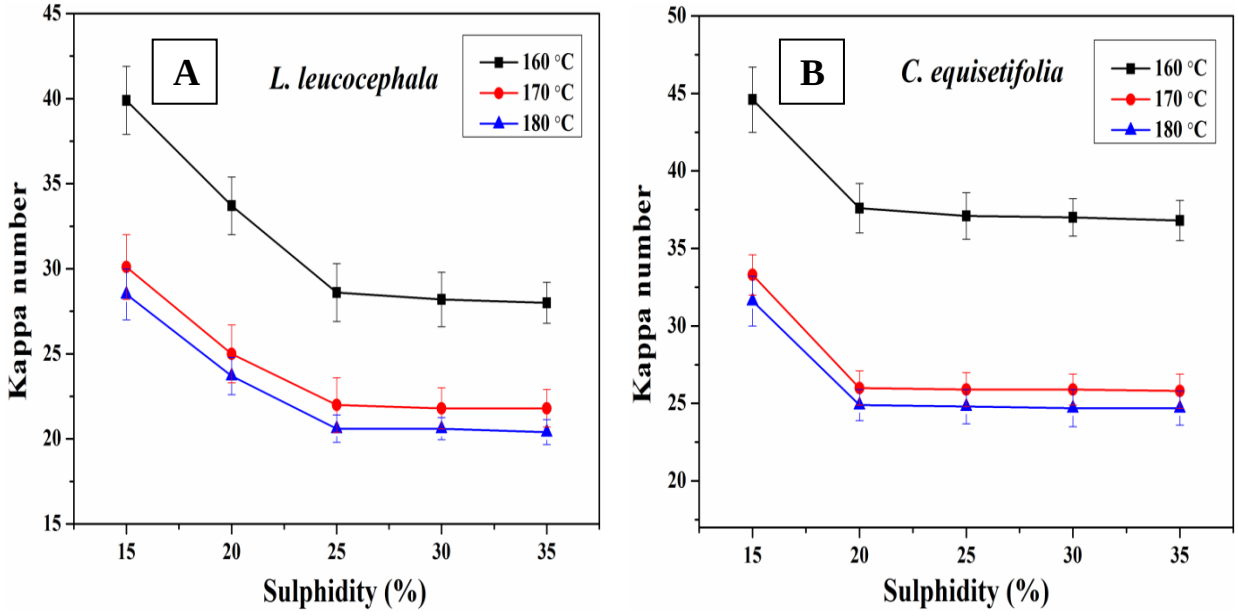


Figure 4.5: Curves of pulp kappa number *vs*. different sulphidity levels at maximum cooking temperatures during kraft pulping of *L. leucocephala* (A) and *C. equisetifolia* (B).

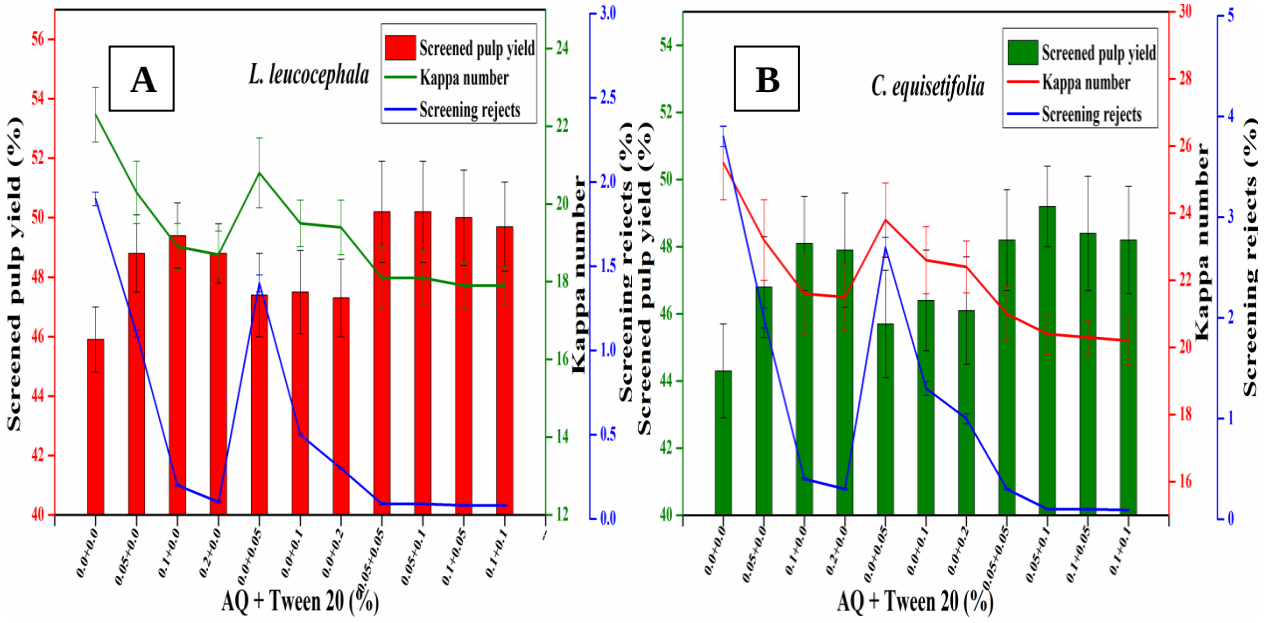


Figure 4.6: Effect of AQ and Tween 20 on screened pulp yield, screening rejects and pulp kappa number of *L. leucocephala* (A) and *C. equisetifolia* (B).

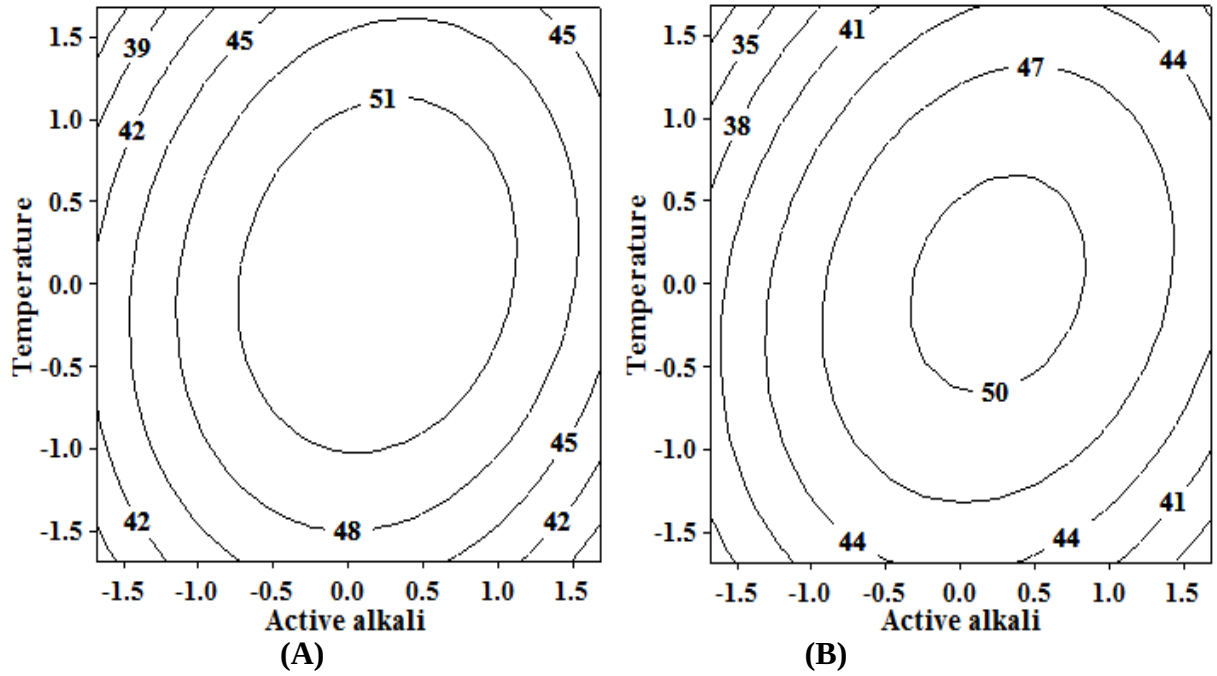


Figure 4.7: Contour plots of the combined effect of alkali level and maximum temperature on screened pulp yield of *L. leucocephala* (A) and *C. equisetifolia* (B), at a constant cooking time of 90 min.

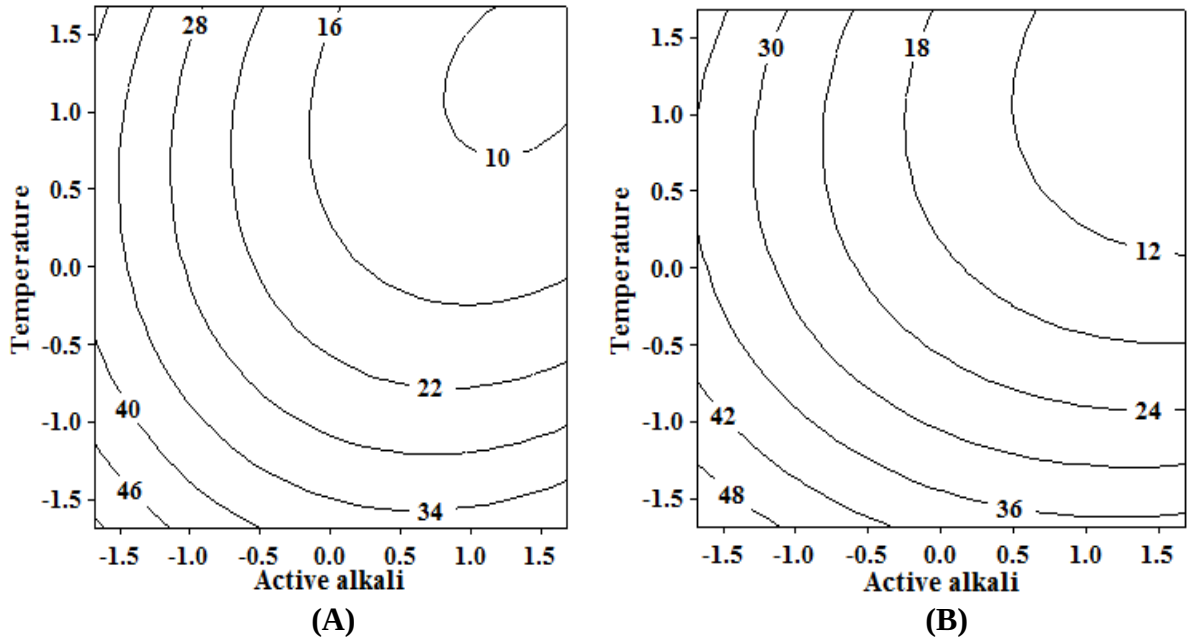


Figure 4.8: Contour plots of the combined effect of alkali level and maximum temperature on pulp kappa number of *L. leucocephala* (A) and *C. equisetifolia* (B), at a constant cooking time of 90 min.

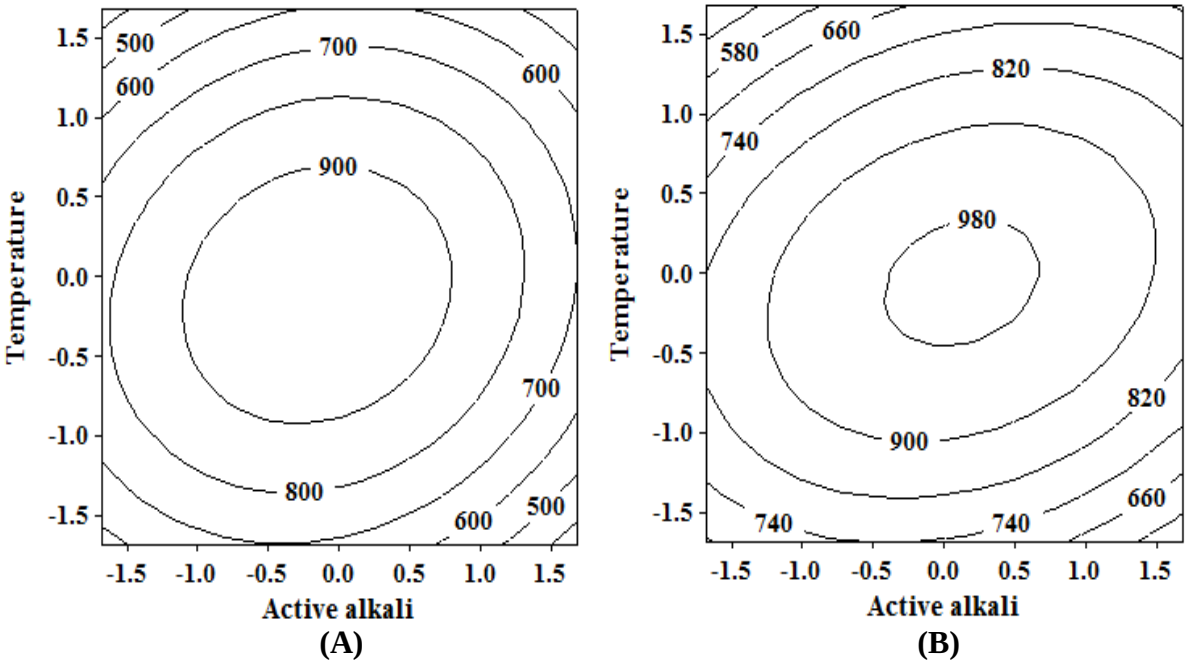
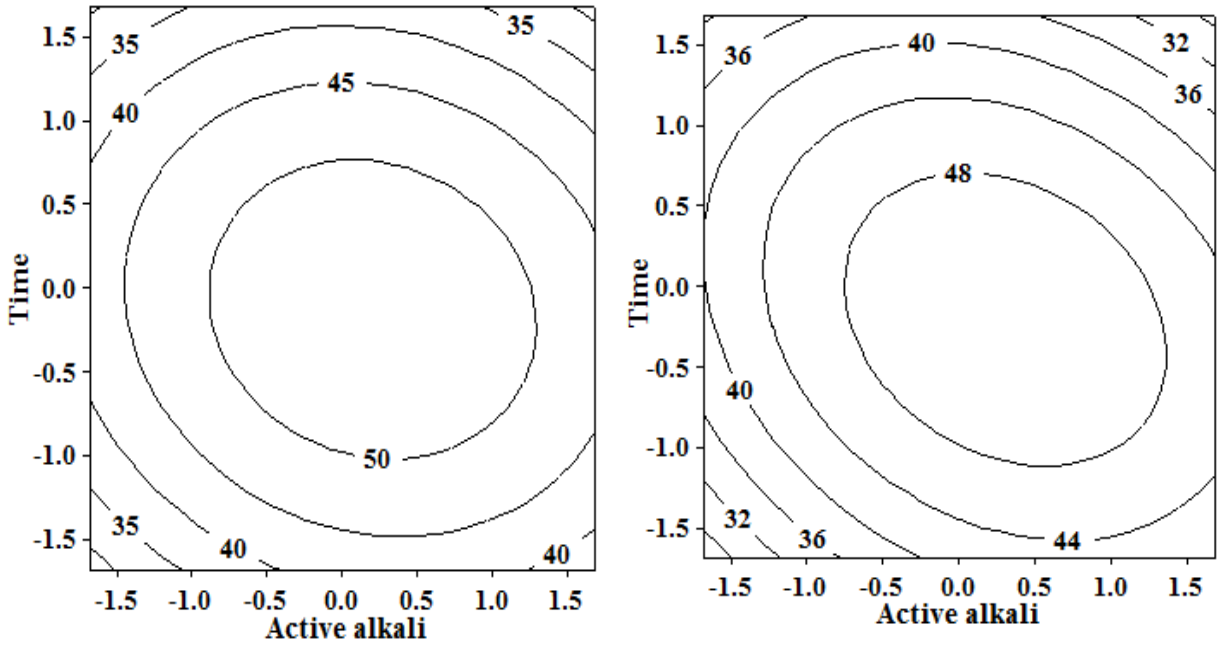
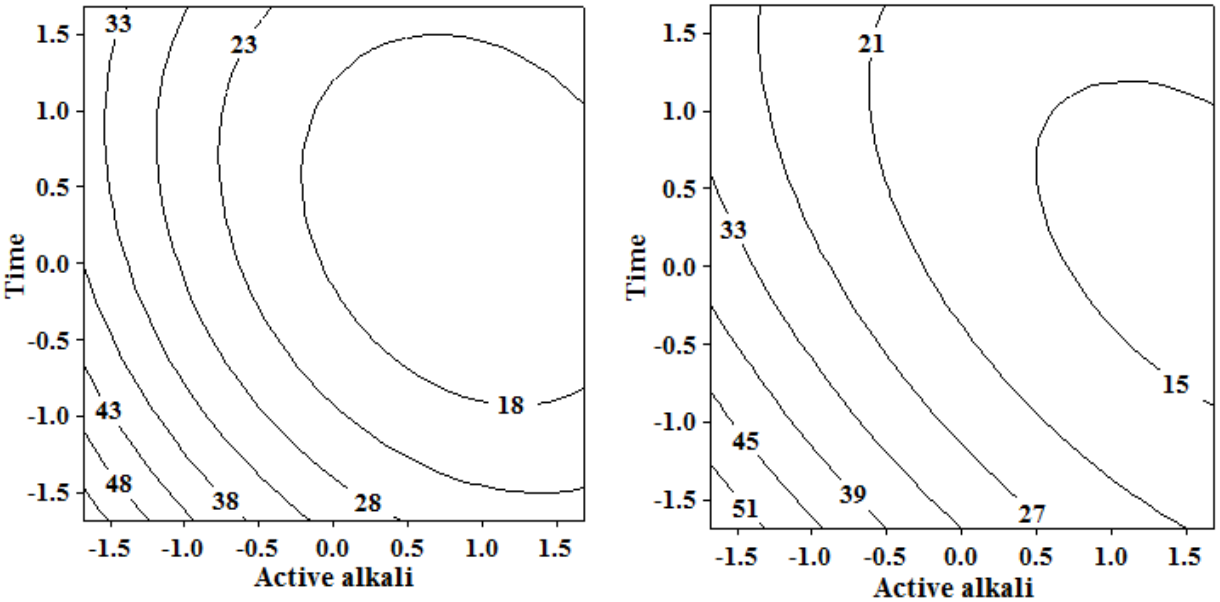


Figure 4.9: Contour plots of the combined effect of alkali level and maximum temperature on pulp viscosity of *L. leucocephala* (A) and *C. equisetifolia* (B), at a constant cooking time of 90 min.



(A) (B)

Figure 4.10: Contour plots of the combined effect of alkali level and cooking time on screened pulp yield of *L. leucocephala* (A) and *C. equisetifolia* (B), at a constant maximum temperature of 170 °C.



(A) (B)

Figure 4.11: Contour plots of the combined effect of alkali level and cooking time on pulp kappa number of *L. leucocephala* (A) and *C. equisetifolia* (B), at a constant maximum temperature of 170 °C.

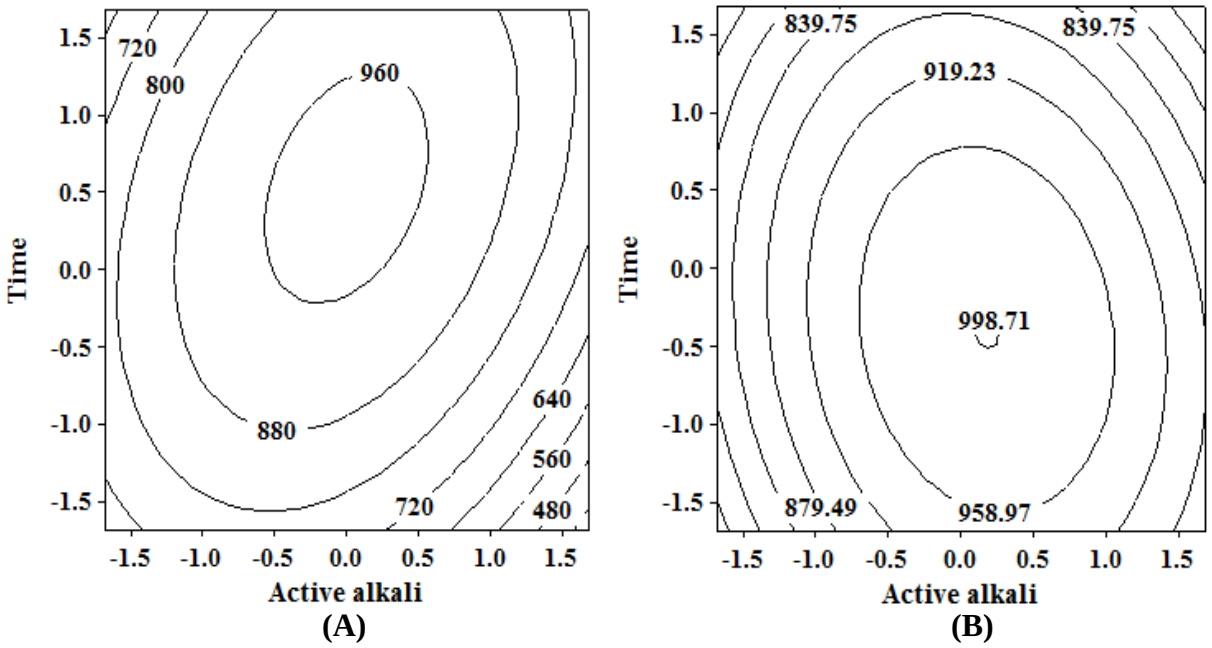


Figure 4.12: Contour plots of the combined effect of alkali level and cooking time on pulp viscosity of *L. leucocephala* (A) and *C. equisetifolia* (B), at a constant maximum temperature of 170 °C.

