STUDIES ON ALKALI-TOLERANT WHITE ROT FUNGI FOR BIOBLEACHING OF KRAFT PULP OF *A. CADAMBA*

A THESIS

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CANDIDATE'S DECLARATION

I hereby certify that the work which is being presented in the thesis entitled **STUDIES ON ALKALI-TOLERANT WHITE ROT FUNGI FOR BIOBLEACHING OF KRAFT PULP OF *A. CADAMBA*** in fulfilment of the requirements for the award of the degree of Doctor of Philosophy submitted in the **Indian Institute of Technology Roorkee, Roorkee** is an authentic record of my own work carried out during a period from January 2004 to December 2008 under the supervision of Dr. J.S. Upadhyaya, Professor, Dr. Dharm Dutt Associate Professor and Dr. C.H. Tyagi, Associate Professor, Department of Paper Technology, Indian Institute of Technology Roorkee, Roorkee.

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

Mohan lal

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

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The Ph.D. Viva-Voce Examination of **MOHAN LAL,** Research Scholar has been held on 8.5.2009

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ABSTRACT

The pulp and paper industry is the sixth largest energy consumer in the industrial sector and intensive in terms of consumption of raw materials, energy, water and capital requirement. The pulp and paper industry is also facing the problem of an acute shortage of cellulosic fibrous raw materials and soaring environmental cost with other associated issues and challenges like, tough competition from import, obsolete technology, minimum profit level and high energy cost. Due to constant pressure from government statutory bodies and public awareness to mitigate emissions to air and water and decrease in energy cost keeping in view the economic viability of the mill, it is necessary to develop environmental benign technology with energy conservation. Application of biotechnology in pulp and paper industry is helpful to solve the above mentioned purposes to some extent. The use of microbial enzymes for prebleaching of pulp is effective to reduce kappa number before bleaching and thereby saving bleaching chemicals and reducing pollution load. Most of the enzymes available in market are active at slightly acidic conditions and temperature below 70 ^{0}C and are cost effective. The pulp produced after brown stock washing is having high temperature and alkaline in nature. Aiming this, the present investigation focuses at developing thermophilic and alkali tolerant enzymes from white rot fungi (*Coperinellus dissiminatus*) for biobleaching of *A. cadamba* and their impact on pulp and paper properties and pollution load generated during bleaching

The main objectives of the present study are here as under:

- To isolate and screen thermo-alkaline xylanases producing white rot fungi.
- To produce xylanases from screened isolates under optimum condition of liquid state fermentation using low cost substrate for subsequent application in biobleaching.
- To study anatomical, morphological and chemical characterization of *A. cadamba*. Variation in basic density and chemical composition of *A. cadamba* (12 years old)

along the height on α-cellulose, lignin and alcohol-benzene soluble has also been studied.

- Optimization of various operating parameters of kraft pulping of *A. cadamba* and the effect of anthraquinone at optimum pulping condition on pulp yield and kappa number has also been studied.
- To study the impact of enzymatic bleaching on different bleaching sequences with regard to pulp viscosity, optical properties and mechanical strength properties and pollution load generated during bleaching

15 strains of white rot fungi were isolated by enriched culture technique using wheat bran as a sole substrate from 30 decaying wood samples. The isolates were purified on wheat bran agar medium (2% wheat bran + 2% agar). For the screening of alkali-tolerant xylanases, the enzymes were produced under liquid state fermentation conditions at 40 ^{0}C and pH levels varying from 4.0 to 11.0 and incubation period 8th day using 2% wheat bran as a core substrate. Out of 15, only two were found active at pH above 9.0. Based on spore print, fruiting bodies and microscopic examination both isolates were identified as different strains of *Coprinellus disseminates*. They were designated as fungal strains MLK01 and MLK07. Both of the fungal strains were screened for extracellular enzymes and found positive for xylanase, cellulase, laccase, lignin peroxidase, amylase, mannanase and protease. The effect of fermentation conditions (incubation period and pH) on enzyme production by the test strains *C. disseminates* MLK01 and MLK07 under L.S.F. were studied. The effect of different sugars (glucose, xylose, galactose and xylan), urea, lignocelluloses as substrates (wheat bran, sugarcane bagasse, wheat straw, saw dust of wood and ground nut shell) were also studied. The crude xylanases from both of strains were analyzed for pH and temperature optima. The effect of different metal ions at 1.0 mM concentration was also studied. Both of the fungal strains MLK01 and MLK07 produced maximum xylanases between 8th to 10th days of incubation. Cellulases activity was found to decrease after 7th day of incubation while lignin

peroxidase activity increased with increasing the incubation days up to 12 days for strain MLK01. Cellulases and lignin peroxidase activities were observed maximum at 11th and 13th day of incubation for strain MLK07. All the sugars, repressed the xylanase as well as cellulases activity while lignin peroxidase activity and fungal growth was increased. Wheat bran was found to be the best and cheaper substrate for xylanases production. Additional nitrogen source (urea) found represses the xylanase and lignin peroxidase induction on the other hand, it enhanced the fungal growth and cellulase secretion for both of fungal strains. Xylanase from MLK01 showed the maximum xylanase activity at pH 7.5 and temperature 75 ^{0}C, while xylanase from MLK07 showed maximum xylanase activity at pH 8.0 and temperature 65 ^{0}C. Both the xylanase activity was inhibited by $HgCl_2$ and $CuSO_4$ at 1.0 mM concentration whereas; $ZnSO_4$ and $FeSO_4$ found to be enhanced xylanases activity. The enzymes extracted from fungal strains MLK01 and MLK07 were designated as enzyme-A and enzyme-B respectively.

In order to assess the suitability of *A. cadamba* for pulp and paper making, anatomical, morphological and chemical characterization of *A. cadamba was* done. Chemical characterization includes water soluble, 1% NaOH, alcohol benzene soluble, holocellulose, lignin, ash and pentosan. *A. cadamba* contains 20.6% lignin, 19 %, pentosan 76.20%, holocellulose, 20.56% hemicellulose and 44.3% α- cellulose. The variation in basic density and chemical composition (alcohol-benzene soluble, holocellulose, lignin and α–cellulose) of *A. cadamba* was done along the height i.e. five feet long cylindrical piece from 5, 25 and 40 ft height from base. Average fiber length 1.43 mm, fiber width 38.12 μm, lumen diameter 26.10 μm and cell wall thickness 5.51 was observed.

Screened chips of *A. cadamba* were digested at different cooking conditions. Based on experimental results, the optimum cooking condition for *A. cadamba* was found as: active alkali 16% (as Na_2O), sulphidity 20%, temperature 165 ^{0}C, time at temperature 90 min and liquor to wood ratio of 3.5:1. *A. cadamba* produced 48.74% screened pulp at kappa number

22.5. The addition of 0.1 % AQ increases screened pulp yield by 0.38 % but significantly reduces the kappa number by 6.5 units (28.8%). The effect of aging of *A. cadamba* (2, 3, 4 and 12 years) on screened pulp yield, kappa number and screening rejects was studied. The Baeur-McNett fiber classification of *A. cadamba* using mess size +20, +48, +100 and +200 of kraft-AQ pulp at optimum cooking condition was carried out. The unbleached pulp of *A. cadamba* was beaten in PFI mill at different beating levels to optimize various mechanical strength properties like, tear index, tensile index, burst index, double fold, thickness and stretch. The scanning electron microscopic studies of unbleached kraft-AQ fibres were carried out.

The unbleached kraft-AQ pulp of *A. cadamba* was optimized for various operating parameters, like enzyme doses, consistency and reaction time during prebleaching with enzyme-A (strain MLK01) and enzyme-B (strain MLK07) separately and its impact on release of chromophores and reducing sugars in filtrate were studied. The enzymatically prebleached pulps were subjected to alkali extraction with 2% NaOH separately with enzyme-A and B and evaluated for pulp kappa number and viscosity.

The impact of enzymatic prebleaching during different CEHH bleaching sequences, carried out at pulps of different kappa numbers, different chlorine demands and at different pH levels during enzymatic prebleaching, on pulp viscosity, brightness, PFI revolutions to get a beating level of 35 ^{0}SR, optical and mechanical strength properties and effluent characteristics like, COD, colour and AOX were studied. The bleach boosting effect of enzymatic treatment on 24 kappa number pulp was found better than that of pulp of kappa number 16. At 2% chlorine demand, viscosity, mechanical strength properties and pollution load were observed to improve while brightness was found to decrease compared to 4% chlorine demand. The kraft-AQ pulp of *A. cadamba* was prebleached at different pH levels i.e. 5.0, 6.0, 8.0 and 9.0 with 4% chlorine demand. The brightness of bleached pulp, COD and colour of combined effluent generated during ^{A}XECEHH and ^{B}XECEHH bleaching

sequences decreased with increasing the pH. On the other hand, pulp viscosity increased with increasing pH and found maximum at pH 9.0. AOX was also found to decrease with increasing the pH.

The kraft-AQ pulp of *A. cadamba* of kappa number 16 was delignified with O_2 at consistency 15%, pH 11.0 maintained with 2% NaOH (as such), oxygen pressure 5 kg/cm^2, temperature 110 ^{0}C and reaction time 90 min in presence of carbohydrate stabilizer i.e. Epsom salt (0.1% $MgSO_4$) followed by alkali extraction with 2% NaOH at 70 ^{0}C and 10% consistency for 90 min. After oxygen delignification, the kappa number and pulp viscosity reduced by 38.12 and 18.70% respectively whereas brightness improved by 22.39%. The oxygen delignified pulp was subjected to bleach by ECF bleaching sequences i.e., ODED, O^AXEDED and O^BXEDED; ODEDP, $O^AXEDEDP$ and $O^BXEDEDP$ and ODEP, O^AXEDEP and O^BXEDEP bleaching sequences. The brightness improvement in various bleaching sequences in ascending order was as: ODED< ODEDP<ODEP for control; O^AXEDEP< $O^AXEDEDP$< O^AXEDEP for enzyme A and O^BXEDED< $O^BXEDEDP$< O^BXEDEP for enzyme B. All the ECF bleaching sequences with enzymatic prebleaching after O_2 delignification required more PFI revolutions to get 35 ^{0}SR beating level compared to control. In all the ECF bleaching sequences, tensile index, burst index and double fold improved except tear index with enzymatic treatment. Both the Enzyme A and B reduce AOX load and increase COD and colour in the combined effluent of respective bleaching sequences compare to control. The reduction in AOX was found decreases sharply after ODL.

The introduction of enzyme-A and B in TCF bleaching sequences of kraft-AQ pulp of *A. cadamba* i.e. O^AXEQPP and O^BXEQPP improves the brightness and pulp viscosity and increases PFI revolutions to get a beating level of 35 ^{0}SR over OQPP bleaching sequence. All the mechanical strength properties except tear index improve in O^AXEQPP and O^BXEQPP bleaching sequences while COD shows an increasing trend while colour which decreases in both the bleaching sequences compared to OQPP bleaching sequence.

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ABBREVATIONS

2, 4- DCP	2, 4 Di Chloro Phenol
A. cadamba	*Anthocephalus cadamba*
AOX	Adsorbable Organic Halides
AQ	Anthraquinone
BOD	Biochemical Oxygen Demand
BSA	Bovine Serum Albumin
C	Chlorination stage
CED	Cupriethylenediamine
ClO_2	Chlorine Dioxide
CMC	Carboxymethylcellulose
COD	Chemical Oxygen Demand
D	Chlorine Dioxide Stage
DNS	3,5-dinitrosalicylic Acid
E	Alkali Extraction Stage
ECF	Elemental Chlorine Free
EDTA	Ethylenediaminetetraacetate
FA	Fungal Agar
g	Gram
GNS	Ground Nut Shell
h	Hour
H	Hypochlorite Stage
HexA	Hexenuronic Acid
kDA	Kilo Dalton
L	Litre
LCC	Lignin-Carbohydrate Complex
LSF	Liquid State Fermentation
LYEA	Lactose Yeast Extract Agar
m	Metre
MEA	Malt Extract Agar

MGYP	Malt Extract Glucose Yeast Extract Peptone
N.S.S	Nutrient Salt Solution
O	Oxygen Stage
O.D.	Oven Dry
ODL	Oxygen Delignification
ONP	Office News Paper
P	Peroxide Stage
PDA	Potato Dextrose Agar
POPs	Persistent Organic Pollutants
Q	Chelation Stage
SEM	Scanning Electron Microscopy
SSF	Solid State Fermentation
TCDD	Tetrachlorodibenzodioxin
TCDDF	Tetrachlorodibenzofuran
TCF	Total Chlorine Free
TOCL	Total Organic Chloride
UV	Ultra Violet
Z	Ozone Stage

INTRODUCTION

World demand for paper has increased at an average annual rate of 4.7% over the past 40 years. Although future growth will reduce to 2-3% as the existing wood resources may be inadequate to meet this growing demand for paper especially in the Asia-Pacific region and Eastern Europe [2]. The world consumption of paper and paper board in 2006 was 380.28 million tonnes [35] of which 36% consumed in Asia and 64% in rest of the world (Figure 1). The world demand for paper and paperboard as per forecast is likely to grow by 2.1% annually in the long term and has been estimated to reach 490 million tonnes by the year 2020, according to a recent paper demand and supply study [16]. China and India will be the most rapidly growing production areas within Asia, accounting for 39% and 8% of the world's incremental production through 2020 [34]. The consumption of paper in India is one of the lowest i.e. 7 kgs/person compared to average world consumption i.e. 50 kgs/person [35] and will be expected to increase 9 kgs/person by 2010. The per capita consumption of paper and paper board of five selected countries of Asia was 11 kgs/ person while in China; it was 49 kgs/person in 2006 and will be expected to increase by14 and 66 kgs/person for five selected Asian countries and China (Figure 2). The consumption of paper will increase with increasing the population and literacy rate. Indian population is expected to grow by 1.2% per year reaching 1.3 billion by 2020. The share of urban population will grow steadily. Literacy rate is expected to grow over 70% by 2020 (Figure 3). Demand for paper in India is projected to grow at a compounded annual growth rate of 6.10% from 2004-05 to an estimated 7.4 million tonnes by 2008-09 and 11.87 million tonnes by 2020 (Table 1) [1, 25].

According to estimation 570 mills are in operation, while numerous small mills are not working at their full capacity. According to the current report on the market published by the British consultancy firm Hawkins Wright, the average capacity of a paper machine is

about 14,000 tonnes/year against a global average of 42, 000 tonnes/year. Likewise, the average paper machine speed at Indian mills is 200-260 m/min which is below the global average of 600 to 700 m/min. Most of the Indian mills are small, only 34 Indian mills have a capacity of over 33,000 tonnes/annum. The number of Indian mills capacity wise is shown in Figure 4. (23) The production capacity of Indian paper industry is divided in to three main fiber groups i.e., recycled fibers, agro-base fibers and forest based fibers but less than 10 % of mills are forest based (Figure 5) [24].

Total forest area in 2005 was estimated to be around 30% of the planet's land area, just under 40 million km^2. This corresponds to an average of 0.62 ha (6200 m^2) per capita (Table 2) [41]. India has total land area of 328.8 million ha. Agriculture land occupies 47% (154.7 million ha) of the total land area, while uncultivated, non-agricultural and barren land accounts for 30% (99.3 million ha) of the land area. Forest and woodlands occupy around 20.6%. Forest cover in India as reported is 67.8 million ha i.e., 20.6% of the country's surface area, which translates into per capita forest area of only 0.8 ha/person, one of the lowest in the world [22]. The paper industry's wood demand is expected to grow from 5.8 million tonnes to 9 million tonnes by 2010 and 13 million tonnes by 2020 (Figure 6). The current forest plantations in India are estimated at 32.5 million tonnes ha of which 90% is based on hardwoods, mainly eucalyptus and acacia (Figure 7) [25]. The country's fuel wood requirement alone is 280 million tonnes/year, and this will rise to 356 million tonnes/yr by 2010 [12]. Total fiber consumption for the production of paper and paperboard in India will nearly be doubled between 2006 and 2016, growing from 7.4 million tonnes/year to 13.7 million tonnes/year. India's total wood fiber deficit as per forecast will increase at an 11.3% annual rate by 2016 [22]. Hardwood pulp production was 0.9 million tonnes in 2000 and is expected to increase 1.5 million tonnes by 2015 and 1.8 million tonnes by 2020. The growth could be higher or lower, depending on development of plantations in India.

The pulp and paper industry uses only 3-4% of total wood. As per the existing forest policy, the paper industry cannot use wood from any of the national forest reserves [15]. Therefore, the plantation is being done by farmers on private lands and the product being sold to pulp and paper industry. The sector is not optimistic about meeting its requirement via farm forestry, as envisaged in the revised National Forest Policy (1988) because:

- land holdings are small and fragmented;
- indebtedness of farmers, which forces them to dispose the produce indiscriminately;
- lack of location specific intercropping systems.

The strategy suggested by this sector is the adoption of policy to allow captive plantations by industries. Following advantages are foreseen in case this strategy is being adopted:

- intensively managed plantations, based on selected genetically superior planting stock in areas close to the manufacturing units with short rotation harvesting cycles will provide high quality pulpwood at optimum cost;
- uniformity in raw material properties will facilitate standardization of manufacturing processes, resulting in higher recoveries and better product;
- productivity in intensively managed plantations based on selected clones will be much higher. Industry has demonstrated that 23-39 m^3/ha/year can be obtained compared to 6-10 m^3/ha/year from eucalyptus plantations based on traditional seed route, established by forest departments/corporations.

It is estimated that the extent of degraded/wasteland to be reclaimed is 130 million ha. A very small percentage of degraded land, if made available to the industry, it is felt by the industrialists that the shortage of raw material can be fulfilled up to same extent [26]. About 0.6 million ha land for plantation would be required to meet the paper industry demand keeping in view the present scenario and projected demand. India has about 130 million ha of

waste land and 32 million ha of degraded forest land, small part of which could be allocated for plantations [3]. There is strong opposition to this demand by environmentalists and non-government organizations. They feel that leasing land to industries to raise plantations will lead to:

- destruction of biodiversity;
- threat to the livelihood security of tribal, etc., depending on forest.

Plantation of fast growing and high yielding hardwoods species are the best alternative for supplying pulp and paper making fibers to Indian paper industry. Use of hardwood species for wood free papers and card boards will be helpful to solve multifold purposes like:

- hardwood species combine best possible combinations of critical paper properties like, tensile-tear, opacity-brightness, smoothness-bulk-stiffness and formation-retention-drainage etc.
- consistent raw material quality, available around the year
- good runnability, low long fiber addition with high filler content-totally cost efficient i.e. economical viability will be increased.
- withstands lumen collapse-possible to tailor properties with refining
- high yield, easy to debark

Besides forest based raw materials, the Indian paper industry is dependent on two major raw materials i.e. agriculture residues and secondary fibers (37). The pulp and paper industry uses 39% of forest based fiber, 31% agro residue based fibers and 30% fiber is derived from waste paper (20). There are serious constraints for the growth of pulp and paper industry based on agriculture residues and waste paper because of:

- narrow product range and low product quality;
- uneconomic size (with reference to production rate);

- obsolete technology;
- lack of chemical recovery and effluent treatment facilities;
- fluctuation in international prices of waste paper;
- uncertainties about long term availability of bagasse and cereal straw, because of their alternative uses.

In view of these constraints, this sector strongly favours review of policies and measures to ensure wood supplies for better quality of the product with minimum complications in processing it at the optimum cost. They have serious doubts about sustained supplies from natural forests and forest plantations in view of:

- intense biotic pressure on forests and plantations leading to illicit removal of firewood, excessive and uncontrolled grazing, fires, poor productivity, etc.;
- competitive demands on available supplies.

In present scenario, non-wood fibered pulp represents only 7% of the total world pulp production [27] but at over 10 million tonnes per annum; it still represents a substantial quantity. About 70 % of this non-wood pulp production occurs in India and China, where domestic wood fiber fails to sustain a continuous supply of fibers to pulp and paper industry. The estimated total availability of non-woody fibrous plant is 2300 million tonnes of which about 50 % are straws.

Waste paper based industry accounts for about one third of Indian paper capacity. The recovery of waste paper has increased from 650 00 tonnes in 1995 to 850 000 tonnes in 2000. Most of the paper is recovered, but due to alternative uses the recovery rate for paper industry is still only about 20 %. This is low by international standards: Thailand (42%), China (33%), and Germany (71%) [9].

The paper mills in India utilizing rice and wheat straw are typically very small with capacity up to 70-80 tonnes per day or 24,000 tonnes /year. The total consumption of rice and

wheat straws for pulp and paper production has been in the range of 0.4-0.5 million tonnes per year and out of which rice straw alone contributes 70% as a raw material to be used in this category.

India is the largest consumer and the second largest producer of sugarcane next to Brazil with a production of more than 300MT of sugarcane in 2001–2002 (accounting for around 10–12% of world's sugar production) [10, 21]. In India, about 4 million ha of land is under sugarcane farming with an average yield of 70 tonnes/ha [21]. The Indian sugar industry is the second largest agro-processing industry in the country after cotton textiles [38]. With an estimated production of 18.6MT in sugar year or SY2006 (sugar year is from October to September), India's sugarcane cultivation area of 4–4.5 million ha accounts for 2.7% of India's cropped area. Sugar industry accounted for around 1% of GDP of the country during the financial year 2005–2006 (FY2005) [30]. It was estimated that, after meeting the requirement of sugar industry, about 7.2 million tonnes of bagasse only may be made available to paper industry. Because of the scattered nature of sugar industry and total captive consumption of bagasse as fuel in sugar mills of capacity 1,200 tonnes per day and also high cost involved in collection, transportation and storage, it was presumed that it would not be possible to utilize the entire quantity of potentially available bagasse in the paper industry. Under these circumstances, the Development Council concluded that total availability would continue to be 7.2 million tonnes per annum, surplus quantity that could be released to paper industry would progressively increase from 15% in 1990 to 50% in year 2000 and 70% in 2015. The bagasse based paper production estimated by the Development Council that average recovery of 1 tonne of paper from 6 tonnes of bagasse.

In the few years, considerable attention has been paid to reduce the effluent generated during pulping as well as bleaching operation in a pulp and paper industry. A major part of the efforts has been focused on reducing emissions from bleaching processes. In most of the mills of India, bleachable grade pulp of kappa number around 24 is being produced from

hardwoods and pulp of kappa number about 16 is produced from bagasse. In India most of the mills uses conventional bleaching sequences, the pulps are being bleached by conventional either CEH or CEHH bleaching sequences, to the brightness level of around 80% (ISO) and is appropriate for most of the end uses. Little doses of hydrogen peroxide in alkaline extraction stage or in final bleaching stage are being used in some of the mills [31]. The AOX generated in conventional CEH/CEHH bleaching sequences of a 24 kappa number pulp to a brightness of 80% (ISO) is about 4-5 kg per tonnes of pulp. The large amount of energy and chemicals are wasted for the treatment of the effluent to bring down its value to the acceptable level of 2 kg per tonne of pulp as recommended by Central Pollution Control Board [36]. The first point in which 2,3,7,8-TCDD, was found out that the C-stage was generally 2,3,7,8-TCDF and 1,2,7,8-TCDF congeners were always present [17,19, 33]. The E-stage filtrate was found to have the highest concentrations of dioxins [29] known as changing the blood chemistry and causing liver damage, skin disorders, lung lesions and tumor types at numerous sites within the body, liver and thyroid included [14, 18, 28].

Delignification after pulping takes place in the chlorination stage of bleaching process. A major portion of lignin can be removed by oxygen delignification before the start of actual bleaching operation. The dissolved lignin during oxygen delignification is taken to recovery section. In oxygen delignification stage, approximately 50 % of the lignin left after cooking stage can be removed [6, 8, 13]. Since, the lignin remaining in the pulp after oxygen delignification is low and so the chlorination becomes less intensive. The level of AOX, TOCl, chlorinated dioxins, chlorophenols, chlorinated organo compounds and chloroform in pulp bleaching effluents can considerably be decreased by oxygen delignification stage than that of conventional bleaching sequence.

Biotechnology applications for pulp and paper industry have been developed for the past twenty years. The first introduction of enzyme at mill scale took place at 1980s, rapidly after the discovery and validation of the xylanase aided bleaching concept. Originally, the main

aim in the enzyme aided bleaching, is to reduce the chlorine chemical consumption in bleaching to reduce environmental discharge load and to increase the brightness of pulp. Xylanase enhances pulp bleaching by reducing the amount of chlorine required and generation of organochlorine compounds [32]. When combined with TCF bleaching sequences, enzymes can be used to increase the final brightness of pulp. Reduction in chlorine equivalents has been reported from laboratory work and mill trials by using enzyme pretreatment [39, 4, 5]. Subsequently the AOX load of the bleaching effluent has been reduced by nearly the same percentage [4]. The chemical oxygen demand of the effluent is due to higher yield loss enzymes also in improving the drainability of pulp [5].

The development in xylanase bleaching is focusing on improved enzyme properties and improved enzyme performance. Improved properties include higher pH and temperature tolerance of the enzymes, to make the enzyme treatment operations more compatible with existing mill operations. Improved enzyme- performance is being approached by tailoring the enzyme action more closely to the hemicellulose structure of the pulp, to result in a greater bleaching benefit or higher pulp yield.

In the present investigation, *Anthocephalus cadamba,* belong to family *Rubiaceae* has been used for biobleaching studies. *Anthocephalus cadamba* is commonly known as Kadam (Indian, French and trade name); common bur-flower tree (Eng.); kaatoan bangkal (Philippines); mai sa kho (Laos); kalempajan, jabon (Indonesia); kalempayan (Malaysia); thkoow (Cambodia). *A. cadamba* is a moderated sized graceful deciduous tree grown as an ornamental. *A. cadamba* is grown as soil improver as its leaves improve some physical and chemical properties of soil under its canopy on decomposition. *A. cadamba* is suitable for the up gradation of the soil as it increases in the level of soil organic carbon, cation exchange capacity, available plant nutrients and exchangeable bases [7, 40].

Keeping in view the discussions in the foregoing pages, the present investigation has been carried out with the following objectives. To keeping in mind the above facts, the following objectives were selected for present study:

1. To isolate and purify the thermo-alkaline xylanases producing white rot fungi from their natural habitats.
2. To characterize the screened isolates for different extracellular enzymes by plate assay methods and nutrient requirements.
3. To optimize the enzyme production parameters with effects of different carbon sources/substrates and nitrogen source under laboratory conditions.
4. To characterize the produced xylanases with respect to pH stability, thermo stability and metal ions stability.
5. To identify the fast growing hardwood species as a potential fibrous raw material (*Anthocephalus cadamba*) for the production of chemical grade pulp.
6. To study the raw material anatomically, morphologically and chemically for the suitability of chemical grade pulp.
7. To optimize the different operating parameters for the production of kraft and kraft-AQ pulp under laboratory conditions.
8. To study the biobleaching effect of produced xylanases in different chemical bleaching sequences including conventional, elemental chlorine free (ECF) and total chlorine free (TCF) bleaching sequences under laboratory conditions.

9. To study the effect of enzyme-aided bleaching on brightness, viscosity, PFI revolutions during beating and mechanical strength properties like, tear index, tensile index, burst index and double fold.

10. To study the effect of enzyme-aided bleaching on COD, colour and AOX in combined bleach effluent

Table1: Paper supply and demand scenario for India (GDP growth: base scenario 6 % annum, conservative scenario 5 %/annum) [1, 25]

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

Table 2: Forest cover by sub region 2005 and distribution [41]

Region/sub region	Forest area (*1 000 ha*)	% of land area	% of global forest area
Eastern and Southern Africa	226534	27.8	5.73
Northern Africa	131048	8.6	3.32
Western and Central Africa	277829	44.1	7.03
Total Africa	635412	21.4	16.08
East Asia	244862	21.3	6.20
South and Southeast Asia	283127	33.4	7.16
Western and Central Asia	43588	4.0	1.10
Total Asia	571577	18.5	14.46
Total Europe	1001394	44.3	25.34
Caribbean	5974	26.1	0.15
Central America	22411	43.9	0.57
North America	677464	32.7	17.14
Total North and Central America	705849	32.9	17.86
Total Oceania	206254	24.3	5.22
Total South America	531540	47.7	21.04
World	3952025	38.3	100.00
1 hectare (ha) = 10 000 square meters (m^2) = 0.01 square kilometers (km^2)			

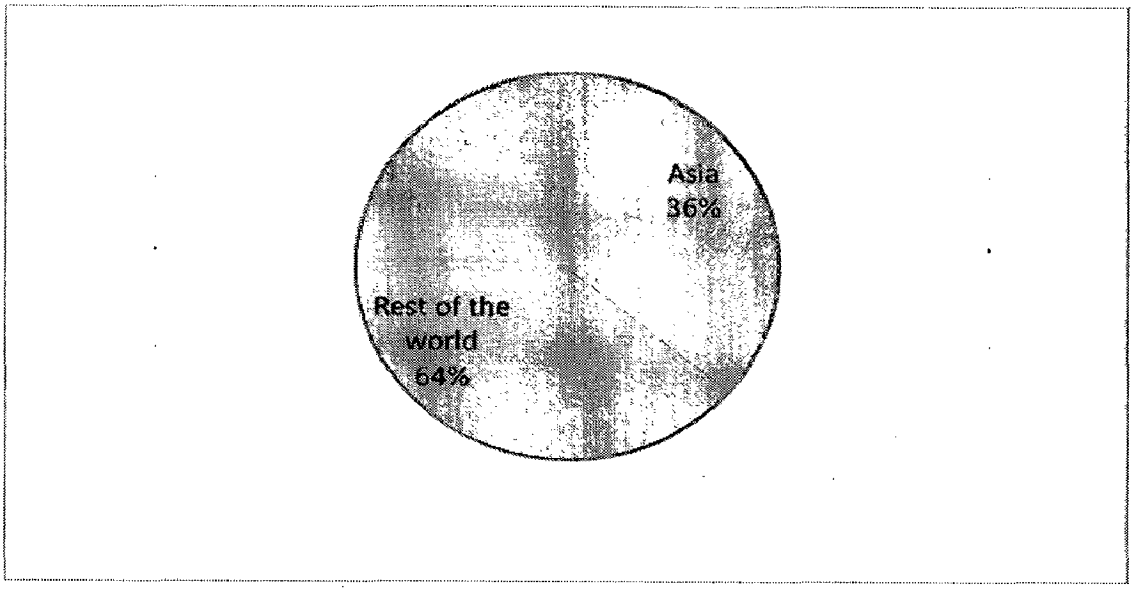

Figure 1: Total world paper and paperboard consumption in 2006 [35]

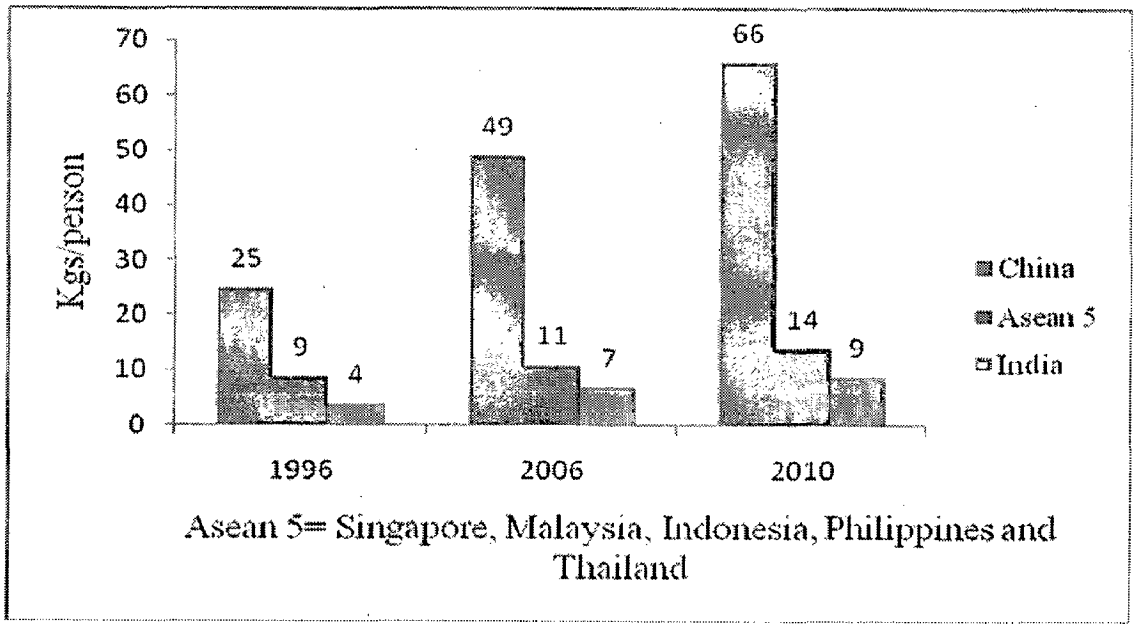

Figure 2: Per capita consumption of paper and paper board in Asia [1, 25]

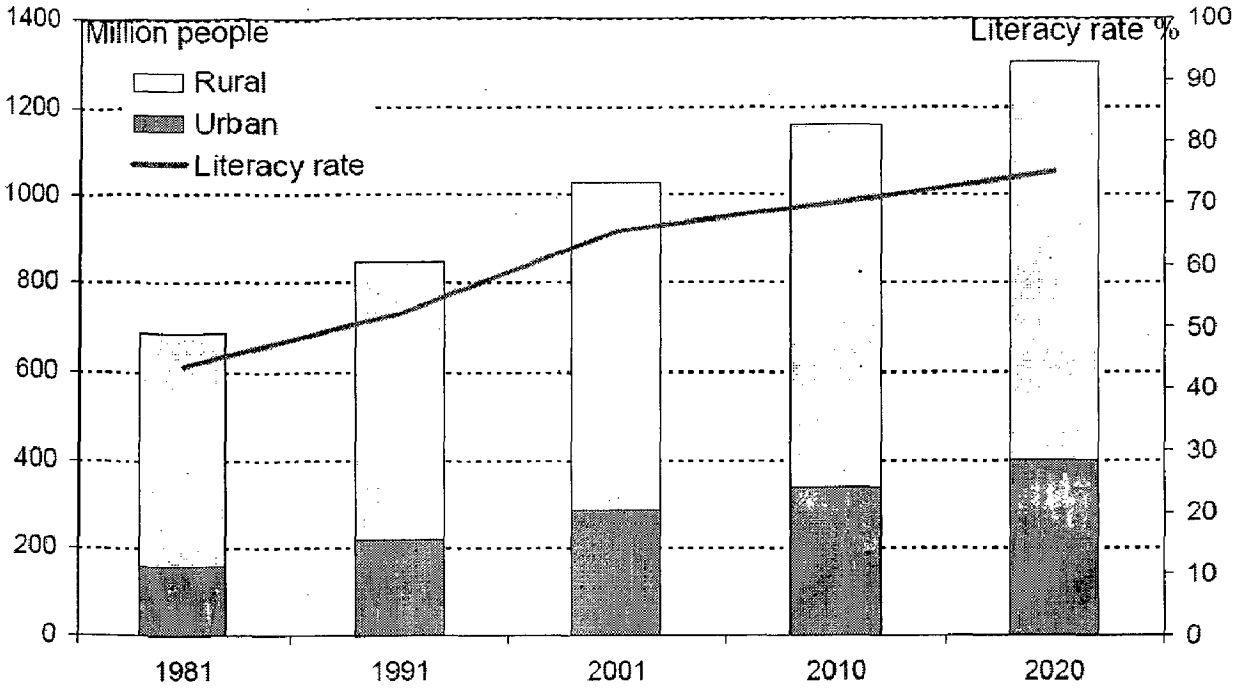

Figure 3: Indian population and literacy rate [25]

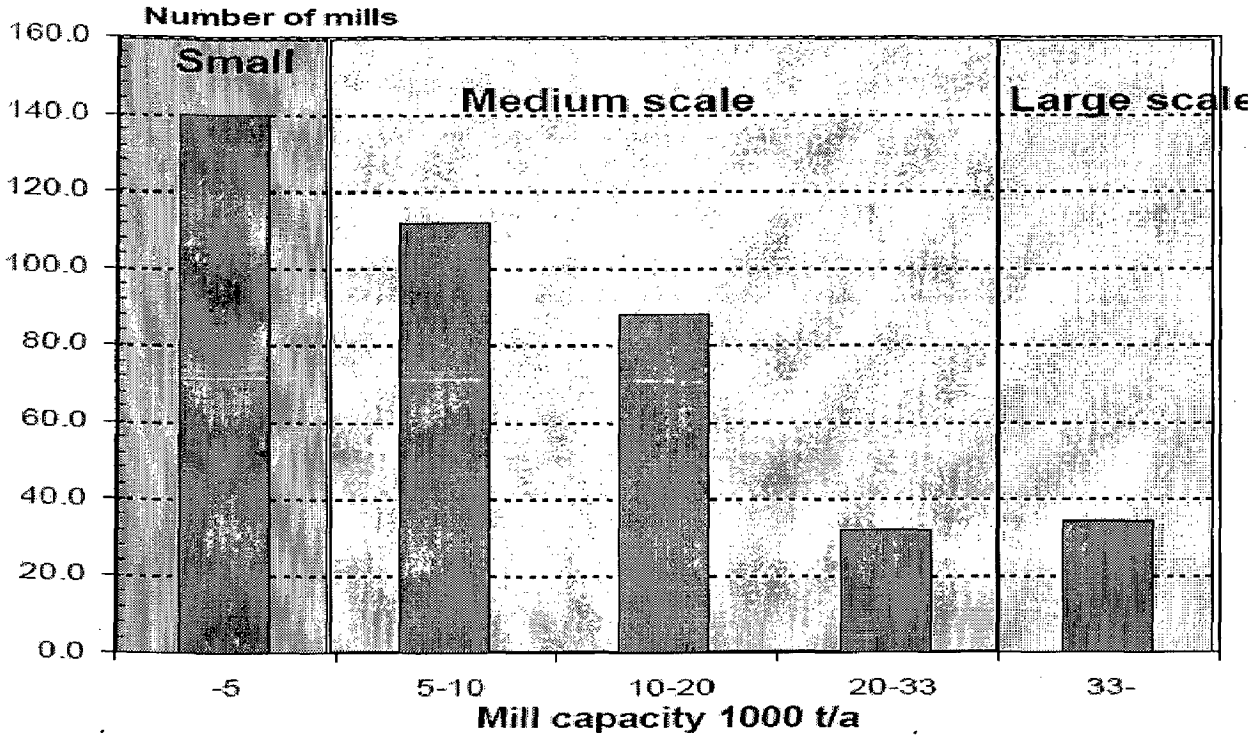

Figure 4: Indian paper mills structure in 2001 [23]

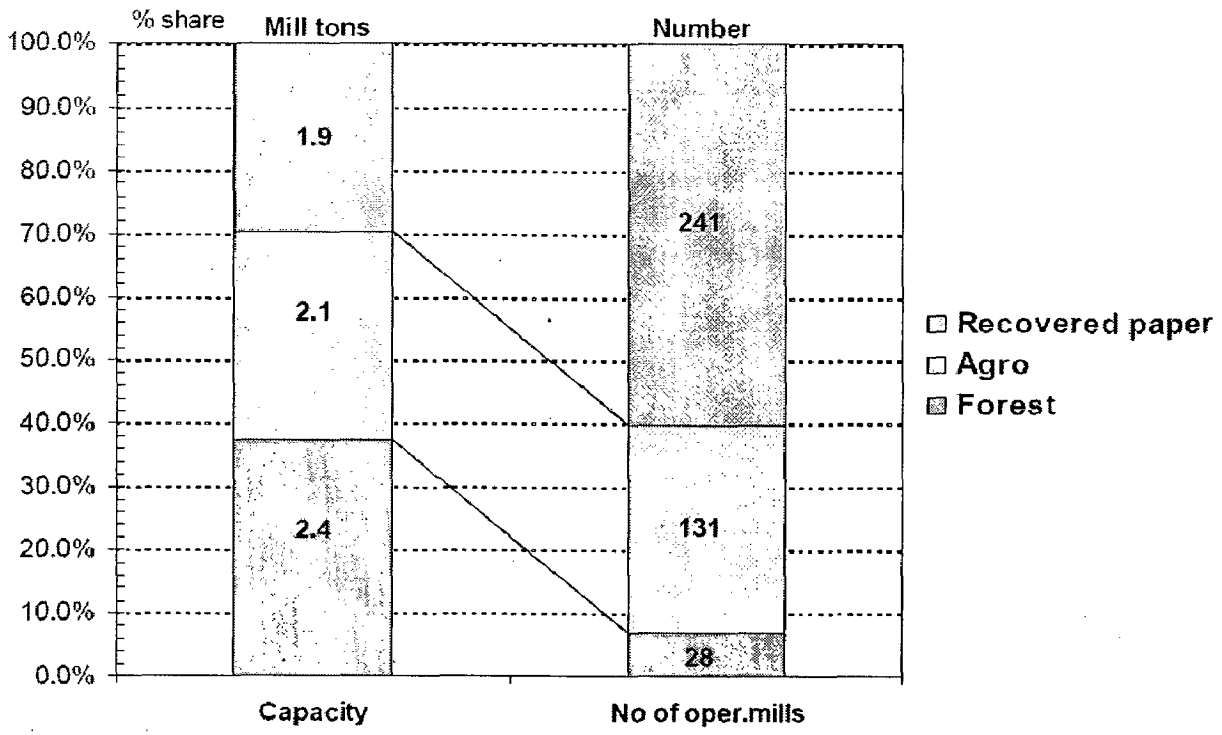

Figure 5: Structure of Indian paper industry based on fibrous raw materials in 2001 [24]

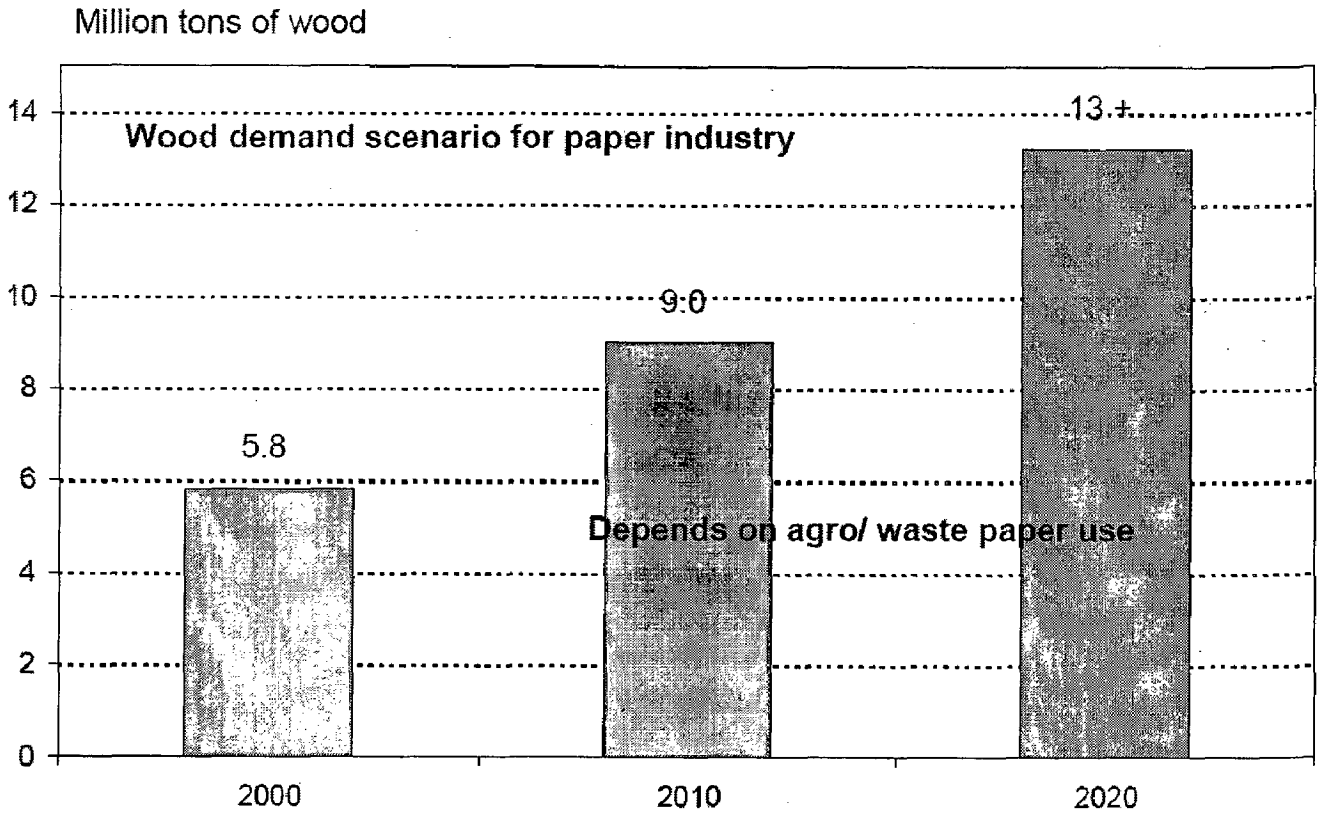

Figure 6: Industrial wood demand scenario for India

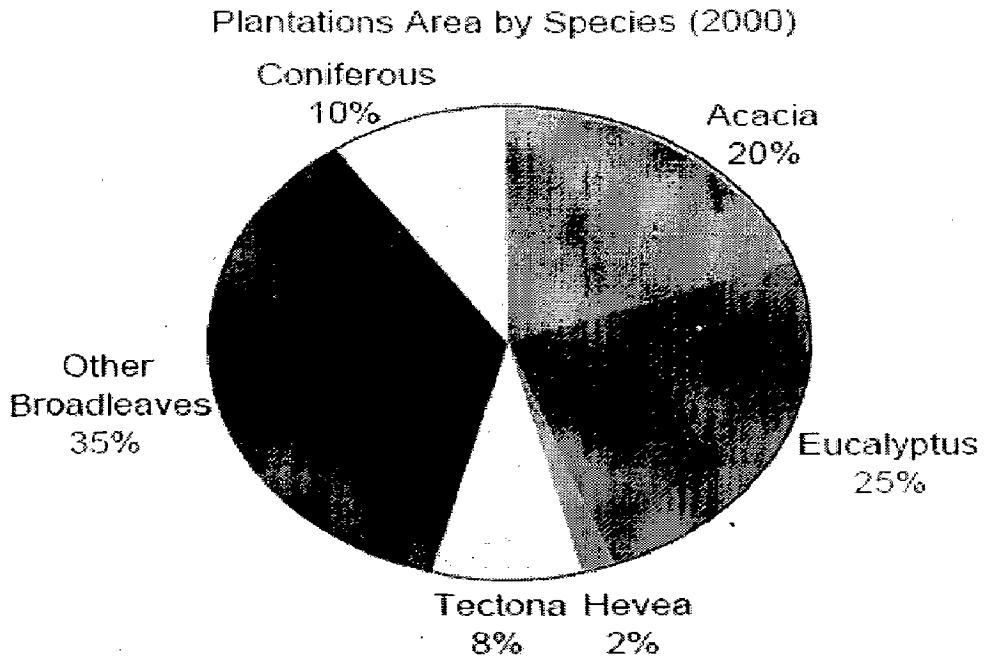

Figure 7: Forest plantation in India [25]

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CHAPTER: 2

STUDIES ON WHITE-ROT FUNGI FOR PREPARATION OF ENZYMES

2.1 INTRODUCTION

The secretion of extracellular metabolites is the inherent practice of microorganisms, which make them unique in the nature. The diversity of microorganisms in nature is surprising. More than 400,000 of microorganisms are known in the nature, and this is just a fraction of the probable number. It is expected that there are four to five million different species of microorganism. As a result, microorganisms can be found in almost every biotope around the world. The enzyme industry is keen to exploit this diversity by gathering soil and water samples from the four corners of the Earth. Presently, the industrial enzyme companies sell enzymes for a wide variety of applications.

According to The Freedonia Group, USA (2007) the global demand for enzymes is expected to grow 7.6% per year and the total market will be reached up to $6 billion by 2011, driven by continued strong growth in pharmaceutical enzyme demand, double-digit increases in demand for biocatalysts for pharmaceutical and other fine chemical production, and the rapid extension in bio-ethanol production from grains and lignocelluloses. According to them, the highest growth will be in the Asia/Pacific and other developing nations because increasing per capita income will give rise to make more thorough use of enzymes, while the North America and Western Europe shall be trailing behind. Descending pricing pressure mainly in the market will grip down further growth, although the introduction of more efficient, higher priced enzymes will enhance average unit pricing values [2].