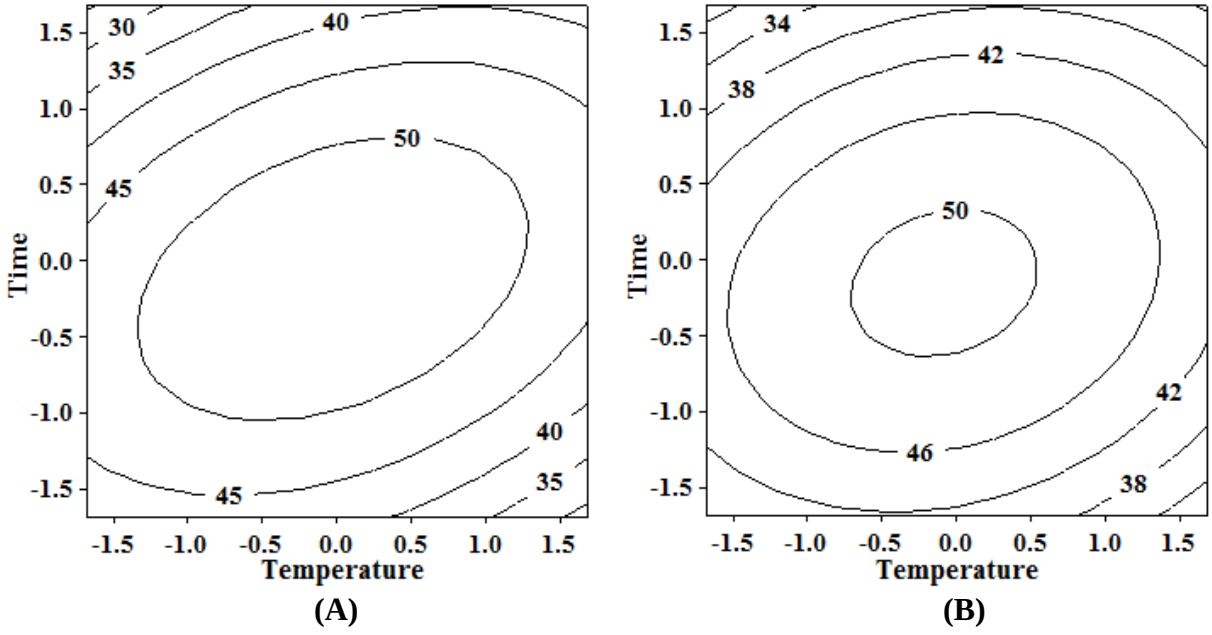


Figure 4.13: Contour plots of the combined effect of maximum temperature and cooking time on screened pulp yield of *L. leucocephala* (A) and *C. equisetifolia* (B), at a constant alkali level of 17%.

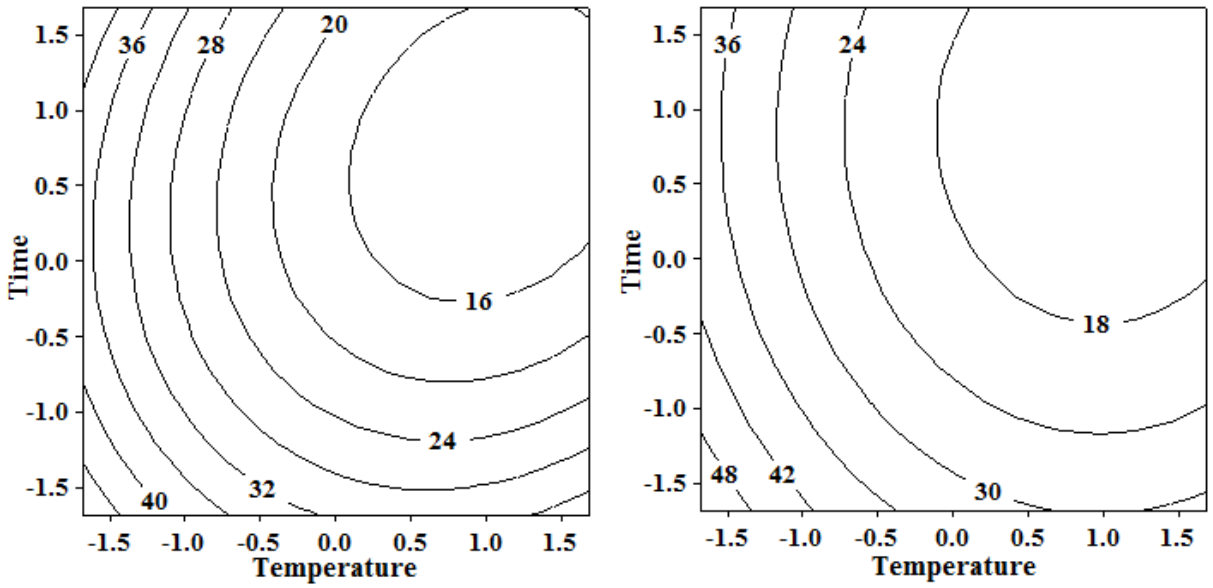


Figure 4.14: Contour plots of the combined effect of maximum temperature and cooking time on pulp kappa number of *L. leucocephala* (A) and *C. equisetifolia* (B), at a constant alkali level of 17%.

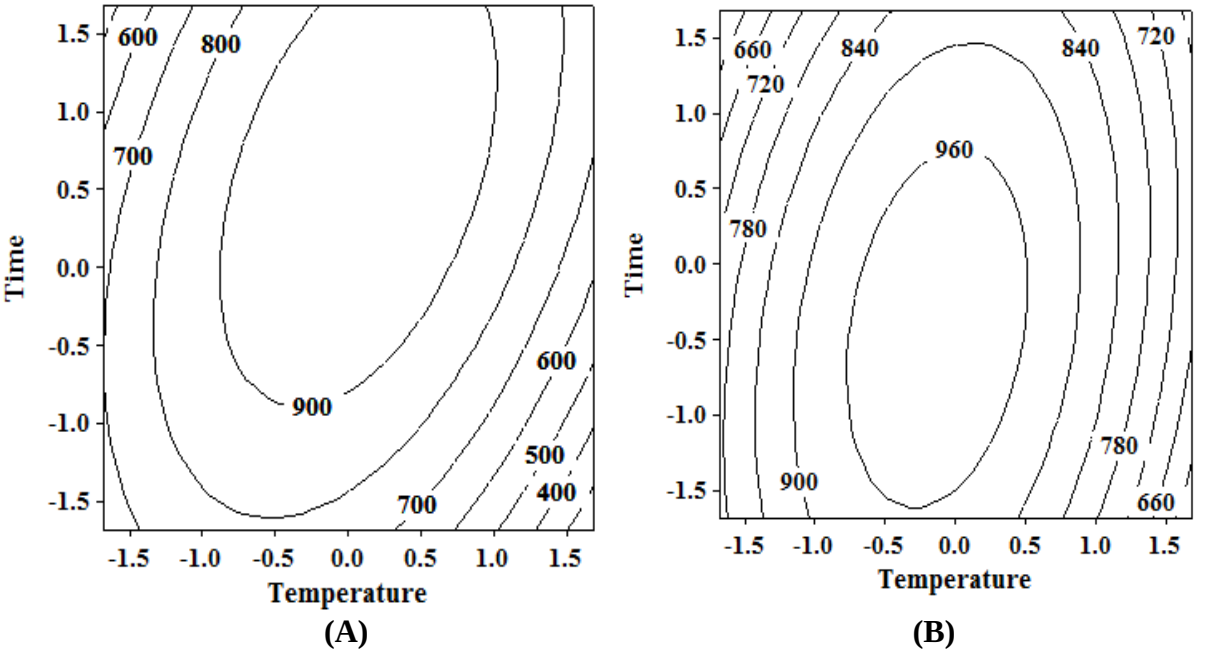


Figure 4.15: Contour plots of the combined effect of maximum temperature and cooking time on pulp viscosity of *L. leucocephala* (A) and *C. equisetifolia* (B), at a constant alkali level of 17%.

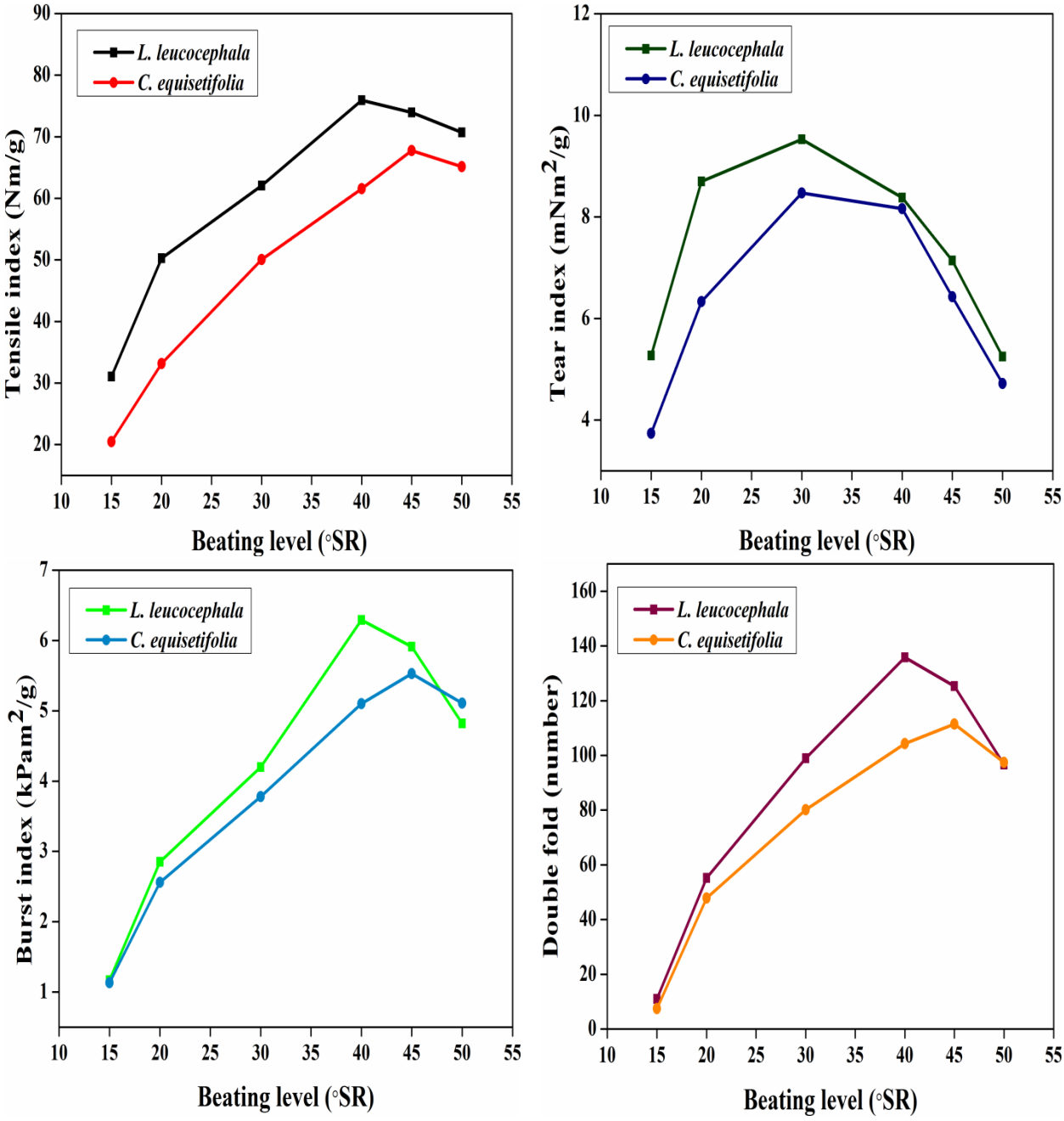
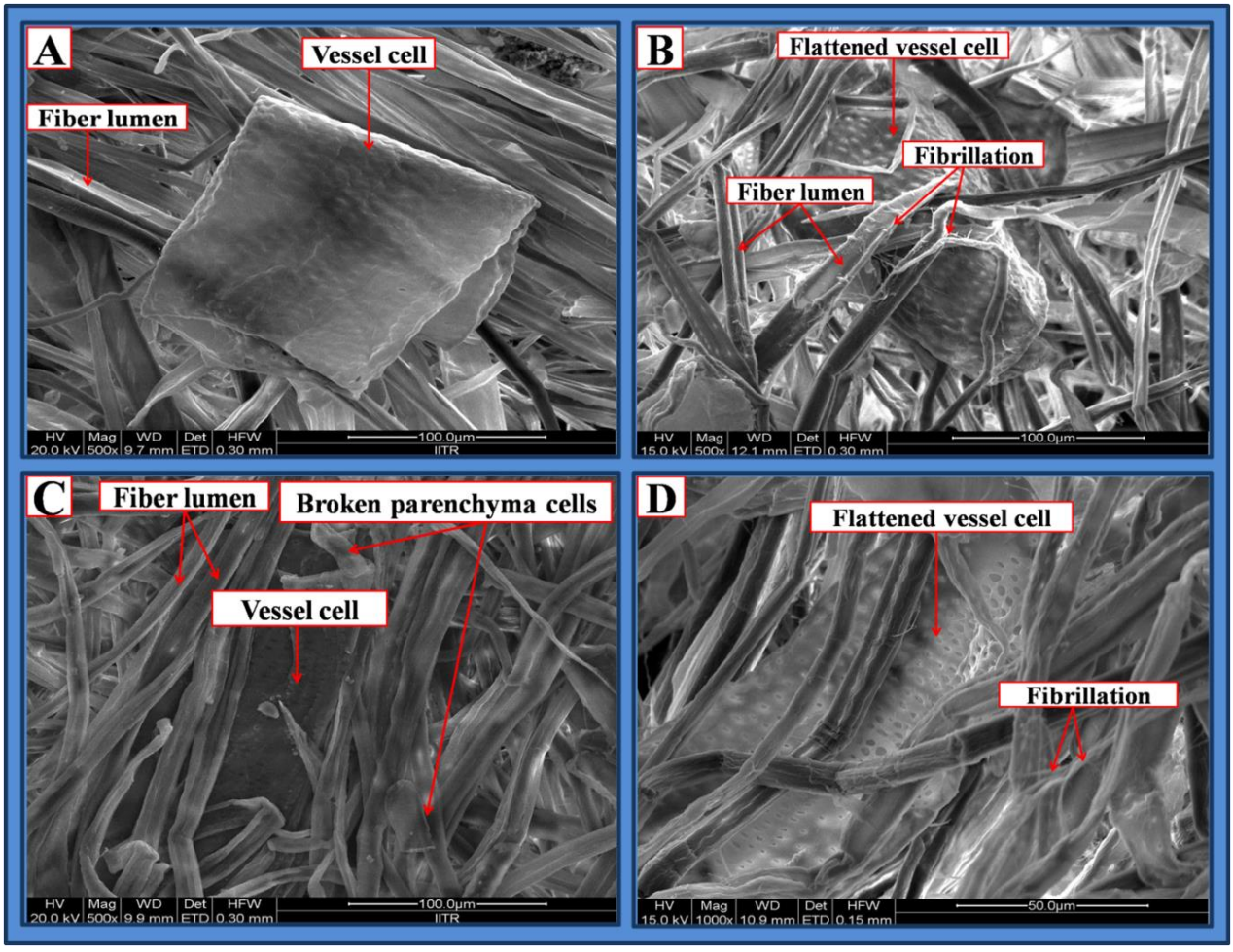


Figure 4.16: Mechanical strength properties of *L. leucocephala* and *C. equisetifolia* kraft pulp at different beating levels



Photomicrograph 4.1: Unbeaten kraft pulp of *L. leucocephala* showing flattened fibers and a vessel element (A); beaten kraft pulp (40 °SR) of *L. leucocephala* showing fibrillation and entangled vessel element (B); unbeaten kraft pulp of *C. equisetifolia* showing flattened fibers, vessel cell and parenchyma cell (C); beaten kraft pulp (45 °SR) of *C. equisetifolia* showing fibrillation (D).

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ANNEXURE 4

ABBREVIATIONS

%	Percent
°C	Degree celcius
°SR	Degree shoper reigler
A	Active alkali (as Na_2O)
AHQ	Anthrahydroquinone
AQ	Anthraquinone
CCD	Central composite design
CE	Coefficient
etc.	et cetera
G	Grams
g/m^2	Gram per meter square
i.e.	That is
LCC	Lignin carbohydrate complex
Min	Minutes
O.D.	Oven dry
R^2	Determination coefficient
R^2_{adj}	Adjusted determination coefficient
R^2_{pred}.	Predicted determination coefficietnt
Rpm	Revolutions per minute
RSM	Response surface methodology
SEM	Scanning electrone microscopy
T	Temperature
T	Time

CHAPTER 5

BLEACHING STUDIES OF *L. LEUCOCEPHALA* AND *C. EQUISETIFOLIA* KRAFT PULPS

5.1. INTRODUCTION

Paper production is a complex multi-variant process. In this process, removal of lignin from the raw material by pulping is the first step in the manufacturing of chemical pulp. Although, most of the lignin is removed during pulping process, some residual lignin bound with carbohydrate moieties [128] remains in pulp that is removed in a subsequent multistage bleaching process [36]. Bleaching is a multistage chemical process applied to the pulp for enhancing the brightness by removing the residual lignin. It also removes the unwanted particles from the pulp that contaminate the fibers. The primary objective of any bleaching sequence aims at achieving high pulp brightness, and the secondary objective aims at producing pulp of brightness stability and cleanliness along with high cellulose content. The chemicals commonly used in bleaching process involve oxidants (oxygen, ozone, chlorine, chlorine dioxide and hydrogen peroxide), alkali (sodium hydroxide) and sodium hydrosulphite [24]. These bleaching chemicals are applied in different bleaching sequences with intermediate washing or extraction stage between the two bleaching stages because the single stage bleaching is not sufficient for complete removal of bleach effluent or decolorisation of residual lignin.

In India, the conventional bleaching sequences such as CEH, CEHH and CHH are the most commonly used bleaching sequences. Usually, multistage bleaching sequence contains chlorine-based chemicals and alkali in extraction stage during the bleaching of kraft pulp. The use of chlorine in the bleaching process generates various toxic chlorinated organic pollutants such as chlorobenzene, 1,1,1-trichloroethane, trichlorophenol, 1,1-dichloroethane, 1,1,2,2-tetrachloroethane, chloroform, methylene chloride, tetrachloroethylene and 2,3,7,8-dibenzofurans. The effluents generated during chlorination stage generally, contain 2,3,7,8-TCDD and 2,3,7,8- TCDF [105, 109, 53] while the effluents generated in extraction (E) stage contain the highest concentration of dioxins [94]. The dioxins are carcinogenic and cause liver damage, lung lesions and tumours and alter the blood chemistry [78, 79, 51]. Chlorinated organic compounds induce genetic defects and cancer [72] in animals and create hazardous environmental impacts. The quantitative measurement of organo-chlorine compounds

generated in the bleaching process is done by the AOX measurement which is closely related to TOCL. The Central Pollution Control Board has forced the Indian pulp and paper mills for reducing the AOX level in a fixed time frame. The range of AOX in final discharge for wood based mills is 0.60-9 mg/L. The effluent discharge from the bleach plant is responsible for 60-70% BOD and 80-90% color load of the entire mill [86]. The chlorine consumption of wood-based mills is 60-100 kg/t of pulp, and about 100 kg of color substances and 2-4 kg of organochlorines are estimated to be produced in bleach plant effluents by the production of one tonne of pulp [68].

Due to growing environmental concerns and legislative pressures, the pulp and paper industry is forced to modify its current pulping, bleaching and effluent treatment technologies. The focus is on the alternative bleaching technologies which can reduce the AOX and TOCL in bleach plant effluents. Pollution load in bleach plant effluents can be mitigated by modifying the conventional pulping process [12, 125], bio-pulping [32, 31, 102], organosolv pulping [58], bio-bleaching [39, 38] and use of cooking additives [69, 55]. Many researchers used different methods for reduction in kappa number of kraft pulp in order to reduce the bleaching chemicals consumption. These methods include; high sulphidity cooking, low concentration of dissolved organics especially in the final phase of the cook, levelling out alkali profile throughout the cook and using wood chips from trees of suitable age [37]. Other alternatives to attain these objectives are replacement of ClO_2 by Cl_2, use of H_2O_2, dimethyldioxiranes, peracetic acid, oxygen [70, 114], enzymes [32] and O_3.

This has led to a trend towards ECF and TCF bleaching methods [83, 18]. Chlorine dioxide (ClO_2) is the main bleaching agent used in ECF processes, while oxygen, ozone, hydrogen peroxide and various other peroxygens are the main bleaching agents used in TCF processes. By adopting the ECF and TCF bleaching, the pulp and paper industry has undergone the technological revolution which launched the production of eco-friendly paper and mitigation of environmental emissions [36, 88]. A considerable reduction has been noticed in the discharge of chlorinated organic materials by adopting the ClO_2 in bleaching in place of elemental chlorine (Cl_2) [106, 107]. By adopting the ECF in bleaching of kraft pulp, a 48-65% reduction in AOX has been reported [52]. ClO_2 acts as an oxidizer which is 2.5 times more powerful than Cl_2 and it attacks lignin selectively while preserving cellulose. Hence, it produces brighter and stronger pulp as compared to Cl_2 bleaching [84]. Although, the use of ECF and TCF bleaching sequences reduces the significant amount of discharge of pollutants

during bleaching process but have some disadvantages also like high cost of chemicals. Today, general concern to remove chlorine from the bleaching process has shifted the focus towards the bio-bleaching process. Bio-bleaching process is carried out in presence of microorganisms or enzymes, and is a good alternative for the production of cleaner pulp [28, 104]. Most of the enzymes used in bio-bleaching process are crude enzymes which contain xylanase as main activity, and enriched with other enzymes like lipase, mannanase, α-galactosidage, laccase and cellulase etc. [3, 10, 94]. The presence of these enzymes in the crude enzyme mixture increases the bleaching effect with improving drainability and refinability [57]. To obtain good results in bio-bleaching; the enzyme dose, pH, reaction time and pulp consistency must be optimized. Many enzymes have been used in bio-bleaching but xylanase and laccase have been employed predominantly. The xylanase enzyme removes the bond present between residual lignin and hemicellulose (primarily xylan); makes the lignin more accessible for ligninolytic enzymes [27].

Many xylanase producing microbes have been studied for their efficacy in bleaching process [20, 127, 94]. Xylanase acts as bleach boosting agent in bleaching process and used in the prebleaching of pulps [95, 10, 3]. Enzymatic prebleaching at the mill scale for the first time was done in 1988. Xylanase treatment can be combined to conventional, ECF and TCF bleaching sequences [108]. The use of enzymes in the prebleaching stage reduces the amount of toxic compounds [41, 40, 116] in bleach liquor with the increase in COD and color of the effluent [89, 63]. Senior and Hamilton observed 40% reduction in AOX of effluent generated after the pretreatment of soft wood pulp with enzyme for 100% ClO_2 replacement [96]. Jain and co-workers reported 15.09% reduction in AOX with 32.19 and 107.89% increment in COD and BOD of effluent generated after the xylanase treatment of eucalyptus kraft pulp [40]. Pulp kappa number, viscosity and chemical demand are among the most important factors for evaluating the efficacy of an enzyme in bio-bleaching [77]. The utilization of xylanase can lead to 2-4 units reduction in kappa number and 5-7 kg replacement of dioxide per tonne of kraft pulp [80]. Many researchers used xylanase in different bleaching sequences for bleaching of wood and non-wood raw materials [90, 91, 117, 112].

Many hypothesis related to bleaching mechanism of xylanase have been put forward but the exact mechanism remains unclear till now. According to one report, xylanase aided bleaching is the result of depolymerisation but not necessarily solubilisation of hemicellulose [75] while the another report proposed that xylanase selectively removes the re-precipitated

xylan from the fiber surface, and enhances extraction [44]. During pulping process, the dissolved xylan and lignin re-precipitate on to the fiber surface as the alkali concentration decreases [61] in the later phase. The re-precipitated xylan and lignin are chemically linked to each other. Xylanase hydrolyzes the re-precipitated xylan on to the fiber surface, increases the permeability of fiber and makes easier the penetration of bleaching chemicals into the pulp and access to lignin [92, 22]. Therefore, xylanase improves fibrillation, water retention and the freeness of pulp while reducing the beating time [10] which ultimately result into better strength of paper and saving of energy. The utilization of xylanase in bio-bleaching of pulp results into high brightness and savings of bleaching chemicals [7]. The alkali and thermo-tolerant xylanase is gaining importance because of its effective implication in pulp pretreatment and improvement in the efficiency of chlorinated bleaching sequences and pollution control [15]. The presence of cellulase enzyme in crude enzyme mixture reduces the cellulose viscosity during bio-bleaching. Cellulose degradation during bleaching may result into significant loss in strength properties of final product [24]. Hence, cellulase free xylanase enzyme is desirable for bio-bleaching application. Pretreatment of kraft pulp with cellulase free xylanase partially hydrolyzes the xylan, and results into the improvement in pulp viscosity [74, 21]. Moreover, the soda and kraft pulping are highly alkaline processes; hence the enzymes active in alkaline region are desirable for bio-bleaching of alkaline pulp [76]. The pulp obtained after cooking and washing steps is warm; consequently temperature stability is another desirable factor for xylanase enzyme used in bio-bleaching process [105, 10].

The present study is done to analyze the effect of crude xylanase extracted from *C. cinerea* RM-1 employed in different bleaching sequences and its effect on optical properties, strength properties and pollution load generated during bleaching of kraft pulps of *L. leucocephala* and *C. equisetifolia*.

5.2. MATERIALS AND METHODS

5.2.1. Materials

Crude xylanase produced by test strain *C. cinerea* RM-1 under optimized conditions as described in chapter 2 was used in bio-bleaching experiments. The unbleached kraft pulps of *L. leucocephala* and *C. equisetifolia* produced under optimized conditions as described in chapter 4 were used in the present study.

5.2.2. Methods

5.2.2.1. Optimization of reaction conditions for xylanase pretreatment of kraft pulps of *L. leucocephala* and *C. equisetifolia*

The reaction conditions i.e. enzyme dose, reaction time and pulp consistency were optimized during enzymatic prebleaching (with crude xylanase) of kraft pulps of *L. leucocephala* and *C. equisetifolia*. The 100 g of unbleached oven dry kraft pulps of *L. leucocephala* and *C. equisetifolia* each were disintegrated in disintegrator (L&W) before bleaching experiments. These pulps were treated in polythene bags with different doses of crude xylanase, varying from 2 to 25 IU/g, and maintained separately at consistency 6% and incubation time 120 min at fixed temperature 55±2 °C and pH 8.3. The pulps were mixed well by vigorous shaking after every 15 min. Similarly, reaction time from 0 to 180 min was optimized by treating the 100 g of kraft pulps of *L. leucocephala* and *C. equisetifolia* each with optimized dose of crude xylanase i.e. 5 IU/g for *L. leucocephala* and 10 IU/g for *C. equisetifolia*. In another set, pulp consistency was varied from 2 to 12% during enzymatic prebleaching of *L. leucocephala* and *C. equisetifolia* each while keeping other conditions constant as described above.

At the end of each set, the pulps were filtered through four-layered cheese cloth and the filtrates were collected separately. The filtrates were then analyzed for release of reducing sugars by measuring absorbance at wavelength 540 nm [66], and for release of chromophores at wavelength 237, 280 and 465 nm [33, 77]. The experiments were performed similarly with controls which were treated with phosphate buffer in place of enzyme. The pulps of both the raw materials were then washed with tap water, and were extracted with 2% NaOH at 70±2 °C for 90 min at pH 11.5. After completion of extraction, the pulps were washed with tap water (hardness 320 ppm) and squeezed, and analyzed for kappa number (T236 cm-85), viscosity (T230 om-04) and brightness (%), ISO (T452 om-02).

5.2.2.2. Effect of xylanase pretreatment on multi-step bleaching process

The effect of xylanase treatment on kraft pulps of *L. leucocephala* and *C. equisetifolia* was studied under multistage conventional, ECF and TCF bleaching sequences. For each bleaching sequence, 100 g (oven dry) disintegrated and unbleached kraft pulps of *L. leucocephala* and *C. equisetifolia* were taken, separately. The bleaching experiments were performed in air tight polythene bags in temperature controlled water bath except chlorination

stage which was performed in air tight plastic bottle at room temperature. All bleaching experiments were carried out in triplicates. After each stage, the pulp samples were washed with water properly, and analyzed for further analysis.

5.2.2.2.1. Conventional bleaching

The kraft pulps of *L. leucocephala* and *C. equisetifolia* were bleached by CEHH, XECEHH, CEHHP and XECEHHP bleaching sequences. The prebleaching stage (X) was performed at an enzyme dose of 5 IU/g for *L. leucocephala* and 10 IU/g for *C. equisetifolia*, pH 8.3, reaction time 120 min, temperature 55±2 °C and pulp consistency 8%. The enzyme treated pulps were extracted (E_1 stage) with 2% NaOH, at pH 11.5, reaction time 90 min, temperature 70±2 °C and pulp consistency 10%. After prebleaching, the pulps were washed and bleached by CEHH bleaching sequence using different chlorine charges. The total chlorine demand (TCD) was calculated as under:

$$\text{TCD, \%} = 0.25 \times \text{kappa number}$$

50% of TCD was charged in 'C' stage and the remaining 50% was charged in hypochlorite 1st (H_1) and 2nd (H_2) stages in a ratio of 70:30, respectively. The chlorination stage was carried out at conditions: pH 2, reaction time 30 min at ambient temperature and consistency 3%. The pulps were filtered through four-layered cheese cloth and the collected filtrates were analyzed for residual chlorine while the remaining filtrates were kept at 4 °C for further investigation.

The extraction stage (E_2) was conducted at the same conditions as in the previous stage (E_1) except the total alkali charge that was applied as half of the total charge applied in the previous stage (E_1). The hypochlorite i.e. H_1 and H_2 stages were performed at pH 11.0, reaction time 60 min, temperature 70±2 °C and consistency 10%. The P-stage of bleaching sequence CEHHP was performed using 2% H_2O_2, 0.1% $MgSO_4$ (as carbohydrate stabilizer) and 0.5% EDTA (to mask the activities of d-block elements) at conditions: pH 11.5, consistency 10%, temperature 90±2 °C and reaction time 120 min.

5.2.2.2.2. ECF bleaching

The kraft pulps of *L. leucocephala* and *C. equisetifolia* were bleached by ODED, XODED, ODEP and XODEP bleaching sequences. The xylanase pretreated and untreated (control) pulps were subjected to oxygen delignification (O stage) in a rotary autoclave heated by ethylene glycol bath (CCL Digester-Feronics, Roorkee, India) at a pressure of 5 kg/cm^2. The pulps were mixed with 0.1% EDTA, 0.1% $MgSO_4$ and 2.0% NaOH. The bleaching conditions

during the oxygen delignification were fixed at pulp consistency 15%, pH 11.0, temperature 110 °C and reaction time 90 min. In chlorine dioxide (D) stage, the pulps were treated with sodium chlorite solution (20 g/L strength) [56]. The treatment was given as follows:

$$\text{Total } ClO_2, \% = 0.25 \times \text{kappa number}$$

50% of the total ClO_2 was charged in 'D_1' stage and the remaining 50% was charged in 'D_2' stage at the conditions described in Table 5.7. The alkali extraction stage (E) was performed as described above, in procedure adopted for conventional bleaching. For the peroxide stage (P), the calculated amount of hydrogen peroxide was added to the pulp samples. The concentration of peroxide added to the pulp samples was determined by iodometric titration [123]. The bleaching conditions in P stage were maintained as described in the Table 5.7.

5.2.2.2.3. TCF bleaching

The kraft pulps of *L. leucocephala* and *C. equisetifolia* were bleached using $O(E_{OP})P$ and $XO(E_{OP})P$ bleaching sequences. The xylanase treated and untreated pulps were delignified with oxygen in a rotary autoclave heated by ethylene glycol at the conditions described in procedure adopted during ECF bleaching. The peroxide and oxygen reinforced extraction stage (E_{OP}) were performed with 3% NaOH, 0.5% H_2O_2 charge, 5 kg/cm^2 O_2 pressure and 0.1% $MgSO_4$ at pH 11.5, pulp consistency 10% and temperature 70 °C for 60 min. Following extraction, the pulps were washed and then subjected to peroxide stage (P) at the conditions described in Table 5.10.

5.2.2.2.4. Preparation and evaluation of laboratory hand sheets

The bleached kraft pulps of *L. leucocephala* and *C. equisetifolia* were beaten at a beating level of 40±1 °SR and 45 °SR, respectively, in PFI mill (T 200 sp-96). Laboratory handsheets of 60 g/m^2 were prepared (T 221 cm-99) and evaluated for various properties such as brightness (%) ISO, tensile index (T 494 om-01), tear index (T 414 om-98), burst index (T 403 om-97) and double fold (T 423 cm-98). The pulps were also evaluated for bleached pulp yield (T 222 om-88), bleaching losses and viscosity (T 230 om-04).

5.2.2.2.5. Analysis of combined bleach effluent

The effluents collected after each bleaching stage were mixed in equal amounts at the end of each bleaching sequence and were analyzed for various effluent characteristics. The COD load was determined by closed reflux titrimetric method (using Thermoreactor CR 2010)

[118, 126]. The color was determined by cobaltiplatinate method by measuring the wavelength at 465 nm. AOX was measured by column method [119].

5.2.2.2.6. SEM studies

The unbleached (control) and xylanase prebleached kraft pulps of *L. leucocephala* and *C. equisetifolia* were examined under FEI Quanta 200 F microscope for detailed morphological studies. The SEM analysis of pulp samples was carried out as per procedure laid down in paragraph 3.2.2.1. of Chapter 3.

5.2.2.2.7. Statistical analysis

All experiments were carried out independently in triplicate, and the results were the mean ± standard deviation of three replicate experiments.

5.3. RESULTS AND DISCUSSION

5.3.1. Optimization of different reaction conditions for xylanase pretreatment of *L. leucocephala* and *C. equisetifolia* kraft pulps

In this study, an attempt has been made to establish the applicability of crude xylanase enzyme produced from the fungus *C. cinerea* RM-1 for the perspective of *L. leucocephala* and *C. equisetifolia* kraft pulps prebleaching.

5.3.1.1. Optimization of xylanase doses for pretreatment

Table 5.1 illustrates the effect of different xylanase doses varying from 0 to 25 IU/g at reaction time 120 min, pH 8.3, pulp consistency 6% and temperature 55 °C. Figures 5.1A and B show the effect of xylanase enzyme at different doses after XE stage on kappa number and brightness of *L. leucocephala* and *C. equisetifolia* kraft pulps, respectively. The kappa number of *L. leucocephala* pulp decreases by 1.85 unit (11.2%) and brightness increases by 8.2% ISO as compared to their respective controls up to an enzyme dose of 5 IU/g (Figure 5.1A) and after that, both kappa number and brightness remain almost constant. Similarly, the kappa number of *C. equisetifolia* pulp decreases by 2.6 unit (15.0%) and brightness increases by 6.6% ISO, as compared to controls, upto an enzyme dose of 10 IU/g (Figure 5.1B). After that, the kappa number and brightness do not reduced/improved significantly.

In the early phase of the kraft pulping, the 4-O-methylglucuronic acid side groups in hemicellulose are converted into HexA which is responsible for the yellowing of pulp and high kappa number [26, 124]. It has been established that xylanase enhances the release of

xylooligosaccharides branches with HexA which may be correlated with the reduction in kappa number and improvement in pulp brightness after XE-stage [120]. Singh and co-workers observed 16.33% improvement in brightness and 24.38% reduction in kappa number of wheat straw soda-AQ pulp by using crude xylanase from the strain of *C. disseminatus* SH1-1163 at 10 IU/g enzyme dose, 55 °C temperature, 6.4 pH, 180 min reaction time and 10% consistency [104]. Dwivedi and coworkers observed 21% reduction in kappa number and 8% improvement in pulp brightness of mixed wood pulps of *Eucalyptus* and Poplar (60:40) by using a concoction of xylanase and laccase (22:1) at an enzyme dose of 8 IU/g, pulp consistency 10%, temperature 55 °C and pH 9.0 for 180 min [25].

Figure 5.2 shows the release of reducing sugars from *L. leucocephala* and *C. equisetifolia* kraft pulps after different doses of enzyme treatment. Both the curves could be estimated by two slopes. The part of curves with steeper slope, up to a xylanase dose of 15 IU/g for *L. leucocephala* and *C. equisetifolia*, is related to the rapid release of sugars while the gentler slope is related to the slow release of sugars. The release of lignin, chromophores and reducing sugars is correlated phenomenon. After the pretreatment of kraft pulps from *L. leucocephala* and *C. equisetifolia* with xylanase enzyme, the xylose and other reducing sugars are released from the hemicellulosic layer of fiber. This ultimately results in an increase in the free sugar content. Xylan is sandwiched between lignin and cellulosic layer of fiber. Lignin and phenolic compounds are set free by hydrolysing the xylan, and as a result, the reducing sugars are also released [49]. The reducing sugars release continuously because of the hydrolysis of soluble oligosaccharides by xylanase treatment. Therefore, the measurement of chromophores release is a better indicator for understanding the enzyme action on to the pulp as oligosaccharides are released by the initial depolymerisation of the xylan deposited on the fiber surface [29].

Figures 5.3A and B show the release of chromophores or phenolic compounds at different wave length i.e. 237, 280 and 465 nm after enzyme treatment of *L. leucocephala* and *C. equisetifolia* kraft pulps, respectively. At a wave length of 237 nm, the chromophores release rapidly up to a xylanase dose of 5 IU/g and 10 IU/g, respectively, for kraft pulps of *L. leucocephala* and *C. equisetifolia*. Beyond that, the release of chromophores or phenolic compounds becomes slow down. Similar trend was observed by many researchers [77, 33, 49, 9]. The release of chromophores into effluent indicates the degradation of lignin-carbohydrate complex (LCC) and lignin-hemicellulose linkages [23]. At a wave length of 280 nm, the

absorbance increases rapidly up to a xylanase dose of 5 IU/g for *L. leucocephala* and 10 IU/g for *C. equisetifolia.* Beyond that, the absorbance continues to be increased but with a slower rate due to the carbohydrate degradation products which also impart color to the effluent [132, 133]. It is indicated that further increase in enzyme dose (beyond 5 IU/g for *L. leucocephala* and 10 IU/g for *C. equisetifolia*); only small amount of additional lignocellulosic complex is attacked. The peak at 280 nm shows the presence of lignin compounds released in the bleaching effluent [54, 73]. After enzyme treatment, the alkaline extraction facilitates the dissolution of the modified lignin-carbohydrate fragments remaining in the pulp which are previously loosens by the attack of enzymes [19]. Hence, an alkaline extraction stage is carried out after xylanase treatment for observing the removal of lignin from kraft pulps of *L. leucocephala* and *C. equisetifolia* [99, 45, 77, 54, 4, 19].

No significant gain in brightness and in the release of chromophores is recorded beyond an enzyme dose of 5 IU/g for *L. leucocephala* and 10 IU/g for *C. equisetifolia*. This indicates that the xylanase doses of 5 IU/g for *L. leucocephala* and 10 IU/g for *C. equisetifolia* are the optimum doses of enzyme for degradation of LCC, and beyond that only small amount of additional LCC is attacked.

5.3.1.2. Optimization of reaction time for xylanase pretreatment

Table 5.2 illustrates the effect of different reaction time varying from 0 to 180 min at pH 8.3, pulp consistency 6%, temperature 55 °C and xylanase dose of 5 IU/g for *L. leucocephala* and 10 IU/g for *C. equisetifolia* kraft pulps. Figures 5.4A and B show the effect of xylanase treatment at different reaction time after XE stage on kappa number and brightness of *L. leucocephala* and *C. equisetifolia* pulps, respectively. The kappa numbers of *L. leucocephala* and *C. equisetifolia* pulps decrease by 2.16 units (12.9%) and 2.87 units (16.48%) while the respective increments in brightness are 10.1 and 7.65% as compared to their respective controls when reaction time was increased from 0 to 120 min. Beyond that, no significant decrease in kappa number or increase in brightness is observed.

Enzyme dose and reaction time are inter-related factors of enzyme activity. The same bleach boosting effect might be attained in shorter time by increasing the enzyme dose [4]. Agnihotri and co-workers produced xylanase from *Coprinellus disseminatus* SW-1 NTCC 1165 and found 29.1% reduction in kappa number along with 9.42% ISO improvement in brightness after XE stage of sugarcane bagasse soda-AQ pulp at an enzyme dose of 8 IU/g, consistency 10%, temperature 55 °C, pH 6.4 and reaction time 120 min [1]. A cellulase free crude xylanase

from strain *Bacillus subtilis* mitigated the kappa number by 4.62% and improved the brightness of kraft pulp by 3.3% ISO at an enzyme dose of 6 IU/g, consistency 10%, temperature 55 °C and reaction time 120 min [93].

The release of reducing sugars at different reaction time of crude xylanase treatment is found in increasing order with the increasing reaction time but it reaches to maximum at the reaction time of 120 min in case of both the raw materials (Figure 5.5). Figures 5.6A and B depict the spectrophotometric analysis of filtrates of *L. leucocephala* and *C. equisetifolia* kraft pulps, respectively, at different reaction time (0 to 180 min) and wave length (237, 280 and 465 nm) after enzyme treatment. The curves indicate that the release of chromophores increases with increasing reaction time from 0 to 120 min at all the wave lengths. Beyond an incubation period of 120 min there is no significant gain in the release of chromophoric groups. Many researchers found the similar trend of release of chromophores after xylanase treatment [1, 48].

5.3.1.3. Optimization of pulp consistency for xylanase pretreatment

Table 5.3 shows the effect of pulp consistency varying from 2 to 12% on kraft pulps of *L. leucocephala* and *C. equisetifolia* while keeping other conditions constant as described in Table 5.3. The kappa numbers of *L. leucocephala* and *C. equisetifolia* kraft pulps reduce by 1.7 units (10.8%) and 2.2 units (13.1%), respectively, with increasing pulp consistency up to 8% (Figures 5.7A and B). Similarly, the brightnesses increase by 10.2 and 10.7% ISO at 8% consistency for *L. leucocephala* and *C. equisetifolia*, respectively. Beyond a consistency of 8%, the decrease in kappa number or increase in brightness is insignificant. It is difficult to decrease kappa number at higher consistency due to limitations of mixing equipments. Many researchers used 10% pulp consistency for prebleaching of kraft pulp of *Eucalyptus* sp. [65, 47, 93] and soda-AQ pulp of *Eucalyptus grandis* [110]. Chauhan and co-workers found 6% consistency as an optimum for xylanase pretreatment of rice straw pulp with a gain of 5.1 points in brightness [16].

The curves of reducing sugars show that release of reducing sugars increases with increasing pulp consistency for both the raw materials but the maximum increase is observed at 8% pulp consistency. Beyond that, only small increase in reducing sugars is observed (Figure 5.8). Figures 5.9A and B depict the spectrophotometric analysis of *L. leucocephala* and *C. equisetifolia* pulps after enzyme treatment at different pulp consistencies (0 to 12%) and wave lengths (237, 280 and 465 nm). The curves illustrate that the release of chromophores improves

with increasing pulp consistency up to 8% and after that, the release of chromophoric groups is insignificant.

The efficiency of enzymes for bleaching of pulp is determined by several factors. The extent of release of reaction product is mainly dependent on substrate characteristics but enzyme specificities like the molecular weight and ionic characteristics also play an important role [96]. Therefore, the interaction of any pulp will be different according to the characteristics features of the enzyme. Moreover, the cellulosic fibers develop mobile and immobile layers when merged in water [74]. The mobile layer is progressively reduced with increasing the consistency of pulp, leaving only the thin envelop of immobile layer. Due to this, the diffusion path length of reactant to the fiber decreases considerably [59, 87, 46]. The pulp consistency is an important parameter because the surface area of the pulp determines the amount of water required for efficient result. A xylanase dose of 5 IU/g for *L. leucocephala* and 10 IU/g for *C. equisetifolia*, a reaction time of 120 min and pulp consistency of 8% may be taken as optimum conditions for enzyme prebleaching using crude xylanase obtained from *C. cinerea* RM-1.

5.3.2. Effect of xylanase pretreatment on different bleaching sequences of *L. leucocephala* and *C. equisetifolia* kraft pulps

The effect of crude xylanase extracted from fungus *C. cinerea* RM-1 is studied on optical properties, viscosity, pulp beating, bleaching losses, TCD, mechanical strength properties and combined effluent properties of kraft pulps of *L. leucocephala* and *C. equisetifolia* during conventional (CEHH and CEHHP), ECF (ODED and ODEP) and TCF ($O(E_{OP})P$) bleaching sequences.

5.3.2.1. Effect of xylanase pretreatment on kappa number and brightness

Tables 5.4 and 5.5 illustrate the effect of crude xylanase pretreatment for kraft pulps of *L. leucocephala* and *C. equisetifolia*, respectively, on kappa number and brightness during CEHH and CEHHP bleaching sequences. Crude xylanase pretreatment followed by alkali extraction mitigates kappa numbers of unbleached pulps of *L. leucocephala* and *C. equisetifolia* by 2.57 units (15.37%) and 2.99 units (17.12%), respectively. Xylanase pretreatment increases the brightness of CEHH and CEHHP bleached *L. leucocephala* kraft pulp by 1.5 and 1.9%, respectively. Similarly, the brightness of kraft pulp of *C. equisetifolia* after XECEHH and XECEHHP bleaching sequences improves by 1.0 and 2.1%, respectively, as compared to pulp brightness of CEHH and CEHHP bleaching sequences. Tables 5.7 and 5.8 show the effect of

xylanase pretreatment for kraft pulps of *L. leucocephala* and *C. equisetifolia*, respectively, on kappa number and brightness during ODED and ODEP bleaching sequences. The kappa numbers of unbleached kraft pulps of *L. leucocephala* and *C. equisetifolia* decrease by 5.7 units (34.15%) and 5.2 units (29.8%), respectively, after introduction of O_2 (pressure 5 kg/cm^2). The introduction of O_2 after xylanase treatment reduces the kappa number of unbleached kraft pulps of *L. leucocephala* and *C. equisetifolia* by 8.4 units (50.48%) and 7.9 units (45.59%), respectively. Xylanase pretreatment increases the brightness of *L. leucocephala* kraft pulp by 3.3 and 3.6% ISO after XODED and XODEP bleaching sequences, respectively, in comparison with their respective controls. Similarly, The brightness of kraft pulp of *C. equisetifolia* after XODED and XODEP bleaching sequences improves by 2.2 and 3.3% ISO as compared to pulp brightness of ODED and ODEP bleaching sequences. The brightness of kraft pulps of *L. leucocephala* and *C. equisetifolia* increases by 2.3 and 3.1%, respectively, after XO(E_{OP})P bleaching sequence as compared to control O(E_{OP})P) (Table 5.10).