Presently, the technical industries, dominated by the detergent, starch, textile and fuel alcohol industries, account for the majority of the total enzymes market, with the feed and food enzymes together adding only about 35%. However, sales in some of the main technical industries have stagnated presently. While sales in both the food and feed industries are

increasing day-by-day with annual growth rate of approximately 4–5% being forecasted [23]. Hydrolytic enzymes constitute around 75% of the markets for industrial enzymes, with the glycosidases, including cellulases, amylases and hemicellulases, constituting the second major group after proteases [8].

Xylanases constitute the major marketable proportion of hemicellulases but represent only a small percentage of the total enzyme sales. The sales figures are expected to increase, however, as these enzymes are drawing attention of industrialist and creating more interest due to their possible use in different industries including pulp and paper, juice processing, textile, and food and feed. According to United States Patent and Trademark Office more than 468 patents has been obtained by individually or commercial firms since 2001 with reference to xylanases. The major commercial hemicellulases and manufacturing firms are listed in Table 2.1

Xylanase is a hydrolase, catalysed the hydrolysis of xylan. Xylan is the second most abundant structural polysaccharide in nature. Its whole degradation requires the mutual action of a variety of hydrolases i.e. the endoxylanases (EC 3.2.1.8), which randomly cleave β-1, 4-linked xylose; the β-xylosidases (EC 3.2.1.37), which hydrolyze xylooligomers; and the different side-branch splitting enzymes, including α-glucuronidase and α-arabinosidase, acetylxylan esterase, and acetyl esterase, which liberate other sugars included glucuronic acid and arabinose that are attached as branches to the backbone [9]. The hypothetical structure of xylan attack by xylanolytic enzymes is given in Figure 2.1 [54].

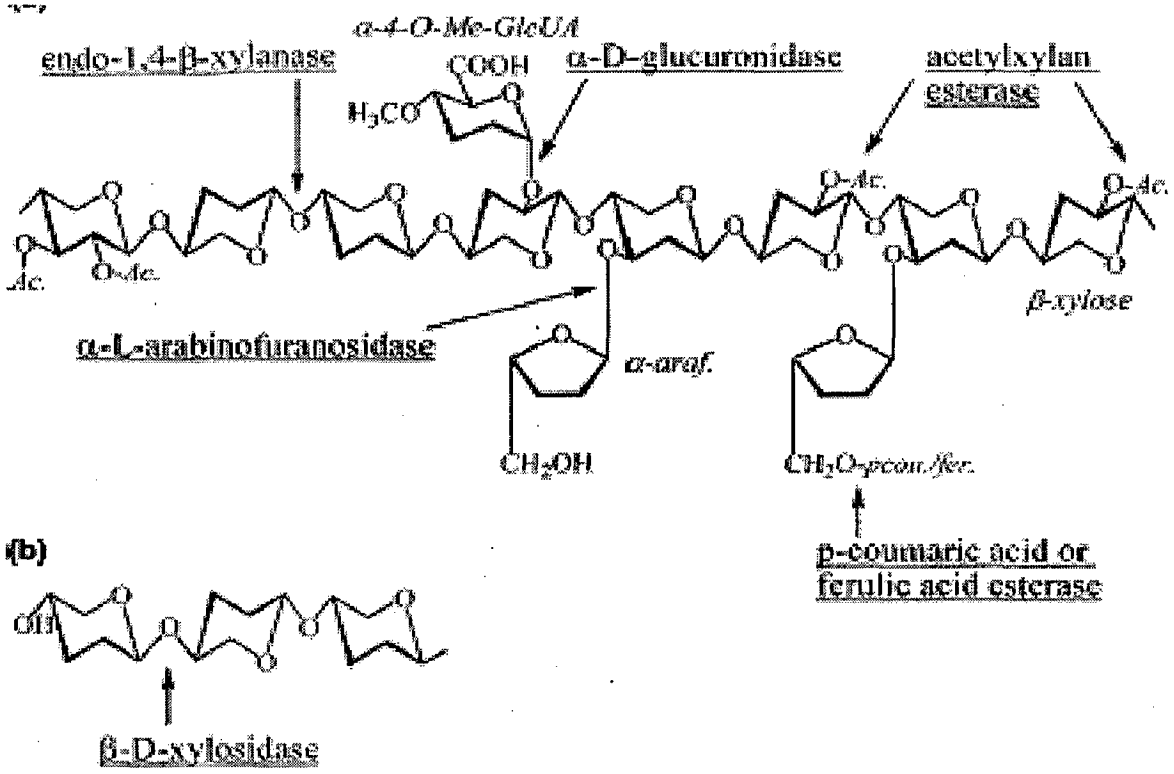


Figure 2.1: Structure of xylan showing sites of xylan attack by xylanolytic enzymes. The backbone of substrate is composed of 1, 4- β-linked xylose residues [54].

Xylanases from alkalophilic and thermophilic fungi are receiving considerable interest because of their application in pulp and paper industry for biobleaching, in which the enzymatic removal of xylan from lignin-carbohydrate complexes facilitates the leaching of lignin from the fiber cell wall, obviating the need for chlorine for pulp bleaching. The majority of xylan-degrading enzymes from thermophilic fungi are endoxylanases [43].

Thermophilic fungi are the principal components of the micro-flora that develops in heaped masses of plant material, piles of agricultural and forestry products, and other accumulations of organic matter wherein the warm, humid, and aerobic environment provides the basic conditions for their development [1]. They constitute a heterogeneous physiological group of various genera in the Phycomycetes, Ascomycetes, Fungi Imperfecti, and Mycelia Sterilia [46]. Maheshwari and Kamalam, have reported that *Melanocarpus albomyces* produces thermostable xylanase and fungus is able to be grown at 30, 40 and 50 ^{0}C while, 50 ^{0}C is found the best temperature for growth. The maximum stability of enzyme activity is found at 40 ^{0}C [43]. Another fungus, *Thermoascus aurantiacus* produces xylanase and L-xylosidase with

maximum activities at 80 ^{0}C and 75 ^{0}C respectively. More than 90% of xylanase and 60% of L-xylosidase activities are retained over 84 days at 50 ^{0}C [24]. Among genus *Aspergillus*, it has been reported that *Aspergillus sydowii* MG 49 produces two xylanases with optimal activity at 60 ^{0}C and a stability in the range of 40 to 70 ^{0}C. However, xylanase activity is found to be declining sharply around at 70 ^{0}C [20]. Kitpreechavanich *et al.*, has reported xylanase stability at 65 ^{0}C, produced by *Aspergillus fumigatus* [37]. A strain of *Aspergillus niger* reported by John *et al.*, produces two enzymes with a broad temperature activity and maximal activity between 65 and 80 ^{0}C [32]. Recently, Castro *et al.*, has reported a thermo tolerant strain of *Aspergillus* which produces xylanase active at 80 ^{0}C and pH 6.5 [10].

The main source of xylanases is microorganisms, which produces extracellular xylanases in presence of suitable inducer such as, xylan. The basic factors for efficient production of xylanolytic enzymes are the choice of an appropriate inducing substrate and optimum medium composition [38]. The induction of enzyme production using purified substrate makes the processes expensive. The use of agricultural residues rich in hemicellulose increases xylanase production, lowering the cost of biobleaching of pulp [4].

A variety of inducers have been used for induction of xylanases such as, sawdust, corn cob, wheat bran, and sugar beet pulp and sugarcane bagasse. In *Melanocarpus albomyces* and *Thermomyces lanuginosus*, xylose, the monomeric unit of xylan, can also induce xylanase. Xylanases are often co-induced with cellulases by pure cellulose, as in *T. aurantiacus*, *Chaetomium thermophile* var. *coprophile*, and *H. insolens*. Wheat bran is found to be the best substrate for xylanase production by a thermophilic *Bacillus licheniformis* grown on solid substrate [4].

The most of the xylanases are single polypeptides. Molecular mass of xylanases covers a broad range, from 21 to 78 kDa. However, Dusterhft *et al.*, has reported a xylanase II from *Humicola insolens*, with extraordinary molecular mass of 7.0 kDa [16]. The carbohydrate contents of the three xylanases of *Talaromyces byssochlamydoides* vary from 14- 37%. Two

endo-xylanases of *T. emersonii* are remarkable in the way that they do not have action on xylan unless the arabinose substituents are removed and also in their ability to hydrolyze aryl β-D-xylosides. Xylanases have not shown cooperative relations in the hydrolysis of xylan [22, 49].

White rot fungi, which use cellulose as a carbon source, possesses the unique ability to degrade lignin completely to carbon dioxide to access the cellulose molecule. Scientists hope that an understanding of how white rot fungus degrades wood will lead to its successful application in enzyme industry as well as in hazardous waste remediation. The lignin degradation enzyme system of white rot fungi is extracellular and unusually nonspecific. The enzymatic mechanism of wood degrading fungi is clarified to a great extent. The discovery of lignin peroxidase by Tuor *et al.,* [56] from *P. chrysosporium* triggers research on biodegradation of lignin. Many efforts have been made to investigate the application of these fungi for the removal of lignin in the pulping and bleaching process. It was first reported by Kirk and Yang [36] that *P. chrysosporium* is able to partially delignify unbleached kraft pulp.

Coprinus disseminates is a member of family Coprinaceae, generally, grows with many basidiocarps on and around trees or on soil having plant debris. The velar spherocysts and typical spore-shape together with the long pileocystidia with cylindrical neck and rounded apex is special characteristics of species. *Coprinus disseminatus* is scientifically classified as Fungi, Basidiomycota, Agaricomycotina, Agaricomycetes, Agaricomycetidae, Agaricales, Coprinaceae, *Coprinus.* The genus *Coprinus* has been characterized by dark spores possessing an apical germ pore, well-developed paraphyses, the deliquescent nature of lamellae, and inaequihymeniferous development of basidia. In most species, autolysis of lamellae and pilei can be detected. Morphological and developmental characters based on the above-mentioned ones have been used to define coprinoid taxa. Several species have been well-known for a model system in the studies of mating compatibility, speciation, molecular biology and fruit body development. Kwan *et al.,* investigated the Phylogeographic divergences of four coprinoid

species, *Coprinus comatus*, *Coprinellus disseminatus*, *Coprinellus micaceus* and *Coprinopsis lagopus* using nuclear ITS sequences. Each taxon shows genetic variation that corresponds with the geographic origins of collections [39].

2.2 MATERIALS AND METHODS

2.2.1 Isolation of fungi

A total of 30 decaying wood samples were collected from different sites in the vicinity of Saharanpur located near the foothills of Shivalik hills in western Uttar Pradesh (India). Out of 30 wood samples, 15 strains of wood rotting white rot fungi were isolated by enrichment technique, in which the decaying wood samples were kept in 9 mm diameter glass Petri plates enriched with moist wheat bran. These wood samples were incubated at 40 ^{0}C in a BOD incubator and the growth was observed every day. The moisture of plates was maintained with sterile tap water. Growing fungal cultures were isolated on wheat bran agar medium (2% wheat bran powder + 2% agar powder) and examined under light microscope. When white rot basidiomycetes observed, these were purified and stabilized on same medium by frequently sub culturing.

The purified cultures of fungi were maintained at 4 ^{0}C in Potato Dextrose Agar (PDA) slants (HiMedia) in screw capped culture tubes. These were frequently sub cultured after every 2-3 months. The spores were preserved in 15% sterile glycerol and stored at -20 ^{o}C in deep freeze for further studies.

2.2.2 Effect of pH on growth and xylanase activity

In the contrast of screening alkali-tolerant/alkalophilic fungi, the method described by Nagai *et al.*, [47] was adopted. The isolated fungi were cultivated in L.S.F. conditions by shake flask culture method using 2% wheat bran (w/v) as substrate, 40 mL of nutrient salt solution having 1.5 g/L KH_2PO_4, 4 g/L $NaNO_3$, 0.5 g/L $MgSO_4$, 0.5 g/L KCl and 1 g/L yeast extract in distilled water with 0.04 mL/L trace elements solution (200 µg/L $FeSO_4$. $7H_2O$, 180 µg/L

$ZnSO_4$. $7H_2O$, 20µg/L $MnSO_4$. $7H_2O$). The pH of flasks was adjusted to 5.0 to 11.0 by 1.0 N NaOH by mixing 90% of nutrient salt solution and 10% of appropriate buffer solution according to pH. These were autoclaved at 15 Pa for 15 min and the pH of flasks was readjusted with 0.1 N NaOH. The flasks were incubated at 40 ^{0}C for 10 days in incubator shaker (Model: Innova, 43 Brunswick, USA) at 100 rpm. After 10 days of incubation, the fermentation broth was filtered through cheese cloth followed by centrifugation at 15,000 rpm for 1 h at 4 ^{0}C. The supernatants obtained were analyzed for xylanase activity at different pH levels and two fungal strains namely MLK 01 and MLK 07 were selected for further study.

2.2.3 Identification of cultures

The two selected fungal strains were sent to Plant Pathology Laboratory, Forest Research Institute (FRI), Dehradun (India) for further identification. The clean and dry glass slides (15 mm) were covered with a thin smear of potato dextrose agar medium and these glass slides were kept in glass petri plates (9 cm diameter) and autoclaved. The fresh cultures of two selected strains were placed with sterile needle on glass slides at center separately and incubated at 40 ^{0}C. The fungal growths were examined under light microscope at different magnifications. The fungal cultures were further grown on wheat straw powder in 250 mL Erlenmeyer flasks in highly humid condition for examination of fruiting bodies.

2.2.4 Effect of different agar media on fungal growth

Six agar media including wheat bran agar (WBA), malt extract agar (MEA), potato dextrose agar (PDA), fungal agar (FA), lactose yeast extract agar (LYEA) and malt glucose yeast peptone agar (MGYP) were prepared to observe its effect on fungal growth. The pH of the media was maintained at 10.0 with 1.0 N NaOH after autoclaving. A disc of 6 mm diameter from 4 day old culture of test fungi was aseptically inoculated in center of each Petri plate. These were incubated at 40 ^{0}C in a BOD incubator and growth was measured after 36 and 72 h of incubation respectively in terms of colony diameter (cm). The appearances of fungi were observed with naked eye every day.

2.2.5 Screening for extracellular enzymes

The two selected fungal cultures were screened for the following enzyme activities: i) amylases, ii) cellulases, iii) xylanases, iv) mannanases, v) laccases, vi) ligninases, and vii) proteases

Screening of fungal cultures for the various extracellular enzyme activities were carried out on agar medium using 1% xylan (Sigma) for xylanase, 1% CMC (HiMedia) for cellulase, 1% mannan (HiMedia) for mannanase , 1% soluble starch (HiMedia) for amylase, 1% gelatin for protease, 0.02% tannic acid for ligninase, 10 mM guaiacol (HiMedia) for laccase. The media were supplemented with 1% yeast extract. A 6 mm diameter disc from 4-day old culture of each test fungi was inoculated at the center of the each Petri plate and incubated at 40 ^{0}C for a period of 3 day. The Petri plates were flooded with 1% iodine in 0.2% KI solution to enhance the visibility of amylase activity. In the same way, cellulases, mannanases and xylanases, the Petri plates were flooded with an aqueous solution of 0.1% Congo red. After 30 min, these Petri plates were frequently washed with 1.0 N NaCl solution to enhance the visibility of cellulases, mannanases and xylanases activities. The activities of laccases, ligninases and proteases were observed visibly without staining.

2.2.6 Preparation of extracellular enzymes under liquid state fermentation conditions

Both the fungal strains were cultivated separately under LSF condition in 250 mL Erlenmeyer conical flasks using 0.8 g wheat bran powder, 40 mL of nutrient salt solution having 1.5 g/L KH_2PO_4, 4 g/L $NaNO_3$, 0.5 g/L $MgSO_4$, 0.5 g/L KCl and 1 g/L yeast extract in distilled water with 0.04 mL/L trace elements solution (200 µg/L $FeSO_4$. $7H_2O$, 180 µg/L $ZnSO_4$. $7H_2O$, 20µg/L $MnSO_4$. $7H_2O$). The desired pH of nutrient salt solution was adjusted with NaOH /H_2SO_4 with the help of microprocessor controlled pH meter (Knick, Germany, Model-761 Calimatic). Each flask was plugged with cotton and sterilized at 15 Pa for 15 min. The pH of each flask was checked on cooling at room temperature and readjusted again aseptically. Two discs of 6 mm diameter of 4-d old culture of both the fungal strains were aseptically inoculated

in separate flask. These flasks were incubated at 40 ^{0}C with shaking at 100 rpm. The shaking was stopped before 24 hrs of harvesting to preserve the lignin peroxidase activity.

2.2.7 Harvesting of enzyme

After optimizing incubation time under LSF conditions, the flasks were taken out from incubator and contents were filtered through cheese cloth followed by vacuum filteration with microfilter of 0.45 μm at room temperature and the lignin peroxidase activity was determined immediately. The rest of the filtrate was centrifuged at 15000-x g (Sigma centrifuge mode 2K15) for 1 h at 4 ^{0}C. The dark brown coloured supernatant was stored at –20 ^{0}C until used and pallets were analyzed for mycelial growth.

2.2.8 Estimation of fungal growth in terms of mycelial protein concentration

The growth of fungus was determined as described by Ball and McCarthy [6]. The pellets retained on cheese cloth were washed with distilled water for four times to remove the attached wheat bran powder. Washed pallets then boiled in 20 mL of 1.0 M NaOH for 10 min. After cooling at room temperature, required solution of 1.0 M NaOH was added to make up a final volume of 20 mL. The protein concentration of filtrate was determined by Lowry's method [42] using bovine serum albumin as standard. Fungal growth has been expressed in terms of mycelia protein concentration (mg/mL).

2.2.9 Xylanase assay

The xylanase activity was determined by measuring the release of reducing sugars using birch wood xylan (Sigma Chemicals Co.) as a substrate by 3, 5 dinitrosalicylic acid reagent (DNS) method [45]. 1.6 mL of enzyme preparation was added in a sterile tube, which contained 0.4 mL of substrate suspension (10 mg/mL birch wood xylan in 0.1 M potassium phosphate buffer). Proper controls, in which substrate and/or enzyme preparation had been omitted, were included. The assay mixture was incubated at 55 ^{0}C for 15 min with constant shaking at 100 rpm. Then, assay mixtures was cooled and centrifuged at 10,000 x g. 1 mL of supernatant was poured in a fresh tube which contained 3 mL of 3, 5-dinitrosalicylic acid (DNS) reagent and was kept for 5 min on boiling water

bath. Optical density was measured at 540 nm in a double beam UV-visible spectrophotomet (Systronics UV-Visible double beam spectrophotometer model - 2201). The enzyme activity expressed as μ moles of D-xylose equivalents released min $^{-1}$ at 55 ^{0}C (IU). The blank w maintained in same manner using distilled water in place of enzymatic reaction products.

2.2.10 Estimation of cellulase activity

The cellulase activity in terms of CMCase was determined by DNS method [45]. Two mL crude enzyme preparation diluted to 10 times with distilled water was taken in screw capp universal sterile tubes containing 2 mL of 2% (w/v) carboxyl methyl cellulose (CMC) (make BDH as a substrate maintained at pH 4.8 with 0.05 M citrate buffer. The reaction mixture was incubate in water bath at constant temperature of 50 ^{0}C for 30 min. The reaction mixture was centrifuged 5000 x g (Remi centrifuge, R 8 C) for 2 min after cooling in ice bath. One mL of supernatant and mL of 3, 5 dinitro salicylic acid (DNS) reagents was mixed thoroughly and kept on boiling wat bath for 5 min. The contents were cooled rapidly under tap water and the optical density wa measured at 575 nm in a double beam Spectrophotometer (Systronics UV-Visible double bea spectrophotometer model - 2201). The same was repeated as control using distilled water in place crude enzyme preparation. Reducing sugars were measured by comparing with the standard curv prepared for D-glucose. The enzyme activity is expressed as μ moles of D-glucose equivalen released min $^{-1}$ at 50 ^{0}C (IU).

2.2.11 Estimation of lignin peroxidase

Lignin peroxidase activity was measured spectrophotometerically as described by Merce [44] using 2, 4-di-chlorophenol as a substrate. One mL of reaction mixture was taken, whic contained 200 μL of 50 mM 2, 4-4-di-chlorophenol; 200 μL of 1 mM 4-aminoantipyrine and 20 μL crude enzyme preparation maintained at pH 6.5 with 200 μL of 100 mM phosphate buffer wa taken in a cuvette of 2 mL capacity for determination of peroxidase activity. Now 200 μL of 50 mM H_2O_2 was being added in to the reaction mixture and immediately measured the absorbance at 51 nm after every 30 s until constant with double beam Spectrophotometer (Systronics UV-Visibl

double beam spectrophotometer model - 2201). The same was repeated as control using distilled water in place of crude enzyme preparation. The enzyme activity was expressed as the amount of enzyme produced with an increase of 1.0 absorbance unit per 30 s.

2.2.12 Optimization of incubation period

Two sets of fifteen Erlenmeyer flasks of capacity 250 mL were prepared for each fungal strain as described earlier in the paragraph 2.2.6 for the optimization of incubation period. 2% wheat bran and 40 mL of nutrient salt solution was added in each flask and maintained at pH 10 with $NaOH/H_2SO_4$. These flasks were autoclaved at 15 Pa for 15 min. Two discs of 6 mm diameter of 4-d old cultures of both the fungi were aseptically inoculated separately in each set. These were incubated at 40 ^{0}C and were harvested periodically after 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 18 and 20th day. The fungal growth estimated as per protocol described in paragraph 2.2.8 and their extracellular xylanase, cellulases and lignin peroxidase activities were estimated as per the protocols described in paragraphs 2.2.9-2.2.11 respectively.

2.2.13 Optimization of pH

Two sets of eight Erlenmeyer flasks of capacity 250 mL were prepared for each fungal strain as described in paragraph 2.2.6 for the optimization of pH level. 2% wheat bran and 40 mL of nutrient salt solution was added in each flask and varying different pH levels i.e. 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, 11.0 and 12.0 using different buffer solutions as described by Nagai separately [47]. These flasks were autoclaved at 15 Pa for 15 min. The pH of each flask was checked and readjusted aseptically in laminar air flow chamber after cooling at room temperature. Two discs of 6 mm diameter of 4-d old cultures of both the fungi were aseptically inoculated separately. These flasks were incubated at 40 ^{0}C and harvested after 8th day of incubation. The fungal growth estimated as per protocol described in paragraph 2.2.8 and their extracellular xylanase, cellulases and lignin peroxidase activities were estimated as per the protocols described in paragraphs 2.2.9-2.2.11 respectively.

2.2. 14 Effect of different sugars on the production of extracellular enzymes under L.S.F. conditions

In the similar way, two sets of five Erlenmeyer flasks of capacity 250 mL were prepared for each fungal strain as described in paragraph 2.2.6 to observe the effect of different sugars i.e. glucose, xylose, galactose and xylan of fungal growth and enzyme induction at different concentrations ranging from 0.5 to 5.0 g/L. Two percent wheat bran and 40 mL of nutrient salt solution was added in each flask while varying the concentrations of glucose, xylose, galactose and xylan from 0.5, 1.0 to 5.0 g/L with an interval of 1.0 g/L. These flasks were autoclaved at 15 Pa for 15 min and pH in each set was adjusted to 10 with $NaOH/H_2SO_4$. Two discs of 6 mm diameter of 4-d old cultures of both the fungi were aseptically inoculated separately. These flasks were incubated at 40 ^{0}C and harvested after 8th day of incubation. The fungal growth estimated as per protocol described in paragraph 2.2.8 and their extracellular xylanase, cellulases and lignin peroxidase activities were estimated as per the protocols described in paragraphs 2.2.9-2.2.11 respectively.

2.2.15 Effect of different concentrations of urea on the production of extracellular enzymes under L.S.F. condition

Two sets of ten Erlenmeyer flasks of capacity 250 mL were prepared for each fungal strain as described in paragraph 2.2.6 for the optimization of urea. 0.8 g wheat bran and 40 mL of nutrient salt solution was added in each flask while varying the concentrations of urea from 0 to 5.0 g/L with an interval of 1 g/L. These flasks were autoclaved at 15 Pa for 15 min and pH in each set was adjusted to 10 with $NaOH/H_2SO_4$. Two discs of 6 mm diameter of 4-d old culture of both the fungi were aseptically inoculated separately. These flasks were incubated at 40 ^{0}C and harvested after 8th day of incubation. The fungal growth estimated as per protocol described in paragraph 2.2.8 and their extracellular xylanase, cellulases and lignin peroxidase activities were estimated as per the protocols described in paragraphs 2.2.9-2.2.11 respectively.

2.2.16 Effects of different substrates

Two sets of five Erlenmeyer flasks of capacity 250 mL were prepared for each fungal strain as described in paragraph 2.2.6 for the optimization of different lignocelluloses wastes like, wheat

bran (WB), sugarcane bagasse (BG), wheat straw(WS), saw dust (SD) of wood and ground nut shell (GNS). These lignocelluloses wastes were milled separately into powder in a laboratory Wiley mill and a fraction retained on +100 mesh size was used as substrate. 0.8 g of above mentioned lignocelluloses wastes were added separately in each flask containing 40 mL of nutrient salt solution. These flasks were autoclaved at 15 Pa for 15 min and pH in each set was adjusted to 10 with $NaOH/H_2SO_4$. Two discs of 6 mm diameter of 4-d old culture of both the fungi were aseptically inoculated separately. These flasks were incubated at 40 ^{0}C and harvested after 8th day of incubation. The fungal growth estimated as per protocol described in paragraph 2.2.8 and their extracellular xylanase, cellulases and lignin peroxidase activities were estimated as per the protocols described in paragraphs 2.2.9-2.2.11 respectively.

2.2.17 Mass production of enzymes under L.S.F. condition

The mass production of enzymes from both the fungal strains was carried out in Bench Loop Fermentor (INFORS-AG-CH-4103 Bottmingem/ Switzerland) having vessel capacity of 2 L. The composition of medium for both the fungal strains used for L.S.F. was: 30g wheat bran and 1.5 L nutrient salt solution maintained at pH 10 and temperature 40 ^{0}C. The optimum xylanase activity was produced on 6th day. The contents after fermentation were filtered through 4 layer of cheesecloth and filtrate was used for their extracellular xylanases, cellulases and lignin peroxidase activities were estimated as per the protocols described in paragraphs 2.2.9-2.2.11 respectively.

2.2.18 Characterization of xylanases

2.2.18.1 Effect of reaction pH on xylanase activity

The xylanase activities of both the fungal strains were measured at various pH levels ranging from 4.0 to 10.0 with an interval of 0.5 while keeping other variables constant such as, incubation period 15 min, temperature 55 min and birch wood xylan concentration 10 mg/mL. The pH levels from 4-6 were maintained by using sodium silicate buffer solution, pH levels 6.5 to 8.0 with phosphate buffer solution and pH levels 8.5 -10 with NaOH, glycine solution. The xylanase activity was measured by DNS method [45]. Maximum value obtained was taken as

100% activity and xylanase activity measured at different reaction pH expressed as relative (%) activity.

2.2.18.2 Effect of temperature on xylanase activity

The xylanase activities of both the fungal strains were measured at different temperatures ranging from 45-90 ^{0}C with an interval of 5 ^{0}C for 1 h while keeping other variables constant like, pH 7.5 for fungal strain MLK 01 and pH 8.0 for fungal strain MLK 07 and birch wood xylan concentration 10 mg/mL. Other assay conditions were the same as per protocol as described above. The xylanase activity was measured by DNS method [45]. Maximum value obtained was taken as 100% activity and xylanase activities measured at different temperatures expressed as relative (%) activity.

2.2.18.3 Effect of different metal ions on xylanase activity

The effect of different metals ion i.e. $HgCl_2$, $ZnSO_4$. $7H_2O$, NaCl, KCl, NiO, $MgCl_2$. $6H_2O$, $CuSO_4$. $5H_2O$, Pb $(NO_3)_2$, $FeSO_4$. $7H_2O$, $MnSO_4$ $7H_2O$ and $CaCl_2$ on the xylanase activity were observed while keeping other variables constant like, incubation period 15 min, pH 7.5 at 65 ^{0}C for fungal strain MLK 01 and 8.0 pH at 75 ^{0}C for fungal strain MLK 07, and birch wood xylan concentration 10 mg/mL. The metal ions at a concentration of 1.0 mM were added in assay mixture. A control was also repeated without metal ion. The xylanase activity of control was taken as 100% and xylanase activity measured at different metal ions expressed as relative (%) activity. The xylanase activity was measured DNS method [45].

2.3.0 RESULTS AND DISCUSSION

2.3.1 Isolation of white rot fungi

The white rot fungi are isolated from decaying wood samples by enriched culture technique by using wheat bran as a core substrate. After a successive degradation of substrate, a white, cord like fruiting body is observed on decaying wood sample (Photograph 2.1 A) which is further purified on wheat bran agar medium containing 2% wheat bran powder and 2% agar without any nutrient (Plate 2.1B). Wheat bran contains 71% fiber carbohydrate, a number of

amino acids and trace elements necessary for growth of white rot fungi [57]. Thus, out of 30 wood samples 15 white rot fungi are isolated and purified. The microscopic examination reveals that the purified isolates on onset of maturity of fruiting bodies, disperse brown coloured basidiospores (Plates 2.1 C and D) validating them as white rot basidiomycetes.

2.3.2 Screening of alkali-tolerant white rot fungi

Table 2.2 shows that two fungal isolates namely MLK01 and MLK07 out of 15 white rot fungi show good growth at pH range varying from 5.0 to 10.0. On the other hand, rest of the fungal isolates grows well at pH levels ranging from 5 to 7. The xylanase assay of culture supernatants was performed at different pH levels using different buffers by standard methods as described in materials and methods. Table 2.3 shows xylanase assays of 15 fungal isolates at different pH levels ranging from 5 to 9.5. The xylanases isolated from fungal strains namely MLK01 and MLK07 are active at pH levels varying from 5.0 to 9.5. Whereas, xylanases isolated from rest of the fungal are active at pH levels 5 to 8.0. Therefore, fungal isolates namely MLK01 and MLK07 are more alkaline tolerant compared to rest of the fungal isolates and are selected for further studies. Both the fungal strains are found to grow well up to pH 10 but the xylanases are active at pH 8.5. The reason is that the plasma membrane of microorganisms is unstable above pH 8.5 and the cell wall of alkaliphiles which contains acidic polymers functioning as negative charged matrix may reduce the pH at the cell surface. Horikoshi reported that α-galactosidase, isolated from *Micrococcus* sp. strain 31-2- an alkaliphile, had its optimal activity at pH 7.5 indicating that the internal pH of bacterium is almost neutral [28].

2.3.3 Identification of alkali-tolerant white rot fungi

Pathology Division, Forest Research Institute, Dehradun (India) confirms both the fungal isolates (MLK01 and MLK07) as different strains of *Coprinellus disseminatus* (Pers.:Fr.) Lange (*Coprinus disseminatus* (Pers.:Fr.) Gray on the basis of fruiting bodies characteristics, spore print and microscopic examination. Microscopic examination shows the presence of

pileocystidea with cylindrical neck and rounded apex (Plates 2.2 A), dark brown spores with apical germ pores (Plates 2.1 D), hymeneal layers (Plates 2.2 C), dipolar mating (Plates 2.2 B) and basidium with tetra basidiospores (Plates 2.2 A-D). The mating system in *Coprinellus disseminatus* was determined by Lange [40].

2.3.4 Screening for extracellular enzymes

Table 2.4 and Plate 2.3 shows the plate assay of different extracellular enzymes i.e. xylanases, cellulases, mannanases, amylases, proteases, laccase and lignin peroxidase on agar plate by both the fungal strains i.e. MLK01 and MLK07. Generally, the extracellular enzymes are inducible, thus, the enzyme activities of different extracellular enzymes are determined on agar media containing their respective substrates as described earlier in paragraph 2.2.5. Xylanases, mannanases and cellulases, positive test show reddish zone around their growing margin (plate 2.3) after staining with 0.1% congored. The activity of mannanases and cellulases is found poor whereas, the activity of xylanases is good in both the fungal strains. A yellowish hollow zone around fungal growth indicating the ability of both strains (MLK01 and MLK07) to hydrolyze starch (Plate 2.3). The proteases which have poor activity on gelatin agar media show halo appearance around the colony of both the fungal strains. Laccase and lignin peroxidase show radish and dark brown appearance around the growing cultures of both the fungal strains on agar- guaiacol and agar- tannic acid media respectively. The activity of laccase is average in case of strain MLK01 and good in case of strain MLK07. On the other hand, both the strains show good lignin peroxidase activity.

2.3.5 Effect of different fungal agar media on the growth and appearance of *Coprinellus disseminatus*

Table 2.5 shows the growth and appearance of both the fungal isolates i.e. MLK01 and MLK07 on different agar media at pH 10. The descending growth pattern for strain MLK01 on different agar media after 36 hours is MEA>FA>WBA>MGYP>PDA>LYEA and after 72 hours FA=MEA=WBA=MGYP>PDA>LYEA. The descending growth pattern for strain MLK07 on different agar media after 36 hours is MEA>FA>MGYP>WBA>PDA>LYEA and

after 72 hours FA=MEA=WBA=MGYP>PDA>LYEA. The growth diameter of fungal isolates MLK01 and MLK07 on wheat bran agar medium is 6.0 and 6.2 cm respectively after 36 h and 9.0 cm for both the strains after 72 hours. The appearance is white concave with fast growth in both the cases. Strain MLK07 starts mating after 72 hours of growth and dark brown coloured basidiospores appear on onset of fruiting bodies. The vigorous growth after 72 h indicates the fast growing nature of fungi. While, the poorest growth of both the fungal isolates is observed on lactose yeast extract agar medium because both the fungal isolates are unable to utilize lactose as a carbon source. However, the growth of both the fungal strains is good on malt extract agar medium after 36 hours and both the fungal isolates on wheat bran agar medium attain the same growth as on malt extract agar medium after 72 h. Wheat bran is a cheap and easily available substrate compared to other commercial media as described above and it contains 71% fiber carbohydrate, a number of amino acids and trace elements necessary for enzyme induction [57]. Keeping this in view, wheat bran agar medium is selected for further studies. Plates 2.4 A-F show the growth and appearance of both strains on different agar media.

2.3.6: Effect of incubation period on growth and extracellular enzyme production

Table 2.6 shows the effect of incubation periods varying from 3 to 20 day on growth and enzyme production by both the fungal strains while keeping other variables constant. Fungal strain MLK01 produces maximum xylanase activity of 28.04 IU/mL on 8^{th} day of incubation and maximum cellulase activity of 0.64 IU/mL on 7^{th} day of incubation (Figures 2.2 A-B). The maximum fungal growth in terms of protein concentration in fungal mycelia is 0.48 mg/mL on 9^{th} day of incubation (Figure 2.2 D). In the similar way, the fungal strain MLK07 produces maximum xylanase (30.76 IU/mL) and cellulase (0.68 IU/mL) activities on 9^{th} and 11^{th} day of incubation respectively (Figures 2.3 A-B). Whereas, the maximum protein concentration in fungal mycelia is observed 0.38 mg/mL on 7^{th} day of incubation in order to reflect the growth of fungi (Figure 2.3D). It indicates that the extracellular xylanase and cellulases are produced in chorus along with fungal growth when wheat bran was used as a core substrate. Steiner also

reported that fungal xylanase are generally associated with cellulases [53]. It means that the xylanase and cellulase production depends on growth of fungi up to some extent because cellulases and xylanase are a part of primary metabolites. Therefore, cellulases and xylanase which are produced during exponential phase of growth begin to decrease on the onset of death phase with fungal growth. Beside this, the metabolic enzymes such as, proteases and transglycosidases secreted by xylanase producing microorganisms may hydrolyze xylanase and cellulase which can affect the enzyme yield adversely [30].

Figures 2.2 C and 2.3 C reveal that the maximum peroxidase activity is 0.73 and 0.33 IU/mL on 12^{th} and 13^{th} day of incubation for strains MLK01 and MLK07 respectively. It means that, the production of lignin degrading enzymes is a secondary metabolic event and produced in the stationary phase of growth. Because of the basidiomycetes are considered as slow growing fungi. del, Rio found 40 days as the optimum period for fungal growth on *Eucalyptus globulus* wood chips [13].

2.3.7 Effect of initial pH on growth and extracellular enzyme production

Table 2.7 reveals the effect of pH varying from 5 to12 on growth and enzyme production for both the fungal strains while keeping other variables constant. Both of the fungal strains exhibit a broad range of pH for growth and production of extracellular enzymes i.e. between pH 5.0 to 11.0. Both the fungal strains have the maximum growth at alkaline pH level. The higher fungal biomass achieved at higher pH might have been due to the effect of pH on nutrients availability [51]. The xylanase activity of both the fungal strains increases with increasing pH of the medium (Figures 2.4A and 2.5A) and no growth is observed at pH 12.0 (Figures 2.4D and 2.5D). The both of fungal strains MLK01 and MLK07 show maximum xylanase activity of 26.32 and 31.45 IU/mL respectively at pH 10. The cellulases and lignin peroxidase activity follow the reverse pattern and gradually decrease with increasing the pH of medium. Figures 2.4 B & C and 2.5 B & C reveal that the maximum cellulases and lignin peroxidase activity is observed at pH 5.0. The reason is that the degrading wood, a natural habitat for growth of white

rot fungi has an acidic environment. Kirk *et al.*, [36] found optimum pH 4.0-4.5 for lignin degradation but probably broader for growth of *P. chrysosporium*. Thus, both the fungal strains are considered as alkali-tolerant fungi according to the definition given by Nagai *et al.*, [47].

This pH range is significantly higher than that reported for other basidiomycetes [3]. The alkali tolerant property makes the enzyme available for direct application on the alkaline kraft pulp without any intervening pH adjustment thus; these enzyme preparations would be able to fulfill the current industry requirements.

2.3.8 Effect of birch wood xylan on the growth and enzyme production

The suitable inducer and optimum medium composition are the key factors for efficient production of xylanolytic enzymes [38]. In this connection, we had examined xylan as substrates for the induction of xylanases. Table 2.8 shows that the birch wood xylan significantly suppress the xylanase as well as cellulase activity at different concentration ranging from 1.0 to 5.0 g/L. while, fungal growth and lignin peroxidase activity are found to be increased up to some extent with increasing the birch wood xylan concentration in medium.

Figures 2.6 A & 2.7A reveal that the xylanase activity of both the fungal strains decrease with increasing birch wood xylan concentration but the enzyme activity remains almost constant between xylan doses from 2-4 g/L for both the fungal strains. The minimum xylanase activity of 8.54 and 8.98 IU/mL are observed at 5.0 g/L birch wood xylan respectively for MLK01 and MLK07. In the same way, cellulases activity decrease gradually with increasing the dose of xylan (Figures 2.6B and 2.7B). The lignin peroxidase activity of both the fungal strains increases with increasing birch wood xylan concentration up to 2.0 g/L and then declines gradually (Figures 2.6C and 2.7C) On the other hand, fungal growth increases up to 4.0 and 3.0 g/L birch wood xylan concentrations for fungal strains MLK 01 and MLK07 respectively and then declines. In fact xylan being a high molecular mass polymer cannot penetrate the cell wall of fungi. The low molecular mass fragments of xylan play a key role in the regulation of xylanase biosynthesis. These fragments include xylose, xylobiose, xylooligo-saccharides,

hetero-disaccharides of xylose and glucose and their positional isomers which may act as a catabolic repressor hence, repressed the extracellular xylanase production [38]. However, some other researchers found xylan as an inducer for xylanase production [25, 30, 50].

2.3.9 Effect of reducing sugars on the growth and enzyme production

Tables 2.9 and 2.10 represent the effect of reducing sugars on the growth and enzyme production by both the fungal strains i.e. MLK01 and MLK07 respectively. Figures 2.8 A&B□ 2.13 A&B reveal that all the tested reducing sugars including D-glucose, D-xylose and D-galactose repress the xylanase as well as cellulase synthesis in both the fungal strains under LSF conditions. D-xylose is found to be major catabolic repressor at different concentration for both of the fungal strains with minimum xylanase activity i.e. 3.98 and 5.98 IU/mL at 5.0 g/L of dose respectively. The lignin peroxidase activity and fungal growth is found maximum at a glucose dose of 2 g/L in case of fungal strain MLK01 and 4 g/L in case of strain MLK07. The lignin peroxidase activity and fungal growth increase with increasing D-xylose dose up to 3 and 4 g/L respectively for fungal strain MLK01 and beyond that there are no significant changes in lignin peroxidase activity as well as in fungal growth are observed. The lignin peroxidase activity in case of fungal strain MLK07 increases up to D-xylose dose of 3 g/L and then declines. Whereas, the growth of fungal strain MLK01 increases up to D-xylose dose of 5 g/L and then found constant. The addition of D-galactose in the medium represses the fungal growth and extra cellular enzymes activity of both the fungal strains. It means that D- galactose is a poor medium for both the fungal growth as well as enzyme production (Figure 2.8 C& D - 2.13 C& D).

Generally, the reducing sugars act as a catabolic repressor for xylanase as well as cellulase production. Catabolic repression by glucose and xylose was also reported by other authors [34]. While de Souza *et al.*, [12] found resistance of *Aspergillus tamari* to catabolic repression with glucose when wheat bran used as a soul substrate in SSF condition. Some other researcher reported cellulase and xylanase induction by homo and hetero-disaccharides composed of glucose and xylose [29]. The growth of both the fungal strains as lignin peroxidase

activity is found to increase with the addition of D-xylose and D-glucose in comparison to control. From the above results, it has been concluded that the lower dose of reducing sugars consumed during primary metabolic events, and increases the fungal mycelium growth. On the other hand, lignin peroxidase produced as secondary metabolites when the culture medium is deficient of carbon, sulphur or nitrogen; the lignin peroxidase activity increases due increased mycelia growth [31]. The similar results were reported in case of *P. chrysosporium* [36].

2.3.10 Effect of different concentration of urea on the growth and enzyme production

Table 2.11 shows the effect of different concentrations of urea varying from 1-5 g/L on fungal growth and extracellular cellular enzyme production while keeping other variables constant. The xylanase and lignin peroxidase activities decrease with increasing the dose of urea (Figures 2.14 A & C and 2.15 A & C). The minimum xylanase activity for both strains is found 10.08 and 10.38 IU/mL respectively at 5.0 g/L dose of urea. Whereas, the minimum lignin peroxidase activities for both strains are found 0.11 and 0.18 U/mL respectively at the same dose of urea. It means that the high dose of urea represses the lignin peroxidase induction. The lignin peroxidase activity decreases with increasing urea dose which may be due to consumption of lower dose of urea during primary growth of fungi while lignin peroxidase is produced as secondary metabolite. Fem and Kirk [18] reported that urea repressed 57% lignolytic activity in *Phanerochaete chrysosporium.* Eva Kachilishvili *et al.,* [33] reported that the yield of hydrolytic enzymes and laccase produced by four white rot fungi namely *Funalia trogii* IBB146, *Lentinus edodes* IBB363, *Pleurotus dryinus* IBB 903 and *P. tuberregium* IBB624 increased and manganese peroxidase activity repressed by supplementation of medium with an additional nitrogen source during solid state fermentation state of wheat straw and beech tree leaves.

On the other hand, fungal growth as well as cellulase activity of both the fungal strains MLK01 and MLK07 increase with increasing dose of urea (Figures 2.14B & D and 2.15 B & D). The cellulases activity of fungal strains MLK01 (from 0.42 to 0.87 IU/mL) and MLK07 (from 0.42 to 0.86 IU/mL) increases about two times i.e. when urea dose was increased from 0-

5.0 g/L. The wood is a natural site for the growth of white rot fungi and has very low nitrogen content i.e. 0.303% to 0.073% dry weight [14]. Addition of nitrogen to the wood leads to cellulose degradation.

2.3.11 Effect of different lignocelluloses as substrate on the growth and enzyme production

Table 2.12 shows the effect of different lignocelluloses wastes on fungal growth and extracellular enzyme produced by both the fungal strains i.e. MLK01 and MLK07. The descending order for xylanase and cellulase activity of fungal strain MLK01 on different lignocelluloses is found as: wheat bran>ground nut shell>bagasse>wheat straw>wood dust and for strain MLK07 as: wheat bran>bagasse >ground nut shell >wheat straw>wood dust. The maximum xylanase activity of 26.24 IU/mL for strain MLK01 and 30.43 IU/mL for strain MLK07 and cellulase activity of 0.32 IU/mL for strain MLK01 and 0.36 IU/mL for strain MLK07 is observed on wheat bran powder. Figures 2.16 A-B and 2.17 A-B show the results. The yield of xylanase in a fermentation process is governed by the few key factors in addition to the parameters like, incubation period, pH and temperature. When xylanase fermentation is carried out on complex heterogeneous substrates, various factors have a combined effect on the level of xylanase expression. They consist of openness and accessibility of substrate, rate and amount of release of the xylo-oligosaccharides and their chemical nature and amount of xylose released which acts as the carbon source and as an inhibitor of xylanase synthesis in most of the cases. Usually, the slow release of the inducer molecules and the possibility of the culture filtrate converting the inducer to its non-metabolizable derivative are believed to boost up the level of xylanase activity [38]. Archana and Satyanarayana, [4] reported wheat bran is the best substrate for xylanase production by a thermophilic *Bacillus licheniformis* grown under SSF conditions.

Figures 2.16 C-D and 2.17 C-D show the descending order for lignin peroxidase and fungal growth of strain MLK01 on different lignocelluloses as: wheat bran> bagasse > wood

dust> ground nut shell>wheat straw and for strain MLK07 as: wheat bran>bagasse > wheat straw > wood dust>ground nut shell.

2.3.12 Bulk production of extracellular enzymes for biobleaching

The mass production of extracellular enzymes in a fermentor of 2.5 L capacity with 2 L working capacity was carried out under optimized conditions. Table 2.13 reveals that the optimum enzyme activity of both the fungal strains i.e. 30.32 IU/mL for strain MLK01 and 36.87 IU/mL for strain MLK07 observed on 6^{th} day of incubation with minimum contamination of cellulase (0.32 IU/mL for strain MLK01 and 0.25 IU/mL for strain MLK07). The lignin peroxidase activity and protein broth are 0.25 IU/mL and 1.76 mg/mL respectively for strain MLK01 and 0.15 IU/mL and 1.89 mg/mL respectively for strain MLK07. Therefore, bulk enzymes for commercial purpose can be obtained on 6^{th} day of incubation which is quite acceptable for a commercial venture.

2.3.13 Effect of pH on xylanase activity

Table 2.14 shows the effect of pH on xylanase activity of both the fungal strains produced under optimum LSF condition. The crude xylanases obtained from both the fungal strains are active over a wide pH range of 4 to 9 with a maximal activity of strain MLK01 at pH 7.5 and for strain MLK07 at pH 8.0. Xylanases from fungal strain MLK01 and strain MLK07 retain 67.56 and 60.16% of their relative activities at pH 5.0. On the other hand, at pH 9.0 enzymes obtained from both of fungal strains retain 60.49 and 74.09 % of their relative activities (Figure 2.18). Both of the xylanases found to loose about 70-75% of their activities at pH 9.5 while maintain about 30% of their activities at pH 4.0. The above results indicate that xylanases produced from both the strains are alkaline in nature. However, in the harsh conditions such as, change in pH, high temperature or presence of high concentration of metal ions proteins loose their basic structure and subsequently, loose active site which may result in loss of enzyme activity. Since, enzymes are proteins, the ionic character of the amino and carboxylic acid groups on the protein surface are likely to be affected by pH changes and the catalytic property

of the enzyme is markedly influenced. The pH activity profiles of the enzyme are highly dependent on pKa value of the catalytic residues which are themselves dependent on the local environment and hence, on the nature of the amino-acids in the vicinity of the catalytic residues. Lower the pKa value, higher the pH stability [11]. Gupta *et al.*, [26] reported an alkalistable xylanase from a haloalkalophilic *Staphylococcus* sp. which exhibits dual pH optima of 7.5 and 9.5. Broad pH optima of 7.5–9.0, 7.0–9.5 and 6.0–10.0 have been reported in xylanase from *Micrococcus* sp. AR-135 [19] and *Bacillus* sp. C-125 [27] respectively. Thus, xylanases isolated from strains MLK01 and MLK07 can be used commercially in pulp and paper industry as the pulp is processed normally under highly alkaline conditions in a kraft mill. Therefore, xylanase that are active at alkaline pH are needed in order to make enzymatic process technically and economically more feasible [5, 48].