This improvement in brightness and reduction in kappa number of *L. leucocephala* and *C. equisetifolia* pulps after xylanase treatment, possibly result from the partly degradation of xylan on the fiber surface and destruction of LCC; thus improving the accessibility of bleaching chemicals with facilitating the removal of dissolved lignin during washing and bleaching steps [95, 129]. Xylanase treatment increases the penetration of bleaching chemicals into the pulp. It facilitates the movement of the small lignin fragments outward and thus, increases the brightness and reduces the kappa number of pulp at the same or less bleaching reagent consumption [117]. The pretreatment of wheat straw pulp with xylanase produced by *A. niger* An76, showed 4-5% ISO improvement in brightness in comparison with control (CEH) [127]. The pretreatment of birch wood kraft pulp with cellulase free xylanase produced from *Streptomyces thermoviolaceus* reduced the kappa number by 18% [29]. A 30% reduction in kappa number was noticed for kraft pulp of *Eucalyptus* by treating with xylanase from *Staphylococcus* sp. SG-13 and 8% hypochlorite [33]. Singh and co-workers observed 5.17 and 2.58% ISO improvement in brightness of soda–AQ pulp of wheat straw after bio-bleaching with two xylanases; enzyme-A and enzyme-B, during CEHH bleaching at 4.5% chlorine charge [104]. They also observed that the pretreatment of wheat straw soda-AQ pulp with cellulase free xylanases i.e. enzyme-A and enzyme-B reduced the kappa number by 24.38 and 27.94%, respectively, as compared to control [104]. A 50% reduction in the kappa number of unbleached pulp of *Melocanna baccifera* after ODL was observed at 0.50 mPa O_2 pressure

[111]. The kappa numbers of unbleached hardwood and unbleached bagasse pulp were mitigated by 59 and 60%, respectively, after ODL bleaching sequence at an O_2 pressure of 0.50 mPa [81]. Bio-bleaching of bagasse pulp with xylanase produced by *T. lanuginosus* SSBP increased the pulp brightness by 4.5% as compared to control (DED) [13] while the brightness of bagasse and softwood kraft pulps was increased by 3.1 and 5.1% as compared to controls after ECF bleaching using a commercial enzyme (xylanase-P) [62].

5.3.2.2. Effect of xylanase pretreatment on total chlorine demand and pulp viscosity

The TCD of kraft pulp of *L. leucocephala* bleached by XECEHH and XECEHHP reduces by 15.3% in comparison with TCD of both CEHH and CEHHP bleaching sequences (Table 5.4). Similarly, TCD of kraft pulp of *C. equisetifolia* bleached by XECEHH and XECEHHP reduces by 17.16% in comparison with TCD of both CEHH and CEHHP bleaching sequences (Table 5.5). As a result of xylanase pretreatment, the requirement of ClO_2 charge applied to the pulp of *L. leucocephala* is reduced by 24.64% during XODED and XODEP bleaching sequences as compared to controls (ODED and ODEP) (Table 5.7). Similarly, for *C. equisetifolia*, it is reduced by 22.22% as compared to controls (Table 5.8). Enzymatic prebleaching of hardwood and softwood pulps showed about 35-41% and 10-20% reduction, respectively, in the active chlorine at the chlorination stage [95, 8, 115]. The enzymatic prebleaching of wheat straw pulp prior to H, CH or CEH bleaching with xylanase produced by *A. niger* An76 reduced the chlorine consumption by 20-30% [127]. Treatment of kraft pulp with xylanase-pectinase enzyme mixture obtained from *Bacillus pumilus* showed 25% reduction in active chlorine consumption in subsequent bleaching stages while maintaining the same brightness level [47].

The viscosity of kraft pulp of *L. leucocephala* after XECEHH and XECEHHP bleaching sequences improves by 6.07 and 5.13%, respectively, over viscosity of CEHH and CEHHP bleached pulp (Table 5.6). In the same manner, the viscosity of kraft pulp of *C. equisetifolia* after XECEHH and XECEHHP bleaching sequences improves by 2.76 and 4.23%, respectively, over viscosity of CEHH and CEHHP bleached pulp (Table 5.6). Xylanase treatment before ODED and ODEP bleaching sequences, improves the viscosity of *L. leucocephala* kraft pulp by 2.84 and 1.88%, respectively (Table 5.9). In the same manner, the viscosity of *C. equisetifolia* kraft pulp improves by 1.86 and 2.74% after XEODED and XEODEP bleaching sequences over viscosity of ODED and ODEP bleached pulp. The viscosity of kraft pulps of *L. leucocephala* and *C. equisetifolia* after XO(E_{OP})P bleaching

sequence improves by 2.5 and 1.5%, respectively, over viscosity of control pulps ($O(E_{OP})P$) (Table 5.11).

The improvement in viscosity of xylanase pretreated pulps reflects the selective hydrolysis of low DP xylan and enrichment of high molecular weight carbohydrates [74, 88, 101, 121]. Similar results were observed by Kaur and co-workers who found the slight increment in viscosity of soda-AQ pulps of lemon and sofia grasses after crude xylanase pretreatment during conventional, ECF and TCF bleaching sequences [48].

The crude enzyme filtrate used in this study has a high xylanase activity with negligible cellulase contamination, and the enhancement in viscosity of xylanase pretreated pulps under this study confirms that the cellulase contamination in the crude enzyme extract has no adverse effect on pulps of *L. leucocephala* and *C. equisetifolia*. Many studies reported that the nonspecific endoglucanases affected the viscosity of softwood kraft pulp negatively, indicating the degradation of high molecular weight cellulose chains [122, 103].

5.3.2.3. Effect of xylanase pretreatment on pulp beating and bleaching losses

The effect of xylanase treatment on beating response for kraft pulps of *L. leucocephala* and *C. equisetifolia* during CEHH and CEHHP bleaching sequences is shown in Table 5.6. XECEHH and XECEHHP bleached kraft pulp of *L. Leucocephala* requires respectively, 22.85 and 23.32% lesser PFI revolutions for reaching the same beating level (40 °SR) as compared to controls. Similarly, the kraft pulp of *C. equisetifolia* requires 22.55 and 24.42% lesser PFI revolutions after XECEHH and XECEHHP bleaching sequences**,** respectively, for reaching the same beating level (45 °SR) as compared to controls. Table 5.9 illustrates the effect of xylanase treatment on beating response for kraft pulps of *L. leucocephala* and *C. equisetifolia* during ODED and ODEP bleaching sequences. XODED and XODEP bleached kraft pulps of *L. Leucocephala* require 17.07 and 12.01% lesser PFI revolutions, respectively, for reaching the targeted beating level (40 °SR) as compared to controls. Similarly**,** kraft pulps of *C. equisetifolia* require 15.1 and 10.11% lesser PFI revolutions after XODED and XODEP**,** respectively, as compared to controls (ODED and ODEP), for reaching the same beating level (45 °SR). Table 5.11 shows the effect of xylanase treatment on beating response for kraft pulps of *L. leucocephala* and *C. equisetifolia* during $O(E_{OP})P$ bleaching sequence. $XO(E_{OP})P$ bleached kraft pulps of *L. leucocephala* and *C. equisetifolia* show 10.53 and 8.08% lesser PFI revolutions as compared to their respective controls ($O(E_{OP})P$).

The results show the reduction in PFI revolution after xylanase treatment compared to their respective controls, and it exhibits a decreasing order with respect to conventional, ECF and TCF bleaching sequences to attain the fixed level of beating. This may be due to the presence of lesser quantity of low DP xylan in the pulps bleached by ECF and TCF bleaching sequences because oxygen delignification during ECF and TCF has detrimental effect on xylan [90]. It is worth mentioning here that the hemicellulose (xylan) content is favorable for beating of pulp [2]. Enzyme treatment of pulp increases the swelling and absorption capability of fibers in alliance with the improvement in refining properties and reduction in refining energy [131, 17, 43]. Enzyme treatment of pulp can degrade the fine fibers and make the pulp easier to be beaten or refined along with improvement in fiber drainage and pulp physical strength [130]. Xylanase pretreated pulp shows a decrease in refining energy consumption (12.5 to 18%) and PFI mill revolutions (1000r to 4500r) while reaching to the same freeness level [129].

The bleaching losses of kraft pulp of *L. leucocephala* during XECEHH and XECEHHP bleaching sequences are 1.1 and 1.3% lesser as compared to CEHH and CEHHP bleaching sequences, respectively (Table 5.4). Similarly, the respective values for bleaching losses of kraft pulp of *C. equisetifolia* are 1.2 and 1.0% lesser as compared to CEHH and CEHHP bleaching sequences (Table 5.5). The bleaching losses in *L. leucocephala* kraft pulp decrease by 1.4 and 1.5% after XODED and XODEP bleaching sequences as compared to ODED and ODEP (Table 5.7). While the value of bleaching losses in *C. equisetifolia* kraft pulp reduces by 0.5% after XODED and XODEP bleaching sequences compared to controls (Table 5.8). The bleaching losses in *L. leucocephala* and *C. equisetifolia* pulps during XO(E_{OP})P bleaching sequence are 0.9 and 0.6% lesser compared to O(E_{OP})P (Table 5.10).

The results show that xylanase pretreated pulps show lesser bleaching losses as compared to untreated pulps. This may be because of lower amount of chemical charge applied to the xylanase pretreated pulps which is beneficial for bleaching yield. On the other hand, the higher chemical charge applied to the control pulps has a detrimental effect on pulp yield and results into higher amount of bleaching losses.

5.3.2.4. Effect of xylanase pretreatment on mechanical strength properties

Table 5.6 illustrates the effect of xylanase treatment on mechanical strength properties during CEHH and CEHHP bleaching sequences of *L. leucocephala* and *C. equisetifolia* kraft pulps. Figures 5.10A and B show the effect of xylanase treatment on tensile, tear, burst indexes and double fold of kraft pulps of *L. leucocephala* and *C. equisetifolia*, respectively, during

CEHH and CEHHP bleaching sequences. The tensile, tear, burst indexes and double fold of kraft pulps of *L. leucocephala* are increased by 12.15, 7.73, 10.56 and 10.96%, respectively, after XECEHH bleaching sequence as compared to CEHH bleaching sequence whereas the respective improvements are 15.68, 9.98, 17.24 and 15.28% after XECEHHP bleaching sequence as compared to CEHHP bleaching sequence (Figure 5.10A). Likewise, the tensile index, tear index, burst index and double fold of kraft pulp of *C. equisetifolia* are increased by 20.18, 3.15, 13.74 and 13.33%, respectively, after XECEHH bleaching sequence as compared to CEHH bleaching sequence whereas the respective improvements are 17.17, 3.09, 14.43 and 16.07% after XECEHHP bleaching sequence as compared to CEHHP bleaching sequence (Figure 5.10B).

Table 5.9 illustrates the effect of xylanase treatment on mechanical strength properties of *L. leucocephala* and *C. equisetifolia* kraft pulps during ODED and ODEP bleaching sequences. For *L. leucocephala,* tensile, tear, burst indexes and double fold are increased by 11.43, 4.91, 9.89 and 9.30%, respectively, after XODED as compared to ODED bleaching sequence whereas the respective improvements are 9.80, 5.45, 7.97 and 7.59% after XODEP bleaching sequence as compared to ODEP bleaching sequence (Figure 5.13A). For *C. equisetifolia,* the tensile index, tear index, burst index and double fold are increased by 11.22, 5.12, 12.66 and 7.69%, respectively, after XODED bleaching sequence as compared to ODED bleaching sequence whereas the respective improvements are 11.57, 4.72, 11.86 and 10.67% after XODEP bleaching sequence as compared to ODEP bleaching sequence (Figure 5.13B). Table 5.11 illustrates the effect of xylanase treatment on mechanical strength properties of *L. leucocephala* and *C. equisetifolia* kraft pulp during O(E_{OP})P bleaching sequence. For *L. leucocephala,* the tensile index, tear index, burst index and double fold are increased by 2.05, 3.71, 1.55 and 6.25%, respectively, after XO(E_{OP})P compared to O(E_{OP})P bleaching sequence whereas the respective improvements are 3.2, 3.17, 0.97 and 7.46% for *C. equisetifolia* (Figures 5.16A and B).

Xylanase treatment facilitates the removal of lignin in association with degradation of hemicellulose into the pulp; thus the fibrillation extent of the fibers is increased due to loosening and softening of fibers by xylanase treatment [129]. Enzyme treatment of pulp improves the fiber flexibility as well as internal and external fibrillation [42]. The improvement in flexibility enhances the inter fiber bonding in the pulp [35]. Enzymes facilitate refining by softening the fiber walls, and they break the impermeable primary wall of fibers and increase

access to cellulose fibers thus resulting into better physical properties [22]. The enzyme pretreated fibers are softer, more porous and have lower fine content and higher fibrillation extent as compared to untreated fibers [129]. Xylanase pretreatment of pulp hydrolyzes the microfibers, degrades the xylan and residual LCC structure in accordance with the improvement in fiber flexibility and fiber bonding capability. Therefore, the enzyme pretreated fibers have higher fiber bonding capability and pulp strength properties. Xylanase degrades the xylan of the amorphous region on the fiber surface, thus increases the relative amount of crystalline area of pulp fiber. Increase in crystallinity contributes to the enhanced fiber flexibility and physical strength properties, and avoids the excessive cutting of fibers [129]. The fiber length and torsion of xyalanse treated pulp were also increased as compared with the untreated pulp. This may be the reason of higher physical strength properties of xylanase pretreated pulp [129]. Reduction of chlorine demand for xylanase pretreated pulps during conventional bleaching can hence be a possible reason for improved strength properties as higher chlorine charge proved to be detrimental for paper strength [127]. In XODED and XODEP bleached kraft pulps of *L. leucocephala,* the ClO_2 charge is 24.64% lesser as compared to controls i.e. ODED and ODEP bleaching sequences while for *C. equisetifolia,* the respective ClO_2 charge is 22.2% lesser as compared to controls (ODED and ODEP). This may be the strong reason for higher strength properties of xylanase pretreated pulps because the high chemical charge (ClO_2) applied to the controls pulps (ODED and ODEP) may have detrimental effect on pulps and results into reduced strength properties.

Yang and co-workers showed that the brightness, tensile index, tearing index, bursting index and folding number of xylanase pretreated APMP pulp of poplar were improved by 2.1 ISO, 16.8, 8.8, 8.9 and 25%, respectively, as compared to control (untreated pulp) [129]. The bio-bleaching of eucalyptus kraft pulp with xylanase enzyme produced from *Streptomyces* sp. QG-11-3, showed an improvement of 63 and 8% in tensile strength and burst factor, respectively, prior to conventional (CEHH) bleaching [9]. The bio-bleaching of *Eucalyptus globulus* kraft pulp with xylanase, increased the tear index while leaving tensile index unaffected [89] and bio-bleaching of wheat straw pulp with xylanase obtained from *Streptomyces cyaneus* SN32, had shown an increment in tear index and burst factor [71]. Xylanase hydrolyzes the hemicellulose selectively without lowering the fiber strength [50]. The contamination of cellulase enzyme present in the crude enzyme mixture could also have played an important role for improving the strength properties of enzymatically treated pulps. Bolaski

and co-workers reported the fibrillation of fibers and enhancement in strength properties by cellulase treatment [14]. Cellulase treatment improved the brightness, tensile index, tearing index, bursting index and folding number of poplar APMP pulp by 1.2%, 23.7%, 14.8%, 14.6% and 50% ISO respectively while reducing the PFI mill revolutions (from 1000r to 5500r) and beating energy consumption (from 12.5% to 22.0%) [129]. On the contrary, some researchers observed the reduction in the mechanical strength properties of enzymatically treated pulps of *D. strictus* as compared to their respective controls [85].

5.3.2.5. Effect of xylanase pretreatment on combined bleach effluent characteristics

Table 5.6 shows the effect of enzyme treatment on AOX, COD and color of combined effluents generated during CEHH and CEHHP bleaching sequences for kraft pulps of *L. leucocephala* and *C. equisetifolia*. For *L. leucocephala* pulp, the AOX level is reduced by 33.33 and 39.01% and for *C. equisetifolia* pulp, it is reduced by 23.85 and 28.93% after XECEHH and XECEHHP bleaching sequences, respectively, as compared to their respective controls (CEHH and CEHHP) (Figures 5.11A and B). The COD load and color values of combined effluent of kraft pulp of *L. leucocephala* during XECEHH bleaching sequence are increased by 15.16 and 12.52%, respectively, as compared to CEHH bleaching sequence whereas the improvement in COD and color values are 11.33 and 10.1%, respectively, during XECEHHP bleaching sequence as compared to CEHHP bleaching sequence (Figure 5.12A). The COD load and color values of combined effluent of kraft pulp of *C. equisetifolia* during XECEHH bleaching sequence are increased by 8.0 and 10.31%, respectively, as compared to CEHH bleaching sequence whereas the improvement in COD load and color values are 7.31 and 9.93%, respectively, during XECEHHP bleaching sequence as compared to CEHHP bleaching sequence (Figure 5.12B).

Table 5.9 shows the effect of enzyme treatment on AOX, COD and color of combined effluents generated during ODED and ODEP bleaching sequences for kraft pulps of *L. leucocephala* and *C. equisetifolia*. The AOX of combined effluent of XODED and XODEP bleached kraft pulps of *L. leucocephala* shows a significant decrease of 43.33 and 48.86%, respectively, as compared to their respective controls (Figure 5.14A). As would be expected, the AOX of combined effluent of kraft pulp of *C. equisetifolia* bleached by XODED and XODEP also shows reduction i.e. 37.5 and 38.71%, respectively, as compared to their controls (Figure 5.14B). The COD load and color values of combined effluents of each bleaching sequence i.e. XODED and XODEP for kraft pulp of *L. leucocephala* and *C. equisetifolia* are on

higher side than ODED and ODEP (Figure 5.15). For *L. leucocephala*, the percent increment in COD and color values of combined effluent are 11.35 and 13.18%, respectively, during XODED bleaching sequence as compared to ODED while during XODEP the respective increments are 14.92 and 12.43% as compared to ODEP (Figure 5.15A). Similarly, The COD load and color values of combined effluent of kraft pulp for *C. equisetifolia* are increased by 13.71 and 12.93%, respectively, during XODED bleaching sequence as compared to ODED whereas during XODEP the respective increments are 12.88 and 13.94% as compared to ODEP (Figure 5.15B). Table 5.11 demonstrates the effect of enzyme treatment on COD and color of combined effluents generated during XO(E_{OP})P bleaching sequence for kraft pulps of *L. leucocephala* and *C. equisetifolia*. COD and color values of combined effluent for kraft pulp of *L. leucocephala* are increased by 13.91 and 12.61%, respectively, during XO(E_{OP})P as compared to O(E_{OP})P bleaching sequence (Figure 5.17A). Similarly, COD and color values of combined effluent for kraft pulp of *C. equisetifolia* are increased by 13.12 and 12.62%, respectively, during XO(E_{OP})P as compared to O(E_{OP})P bleaching sequence (Figure 5.17B).

The chlorine reacts with residual lignin present in fibers and produces the organochlorine compounds during bleaching process [6]. The xylanase pretreatment reduces the TCD in bleaching sequences; this results into the low toxicity of the spent bleach liquor [113]. Hence, the bio-bleaching using xylanase enzyme reduces the amount of chlorophenols and AOX in the bleaching effluent [4, 116]. Senior and Hamilton reported the reduction of about 20-45% in AOX level generated after xylanase pretreatment of pulp [96]. Bio-bleaching of kraft pulp by xylanase enzyme replaced the chlorine dioxide by 5-7 kg per tonne of kraft pulp [80]. The effluent AOX of eucalyptus kraft pulp was reduced by 15.09% after pretreatment with commercial xylanase [40]. Many researchers observed the improvement in pulp properties, reduction in chlorine demand and AOX generation in bleach effluent by the xylanase pretreatment of kraft pulps [100, 113]. Enzymes dissolve the carbohydrate bonds present in pulp; this leads to an increase in the concentration of hydrolyzed xylan and residual lignin carbohydrate complexes in the effluent which ultimately results into an increase in COD and color. Many researchers observed the similar results [88, 63, 121]. Bio-bleaching of eucalyptus kraft pulp with commercial xylanase showed 32.19 and 107.89% increment in COD and BOD load of combined bleach effluent, respectively, as compared to control [40, 41]. Bio-bleaching of *E. globulus* pulp with xylanase showed an increase of 27.76% in the color of bleaching effluent of OD_1PD_2 sequence [121].

Chlorine dioxide is a stronger bleaching agent compared to elemental chlorine, due to its lower atomic chlorine content than chlorine [98]. It acts as an oxidative reagent during bleaching process and reduces the amount of AOX formation in the effluents [64, 67]. ECF bleaching effectively reduces the chloroform and total organic halides (AOX) formation by 90% [82]. $MgSO_4$ prevents the degradation of cellulose fibers during oxygen bleaching which causes pulp degradation and reduction in paper strength. Guay and co-workers suggested that hydroxyl free radicals ($OH^{\bullet}$) are formed during oxygen delignification; hemicellulose polymer protects the cellulose from degradation caused by hydroxyl free radicals ($OH^{\bullet}$) [30]. Use of sodium salts of ethylenediamine tetraacetic acid (EDTA) mitigates the degradation of polysaccharides and masks the activities of transition metals by forming polydendate ligand molecules. The xylanase treated pulps of *L. leucocephala* and *C. equisetifolia* result into an increase in residual ClO_2 after ECF bleach sequences. It can be concluded that the treated pulps require lesser amount of chemicals and reach the chlorine saturation point sooner than the control pulps [34]. Moreover, it explains the lower level of AOX obtained after bio-bleaching of the two pulps. The COD of bleaching effluent of *E. globulus* pulp generated during ODPD sequence was increased by 51.13%, after xylanase pretreatment [121]. Many researchers observed the similar trends for COD increment after xylanase pretreatment [63, 41, 5]. Many researchers observed that xylanase pretreatment increased the color of bleaching effluents, and explained that it was due to the solubilisation of xylan and more lignin in xylanase treated pulp as compared to control [88, 121].

5.3.3. SEM studies

The unbleached (control), xylanase pretreated and oxygen delignified pulps of *L. leucocephala* and *C. equisetifolia* are observed under SEM. The unbleached and untreated samples of *L. leucocephala* and *C. equisetifolia* pulps show smooth fibers without any external fibrillation and swelling (Photomicrographs 5.1A and 5.2A). The xylanase pretreated pulps of *L. leucocephala* and *C. equisetifolia* show significant changes on the surface of fibers (Photomicrographs 5.1B and 5.2B). The xylanase pretreated pulp fibers show cracks, delamination of cell wall due to peeling, swelling and external fibrillation on their surface. Xylanase hydrolyzes the redeposited xylan which is attached on to the fiber surface during kraft pulping. The elimination of the deposited xylan increases the flow of bleaching chemicals into the fiber; this explains the bleach boosting effect of the xylanase [89, 95]. The observations are in accordance with the views given by other researchers [13, 89, 29]. Beg and co-workers

explained that xylanase obtained from *Streptomyces* sp. QG-11-3 enhanced the porosity, swelling and fibrillation in the fibers of eucalyptus pulp compared to control [9]. Li and co-workers observed the cracks and peelings on to the fiber surface after xylanase pretreatment [60]. Photomicrographs 5.1C and 5.2C show the untreated and unbleached pulps of *L. leucocephala* and *C. equisetifolia,* respectively, beaten at 40 ±1 °SR and 45±1 °SR, respectively. Photomicrographs 5.1D and 5.2D represent the xylanase pretreated pulps of *L. leucocephala* and *C. equisetifolia* beaten at 40±1 °SR and 45±1 °SR, respectively. Photomicrographs 5.1E and 5.2E show the oxygen delignified pulps of *L. leucocephala* (40±1 °SR) and *C. equisetifolia (*45±1 °SR), respectively.

These Photomicrographs show that the xylanase pretreated fibers are rougher and contain more fibril after beating as compared to untreated and oxygen delignified fibers. Therefore, it can be concluded that xylanase treatment saves the refining energy as the xylanase pretreated fibers would require lesser PFI revolution for reaching to a fixed °SR in comparison to untreated fibers [129, 11].

Table 5.1: Optimization of enzyme dose for prebleaching of kraft pulps of *L. leucocephala* and *C. equisetifolia*

Raw material	Enzyme dose (IU/g)	* Kappa number	*Brightness (%) ISO	Reducing sugars released (mg/g)	Chromophores released (optical density)		
					237 nm	280 nm	465 nm
L. leucocephala	0	16.50±0.30	45.15±1.0	0.19±0.01	0.051±0.002	0.035±0.001	0.016±0.001
	2	16.14±0.28	48.92±1.2	0.77±0.02	0.160±0.010	0.094±0.001	0.089±0.001
	5	14.65±0.38	53.39±1.5	2.16±0.11	0.315±0.018	0.196±0.011	0.177±0.009
	10	14.69±0.28	53.15±1.5	4.22±0.15	0.357±0.006	0.243±0.010	0.215±0.008
	15	14.67±0.37	53.00±1.1	6.37±0.12	0.396±0.016	0.252±0.001	0.231±0.001
	20	14.71±0.25	52.30±1.4	7.14±0.29	0.410±0.006	0.267±0.001	0.256±0.004
	25	14.74±0.16	52.10±1.0	7.52±0.24	0.401±0.010	0.273±0.003	0.269±0.010
C. equisetifolia	0	17.32±0.21	42.15±1.5	0.11±0.01	0.019±0.001	0.013±0.002	0.011±0.004
	2	16.50±0.18	43.82±1.1	0.60±0.02	0.295±0.013	0.245±0.010	0.237±0.010
	5	15.71±0.25	45.89±1.3	1.50±0.05	0.406±0.013	0.327±0.010	0.317±0.003
	10	14.72±0.24	48.72±1.4	3.04±0.08	0.460±0.010	0.349±0.016	0.333±0.009
	15	14.85±0.25	48.76±1.1	4.23±0.10	0.474±0.006	0.368±0.011	0.358±0.015
	20	15.08±0.24	48.52±1.3	4.63±0.07	0.489±0.008	0.417±0.016	0.404±0.035
	25	15.09±0.38	48.48±1.5	4.94±0.08	0.504±0.007	0.421±0.016	0.415±0.012

* refers values after XE stage

± refers standard deviation

Enzymatic prebleaching conditions (X-stage): reaction time = 120 min, pH = 8.3, consistency = 6% and temperature = 55±2 °C

Extraction stage: 2% NaOH at 70±2 °C temperature for 90 min, 11.5 pH

Table 5.2: Optimization of reaction time for prebleaching of kraft pulps of *L. leucocephala* and *C. equisetifolia*

Raw material	Reaction time (min)	* Kappa number	*Brightness (%) ISO	Reducing sugars released (mg/g)	Chromophores released (optical density)		
					237 nm	280 nm	465 nm
L. leucocephala	0	16.70±0.22	44.15±1.2	0.0	0.0	0.0	0.0
	30	16.49±0.28	47.85±1.3	0.53±0.02	0.054±0.009	0.036±0.004	0.024±0.003
	60	15.67±0.16	49.27±1.5	1.16±0.04	0.149±0.009	0.111±0.011	0.109±0.008
	90	15.03±0.09	51.62±1.4	1.89±0.08	0.301±0.014	0.161±0.005	0.160±0.019
	120	14.54±0.29	54.25±1.6	2.27±0.06	0.335±0.025	0.209±0.029	0.190±0.022
	150	14.50±0.22	54.10±1.2	2.35±0.06	0.342±0.015	0.214±0.027	0.226±0.019
	180	14.56±0.16	53.87±1.3	2.42±0.05	0.346±0.014	0.220±0.016	0.230±0.017
C. equisetifolia	0	17.42±0.33	41.50±1.1	0.0	0.0	0.0	0.0
	30	16.65±0.18	42.63±1.4	0.81±0.02	0.131±0.003	0.125±0.001	0.102±0.001
	60	15.48±0.21	44.35±1.4	1.61±0.06	0.279±0.007	0.244±0.008	0.230±0.013
	90	15.02±0.37	46.76±1.3	2.74±0.09	0.423±0.018	0.324±0.017	0.329±0.018
	120	14.55±0.36	49.15±1.5	3.10±0.06	0.465±0.009	0.361±0.018	0.340±0.011
	150	14.50±0.18	49.21±1.6	3.19±0.07	0.461±0.008	0.356±0.008	0.338±0.010
	180	14.52±0.18	49.21±1.5	3.28±0.07	0.465±0.009	0.360±0.008	0.343±0.011

* refers values after XE stage

± refers standard deviation

Enzymatic prebleaching conditions (X-stage): enzyme dose 5 IU/g for *L. leucocephala* and 10 IU/g for *C. equisetifolia*, pH = 8.3, consistency = 6% and temperature = 55±2 °C

Extraction stage: 2% NaOH at 70±2 °C temperature for 90 min, 11.5 pH

Table 5.3: Optimization of pulp consistency for prebleaching of kraft pulps of *L. leucocephala* and *C. equisetifolia*

Raw material	Pulp consistency (%)	* Kappa number	*Brightness (%) ISO	Reducing sugars released (mg/g)	Chromophores released (optical density)		
					237 nm	280 nm	465 nm
L. leucocephala	2	15.97±0.16	46.06±1.3	0.81±0.01	0.198±0.011	0.095±0.001	0.067±0.002
	4	15.29±0.18	48.63±1.2	1.57±0.07	0.249±0.014	0.144±0.008	0.117±0.010
	6	14.65±0.18	53.33±1.6	2.18±0.03	0.326±0.009	0.198±0.015	0.171±0.019
	8	14.24±0.08	56.29±1.5	2.26±0.05	0.382±0.013	0.233±0.018	0.225±0.015
	10	14.25±0.21	56.26±1.4	2.38±0.03	0.388±0.006	0.236±0.004	0.237±0.013
	12	14.21±0.21	56.25±1.5	2.48±0.01	0.382±0.009	0.241±0.011	0.245±0.009
C. equisetifolia	2	16.48±0.25	41.15±1.0	0.85±0.02	0.209±0.012	0.089±0.004	0.087±0.003
	4	15.24±0.28	45.37±1.4	1.68±0.04	0.272±0.009	0.185±0.009	0.177±0.009
	6	14.86±0.29	48.97±1.6	2.75±0.09	0.468±0.022	0.338±0.026	0.335±0.008
	8	14.33±0.23	51.87±1.5	3.16±0.04	0.507±0.012	0.388±0.006	0.396±0.008
	10	14.30±0.09	51.8±1.2	3.27±0.05	0.514±0.007	0.381±0.006	0.402±0.005
	12	14.28±0.13	51.82±1.2	3.41±0.05	0.512±0.016	0.384±0.009	0.393±0.008

* refers values after XE extraction
± refers standard deviation
Enzymatic prebleaching conditions (X-stage): enzyme dose 5 IU/g for *L. leucocephala* and 10 IU/g for *C. equisetifolia*, reaction time = 120 min, pH = 8.3 and temperature = 55±2 °C
Extraction stage: 2% NaOH at 70±2 °C temperature for 90 min, 11.5 pH

Table 5.4: Effect of xylanase pretreatment on conventional bleaching sequences of kraft pulp of *L. leucocephala*

Particular	Bleaching sequence						
	CEHH		**XECEHH**		**CEHHP**		**XECEHHP**
Xylanase (X) stage							
#Enzyme dose (IU/g)	–		5		–		5
Extraction (E_1) stage							
#NaOH applied (%)	–		2		–		2
Kappa number of xylanase treated pulp	–		14.15±0.09		–		14.15±0.09
Chlorination (C) stage							
#Cl_2 applied as available Cl_2 (%)	2.09		1.77		2.09		1.77
#Cl_2 consumed as available Cl_2 (%)	2.06		1.75		2.08		1.72
Amount of Cl_2 consumed (%)	98.6		98.9		99.5		97.2
Extraction (E_2) stage							
#NaOH applied (%)	2		1		2		1
Hypochlorite (H_1) stage							
#$Ca(OCl)_2$ applied as available Cl_2 (%)	1.46		1.24		1.46		1.24
#$Ca(OCl)_2$ consumed as available Cl_2 (%)	1.40		1.21		1.41		1.22
Amount of $Ca(OCl)_2$ consumed (%)	95.9		97.6		96.6		98.4
Hypochlorite (H_2) stage							
#$Ca(OCl)_2$ applied as available Cl_2 (%)	0.63		0.53		0.63		0.53
#$Ca(OCl)_2$ consumed as available Cl_2 (%)	0.58		0.49		0.59		0.50
Amount of $Ca(OCl)_2$ consumed (%)	92.1		92.5		93.7		94.3
Peroxide (P) stage							
#H_2O_2 applied (%)	–		–		2		2
#H_2O_2 consumed (%)					1.99		1.94
#$MgSO_4$ applied (%)					0.1		0.1
# EDTA applied (%)					0.5		0.5
#Total Cl_2 applied (%)	4.18		3.54		4.18		3.54
#Total Cl_2 consumed (%)	4.04		3.45		4.08		3.44
Amount of total Cl_2 consumed (%)	96.65		97.46		97.61		97.18
Total residual Cl_2 (%)	3.35		2.54		2.39		2.82
Bleaching losses (%)	8.5		7.4		8.9		7.6
Bleached pulp yield (%)	45		46.1		44.6		45.9
Pulp brightness (%) ISO	76.0±1.1		77.5±1.0		80.6±0.92		82.5±1.1
Pulp viscosity (cm^3/g)	492.5±15.5		524.3±12.6		425.5±9.5		448.5±8.7
Bleaching conditions	**X**	**E_1**	**C**	**E_2**	**H_1**	**H_2**	**P**
Consistency (%)	8	10	3	10	10	10	10
pH	8.3	11.5	2	11.5	11	11	11.5
Temperature (°C)	55±2	70±2	Ambient	70±2	70±2	70±2	90±2
Reaction time (min)	120	90	30	90	60	60	120
Unbleached pulp kappa number = 16.72±0.28, unbleached pulp brightness = 44.03±1.1 and unbleached pulp viscosity = 987.1±17.21 cm^3/g							

± refers standard deviation; # refers chemical charge on O.D. pulp basis

Table 5.5: Effect of xylanase pretreatment on conventional bleaching sequences of kraft pulp of *C. equisetifolia*

Particular	Bleaching sequence			
	CEHH	**XECEHH**	**CEHHP**	**XECEHHP**
Xylanase (X) stage				
#Enzyme dose (IU/g)	–	10	–	10
Extraction (E_1) stage				
#NaOH applied (%)	–	2	–	2
Kappa number of xylanase treated pulp	–	14.47±0.06	–	14.47±0.06
Chlorination (C) stage				
#Cl_2 applied as available Cl_2 (%)	2.19	1.81	2.19	1.81
#Cl_2 consumed as available Cl_2 (%)	2.17	1.80	2.15	1.79
Amount of Cl_2 consumed (%)	99.1	99.5	98.2	98.9
Extraction (E_2) stage	2	1	2	1
#NaOH applied (%)				
Hypochlorite (H_1) stage				
#$Ca(OCl)_2$ applied as available Cl_2 (%)	1.53	1.27	1.53	1.27
#$Ca(OCl)_2$ consumed as available Cl_2 (%)	1.52	1.23	1.51	1.21
Amount of $Ca(OCl)_2$ consumed (%)	99.3	96.9	98.7	95.3
Hypochlorite (H_2) stage				
#$Ca(OCl)_2$ applied as available Cl_2 (%)	0.66	0.54	0.66	0.54
#$Ca(OCl)_2$ consumed as available Cl_2 (%)	0.62	0.51	0.64	0.50
Amount of $Ca(OCl)_2$ consumed (%)	93.9	94.4	96.9	92.6
Peroxide (P) stage				
#H_2O_2 applied (%)	–	–	2	2
#H_2O_2 consumed (%)			1.98	1.96
#$MgSO_4$ applied (%)			0.1	0.1
# EDTA applied (%)			0.5	0.5
Total Cl_2 applied (%)	4.37	3.62	4.37	3.62
Total Cl_2 consumed (%)	4.31	3.54	4.30	3.50
Amount of total Cl_2 consumed (%)	98.6	97.8	98.4	96.7
Total residual Cl_2 (%)	1.4	2.2	1.6	3.3
Bleaching losses (%)	8.2	7.0	8.8	7.8
Bleached pulp yield (%)	43.1	44.3	42.5	43.5
Pulp brightness (%) ISO	75.2±0.80	76.2±1.0	79.0±0.78	81.1±0.83
Pulp viscosity (cm^3/g)	510.5±7.6	525.0±8.9	485.0±7.2	506.4±11.4

Bleaching conditions	**X**	**E_1**	**C**	**E_2**	**H_1**	**H_2**	**P**
Consistency (%)	8	10	3	10	10	10	10
pH	8.3	11.5	2	11.5	11	11	11.5
Temperature (°C)	55±2	70±2	Ambient	70±2	70±2	70±2	90±2
Reaction time (min)	120	90	30	90	60	60	120

Unbleached pulp kappa number =17.46±0.26, unbleached pulp brightness = 41.49±1.0% and unbleached pulp viscosity = 995±18.5 cm^3/g

± refers standard deviation; # refers chemical charge on O.D. pulp basis

Table 5.6: Effect of xylanase pretreatment on beating, mechanical strength properties and combined effluent characteristics generated during conventional bleaching of kraft pulps of *L. leucocephala* and *C. equisetifolia*

Particular	CEHH	XECEHH	Diff. (%)	CEHHP	XECEHHP	Diff. (%)
L. leucocephala						
Pulp brightness (%) ISO	76±1.1	77.5±1.0	+1.5	80.6±0.92	82.5±1.1	+1.9
Pulp viscosity (cm^3/g)	492.5±15.5	524.3±12.6	+6.07	425.5±9.5	448.5±8.7	+5.13
Total chlorine demand (%)	4.18	3.54	−15.31	4.18	3.54	−15.31
PFI revolution (number)	6500	5015	−22.85	6110	4685	−23.32
Beating level (°SR)	40±1	40±1	−	40±1	40±1	−
Tensile index (Nm/g)	64.25	73.14	+12.15	62.1	73.65	+15.68
Tear index (mNm^2/g)	8.0	8.67	+7.73	7.4	8.22	+9.98
Burst index ($kPam^2/g$)	6.35	7.10	+10.56	6.0	7.25	+17.24
Double fold (number)	65	73	+10.96	61	72	+15.28
AOX (kg/t)	3.15	2.10	−33.33	3.82	2.33	−39.01
COD (mg/L)	1612	1900	+15.16	1675	1889	+11.33
Color (PCU)	2515	2875	+12.52	2576	2865	+10.1
C. equisetifolia						
Pulp brightness (%) ISO	75.2±0.80	76.2±1.0	+1.0	79.0±0.78	81.1±0.83	+2.1
Pulp viscosity (cm^3/g)	510.5±7.6	525.0±8.9	+2.76	485.0±7.2	506.4±11.4	+4.23
Total chlorine demand (%)	4.37	3.62	−17.16	4.37	3.62	−17.16
PFI revolution (number)	8070	6250	−22.55	7515	5680	−24.42
Beating level (°SR)	45±1	45±1	−	45±1	45±1	−
Tensile index (Nm/g)	54.36	68.10	+20.18	55.25	66.70	+17.17
Tear index (mNm^2/g)	5.85	6.04	+3.15	5.64	5.82	+3.09
Burst index ($kPam^2/g$)	5.15	5.97	+13.74	4.92	5.75	+14.43
Double fold (number)	52	60	+13.33	47	56	+16.07
AOX (kg/t)	3.48	2.65	−23.85	3.56	2.53	−28.93
COD (mg/L)	1725	1875	+8.0	1775	1915	+7.31
Color (PCU)	2610	2910	+10.31	2612	2900	+9.93

± refers standard deviation; +/− refers % improvement/reduction`

Table 5.7: Effect of xylanase pretreatment on ECF bleaching sequences of kraft pulp of *L. leucocephala*

Particular	Bleaching sequence			
	ODED	**XODED**	**ODEP**	**XODEP**
Xylanase (X) stage				
#Enzyme dose (IU/g)	–	5	–	5
Oxygen (O) stage				
O_2 pressure (kg/cm^2)	5	5	5	5
#EDTA applied (%)	0.1	0.1	0.1	0.1
#$MgSO_4$ applied (%)	0.1	0.1	0.1	0.1
#NaOH applied (%)	2	2	2	2
Kappa number after O_2 treatment	11.01±0.58	8.28±0.16	11.01±0.58	8.28±0.16
Chlorine dioxide (D_1) stage				
#ClO_2 applied as available Cl_2 (%)	1.38	1.04	1.38	1.04
#ClO_2 consumed as available Cl_2 (%)	1.36	0.99	1.36	0.99
ClO_2 consumed as Cl_2 basis (%)	98.6	95.2	98.6	95.2
Extraction (E) stage				
#NaOH applied (%)	2	2	2	2
Chlorine dioxide (D_2) stage				
#ClO_2 applied as available Cl_2 (%)	1.38	1.04	–	–
#ClO_2 consumed as available Cl_2 (%)	1.32	0.93		
ClO_2 consumed as Cl_2 basis (%)	95.7	89.4		
Peroxide (P) stage				
#H_2O_2 applied (%)	–	–	2	2
#H_2O_2 consumed (%)			1.98	1.95
#$MgSO_4$ applied (%)			0.1	0.1
# EDTA applied (%)			0.5	0.5
#Total ClO_2 applied (%)	2.76	2.08	1.38	1.04
#Total ClO_2 consumed (%)	2.68	1.92	1.36	0.99
Amount of total ClO_2 consumed on Cl_2 basis (%)	97.1	92.3	98.6	95.2
Total residual ClO_2 on Cl_2 basis (%)	2.9	7.7	1.4	4.8
Bleaching losses (%)	7.9	6.5	8.0	6.5
Bleached pulp yield (%)	45.6	47	45.5	47
Pulp brightness (%) ISO	80.2±0.9	83.5±1.0	80.7±1.1	84.3±0.9
Pulp viscosity (cm^3/g)	527.1±10.5	542.5±13.4	515.5±12.8	525.4±1.0

Bleaching conditions	**O**	**X**	**D_1**	**E**	**D_2**	**P**
Consistency (%)	15	8	10	10	10	10
pH	11.0	8.3	3.2	11.5	3.2	11.5
Temperature (°C)	110±2	55±2	70±2	70±2	70±2	90±2
Reaction time (min)	90	120	120	90	120	120

Unbleached pulp kappa number = 16.72±0.28, unbleached pulp brightness = 44.03±1.1 and unbleached pulp viscosity = 987.1±17.21 cm^3/g

± refers standard deviation; # refers chemical charge on O.D. pulp basis

Table 5.8: Effect of xylanase pretreatment on ECF bleaching sequences of kraft pulp of *C. equisetifolia*

Particulars	Bleaching sequences			
	ODED	**XODED**	**ODEP**	**XODEP**
Xylanase (X) stage				
#Enzyme dose (IU/g)	-	10	-	10
Oxygen (O) stage				
O_2 pressure (kg/cm^2)	5	5	5	5
#EDTA applied (%)	0.1	0.1	0.1	0.1
#$MgSO_4$ applied (%)	0.1	0.1	0.1	0.1
#NaOH applied (%)	2	2	2	2
Kappa number after O_2 treatment	12.25±0.47	9.5±0.28	12.25±0.47	9.5±0.28
Chlorine dioxide (D_1) stage				
#ClO_2 applied as available Cl_2 (%)	1.53	1.19	1.53	1.19
#ClO_2 consumed as available Cl_2 (%)	1.52	1.17	1.52	1.17
ClO_2 consumed as Cl_2 basis (%)	99.3	98.32	99.3	98.32
Extraction (E) stage				
#NaOH applied (%)	2	2	2	2
Chlorine dioxide (D_2) stage				
#ClO_2 applied as available Cl_2 (%)	1.53	1.19	–	–
#ClO_2 consumed as available Cl_2 (%)	1.50	1.15		
ClO_2 consumed as Cl_2 basis (%)	98	96.6		
Peroxide (P) stage				
#H_2O_2 applied (%)	–	–	2	2
#H_2O_2 consumed (%)			1.99	1.94
#$MgSO_4$ applied (%)			0.1	0.1
# EDTA applied (%)			0.5	0.5
#Total ClO_2 applied (%)	3.06	2.38	1.53	1.19
#Total ClO_2 consumed (%)	3.02	2.32	1.52	1.17
Amount of total ClO_2 consumed on Cl_2 basis (%)	98.7	97.5	99.4	98.3
Total residual ClO_2 on Cl_2 basis (%)	1.3	2.5	0.6	1.7
Bleaching losses (%)	7.0	6.5	7.5	7.0
Bleached pulp yield (%)	44.3	44.8	43.8	44.3
Pulp brightness (%) ISO	80±0.7	82.2±0.9	80.2±0.8	83.5±1.0
Pulp viscosity (cm^3/g)	545±13.5	555.3±11.6	535.5±10.8	550.6±13.5

Bleaching conditions	**O**	**X**	**D_1**	**E**	**D_2**	**P**
Consistency (%)	15	8	10	10	10	10
pH	11.0	8.3	3.2	11.5	3.2	11.5
Temperature (°C)	110±2	55±2	70±2	70±2	70±2	90±2
Reaction time (min)	90	120	120	90	120	120