2.3.14 Effect of temperature on xylanase activity

The effect of temperature on xylanase activity is reported in Table 2.15 and Figure 2.19. The maximum xylanase activity of strain MLK01 and strain MLK07 is found at 75 and 65 ^{0}C temperature respectively. Xylanase from strain MLK01 shows 70.04 % of its relative activity at 45 ^{0}C. On the other hand, xylanase from strain MLK07 exhibits 84.95% of its relative activity at 45 ^{0}C. Xylanase from fungal strain MLK01 retains 94.11% of its relative activity at 85^{0}C. In the same way, xylanase from strain MLK07 retains 65.07% of its relative activity at 75^{0}C. It means xylanase obtained from MLK01 strain is more thermo-tolerant compared to MLK07. Even then, both the xylanases produced from strains MLK01 and MLK07 are considered as thermozyme [41]. Thermo stability of enzymes seems to be a property acquired by a protein through a combination of many small structural modifications that are achieved with the exchange of some amino acids. The variation of the canonical forces e.g. hydrogen bonds, ion-pair interactions, hydrophobic interactions found in thermozymes provide resistance to them at high temperature [52]. Thermozymes present following key biotechnological rewards over mesophilic enzymes: a). they are easier to purify by heat treatment, b). they have a higher resistance to chemical

denaturants such as, solvents and guanidinium hydrochloride, and c). they withstand with higher substrate concentrations. Therefore, because of their stability at higher temperature, thermozyme reactions are less susceptible to microbial contamination and often exhibit higher reaction rates than mesozyme catalyzed reactions. In view of these important advantages, thermozymes are attracting much industrial interest [7]. Gilbert *et al.,* [21] isolated and characterized a xylanase from the thermophilic ascomycete *Thielavia terrstris* 255B.

2.3.15: Effect of metal ions on xylanase activity

In pulp and paper industry, pulp itself, and process water contains a number of metal ions which affect the enzyme accessibility on pulp during biobleaching. Aiming at this, we examined the effect of some common metal ions on xylanase activity. Table 2.16 shows that the metal ions like, Zn^{2+} and Fe^{3+} are strong stimulators; they enhance the relative xylanase activity from 179.4 to 200.2% in case of both the fungal strains. The descending order of metal ions which stimulates xylanase activity are: Zn^{2+}, $Na^{+} > Fe^{+2} > Ni^{2+} > Na^{+} > K^{+} > Pb^{2+} > Ca^{+2} > Mn^{+2} > Mg^{2+}$ for strain MLK01 and $Fe^{+2} > Zn^{2+}$ $Ni^{2+} > K^{+} > Na > Mg^{+2} > Mn^{+2} > Pb^{+2} > Ca^{+2}$ for strain MLK07. These ions may bind to the enzyme, causing conformational changes that result in increased enzyme activity. Khandeparkar and Bhosle [35] also reported similar results in case of *Enterobacter* sp. MTCC 5112. On the other hand, Hg^{2+} inhibits about 80% relative enzyme activity in both the strains. Cu^{2+} is found to inhibit the relative enzyme activity by 17.8% and 29.5% for strains MLK01and MLK07 respectively. Activation of enzyme also depends on maintaining the integrity of sulphydryl (-SH) groups in the enzyme protein since these groups constitute the active center of the enzyme. When sulphydryl (-SH) groups are oxidized to the disulphide (-S-S-) for, the enzyme becomes inactive. The inhibition of the enzyme activity by metal ions which reacts with sulphydryl groups of enzyme such as, Hg^{2+} ions, suggested that there was an important cysteine residue in or close to the active site of the enzyme. Similar results are also reported by others researchers [17,19, 26].

Table 2.1: Commercial hemicellulases for enzyme-aided bleaching [55]

Brand name	Firms	Enzyme type	pH-optima	Temperature optima ^{0}C
Cartazyme SR-10	Sandoz	Xylanase	4 - 5	60 - 80
Cartazyme PS-10	Sandoz	Xylanase	7 - 9	60 - 70
Cartazyme HS-10	Sandoz	Xylanase	4 - 5	40 - 60
Ecopulp X-200	Primalco	Xylanase	5 - 6	50 - 55
Ecopulp X-200/4	Primalco	Xylanase	3 - 4.5	45 - 55
Ecopulp TX-100	Primalco	Xylanase	6 - 8	50 - 80
Ecopulp TX-200	Primalco	Xylanase	6 - 8	50 - 80
Ecopulp XM	Primalco	Xylanase Mannase	5 - 6	50 - 55
Ecopulp X-100	Primalco	Mannase	5 - 6	50 - 55
Ecozyme	Zeneca	Xylanase	7 - 9.5	65
GS 35	Iogen	Xylanase	5.2 - 7.8	47 - 58
HS 70	Iogen	Xylanase	5.3 - 7.5	45 - 55
Irgazyme 40 x 4	Genencor International	Xylanase	6 - 7	50 - 60
Irgazyme 40	Genencor International	Xylanase	6 - 7	50 - 60
Irgazyme 10A x 4	Genencor International	Xylanase	4 - 5	50 - 60
Optipulp-L 1000	Solvay Interox	Xylanase	6.5	55
Pulpzyme HC	Novo Nordisk	Xylanase	6 - 9.5	60

Table 2.2: Relative growth of xylanases producing white rot fungal strains at various pH levels on wheat bran under LSF conditions

Strains	Initial pH levels for fungal growth and xylanase production						
	pH 5.0	pH 6.0	pH 7.0	pH 8.0	pH 9.0	pH 10	pH 11.0
MLK 01[a]	++	++	+++	+++	+++	+++	+
MLK 02	+++	+++	+++	++	–	–	–
MLK 03	+++	+++	+++	+	–	–	–
MLK 04	+++	+++	++	+	–	–	–
MLK 05	+++	+++	++	+	–	–	–
MLK 06	+++	+++	++	+	+	–	–
MLK 07[a]	++	++	++	+++	+++	+++	+
MLK 08	+++	+++	+++	++	–	–	–
MLK 09	+++	+++	+++	++	–	–	–
MLK 10	+++	+++	+++	+	–	–	–
MLK 11	+++	+++	+++	++	–	–	–
MLK 12	+++	+++	+++	+++	–	–	–
MLK 13	+++	+++	++	+	–	–	–
MLK 14	+++	+++	+++	+++	–	–	–
MLK 15	+++	+++	+++	++	–	–	–

\+ Poor growth/activity
++ Average growth/activity
+++ Good growth/activity
– No growth/activity
a = Out of fifteen, two fungal isolates MLK01 and MLK07 were selected for further studies based on pH stability

Table 2.3: Relative xylanases activity of white rot fungal strains at various pH levels produced on wheat bran under LSF conditions

Strains	Initial pH levels for fungal xylanase activity						
	pH 5.0	pH 6.0	pH 7.0	pH 8.0	pH 8.5	pH 9.0	pH 9.5
MLK 01[a]	++	+++	+++	+++	++	++	+
MLK 02	+++	+++	+++	+++	++	++	–
MLK 03	+++	+++	+++	+	–	–	–
MLK 04	+++	+++	++	+	–	–	–
MLK 05	+++	+++	++	+	–	–	–
MLK 06	+++	+++	++	+	+	–	–
MLK 07[a]	++	++	+++	+++	+++	++	++
MLK 08	+++	+++	+++	++	+	-	–
MLK 09	+++	+++	+++	++	+	-	–
MLK 10	+++	+++	+++	+	–	–	–
MLK 11	+++	+++	+++	++	+	–	–
MLK 12	+++	+++	+++	+++	+	–	–
MLK 13	+++	+++	++	+	–	–	–
MLK 14	+++	+++	+++	+++	+	-	–
MLK 15	+++	+++	+++	++	+	–	–

\+ Poor activity
++ Average activity
+++ Good activity
– No activity
a = Out of fifteen, two fungal isolates MLK01 and MLK07 were selected for further studies based on pH stability

Table 2.4: Results of plate assay of different extra-cellular enzymes for fungal strains MLK01 and MLK07.

Sl. No.	Assay	MLK01	MLK07
1	Xylanases	+++[a]	+++[a]
2	Cellulases	+	+
3	Mannanase	+	+
4	Laccase	++	+++
5	Amylases	++	+++
6	Lignin peroxidase	+++[a]	++[a]
7	Proteases	+	+

\+ Poor activity
++ Average activity
+++ Good activity
a = Based on performance of plate assay of extracellular enzymes, both strains were selected for xylanase and lignin peroxidase estimation.

Table 2.5: Growth and appearance of strain MLK01 and strain MLK07 on different agar medium at initial pH 10.

	Growth Media	Strain MLK-01		Strain MLK-07	
		Growth, cm	Appearance	Growth, cm	Appearance
After 36 h	WBA	6.0	Dull white, lateral	6.2	White, lateral
	FA	6.4	White, lateral	7.3	do
	MEA	6.5	Cottony white	7.5	Cottony white
	LYEA	2.2	Dense, irregular	3.6	Dense, irregular
	PDA	4.3	White, lateral	5.3	White, lateral
	MGYP	5.6	do	6.7	do
After 72 h	WBA	9.0	Floppy, aerial, white, concave, vigorous, spore print is black	9.0	Dull white, rings, Aerial, vigorous, mating start
	FA	9.0	Concave, Floppy, aerial, white	9.0	Lateral, dull white, mating start
	MEA	9.0	Floppy, cotton white, concave, dense, spore print is black	9.0	Dull white, slight rings, mating start
	LYEA	3.6	Disperse, irregular, dull white, weak	4.0	Weak but floppy growth, a yellowish drop appeared
	PDA	7.9	Dense rings, creamy white	8.4	Strong rings, creamy white, mating start
	MGYP	9.0	Floppy, aerial, white, concave, spore print is black	9.0	Strong rings, creamy white, mating start

Abbreviation used:
WBA = wheat bran agar, FA = fungal agar, MEA = malt extract agar, LYEA = lactose yeast extract agar, PDA = potato dextrose agar, MGYP = malt extract glucose yeast extract peptone

Table 2.6: Effect of incubation period on the growth and extracellular enzymes production by *Coprinellus disseminatus* strains MLK01 and MLK07 under LSF conditions.

Incubation period, day	Xylanase activity, IU/mL	CMCase activity, IU/mL	Lignin Peroxidase activity, U/mL	Fungal growth as mycelial protein, mg/mL
***C. disseminatus* strain MLK01**				
3	5.27 ±2.2	0.32±0.03	0.11±0.02	0.22±0.04
4	9.93±3.8	0.35±0.02	0.15±0.02	0.31±0.02
5	12.21±2.2	0.42±0.02	0.22±0.03	0.32±0.03
6	19.16±2.6	0.46±0.01	0.24±0.02	0.34±0.02
7	24.21±1.4	0.64±0.02	0.34±0.04	0.37±0.01
8	28.04±1.8	0.62±0.02	0.40±0.03	0.39±0.01
9	27.66±1.8	0.48±0.01	0.55±0.01	0.48±0.02
10	27.21±1.1	0.44±0.03	0.68±0.03	0.43±0.04
11	21.65±2.3	0.42±0.04	0.71±0.02	0.32±0.03
12	20.52±2.6	0.40±0.03	0.73±0.03	0.31±0.02
13	18.80±2.1	0.39±0.02	0.62±0.04	0.28±0.01
14	17.45±2.0	0.38±0.03	0.61±0.02	0.26±0.02
15	15.80±2.6	0.33±0.04	0.50±0.02	0.24±0.05
18	11.08±2.4	0.31±0.02	0.49±0.03	0.22±0.04
20	10.80±1.2	0.28±0.03	0.49±0.02	0.19±0.03
***C. disseminatus* strain MLK07**				
3	7.54±2.0	0. 20±0.02	0.09±0.01	0.15±0.02
4	9.76±2.7	0.35±0.05	0.11±0.01	0.22±0.03
5	11.21±2.3	0.42±0.03	0.20±0.02	0.25±0.02
6	18.19±2.0	0.43±0.03	0.24±0.01	0.36±0.01
7	22.60±1.5	0.46±0.02	0.25±0.03	0.38±0.05
8	30.50±2.8	0.53±0.01	0.27±0.02	0.38±0.04
9	30.76±2.1	0.54±0.05	0.29±0.01	0.34±0.03
10	30.21±1.6	0.65±0.01	0.30±0.03	0.33±0.02
11	27.87±2.4	0.68±0.02	0.31±0.02	0.29±0.03
12	22.21±2.2	0.43±0.02	0.33±0.01	0.27±0.02
13	15.80±1.8	0.22±0.03	0.33±0.03	0.25±0.05
14	15.45±1.2	0.20±0.01	0.32±0.03	0.24±0.04
15	13.80±2.0	0.14±0.04	0.29±0.02	0.23±0.01
18	11.80±2.8	0.11±0.03	0.23±0.02	0.21±0.01
20	9.86±2.0	0.09±0.02	0.21±0.03	0.19±0.02

Conditions for fermentation: Initial pH = 10.0, temperature = 40 °C, substrate = 2% wheat bran (w/v).
Conditions for xylanase assay: pH = 7.5, temperature = 55 °C, incubation time = 15 min, substrate = 10 mg/mL birch wood xylan (Sigma).
Conditions for cellulase assay: pH = 6.0, temperature = 50 °C, incubation time = 30 min, substrate = 2% CMC (HiMedia).
Conditions for lignin peroxidase assay: pH = 6.5, temperature = 25 °C, incubation time = 20 min, substrate = 50 mM 2, 4-DCP (HiMedia)

Table 2.7: Effect of initial pH on the growth and extracellular enzymes production by *Coprinellus disseminatus* strains MLK01 and MLK07 under LSF conditions

Initial pH(After autoclave)	Xylanase activity, IU/mL	CMCase activity, IU/mL	Lignin peroxidase activity, U/mL	Fungal growth as mycelial protein, mg/mL
***C. disseminatus* strain MLK01**				
5.0 (6.28)*	16.22±1.2	0.85±0.02	0.55±0.01	0.31±0.02
6.0 (7.05)*	19.88±1.6	0.82±0.03	0.52±0.00	0.35±0.01
7.0 (7.14)*	21.76±1.1	0.76±0.01	0.49±0.02	0.36±0.02
8.0 (7.50)*	22.08±1.2	0.74±0.04	0.44±0.03	0.38±0.03
9.0 (7.85)*	24.02±2.8	0.62±0.02	0.36±0.01	0.41±0.05
10.0(8.60)*	26.32±1.2	0.58±0.03	0.35±0.01	0.42±0.04
11.0(8.93)*	25.66±2.8	0.25±0.02	0.21±0.03	0.42±0.02
12.0	nd	nd	nd	nd
***C. disseminatus* strain MLK07**				
5.0 (6.30)*	20.43±2.2	0.62±0.05	0.35±0.01	0.17±0.04
6.0 (7.20)*	21.33±1.4	0.63±0.01	0.22±0.00	0.19±0.03
7.0 (7.25)*	22.76±2.1	0.46±0.03	0.24±0.02	0.22±0.06
8.0 (7.60)*	25.08±1.0	0.33±0.02	0.19±0.03	0.28±0.04
9.0 (7.90)*	28.12±1.2	0.24±0.04	0.16±0.01	0.36±0.02
10.0 (8.64)*	31.45±2.6	0.25±0.01	0.15±0.01	0.38±0.03
11.0 (8.90)*	25.43±2.1	0.18±0.02	0.13±0.02	0.40±0.01
12.0	nd	nd	nd	nd

Conditions for fermentation: Incubation period = 8 d for strain MLK01 and 9 d for strain MLK07, temperature = 40 °C, substrate = 2% wheat bran (w/v).
Conditions for xylanase assay: pH = 7.5 for strain MLK01 and 8.0 for strain MLK07, temperature = 55 °C, incubation time = 15 min, substrate = 10mg/mL birch wood xylan (Sigma).
Conditions for cellulase assay: pH = 6.0, temperature = 50 °C, incubation time = 30 min, substrate = 2% CMC (HiMedia).
Conditions for lignin peroxidase assay: Buffer pH = 6.5, temperature = 25°C, incubation time = 20 min, substrate = 50 mM 2, 4-DCP (HiMedia)
* values in parenthesis shows pH after fermentation, nd = not detected

Table 2.8: Effect of different concentration of birch wood xylan on the growth and extracellular enzymes production by *Coprinellus disseminatus* strains MLK01 and MLK07 under LSF conditions

	Dose, g/L	Xylanase activity, IU/mL	CMCase activity, IU/mL	Lignin Peroxidase activity, U/mL	Fungal growth as mycelial protein, mg/mL
birch wood xylan	***C. disseminatus* strain MLK01**				
	Control	29.20±1.8	0.50±0.03	0.36±0.03	0.32±0.03
	1.0	14.35±2.1	0.42±0.03	1.02±0.02	0.41±0.02
	2.0	11.37±2.0	0.39±0.01	1.21±0.01	0.53±0.04
	3.0	10.60±1.8	0.38±0.02	0.94±0.02	0.54±0.03
	4.0	10.17±1.2	0.32±0.02	0.45±0.02	0.59±0.05
	5.0	08.54±0.6	0.28±0.01	0.26±0.03	0.46±0.04
	***C. disseminatus* strain MLK07**				
	Control	32.86±1.8	0.25±0.02	0.36±0.03	0.33±0.04
	1.0	16.43±2.3	0.19±0.03	0.42±0.02	0.44±0.02
	2.0	13.37±1.8	0.16±0.01	0.48±0.01	0.48±0.03
	3.0	12.60±2.2	0.13±0.04	0.44±0.02	0.54±0.04
	4.0	10.27±1.4	0.10±0.02	0.35±0.02	0.41±0.01
	5.0	8.98±1.5	0.08±0.03	0.32±0.03	0.36±0.03

Conditions for fermentation: Initial pH = 10.0, pH after fermentation = 8.60 ± 0.5, incubation period = 8 d for strain MLK01 and 9 d for strain MLK07, temperature = 40 °C, substrate = 2% wheat bran (w/v).
Conditions for xylanase assay: pH = 7.5 for strain MLK01 and 8.0 for strain MLK07, temperature = 55 °C, incubation time = 15 min, substrate = 10 mg/mL birch wood xylan (Sigma).
Conditions for cellulase assay: pH = 6.0, temperature = 50 °C, incubation time = 30 min, substrate = 2% CMC (HiMedia).
Conditions for lignin peroxidase assay: pH = 6.5, temperature = 25°C, incubation time = 20 min, substrate = 50 mM 2, 4-DCP (HiMedia)

Table 2.9: Effect of reducing sugars at different concentrations on the growth and production of extracellular enzymes by *Coprinellus disseminatus* MLK01 under LSF conditions

	Dose, g/L	Xylanase activity, IU/mL	CMCase activity, IU/mL	Lignin peroxidase activity, U/mL	Fungal growth as mycelial protein, mg/mL
D-glucose	Control	27.10±1.2	0.41±0.03	0.46±0.05	0.42±0.04
	1.0	15.37±4.1	0.26±0.04	1.02±0.04	0.45±0.05
	2.0	12.87±2.2	0.16±0.02	1.21±0.03	0.48±0.02
	3.0	12.40±1.6	0.10±0.03	0.94±0.03	0.44±0.01
	4.0	10.12±2.6	0.09±0.02	0.85±0.02	0.41±0.03
	5.0	09.23±1.1	nd	0.77±0.03	0.36±0.02
D-xylose	1.0	11.86±1.3	0.28±0.05	0.64±0.05	0.55±0.04
	2.0	10.65±1.7	0.19±0.04	0.87±0.02	0.59±0.06
	3.0	08.09±0.8	0.17±0.03	0.95±0.03	0.62±0.04
	4.0	04.76±1.0	0.12±0.02	0.94±0.04	0.63±0.03
	5.0	03.98±0.3	0.10±0.02	0.94±0.02	0.63±0.02
D-galactose	1.0	15.07±0.6	0.17±0.05	0.60±0.03	0.40±0.05
	2.0	14.98±0.8	0.15±0.04	0.42±0.03	0.36±0.02
	3.0	10.00±1.1	0.12±0.03	0.31±0.04	0.32±0.03
	4.0	05.45±0.5	0.11±0.04	0.25±0.01	0.21±0.02
	5.0	01.89±0.3	nd	0.20±0.03	0.10±0.05

Conditions for fermentation: Initial pH = 10.0, pH after fermentation = 8.60 ± 1.0, incubation period = 8 d, temperature = 40 ^{0}C, substrate = 2% wheat bran (w/v).
Conditions for xylanase assay: pH = 7.5, temperature = 55 ^{0}C, incubation time = 15 min, substrate = 10 mg/mL birch wood xylan (Sigma).
Conditions for cellulase assay: pH = 6.0, temperature = 50 ^{0}C, incubation time = 30 min, substrate = 2% CMC (HiMedia).
Conditions for lignin peroxidase assay: pH = 6.5, temperature = 25 ^{0}C, incubation time = 20 min, substrate = 50 mM 2, 4-DCP (HiMedia), nd = not detected

Table 2.10: Effect of reducing-sugars at different concentrations on the growth and production of extracellular enzymes by *Coprinellus disseminates* strains MLK07 under LSF conditions

	Dose, %	Xylanase activity, IU/mL	CMCase activity, IU/mL	Lignin peroxidase activity, U/mL	Fungal growth as mycelial protein, mg/mL
D-glucose	Control	32.87±2.0	0.27±0.03	0.36±0.04	0.32±0.03
	1.0	18.36±2.1	0.12±0.01	0.42±0.03	0.34±0.04
	2.0	16.76±2.8	0.09±0.03	0.51±0.03	0.42±0.03
	3.0	14.60±1.7	0.08±0.02	0.54±0.03	0.51±0.02
	4.0	13.14±2.2	0.05±0.02	0.65±0.02	0.53±0.02
	5.0	11.26±2.3	nd	0.47±0.03	0.36±0.04
D-xylose	1.0	18.86±1.2	0.18±0.02	0.54±0.04	0.38±0.03
	2.0	12.65±1.8	0.15±0.01	0.65±0.02	0.41±0.05
	3.0	10.09±1.4	0.10±0.03	0.87±0.04	0.46±0.03
	4.0	08.76±1.2	0.08±0.02	0.73±0.04	0.57±0.02
	5.0	05.98±0.5	0.06±0.02	0.62±0.02	0.62±0.01
D-galactose	1.0	08.97±1.1	0.08±0.02	0.32±0.03	0.24±0.04
	2.0	06.98±1.3	0.05±0.01	0.27±0.03	0.21±0.03
	3.0	05.07±1.2	0.04±0.01	0.21±0.04	0.18±0.02
	4.0	03.85±1.0	0.02±0.01	0.12±0.01	0.13±0.01
	5.0	03.02±0.6	nd	0.10±0.01	0.10±0.03

Conditions for fermentation: Initial pH = 10.0, pH after fermentation = 8.64 ± 1.0, incubation period = 9 d, temperature = 40 °C, substrate = 2% wheat bran (w/v).
Conditions for xylanase assay: pH = 8.0, temperature = 55 ^{0}C, incubation time = 15 min, substrate = 10 mg/mL birch wood xylan (Sigma).
Conditions for cellulase assay: pH = 6.0, temperature = 50 ^{0}C, incubation time = 30 min, substrate = 2% CMC (HiMedia).
Conditions for lignin peroxidase assay: pH = 6.5, temperature = 25 ^{0}C, incubation time = 20 min, substrate = 50 mM 2, 4-DCP, nd = not detected

Table 2.11: Effect of different concentrations of urea on the growth and production of extracellular enzymes by *Coprinellus disseminatus* strains MLK01 and MLK07 under LSF conditions.

	Dose, g/L	Xylanase activity, IU/mL	CMCase activity, IU/mL	Lignin peroxidase activity, U/mL	Fungal growth as mycelial protein, mg/mL
Urea	***C. disseminatus* strain MLK01**				
	Control	25.20±1.0	0.42±0.06	0.23±0.03	0.32±0.02
	1.0	19.34±1.2	0.68±0.04	0.17±0.02	0.43±0.04
	2.0	17.50±1.1	0.72±0.05	0.15±0.05	0.54±0.02
	3.0	15.12±1.7	0.78±0.03	0.13±0.03	0.58±0.03
	4.0	12.67±1.8	0.82±0.04	0.12±0.04	0.66±0.01
	5.0	10.08±1.0	0.87±0.02	0.11±0.01	0.66±0.02
	***C. disseminatus* strain MLK07**				
	Control	32.2±2.2	0.40±0.04	0.42±0.03	0.38±0.01
	1.0	13.32±2.0	0.57±0.03	0.36±0.02	0.66±0.02
	2.0	12.5±1.5	0.68±0.04	0.31±0.05	0.68±0.01
	3.0	12.14±2.1	0.77±0.02	0.27±0.03	0.70±0.03
	4.0	11.37±1.8	0.83±0.03	0.21±0.04	0.76±0.02
	5.0	10.38±2.1	0.86±0.02	0.18±0.01	0.83±0.03

Conditions for fermentation: Initial pH = 10.0, pH after fermentation = 8.60 ± 0.5, incubation period = 8 d for strain MLK01 and 9 d for strain MLK07, temperature = 40 °C, substrate = 2% wheat bran (w/v).
Conditions for xylanase assay: pH = 7.5 for strain MLK01 and 8.0 for strain MLK07, temperature = 55 ^{0}C, incubation time = 15 min, substrate = 10 mg/mL birch wood xylan (Sigma).
Conditions for cellulase assay: pH = 6.0, temperature = 50 ^{0}C, incubation time = 30 min, substrate = 2% CMC (HiMedia).
Conditions for lignin peroxidase assay: pH = 6.5, temperature = 25 ^{0}C, incubation time = 20 min, substrate = 50 mM 2, 4-DCP (HiMedia)

Table 2.12: Effect of different substrates on the growth and extracellular enzymes production by *Coprinellus disseminatus* strains MLK01 and MLK07 under LSF conditions

Growth Substrate, 2% (w/v)	Xylanase activity, IU/mL	CMCase activity, IU/mL	Lignin peroxidase activity, U/mL	Fungal growth as mycelial protein, mg/mL
***C. disseminatus* strain MLK01**				
Wheat bran	26.24±2.5	0.32±0.05	0.32±0.03	0.32±0.07
Bagasse	16.02±2.8	0.25±0.07	0.32±0.06	0.21±0.05
Wheat straw	12.88±2.8	0.20±0.04	0.28±0.05	0.15±0.06
Wood dust	10.76±3.3	0.21±0.05	0.30±0.03	0.16±0.04
Groundnut shell	18.08±2.8	0.27±0.05	0.29±0.07	0.27±0.08
***C. disseminatus* strain MLK07**				
Wheat bran	30.43±1.2	0.36±0.02	0.32±0.03	0.32±0.03
Bagasse	24.52±2.6	0.15±0.03	0.22±0.02	0.41±0.02
Wheat straw	12.22±1.5	0. 26±0.03	0.18±0.02	0.13±0.03
Wood dust	9.96±2.3	0. 21±0.02	0.20±0.03	0.11±0.01
Groundnut shell	16.21±2.6	0. 24±0.02	0.19±0.03	0.15±0.02

Conditions for fermentation: Initial pH = 10.0, pH after fermentation = 8.60 ± 0.5, incubation period = 8 d for strain MLK01 and 9 d for strain MLK07, temperature = 40 °C, substrate = 2% (w/v).
Conditions for xylanase assay: pH = 7.5 for strain MLK01 and 8.0 for strain MLK07, temperature = 55 ^{0}C, incubation time = 15 min, substrate = 10 mg/mL birch wood xylan (Sigma).
Conditions for cellulase assay: pH = 6.0, temperature = 50 ^{0}C, incubation time = 30 min, substrate = 2% CMC (HiMedia).
Conditions for lignin peroxidase assay: pH = 6.5, temperature = 25 ^{0}C, incubation time = 20 min, substrate = 50 mM 2, 4-DCP (HiMedia)

Table 2.13: Mass production of extracellular enzymes under optimized conditions in bench fermentor

Sl. No	Particulars	Strain MLK01	Strain MLK07
1	Xylanase activity, IU/mL	30.32	36.87
2	CMCase, activity, IU/mL	0. 32	0.25
3	Lignin peroxidase activity, U/mL	0.25	0.15
4	Protein in broth, mg/mL	1.76	1.89
Conditions for strain MLK01 1. Incubation period = 6 d 2. Fermentation pH = 10.0 3. Shaking = 100 rpm 4. Fermentation temperature = 40 ^{0}C 5. Substrate concentration = 2% wheat bran			
Conditions for strain MLK07 1. Incubation period = 6 d 2. Fermentation pH = 10.0 3. Shaking = 100 rpm 4. Fermentation temperature = 40 ^{0}C 5. Substrate concentration = 2% wheat bran			

Table 2.14: Effect of reaction pH on xylanase activity

Sl. No	Reaction pH	Relative xylanase activity, %	
		Strain MLK-01	Strain MLK-07
1	4.0	30.10±0.5	28.70±0.3
2	4.5	48.30±0.8	45.87±0.6
3	5.0	67.56±0.7	60.16±0.5
4	5.5	80.65±0.8	71.67±0.3
5	6.0	91.97±0.9	79.44±0.4
6	6.5	95.04±0.8	88.23±0.8
7	7.0	98.98±0.7	94.66±0.9
8	7.5	100.00±1.0	97.70±1.2
9	8.0	93.56±0.9	100.00±1.5
10	8.5	85.43±0.7	95.90±0.8
11	9.0	60.49±0.8	74.09±0.3
12	9.5	25.15±0.4	30.65±0.2
13	10.0	nd	nd

nd = Not detected

Table 2.15: Effect of temperature on xylanase activity and stability

Sl. No.	Temperature, ^{0}C	Relative xylanase activity, %	
		Strain - MLK01	Strain - MLK07
1	45	70.04±1.0	84.95±0.8
2	55	85.09±0.9	99.61±0.6
3	65	89.03±0.7	100.00±0.5
4	70	90.32±0.5	93.14±0.6
5	75	100.00±0.7	65.07±0.5
6	80	95.78±0.8	41.20±0.4
7	85	94.11±0.9	nd
8	90	35.21±0.4	nd
9	95	16.88±0.3	nd

nd = Not detected

Table 2.16: Effect of metal ions on xylanase activity at 1.0 mM concentration

Sl. No	Metal ions concentrations, 1.0 mM	Relative xylanase activity, %	
		Strain - MLK01	Strain - MLK07
1	Control	100.0±1.5	100.00±1.0
2	$HgCl_2$	17.6±0.5	20.20±0.4
3	$ZnSO_4.7H_2O$	200.0±2.5	185.0±0.8
4	NaCl	158.8±1.6	150.0±0.8
5	KCl	141.1±1.4	160.1±1.3
6	NiO	164.6±1.3	185.2±1.3
7	$MgCl_2.6H_2O$	112.8±1.4	132.5±1.5
8	$CuSO_4.5H_2O$	82.2±1.0	70.5±1.6
9	$Pb(NO_3)_2$	135.3±1.2	128.4±1.5
10	$FeSO_4.7H_2O$	179.4±1.2	200.2±1.2
11	$MnSO_4.7H_2O$	117.6±1.3	132.2±2.0
12	$CaCl_2$	125.6±1.3	112.3±1.6

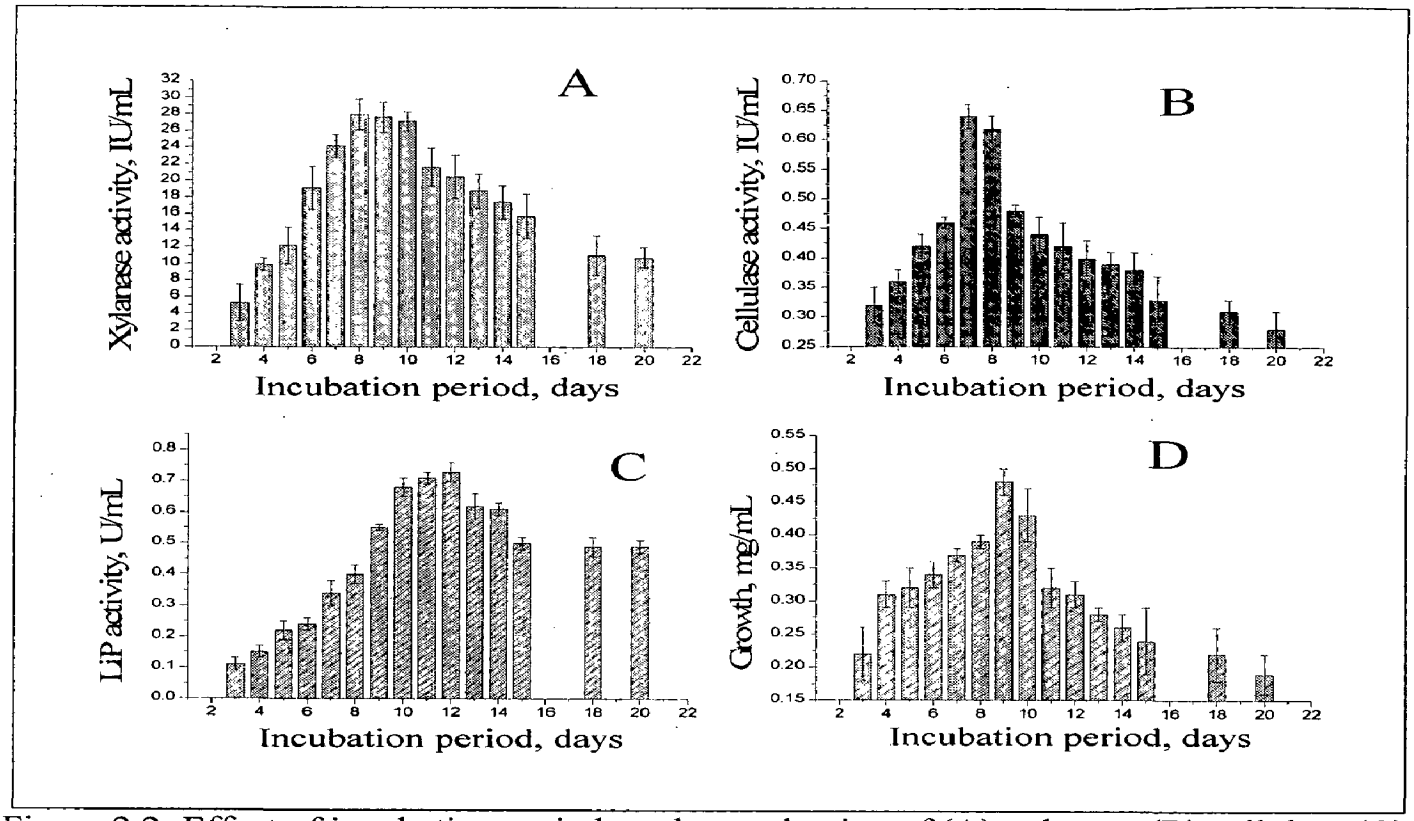


Figure 2.2: Effect of incubation period on the production of (A) xylanase (B) cellulase (C) lignin peroxidase and (D) growth of strain MLK01 at initial pH 10 with 2% wheat bran as substrate at 40 ^{0}C

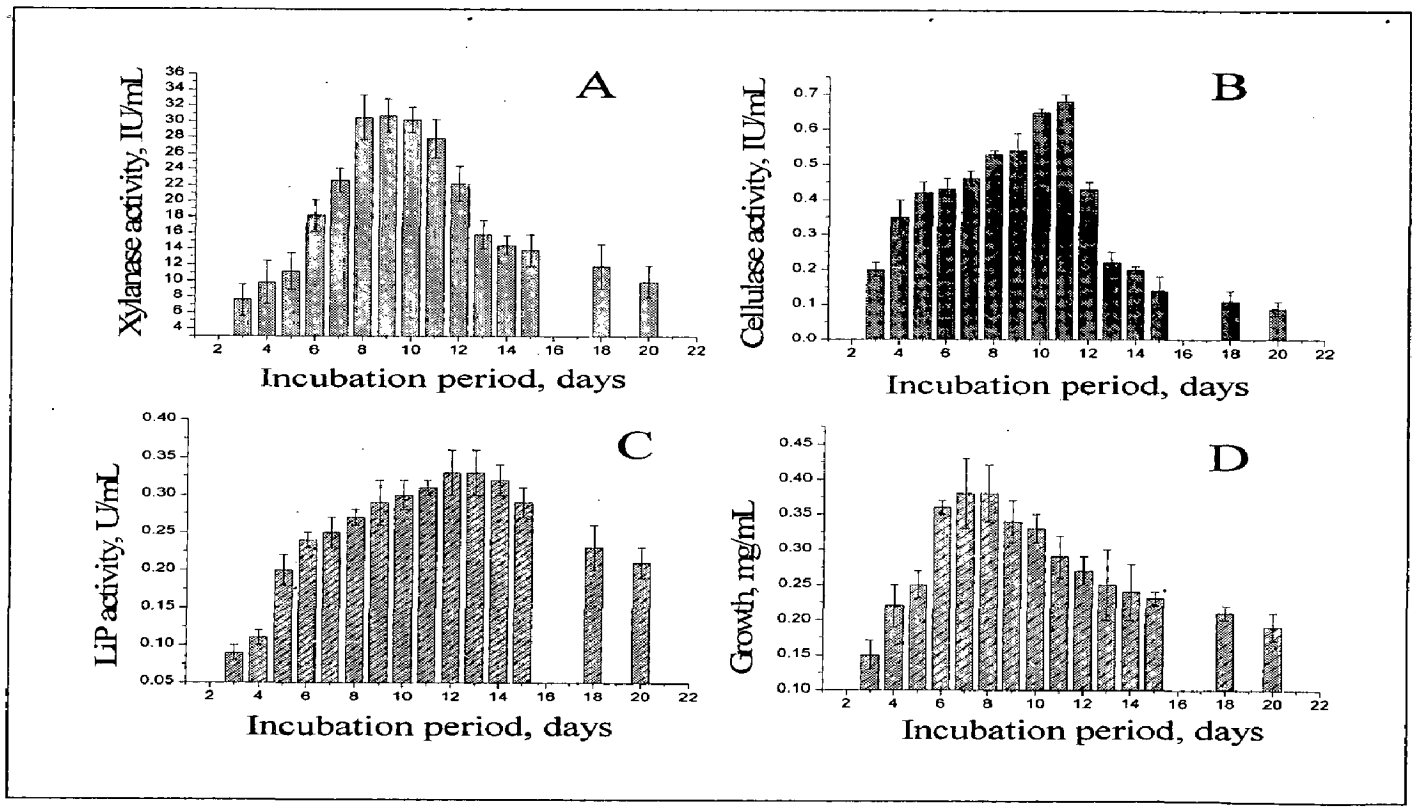


Figure 2.3: Effect of incubation period on the production of (A) xylanase (B) cellulase (C) lignin peroxidase and (D) growth of strain MLK07 at initial pH 10 with 2% wheat bran as substrate at 40 ^{0}C

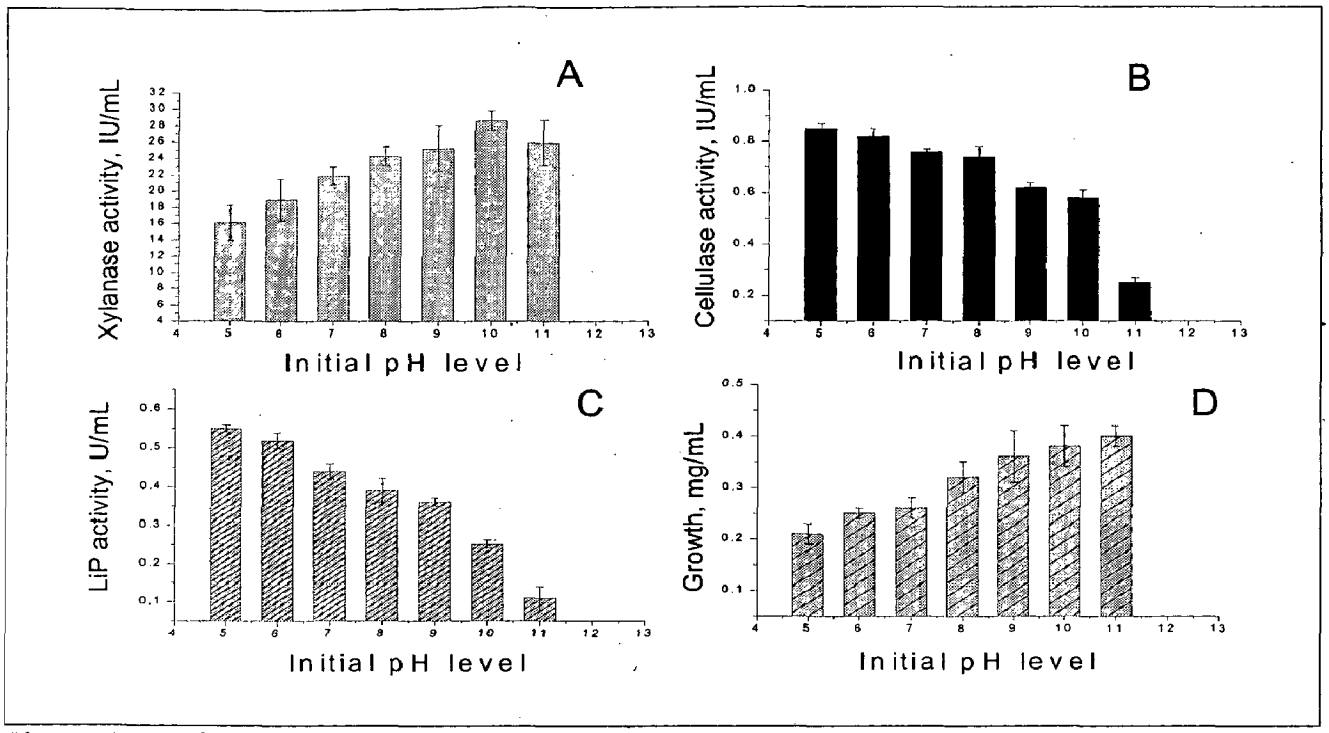


Figure 2.4: Effect of initial pH on the production of (A) xylanase (B) cellulase (C) lignin peroxidase and (D) growth of strain MLK01 at initial pH 10 with 2% wheat bran as substrate after 8 days of incubation at 40 ^{0}C

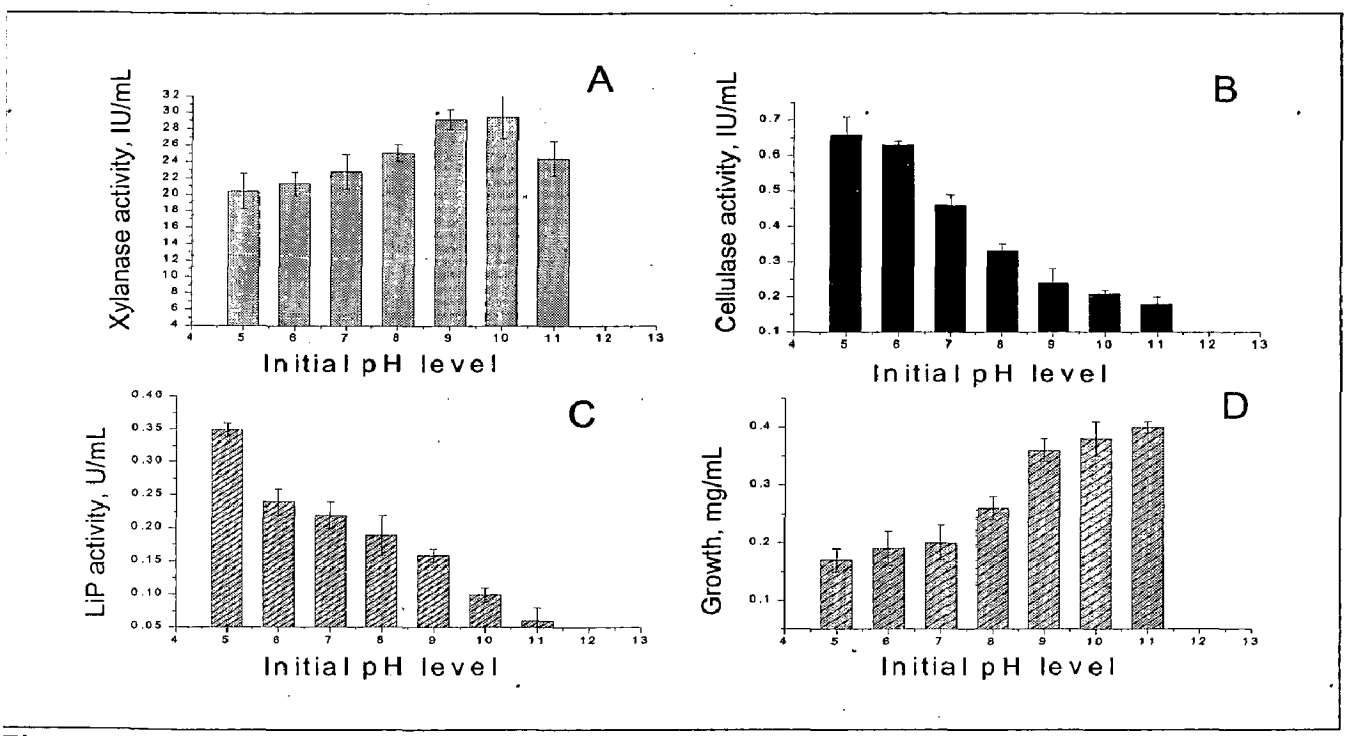


Figure 2.5: Effect of initial pH on the production of (A) xylanase (B) cellulase (C) lignin peroxidase and (D) growth of strain MLK07 after 8 days with 2% wheat bran as substrate after 9 day of incubation at 40 ^{0}C

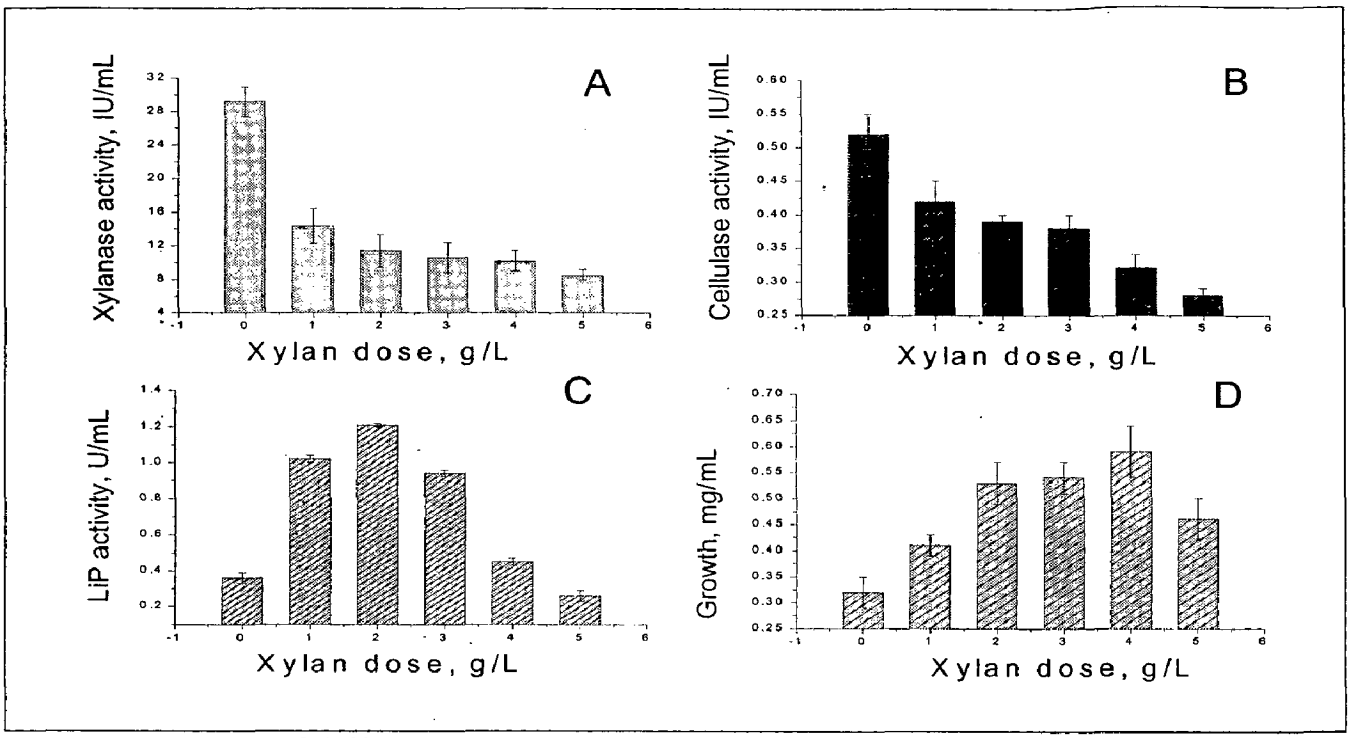


Figure 2.6: Effect of different concentration of xylan on the production of (A) xylanase (B) cellulase (C) lignin peroxidase and (D) growth of strain MLK01 at initial pH 10 with 2% wheat bran as substrate after 8 day of incubation at 40 ^{0}C