Unbleached pulp kappa number = 17.46±0.26, unbleached pulp brightness = 41.49±1.0 and unbleached pulp viscosity = 995±18.5 cm^3/g

± refers standard deviation; # refers chemical charge on O.D. pulp basis

Table 5.9: Effect of xylanase pretreatment on beating, mechanical strength properties and combined effluent characteristics generated during ECF bleaching of kraft pulps of *L. leucocephala* and *C. equisetifolia*

Particular	ODED	XODED	Diff. (%)	ODEP	XODEP	Diff. (%)
L. leucocephala						
Pulp brightness (%) ISO	80.2±0.9	83.5±1.0	+3.3	80.7±1.1	84.3±0.9	+3.6
Pulp viscosity (cm^3/g)	527.1±10.5	542.5±13.4	+2.84	515.5±12.8	525.4±1.0	+1.88
PFI revolution (number)	5794	4805	−17.07	4456	3921	−12.01
Beating level (°SR)	40±1	40±1	−	40±1	40±1	−
Tensile index (Nm/g)	70.5	79.6	+11.43	69	76.5	+9.80
Tear index (mNm^2/g)	8.32	8.75	+4.91	8.15	8.62	+5.45
Burst index (kPa m^2/g)	6.65	7.38	+9.89	6.35	6.9	+7.97
Double fold (number)	78	86	+9.30	73	79	+7.59
AOX (kg/t)	1.2	0.68	−43.33	1.31	0.67	−48.86
COD (mg/L)	1250	1410	+11.35	1215	1428	+14.92
Color (PCU)	2036	2345	+13.18	2100	2398	+12.43
C. equisetifolia						
Pulp brightness (%) ISO	80±0.7	82.2±0.9	+2.2	80.2±0.8	83.5±1.0	+3.3
Pulp viscosity (cm^3/g)	545±13.5	555.3±11.6	+1.86	535.5±10.8	550.6±13.5	+2.74
PFI revolution (number)	7020	5960	−15.1	5490	4935	−10.11
Beating level (°SR)	45±1	45±1	−	45±1	45±1	−
Tensile index (Nm/g)	65.25	73.50	+11.22	65	73.50	+11.57
Tear index (mNm^2/g)	6.12	6.45	+5.12	5.86	6.15	+4.72
Burst index (kPa m^2/g)	5.45	6.24	+12.66	5.20	5.9	+11.86
Double fold (number)	72	78	+7.69	67	75	+10.67
AOX (kg/t)	1.52	0.95	−37.5	1.55	0.95	−38.71
COD (mg/L)	1316	1525	+13.71	1285	1475	+12.88
Color (PCU)	2155	2475	+12.93	2160	2510	+13.94

± refers standard deviation; +/− refers % improvement/reduction

Table 5.10: Effect of xylanase pretreatment on TCF bleaching sequences of kraft pulps of *L. leucocephala* and *C. equisetifolia*

Particular	***L. leucocephala***		***C. equisetifolia***	
	O(E_{OP})P	**XO(E_{OP})P**	**O(E_{OP})P**	**XO(E_{OP})P**
Xylanase (X) stage				
#Enzyme dose (IU/g)	–	5	–	10
Oxygen (O) stage				
O_2 pressure (kg/cm^2)	5	5	5	5
#EDTA applied (%)	0.1	0.1	0.1	0.1
#$MgSO_4$ applied (%)	0.1	0.1	0.1	0.1
#NaOH applied (%)	2.0	2.0	2.0	2.0
Kappa number after O_2 treatment	11.01±0.58	8.28±0.16	12.25±0.47	9.5±0.28
Extraction (E_{OP}) stage				
#NaOH applied (%)	3.0	3.0	3.0	3.0
#H_2O_2 applied (%)	0.5	0.5	0.5	0.5
O_2 pressure (kg/cm^2)	5	5	5	5
#$MgSO_4$ applied (%)	0.1	0.1	0.1	0.1
Peroxide (P) stage				
#H_2O_2 applied (%)	2.0	2.0	2.0	2.0
#H_2O_2 consumed (%)	1.99	1.97	1.98	1.94
#$MgSO_4$ applied (%)	0.1	0.1	0.1	0.1
# EDTA applied (%)	0.5	0.5	0.5	0.5
#Total H_2O_2 applied (%)	2.5	2.5	2.5	2.5
Bleaching losses (%)	6.5	5.6	6.0	5.4
Bleached pulp yield (%)	47.0	47.9	45.3	45.9
Pulp brightness (%) ISO	74.2±0.8	76.5±0.7	73±1.1	76.1±0.8
Pulp viscosity (cm^3/g)	534.5±11.5	548.2±12.6	550±10.6	558.5±13.8
Bleaching conditions	**X**	**O**	**(E_{OP})**	**P**
Consistency (%)	8	15	10	10
pH	8.3	11.0	11.5	11.5
Temperature (°C)	55±2	110±2	70±2	90±2
Reaction time (min)	120	90	60	120
Unbleached pulp kappa number = 16.72±0.28 (*L. leucocephala*) and 17.46±0.26 (*C. equisetifolia*), unbleached pulp brightness = 44.03±1.1% (*L. leucocephala*) and 41.49±1.0% (*C. equisetifolia*), unbleached pulp viscosity = 987.1±17.21 cm^3/g (*L. leucocephala*) and 995±18.5 cm^3/g (*C. equisetifolia*)				

± refers standard deviation; # refers chemical charge on O.D. pulp basis

Table 5.11: Effect of xylanase pretreatment on beating, mechanical strength properties and combined effluent characteristics generated during TCF bleaching of kraft pulps of *L. leucocephala* and *C. equisetifolia*

Particular	*L. leucocephala*			*C. equisetifolia*		
	$O(E_{OP})P$	**$XO(E_{OP})P$**	**Diff. (%)**	**$O(E_{OP})P$**	**$XO(E_{OP})P$**	**Diff. (%)**
Pulp brightness (%) ISO	74.2±0.8	76.5±0.7	+2.3	73±1.1	76.1±0.8	+3.1
Pulp viscosity (cm^3/g)	534.5±11.5	548.2±12.6	+2.5	550±10.6	558.5±13.8	+1.52
PFI revolution (number)	5700	5100	−10.53	7055	6485	−8.08
Beating level (°SR)	40±1	40±1	−	45±1	45±1	−
Tensile index (Nm/g)	67.0	68.4	+2.05	60.5	62.5	+3.2
Tear index (mNm^2/g)	8.30	8.62	+3.71	5.81	6.0	+3.17
Burst index (kPa m^2/g)	6.35	6.45	+1.55	5.1	5.15	+0.97
Double fold (number)	75	80	+6.25	62	67	+7.46
COD (mg/L)	1300	1510	+13.91	1325	1525	+13.12
Color (PCU)	2011	2300	+12.61	2250	2575	+12.62

± refers standard deviation; +/− refers % improvement/reduction

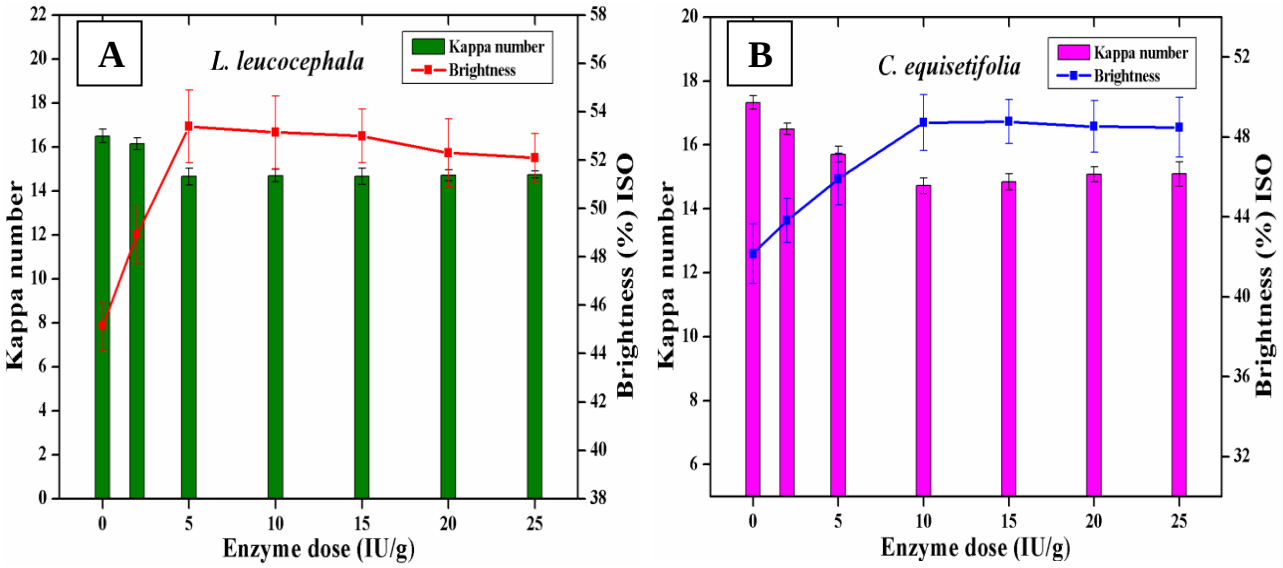


Figure 5.1: Effect of enzyme dose on kappa number and brightness during enzymatic pre-bleaching of kraft pulps of *L. leucocephala* (A) and *C. equisetifolia* (B).

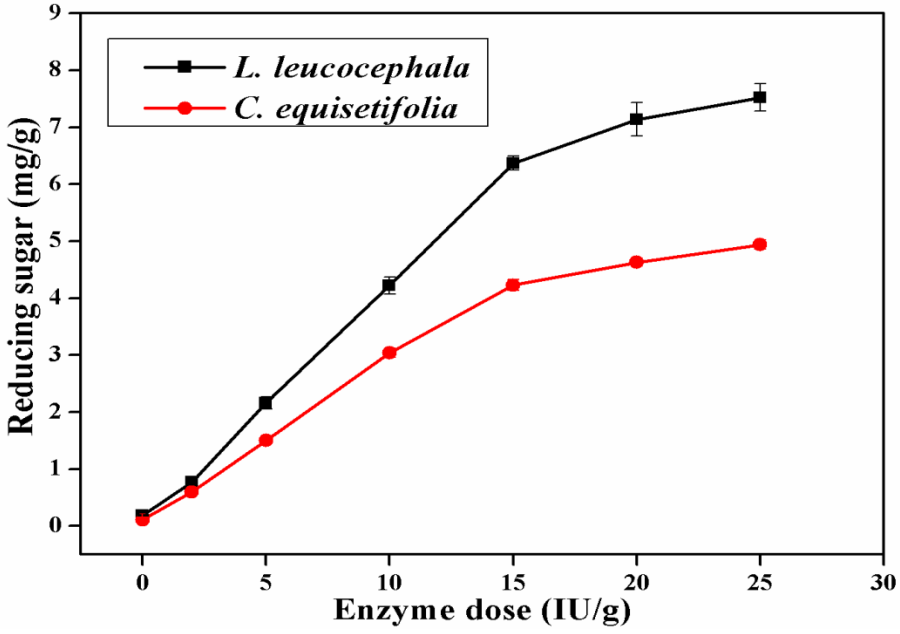


Figure 5.2: Effect of enzyme dose on reducing sugar released during enzymatic pre-bleaching of kraft pulps of *L. leucocephala* and *C. equisetifolia.*

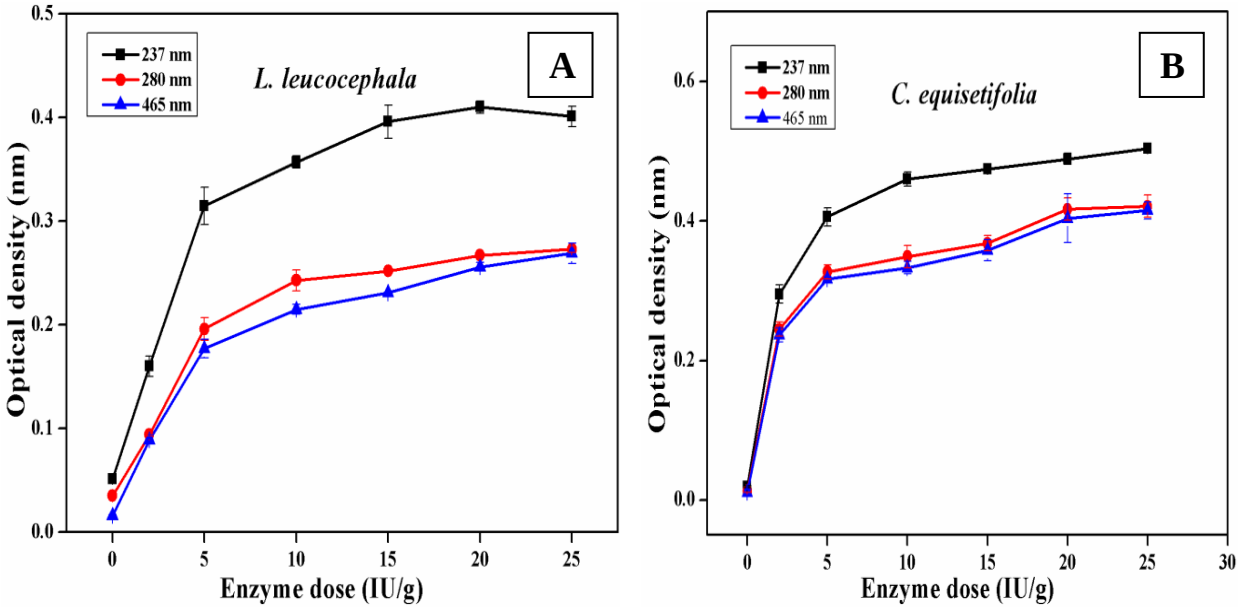


Figure 5.3: Effect of enzyme dose on release of chromophoric groups from kraft pulps of *L. leucocephala* (A) and *C. equisetifolia* (B) during enzymatic prebleaching.

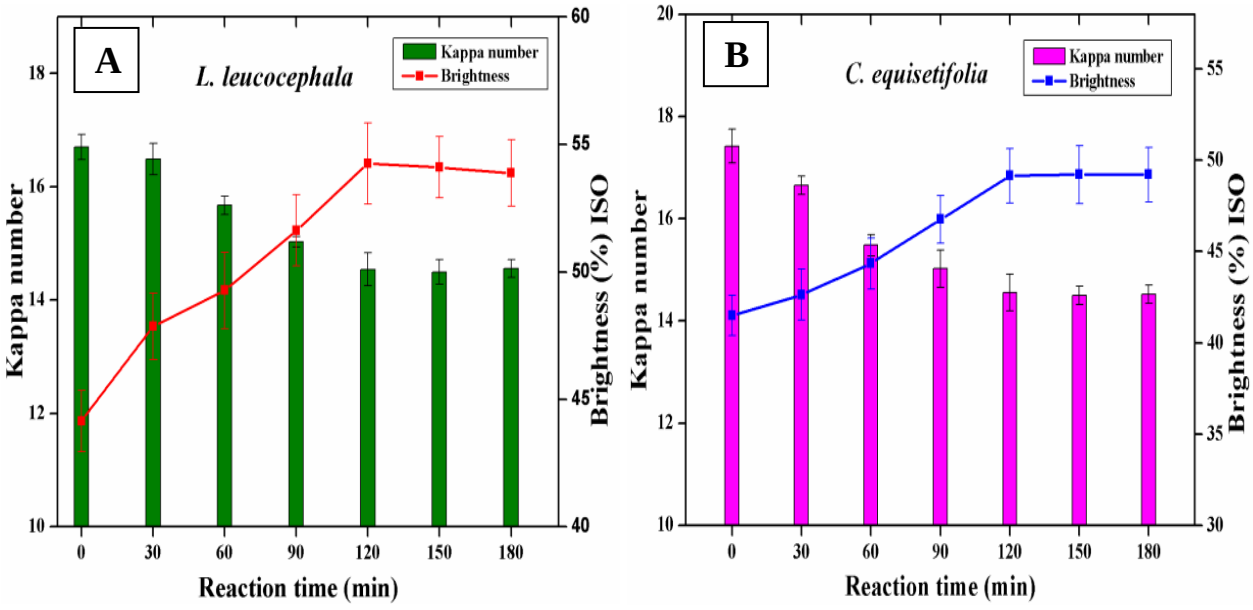


Figure 5.4: Effect of reaction time on kappa number and brightness during enzymatic pre-bleaching of kraft pulps of *L. leucocephala* (A) and *C. equisetifolia* (B).

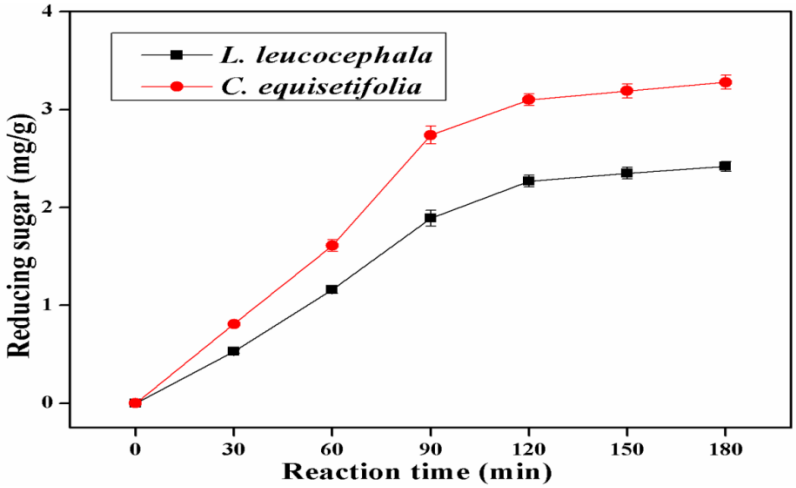


Figure 5.5: Effect of reaction time on reducing sugar released during enzymatic pre-bleaching of kraft pulps of *L. leucocephala* and *C. equisetifolia.*

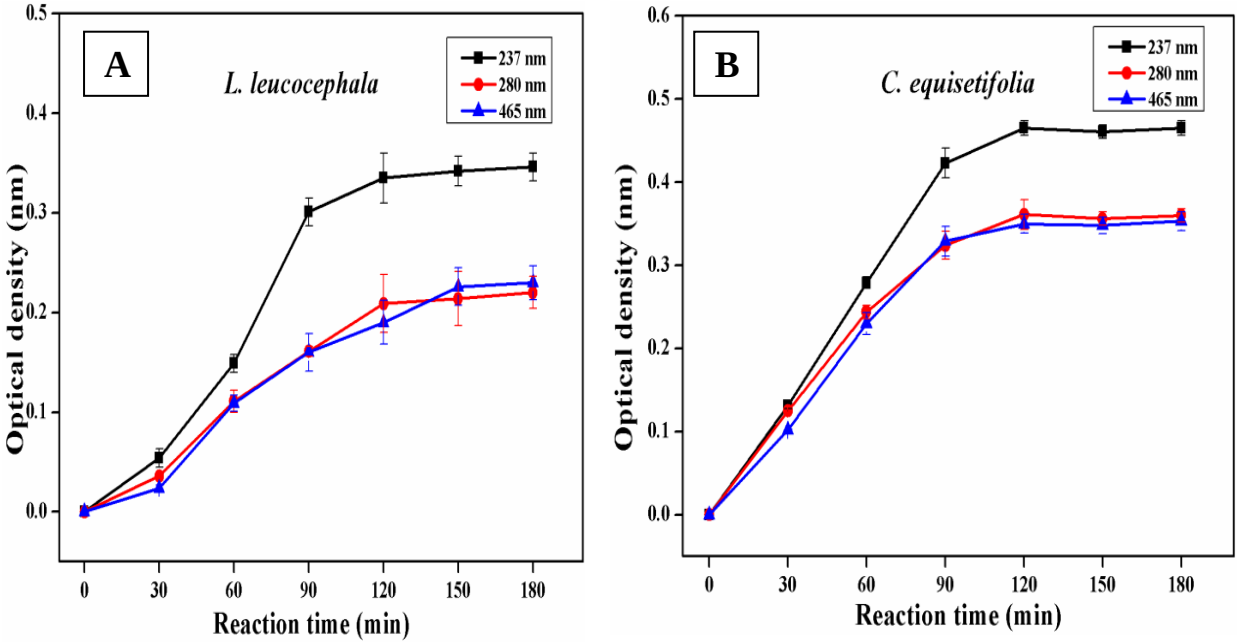


Figure 5.6: Effect of reaction time on release of chromophoric groups from kraft pulps of *L. leucocephala* (A) and *C. equisetifolia* (B) during enzymatic prebleaching.

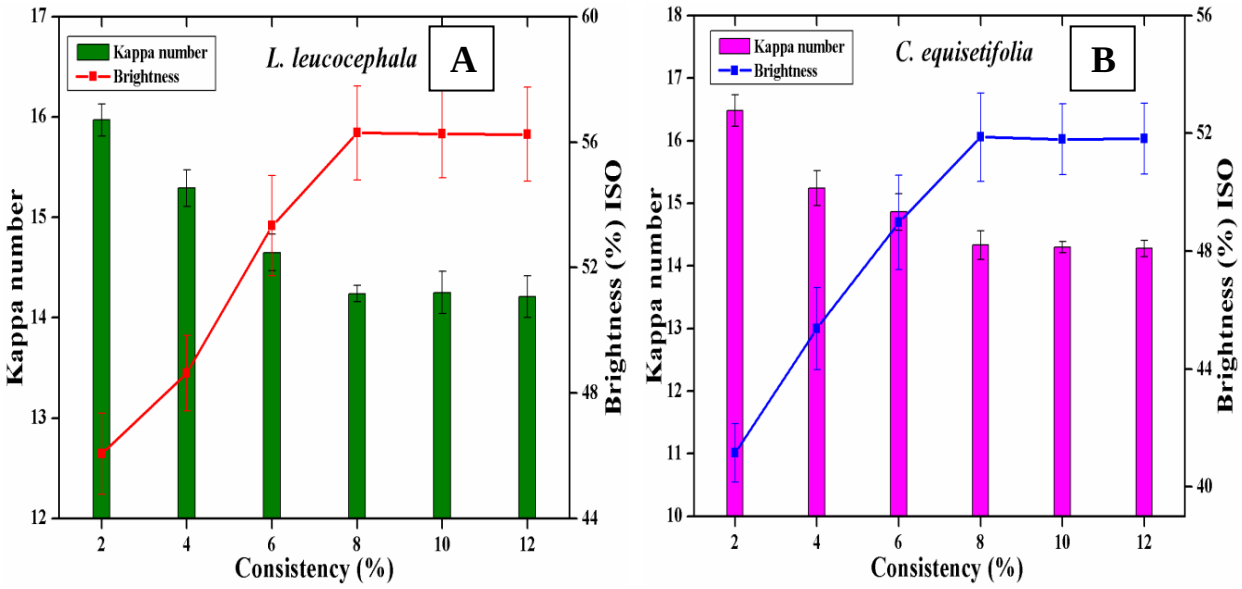


Figure 5.7: Effect of pulp consistency on kappa number and brightness during enzymatic pre-bleaching of kraft pulps of *L. leucocephala* (A) and *C. equisetifolia* (B).

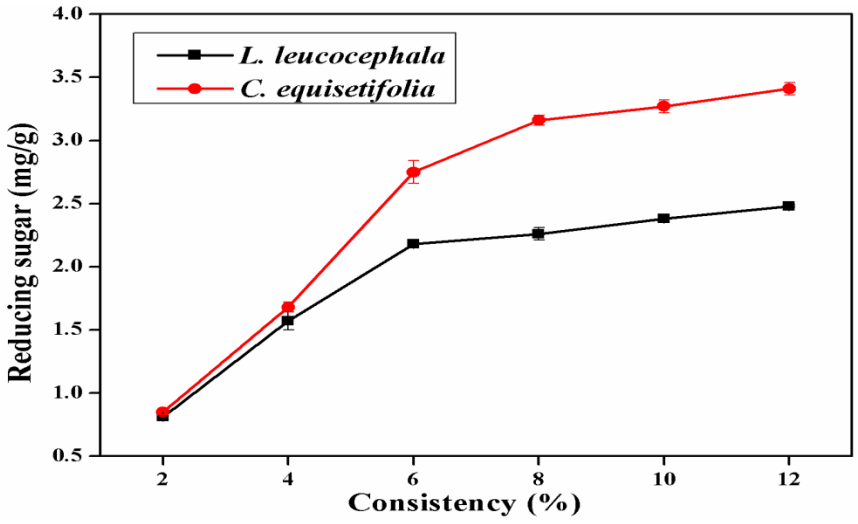


Figure 5.8: Effect of pulp consistency on reducing sugar released during enzymatic pre-bleaching of kraft pulps of *L. leucocephala* and *C. equisetifolia.*

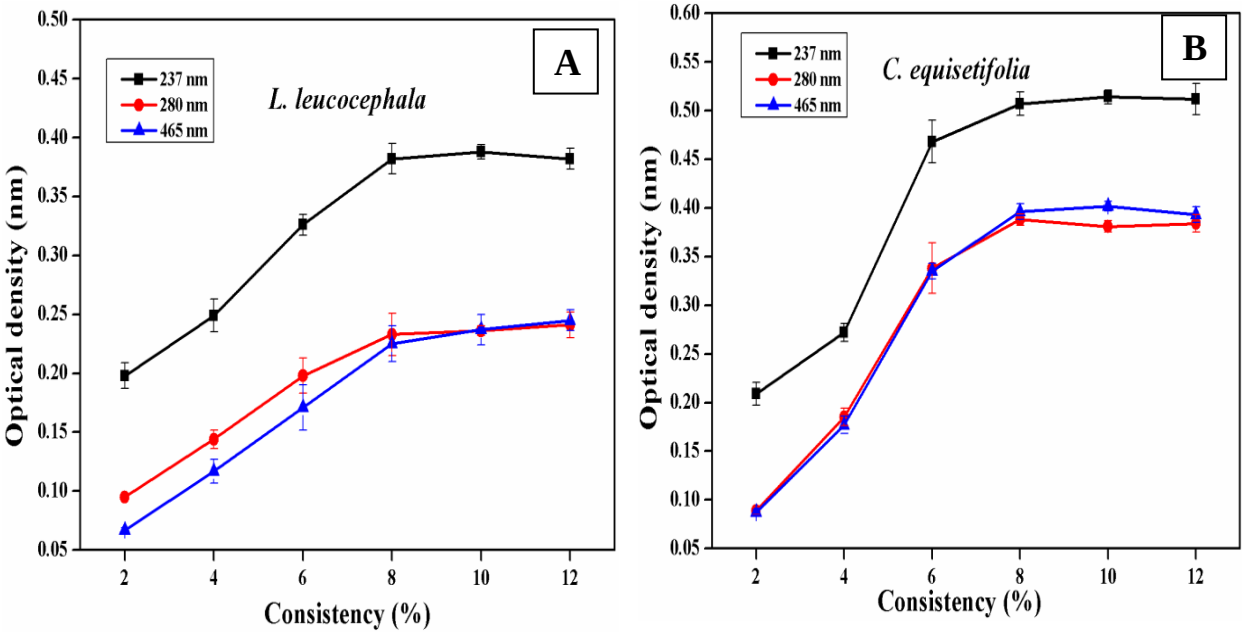


Figure 5.9: Effect of pulp consistency on release of chromophoric groups from kraft pulps of *L. leucocephala* (A) and *C. equisetifolia* (B) during enzymatic pre-bleaching.

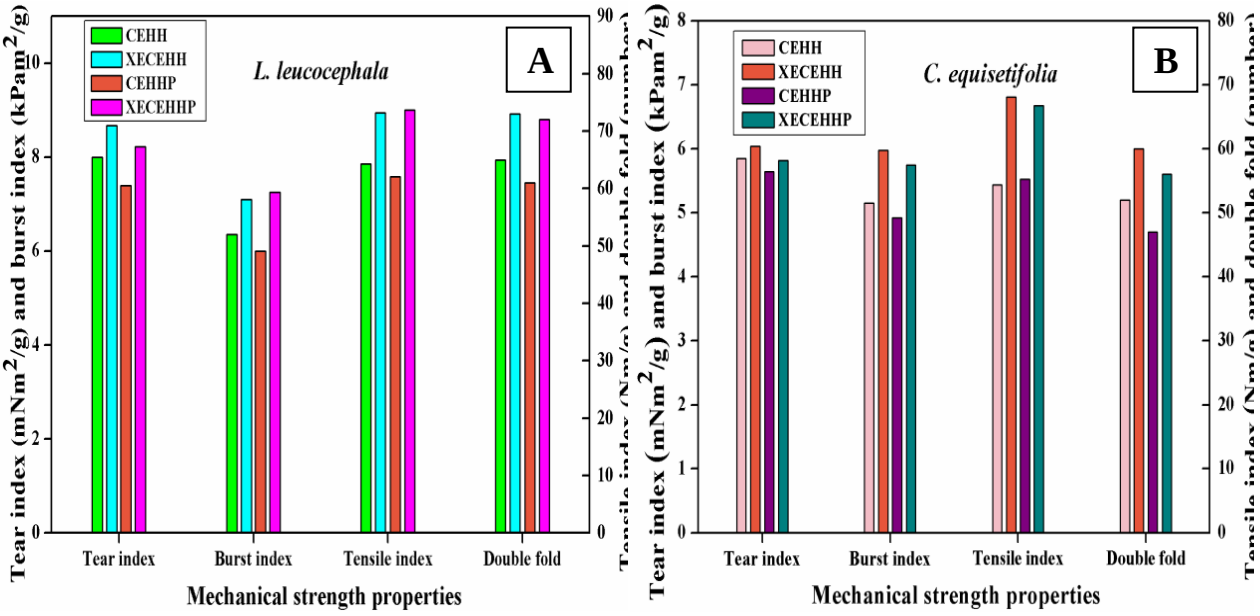


Figure 5.10: Comparison of mechanical strength properties of kraft pulps of *L. leucocephala* (A) and *C. equisetifolia* (B) beaten at 40±1 and 45±1 °SR, respectively, bleached by CEHH, XECEHH, CEHHP and XECEHHP sequences.

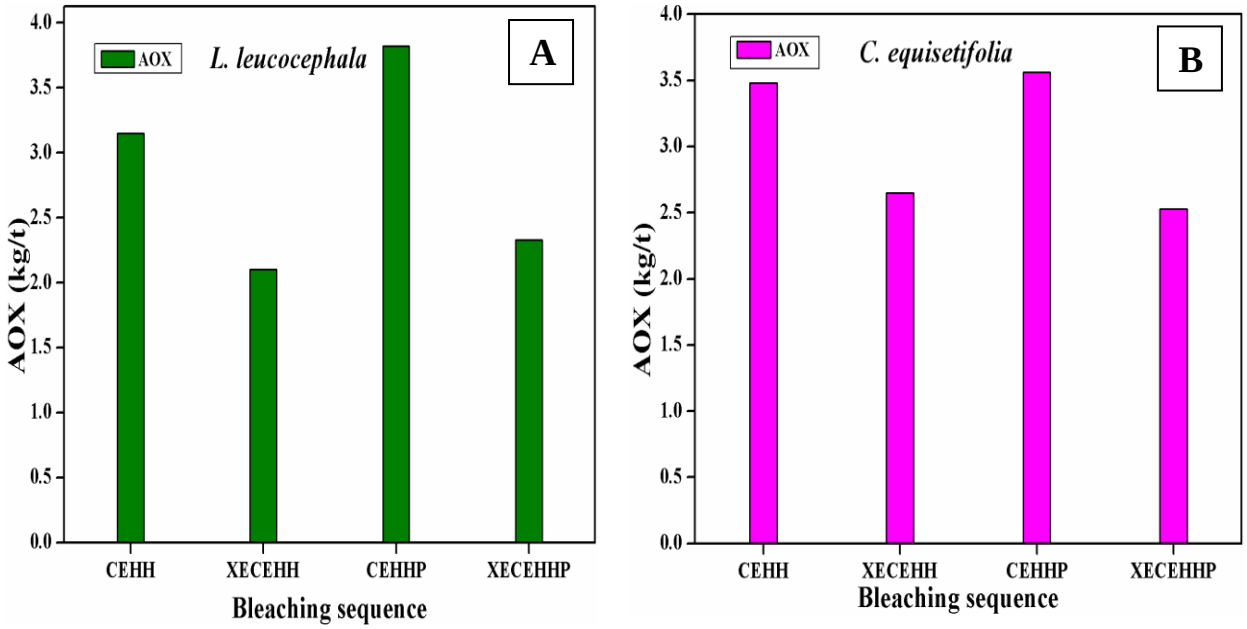


Figure 5.11: Comparison of AOX formation during CEHH, XECEHH, CEHHP and XECEHHP bleaching sequences for kraft pulps of *L. leucocephala* (A) and *C. equisetifolia* (B).

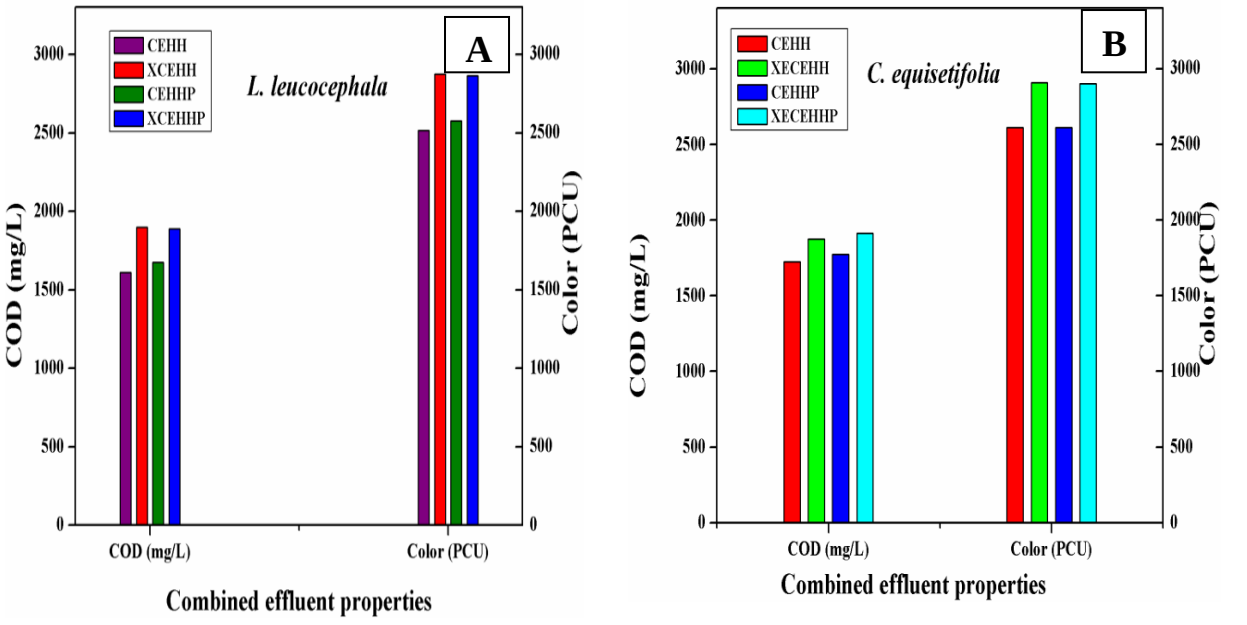


Figure 5.12: Comparison of combined bleach effluent characteristics generated during CEHH, XECEHH, CEHHP and XECEHHP bleaching sequences for kraft pulps of *L. leucocephala* (A) and *C. equisetifolia* (B).

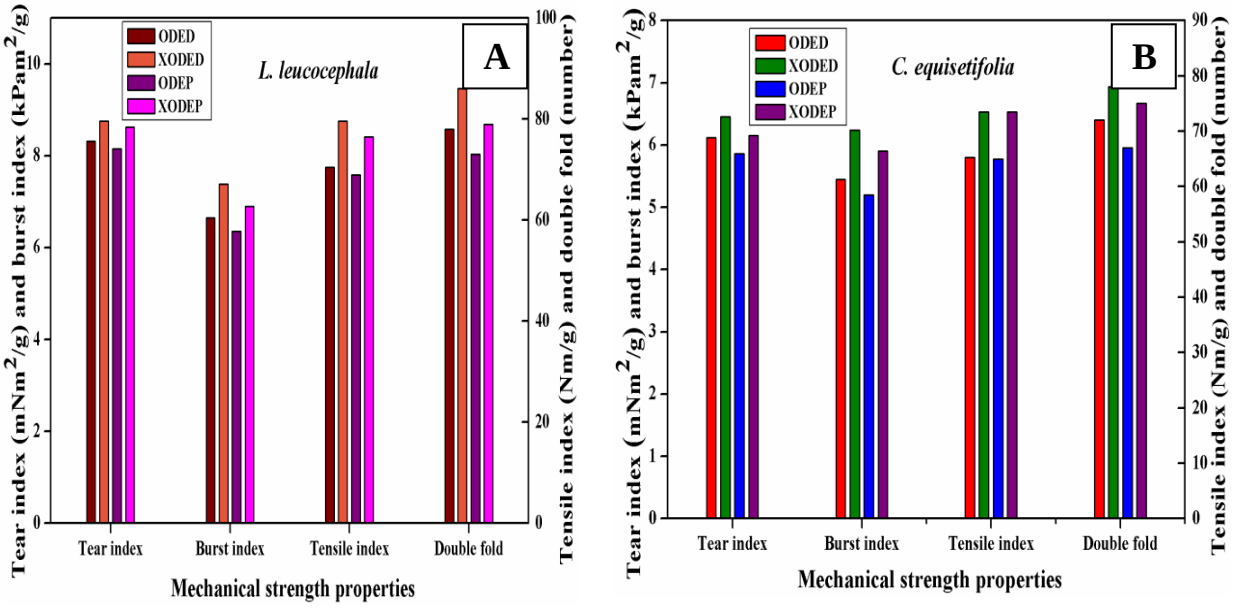


Figure 5.13: Comparison of mechanical strength properties of kraft pulps of *L. leucocephala* (A) and *C. equisetifolia* (B) beaten at 40±1 and 45±1 °SR, respectively, bleached by ODED, XODED, ODEP and XODEP sequences.

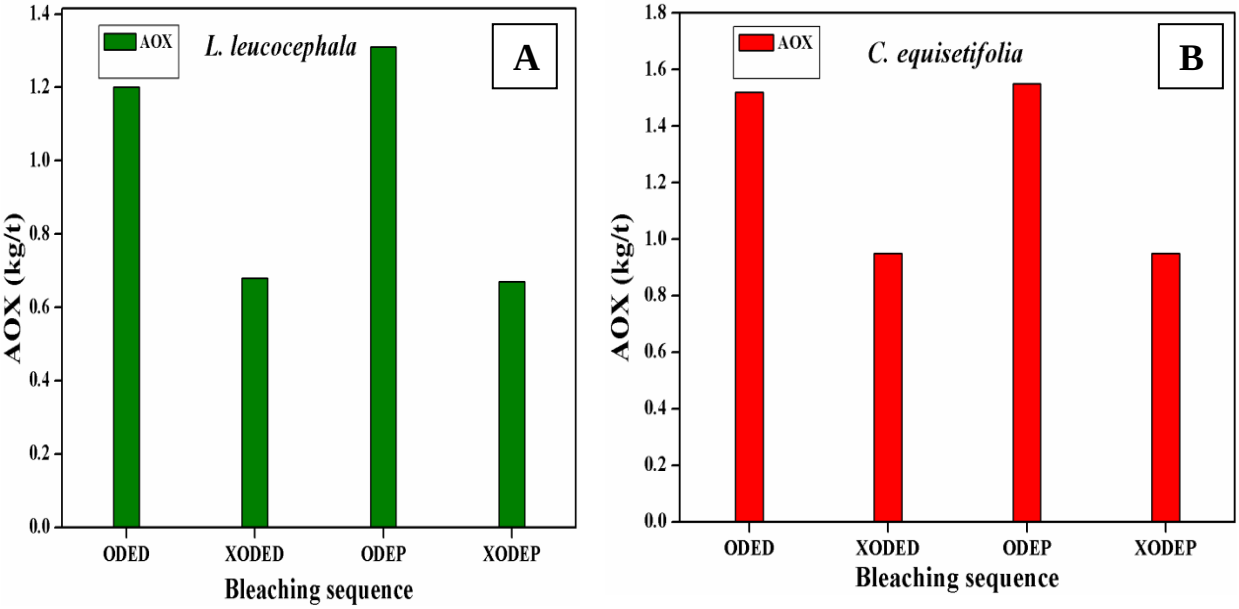


Figure 5.14: Comparison of AOX formation during ODED, XODED, ODEP and XODEP bleaching sequences for kraft pulps of *L. leucocephala* (A) and *C. equisetifolia* (B).

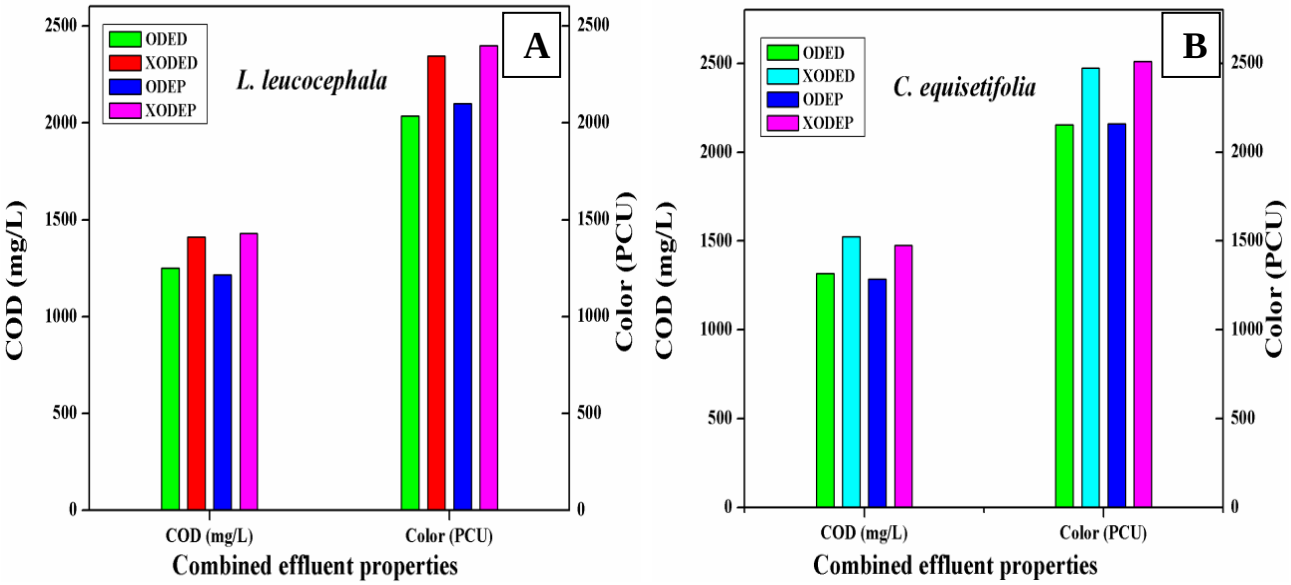


Figure 5.15: Comparison of combined bleach effluent characteristics generated during ODED, XODED, ODEP and XODEP bleaching sequences for kraft pulps of *L. leucocephala* (A) and *C. equisetifolia* (B).

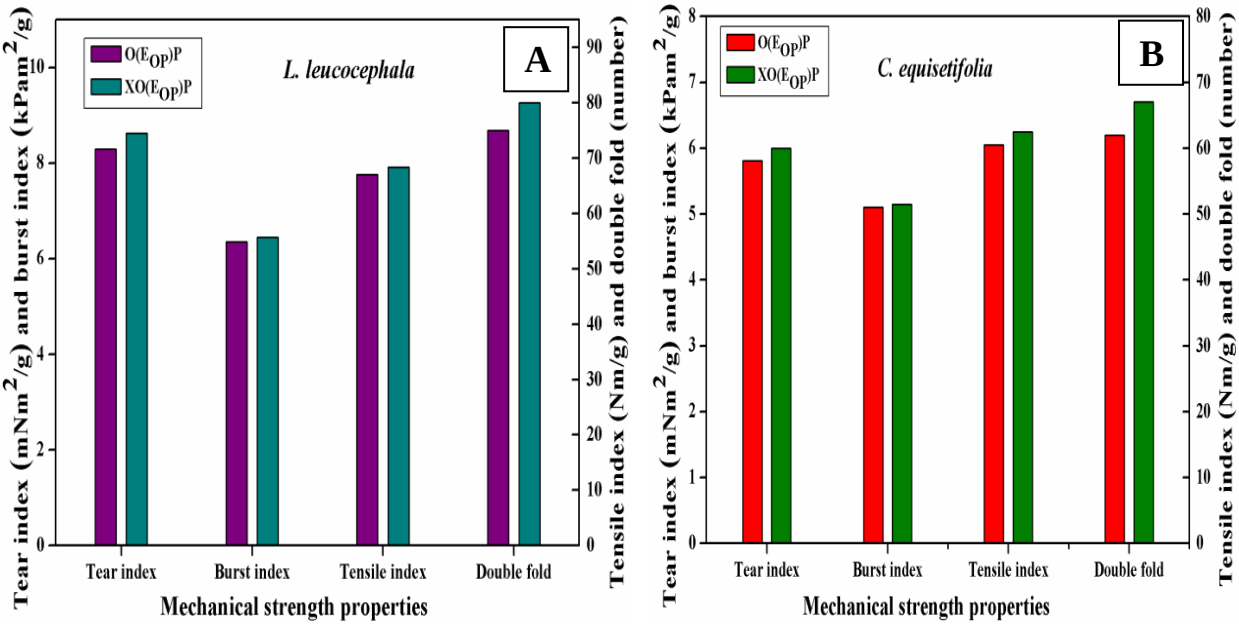


Figure 5.16: Comparison of mechanical strength properties of kraft pulps of *L. leucocephala* (A) and *C. equisetifolia* (B) beaten at 40±1 and 45±1 °SR, respectively, bleached by O(E_{OP})P and XO(E_{OP})P sequences.