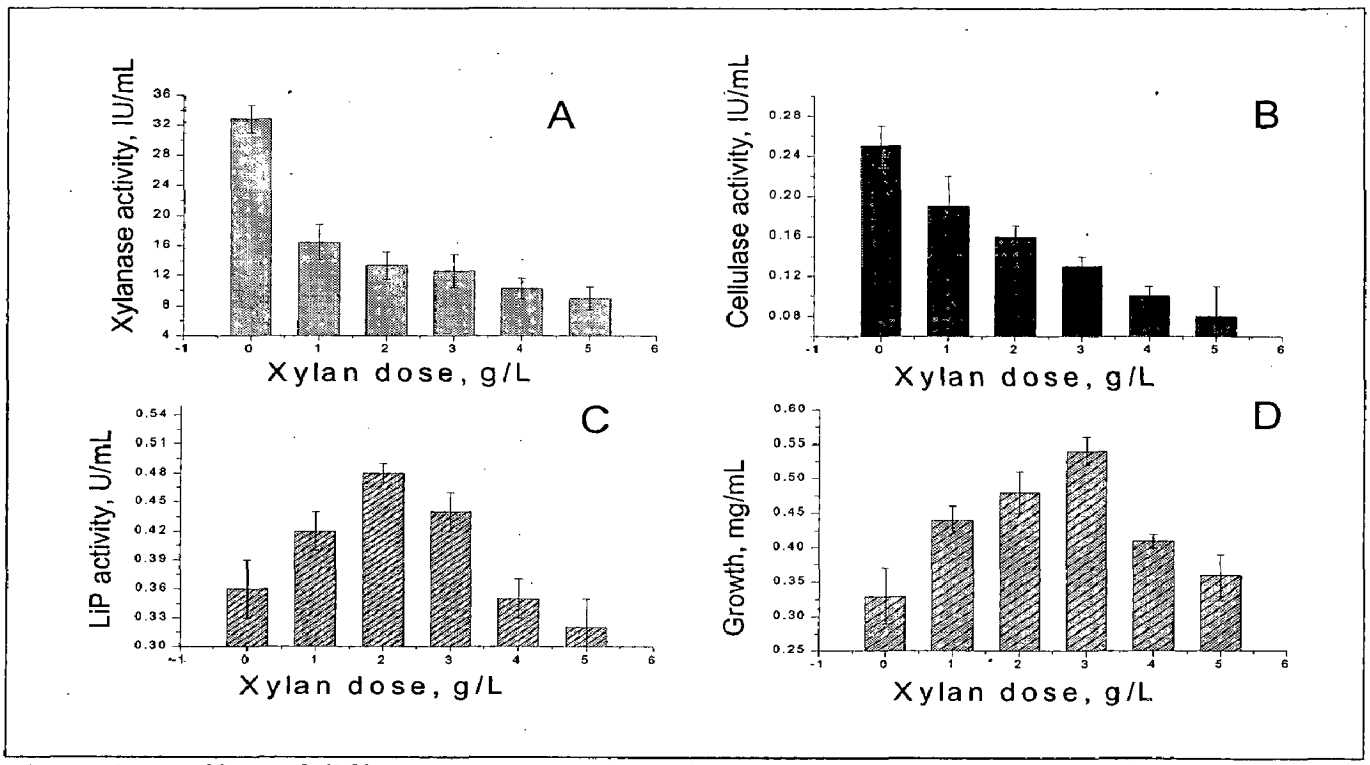


Figure 2.7: Effect of different concentration of birch wood xylan on the production of (A) xylanase (B) cellulase (C) lignin peroxidase and (D) growth of strain MLK07 at initial pH 10 with 2% wheat bran as substrate after 9 day of incubation at 40 ^{0}C

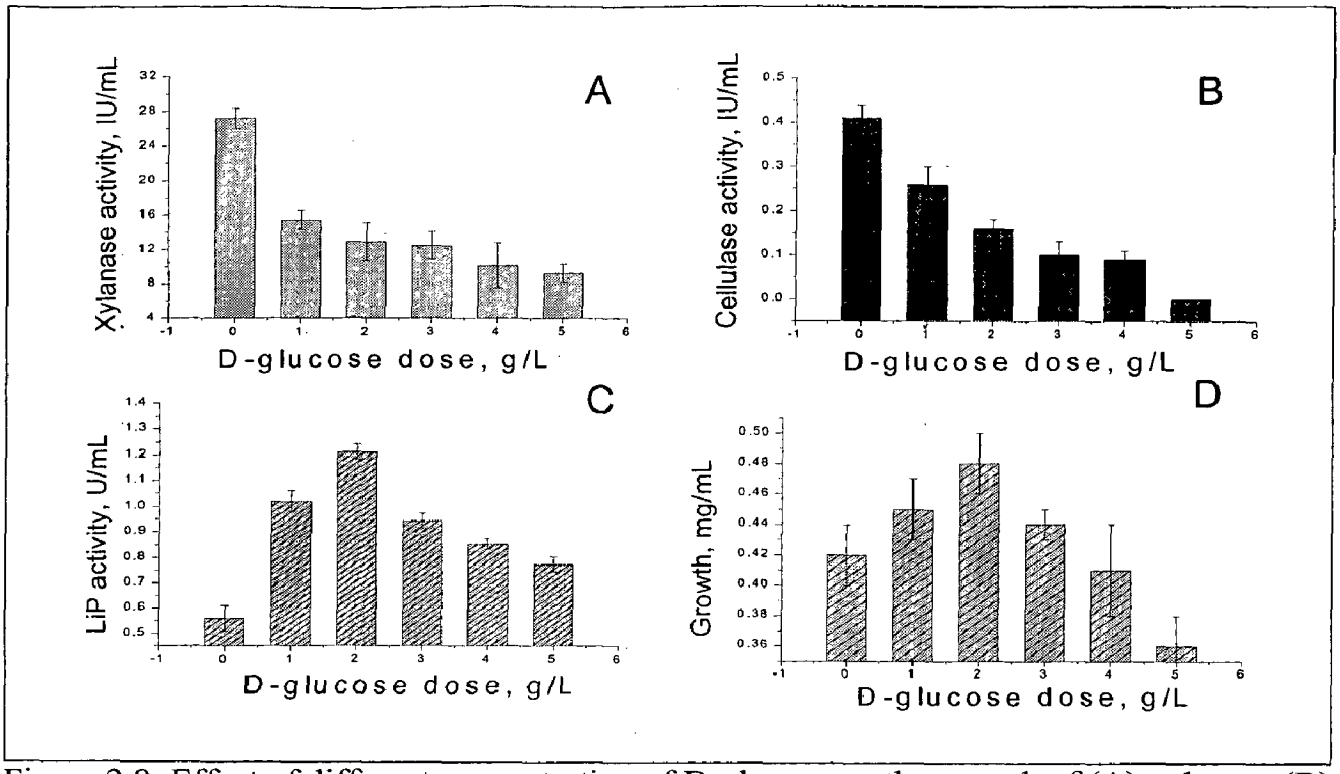


Figure 2.8: Effect of different concentration of D-glucose on the growth of (A) xylanase (B) cellulase (C) lignin peroxidase and (D) growth of strain MLK01 at initial pH 10 with 2% wheat bran as substrate after 8 day incubation at 40 ^{0}C

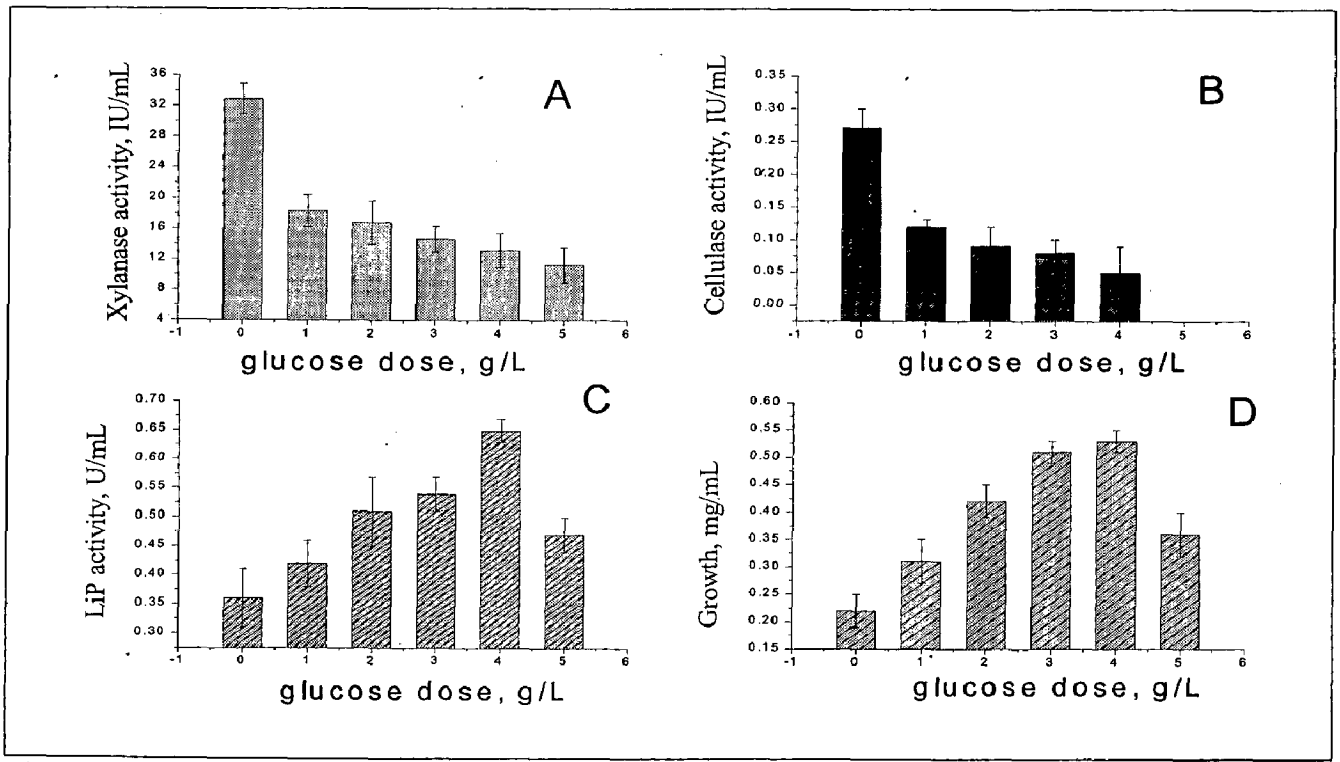


Figure 2.9: Effect of different concentration of D-glucose on the production of (A) xylanase (B) cellulase (C) lignin peroxidase and (D) growth of strain MLK07 at initial pH 10 with 2% wheat bran as substrate after 9 day of incubation at 40 ^{0}C

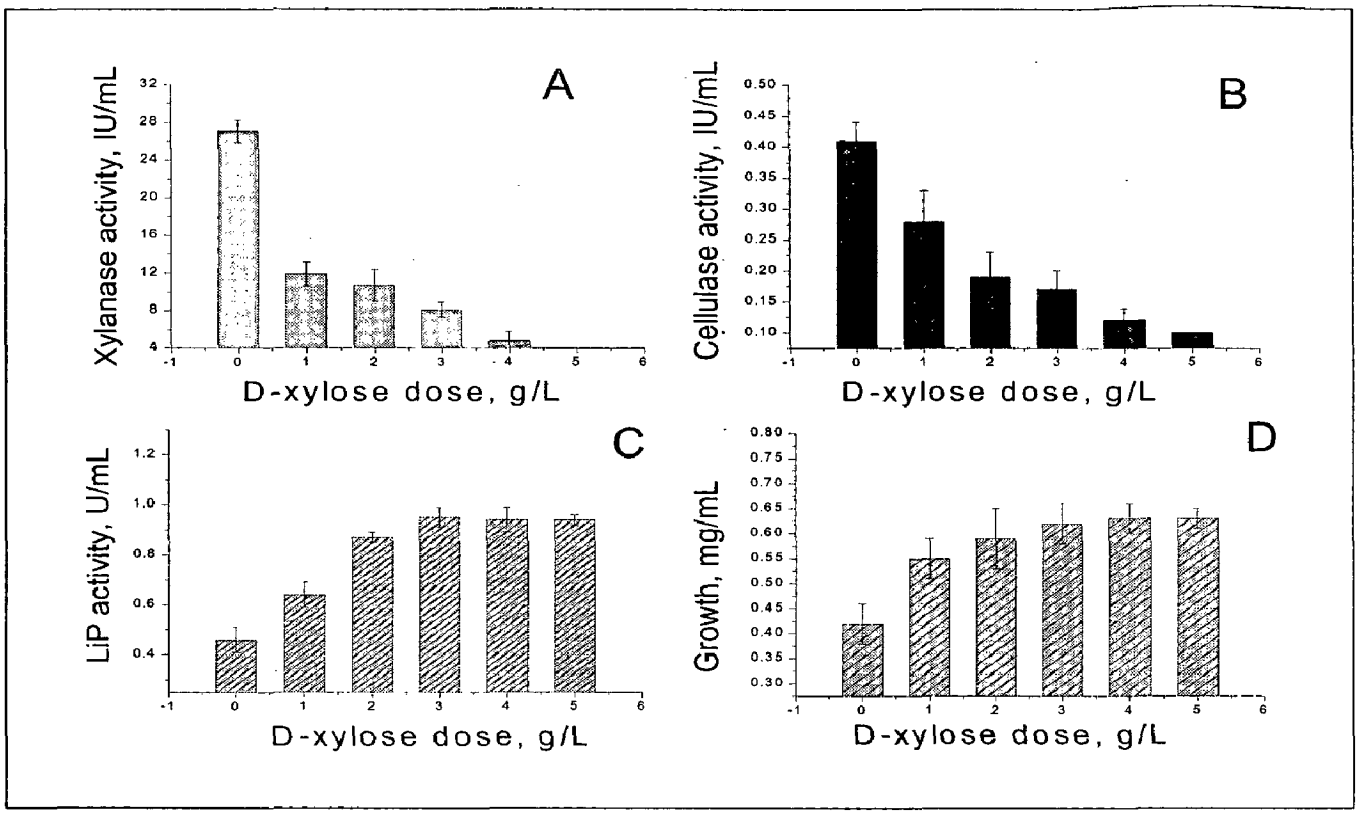


Figure 2.10: Effect of different concentration of D-xylose on the production of (A) xylanase (B) cellulase (C) lignin peroxidase and (D) growth of strain MLK01 at initial pH 10 with 2% wheat bran as substrate after 8 day of incubation at 40 ^{0}C

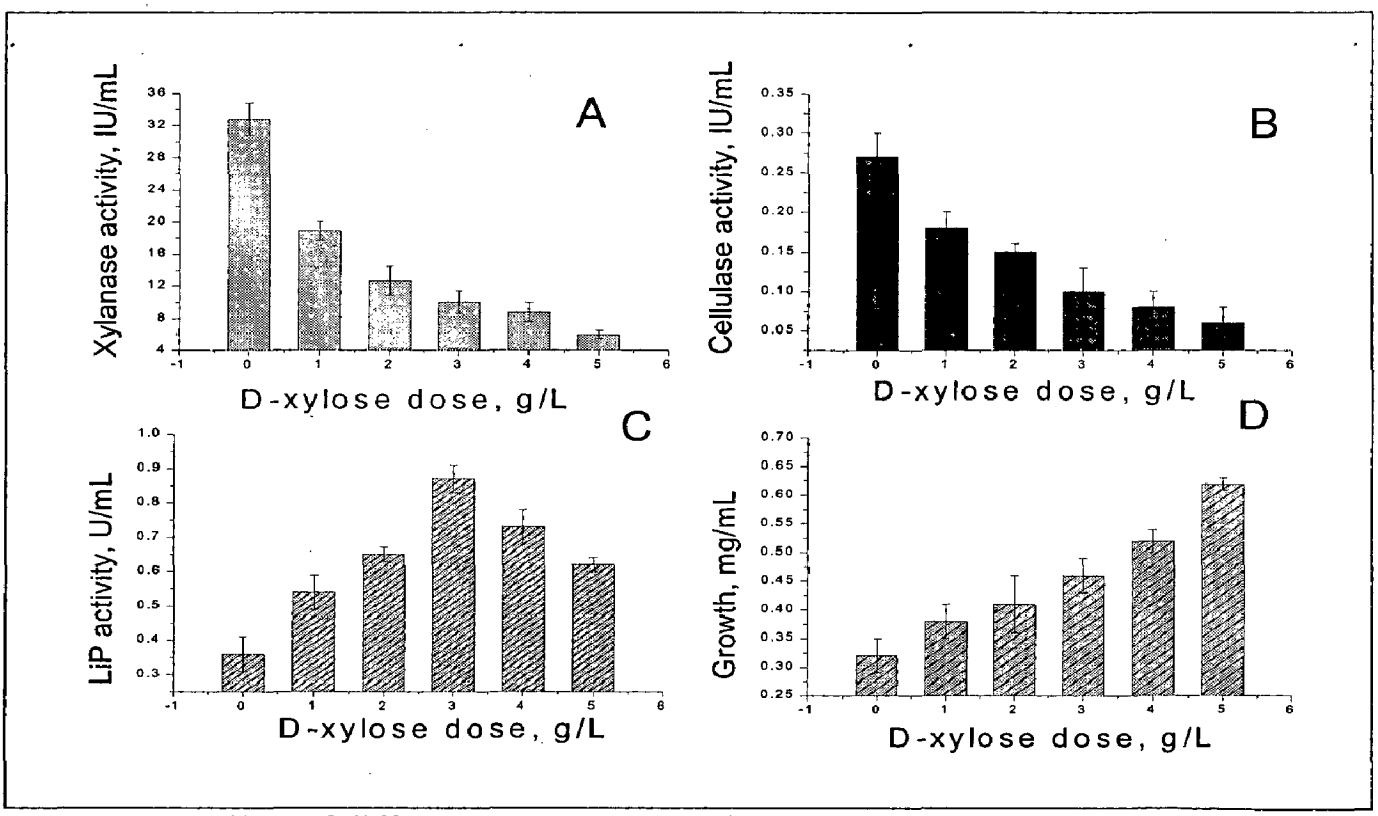


Figure 2.11: Effect of different concentration of D-xylose on the production of (A) xylanase (B) cellulase (C) lignin peroxidase and (D) growth of strain MLK07 at initial pH 10 with 2% wheat bran as substrate after 9 day of incubation at 40 ^{0}C

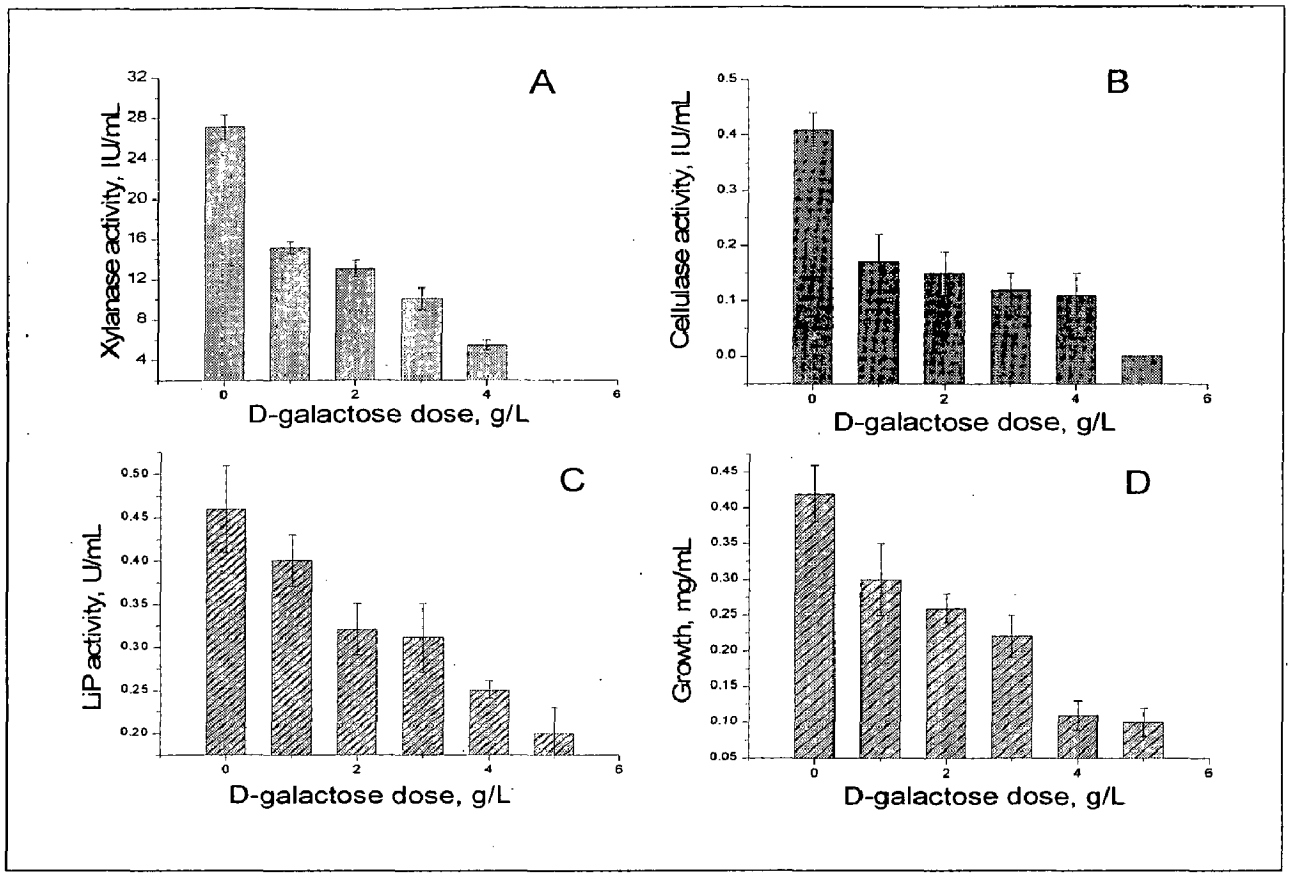


Figure 2.12: Effect of different concentration of D-galactose on the production of (A) xylanase (B) cellulase (C) lignin peroxidase and (D) growth of strain MLK01 at initial pH 10 with 2% wheat bran as substrate after 8 day of incubation at 40 ^{0}C

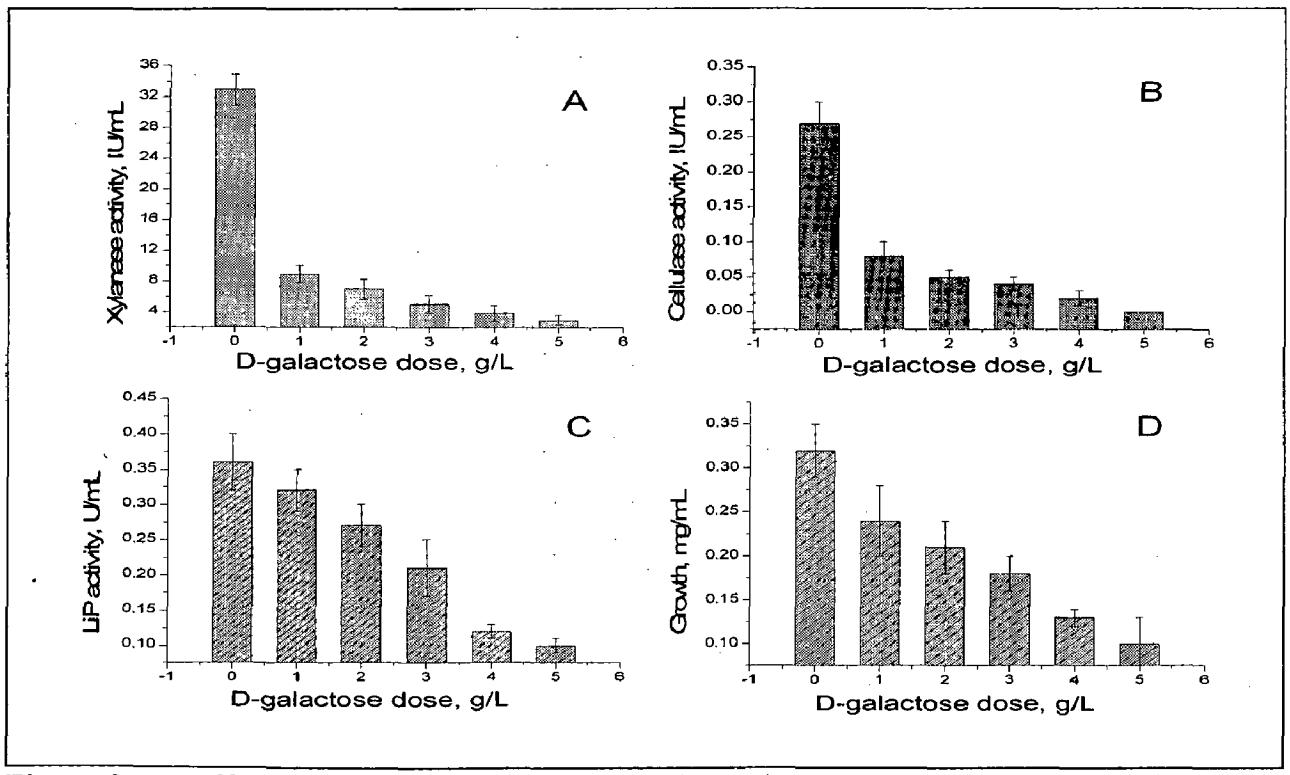


Figure 2.13: Effect of different concentration of D-galactose on the production of (A) xylanase (B) cellulase (C) lignin peroxidase and (D) growth of strain MLK07 at initial pH 10 with 2% wheat bran as substrate after 9 day of incubation at 40 ^{0}C

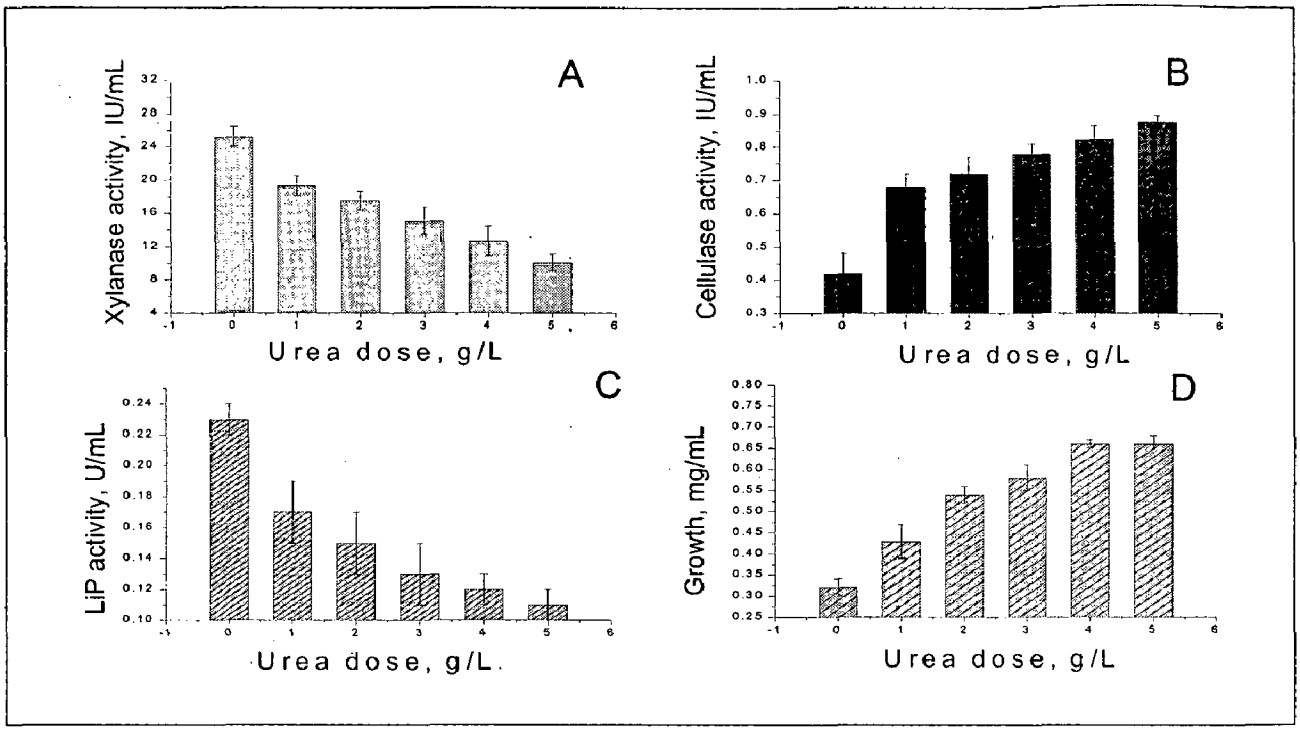


Figure 2.14: Effect of different concentration of urea on the production of (A) xylanase (B) cellulase (C) lignin peroxidase and (D) growth of strain MLK01 at initial pH 10 with 2% wheat bran as substrate after 8 day of incubation at 40 ^{0}C

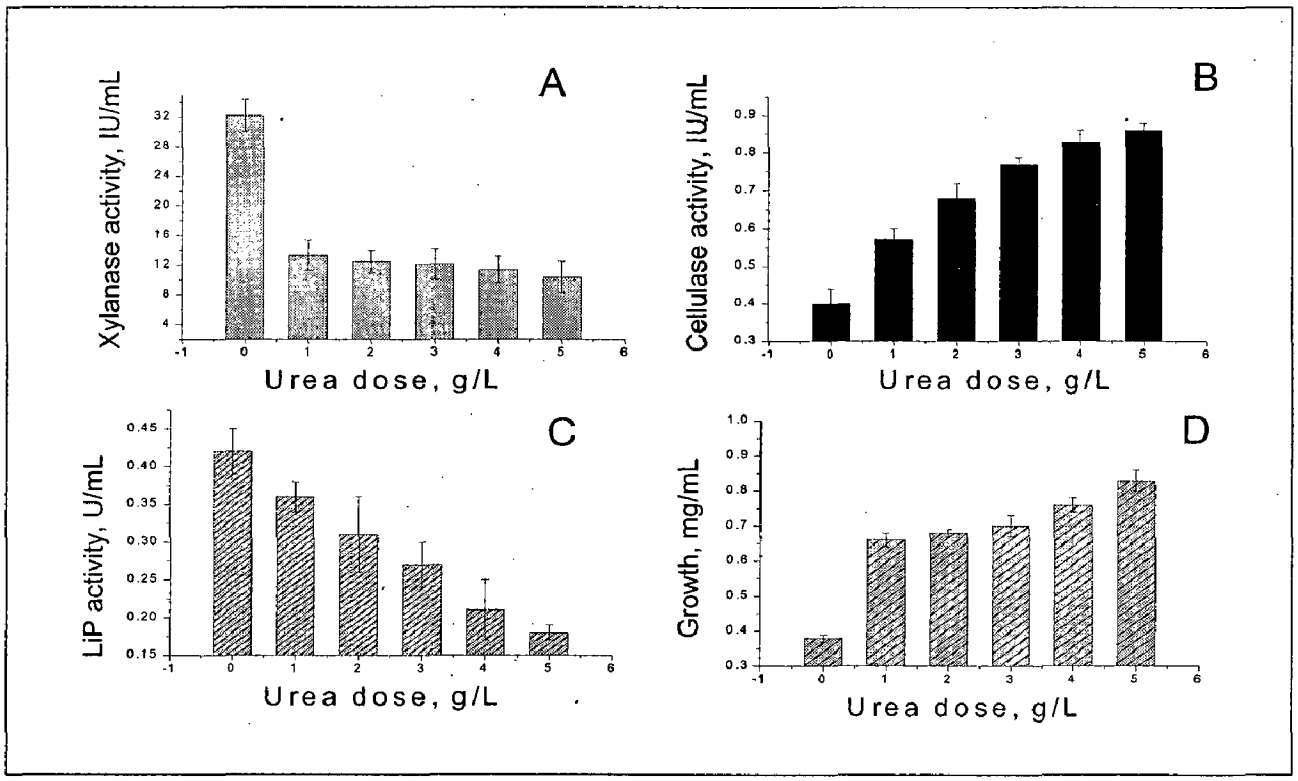


Figure 2.15: Effect of different concentration of urea on the production of (A) xylanase (B) cellulase (C) lignin peroxidase and (D) growth of strain MLK07 at initial pH 10 with 2% wheat bran as substrate after 9 day of incubation at 40 ^{0}C

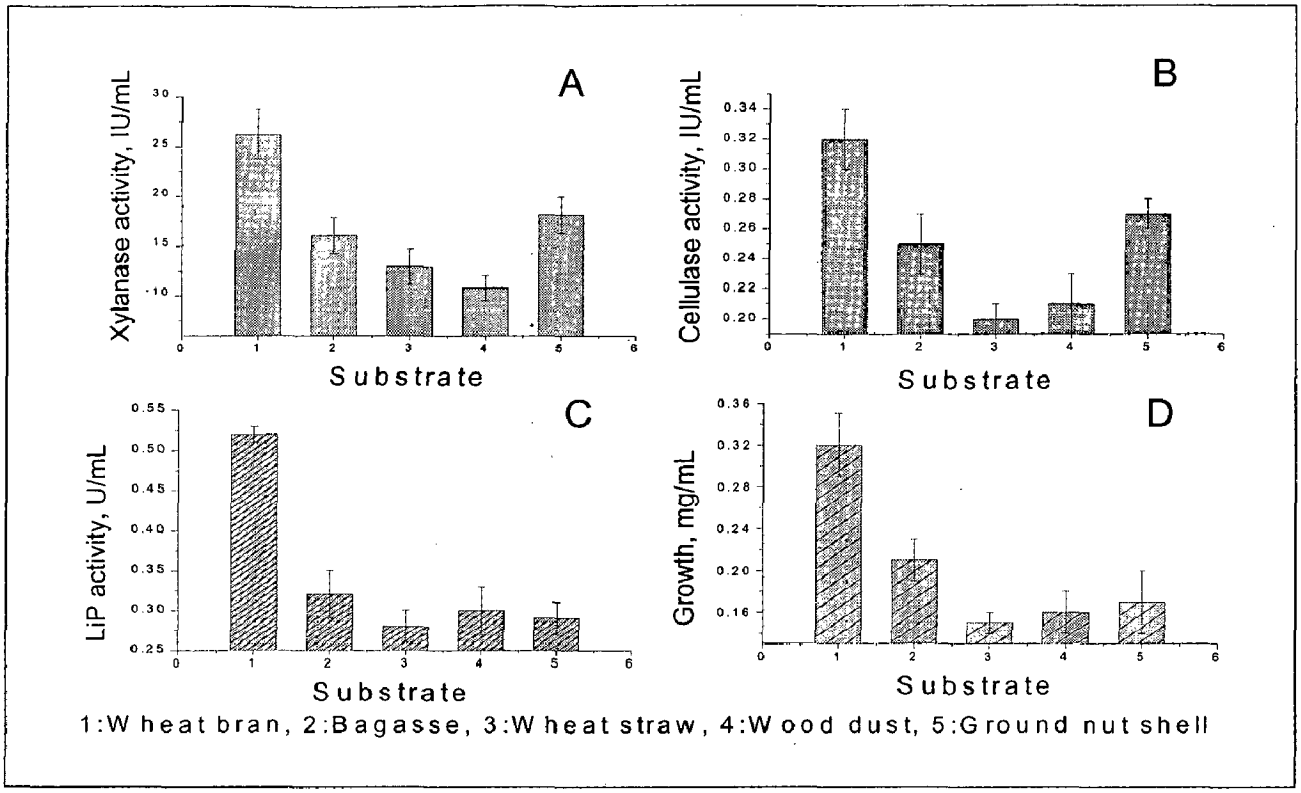


Figure 2.16: Effect of different lignocellulosic substrates on the production of (A) xylanase (B) cellulase (C) lignin peroxidase and (D) growth of strain MLK01 at initial pH 10 after 8 day of incubation at 40 ^{0}C

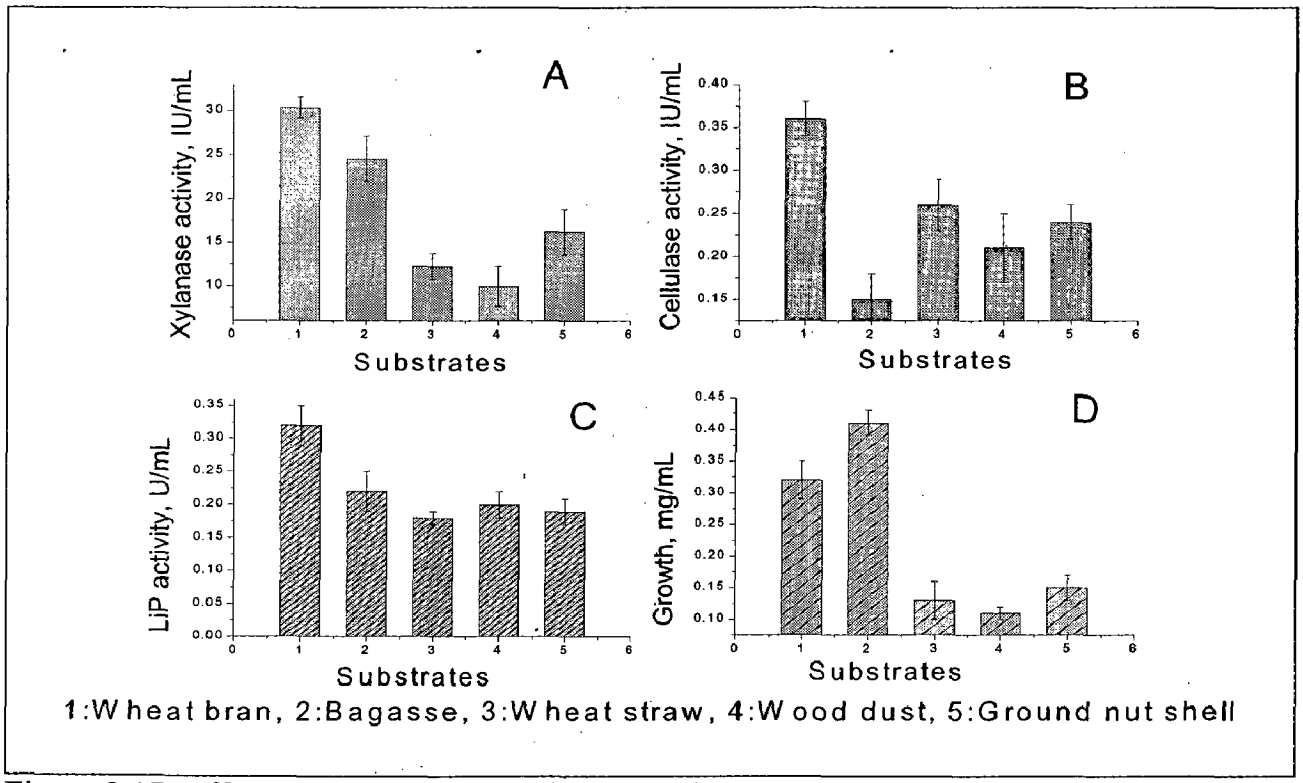


Figure 2.17: Effect of different lignocellulosic substrates on the production of (A) xylanase (B) cellulase (C) lignin peroxidase and (D) growth of strain MLK07 at initial pH 10 with 2% substrate after 9 day of incubation at 40 ^{0}C