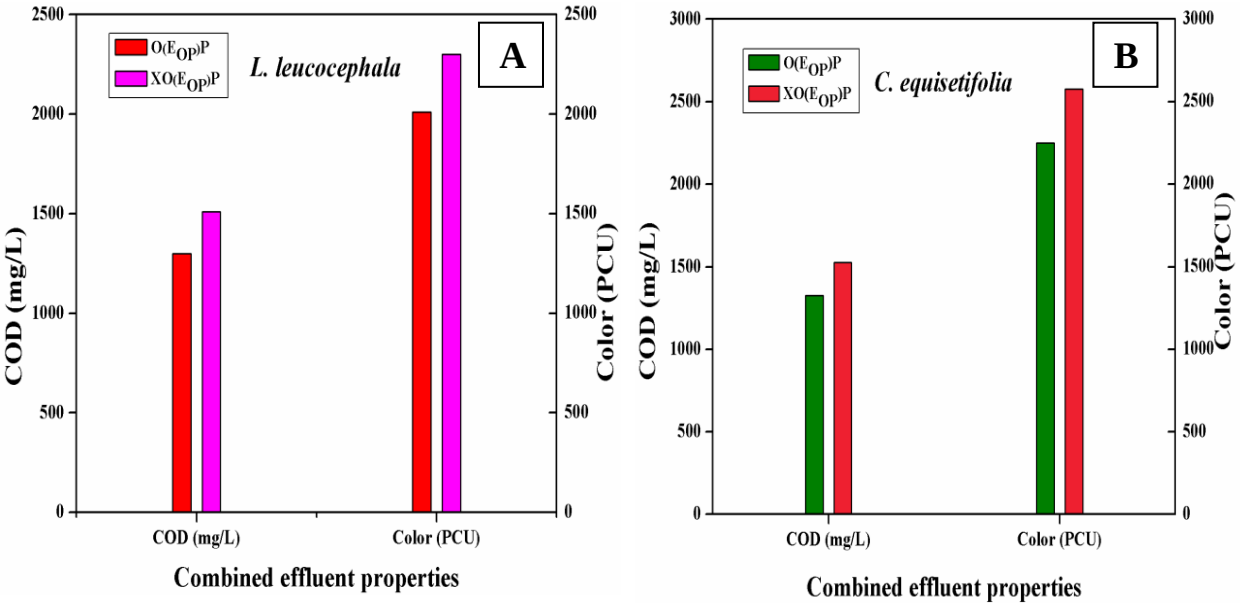
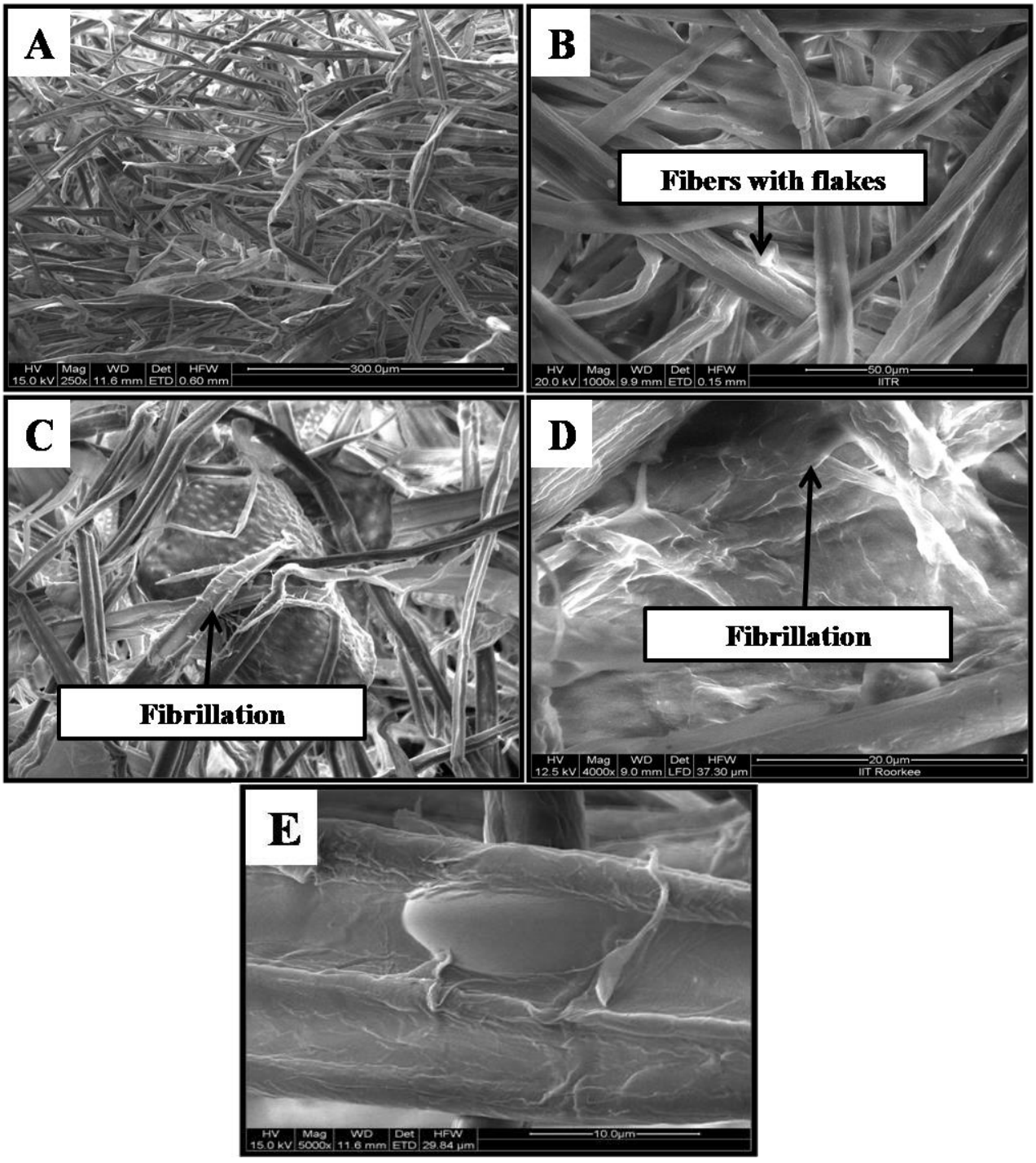
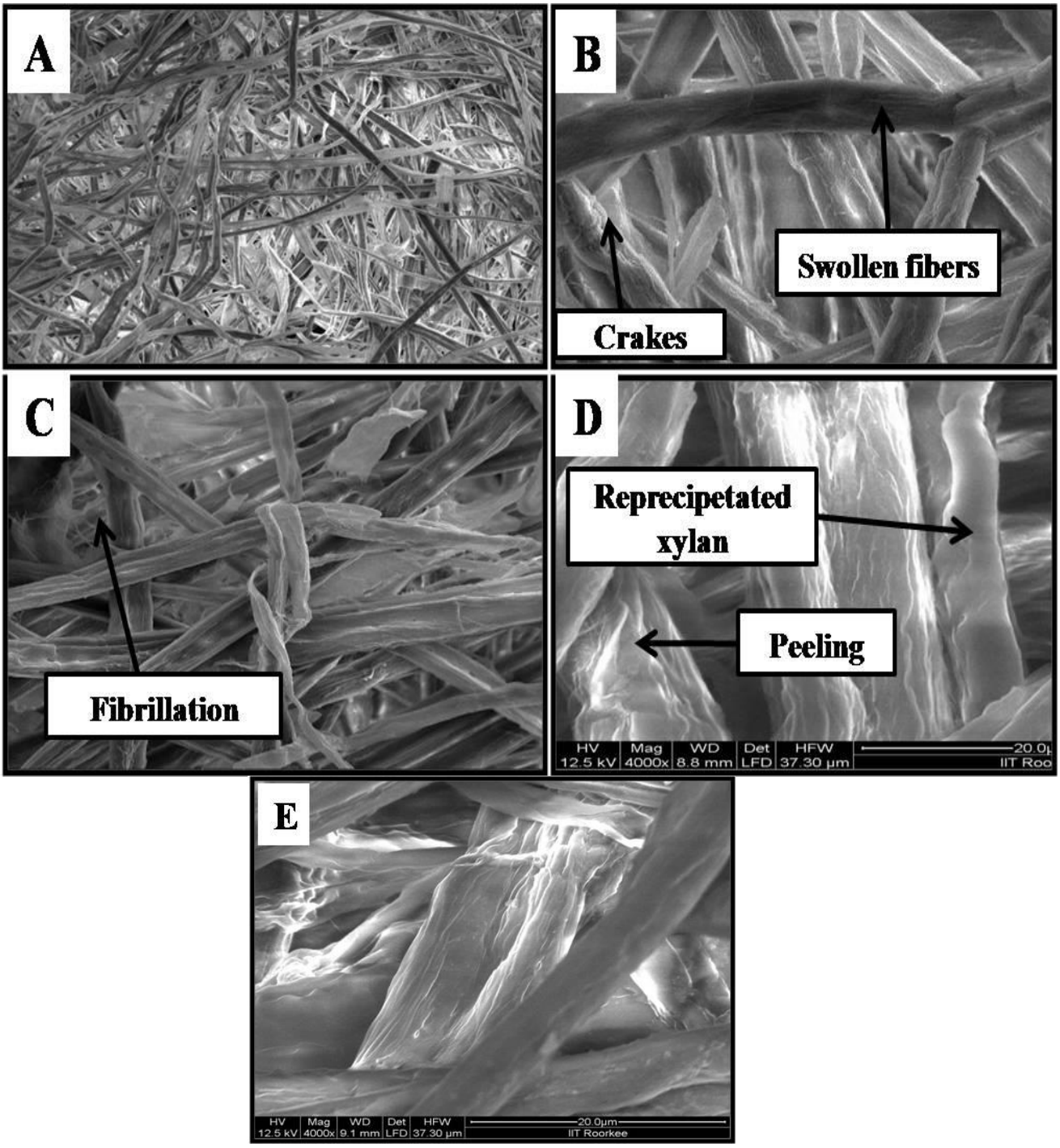


Figure 5.17: Comparison of combined bleach effluent characteristics generated during O(E_{OP})P and XO(E_{OP})P bleaching sequences for kraft pulps of *L. leucocephala* (A) and *C. equisetifolia* (B).



Photomicrograph 5.1: *L. leucocephala*: unbleached pulp showing fibers with a smooth surface (A); enzyme treated pulp showing swollen fibres with rough surface and cracks (B); untreated pulp after beating at a level of 40 °SR showing fibrillation (C); enzyme treated pulp after beating at a level of 40 °SR showing profuse fibrillation and reprecipitated xylan (D); oxygen delignified pulp after beating at a level of 40 °SR (E).



Photomicrograph 5.2: *C. equisetifolia*: unbleached pulp showing fibers with a smooth surface (A); enzyme treated pulp showing swollen fibres with rough surface and cracks (B); untreated pulp beaten at 45 °SR showing fibrillation (C); enzyme treated pulp beaten at 45 °SR showing profuse fibrillation, reprecipitated xylan and peeling (D); oxygen delignified pulp beaten at 45 °SR (E).

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ANNEXURE 5

ABBREVIATIONS

%	Percent
°C	Degree celcius
°SR	Degree shoper reigler
AOX	Adsorable organic halides
AQ	Anthraquinone
BOD	Biological oxygen demand
C	Chlorination stage
COD	Chemical oxygen demand
D	Chlorine Dioxide stage
E	Alkali extraction stage
ECF	Elemental chlorine free
EDTA	Ethylenediaminetetraacetate
etc.	et cetera
g	Grams
g/L	Grams per liters
H	Hypochlorite stage
HeXA	Hexenuronic acid
ISO	International Organization of Standardization
IU/g	International unit per gram
Kg	Kilograms
LCC	Lignin carbohydrate complex
mg/L	Milligrams per litre
min	Minutes
nm	Nanometers
O	Oxygen stage
P	Peroxide stage
PCU	Platinum cobalt unit
r	Revolutions
SEM	Scanning electrone microscopy
sp.	Species
TCDD	Tetrachlorodibenzodioxin
TCDF	Tetrachlorodibenzofuran
TCF	Total chlorine free
TOCL	Total organic chlorine
X	Enzyme stage

CHAPTER 6

CONCLUSIONS AND SUGGESTIONS

6.1. CONCLUSIONS

The followings are the major conclusions drawn from the present study:

1. Among 32 fungal strains isolated from different sources, *C. cinerea* RM-1 NFCCI-3086 is selected as the potent producer of xylanase as well as good producer of cellulase.
2. SSF is selected as it produces higher levels of xylanase as well as cellulase production than SmF.
3. The combination of wheat bran and corn cob (7:3) is found to be the most preferred solid support for xylanase production based on its privilege of providing high xylanase and negligible cellulase activity, for utilization in bio-bleaching experiments while sugarcane bagasse is selected for cellulase production as it produces the highest cellulase activity among all agro-residues tested.
4. As a result of OVAT optimization, the various components of the NSS medium are modified as: for xylanase production, NH_4Cl (4 g/L) is replaced by $(NH_4)_2SO_4$ (3 g/L) and Tween 80 (0.1 g/L) is also added. For cellulase production, NH_4Cl (4 g/L) and yeast extract (1 g/L) have been replaced by $(NH_4)_2SO_4$ (3 g/L) and peptone (1 g/L), respectively, with the addition of lactose (1 g/L) and Tween 80 (0.2 g/L).
5. The various optimized culture conditions through PB design under SSF for crude enzyme production by fungal strain RM-1 are: substrate concentration (5 g/flask), particle size (600 µm), inoculum size (3 disks/flask), inoculum age (5 days) and moisture ratio (66.67%), for xylanase production. For cellulase production, the optimized conditions are substrate concentration (5 g/flask), particle size (300 µm), inoculum size (3 disks/flask), inoculum age (5 days) and moisture ratio (66.67%).
6. Two models are obtained each for crude xylanase and crude cellulase production, as the results of central composite experimental designs, and statistical analysis is confirmed the significance of both the models.
7. Using optimum conditions i.e. pH 5.8, incubation period 7.3 days and incubation temperature 38.5 °C, the strain RM-1 produces maximum xylanase (1038.5 IU/mL) with negligible cellulase (0.465 IU/mL) by fermentation of wheat bran+corn cob (7:3).

While maximum cellulase (6.5 IU/mL) activity is obtained on sugarcane bagasse as the solid substrate, and the optimum conditions are found as pH 4.3, incubation period 5.7 days and incubation temperature 34 °C.

8. The crude xylanase produced by *C. cinerea* RM-1 is stable in pH range of 4.3 to 8.3 along with a 70% of its maximum activity. It shows good activity in the temperature range of 55-75 °C. This indicates that the crude xylanase so obtained is thermo and alkali-tolerant and can be used for further bio-bleaching studies.
9. SEM studies of *L. leucocephala* and *C. equisetifolia* show that the fibers of both the raw materials exhibit a relatively broad middle region and a bit abrupt to gradual tapering pointed ends. Vessels are large sized, mostly solitary and might be present in radial multiples. The vessel cells have dense lignified thickenings in the secondary wall; these are arranged as annular rings with simple and oblique perforation plate.
10. Proximate chemical analysis of *L. leucocephala* and *C. equisetifolia* depicts that these raw materials are comparable to E. *tereticornis* and *T. orientals* and could satisfactorily be used for pulp and paper production.
11. The wood chips of *L. leucocephala* and *C. equisetifolia* are used successfully for the production of kraft pulps. *L. leucocephala* produces a screened pulp yield of 53.54% with kappa number 16.7 and pulp viscosity of 950.5 cm^3/g using the best optimum cooking conditions like active alkali charge 17.5%, maximum cooking time 90.6 min and temperature 169.8 °C, sulphidity 25%, AQ 0.05%, Tween 20 0.05% and liquor to wood ratio 3:1. While *C. equisetifolia* produces a screened pulp yield of 52.4% with kappa number 18.1 and viscosity 974.5 cm^3/g under the most suitable pulping conditions which are: alkali charge 17.9%, temperature 170.2 °C, cooking time 82.1 min, sulphidity 20%, AQ 0.05%, Tween 20 0.1% and liquor to wood ratio 3:1.
12. Six models are obtained, as the results of central composite experimental designs, and statistical analysis confirms the significance of all the models.
13. The mechanical strength properties are found optimum at a beating level of 40±1 °SR for *L. leucocephala* and 45±1 °SR for *C. equisetifolia*. The strength properties of *L. leucocephala* kraft pulp are found superior to that of *C. equisetifolia*.
14. The +20 fraction of *L. leucocephala* kraft pulp separated by Bauer-McNett fiber classifier is found more than the double value as compared to *C. equisetifolia* and it mainly consists of long sclerenchymatous fiber. It indicates that the kraft pulp of *L.*

leucocephala is stronger than the *C. equisetifolia* pulp. While +48 fraction of *L. leucocephala* is about 9.8% less as compared to *C. equisetifolia* pulp. The +100 fraction which constitutes the short fibers, parenchyma cells and vessels is less for *L. leucocephala* as compared to *C. equisetifolia*. +200 fraction is 2.4% more in case of *L. leucocephala* than *C. equisetifolia.*

15. SEM studies of *L. leucocephala* and *C. equisetifolia* kraft pulps indicate that the fibers exhibit fibrillation after beating, and vessel elements are entangled with beaten fibers of *L. leucocephala* and *C. equisetifolia*.
16. The crude xylanase produced by *C. cinerea* RM-1 NFCCI-3086 using the wheat bran and corn cob (7:3) as the substrate, has successfully been used in bio-bleaching of *L. leucocephala* and *C. equisetifolia* kraft pulps. A xylanase dose of 5 IU/g for *L. leucocephala* and 10 IU/g for *C. equisetifolia*, a reaction time of 120 min and pulp consistency of 8% are found to be optimum at temperature of 55 °C and pH 8.3 for enzymatic pre-bleaching. Xylanase also causes the release of reducing sugars along with chromophores from the kraft pulps of *L. leucocephala* and *C. equisetifolia* and depicts the bio-bleaching potential of xylanase obtained from *C. cinerea* RM-1.
17. Xylanase pretreated pulps of *L. leucocephala* and *C. equisetifolia* show the reduction in kappa number and improvement in brightness during all bleaching sequences as compared to untreated pulps.
18. The total chlorine demand is reduced by 15.3 and 17.16% for kraft pulps of *L. leucocephala* and *C. equisetiolia,* respectively, bleached by XECEHH and XECEHHP sequence as compared to CEHH and CEHHP bleaching sequences. Similarly, the total chlorine demand of kraft pulps bleached by XODED and XODEP is reduced by 24.64 and 22.22% for *L. leucocephala* and *C. equisetiolia,* respectively as compared to controls (ODED and ODEP).
19. The AOX in combined bleach effluents of *L. leucocephala* and *C. equisetifolia* pulps bleached by different conventional and ECF bleaching sequences reduces as a result of xylanase pretreatment as compared to their controls.
20. A gain in viscosity is noticed as a result of xylanase pretreatment in conventional, ECF and TCF bleaching sequences of kraft pulps of *L. leucocephala* and *C. equisetiolia*. This indicates that the cellulase contamination in the crude enzyme extract has no adverse effect on the pulps of two raw materials.

21. The xylanase pretreated kraft pulps of *L. leucocephala* and *C. equisetiolia* show the reduction in PFI revolution to attain the same beating level as compared to their respective controls. This indicates that xylanase treatment helps in increasing the fibrillation, swelling and absorption capability of *L. leucocephala* and *C. equisetiolia* fibers in alliance with the improvement in the refining properties and reduction in refining energy.
22. The All mechanical strength properties are increased slightly as a result of xylanase pretreatment in conventional, ECF and TCF bleaching sequences of kraft pulps of *L. leucocephala* and *C. equisetiolia*.
23. COD and color values of combined bleach effluents from all bleaching sequences, are also increased by xylanase pretreatment. This might be due to the solubilization of residual LCC by the xylanase treatment.

Therefore, it is concluded that *C. cinerea* RM-1 NFCCI-3086 produces high amount of crude xylanase (1038.5 IU/mL) with a negligible cellulase (0.465 IU/mL) contamination, by using cheap agro-residues, wheat bran and corn cob (7:3) under solid state fermentation, and this enzyme can successfully be used in bio-bleaching of *L. leucocephala* and *C. equisetiolia* kraft pulps, without any purification. Moreover, this strain produces high amount of crude cellulase (6.5 IU/mL) by using a different agro-residue, sugarcane bagasse, as the substrate, and this enzyme can be used in other applications.

6.2. SUGGESTIONS FOR FUTURE WORK

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

- It is recommended to take industrial trials with crude xylanase obtained from *C. cinerea* RM-1 using *L. leucocephala* and *C. equisetiolia* to validate the laboratory results. Also, studies should be carried out on pulps produced by different methods and different raw materials to see the effect of xylanase produced by *C. cinerea* RM-1.
- Realistic cost estimates must be made for the commercialization of bio-bleaching technology.
- *C. cinerea* RM-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.

- Further work on purification and characterization of the xylanases and cellulases obtained from *C. cinerea* RM-1 is suggested for a better understanding of their enzyme system.
- Biobleaching process may be carried out at higher temperature and pH levels for checking the viability of crude xylanase obtained from *C. cinerea* RM-1 in extreme conditions.
- It is recommended to use the crude xylanase with other bleaching sequences also.
- It is recommended to boost up this technology in Indian paper industry in order to explore the possibilities of achieving the desired standard norms in respect of the AOX levels.

RESEARCH PUBLICATIONS

1. Poonam and Dharm Dutt, Anthraquinone and Tween 20 based kraft pulping technology for *L. leucocephala* and *C. equisetifolia, IJESR*, 4(11):808-811(2014).
2. Poonam, Dharm Dutt and A.K. Vidyarthi, Optimization of kraft pulping for *Casuarina equisetifolia* by response surface methodology, *Cellulose Chemistry and Technology*, (Communicated).
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Thesis Title

स्टडीज ऑन एन्ज़ीमैटिक आस्पेक्ट्स ऑफ़ माइक्रोबियल ऑरिजिन एंड बायो- ब्लीचिंग ऑफ़ हार्डवुड़स.

STUDIES ON ENZYMATIC ASPECTS OF MICROBIAL ORIGIN AND BIO-BLEACHING OF HARDWOODS.