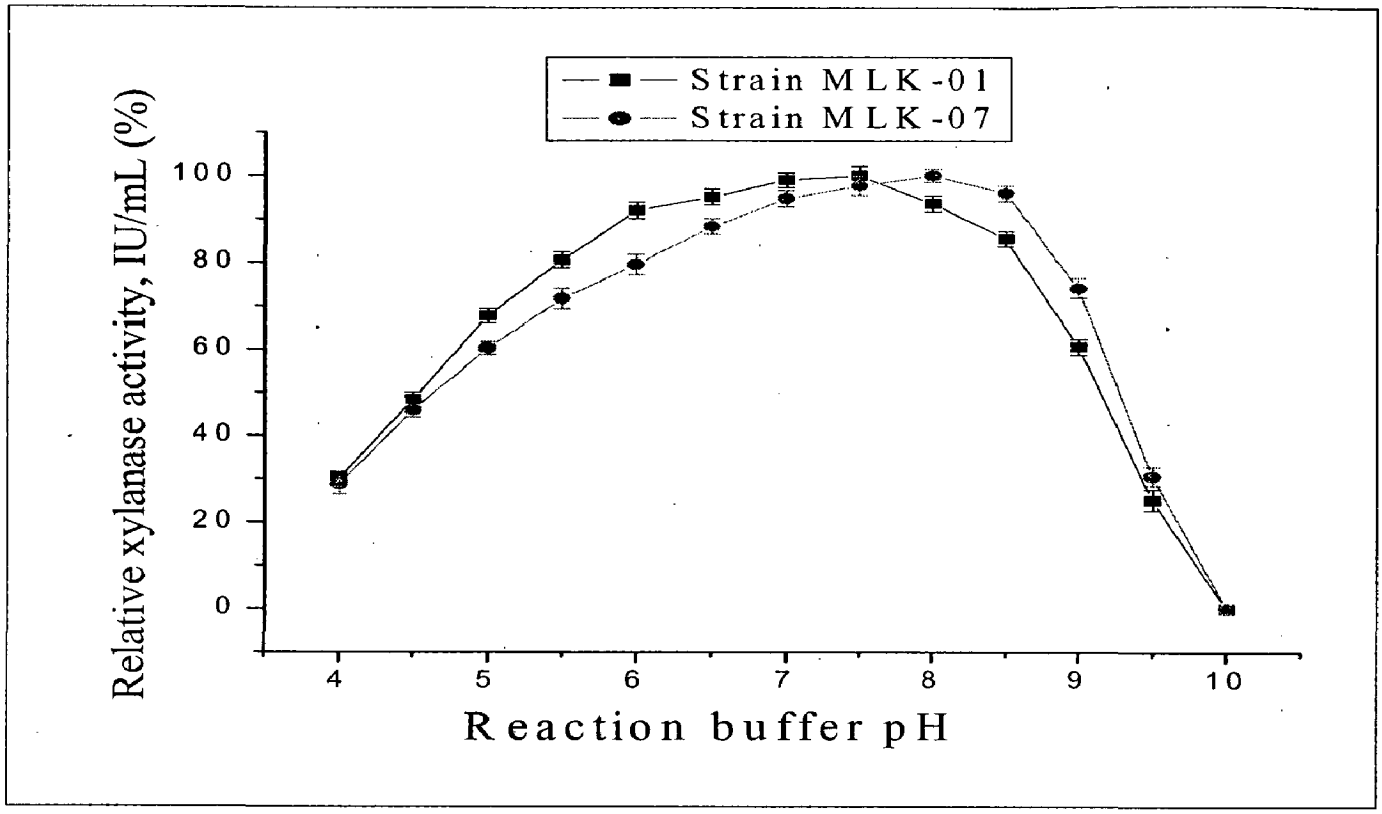


Figure 2.18: Effect of reaction buffers of different pH on xylanase activity

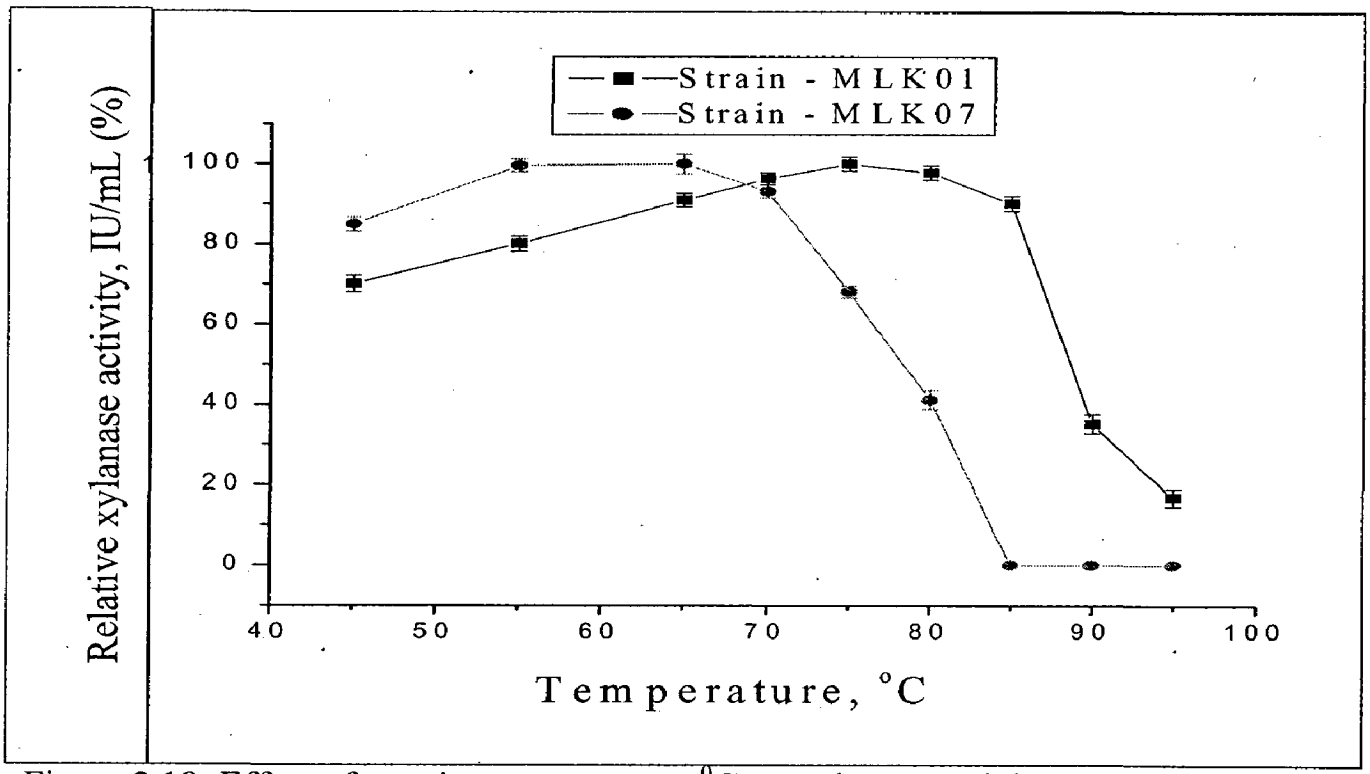


Figure 2.19: Effect of reaction temperature, ^{0}C on xylanase activity

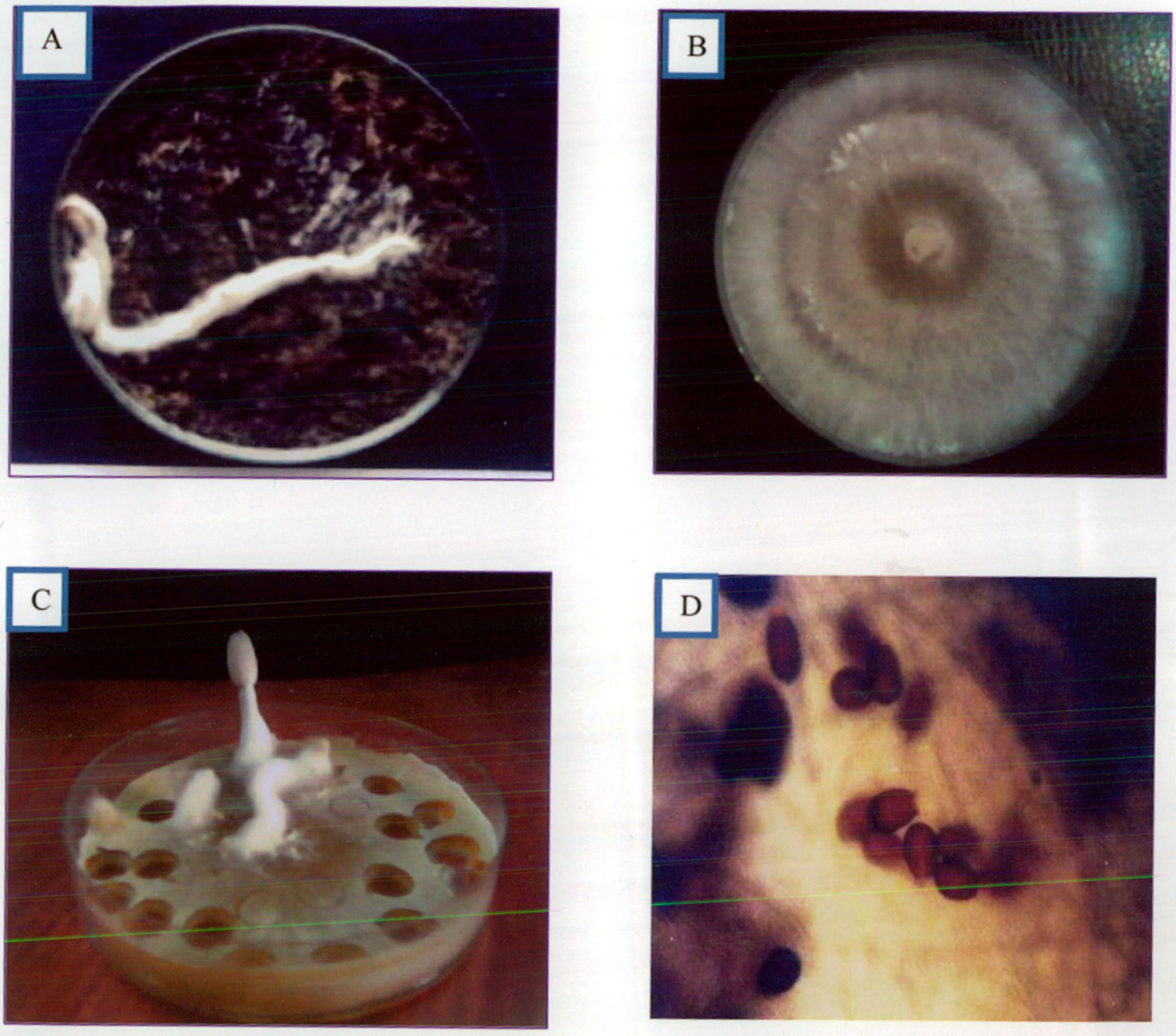
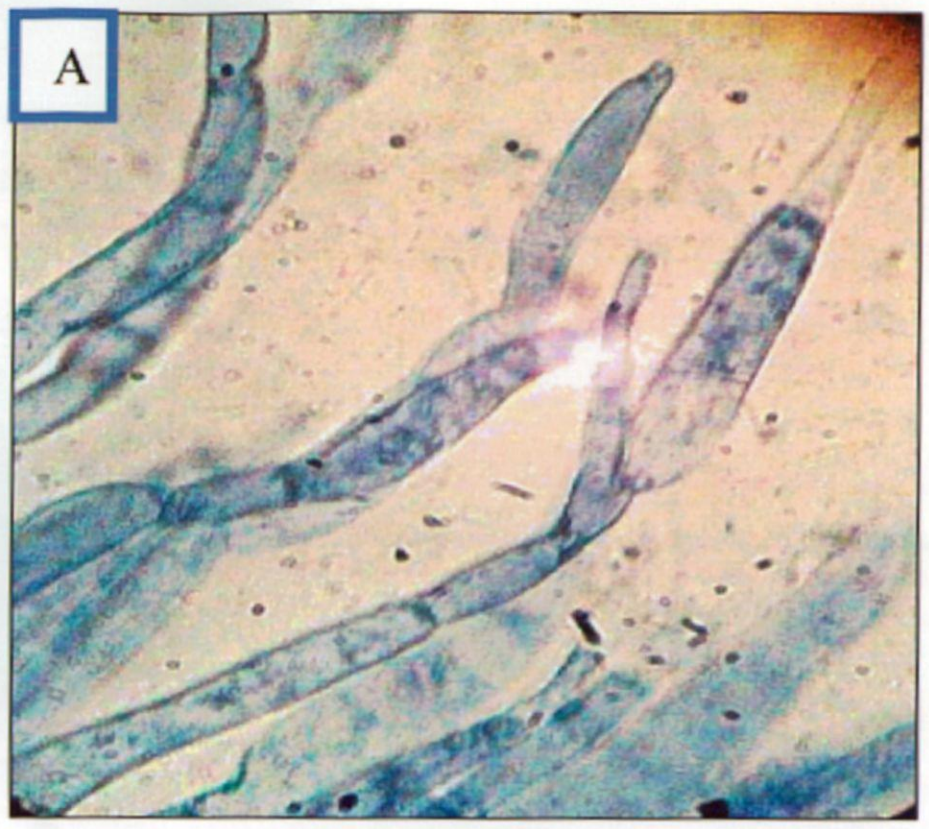
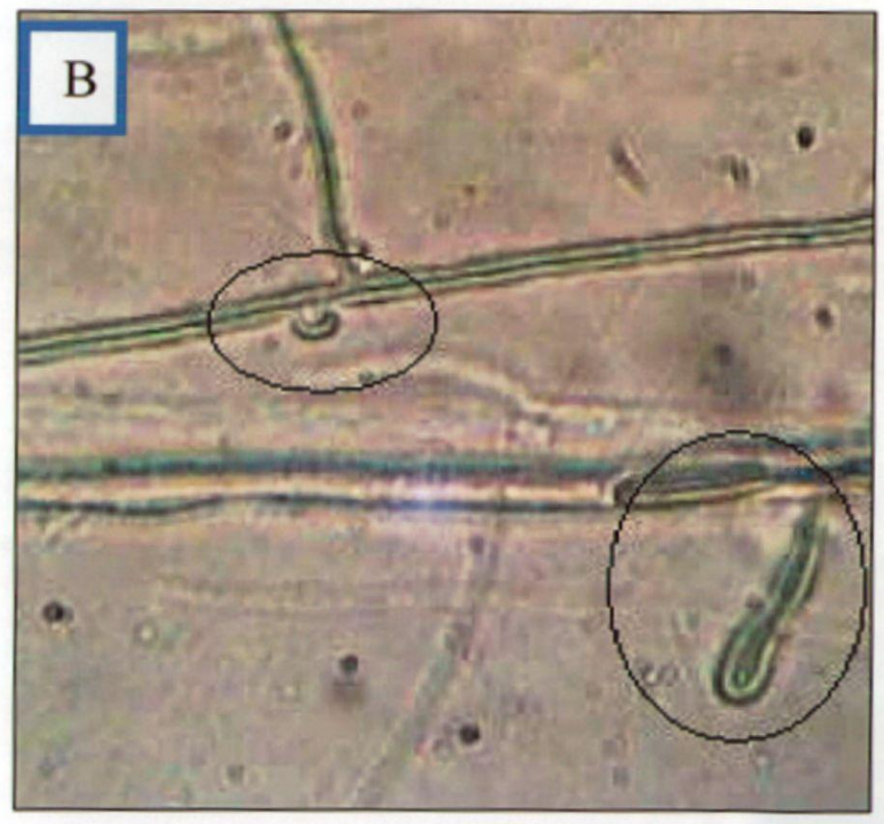
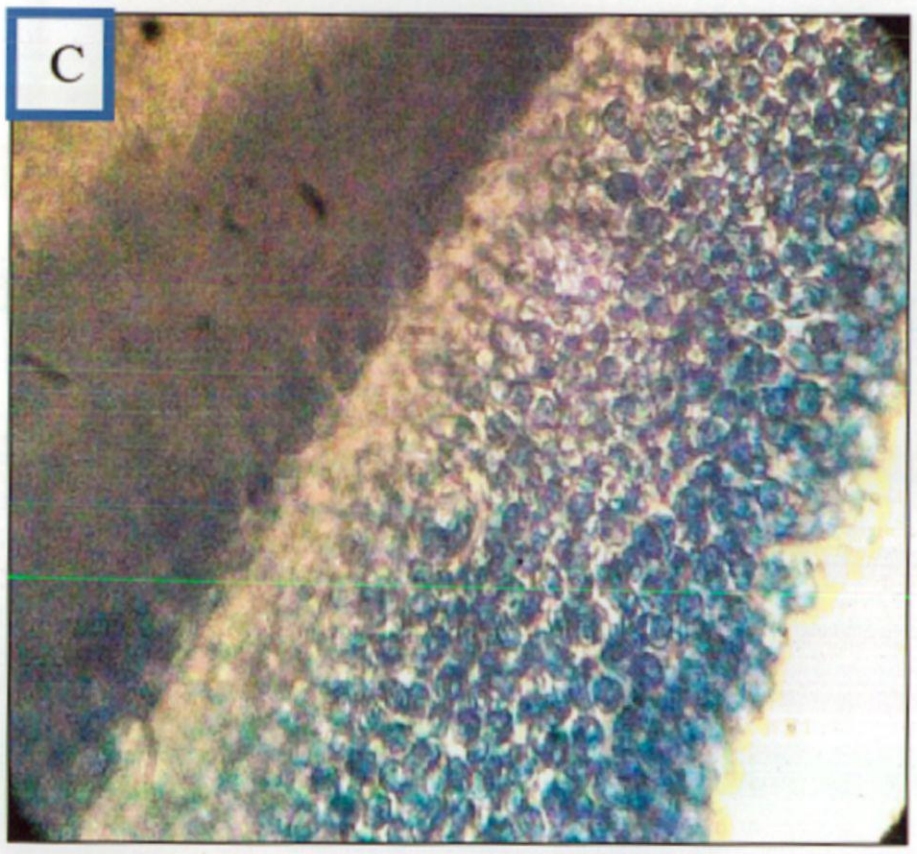


Plate 2.1: (A) White fruiting body appeared on decaying wood in wheat bran enriched medium (B) 4-d old culture of white rot fungi on wheat bran agar medium at initial pH 10.0 (C) fruiting body on wheat bran agar in laboratory conditions (D) microphotograph of basidiospores at 40X10 magnification.







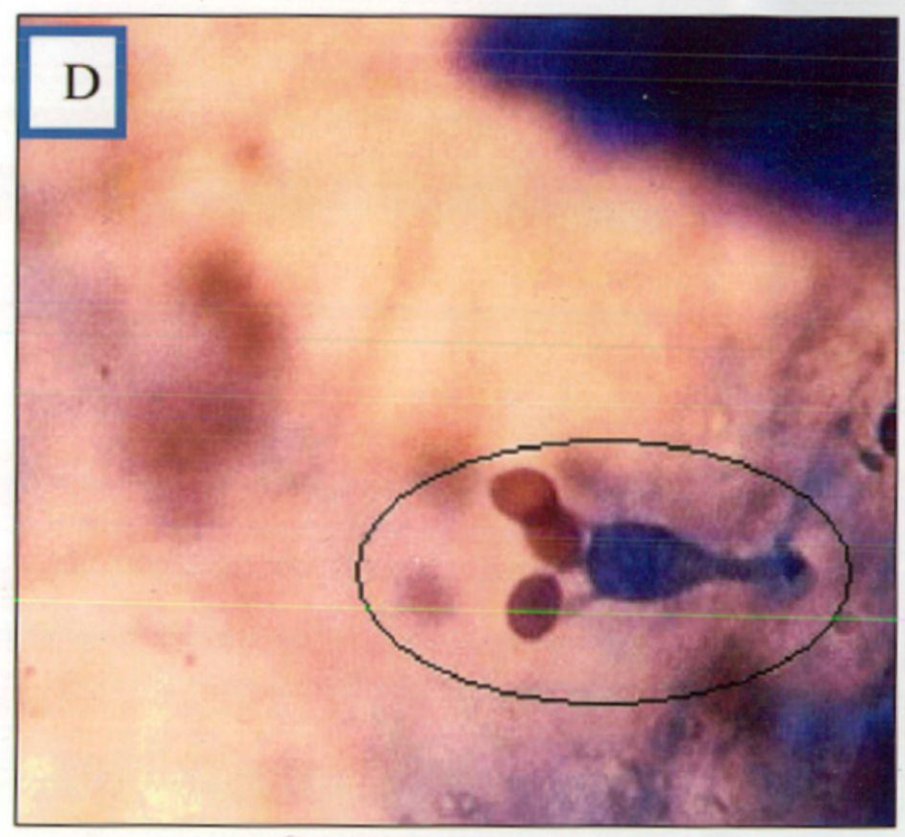


Plate 2.2: Microphotograph shows (A) pileocystidea (40X), (B) bipolar mating system (40X), (C) hymeneal layer (40X), (D) basidium with attached spores in *Coprinellus*

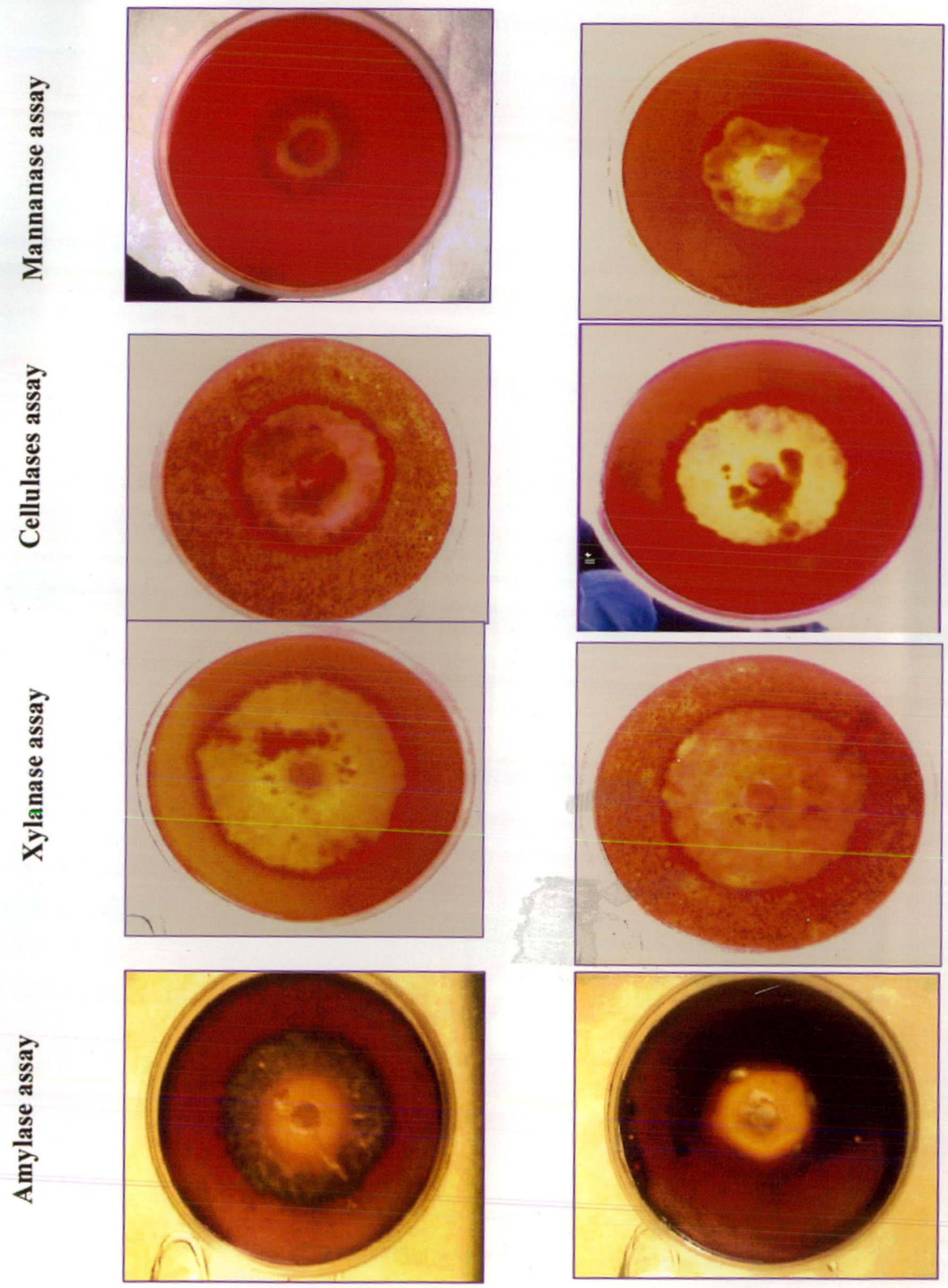
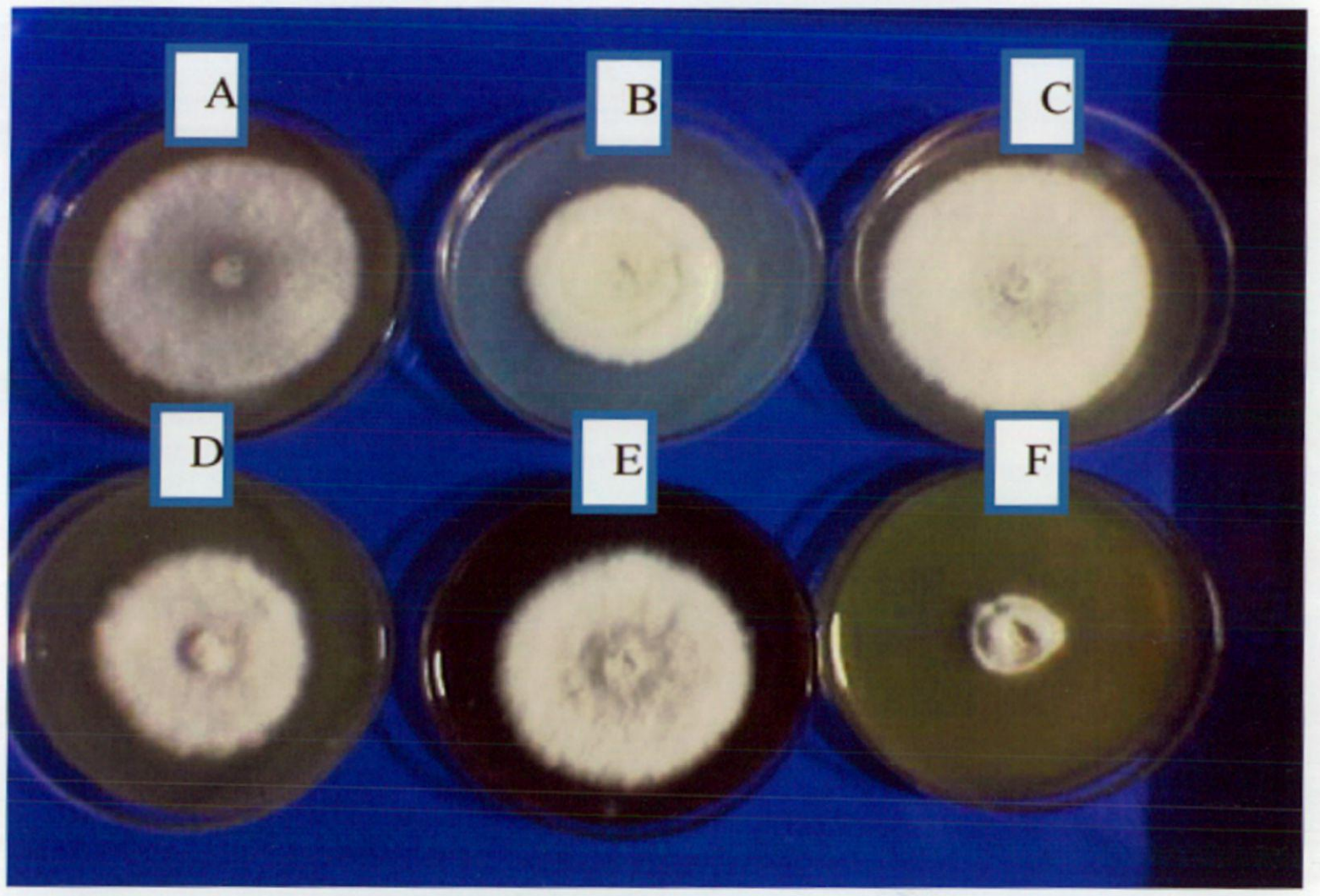
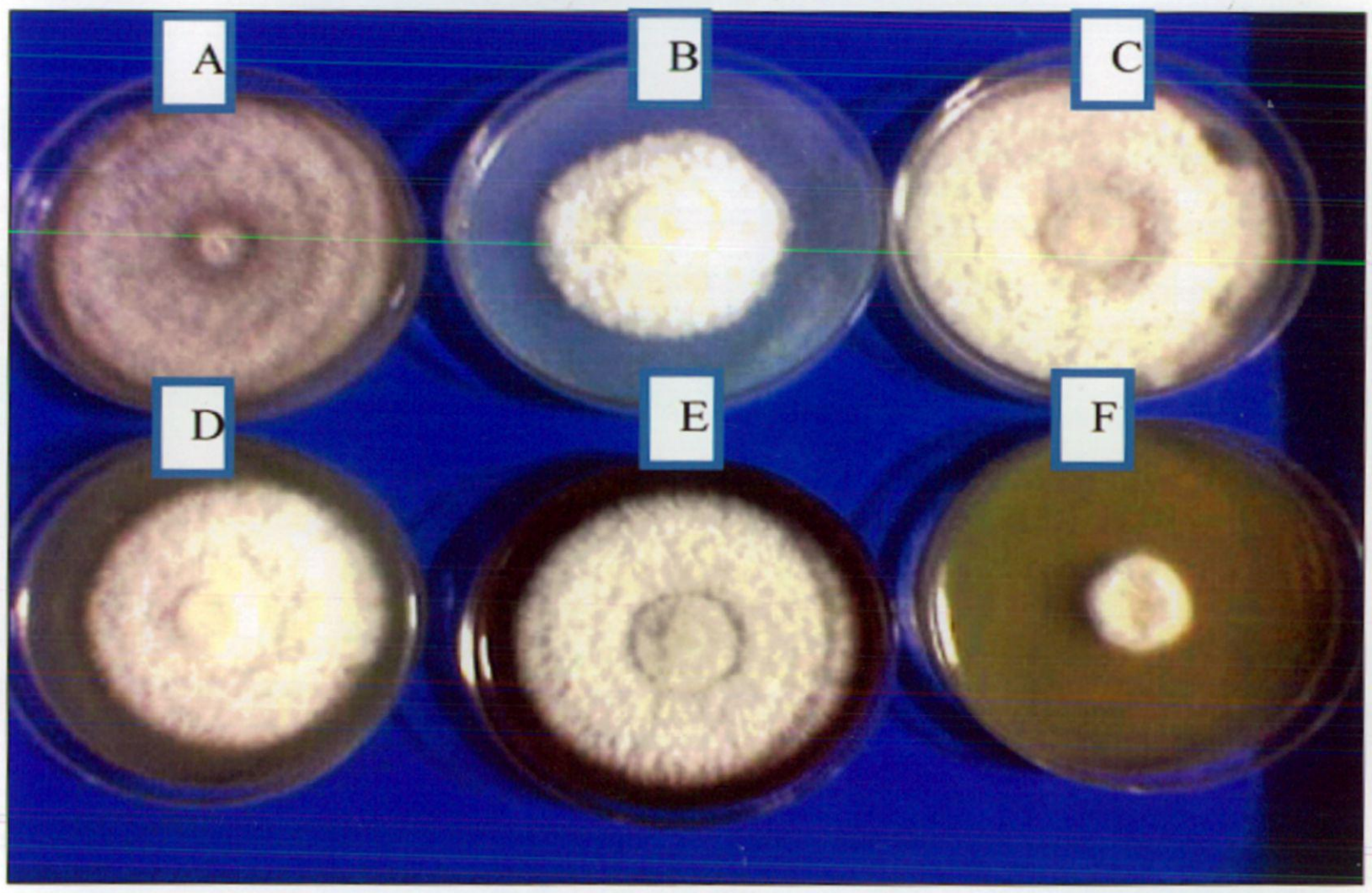


Plate 2.3: Plates show mannanase, cellulase, xylanase and amylase positive test respectively by both the strains of *Coprinellus disseminatus* MLK01 and MLK07



Strain MLK01



Strain MLK07

Plate 2.4: Photographs show the growth of *Coprinellus disseminatus* after 36 h on different agar media: (A) wheat bran agar (B) potato dextrose agar (C) malt extract agar (D) malt extract glucose yeast peptone agar (E) fungal agar (F) lactose yeast extract agar.

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ANATOMY, FIBER MORPHOLOGY AND CHEMICAL COMPOSITION OF *A. CADAMBA*

3.0 INTRODUCTION

The fundamental and structural unit of a plant is cell. A living plant cell consists of protoplasm, which is surrounded by cell wall. At maturity, the protoplasm disappears and living hollow quill shaped structure known as fiber. The cell wall of fiber is a dynamic structure that is integrally involved in the development and life of the plant [19]. It provides rigid barriers that prevents the cell from bursting in its hypotonic environment and allows the protoplast to generate the outward hydrostatic pressure called turgor [44]. Variation in the chemical composition and structure of the cell wall reflects its changing function during cell development [17]. The wall of growing cell commonly called primary wall is viscoelastic, allowing controlled expansion of the cell in response to turgor. The pattern of cellulose microfibril deposition in these expanding wall determined cell shape and ultimately, plant morphogenesis. Specialized, non-extensible secondary walls, which have a higher percentage of cellulose microfibrils, are often deposited after the cessation of cell growth. Incrustation of cell wall and intracellular spaces with lignin during secondary wall formation results in a plant structure that is rigid even in the absence of internal turgor. Cell wall structure may also change in response to the position of a plant part, temperature stresses, wounding or pathogenesis [17].

Beasley et al. and Preston [7, 42] suggested that the cellulose crystallinity and fibril substructure properties that are fundamental for any application of cellulose will ultimately depend on a complete understanding that, how cellulose is made by living cells. The suitability of various woods and fibers for specific industrial applications is ultimately related to their development and molecular properties. The strength of wood depends upon relative number, distribution, length, diameter and thickness of the conducting elements and the fiber

in the xylem. The environmental factors as well as nutrients are highly influence the paper making properties of cellulose fiber. Ogbonnaya, [39] studied on the effects on nitrogen sources on the wood properties of *Gmilina orborea* related to paper making and concluded that, the largest fiber are found with NO_3-N and shortest with NH_4-N. Urea-N produces narrowest cell wall while, NH_4-N produces widest fiber with the thickest cell wall. Gustavsson and Martinsson [5] studied on seasonal changes in biochemical composition of cell wall, morphology and growth in *Phleum pratense L.* and concluded that, in spring the plant growth, in terms of dry matter, is faster than summer. At low temperature both growth and development are slow.

Wood fiber cell wall is a natural composite material and a chemical complex of cellulose, lignin, hemicelluloses and extractives [12]. Table 3.1 and 3.2 shows the chemical composition of five distinct hardwoods and softwoods [51]. The schematic illustration of the arrangement of the wood polymers within the secondary wall of a softwood fiber is shown in Figure 3.1 [2].

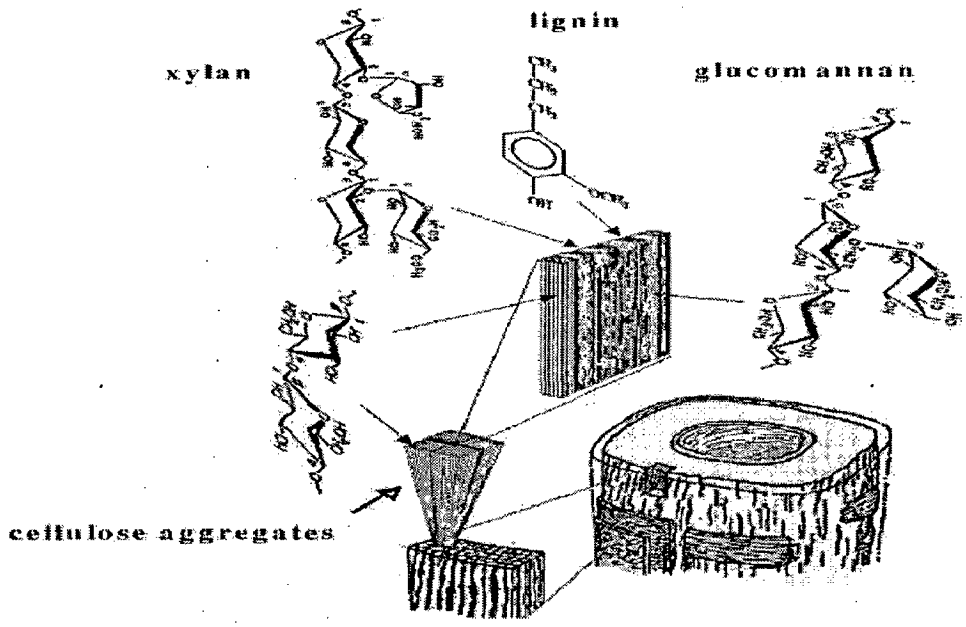

Figure 3.1: Schematic illustration of the secondary wall of a softwood fiber [2]

Cellulose contents in a cell wall vary with cell development and type of organism: primary cell wall contain 1-10% cellulose and secondary wall commonly contain about 50% cellulose, in the case of higher plants. The thickened walls of some algae contain as much as 80% cellulose and the cell wall of cotton seed hair is almost pure cellulose [41]. The degree of polymerization of cotton polymer has been estimated to range from 2000-6000 in the primary wall and proximately 14,000 in the secondary wall [31]. Degree of polymerization of about 14000 have also been found for cellulose from wood, flax, ramie while, cellulose in the secondary wall of algae, *Valonia* has a degree of polymerization of 18,000 [31]. Cellulose polymers associate through H-bonds [45]. The H-bonding of many cellulose molecules to each other results in the formation of microfibrils which can interact to form fibers. Certain cells, like those in cotton ovules, can grow cellulose fibers of enormous lengths. Cellulose fibers usually consist of over 500,000 cellulose molecules. If a fiber consists of 500,000 cellulose molecules with 5000 glucose reside/cellulose molecule, the fiber would contain about 2.5 billion H-bonds. Even if an H-bond is about 1/10 the strength of a covalent bond, the cumulative bonding energy of 2.5 billion of them is awesome. It is the H-bonding that is the basis of the high tensile strength of cellulose. The amount of cellulose is directly related to pulp yield.

The name hemicellulose was given to all of the polysaccharides of the plant cell wall except pectin and cellulose [55], composed of a variety of sugars including xylose, arabinose, mannose, galactose and glucose. Hemicellulose that is primarily xylose or arabinose is referred to as xyloglucans or arabinoglucans, respectively. Hemicellulose molecules are often branched. The number and the composition of the side chain vary greatly between species, tissue and even the same cell at different stages of development [12, 48]. The secondary wall of angiosperms contains heteroxylans as the major hemicellulose; O-acetyl-4-Orthomethyle-γ-glucourono-β-D-xylan with degree of polimerization of 150-200 is predominant in secondary wall of dicot [50]. Xylan in monocot cell wall commonly has side chains of

arabinose, glucuronosyl, or 4-O-methyl-glucuronosyl residues [12]. The secondary wall of gymnosperms contains large amounts of hemicellulosis glucomannans with degree of polymerization of 100-400 and minor amount of arabino-4-O-methyle-glucurono-xylan [50, 55]. The main hemicellulose extracted from dicot primary cell wall is contain about 19% (DP of about 50) xyloglucan and 5% glucuroarabinoxylan. In monocot primary cell wall about 2% xyloglucan present while, arabinoxylan is the main cellulose [12]. During secondary wall formation, the deposition of hemicellulose continues during cell maturation and function in hydrogen bonding with cellulose [12, 32]. Primary wall of dicot contains 24% hemicellulose and mature wall in wood contain 15-35% hemicellulose [50]. Hemicellulose molecules are very hydrophilic. They become highly hydrated and form gels within the cell [8, 13]. The hemicellulose dissolves to a large extent in the chemical pulping; however, a substantial quantity of hemicellulose is always associated with the pulp. It also has a great influence on the swelling behavior of the fiber [3].

Lignin is an aromatic, amorphous substance containing phenolic methoxyl, hydroxyl, and other constituent groups; its chemical structure has not been fully elucidated. Lignin is a complex aromatic polymer with molecular weight of about 11000 that is formed by the three dimensional polymerization of sinnapyl alcohols [54]. Lignin deposition results in wall that is extremely rigid and stress resistant, less permeable to water and more resistant to pathogenic degradation [18, 21, 52]. In this method of determination, lignin (also known as "Klason lignin") is defined as a wood or pulp constituent insoluble in 72% sulfuric acid. The percentage of lignin in the wood is related with the chemical dose and time required for delignification, the higher the lignin content, the higher the chemical dose and longer the cooking cycle required for pulping.

The chemical analysis of a plant is known as proximate chemical analysis which provides important information pertaining to suitability of a plant for pulp and paper making.

Water soluble affects the pulp yield to some extent. Therefore, water soluble in a plant should be as low as possible [6]. The cold-water procedure removes a part of extraneous components, such as inorganic compounds, tannins, gums, sugars, and coloring matter present in wood and pulp. The hot-water procedure removes, in addition, starches. The ethanol-benzene extractable content of the wood consists of low-molecular-weight carbohydrates, salts, waxes, fats, resins, photosterols and non-volatile hydrocarbons. The amount is markedly influenced by seasoning or drying of the wood. The solvent extractable material in pulp may be considered to consist primarily of resin and fatty acids and their esters, waxes, and unsaponifiable substances. They cause pitch problem [29]. Their deposition in the fourdrinier wire and pressing section of paper machine causes slow drainage and paper defects. Hot alkali solution extracts low-molecular-weight carbohydrates consisting mainly of hemicellulose and degraded cellulose in wood and pulp. The solubility of wood indicates the degree of fungus decays or of degradation by heat, light, oxidation, etc. As the wood decay or degrades, the percentage of the alkali-soluble material increases [33, 43]. The solubility of pulp indicates an extent of cellulose degradation during pulping and bleaching processes and has been related to strength and other properties of pulp [4]. Therefore, 1% NaOH soluble of wood should be as low as possible.

The conversion of wood in to marketable paper product is dependent upon the wood density, chemical composition and morphological characteristics of the pulp fiber and on the response of this fiber to processing variables. Horn, concluded that, the tear strength of sheet made from either unbeaten or beaten hardwood fiber is principally dependent upon fiber length [22]. This is in contrast with paper made from softwood pulp in which cross-sectional area and cell wall thickness are the dominant variable [24].

In addition to fiber length, tearing strength also shows a significant relation with fibril angle in unbeaten pulp. The positive correlation of fibril angle with tearing strength would

indicate that fiber extensibility contributes more to tearing strength than does fiber strength. Page shows that fiber strength depends upon fiber angle, regardless of species or fiber type [23]. Bursting and tensile strength of pulps are two properties highly dependent upon fiber-to-fiber bonding and respond to the same morphological as do softwood. This is especially true when pulp has been beaten. It has been shown that the presence of a high percentage of parenchyma cells can reduce sheet strength by inhibiting fiber-to-fiber bond formation [24]. Therefore, the parenchyma cell content of any new fiber source should be a consideration in assessing its potential use in papermaking. Fiber length to width calculated from these derived values has been shown unreliable in providing basic information on strength properties depend upon fiber bonding [39]. However, after beating, the L/T ratio becomes the dominant factor for both bursting strength and tensile strength of sheets made from hardwood. This most probably reflects the greater degree of fiber collapse, which results from beating. The fibers become more flexible and conformable, which in turn provides for more area to be developed for bonding along the fibers length. In addition, beating reduces the identity of parenchyma cells and, in a sense, adds bonding material to the pulp furnish. Therefore, bursting and tensile strength, being dependent upon the formation of fiber-to-fiber bonds, is greatly influenced by fiber length and cell wall thickness [24].

3.2 MATERIALS AND METHODS

3.2.1 Anatomy and morphological studies

A. cadamba was collected from Horticulture Research Institute 7 km away from Department of Paper Technology, Indian Institute of Technology Roorkee, Saharanpur Campus, Saharanpur located in the foothills of Shivalik range of Western Uttar Pradesh (India). The logs of *A. cadamba* were debarked manually. Three samples from *A. cadamba* were taken at 10% (base), 50% (middle) and 90% (top) of its height/length respectively, an approach similar to that followed by Paraskevopoulou [40]. For fiber length determination,

small slivers were obtained and macerated with 10 mL of 67% HNO_3 and boiled in a water bath (100±2 °C) for 10 min. [38]. The slivers were then washed, placed in small flasks with 50 mL distilled water and the fiber bundles were separated into individual fibers using a small mixer with a plastic end to avoid fiber breaking. The macerated fiber suspension was finally placed on a slide (standard, 7.5 cm × 2.5 cm) by means of a medicine dropper [20]. All fiber samples were viewed under a calibrated microscope; a total of 50 randomly chosen fibers were measured from each sample for a total of 150 fiber measurements. For fiber diameter, lumen diameter and cell wall thickness determination, cross-sections were cut on Leitz base sladge Microtome at the same height/length as above and were stained with 1:1 aniline sulphate–glycerin mixture to enhance cell wall visibility (cell walls retain a characteristic yellowish colour). Five derived values were also calculated using fiber dimensions: wall fraction as twice cell wall thickness/fiber diameter, flexibility coefficient as (fiber lumen diameter/fiber diameter) × 100, Runkel ratio as (2 × fiber cell wall thickness/lumen diameter), slenderness ratio (lumen diameter/fiber diameter) and suppleness coefficient (lumen diameter/fiber diameter x 100) [38, 48]. The results are reported in Table 3.3.

3.2.2 Variation in basic density and chemical composition of *A. cadamba* along the height

Three logs (cylindrical pieces) of about five feet long were cut from 5, 25 and 40 ft height from base of *A. cadamba* estimated to be 12 years old. Discs of one inch thick were cut from each end of each log. Nine discs were used for the determination of basic density of wood (T 258 om-02) as per Tappi Standard Test Methods. The remainders after debarking were disintegrated in to chips separately in Veco Plan Chipper at Star Paper Mills Ltd., Saharanpur (India). The wood chips of were milled into powder in a laboratory Wiley mill and a fraction passing through −48 mesh size but retained on +80 mesh size was used for proximate chemical analysis. These samples were analyzed for alcohol-benzene soluble (T 204 cm–97), holocellulose (T 249 cm–00), lignin (T 222 om–02) as per Tappi Standard Test

Methods [49] and α–cellulose by Wise and his co-workers methods [57]. The results are reported in Table 3.4.

3.2.3 Proximate chemical analysis

The logs of *A. cadamba* were disintegrated in to chips in Veco Plan Chipper The wood chips of were milled into powder in a laboratory Wiley mill and a fraction passing through −48 mesh size but retained on +80 mesh size was used for analysis of, water soluble(T 207 cm–99), 1% NaOH solubility (T 212 om–02), alcohol benzene soluble (T 204 cm–97), holocellulose (T 249 cm−00), lignin (T 222 om−02), Ash (T 211 om–02), pentosan (T 223 cm–1) as per Tappi Standard Test methods [49], α–cellulose and hemicellulose by Wise and his co-workers methods [57]. The results of proximate chemical analysis were compared with *E. tereticornis* and *P. deltoids* [15, 27] to assess their suitability for pulp and paper making. The results are reported in Table 3.5.

3.3 RESULTS AND DISCUSSION

Microphotographs: 3.1A shows the T. S. of *A. cadamba* at a magnification of 260X. Wood is diffuse porous and growth ring boundaries are indistinct or absent. Vessels are arranged in an indeterminate pattern, commonly short (2–3 vessels) with radial rows. Average tangential vessel diameter varies between 95-225 μm. Average numbers of vessels/mm^2 5–12. Microphotograph 3.1B shows the L.S. of *A. cadamba* at a magnification of 260X. Axial parenchyma cells are present which are not banded. Axial parenchyma cells are of apotracheal and paratracheal types. Apotracheal axial parenchyma is diffuse-in-aggregates which forms subtle network between ray cells. Paratracheal axial parenchyma is scanty. Average number of cells per axial parenchyma strand ranging from 7 to11. Thin walled tyloses are present in vessels (sparse). Other deposits in heartwood vessels are very little which are yellowish in colour. Microphotograph 3.1C shows the radial view of *A. cadamba* at a magnification of 260X. Radial view of *A. cadamba* shows crystal sand (calcium oxalate) in ray cells, xylem

cells are arranged in storied pattern and are surrounded by laticiferous cells. Ray cells are of two distinct sizes and multiseriate. Ray cells are composed of two or more cell types. Heterocellular ray cells are with square and upright cells restricted to marginal rows, mostly one marginal row of upright or square cells or mostly 2–4 marginal rows of upright or square cells. Disjunctive ray parenchyma has end walls distinct. Crystal-containing ray cells are upright and/or square marginal cells. Ray cells are perforated with disjunctive ray parenchyma cell walls Silica is not observed. Plates 3.1 D & E show the vessel of *A. cadamba* (at a magnification of 40X) having reticulate thickenings of lignin, foraminate, numerous more or less circular pores. Plate 3.1 F shows the fibers at a magnification of 10X. Fibers are very thin-walled, or of medium wall thickness. Fiber pits are common in both radial and tangential walls, simple to minutely ordered or distinctly bordered. Fibers are septate and are evenly distributed.

Table 3.3 shows the morphological characteristics of *A. cadamba* and its comparison with softwoods like, *P. kesiya* and *P. abies* and hardwoods like *E. tereticornis* and *E. camaldulensis*. The average fiber length of *A. cadamba* are 1.43 mm which is 37.8% lesser than *P. kesiya* and *P. abies* and just double the length of *E. tereticornis* and *E. camaldulensis*. The other average fiber dimensions are: fiber width 38.12 μm, lumen width 26.10 μm and cell wall thickness 5.51μm. The average fiber width and cell wall thickness of *A. cadamba* are comparable to those of *P. kesiya* and *P. abies*. The flexibility coefficient of *A. cadamba* is on lower side than that of *P. kesiya* and *P. abies* due to lower lumen diameter and is comparable to those of *E. tereticornis* and *E. camaldulensis*. However, slenderness ratio is more than *E. tereticornis* and slightly lower than *E. camaldulensis*. The low flexibility is expected to have an inevitably negative effect on tensile and bursting strengths as well as on folding endurance [38]. *A. cadamba* fibers have flexibility coefficient 68.47 which is 19.9 % less than *P. kesiya* and *P. abies* and compared to hardwoods. Therefore, paper made from *A. cadamba* fibers are expected to have increased mechanical strength and thus be suitable for writing, printing,

wrapping and packaging purposes [35, 47). This is partly because long and thin walled fibers readily produce good surface contact and fiber-to-fiber bonding [38]. These results in satisfactory pulp tear indices and bursting strengths for printing and writing purposes [10, 28]. The rigidity coefficient of *A. cadamba* is comparable to those of softwoods like *P. kesiya* and *P. abies*. The concept of using rigidity coefficient (or the inverse of collapsibility of fibers) is based on the assumption that fibers act like a thin-wall cylinder whose collapse pressure is proportional to (2w/*D*) [1], where *w* is wall thickness and *D* is fiber diameter. With thin cell wall, the fibers would easily collapse during sheet making, hence giving a sheet of lower bulk and higher inter-fiber bonding potential in comparison with the wood counterparts. The Runkel ratio of *A. cadamba* fibers are 21.4% more than *P. kesiya* and *P. abies* and slightly lower than *E. tereticornis* and *E. camaldulensis* [16]. The thin walled wide lumen fibers collapse readily to double walled ribbons on pressing and exhibit plastic formation, thus offering more surface contact [27, 46]. The flexible fibers with a good Runkel ratio can thus complement the higher mechanical strength of the *A. cadamba* fibers [26].

Table 3.4 presents the change in chemical composition along the height of *A. cadamba*. There is a general tendency for a decrease in α-cellulose, lignin and ash content as we move from the base of the log to the top. This was expected since mature tissues (at the base) accumulate higher amounts of metabolic products than the younger parts at the top. The lignin content is observed maximum at bottom of *A. cadamba* and decreases gradually from base to top. α-cellulose is slightly on higher side in the middle and lower at the top of *A. cadamba*. The results of alcohol-benzene soluble indicate that the resinous matter in the middle portion is more and decreases gradually at the top than those of wood, as evidenced by the lower levels of extractives soluble in alcohol–benzene. The results on α-cellulose content changes along the stems reported by Neto *et al.,* [35] for kenaf and by Dutt *et al.,* [15] for reed generally agree with our findings. In a detailed study of maize (*Zea mays* L.) stalks, Morrison *et al.,* [34] reported that lignin and cellulose deposition increases with tissue maturation and

maximum rate of lignin deposition following that of cellulose. The statistically significant differences between cellulose and lignin contents especially between the base and top for some species should be taken into account before pulping only if they are associated with specific morphological characteristics such as in the case of reed [36, 48].

Table 3.5 reveals the results of proximate chemical analysis of *A. cadamba* and its comparison with *E. tereticornis* and *P. deltoids*. The water soluble in *A. cadamba* is on lower side compared to *E. tereticornis* and *P. deltoids* except hot water soluble which is marginally higher than *E. tereticornis* and *P. deltoids*. All the soluble materials come under the category of extrectives and these are totally undesirable for pulp and paper making. The water and alcohol benzene soluble affects the pulp yield, paper quality and drainage characteristics of paper machine. *A. cadamba* is comparatively less resinous than that of *P. deltoides* and more resinous than that of *E. tereticornis*, as evidenced by the lower levels of extractives soluble in alcohol–benzene. Therefore, it will create lesser pitch problem and also more homogeneities in the paper sheet [25]. 1% NaOH solubility in *A. cadamba* is on higher side i.e. 20.7 % than that of 16.2, 19.6% % in *E. tereticornis* and *P. deltoids* respectively. It means that degradation of *A. cadamba* due to light and heat and fungal decay is more compared to *E. tereticornis* and *P. deltoides*. The lignin content (20.6%) is lower than normally found in common hardwood, for example *E. tereticornis* [27] and *P. deltoides* [15]. This is an added advantage in that they will be much easier to be chemically pulped. The functional significance of lignin has long been associated with mechanical support for plant organs that enables increased growth in height [9, 14] its lacking will no longer allow plants to be upright [58].

It means that *A. cadamba* needs, in general, milder pulping conditions (lower temperatures and chemical charges) than those of softwoods and hardwoods in order to reach a satisfactory kappa number. It also indicates the potential of *A. cadamba* to undergo bleaching more easily and with the utilization of fewer chemicals. Examples of milder

pulping conditions leading to satisfactory delignification levels are abundant in the literature. Saikia et al. [47] report such conditions for kenaf, [56] for reed and [30] for olive tree pruning. Chemically, the *A. cadamba* is rich in holocellulose (76.2%) and also high in α-cellulose (44.3%) as illustrated in Table 3.5. Both of which are important parameters in determining the suitability of a raw material for papermaking [40]. *A. cadamba* has holocellulose and α-cellulose more than *E. tereticornis* and *P. deltoids* except α-cellulose which is higher (47.4%) in *P. deltoids*. According to the rating system designated by Nieschlag *et al.* [37], plant materials with 34% and over α-cellulose content were characterized as promising for pulp and paper manufacture from a chemical composition point of view. *A. cadamba* contains 20.56% hemicelluloses which are in broad similarity with that of hardwoods [51, 53]. *A. cadamba* contains comparatively lower ash content. This characteristic might not contribute to an abnormal mechanical wear of processing equipments.

Table 3.1: Chemical composition of wood from five selected hardwoods[a] [51]

Cell wall constituent	*Acer rubrum*	*Betula paryrifera*	*Fagus grandifolia*	*Polpules tremuloides*	*Ulmus americana*
Cellulose	45	42	45	48	51
Lignin	24	19	22	21	24
Glucuronoxylan	25	35	26	24	19
Glucomannan	4	3	3	3	4
Pectin, starch, ash, etc	2	1	4	4	2
[a] All values in percent of extractive free wood.					

Table 3.2: Chemical composition of wood from five selected softwoods[a] [51]

Cell wall constituent	*Abies balsamea*	*Picea glauca*	*Pinus strobus*	*Tsuga conadensis*	*Thuja occidentalis*
Cellulose	42	41	41	41	41
Lignin	29	27	29	33	31
Glucuronoxylan	9	13	9	7	14
Glucomannan	18	18	18	16	12
Pectin, starch, ash, etc	2	1	3	3	2
[a] All values in percent of extractive free wood.					

Table 3.3: Morphological characteristics of *A. cadamba* and comparison with *Pinus kesiya, Picca abies, E. tereticornis* and *E.camaldulensis*

Parameters	*A. cadamba*	*Pinus kesiya*	*Picca abies*	*E. tereticornis*	*E. camaldulensis*
Average fiber length (L), mm Variation	1.43±0.05 1.34-1.65	2.3	2.3	0.62	0.75
Fiber width (D), μm Variation	38.12±0.43 37.32-39.22	40.7	41.7	26	18
Average lumen diameter (d), μm Variation	26.10±1.2 24.15-29.30	34.8	35.7	18	12
Average cell wall thickness, (w), μm Variation	5.51±0.16 5.19-5.85	5.9	6.0	4	3
Flexibility coefficient (d/D) x 100	68.47	85.6	85.5	0.69	66.67
Slenderness ratio (L/D)	37.51	0.06	0.06	23.85	41.67
Rigidity coefficient (2w/D)	0.29	0.29	0.29	0.31	0.33
Wall fraction (2w/D) x 100	28.91	28.99	28.77	30.77	33.33
Runkel ratio (2w/d)	0.42	0.33	0.34	0.44	0.50
Co-efficient of suppleness (d/D) x100	68.47	85.50	85.62	69.20	66.67
± Refers to standard deviation					

Table 3.4: Specific gravity, alcohol-benzene solubility, holocellulose, α-cellulose and lignin content of *A. cadamba* along the height of log from the base

Sl. No.	Parameters	Samples of *A. cadamba* collected at different heights from the base (Age of tree 12 years)		
		5 ft	25 ft	40 ft
1	[a]Density, kg/m^3	375±0.01	380±0.005	400±0.030
2	[b]Alcohol-benzene soluble,%	3.27±0.05	3.41±0.04	3.15±0.04
3	[b]Holocellulose,%	81.2±0.11	80.6±0.14	76.8±0.13
4	[b]α- cellulose, %	42.4±0.11	42.6±0.12	41.5±0.10
5	[b]Lignin,%	22.8±0.08	19.4±0.09	14.6±0.09
[a]Oven-dry weight per green (soaked) volume, [b] results are expressed on a moisture- free raw material basis				

Table 3.5: Results of proximate chemical analysis of *A. cadamba* and comparison with *E. tereticornis* and *Populus deltoides*

Sl. No.	Particulars	*A cadamba*	*E. tereticornis*	*Populus deltoides*
I	Cold water solubility, %	2.3±0.06	2.9	3.8
2	Hot water solubility, %	5.0±0.01	7.8	4.5
3	1% NaOH solubility, %	20.7±0.03	16.2	19.6
4	Alcohol: Benzene (1:2 v/v) solubility, %	2.4±0.01	1.4	2.8
5	Lignin, %	20.6±0.06	25.9	21.8
6	Pentason, %	19.0±0.08	16.0	17.3
7	Holocelluloses, %	76.20 ±0.09	65.2	69.3
8	Hemicellulose, %	20.56±0.07		
9	α- cellulose, %	44.3±0.07	43.8	47.4
11	Ash content, %	0.54±0.09	0.50	0.63
± Refers to standard deviation, all results are expressed on extractive free basis				

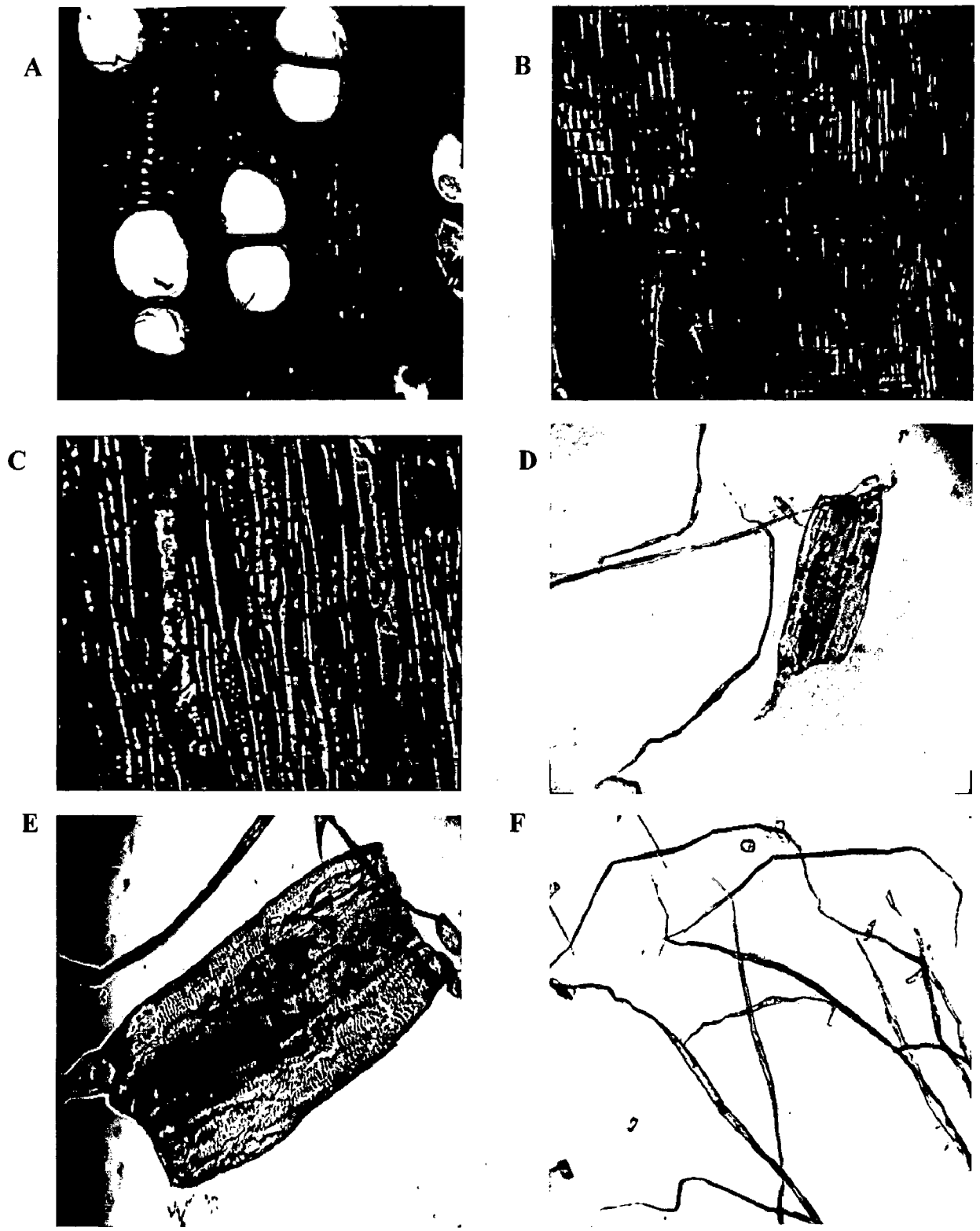

Plate 3.1: (A) T.S. of *A. cadamba* showing vessels, vesicentric tissue and parenchymatous cells (magnification 260X) (B) L.S. of *A. cadamba* showing vessels, axial parenchymatous cells (both apotracheal and paratracheal) (magnification 260X), (C) radial view of *A. cadamba* showing crystal sand (calcium oxalate) in ray cells, xylem cells are arranged in storied pattern and surrounded by laticiferous cells (magnification 260X), (D & E) vessel of *A. cadamba* (magnification 10X & 40X), (F) fibers of *A. cadamba* pulp (magnification 10X).

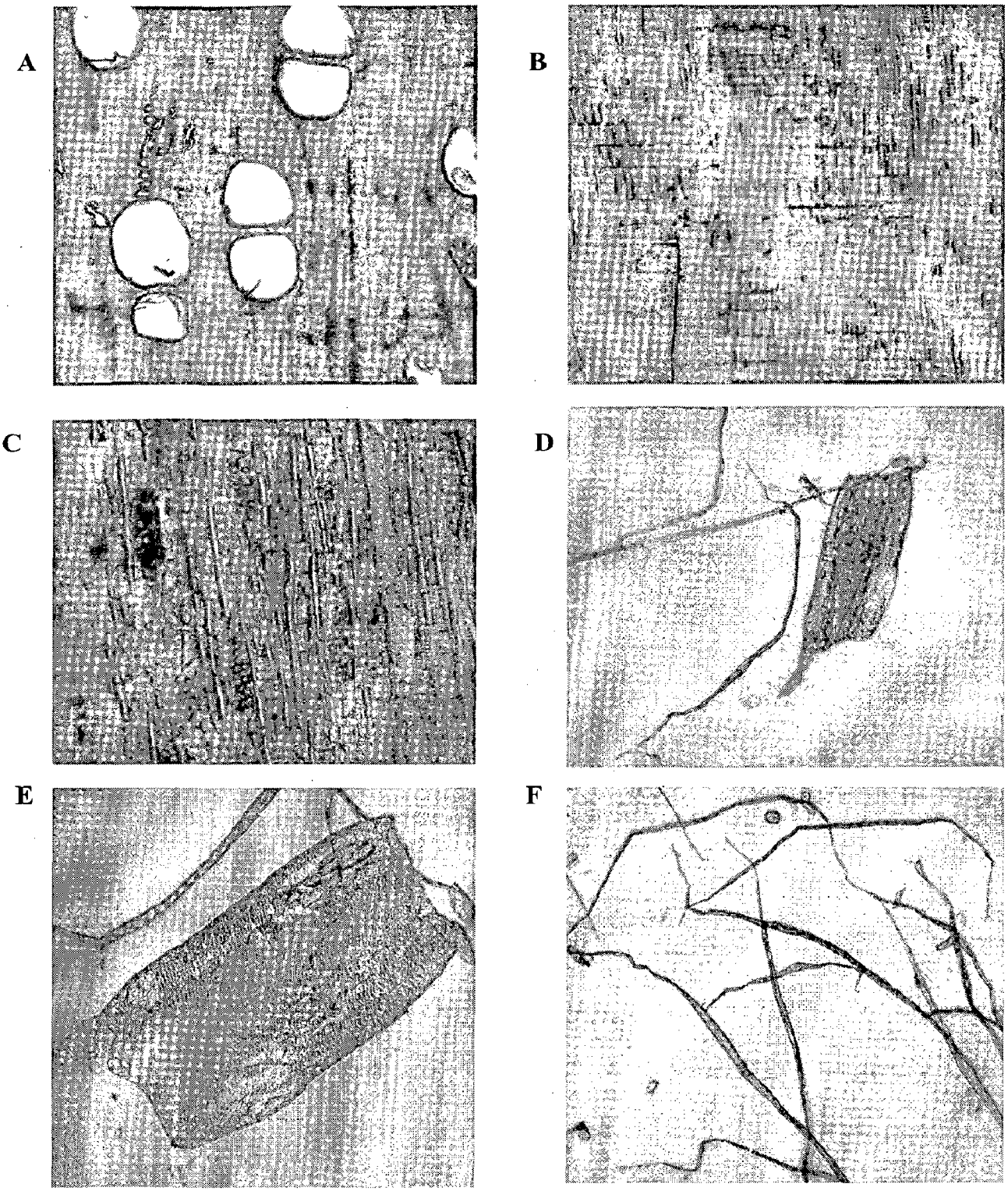

Plate 3.1: (A) T.S. of *A. cadamba* showing vessels, vesicentric tissue and parenchymatous cells (magnification 260X) (B) L.S. of *A. cadamba* showing vessels, axial parenchymatous cells (both apotracheal and paratracheal) (magnification 260X), (C) radial view of *A. cadamba* showing crystal sand (calcium oxalate) in ray cells, xylem cells are arranged in storied pattern and surrounded by laticiferous cells (magnification 260X), (D & E) vessel of *A. cadamba* (magnification 10X & 40X), (F) fibers of *A. cadamba* pulp (magnification 10X).

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KRAFT-AQ PULPING

4.1 INTRODUCTION

Kraft pulping process is most widely used pulping process because any wood species or raw material can be delignified to get chemical grade pulp. In this process wood chips are treated with an aqueous alkaline solution composed essentially of sodium hydroxide and sodium sulphide at high temperatures ranging from 150 to 170 ^{0}C. Lignin, an aromatic polymer composed of dehydropolymerized structural units derived from phenylpropane, and primarily responsible for the cohesion between fibers in wood tissues, is degraded and dissolved almost completely (90–95%) in black liquor (the aqueous solution containing the inorganic and organic reaction by-products), allowing fiber separation. In such process, wood polysaccharides, namely cellulose, hemicelluloses and less abundant polysaccharides such as pectin, is also partially degraded to low molecular weight derivatives or dissolved in the black liquor, partially keeping its polymeric nature [10, 25].

C. F. Dahl was recognized and obtained a patent for his process in 1884 [33]. In an effort to find a substitute for expensive sodium carbohydrate (soda ash) as make up chemical for the soda process chemical cycle by adding sodium sulphate (salt cake) to recovery furnace. The sulphate was reduced to sulphide by the action of the furnace and sulphide was reduced to sulphide by the action of furnace and sulphide was thus introduced in to the liquor system. The superior strength properties of paper modifying this process were found and this new type of paper produced aptly called kraft paper. Kraft is the German world for strength. During pulping the addition of sodium sulphide results in faster pulping and better selectivity for delignification over polysaccharide degradation [16]. As a result, many soda mills were converted to kraft mills because of the greater strength of the pulp. Kraft pulp, however, was dark in color and difficult to bleach compared to the competing sulphite pulp.

The disadvantages of kraft pulping as compared to sulphite pulping are the difficulty in bleaching of the kraft pulp, lower yield due to the carbohydrates losses and sulphur in its reduced form that provides emissions which are extremely odoriferous. Sulphur escapes as hydrogen sulphide, dimethyl sulphide and methyl mercaptans that are very odoriferous gases, even in extremely small amounts. Various methods such as black liquor oxidation and scrubbing of stack gases have been developed to eliminate these odoriferous gases but these methods are expensive. A few parts per million still remain and unfortunately, the human olfactory sense can detect the small of a few parts per billion. Air pollution from kraft mills is thus a serious problem and has recently attracted considerable public attentions, and is under investigation by the environmental protection agency [21, 31].

An attractive way to reduce the plant effluent discharge and improve pulp yield is by modifying the pulping process by adding anthraquinone (AQ) which, allows more lignin to remove in the digester and leaves less amount to remove by bleaching. Many methods of doing this have been developed over the past two decades. Adding AQ to kraft digester and simply extended the cook by manipulating pulping variables are suggested in 1980s [20]. The catalytic effect of AQ and related derivatives in the alkaline pulping process has raised interest in sulphur free pulping process [13]. Unfortunately, numerous studies revealed that the process though simple and efficient, had certain drawbacks, such as lower pulp strength (which is the major concern in softwood pulps), inferior pulp bleachability and higher production cost for the reason that of the significant amount of AQ consumed [5, 13, 17]. In order to obtain a still lower kappa number an additional delignification agent has to be introduced. Sturgeoff and Pitl in 1993 [26] found that the addition of 0.1% AQ to softwood kraft cooks at different sulphidities, with alkali charges chosen to give constant kappa number in the absence of AQ. At 25% sulphidity, AQ reduced the unbleached kappa number of softwood kraft pulp by 18% with no loss in yield and therefore no increase in loading to the recovery furnace. The reduction increases with decreasing sulphidity and it is smaller for

hardwoods. Extended cooking to kappa number 20 in conventional digester without AQ can be done without incurring the strength penalty, has been demonstrated by Tikka [29].

The superiority of kraft pulping has further extended since the introduction of modified cooking technology in the early 1980s [28]. In the meantime, three generations of modified kraft pulping processes (MCC, ITC and compact cooking as examples for continuous cooking and cold-blow, super batch/RDH and continuous batch cooking, CBC, for batch cooking technology) have emerged through continuous research and development [2]. The third generation includes black liquor impregnation, partial liquor exchange, increased and profiled hydroxide ion concentration and low cooking temperature (elements of compact cooking), as well as the controlled adjustment of all relevant cooking conditions in all the processes-related liquors are prepared outside the digester in the tank (as realized in CBC). However, the potential of kraft cooking is not exhausted so far. Therefore, due to the distinct advantages of the kraft pulping technology, including all its modifications, over the sulphite cooking process, the share of sulphite pulps in total fiber production steadily decreased from 60% in 1925 to 20% in 1967 [15] to 9.2% in 1979 [8] and finally to only 3.7% in 2000 [30] (Table. 4.1) New generations of kraft cooking processes shell likely to be introduced, focusing on the improved pulp quality, lowered production cost and efficient energy utilization, further decreasing the impacts on the receiving water, and recovering high added-value wood byproducts [2].

Besides that, the quality and quantity of kraft pulp, within a species can also be influenced by cooking variables, age of plant, growth conditions of plant, chemical composition of soil, temperature and solar radiation intensity of a particular reason. Recently, Akgul and Teniz assessed the role of sodium borohydrate in kraft pulping of brutia pine at the magnitude of 1%, 2%, and 3% (o.d. wood basis). They found that, adding sodium borohydrate in kraft cooks not only increases the pulp yield but also significantly decreases the amount of rejects leading to improved selectivity of the lignin removal and delignification rate. In

addition, kraft-sodium-borohydrate pulps gave higher pulp viscosity and brightness; however, it is worth mentioning the strength properties were little lower compared to the kraft method [1].

Recently, Baptista *et al.,* [3] studies the relationship between lignin structure and delignification degree of *Pinus pinaster* kraft pulps. They concluded that, the delignification degree increases the condensation of the lignin structure, which might have an influence upon the increased pulp colour. The lack of selectivity of kraft pulping process in case of more delignified pulps was also shown.

4.2 MATERIALS AND METHODS

4.2.1 Pulping studies

Screened chips of *A. cadamba* were digested in a Weverk electrically heated rotary digester of 0.02 m^3 capacity having four bombs of one liter capacity each. The chips of *A. cadamba* were digested at different cooking conditions like, maximum temperature from 155 to 175 ^0C, cooking time from 0.5 to 3.5 hour, active alkali from 11 to 18 % (as Na_2O), sulphidity 0-25% and liquor to wood ratio of 3.5:1. Based on experimental results, the optimum cooking conditions for *A. cadamba* was found to be as: active alkali 16% (as Na_2O), sulphidity 20%, temperature 165 ^0C, time at temperature 90 min and liquor to wood ratio of 3.5:1. At optimum cooking conditions, anthraquinone (AQ) was added from 0.0 to 0.2% (based on oven dry raw material basis) to observe its effect on pulp yield and kappa number. The logs of *A. cadamba* harvested after 2, 3, 4 and 12 years of age were reduced into chips in Norman Carthage and Veco plan chippers followed by screening. The screened wood chips in each case were digested at above mentioned cooking conditions. After the completion of cooking, the pulps were washed on a laboratory flat stationary screen having 300 mesh wire bottom for the removal of residual cooking chemicals. The pulp was disintegrated and screened through a Weverk vibratory flat screen with slot size of 0.15 mm and the screened pulp was washed, pressed and crumbled. The pulps were analyzed for kappa number (T 236

cm-85), pulp yield and lignin (T 222 om-88), and screening rejects as per Tappi Standard Test Methods: 2000 [27]. The results are shown in Tables 4.1-4.6 and Figures 4.1-4.7.

4.2.2 Preparation of laboratory hand sheets and their evaluation

The unbleached pulp of *A. cadamba* was disintegrated in PFI mill (Tappi T 200 sp-96) at different beating levels. Laboratory hand sheets of 60 ±2.5 g/m^2 were prepared (T 221 cm-99) and conditioned at 27±2 ^{0}C and relative humidity 65±2%. The laboratory made hand sheet were evaluated for various physical strength properties such as: tear index (T 414 om-98), tensile index (T 494 om-01), burst index (T 403 om-97) and double fold (T 423 cm-98), thickness (T411 om 05), stretch (T404 wd 03), as per Tappi Standard Test Methods: 2000. The results are shown in Tables 4.7 and 4.8 and graphically presented in Figures 4.8 and 4.9.

4.2.3 Scanning electron microscopy

The detailed morphological studies of *A. cadamba* kraft pulp samples were carried out using scanning electron microscopy (SEM, Leo 435 VP, England). Pulp sample was taken and subjected for fixation using 3% (v/v) glutaraldehyde-2% (v/v) formaldehyde (4:1) for 24 h. Following the primary fixation, samples were washed thrice with double distilled water. The samples were then treated with the alcohol gradients of 30, 50, 70, 80, 90 and 100% for dehydration. Samples were kept for 15 min each up to 70% alcohol gradient, thereafter treated for 30 min each for subsequent alcohol gradients. After treating with 100% alcohol, samples were air dried and examined under SEM using gold shadowing technique [9]. Electron photomicrographs were taken at 15.00 kV using detector SE1 and at desired magnifications. The microphotographs are shown in Microphotographs 4.0 A-E.

4.2.4 Bauer-McNett fiber classification

The weighted fiber length of kraft pulp of *A. cadamba* at optimum pulping condition was carried out using Baeur-McNett fiber classifier using mess size +20, +48, +100 and +200. The results are reported in Table 4.9.

4.2.5 Fiber analysis

A suspension of fibers (0.02% consistency) was used for detailed morphological characteristics such as: fiber length, fines, number of shives per meter, curl index, kink index and fiber width by using Hi-Resolution Fiber Quality Analyser (make: Optest Equipment Inc. model: LDA 2002). The results are reported in Table 4.10.

4.3 RESULTS AND DISCUSSIONS:

4.3.1 Influence of temperature and time

Figure 4.1 reveals the curves plotted between residual lignin and reaction time at reaction temperatures varying from 155 to175 ^{0}C , cooking time from 0.5 to 3.5 h, active alkali from 11 to 18 % (as Na_2O), Sulphidity 0-25% and liquor to wood ratio of 3.5:1. The curves can be approximated by two straight lines at each temperature investigated. The curves with steeper slopes are related to rapid solublization of bulk of lignin (bulk delignification), where as, the part of curves with gentler slopes are related to the slow solublization of the residual lignin (residual delignification). Both parts of these curves are having different velocity constants. The bulk delignification corresponds to the removal of easily assessable lignin present in the middle lamella and the residual delignification corresponds to the removal of lignin present in the primary wall, secondary wall layers i.e. S_1, S_2 and S_3 layers and the central inter connections cavity etc. The delignification of wood in alkaline pulping is also associated with the solubilization of significant amounts of hemicelluloses [16, 20].

These curves also clearly indicate that as the temperature decreases from 175 to 155 ^{0}C, the time to reach transition from bulk to residual delignification and the lignin content of the pulp, corresponding to this transition point both increases. Table 4.2 also reveals that at lower temperature range, the residual lignin contents decreased sharply, while at higher temperature, the magnitude of decrease in lignin content is not so pronounced. Moreover, at

higher temperature, the degradation of carbohydrate fractions also increases, thereby reducing the pulp yield [16]. In other words, at the transition point, a lower pulp lignin contents are obtained at 165 ^{0}C. Beyond a temperature of 165 ^{0}C, degradation of carbohydrates contents occurs due to peeling reactions [5, 12, 21, 31]. The nature of curves after transition points are almost horizontal straight lines, clearly indicating that the bulk delignification are over up to these transition points and it is not economical to continue the cooking operation beyond this optimum temperature of 165 ^{0}C. The curves plotted between residual lignin and reaction time (Figure 4.1) and reaction time and kappa number (Figure 4.2) clearly reveal that the drop in kappa number beyond a maximum cooking time of 1.5 h are found to be insignificant and pulp yield reduces sharply (Figure 4.3). Therefore, based on experimental data, a maximum cooking time of 1.5 h and cooking temperature 165 ^{0}C may be considered as an optimum cooking condition for the kraft cooking of *A. cadamba*.

4.3.2 Influence of alkali charge

Table 4.3 shows that screened pulp yield increases with an increase in active alkali from 11 to 16 % (as Na_2O) and then begins to decrease sharply, while both kappa number and screening rejects decline sharply up to an alkali dose of 16 % and beyond that both of these parameters practically remain constant. The screened pulp yield of *A. cadamba* is found to be 48.74 % at kappa number of 22.5. Therefore, active alkali charge of 16 % (as Na_2O) may be considered as an optimum cooking dose for *A. cadamba* (Figure 4.4*)*. During kraft pulping, the consumption of active alkali was found to be constant over a wide range of alkali charge [19]. It was found that the excessive active alkali charge which remains unconsumed during the course of pulping adversely affects the pulping viscosity.

4.3.3 Influence of ageing

Table 4.4 shows the effect of aging of *A. cadamba* on screened pulp yield, kappa number and screening rejects. The unbleached screened pulp yield increases with increase age

of *A. cadamba* between ages 4 -12 years. On the other hand, kappa number and screening rejects decreases with increasing age of *A. cadamba* but decrease in both kappa number and screening rejects between ages 4 -12 years is insignificant. Table 4.4 also indicates that viscosity of unbleached pulp increases up to the age of 3 years and then pulp viscosity is almost constant. The overall increase in pulp viscosity and screened pulp yield is due to progressive polymerization of cellulose [6].

4.3.4 Influence of sulphidity

Table 4.5 reveals the effect of sulphidity on pulp yield, kappa number and lignin content of pulp at distinct reaction times (at temperature). Figure 4.5 reveals that the curves plotted between residual lignin and reaction time at different sulphidity levels indicate that at lower sulphidity levels, the variation in the amount of residual lignin contents is too much in comparison to variation at higher sulphidity levels. There were practically no variation in the amount of residual lignin contents beyond 20% sulphidity level and the curves plotted at 20 and 25 % sulphidity levels coincides with each other. The yield drop of the pulps was studied at 165 ^{0}C using 16% active alkali charge at 20% sulphidity. The cooking temperature has relatively little influence upon the ratio of lignin to carbohydrates solubilized during the course of bulk delignification. This supported the suggestion that in the alkaline delignification of wood there is a definite relationship between the solubilization of lignin and cellulosic portions [20]. The high loss of carbohydrates related to the dissolved lignin found in the residual delignification suggested that commercial kraft pulping should be limited to the range of bulk delignification only. Figure 4.6 indicates that the magnitude of reduction in kappa number was found to be more pronounced at 20% sulphidity and beyond it, the reduction in kappa number is not much pronounced and looks to be almost negligible. Figure 4.7 reveals that the pulp yield beyond a sulphidity level of 20% slightly decreases. Therefore,

20 % sulphidity may be considered as an optimum level, as there is practically no gain of using higher doses of sulphidity.

4.3.5 Influence of anthraquinone

Table 4.6 shows the effect of different doses of AQ (ranging from 0.0 to 0.2% on O.D. wood basis) on pulp yield, kappa number and screening rejects at optimum cooking conditions for kraft pulping. The addition of 0.1 % AQ increases screened pulp yield to a lesser extent i.e. 0.38 % but significantly reduces the kappa number by 6.5 units (28.8%). The increase in pulp yield and reduction in kappa number can be explained based on redox catalytic activity of anthraquinone. Anthraquinone accelerate the delignification by disintegration of β-phenyl-ester bond and stabilize the cellulosic materials by oxidation and termination of aldehyde groups of carbohydrates [22]. In the latter case the carbohydrates are stabilized against the alkaline peeling reactions so called stopping reactions leading to increase in pulp yield. Because anthraquinone goes through a cyclic process, it is typically used at about 0.1% on o. d. wood basis and resulted in a 1-3 % increase in pulp yield [7, 14. 32]. A similar trend has been observed for *E. tereticornis* [4] as well as binary mixture of *E. tereticornis* and *P. roxburghii* [23] in the ratio of 70:30.

4.3.6 Mechanical strength properties

Table 4.7 reveals the mechanical strength properties of *A. cadamba* at 16% active alkali, maximum cooking time 1.5 h and sulphidity 20%. All the mechanical strength properties such as burst index, tensile index, double fold and stretch are found to increase with increasing beating level up to 44 ^{0}SR and beyond a beating level of 44 ^{0}SR all the properties decline except tear index which decreases beyond a beating level of 35 ^{0}SR (Figure 4.8). During beating, removal of primary wall exposes secondary wall layers. However, primary wall is permeable to water but does not participate in bond formation. Therefore, tearing energy required to pull the fibers from the mesh will be slightly more due to hydrogen

bonding between fibers after removal of primary wall. Further, due to cutting action, external and internal fibrillation and brushing action, tear index declines whereas all other properties which depend upon hydrogen bonding improve with pulp beating. Further, addition of 0.1% AQ improves all other mechanical strength properties (Tables 4.7 and 4.8 and Figure 4.9). This is due to the fact that AQ accelerates the delignification rate without degrading carbohydrates.

4.3.7 Fiber classification and morphological characteristics

Table 4.9 shows the results of Bauer-McNett classification of kraft pulp of *A. cadamba*. The +20 fraction contains only 3.07% of the total weight of the fibers. Further, maximum fibers are retained on +48 mess fraction comprising of 64.34% of the total weight of the fibers. The <200 mesh fraction is comprised of 12.69% fines, vessels and parenchymatous cells which are further confirmed with the results obtained from Fiber Quality Analyzer Model V1. 3 REB 19 CV-M4.

Morphological characteristics of *A. cadamba* kraft pulp and its comparison with aspen spruce and *E. tereticornis* pulps are given in Table 4.10. The average length of the *A. cadamba* fiber is comparable to that of aspen but less than about one third to that of spruce. The fines (<0.20mm) which are mainly parenchyma cell and vessels elements, are higher than that of aspen and spruce but lesser than switch grass stem (30.47%) [18]. Similar results were also reported by other researchers [11, 24]. Kinks are usually edge crack zones. They are identified as sudden slope changes during the fiber length calculation while curl is defined as the ratio of the arc to the developed length of the fiber. The mean fiber kink index in *A. cadamba* is lower compared to *E. tereticornis*. On the other hand, kink index and kink per mm length of fiber are on lower side compared to *E. tereticornis* but kink angle is lower in *A. cadamba* compared to *E. tereticornis*.

4.3.8 Scanning electron microscopy

Microphotograph 4.1 A reveals that as kraft pulping loosens the connection between the fibers, firstly along radial planes, and eventually the fibers stick together only along those edges where several cells meet and delignification is still incomplete. Microphotograph 4.1 B shows primary wall and lumen of the fibers and after removal of most of the lignin, the fibers lose their rigidity and collapsability. Microphotographs 4.1 C and D show no sign of external fibrillation or formation of fibrils. During alkaline pulping some part of hemicelluloses (mainly xylan) is solubilized and reprecipitates on to the fiber surface (microphotograph C and D). Microphotograph E shows that the fibers of *A. cadamba* are uniform and straight, intact with a smooth, silky surface and bear an appearance of compactness.

Table 4.1: Global pulp production by category, 2000 [30]

Sl. N0.	Pulp category	Pulp production (MT)
1	Chemical	131.0
2	Kraft	117.0
3	Sulphite	7.0
4	Semichemical	7.2
5	Mechanical	37.0
6	Non-wood	18.0
7	Total virgin fiber	187.0
8	Recovered Fiber	147.0
9	Total fibers	334.0

Table 4.2: Effect of temperature on pulp yield, kappa number and lignin at 16% active alkali (as Na_2O) and 20% sulphidity during kraft pulping of *A. cadamba*

Max. cooking temp, ^0C	Time at temp, h	Pulp yield, %	kappa number	Lignin, %
155	0.5	60.6	58.3±.30	9.11±0.09
	1.0	56.16	45.4±.27	6.85±0.08
	1.5	53.6	32.5±.25	5.06±0.07
	2.0	51.25	27.6±.21	4.65±0.05
	2.5	48.25	24.7±.17	4.35±0.03
	3.0	45.65	22.2±.17	4.12±0.04
	3.5	42.55	20.3±.15	3.90±0.03
165	0.5	56.4	50.2±.31	7.14±0.10
	1.0	52.15	36.6±.30	5.15±0.08
	1.5	49.34	22.5±.26	3.52±0.06
	2.0	46.6	19.2±.24	3.15±0.07
	2.5	44.25	16.8±.22	2.85±0.04
	3.0	42.15	15.3±.16	2.64±0.03
	3.5	39.85	14.4±.14	2.45±0.03
175	0.5	51.4	43.5±.28	6.10±0.07
	1.0	47.56	31.7±.24	4.32±0.07
	1.5	44.75	19.3±.20	3.03±0.04
	2.0	42.25	16.6±.19	2.75±0.03
	2.5	40.25	14.1±.16	2.55±0.03
	3.0	38.15	12.4±.13	2.42±0.03
	3.5	36.1	11.2±.11	2.32±0.03

Table 4.3: Effect of alkali dose as Na_2O on pulp yield, kappa number and screening rejects during kraft pulping

Sl. No.	Parameters	Active alkali doses, % (as Na_2O)					
		13	14	15	16	17	18
1	Screened yield, %	45.75	47.5	48.5	48.74	45.23	40.67
2	Rejects, %	3.53	2.25	1.34	0.6	0.35	0.25
3	Total yield, %	51.33	49.45	47.74	49.34	42.37	37.92
4	Kappa number	42.58±0.16	33.22±0.15	27.75±0.14	22.5±0.12	20.89±0.11	20.96±0.11
5	Lignin, %	5.15±0.09	3.52±0.04	3.15±0.04	2.85±0.03	2.64±0.03	2.45±0.03

Cooking conditions:

1. Time from room to 105 ^{0}C = 45 min
2. Time from 105 ^{0}C to 165 ^{0}C = 45 min
3. Maximum temperature = 165 ^{0}C
4. Time at maximum temperature = 90 min
5. Bath ratio = 1:3.5
6. Sulphidity = 20% (as Na_2O)

Table 4.4: Pulping results of *A. cadamba* of different ages on pulp yield, kappa number and screening rejects during kraft pulping

Parameters	Age of *A. cadamba*			
	2 years	3 years	4 years	12 years
Bulk density, %	155	170	195	235
Screened yield, %	43.6	43.7	47.2	48.74
Screening rejects, %	1.5	2.1	0.35	0.6
Total yield, %	45.1	45.8	46.85	49.34
Kappa number	26.5±0.14	24.4±0.12	23.4±0.11	22.5±0.11
Pulp viscosity, cps	25.2	29.2	28.8	28.9

Cooking conditions:

1. Time from room to 105 ^{0}C = 45 min
2. Time from 105 ^{0}C to 165 ^{0}C = 45 min
3. Maximum temperature = 165 ^{0}C
4. Time at maximum temperature = 90 min
5. Bath ratio = 1:3.5
6. Sulphidity = 20% (as Na_2O)

Table 4.5: Effect of sulphidity on pulp yield, kappa number and lignin at 16% active alkali (as Na_2O) at 165 ^{0}C during kraft pulping of *A. cadamba*

Sulphidity, %	Time at 165 ^{0}C, h	Pulp yield, %	kappa number	Lignin, %
0	0.5	52.25	67.2±35	10.18±0.11
	1.0	49.5	54.1±31	8.23±0.09
	1.5	48.4	43.1±28	6.5±0.06
	2.0	45.3	39.9±25	5.16±0.07
	2.5	43.2	36.8±23	4.75±0.05
	3.0	41.3	35.2±23	4.42±0.04
	3.5	38.0	34.3±22	4.28±0.03
10	0.5	53.8	61.1±34	8.82±0.07
	1.0	50.5	47.5±27	7.05±0.06
	1.5	49	35.4±23	5.32±0.05
	2.0	46.1	33.2±25	4.18±0.04
	2.5	44.1	30.1±21	3.95±0.03
	3.0	41.9	29.8±21	3.8±0.04
	3.5	38.5	27.9±19	3.7±0.03
15	0.5	55.5	55.1±27	7.92±0.07
	1.0	51.65	40.4±24	5.85±0.06
	1.5	49.15	27.5±17	4.12±0.04
	2.0	46.4	25.2±18	3.68±0.04
	2.5	44.15	24.3±16	3.4±0.03
	3.0	42.1	23.4±15	3.08±0.04
	3.5	39.5	21.2±13	2.85±0.03
20	0.5	56.4	50.2±28	7.14±0.06
	1.0	52.15	36.6±24	5.15±0.06
	1.5	49.34	22.5±20	3.52±0.05
	2.0	46.6	21.2±18	3.15±0.04
	2.5	44.25	19.5±18	2.85±0.04
	3.0	42.15	18.2±15	2.64±0.03
	3.5	39.85	16.5±14	2.45±0.03
25	0.5	56.25	45.1±26	6.42±0.04
	1.0	52	32.1±18	4.65±0.05
	1.5	49.25	20.2±13	3.12±0.04
	2.0	46.5	18.2±14	2.85±0.04
	2.5	43	16.2±13	2.62±0.03
	3.0	42.1	14.2±10	2.41±0.04
	3.5	39.5	13.1±11	2.28±0.03

Table 4.6: Effect of AQ doses on pulp yield, kappa number and screening rejects during kraft pulping

Sl. No.	Particulars	AQ doses, %			
		0.0	**0.05**	**0.1**	**0.2**
1	Screened yield, %	48.74	48.85	49.12	48.90
2	Rejects, %	0.6	0.4	0.1	0.1
3	Total yield, %	49.34	49.25	49.22	48.91
4	Kappa number	22.5±0.12	18.2±0.10	16.0±0.05	.15.1±0.05

Cooking conditions:

1. Time from room to 105 ^{0}C = 45 min
2. Time from 105 ^{0}C to 165 ^{0}C =45 min
3. Liquor to wood ratio =1:3.5
4. Sulphidity = 20% (as Na_2O)

Table 4.7: Physical strength properties of kraft pulp at different beating levels

Sl. No.	Particulars	Beating level, ^{0}SR				
		29	**35**	**39**	**44**	**57**
1	Drainage time, s	5.0	5.3	6.5	9.2	15.5
2	Basis weight, g/m^2	59.6	60.2	60.3	59.4	60.1
3	Thickness, µm	108	95	90	85	77
4	Bulk, cm^3/g	1.81	1.57	1.49	1.43	1.28
5	Tensile index, Nm/g	29.6	64.42	85.30	92.57	91.37
6	Burst index, kPam^2/g	2.12	4.36	5.85	6.33	6.30
7	Tear index, mNm^2/g	11.0	13.1	12.5	11.3	9.05
8	Stretch, %	1.2	2.6	3.6	4.0	3.8
9	Double fold, numbers	28	276	785	1115	1125

Cooking conditions:

1. Kappa number = 22.5
2. Viscosity = 28 cps
3. Unbleached brightness = 32 % (ISO)

Pulping conditions:

4. Sulphidity = 20%,
5. Maximum temperature = 165 ^{0}C,
6. Maximum cooking time = 90 min.
7. Active alkali = 16% (as Na_2O

Table 4.8: Physical strength properties of kraft-AQ pulp at different at different beating levels

Sl. No.	Particulars	Beating level, ^{0}SR				
		29	35	39	44	57
1	Drainage time, s	5.1	5.5	6.7	9.5	15.7
2	Basis weight, g/m^2	60.2	60.1	60.0	59.8	60.2
5	Tensile index, Nm/g	30.2	64.87	86.5	93.67	92.50
6	Burst index, kPam^2/g	2.58	5.01	6.23	7.26	6.78
7	Tear index, mNm^2/g	11.3	13.5	12.8	11.8	9.36
8	Stretch, %	1.2	2.6	3.6	4.0	3.8
9	Double fold, numbers	24	250	750	1120	1135
Cooking conditions:						
1. Kappa number		= 16				
2. Viscosity		= 27.8 cps				
3. Unbleached brightness		= 35 % (ISO)				
Pulping conditions:						
4. Sulphidity		= 20%,				
5. Maximum temperature		= 165 ^{0}C,				
6. Maximum cooking time		= 90 min.				
7. Active alkali		= 16% (as Na_2O)				
8. AQ dose		= 1.0%				

Table: 4.9. Results of Baeur-McNett fiber classification of kraft pulp of *A. cadamba*

Sl. No.	Mess size	Fibers retained, %
1	+20	3.07
2	+48	67.34
3	+100	15.91
4	+200	0.99
5	<200	12.69

Table 4.10: Physical characteristics of *A. cadamba* kraft pulp and compression with Aspen and Spruce pulps

Properties (FQA)[a]	*A. cadamba* [b]	Aspen	Spruce	*E. tereticornis*
Mean fiber length (Arithmetic) (L=0.20-4.50 mm)	0.828	0.73	2.26	0.651
Length weighted	0.945	0.96	3.00	—
Weight weighted	1.041	1.07	3.43	—
Fines (L=0.07-0.20 mm), %				25.59
Arithmetic	22.11	17.31	6.06	
Length weighted	3.40	2.60	0.34	5.34
Mean curl index (L=0.50-5.0 mm, Cl=00-10.0) Arithmetic	0.096	na	na	0.141
Mean kink index (L=0.50-5.0 mm, Kl=00-20.0)		na	na	
Kink index	1.65 (1/mm)			2.15
Total kink angle	28.85 degree			29.93
Kinks per mm	0.80 (1/mm)			0.98
Mean width (L=0.30-6.0 mm, W=7-80 µm) Arithmetic	19.0 µm	na	na	na

a = Fiber Quality Analyzer Model V1. 3 REB 19 CV-M4
b= Total fiber count = 8365.00
na= Not available

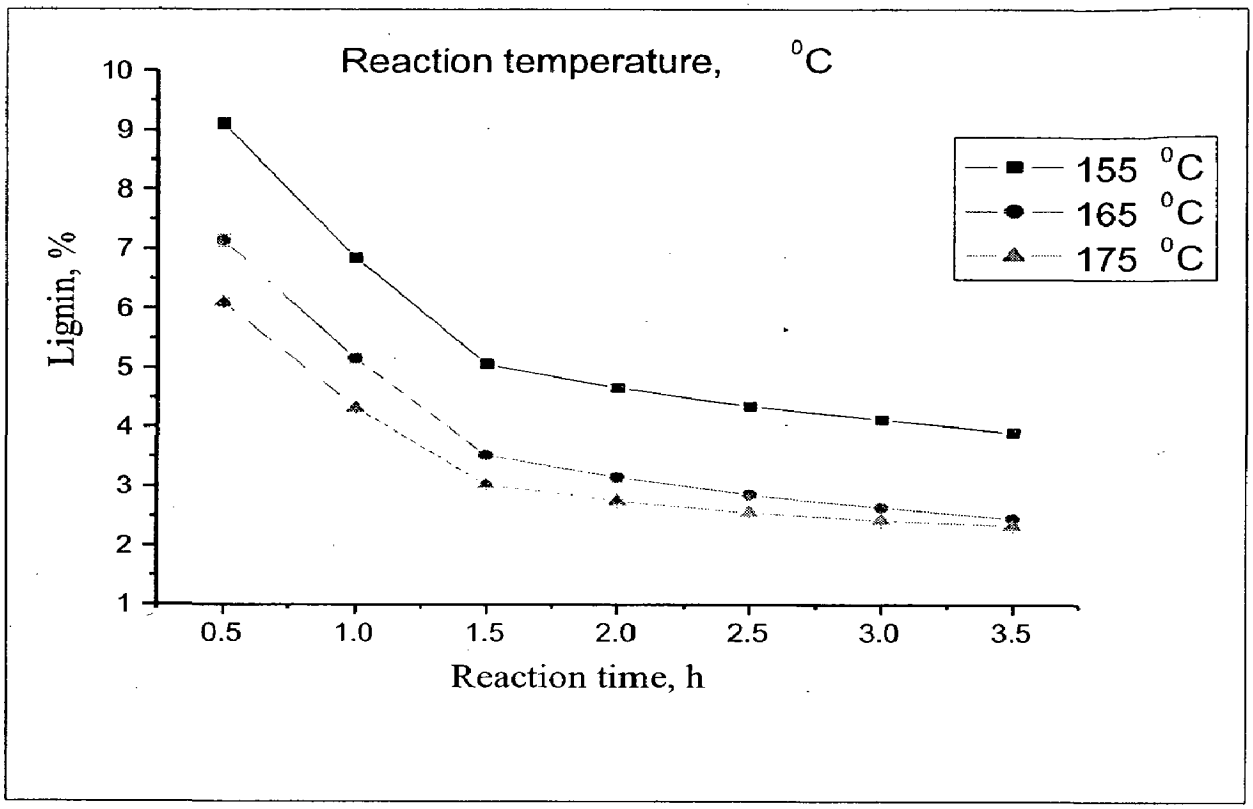


Figure 4.1: Plots of lignin (%) Vs time at different temperatures active alkali16% sulphidity 20% during kraft pulping

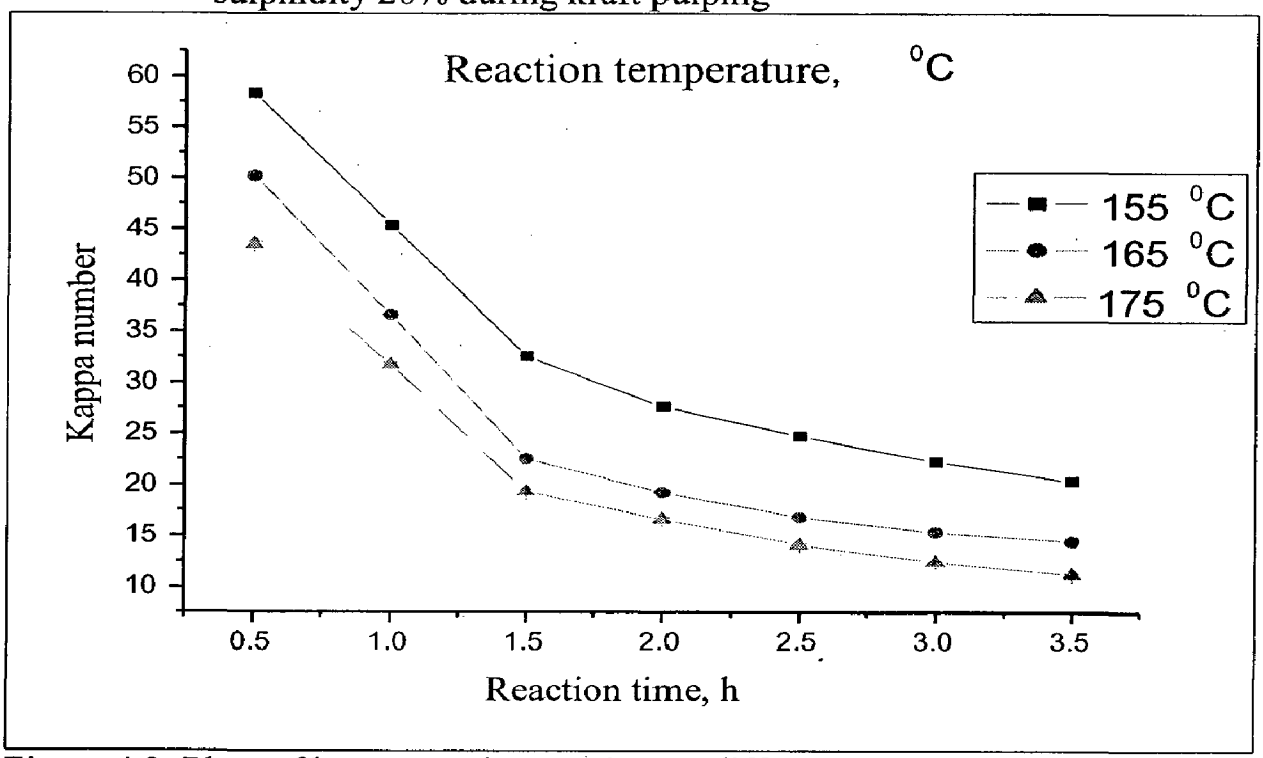


Figure 4.2: Plots of kappa number Vs time at different temperatures, active alkali 16% (as Na_2O), sulphidity 20% during kraft pulping

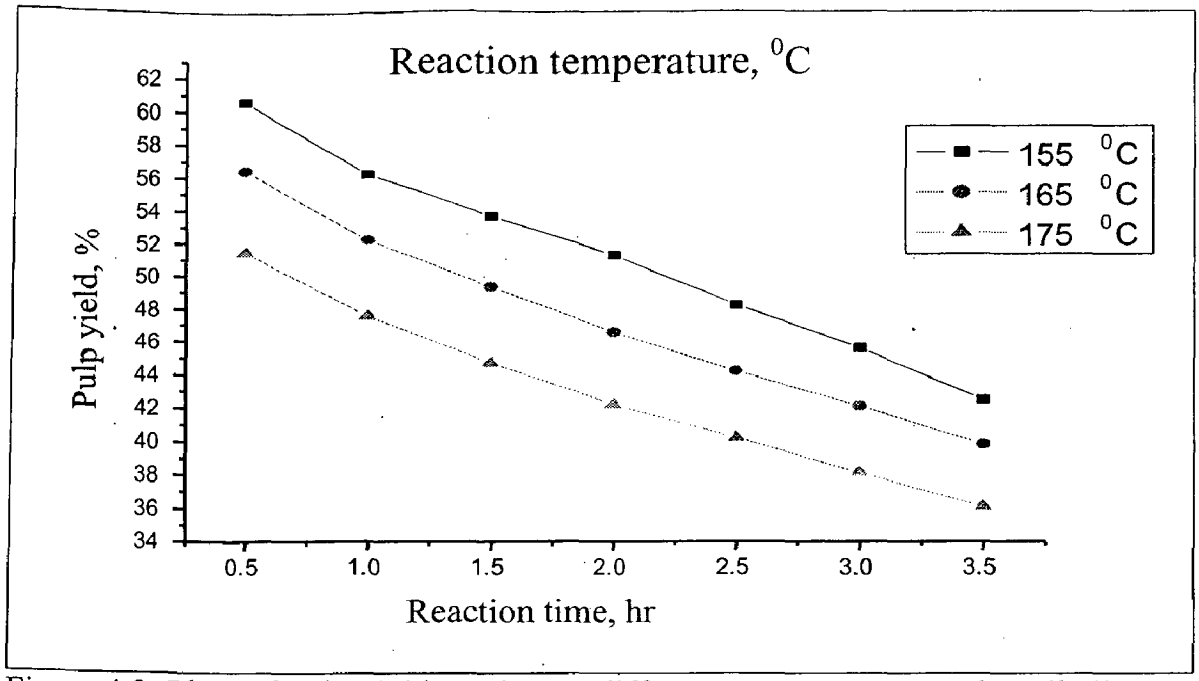


Figure 4.3: Plots of pulp yield Vs time at different temperatures, active alkali 16% (as Na_2O), sulphidity 20% during kraft pulping.

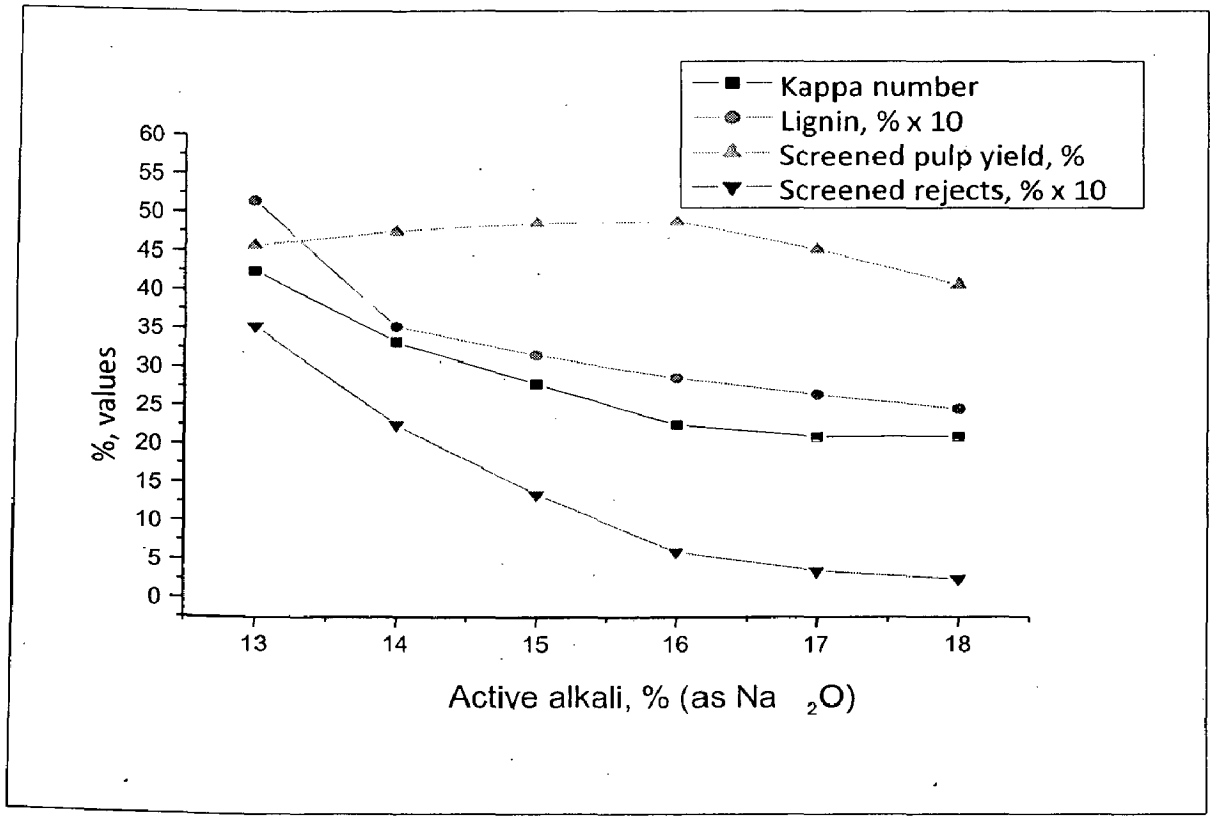


Figure 4.4: Effect of active alkali on screened pulp yield, screening rejects, kappa number and lignin at liquor to wood ratio 3.5:1, maximum cooking temperature 165 ^{0}C, maximum cooking time 90 min and sulphidity 20%

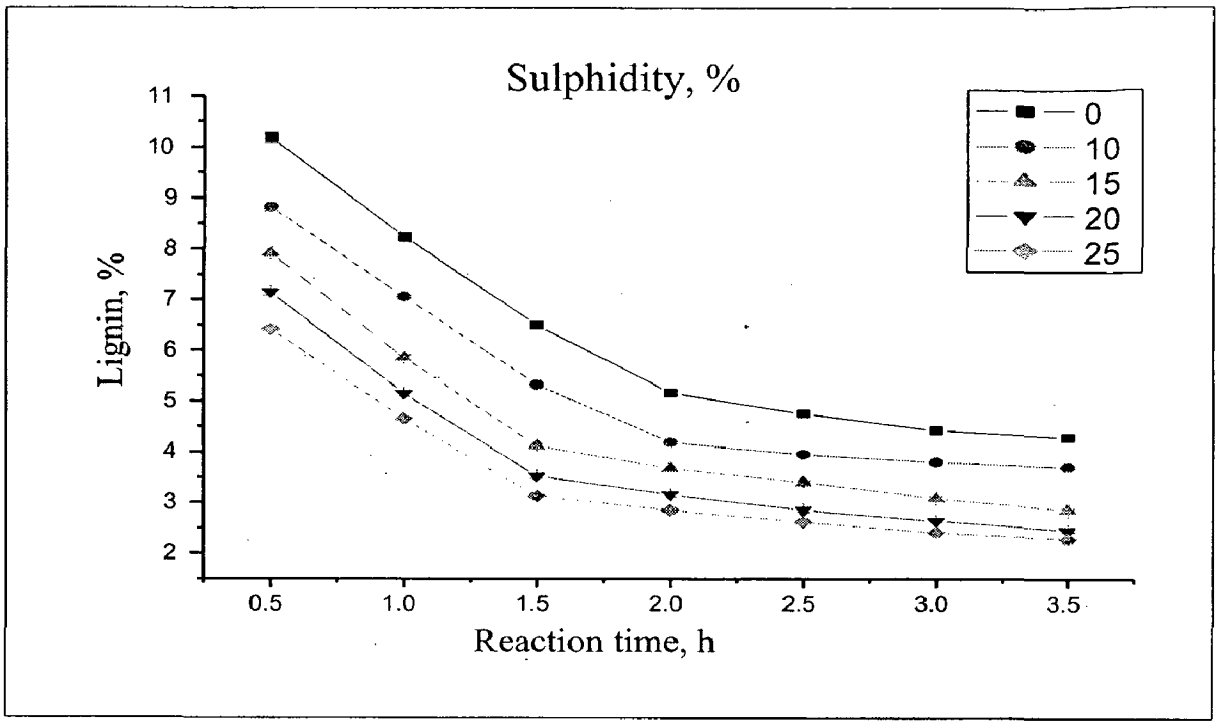


Figure 4.5: Plots of lignin Vs reaction time at different sulphidity levels, maximum temperature 165 ^{0}C and active alkali 16% (as Na_2O) during kraft pulping

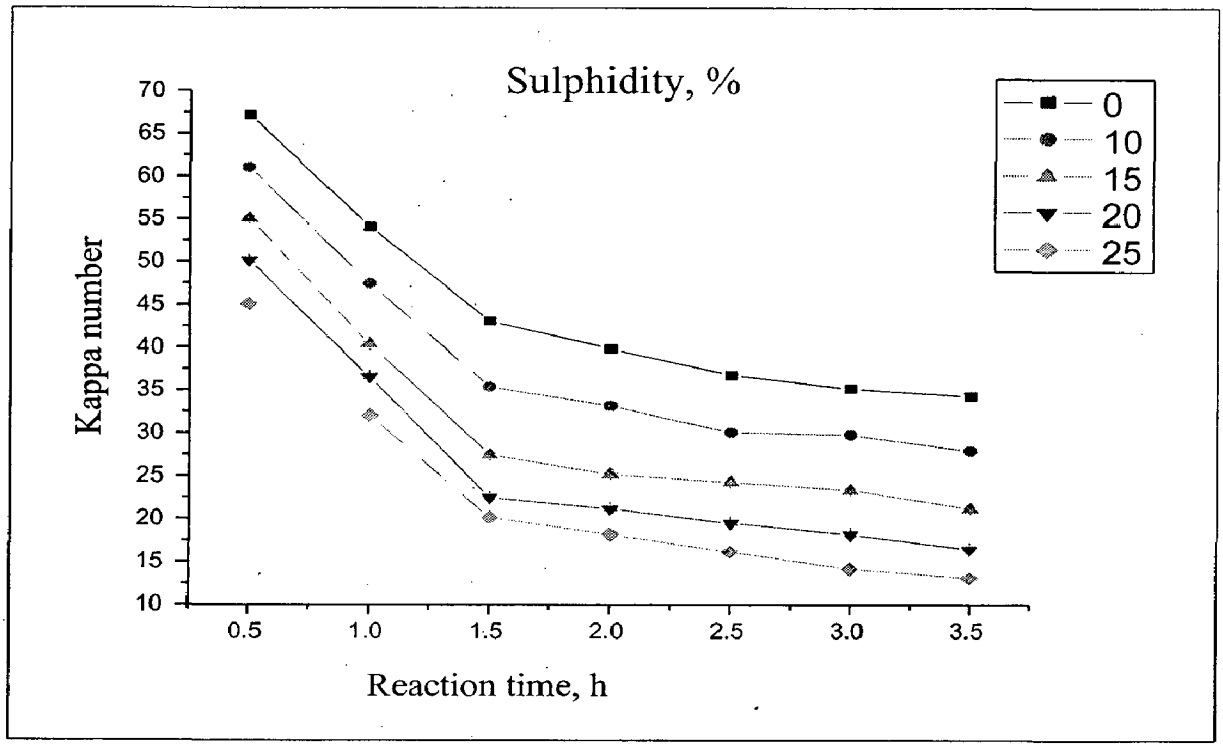


Figure 4.6: Plots of kappa number Vs reaction time at different sulphidity levels, maximum temperature 165 ^{0}C and active alkali 16% (as Na_2O) during kraft pulping

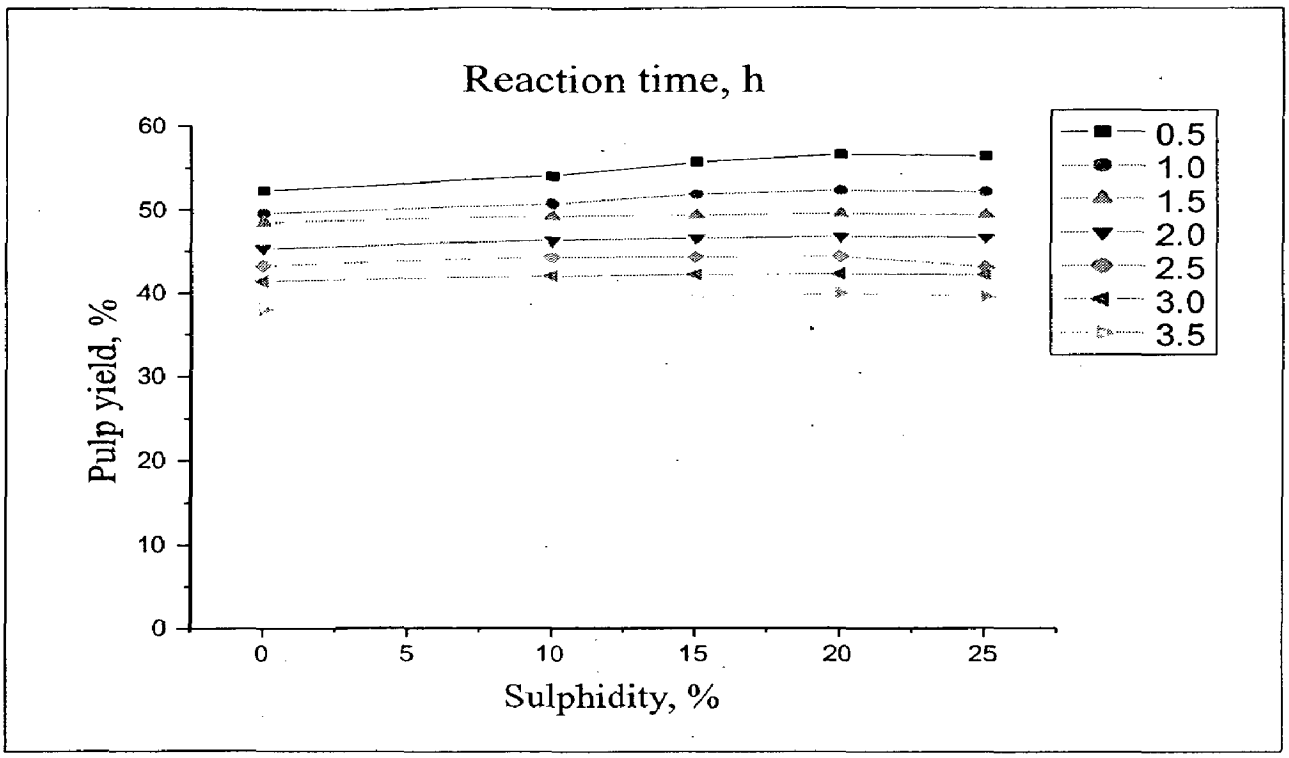


Figure 4.7: Plots of pulp yield Vs sulphidity at different time at temperatures, maximum temperature 165 ^{0}C and active alkali 16% (as Na_2O) during kraft pulping

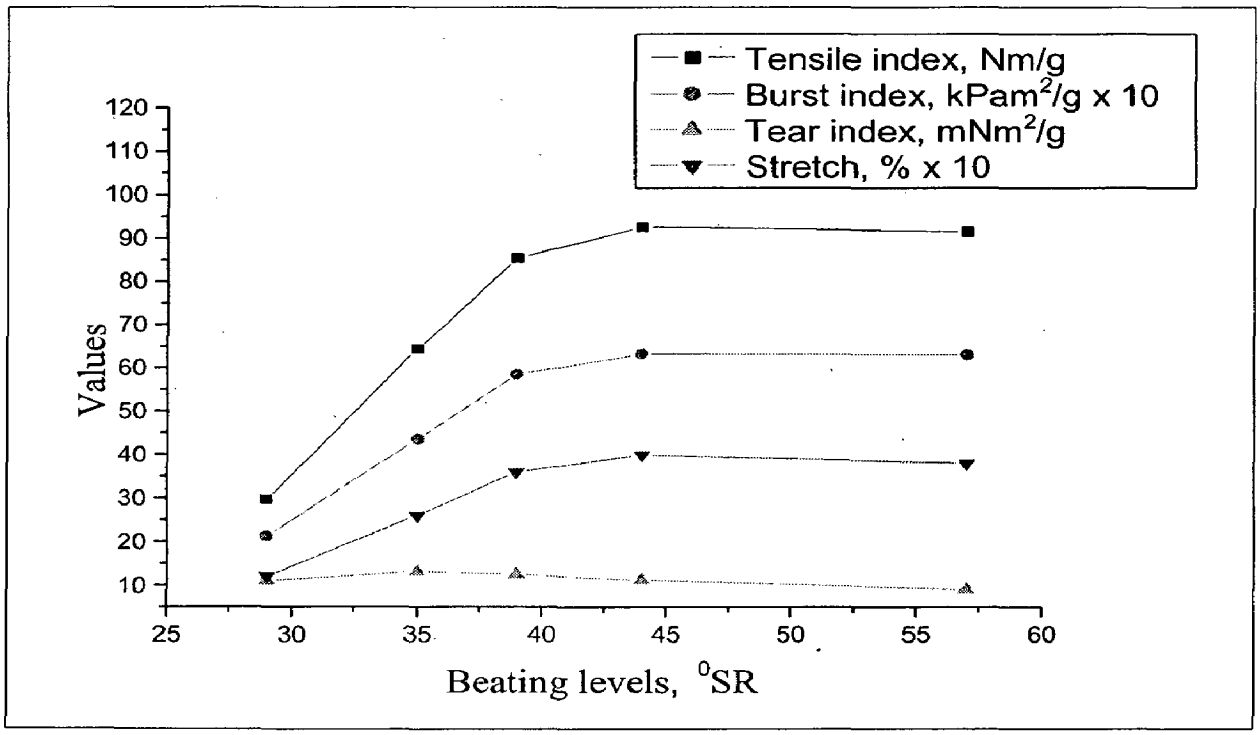


Figure 4.8: Plots of tear index, tensile index, burst index and stretch Vs ^{0}SR at sulphidity 20%, maximum temperature 165 ^{0}C, maximum cooking time 90 min and active alkali 16% (as Na_2O) during kraft pulping

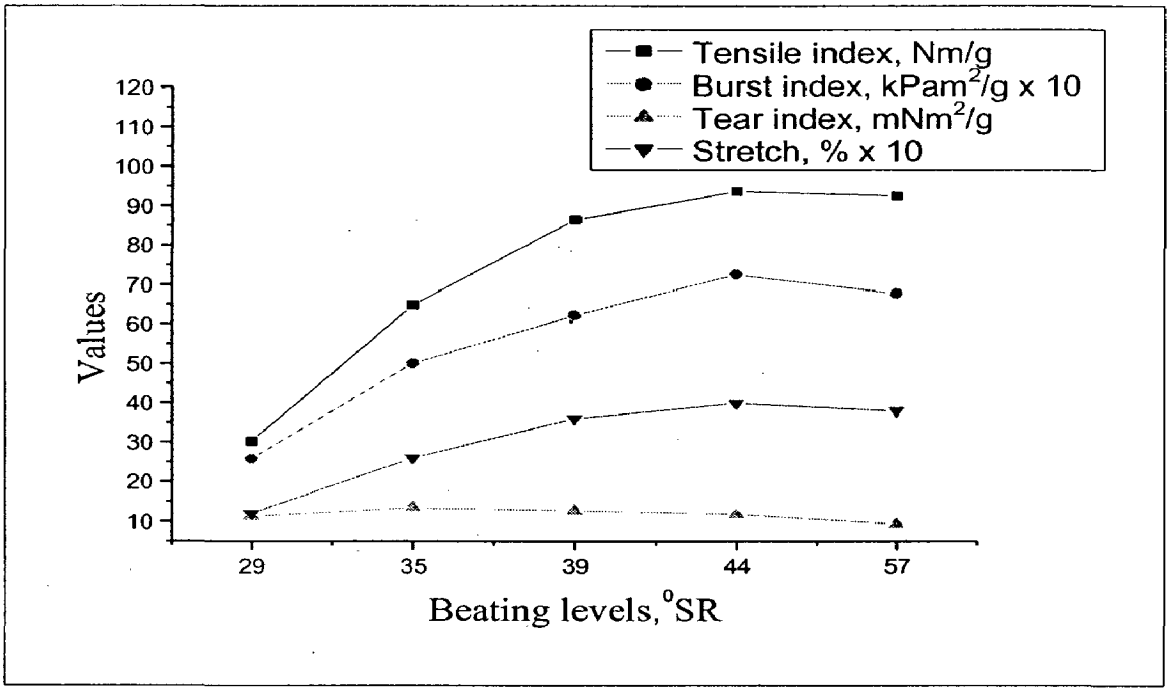


Figure 4.9: Plots of tear index, tensile index, burst index and stretch Vs ^{0}SR at Different sulphidity 20%, maximum temperature 165 ^{0}C, maximum cooking time 90 min and active alkali 16% (as Na_2O), AQ dose 0.1% during kraft pulping

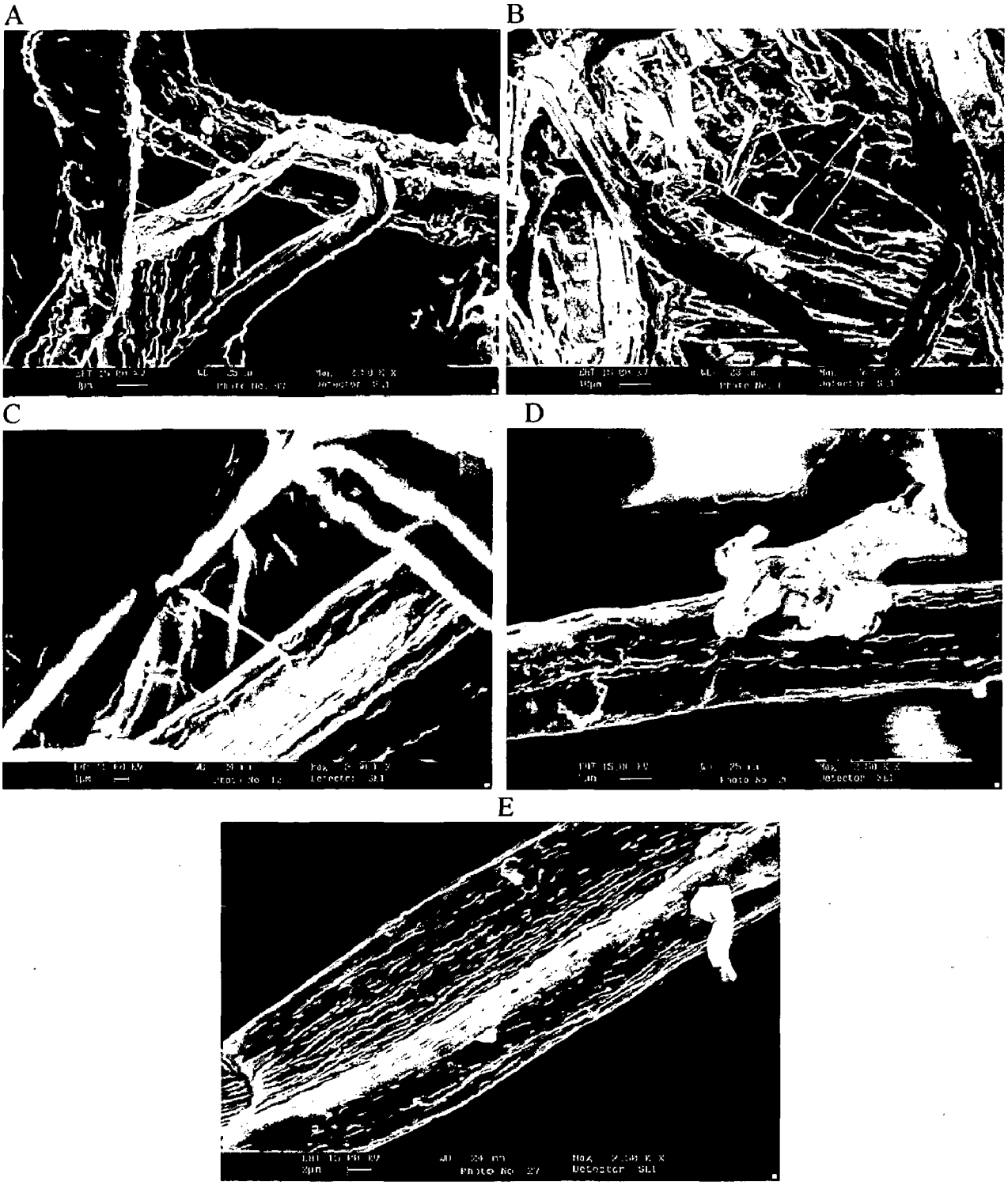
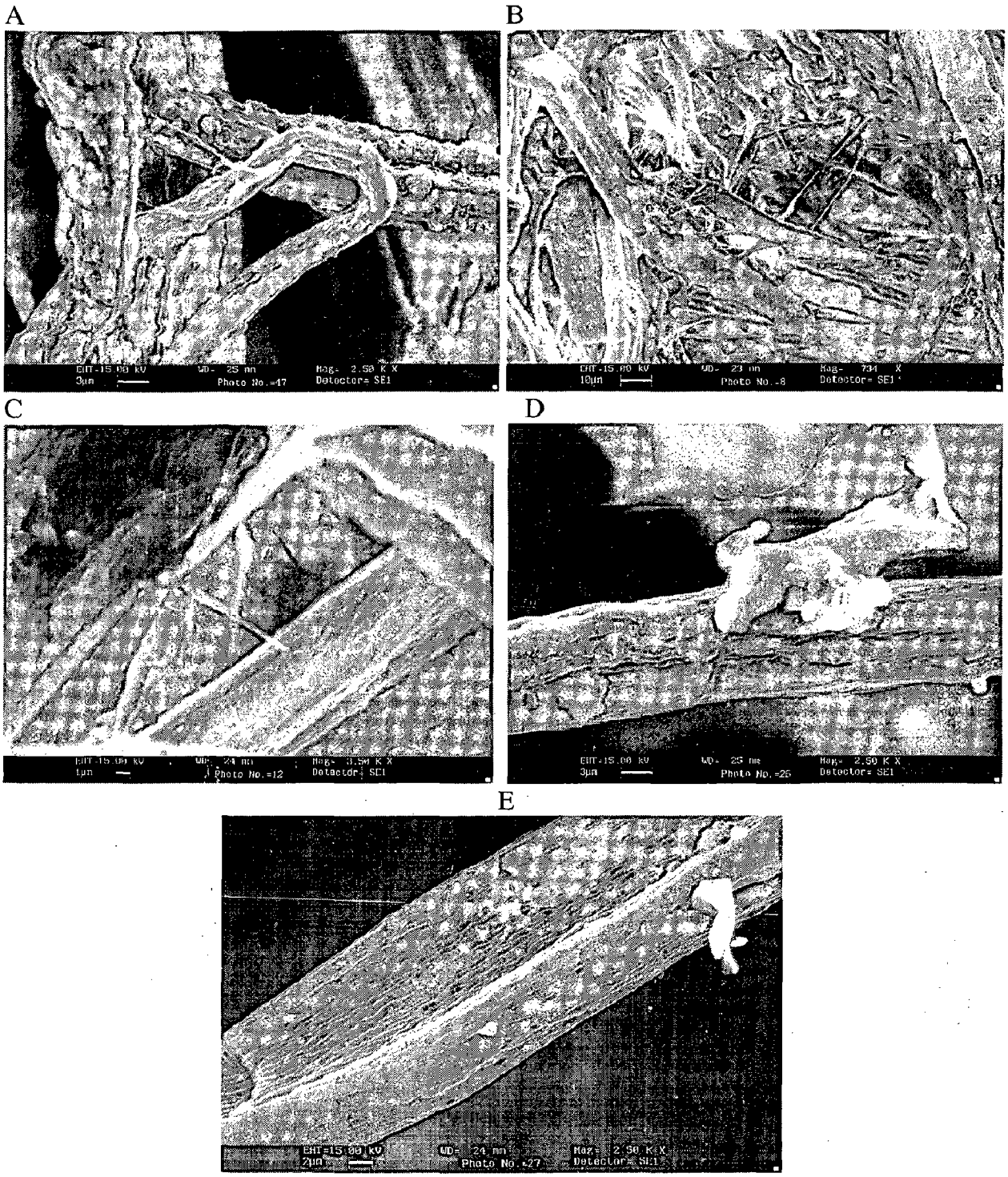


Plate 4.1: (A) Fibers shows the point of incomplete delignification and the flattened fibers after kraft pulping at a magnification of 2.50 K X (B) fibers show lumen of the fibers at a magnification of 734 X (C) (D) fibers shows reprecipitated xylan on fiber surface at a magnification of 2.50 K X (E) complete fibers of with primary wall obtained during kraft pulping at optimum pulping condition (shown by arrow) at a magnification of 3.50 K X.



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BIOBLEACHING STUDIES AND ENVIRONMENTAL ISSUES

5.1 INTRODUCTION

More than 90% of the total annual world production of pulp is obtained by kraft pulping process and it removes most of the constituent lignin, the residual lignin (4-5%) covalently bonded to carbohydrate moieties. Dark brown colour of the kraft pulp is attributed to 3-4% of the residual lignin and its reaction products (quinones), degraded polysaccharides and some extractives [102]. These are subsequently bleached in a multistage chlorine based chemical sequence to increase the brightness of the pulp to marketable grades but produce highly colored toxic chlorinated phenols and dioxins in effluent that are resistant to degradation, thus, causing the serious environmental problems [43, 108]. The major bleaching parameters such as, incoming kappa number, chlorine dosage, pH and temperature of chlorination and extraction stages have considerable effect on the effluent BOD, COD and colour. The formation of chlorinated materials, produced in the chlorination and extraction stages are a function of the amount of chlorine applied to the pulp, which is determined by kappa number of pulp [4, 103]. An increase in the chlorine dosage results an increase in BOD, COD and chlorolignins in the bleach effluent [104].

In a softwood kraft mill using a conventional bleaching sequence, approximately 5 kg of total organically bound chlorine (TOCl) is discharged per tonne of bleached pulp [29] and more than 300 different organic compounds along with a small quantity of highly toxic dioxins have been detected in waste bleach water. Conventionally bleached pulps effluent contains 10-15 μg/g absorbable organic halides [43]. The kappa number of Indian wood based mills vary from 14-25 and kappa number of agro-based mills vary from 18-30. The chlorine consumption of agro-based and wood based mills is 130-200 and 60-100 kg/T of pulp respectively. The AOX range in final discharge of agro based mills with and without chemical

recovery process is 7-11 and 14.2-21.5 mg/L. Where, AOX in final discharge of wood based mills is 0.60-9 mg/L. It was found out that the chlorination stage was generally the first point in which 2,3,7,8-TCDD, 2,3,7,8-TCDF and 1,2,7,8-TCDF congeners were always present [52, 85, 89]. The extraction stage filtrate was found to have the highest concentrations of dioxins [77] known as changing the blood chemistry and causing liver damage, skin disorders, lung lesions and tumor types at numerous sites within the body, liver and thyroid included [49, 69, 70]. Hence, there is great demand for chlorine free bleached pulps [31].

Thus, by far, chlorine is the cheapest and most versatile bleaching agent for the pulp but at the cost of environment. The problems of pulp and paper industry and that of pollution caused by bleach plant effluents can be addressed and solved in several ways [33] : (i) changing the lignin structure by genetic manipulations in forest trees in a way that facilitates pulping and bleaching (ii) increasing the use of recycled paper (iii) decreasing kappa number of pulp prior to commencement of bleaching (iv) replacing partially or completely elemental chlorine with chlorine dioxide (v) developing new biopulping and biobleaching techniques based on lignin-degrading enzymes to remove lignin, and hemicellulose-degrading enzymes to facilitate lignin removal during subsequent chemical bleaching and (vi) purifying bleach plant effluents using physical, chemical and biological techniques [5, 72]. First and last options need extensive research and high capital investments to change the existing infrastructure in the paper mills. The second option, however, of limited value, is under way and several countries including India are encouraging the use of recycled paper. Most of the mills are adopting either third, fourth or both options, decreasing the kappa number by extended delignification and changing conventional bleaching sequence with ECF or TCF bleaching sequence. The fifth option based on applications of enzymes, is new to the paper industry, but the limited capital expenditure associated with "enzyme pretreatment" of the pulp and the use of existing chlorine based bleach infra-structure make it an attractive alternative.

Preventing dioxin formation in the bleach plant has been achieved mainly by decreasing the amount of chlorine used in the first bleaching stage [60]. Oxygen delignification is used to reduce kappa number of pulp by 50% without much affecting the viscosity of pulp [66, 10, , 24], because in an attempt for reduction in kappa number may be expected to increase unexpected degradation of carbohydrate in the pulp and loss of pulp strength [11]. Oxygen delignification decreases the formation of AOX in bleach plant effluent when chlorine based chemicals are used in subsequent bleaching of pulp. Regardless of the bleaching chemicals used oxygen delignification decreases the BOD and COD of the effluent [93]. Oxygen prebleaching decreases the colour of bleach plant effluent 63 to 80% [36, 68].

With the replacement of bleaching sequences using elemental chlorine by chlorine dioxide, the pulp and paper industry has considerably reduced the formation and discharge of chlorinated organic materials in the aquatic environment [86]. The introduction of ECF process has resulted in a decrease of AOX in kraft bleaching ranging from 48-65% [51]. The pulp produced by ClO_2 bleaching is brighter and stronger than that produced by Cl_2 bleaching itself [83]. Substitution of ClO_2 at level of 70-100%, mill can apparently reduce the level of chlorinated organic compounds in mill effluent by 80-90% and reduce dioxins to non-detectable level. In fact, ClO_2 is a superior bleaching agent and will therefore have a prominent place in production of high brightness and high quality pulp during the next decades [83]. ClO_2 substitution decreases effluent colour in proportion to the percent substitution i.e. 1% decrease in colour occurs for 2% substitution [60]. Effluent COD decreases by approximately 10% as ClO_2 substitution increases from 0-100% [58, 60]. The addition of ClO_2 in the first stage of bleaching also reduces the toxicity of the effluent by virtually eliminating dioxins and 12 priority chlorophenols proposed by the U.S. Environmental Protection Agency (EPA) for regulation to non-detectable level [7]. The other benefits of ECF bleaching is that it decreases the formation of chloroform and total chlorinated organic compounds by 90%; efficiently utilizes forest resources; contributes to

eco-system recovery; and its compatible with the minimum impact on emerging technologies [71]. Recent studies reported a 35% reduction in TOCl at 100% substitution of chlorine dioxide [37, 78. In another study, a 63% reduction in the kappa number by oxygen delignification and 52 to 75% reduction in TOCl in the filtrate of C and E-stages of conventional bleaching and 42 to 86% reduction in TOCl in the filtrate of D and E stages was observed with 100% chlorine dioxide bleaching respectively [68]. While a decrease in COD by 20 to 25% occurs at 100% substitution of chlorine by chlorine dioxide [54, 58, 73, 105]. An NCASI review found that a 50 to 80% decrease in colour occurs at 90 to 100% substitution [63]. Therefore, the environmental regulations have made the use of ClO_2 important and moreover, it is economical too, even if total replacement of Cl_2 by ClO_2 is done.

Two recently developed bleaching concepts using ozone and hydrogen peroxide appear to be beneficial from environmental and cost-efficient point of view related to traditional chlorine free bleaching. Studies have shown advantages of using ozone in combination with ClO_2 in DZ and ZD prebleaching stages [26, 55]. The formation of chloro-organic compounds is reduced and the viscosity of pulp is maintained when the ozone (Z) charge is kept low [26]. Ozone can be charged both before and after the ClO_2 addition in ZD and DZ stage respectively [20]. Relative to chemical charge, the DZ treatment gives the most efficient delignification of oxygen delignified kraft pulp [97]. The replacement of chlorine dioxide by ozone in the first stage of ECF bleaching is reported to lower down colour dramatically [110]. Pressurized peroxide bleaching is a possible alternative to chlorine dioxide final bleaching. Pressurized peroxide bleaching (PO)-oxygen pressurized hydrogen peroxide treatment at high temperature is beneficial in several aspects compared to traditional atmospheric hydrogen peroxide bleaching [26].

The traditional, effective approach of using chlorine-containing chemicals as bleaching agents has been challenged, first by environmentalists and then by consumers. In

response to these pressures, alternative bleaching technologies have been developed to partially or completely replace these chemicals [25], particularly elemental chlorine. Environmental, consumer, and regulatory concerns have favoured the use of enzymes in bleaching because they are not only biodegradable but solve multifold problems like, gross habitat alteration, organic enrichment, eutrophication effects and chemical toxicity.

Tolan (1995) has suggested that the enzyme treatment may result in dioxin free bleach process of kraft pulps [94]. The enzyme treatment should not be more expensive than the existing bleaching reagents, although the advantages of biobleaching in reduced waste water purification cost and increased product safety must also be considered. The impact of xylanase pretreatment on kraft pulp bleachability was first demonstrated by Viikari *et al.,* in 1986 and since then the cellulase free xylanase as potential for bleach boosting agent have been recognized and reviewed by several investigators [7, 9, 15, 16, 23, 24, 34, 35, 39, 48, 84, 91, 94, 99, 100, 101, 102, 106, 107]. Currently, the application of hemicellulases prior to chlorine bleaching can reduce 20-30 % consumption of active chlorine input to achieve the required brightness in the final product without affecting the physical strength of the fibres [35, 45]. The successful mill-scale trial of enzyme pretreatment of a softwood kraft pulp prior to bleaching [89, 94] and the commercial potential of such a product is indicated by the use of xylanases pretreatment in full-scale production in 10 mills; 6 in Europe and 4 in Canada [41, 46]. Several patents on xylanases have been obtained by individual scientist and commercial ventures. Pulpzyme TM HB (Novo-Nordisk, Denmark); Cartazyme HS 10 (Sandoz, U.K.); Ecopulp (Alco ICI) and Irgazyme 40 (Ciba Genecor) are the most important commercial preparations in the market [35, 99]. The xylanase enzymes used commercially are made in several-day batches of submerged liquid culture fermentation by bacteria, fungi, or other microbes.

The potential application of xylanases and the need for enzymes that might be more appropriate for the use in pulp bleaching has caused many researchers to look at xylanases in

different organisms and environments. There is need to develop such type of enzymes that can work at alkaline pH and high temperature since, the pulp produced from brown stock washer is having high temperature and alkaline in nature. Because most commercial enzymes of the first generation have not met these requirements, adjustment of pH between 5-7 and cooling of pulp is necessary which generates the corrosion problem and increases the cost of enzymatic bleaching. Most of the xylanases studied are active in slightly acidic conditions ranging between pH 4-6 and temperature below 70 ^{0}C. More thermophilic and alkalophilic xylanases are of great importance because of the prevailing conditions in pulp processing. Thermophilic [13, 87] and alkaline sources [42, 62] for these enzymes have been recently reviewed. Substantial amount of work have been continued in isolating and cloning of new xylanases from all the sources.

The application of ligninases in biobleaching of pulps is comparatively little studied than the pretreatment of pulps with xylanases. Recent studies showed that the enzymes of white rot fungi play an important role in delignification of kraft pulps [29, 47, 71] using MnP deficient strain of *Trametes versicolor* have confirmed the role of laccase and MnP proteins in biobleaching of kraft pulp [1]. However, further researches are necessary to exploit the potential on lignin degrading enzymes for their applications in pulp and paper industry. Logically, the idea sounds good that if the cooperative activity of hemicellulases and ligninases is used together to pretreat unbleached kraft pulps, it should enhance the extractability of lignin.

Xylanase enzymes partially hydrolyze the hemicelluloses portion of pulp. Unlike the conventional bleaching chemicals, these enzymes do not brighten or delignify the pulp. Rather, these enzymes act on the pulp to make the subsequent bleaching by the oxidative chemicals more efficient. The precise mechanism of xylanase action on the pulp is not known. There is evidence that xylanase allows larger molecular weight lignin to be removed from the pulp in the alkali extraction stage, so it is postulated that removal of a portion of the xylan

either releases xylan-bound lignin or increases the ability of higher molecular weight lignin to diffuse through the surface of the fibre. In either case, removing a portion of the xylan from the pulp increases the efficiency of the oxidative chemicals.

Many other enzyme applications are possible based on properties confirmed in the laboratory. These include eliminating caustic chemicals for cleaning paper machines, enhancing kraft pulping, reducing refining time, decreasing vessels picking, facilitating retting, selectively removing fiber components, modifying fiber properties, increasing fiber flexibility, and covalently linking side chains or functional groups [44]. Commercial development of these applications need enhanced knowledge of enzyme mechanisms and actions on fibers, development of improved processes for their use, and changing enzymes to function better under present working conditions.

The present investigation aims at use of crude enzyme preparations, isolated from thermo-alkali-tolerant strains of *Coprinellus disseminatus* MLK01 and MLK07 under L.S.F. conditions and its effect on brightness, viscosity, mechanical strength properties and pollution load was studied during biobleaching of kraft-AQ pulp of *A. cadamba.*

5.2 MATERIALS AND METHODS

The enzyme preparations contain xylanase as their main activity and the enzyme dosing was given according to xylanase activity. The enzyme preparation from strain MLK01 is designated as enzyme-A and enzyme preparation from strain MLK07 as enzyme-B.

5.2.1 Optimization of various operating parameters for enzymatic pre-bleaching

The unbleached kraft pulp of *A. cadamba* was optimized for various operating parameters, like enzyme dose, consistency and reaction time during prebleaching with enzyme-A (Strain MLK01) and enzyme-B (Strain MLK07) separately and its impact on pulp kappa number (T236 cm-85) and viscosity (T230 om-04) as per Tappi Standard Test Methods: 2007 and release of chromophores [67] and reducing sugars [61] in filtrate were studied.

5.2.1.1 Enzyme dose

10 g of unbleached oven dry kraft pulp of *A. cadamba* of kappa number 16, disintegrated in L&W make laboratory disintegrator to loosen fibre bundles or lumps without cutting, was taken in polyethylene bags for optimization of enzyme dose. The consistency of pulp was maintained at 10% and pH 7.5 for enzyme-A and pH 8.0 for enzyme-B with 0.1 N phosphate buffer. These were incubated at constant temperature (75 ±2 ^{0}C for enzyme-A and 65±2 ^{0}C for enzyme-B) in water bath. Different doses of enzyme-A and B i.e. 2, 5, 10, 15, 20 and 30 IU/g of oven dry pulp basis, were added separately in each polyethylene bag and mixed well with vigorous shaking. After 2 h of reaction time, samples were taken out from water bath and filtered through cheese cloth. The filtrate was analyzed for reducing sugars and chromophores. The pulps were washed with 1 L of tap water, squeezed and analyzed for kappa number and pulp viscosity. The results are reported in Table 5.1.

5.2.1.2 Consistency

Similarly, two sets, each of 10 g of disintegrated unbleached kraft pulp of *A. cadamba* was treated with an optimum enzyme dose of 5 IU/g for enzyme-A and 10 IU/g for enzyme-B and pH 7.5 for enzyme-A and pH 8.0 for enzyme-B maintained with 0.1 N phosphate buffer at different pulp consistencies i.e. 2, 5, 10, 15, and 20%. These were incubated at constant temperature (75 ±2 ^{0}C for enzyme-A and 65±2 ^{0}C for enzyme-B) in water bath for two hours and filtered through cheese cloth. The filtrate was estimated for reducing sugars and chromophores. The pulps were washed with 1 L of tap water, squeezed and analyzed for kappa number and pulp viscosity. The results are reported in Table 5.2.

5.2.1.3 Reaction time

In the similar way, two sets, each of 10 g of disintegrated pulp, were treated with an optimum enzyme dose 5 IU/g for enzyme-A and 10 IU/g for enzyme-B at pulp consistency 10% and pH 7.5 for enzyme-A and 8.0 for enzyme-B maintained with 0.1 N phosphate buffer separately. These were incubated in constant temperature (75 ±2 ^{0}C for enzyme-A and 65±2

^{0}C for enzyme-B) in temperature controlled water bath and polyethylene bags were removed after reaction time of 30, 60, 90, 120, 240 and 360 min respectively. These samples were filtered through cheese cloth and filtrates were estimated for reducing sugars and chromophores. The pulps were washed with 1 L of tap water, squeezed and analyzed for kappa number and pulp viscosity. The results are reported in Table 5.3.

5.2.2 Enzymatic prebleaching of pulp in bulk

The unbleached kraft-AQ pulp of *A. cadamba* was prebleached in bulk with crude enzyme preparations (enzyme-A and enzyme-B) obtained from both the strains (MLK01 and MLK07) under optimized conditions like, enzyme dose of 5 IU/g for enzyme-A and 10 IU/g for enzyme-B, 10% pulp consistency and reaction time of 90 min for enzyme-A and 120 min for enzyme-B at temperature of 75 ±2 ^{0}C for enzyme-A and 65±2 ^{0}C for enzyme-B and pH 7.5 for enzyme-A and 8.0 for enzyme-B separately. Subsequently both the pulps were extracted with 2% NaOH at 70 ^{0}C and 10% consistency for 90 min. These were filtered through cheese cloth and the filtrates were collected for further analysis. Then, the pulp was washed with tap water. The enzyme treated pulp after XE stage was evaluated for kappa number, brightness and viscosity.

5.2.3 Chemical bleaching of pulp

The unbleached kraft pulp of *A. cadamba* after enzyme treatment followed by 2% alkali extraction (XE) was subjected to CEHH bleaching sequence. In case of ECF and TCF bleaching sequences, the oxygen delignified pulp of *A. cadamba* was treated with both the enzymes i.e. enzyme-A and enzyme-B separately and extracted with 2% NaOH (as such) (OXE). All the bleaching stages were performed in polythene bags of two kg capacity, in a temperature controlled water bath, except chlorination, that was performed in capped plastic jar at ambient temperature. The pulp and required chemicals were mixed well at desired pulp consistency by shaking the bottles time to time during bleaching experiments. 60 g (on o.d.

pulp basis) unbleached pulp was taken for each experiment. The control was repeated in a similar manner without enzyme pretreatment.

5.2.3.1 Conventional (CEHH) bleaching sequence

The disintegrated pulp slurry was diluted with tap water to maintain 3% consistency. The chlorine demand was calculated based on kappa number of unbleached kraft pulp (UBKP) by using following formula:

% chlorine demand = kappa number of UBKP × 0.25

% chlorine applied in C stage = 50% of total chlorine demand

The chlorination was carried out at 3% consistency, pH 2.0 adjusted with dilute H_2SO_4 and at ambient temperature. The jars were capped tightly in order to avoid leakage of molecular chlorine and contents were mixed well. After 45 min of chlorination, the pulp was filtered through cheese cloth and filtrate was analyzed for residual chlorine. The rest of the filtrate was preserved at 4 ^{0}C for further analysis. The pulp was washed with 2 L of tap water and squeezed with hands.

The chlorinated pulp was extracted with 50% NaOH+ 0.03 (as such) of the chlorine applied in 'C' stage at conditions: consistency 10% and temperature 70 ±2 ^{0}C for 90 min. The pulp was kneaded time to time for proper mixing. After completion of extraction stage, the pulp samples were filtered through cheese cloth and the filtrate was preserved at 4 ^{0}C for further analysis. The pulp was washed with 2 L of tap water and squeezed with hands. Remaining 50% chlorine of the total chlorine demand was charged in H_1 and H_2 stages respectively i.e., 50% in H_1 and 50% in H_2 stage. The hypochlorite Ist and 2nd stages were conducted at 10% consistency, pH 11.3 adjusted with NaOH, and temperature 70 ±2 ^{0}C for 90 min. Similarly, pulp obtained after H_1 and H_2 stages were filtered through cheese cloth and filtrate was analyzed for residual chlorine. The rest of the filtrate was preserved at 4 ^{0}C for further analysis and the pulp was washed with 2 L of tap water, squeezed and crumbled.

5.2.3.1 (a) Effect of enzyme prebleaching on various properties at different kappa number pulps during CEHH bleaching

The unbleached kraft-AQ pulps of *A. cadamba* of kappa numbers 16 and 24 were prebleached with the same doses of enzyme-A and enzyme-B separately at conditions mentioned above. The enzymatically prebleached pulps were bleached by CEHH bleaching sequence at the chlorine demands calculated based on unbleached pulp kappa number. The same was repeated with control without enzyme treatment. The results are reported in Tables 5.4 and 5.5.

5.2.3.1 (b) Effect of enzyme prebleaching on various properties at different total chlorine demands during CEHH bleaching

The unbleached kraft-AQ pulps of *A. cadamba* of kappa numbers 16 was prebleached with the same doses of enzymes-A and B separately at conditions mentioned above. The enzymatically prebleached pulps were further bleached by CEHH bleaching sequence at different total chlorine demands i.e. full and half of the total chlorine demands (4 and 2% on oven dry basis) respectively. The same was repeated with control without enzyme treatment. The results are reported in Table 5.6.

5.2.3.1 (c) Effect of enzyme prebleaching on various properties at different pH during CEHH bleaching

The kraft-AQ pulp of *A. cadamba* was prebleached at different pH levels i.e. 5.0, 6.0, 8.0 and 9.0 while keeping other conditions same as above. The enzymatically prebleached pulps were further bleached by CEHH bleaching sequence and chlorine demand was calculated based on untreated pulp kappa number. The same was repeated with control without enzyme treatment. The results are reported in Tables 5.7 and 5.8.

5.2.4 Oxygen delignification

The kraft-AQ pulps of *A. cadamba* of kappa number 16 was delignified with O_2 in electrically heated Weverk rotary digester of capacity 0.02 m^3 having four bombs of capacity 2L each at conditions: consistency 15%, pH 11.0 maintained with 2% NaOH (as such), oxygen pressure

5 kg/cm^2, temperature 110 ^{0}C and reaction time 90 min in presence of carbohydrate stabilizer Epsom salt (0.1% $MgSO_4$ on o.d. pulp basis). After completion of cooking, the pulps were washed on a laboratory flat stationary screen having 300 mesh wire bottom for the removal of residual cooking chemicals, squeezed and crumbled. The oxygen delignified pulp was evaluated for kappa number, brightness and the viscosity as per TAPPI Standards Test Methods. The oxygen delignified pulp of *A. cadamba* was further prebleached with enzymes-A and B separately at optimum conditions mentioned above. Subsequently both the pulps were extracted with 2% NaOH at 70 ^{0}C and 10% consistency for 90 min. These were filtered through cheese cloth and the filtrate was preserved at 4 ^{0}C for further analysis. Then, the pulp was washed with tap water. The pulps obtained after OXE sequence were evaluated for kappa number, brightness and viscosity and rest of the pulps were subjected to bleach by ECF and TCF bleaching sequences.

5.2.5 ECF bleaching sequences

Total 3.0% of ClO_2 was applied in ODED and OXEDED bleaching sequences (1.8% in D_1 and 1.2% in D_2 stage) maintaining a pulp consistency of 7% and reaction temperature 70 ±2 ^{0}C for 3 h at initial pH 2.5. The pulp was washed through cheese cloth and filtrate was analyzed for residual chlorine. The rest of the filtrate was preserved at 4 ^{0}C for further analysis. The pulp was washed with 2 L of tap water and squeezed with hands. The alkali extraction stage was carried out at 1% NaOH charge (as such), temperature 70 ^{0}C and 10% consistency for 90 min. The results are reported in Table 5.9.

Similarly, the total 2.0% of ClO_2 was applied in single D stage during ODEP and OXEDEP bleaching sequences while keeping other conditions constants as described above. The alkali extraction stage was carried out with 1% NaOH charge (as such) while keeping other conditions are same as mentioned above. Finally, the peroxide stage was performed by using 1.5% H_2O_2 using $MgSO_4$ as a carbohydrate stabilizer, pH 11.4 adjusted with NaOH, consistency 10%, temperature 80 ±2 ^{0}C and reaction time 90 min. H_2O_2 was added in to the

pulp suspension after attaining a temperature of 80 ±2 ^{0}C. After 10 min when temperature of pulp slurry reached near to 80 ±2 ^{0}C, the calculated dose of diluted peroxide was added in each polyethylene bag and these were incubated in water bath for 90 min. After that, the contents were filtered through cheese cloth and the effluent was collected for estimation of residual peroxide. The pulp samples were washed with 2 L of tap water and the effluent was preserved at 4 ^{0}C for further analysis. The results are reported in Table 5.10.

In ODEDP and OXEDEDP bleaching sequences, the total 3.0% of ClO_2 was applied (1.8% in D_1 and 1.2% in D_2 stage) as described above. Alkali extraction stage was carried out at 1% NaOH charge under same conditions as described above. Peroxide treatment was given at 1.0% H_2O_2 was added at same conditions as described above. The pulp samples after each stage were filtered through cheese cloth and residual chemicals were analyzed in filtrate. The pulp samples were then washed with 2L of tap water and effluent was preserved at 4 ^{0}C for further analysis. The results are reported in Table 5.11.

5.2.6 TCF bleaching sequence

Oxygen delignified and OXE semi-bleached kraft-AQ pulps of *A. cadamba* were bleached by rest of the bleaching sequence i.e. QPP. Both the pulps were treated with 1% EDTA at 3% consistency and pH 5.5 adjusted with H_2SO_4 at room temperature for 30 min. Total 3% H_2O_2 was applied in both the peroxide stages (1.5% in each stage) with 0.1% $MgSO_4$ and 0.5% sodium silicate as stabilizing agents maintaining at pH 11.0 of pulp suspension with 2% NaOH, 10% consistency at temperature 80 ^{0}C for 2 h. The samples were kneaded time to time to ensure a uniform mixing of pulp suspension and H_2O_2. The pulp samples were washed through cheese cloth and filtrate was analysed for residual peroxide and filtrate was preserved at 4 ^{0}C for further analysis. The pulp was washed with 2L of tap water. Results are reported in Table 5.12.

5.2.7 Beating and sheet making

The kraft-AQ bleached pulps of *A. cadamba* were beaten in PFI mill at a beating level of 35 ^{0}SR (T248 sp-00) and laboratory hand sheets of 60±2.5 g/m^2 were made on semi-automatic Messmer sheet former. The laboratory made hand sheets were pressed, dried and conditioned at 27± 2 ^{0}C and 65±2 % relative humidity. The pulp pads were prepared on Büchner funnel (T218 sp-02) and tested for brightness (T-452 om-02). Laboratory hand sheets were evaluated for burst index (T-403 om-02) (Burst tester Lorentzen & Wettres, Stockholm, Sweden), tensile index (T-404 wd-03) (Tensile index tester Lorentzen & Wettres, Stockholm, Sweden), double fold (T-423 cm-98) (Folding endurance tester Lorentzen & Wettres, Stockholm, Sweden), tear index (T-414 om-04) (Tear tester Lorentzen & Wettres, Stockholm, Sweden) [90].

5.2.8 Characterization of bleach effluent

The bleach effluent generated from each stage of bleaching sequence was mixed in equal amounts and were analyzed for COD (closed reflux titrimetric method using Thermoreactor CR2010) (Test method No- 508 B) [19], colour (Test method No-204A) as per Standard methods for the examination of water and waste water, American Public Health Association, 1985 [38] and AOX by column method [98] with AOX Analyzer Dextar ECS 1200.

5.2.9 Scanning electron microscopy (SEM) of pulp samples

The detailed morphological studies of kraft-AQ pulp of *A. cadamba* (before and after enzyme treatments) were carried out using scanning electron microscopy model SEM, Leo 435 VP, England by gold shadowing technique. The pulp samples were oven dried at 80 ^{0}C till over night for SEM. The pulp samples were subjected for fixation using 3% (v/v) glutaraldehyde-2% (v/v) formaldehyde (4:1) for 24 h. Following primary fixation, pulp samples were washed thrice with double distilled water. The samples were then treated with

the alcohol gradients of 30, 50, 70, 80, 90 and 100% for dehydration. Samples were kept for 15 min each up to 70% alcohol gradient, thereafter treated for 30 min each for subsequent alcohol gradients. After treatment with 100% alcohol, samples were air dried and examined under SEM using gold shadowing technique [32]. Microphotographs were taken at desired magnifications. Results of SEM are shown in Microphotographs 5.1 and 5.2.

5.2.10 Statistical analysis

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

5.3. RESULTS AND DISCUSSION

The crude enzyme preparations were isolated from two different thermo-alkali-tolerant strains of *Coprinellus disseminatus* namely MLK01 and MLK07 under LSF conditions and designated as enzyme-A and enzyme-B respectively. The crude enzyme-A preparation contains 30.32 IU/mL xylanase, 0.32 IU/mL CMCase and 0.25 U/mL lignin peroxidase activities while crude enzyme-B preparation contains 36.87 IU/mL xylanase, 0.25 IU/mL CMCase and 0.15 U/mL lignin peroxidase activities. The various parameters associated with enzymatic prebleaching i.e., enzyme dose, reaction time and consistency were optimized and their impact on reducing sugars and chromophores released during enzymatic prebleaching were studied. The enzymatically prebleached pulps followed by alkali extraction at various operating parameters were evaluated for kappa number and pulp viscosity.

5.3.1. Optimization of enzyme doses

Table 5.1 reveals the effect of various doses of enzyme-A and enzyme –B i.e. 2, 5, 10, 15, 20 and 25 IU/g (on oven dry basis) while keeping other variables constant i.e. reaction time 120 min, temperature 75 ^{0}C and pH 7.5 for enzyme-A and reaction time 120 min, temperature 65 ^{0}C and pH 8.0 for enzyme-B and 10% consistency for both the cases during kraft-AQ pulp of *A. cadamba*. The reducing sugars released after enzymatic prebleaching is 11.60 mg/g at an enzyme dose of 5 IU/g and beyond that insignificant increase in reducing

sugars is observed. Similarly, reducing sugars released after prebleaching with enzyme-B increases with increasing enzyme doses up to 25 IU/g. The release of chromophores measured spectrophotometerically at different wavelengths i.e. 237 and 280 and 465 nm may be correlated with reduction in kappa number [67]. The concentration of chromophores increases up to an enzyme dose of 5 IU/g for enzyme-A and 10 IU/g for enzyme-B. Release of chromophores is probably a better indicator of the kinetics of the enzyme attack on the pulp as reducing sugar will continue to be generated by xylanase hydrolysis of the soluble oligosaccharides. These oligosaccharides are released by the initial depolymerization of the xylan coating on the fiber surface [35]. The absorbance of filtrate at a wave length of 237 nm is due to release of phenolic compounds, absorbance at a wave length of 465 nm due to release of hydrophobic compounds [9, 39, 48, 53] and absorbance at a wave length of 280 nm due to the presence of lignin in the released colouring matter [53]. Beyond an enzyme dose of 5 IU/g for enzyme-A and 10 IU/g for enzyme-B, the increase in the absorbance at 280 nm supporting the observation by Ziobro that carbohydrate degradation products also attributes to the colouring matter [111, 112].

Both the enzyme prebleached pulps treated at different enzymes doses were subjected to alkali extraction with 2% NaOH (as such) at 70 ^{0}C temperature and10% consistency for 90 min in order to observe its impact on kappa number and viscosity of kraft-AQ pulp of *A. cadamba*. Enzyme-A shows a maximum reduction in kappa number (32.60%) at an enzyme dose of 5 IU/g. After that a slight decrease in kappa number reduction up to an enzyme dose of 25 IU/g is observed. On the other hand, enzyme-B shows a maximum reduction in kappa number i.e. 34.45% at an enzyme dose of 10 IU/g compared to untreated pulp. The maximum pulp viscosity 29.6 cps for enzyme-A at a dose of 5 IU/g and 29.8 cps for enzyme-B at a dose of 10 IU/g is observed which decreases further on increasing enzyme dose up to 25 IU/g. The extraction stage after enzymatic prebleaching is suggested to facilitate the dissolution of lignin-carbohydrate complex (LCC) in pulp that were previously modified by enzymes but

still remain in pulp because of their large molecular weight thus, alkaline treatment swells up the cellulose fiber and increases pore size. Alkaline extraction in combination with enzyme treatment was shown to improve pulp characteristics [21, 22].

The xylanase removes the low degree polymer i.e. xylan from the pulp and thus, improves the viscosity of treated pulp [100]. The reduction in viscosity of pulp at high enzyme dose indicates the presence of both endogluconases and cellobiohydrolase activities in crude enzyme preparation which attacks on amorphous region of cellulose [14]. However, it has been found that, the limited amount of cellulase improve the final properties of pulp. Bajpai *et al.,* also reported that the viscosity of bamboo kraft pulp after treatment with different commercial enzymes is increased [8]. At high enzyme doses there is no significant reduction in kappa number and increase in reducing sugars is observed. It means that at high enzyme dose, high concentration of end product acts as a catabolic repressor which inhibits the enzyme activity. Tenkanen *et al.,* reported that at higher dose of enzyme there was a minor effect on kappa number, reducing sugars and viscosity of birch wood kraft pulp [92].

5.3.2. Optimization of reaction time

Table 5.2 reveals the effect of reaction time varying from 30 to 360 min while keeping other variables constant i.e. enzyme dose 5 IU/g, temperature 75 ^{0}C and pH 7.5 for enzyme-A and enzyme dose 10 IU/g, temperature 65 ^{0}C and pH 8.0 for enzyme-B and 10% consistency in case of both the enzymes during kraft-AQ pulp prebleaching of *A. cadamba.* Reducing sugars released during enzymatic prebleaching with enzymes-A and B separately with different time intervals are found to increase with the increasing reaction time. Enzyme-A releases most of the chromophores and colour between a reaction time of 30 to 90 min and enzyme-B between a reaction time of 90-120 min and beyond that the release of chromophores and colour are not significant. Removal of the reprecipitated xylan by the action of endoxylanases increases the permeability of the fibers and elimination of lignin from pulp fiber, thus, reducing the kappa number of pulp and increasing the chromophores in

filtrate [65]. Similar trend of chromophore release as a result of xylanase pretreatment was reported by Beg *et al.*, [9], Khandparker and Bhosle [48], Shah *et al.*, [80] and Kulkarni and Rao [53].

Enzyme-A reduces maximum kappa number i.e. 32.50% at a reaction time of 90 min and enzyme-B 34.45% at a reaction time of 120 min of enzymatically prebleached kraft-AQ pulp of *A. cadamba* followed by alkali extraction. The maximum viscosity of kraft-AQ pulp of *A. cadamba* for enzyme-A (29.4 cps) and enzyme-B (29.8 cps) is observed at a reaction time of 90 and 120 min respectively. It may be because of the fact that the cellulase present in enzyme preparation requires longer time for full action on cellulose fiber than xylanase. A reaction time of 120 min was considered optimum by many other workers too for achieving the highest biobleaching effect on pulp [9, 35, 48, 80]. The enzyme dose and holding time are inter-related phenomenon. By increasing enzyme dose, for example, the same bleach boosting effect may be achieved in shorter time [8, 50]. Favis *et al.*, reported that the leaching of lignin increase with increasing the temperature [30] and the pH of medium [40].

5.3.3. Optimization of consistency

Table 5.3 describes the effect of consistencies varying from 2, 5, 10, 15 and 20% while keeping other conditions constant i.e. xylanase dose 5 IU/g, temperature 75^0C, reaction time 90 min at pH 7.5 for enzyme-A and xylanase dose 10 IU/g, temperature 65^0C, reaction time 120 min at pH 8.0 for enzyme-B during enzymatic pretreatment of kraft-AQ pulp of *A. cadamba*. The reducing sugars released during enzymatic prebleaching increase with increasing the pulp consistency up to 20% by both the enzyme preparations. The spectrophotometric analysis of filtrates at a wavelength of 237, 280 and 465 nm shows the presence of chromophore and colour which is generated by breakdown of LC bonds of pulp during enzyme treatment. The reducing sugars in filtrate after enzyme treatment are significantly greater than reported by other researchers [9, 35, 39]. It might be due to the presence of other hydrolytic enzymes (cellulase and mannanase) in enzyme preparation which

librates the reducing sugars, reacts with DNS reagent in same manner as xylose and increase the optical density.

Both the enzyme preparations i.e. enzyme-A and enzyme-B reduce the kappa number by 32.50 and 34.45% respectively at 10% consistency during enzymatically prebleaching of kraft-AQ pulp of *A. cadamba* followed by alkali extraction. There is no significant decrease in kappa number on further increase in consistency in both the cases. The viscosity of kraft-AQ pulp of *A. cadamba* is also observed maximum i.e. 29.4 cps for enzyme-A and 29.8 cps for enzyme-B at 10% of consistency. The cellulosic fibers when merged in water; contain mobile and immobile layers surrounding the fibers [64]. As the consistency of pulp increases the mobile layer is progressively eliminated leaving only the thin immobile layer enveloping the fiber, thus decreasing considerably the diffusion path length of reactant to the fiber [56]. Water layer thickness now becomes the rate determining step. However, the higher pulp consistency provides a close interaction between enzyme and pulp fiber. Christov and Prior also suggested that the higher pulp consistency provides a close relation between enzymes and pulp fibers probably because of the reduced volume of the liquid phase which facilitates the enzyme adsorption on the pulp and the subsequent more hydrolysis of hemicellulose [22]. Other researchers also reported 10% optimum consistency for enzyme pretreatment of pulp [9, 39, 65].

Based on above observation enzyme-A (followed by alkali extraction) reduces kappa number from 16 to 10.8, improves brightness 35 to 36.5 % (ISO) and viscosity from 27.8 to 29.4 cps. Similarly, enzyme-B (followed by alkali extraction) reduces kappa number from 16 to 10.5, improves brightness 35 to 36.2 % (ISO) and viscosity from 27.8 to 29.6 cps of unbleached kraft-AQ pulp of *A. cadamba*.

5.3.4 Conventional bleaching

Table 5.4 reveals the results and conditions of CEHH, ^{A}XECEHH and ^{B}XECEHH bleaching sequences on kraft-AQ pulp of *A. cadamba* of kappa number 16. The final brightness and viscosity of CEHH bleached kraft-AQ pulp of *A. cadamba* is 81.9 % (ISO) and 8.5 cps respectively at a chlorine demand of 4%. The prebleaching of kraft-AQ pulp of *A. cadamba* with enzyme-A and B, followed by alkali extraction separately prior to CEHH bleaching, improves the pulp brightness by 4.20 and 3.42% (ISO) and viscosity improves by 6.47 and 7.91% respectively compared to unbleached pulp of kappa number 16 (Figure 5.1). There is a gain in brightness of *A. cadamba* kraft-AQ pulp because enzyme-A and B improve the accessibility of bleaching chemicals by disrupting the xylan chain and thus, facilitates the easier removal of lignin during bleaching [79]. Bajpai and Bajpai evaluated different commercial enzymes for biobleaching of bamboo kraft pulp. They reported that the gain in final brightness under the same total chemical charge was 0.8 to 1.5% (ISO) units with different enzymes [8].

The number of PFI revolutions to achieve a beating level of 35 ^{0}SR of ^{A}XECEHH and ^{B}XECEHH bleached pulps of *A. cadamba* are less by 45.26 and 38.95 % respectively compared to CEHH bleached pulp. Figure 5.2 reveals the results of mechanical strength properties of CEHH, ^{A}XECEHH and ^{B}XECEHH bleaching sequences. The tensile index, burst index and double fold of *A. cadamba* improves by 4.78, 9.61 and 11.11% respectively for ^{A}XECEHH bleached pulp and 10.44, 13.25 and 19.44 % respectively for ^{B}XECEHH bleached pulp compared to CEHH bleaching. On the other hand, the tear index reduces by 10.77 and 6.15 % for ^{A}XECEHH and ^{B}XECEHH bleached pulps compared to control. The mechanical strength properties in case of ^{B}XECEHH bleached pulp are better than that of ^{A}XECEHH bleached pulp. However, the reduction in refining energy during beating of ^{B}XECEHH pulp is less than that of ^{A}XECEHH bleached pulp. It means that the enzyme-B is more effective compared to enzyme-A with respect to development of mechanical strength

properties and enhancement of pulp brightness. The cellulases present in both of the enzyme preparations i.e. enzyme-A and B play an important role in reduction of refining energy as well as improvement in mechanical strength properties of enzymatically treated pulps. Pulp fibrillation by cellulases was recognized as a means to enhance strength properties as early as 1959 by Bolaski and co-workers [12]. It was principally applied to cotton linters and other non-wood pulps. A process patented in 1968 used cellulases from a white-rot fungus, applied at a concentration of 0.1 to 1% (based on the dry wt of pulp), to reduce refining or beating time [109]. Another researcher also reported that xylanase from *Aspergillus niger* when applied on eucalyptus kraft pulp for a given degree of beating e.g. 30 ^{0}SR the number of revolutions was 1909 for the untreated pulp whereas the xylanase treated pulp required only 1595 revolutions [59]. Beg *et al.,* had also reported that the tensile strength and burst factor of eucalyptus kraft pulp improved by 63 and 8% respectively by pretreatment with xylanase produced from *Streptomyces* sp. QG-11-3 prior to CEHH bleaching sequence compared to control [9].

The reduction in AOX in the combined effluent of ^{A}XECEHH and ^{B}XECEHH bleaching sequences is 19.51 and 42.77% respectively compared to CEHH bleaching sequence. On the other hand, both COD and colour of combined effluent increase by 67.17 and 97.53% in case of ^{A}XECEHH bleaching and 58.16 and 83.95% in case of ^{B}XECEHH bleaching compared to control. The results are graphically represented in Figure 5.3. The significant reduction in AOX and increase in colour and COD of combined bleach effluent can be correlated with extraction of more lignin along with xylan by the joint action of ligninase and hemicellulolytic enzymes on the pulp. It has also been demonstrated by various scientists that several oxidative and reductive extracellular enzymes, like lignin peroxidases, manganese peroxidases, laccase and cellobiose: quinone oxidoreductase secreted by white rot fungi degrade the lignin [18, 75]. Senior and Hamilton achieved 40% reduction in AOX while 100% chlorine substituted by chlorine dioxide in case of softwood kraft pulp [79].

Table 5.5 reveals the results and conditions of CEHH, ^{A}XECEHH and ^{B}XECEHH bleaching sequences of kraft-AQ pulp of *A. cadamba* of kappa number 24. Figure 5.4 shows the final brightness and viscosity of CEHH bleached kraft-AQ pulp of *A. cadamba* is 79.9 % (ISO) and 8.2 cps respectively at a chlorine demand of 6%. The ^{A}XECEHH and ^{B}XECEHH bleaching sequences improve the pulp brightness by 9.63 and 10.47% (ISO) and viscosity reduces by 9.76 and 4.87% respectively compared to control. The improvement in brightness is found more in 24 kappa number pulp than 16 kappa number pulp. It is because 24 kappa number pulp has a fairly more xylan content than 16 kappa number pulp thus increasing kappa number offer increase enzyme benefit [95]. The PFI revolutions to get a beating level of 35 ^{0}SR in case of kraft-AQ pulp of *A. cadamba* (kappa number 24) bleached by ^{A}XECEHH and ^{B}XECEHH sequences at chlorine demand of 6% are slightly more than that kraft-AQ pulp of *A. cadamba* of kappa number 16 bleached by ^{A}XECEHH and ^{B}XECEHH bleaching sequences at chlorine demand of 4%. Similarly, the mechanical strength properties such as tensile index, burst index, double fold and tear index of kraft-AQ pulp of *A. cadamba* of kappa number 24 bleached by ^{A}XECEHH and ^{B}XECEHH sequences at chlorine demand of 6% are slightly lower than that kraft-AQ pulp of *A. cadamba* of kappa number 16 bleached by ^{A}XECEHH and ^{B}XECEHH bleaching sequences at chlorine demand of 4% (Figure 5.2 and 5.5). Figure 5.6 shows that the AOX, COD and colour in the combined effluent of ^{A}XECEHH and ^{B}XECEHH bleached pulp (kappa number 24 at total chlorine demand 6%) is on higher side than that of ^{A}XECEHH and ^{B}XECEHH bleached pulp, kappa number 16 at total chlorine demand 4% (Figure 5.6). Jeffrey *et al.*, reported that the pulp of kappa number of 22-26 exhibits more selectivity than that of 27-35 kappa umber pulps [44].

Table 5.6 shows the results and conditions of CEHH, ^{A}XECEHH and ^{B}XECEHH bleaching sequences at different chlorine demands of kraft-AQ pulp of *A. cadamba* of kappa number 16. Figure 5.7 shows that the brightness of ^{A}XECEHH and ^{B}XECEHH bleached pulps at 2% total chlorine demand reduces by 8.63 and 6.64 % respectively compared to ^{A}XECEHH

and ^{B}XECEHH bleached pulps at 4% total chlorine demand. On the other hand, viscosity of ^{A}XECEHH and ^{B}XECEHH bleached pulps at 2% total chlorine demand improves marginally compared to ^{A}XECEHH and ^{B}XECEHH bleached pulps at 4% total chlorine demand. The PFI revolutions to get a beating level of 35 ^{0}SR at chlorine demand of 2% are slightly more than that of pulp bleached at chlorine demand of 4%. Similarly, the mechanical strength properties such tensile index, burst index, double fold and tear index of ^{A}XECEHH and ^{B}XECEHH bleached pulps at 2% chlorine demand improve marginally compared to ^{A}XECEHH and ^{B}XECEHH bleached pulps at chlorine demand of 4% (Figure 5.8). The marginally improved viscosity and mechanical strength properties of ^{A}XECEHH and ^{B}XECEHH pulps at 2% a total chlorine demand may be due to the lower detrimental effect at lower chemical dose on carbohydrates during bleaching compared to ^{A}XECEHH and ^{B}XECEHH bleaching sequences bleached at 4% total chlorine demand.

The AOX of combined effluent of ^{A}XECEHH and ^{B}XECEHH pulps are 1.46 and 1.038 kg/T respectively at total chlorine demand of 4% which reduce to 43.83 and 38.34% in combined effluent of ^{A}XECEHH and ^{B}XECEHH bleached pulps respectively at a chlorine demand of 2%. The COD (2024 mg/L) and colour (1400 PTU) of combined effluent of ^{A}XECEHH bleached pulp at 4% total chlorine demand reduce to 39.03 and 27.71% respectively compared to combined effluent of ^{A}XECEHH bleached pulp at 2% total chlorine demand. In the similar way, the COD (2110 mg/L) and colour (1430 PTU) of combined effluent of ^{B}XECEHH bleached pulp at 4% total chlorine demand reduce to 40.48 and 30.77% respectively compared to combined effluent of ^{B}XECEHH bleached pulp at 2% total chlorine demand. The results are graphically represented in Figure 5.9. The reduction in AOX depends on the total chlorine demand of a bleaching sequence. Viikari *et al.,* observed 16.83% reduction in AOX after xylanase treatment of pulp having kappa 32 at reduced chlorine dose while Dunlop *et al.,* found AOX reduction by 33% in DE(OP)DE(P)D bleaching sequence at reduced chemical charge [27, 101]. The bleach plant effluent colour

also depends on the chlorine usage, a decrease in chlorine compounds also decrease the colour of effluent [28].

Table 5.7 reveals the results and conditions of ^{A}XECEHH bleaching sequence of kraft-AQ pulp of *A. cadamba* prebleached at different pH levels i.e.5-9 keeping the same chlorine demand i.e., 4%. Figure 5.10 shows that the brightness of ^{A}XECEHH bleached pulp decreases from 86.0 to 82.6 % (ISO) whereas; pulp viscosity improves from 7.3 to 8.3 cps when pH varies from 5 to 9 during enzymatic prebleaching with enzyme-A. Similarly, all the mechanical properties such as tensile index, burst index, double fold and tear index of ^{A}XECEHH bleached pulp of kraft-AQ pulp of *A. cadamba* improve with varying pH from 5 to 9 during enzymatic prebleaching with enzyme-A (Figure 5.11). The AOX in combined effluent of ^{A}XECEHH bleached pulp increases with increasing pH from 5 to 9. On the other hand, COD and colour of combined effluent of ^{A}XECEHH bleached pulp decreases with increasing pH. The results are graphically represented in Figure 5.12. In the similar way, kraft-AQ pulp of *A. cadamba* prebleached with enzyme-B at different pH levels (from 5 to 9) and 4% total chlorine demand during ^{B}XECEHH bleaching sequence (Table 5.8) follows the same pattern for optical and mechanical strength properties, viscosity and combined bleach effluent characteristics as in case of ^{A}XECEHH bleaching sequence. The results are graphically represented in Figure 5.13 to 5.15. It has been reported that the mechanical strength properties depends on the removal of xylan from pulp. Jeffries and Lins enzymatically removed about 33% of the xylan from aspen kraft pulp and TMPs using a specific microbial xylanase. In the case of kraft pulp, burst was unchanged while tear decreased by 17% and tensile strength decreased by 10% [45]. Li *et al.*, reported that pretreatment of wheat straw pulp with the xylanase from *Thermomyces lenuginosus* CBS 288.54 was also effective at an alkaline pH as high as 10.0 and reducing the chlorine consumption by 28.3% and improving the pulp brightness by 4.94% (ISO) with a slight decrease in tensile index (1.9%) and breaking length (2.8%) [57]. A number of researchers

showed that enzymatic pretreatment was effective at alkaline pH as high as 10.0 [53, 81, 82, 91].

5.3.5 Elemental chlorine free (ECF) bleaching

Table 5.9 reveals the results and conditions of ODED, $O^{A}XEDED$ and $O^{B}XEDED$ bleaching sequences of kraft-AQ pulp of *A. cadamba* of kappa number 16. An oxygen pressure of 5 kg/cm^2 reduces the kappa number by 43.75%, viscosity by 18.70 and improves the pulp brightness 10.10 % (ISO) of the unbleached pulp. The oxygen delignified pulp when treated with enzyme-A and B separately and followed by alkali extraction shows a reduction in kappa number (16.67 and 15.55% for enzyme-A and B respectively) and improvement in pulp brightness (7.2% for enzyme-A and 5.98% for enzyme-B) and viscosity (5.31% for enzyme-A and 5.98% for enzyme-B). Figure 5.16 reveals that the $O^{A}XEDED$ and $O^{B}XEDED$ bleached pulps show an improvement in final brightness 1.48 and 3.42 % (ISO) respectively. The viscosity of $O^{A}XEDED$ and $O^{B}XEDED$ bleached pulps improves marginally in case of both the bleaching sequences.

The $O^{A}XEDED$ and $O^{B}XEDED$ bleached pulps require 5.76 and 11.53% respectively more PFI revolutions compared to ODED bleached pulp to reach the target beating level of 35 ^{0}SR. It indicates that the enzyme preparation removes the lower DP xylan from the pulp and stuffy, crystalline cellulose fiber remains left in pulp. Secondly, xylan acts as lubricant during beating of pulp. It has been reported that the xylanase solubilizes more sugars from oxygen delignified pulp than brown stock pulps [2]. The results are also correlated with increased viscosity after oxygen delignification. The tensile index, burst index and double fold of $O^{A}XEDED$ bleached pulp shows an improvement of 1.48, 5.73 and 4.44% respectively and 3.42, 15.40 and 15.55% respectively improvement in $O^{B}XEDED$ bleached pulp compared to ODED bleached pulp. On the other hand, 8.95 and 2.98% reduction in tear index of $O^{A}XEDED$ and $O^{B}XEDED$ bleached pulps respectively is observed (Figure 5.17). This is because that the enzyme treatment improves the fibrillation during beating and tensile and

burst strength of paper primarily depends upon fibrillation. Dunlop and Gronberg [27] found improved strength properties of hardwood kraft pulp after xylanase treatment in ODE(OP)DE(P)D bleaching sequence.

Enzyme-A and B reduces AOX levels by 48.28 and 33.78% respectively in the combined effluent of $O^{A}XEDED$ and $O^{B}XEDED$ bleached pulps compared to ODED bleached pulp. Enzyme-A increases COD and colour in the combined effluent of $O^{A}XEDED$ bleaching sequences by 65.83 and 96% respectively and 76.66 and 106% respectively in the combined effluent of $O^{B}XEDED$ bleached pulps compared to ODED bleached pulp. The results are graphically represented in Figure 5.18. It is because the enzyme preparation contains sufficient amount of lignin peroxidase activity which oxidize the lignin structure of pulp exposed after removal of precipitated xylan by xylanase. The colour and COD is found to be increased significantly after enzyme treatment. It is due to the removal of more lignin and may be correlated with increased brightness of enzyme treated pulp. The increase in COD resulted due to the hydrolysis of hemicelluloses by enzyme treatment. The similar results are reported by Vidal *et al.,* [100]. Tolan and Thibault reported that the susceptibility of hardwood pulp is higher than oxygen delignified softwood pulp [96]. It has also been reported that the selectivity of softwood pulp is better than oxygen delignified softwood and hardwood pulp [44].

Table 5.10 reveals the results and conditions of ODEP, $O^{A}XEDEP$ and $O^{B}XEDEP$ bleaching sequences of kraft-AQ pulp of *A. cadamba* at kappa number 16. Figure 5.19 reveals that the final brightness improves by 1.89 and 3.10% respectively in case of $O^{A}XEDEP$ and $O^{B}XEDEP$ bleached kraft-AQ pulp of *A. cadamba* whereas; viscosity improves marginally in both the bleaching sequences compared to ODEP bleaching sequence. The PFI revolutions to achieve a beating level of 35 ^{0}SR, both the $O^{A}XEDEP$ and $O^{B}XEDEP$ bleached pulps respectively require 7.6 and 12.82% more revolutions compared to ODEP bleached pulp. The improvement in brightness of pulp bleached by ODEP, $O^{A}XEDEP$ and $O^{B}XEDEP$ bleaching

sequences is more and requires less PFI revolutions to achieve a beating level of 35 ^{0}SR compared to ODED, O^{A}XEDED and O^{B}XEDED bleaching sequences. All the mechanical strength properties such as tensile index, burst index, tear index and double fold of ODEP, O^{A}XEDEP and O^{B}XEDEP bleaching pulps of kraft-AQ pulp of *A. cadamba* show marginal improvement over ODED, O^{A}XEDED and O^{B}XEDED bleached pulps (Figure 5.20). Enzyme-A and B reduces AOX levels by 54.68 and 37.34% respectively in the combined effluent of O^{A}XEDEP and O^{B}XEDEP bleached pulps compared to ODEP bleached pulp. Enzyme-A increases COD and colour in the combined effluent of O^{A}XEDEP bleaching sequences by 44.44 and 51.85% respectively and 40.21 and 20.37% respectively in the combined effluent of O^{B}XEDEP bleached pulps compared to ODEP bleached pulp. The results are graphically represented in Figure 5.21. The xylanase treated pulp required more PFI revolutions due to removal of xylan. Xylanase generally solubilized more sugars, as well as more of the pulp xylan, from oxygen delignified pulp than from brown stock pulp [2, 3, 88]. Vidal *et al.*, also reported that the increase COD and colour in *Eucalyptus globules* kraft pulp was due to hydrolysis of xylan [100].

Table 5.11 reveals the results and conditions of ODEDP, O^{A}XEDEDP and O^{B}XEDEDP bleaching sequences of kraft-AQ pulp of *A. cadamba* at kappa number 16. Figure 5.22 shows that the final brightness of O^{A}XEDEDP pulp improves by 2.43% while viscosity decreases by 3.43%. Similarly, in O^{B}XEDEDP pulp brightness is increase by 4.13% and viscosity decrease by 5.56% compared to ODEDP bleached pulp. The final brightness and viscosity of ODEDP bleached pulp is 82.3% (ISO) and 9.90 cps respectively and shows an improvement in brightness and viscosity over ODEP and ODED bleaching sequences respectively. However, the PFI revolutions required to get a beating level of 35 ^{0}SR for ODEDP, O^{A}XEDEDP and O^{B}XEDEDP bleached pulps of *A. cadamba* are more or less same compared to ODED, O^{A}XEDED and O^{B}XEDED bleached pulps. Figure 5.23 shows that the mechanical strength properties follow the same pattern as in case of ODED bleaching

sequence. Tensile index, burst index and double fold number increases while tear is found to decrease in O^{A}XEDEDP and O^{B}XEDEDP bleached pulps. Enzyme-A and B reduces AOX levels by 61.15 and 31.33% respectively in the combined effluent of O^{A}XEDEDP and O^{B}XEDEDP bleached pulps compared to ODEDP bleached pulp. Enzyme-A increases COD and colour in the combined effluent of O^{A}XEDEP bleaching sequences by 87.00 and 84.78% respectively, and 61.11 and 28.26% respectively in the combined effluent of O^{B}XEDEDP bleached pulps compared to ODEDP bleached pulp (Figure 5.24).

5.3.6 Total chlorine free (TCF) bleaching

Table 5.12 reveals the results and conditions of OQPP, O^{A}XEQPP and O^{B}XEQPP bleaching sequences of kraft-AQ pulp of *A. cadamba* of kappa number 16. Figure 5.25 shows that final brightness of OQPP bleached pulp is 71.47 % (ISO) with pulp viscosity of 11.5 cps. The O^{A}XEDEDP and O^{B}XEDEDP bleached pulps improve the pulp brightness by 3.61 and 3.36% (ISO) and decrease the viscosity by 6.08 and 8.69% respectively compared to OQPP bleaching sequence. Table 5.12 also reveals that beating is easier with OQPP pulp than that of O^{A}XEDEDP and O^{B}XEDEDP bleached pulps. O^{A}XEQPP and O^{B}XEQPP bleached pulps require 20 and 12% more PFI revolutions to get a same beating level compared to untreated pulps.

Figure 5.25 indicates that the mechanical strength properties such as tensile index, burst index and double fold increases by 5.25, 8.54 and 17.9% respectively with enzyme-A treatment while tear index reduces by 4.63% compared to control. In the same way the tensile index, burst index and double fold increases by 1.69, 5.12 and 12.82% respectively with enzyme-B and tear index reduces by 3.97% with enzyme-B compared to untreated pulp (Figure 5.26).

The COD of combined effluent of O^{A}XEQPP and O^{B}XEQPP bleaching sequences increase 45.83 and 38.67% respectively compared to untreated pulp. Conversely, colour of combined

effluent of $O^{A}XEQPP$ and $O^{B}XEQPP$ bleaching sequences decrease by 7.16 and 15.54% respectively.

Biobleaching technology applications aspects in the field of pulp and paper have been studied and reviewed by a number of researchers. However, the complete mechanism of action of enzyme on pulp fiber and affecting factors is still under investigation. Buchert *et al.*, reported that the action of enzymes on pulps is also depending upon the electrochemical interaction between enzyme and pulp fibers. The surface charge and swelling of fibers affects the action of xylanase [17]. The carboxyl groups in the fiber cell wall are mainly responsible for the swelling property of pulp in the water. The accessibility of pulps towards the enzyme treatment is limited by surface area and porosity of pulp fibers, i.e. the median pore size of the fibers [105].

5.3.7 Scanning electron microscopy (SEM) of pulp

The scanning electron microscopic studies clearly shows that the enzyme, extracted from different strains of *Coprinellus disseminatus* causes great changes in the fiber surface. Plate 5.2 shows the enzyme treated pulp fiber with more porosity, more swelling, more open and rougher surface with more fibrillation which increase the accessibility of treated pulp to the subsequent chemicals. Whereas untreated pulp fiber shows even surface with less swelling and fibrillation (Plate 5.1). These observations indicate the action of xylanase and cellulase on fiber surface which cause the reduction in beating energy in terms of reduced revolution for the target ^{0}SR. Plate 5.3 shows the enzyme treated pulp fiber after oxygen delignification with smooth and compact surface. On the other hand, brown stock pulp after enzyme treatment shows more thickness, more swelling and more heterogeneous surface. The more compact surface lowers the accessibility of fiber to the enzyme and cause more beating energy in ECF and TCF compared to conventional bleached pulps. The observations are in accordance with the findings of other researchers [9, 35, 76].

Table 5.1: Optimization of enzyme dose for prebleaching of kraft-AQ pulp of *A. cadamba*

	Enzyme dose, IU/g	*Reduction in kappa number, %	*Change in viscosity, cps	Reducing sugars released, mg/g	Chromophores released, Optical density		
					237 nm	280 nm	465 nm
Enzyme -A	Control	8.04 ±0.06	28.0±0.10	3.50±0.02	0.050±0.001	0.087±0.004	0.065±0.003
	2	24.40 ±0.16	28.6±0.07	10.0±0.08	0.517±0.002	0.556±0.005	0.568±0.006
	5	32.60 ±0.23	29.6±0.11	11.60±0.15	0.765±0.004	0.769±0.003	0.798±0.005
	10	28.00 ±0.21	29.4±0.10	11.72±0.13	0.742±0.003	0.716±0.007	0.669±0.002
	15	23.20 ±0.18	29.1±0.09	12.00±0.12	0.616±0.005	0.665±0.006	0.666±0.004
	20	21.60 ±0.20	27.0±0.08	12.20±0.11	0.658±0.006	0.647±0.004	0.568±0.003
	25	21.00 ±0.18	24.0±0.10	12.51±0.09	0.554±0.004	0.565±0.003	0.549±0.006
Enzyme -B	Control	6.40 ±0.05	28.2±0.13	2.80±0.02	0.030±0.002	0.067±0.006	0.830±0.007
	2	25.50±0.21	28.4±0.11	10.20±0.07	0.183±0.002	0.276±0.002	0.405±0.005
	5	32.20 ±0.25	28.7±0.08	11.0±0.12	0.266±0.001	0.338±0.003	0.445±0.003
	10	34.45±0.20	29.8±0.14	12.90±0.19	0.734±0.004	0.657±0.005	0.673±0.003
	15	32.00 ±0.26	29.4±0.11	12.25±0.12	0.609±0.004	0.698±0.005	0.623±0.005
	20	31.80 ±0.22	28.9±0.09	12.50±0.15	0.677±0.005	0.658±0.003	0.698±0.003
	25	31.00 ±0.16	26.6±0.10	13.20±0.13	0.675±0.003	0.630±0.004	0.570±0.004

A = Enzyme from strain MLK01, B = Enzyme from strain MLK07
* = Values after alkali extraction with 2% NaOH at 70 ^{0}C temperature for 90 min and 10% consistency
Unbleached pulp kappa number = 16, Unbleached pulp viscosity = 27.8
Operational conditions:
Enzyme A = Reaction time 120 min, pH 7.5, consistency 10%, temperature 75 ^{0}C
Enzyme B= Reaction time 120 min , pH 8.0, consistency 10%, temperature 65 ^{0}C
± Refers to standard deviation

Table 5.2: Optimization of reaction time for prebleaching of kraft-AQ pulp of *A. cadamba*

	Reaction time, min.	*Reduction in kappa number, %	*Change in viscosity, cps	Reducing sugars released, mg/g	Chromophores released, Optical density		
					237 nm	280 nm	465 nm
Enzyme -A	30	18.24 ±0.12	28.0±0.10	10.5±0.07	0.517±0.004	0.556±0.003	0.568±0.007
	60	28.52 ±0.21	29.0±0.07	10.8±0.09	0.547±0.005	0.669±0.006	0.598±0.003
	90	32.50±0.22	29.4±0.08	11.53±0.15	0.727±0.005	0.746±0.005	0.783±0.004
	120	32.64 ±0.26	29.2±0.09	12.0±0.12	0.765±0.003	0.765±0.005	0.796±0.005
	240	334.12±0.20	28.5±0.08	12.9±0.12	0.658±0.006	0.647±0.003	0.568±0.006
	360	33.00±0.21	27.0±0.11	13.60±0.11	0.554±0.005	0.565±0.005	0.549±0.004
Enzyme -B	30	12.87 ±0.06	28.4±0.10	6.70±0.04	0.470±0.007	0.532±0.006	0.450±0.003
	60	23.0±0.14	28.8±0.09	11.50±0.08	0.620±0.006	0.657±0.003	0.657±0.005
	90	25.80 ±0.15	29.3±0.08	12.20±0.13	0.730±0.005	0.723±0.004	0.669±0.005
	120	34.45±0.20	29.8±0.14	12.90±0.19	0.734±0.004	0.657±0.005	0.673±0.003
	240	32.80±0.15	28.4±0.06	13.65±0.15	0.658±0.004	0.589±0.004	0.559±0.004
	360	33.30 ±0.16	27.6±0.09	14.0±0.14	0.654±0.003	0.565±0.003	0.512±0.002

A = Enzyme from strain MLK01, B = Enzyme from strain MLK07
* = Values after alkali extraction with 2% NaOH at 70 ^{0}C temperature for 90 min and 10% consistency
Unbleached pulp kappa number = 16, Unbleached pulp viscosity = 27.8
Operational conditions:
Enzyme A = Enzyme dose 5 IU/g of o.d. pulp, pH 7.5, consistency 10%, temperature 75 ^{0}C
Enzyme B = Enzyme dose 10 IU/g of o.d. pulp, pH 8.0, consistency 10%, temperature 65 ^{0}C
± Refers to standard deviation

Table 5.3: Optimization of consistency for prebleaching of kraft-AQ pulp of *A. cadamba*

	Consistency, %	*Reduction in Kappa number ,%	*Change in viscosity, cps	Reducing sugars released, mg/g	Chromophores released, Optical density		
					237 nm	280 nm	465 nm
Enzyme -A	2	17.00±0.07	27.9±0.08	4.60±0.02	0.315±0.001	0.347±0.001	0.202±0.002
	5	27.20±0.16	28.2±0.06	11.40±0.08	0.693±0.002	0.638±0.002	0.497±0.003
	10	32.50±0.22	29.4±0.08	11.53±0.15	0.727±0.005	0.746±0.005	0.783±0.004
	15	32.00±0.21	30.2±0.11	12.60±0.16	0.815±0.004	0.818±0.005	0.873±0.005
	20	24.80±0.17	30.1±0.09	13.50±0.12	0.880±0.003	0.875±0.004	0.815±0.006
Enzyme -B	2	14.80±0.08	28.0±0.08	6.50±0.04	0.211±0.001	0.343±0.003	0.122±0.002
	5	21.70±0.16	28.4±0.10	11.20±0.11	0.476±0.002	0.556±0.002	0.234±0.002
	10	34.45±0.20	29.8±0.14	12.90±0.19	0.734±0.004	0.657±0.005	0.673±0.003
	15	31.45±0.20	30.2±0.11	13.10±0.20	0.857±0.003	0.746±0.003	0.732±0.005
	20	21.20±0.16	29.8±0.10	13.72±0.17	0.880±0.005	0.732±0.006	0.716±0.004

A = Enzyme from strain MLK01, B = Enzyme from strain MLK07

* = Values after alkali extraction with 2% NaOH at 70^0C temperature for 90 min.

Unbleached pulp kappa number = 16, Unbleached pulp viscosity = 27.8

Operational conditions:

Enzyme A = Reaction time 90 min, pH 7.5, enzyme dose 5 IU/g of o.d. pulp, temperature 75 ^{0}C

Enzyme-B = Reaction time 180 min , pH 8.0, enzyme dose 10 IU/g of o.d. pulp, temperature 65 ^{0}C

± Refers to standard deviation

Table 5.4: Results and conditions of kraft-AQ pulp of *A. cadamba* of 16 kappa number during CEHH, ^{A}XECEHH and ^{B}XECEHH bleaching sequences

Particulars	CEHH	^{A}XECEHH	^{B}XECEHH
Unbleached kappa number	16±0.40		
Unbleached brightness, % (ISO)	35±0.32		
Unbleached viscosity, cps	27.8±0.26		
Enzyme treatment followed by alkali extraction with 2% NaOH (XE$_1$)			
Kappa number	16±0.20	10.8±0.12 (−32.60)	10.5±0.10 (−34.4)
Brightness, % (ISO)	35±0.32	36.5±0.23 (+4.2)	36.2±0.28 (+3.42)
Viscosity, cps	27.8±0.26	29.6±0.25 (+6.47)	30.0±0.32 (+7.91)
Total chlorine charged, % (as available Cl_2)	4.00	4.00	4.00
Chlorination (C) stage			
% Cl_2 applied as available Cl_2 (on o.d. pulp)	2.40	2.40	2.40
% Cl_2 consumed as available Cl_2 (on o.d. pulp)	2.38	2.12	2.11
Brightness, % (ISO)	46.4±0.32	50.6±0.33	51.9±0.31
Alkali extraction (E_2)			
% NaOH applied on o.d. pulp	1.20	1.20	1.20
Hypochlorite (H_1) stage			
% $Ca(OCl)_2$ applied as available Cl_2 (on o.d. pulp)	0.80	0.8	0.8
% $Ca(OCl)_2$ consumed as available Cl_2 (on o.d. pulp)	0.79	0.78	0.78
Hypochlorite (H_2) stage			
% $Ca(OCl)_2$ applied as available Cl_2 (on o.d. pulp)	0.80	0.8	0.8
% $Ca(OCl)_2$ consumed as available Cl_2 (on o.d. pulp)	0.78	0.75	0.76
Final brightness, % (ISO)	81.9±0.28	84.9±0.26 (+3.66)	85.27±0.22 (+4.11)
Final viscosity, cps	8.5±0.12	7.6±0.10 (-10.59)	7.9±0.08 (-7.06)
Pulp beating			
Number of revolutions to achieve 35 ^{0}SR	950	520 (-45.26)	580 (-38.95)
Mechanical strength properties			
Tensile index, Nm/g	56.5	59.2 (+4.78)	62.4 (+10.44)
Burst index, kPa m^2/g	5.81	6.38 (+9.61)	6.58 (+13.25)
Tear index, mNm^2/g	6.5	5.8 (-10.77)	6.1 (-6.15)
Double fold, number	36	40 (+11.11)	43 (+19.44)
Combined bleach effluent characteristics			
AOX, kg/T	1.814	1.460 (-19.51)	1.038 (-42.77)
COD, mg/L	999	1670 (+67.17)	1580 (+58.16)
Colour, PTU	810	1600 (+97.53)	1490(+83.95)

Bleaching conditions

Particulars	Temperature, ^{0}C	Reaction time, min	Initial pH	Consistency, %	Doses
C- stage	Ambient	45	2.5	3	2.4%
E- stages	70±2	90	11.4	10	1.2%
H_1& H_2- stages	70±2	90	11.3	10	0.8%
Enzyme-A	75±2	90	7.5	10	5 IU/g
Enzyme-B	65±2	180	8.0	10	10 IU/g

A = Enzyme extracted from strain MLK01, B= Enzyme extracted from strain MLK07
+/□ = % difference compared to control pulp
± Refers to standard deviation

Table 5.5: Results and conditions of kraft-AQ pulp of *A. cadamba* of 24 kappa number during CEHH, ^{A}XECEHH and ^{B}XECEHH bleaching sequences

Particulars	CEHH	^{A}XECEHH	^{B}XECEHH
Unbleached kappa number	24±0.44		
Unbleached brightness, % (ISO)	33±0.30		
Unbleached viscosity, cps	28.8±0.25		
Enzyme treatment followed by alkali extraction with 2% NaOH (XE_1)			
Kappa number	24±0.44	16.1±0.41(−32.91)	16.0±0.34 (−33.33)
Brightness, % (ISO)	33±0.30	34.0±0.24(+3.03)	34.2±0.28 (+3.63)
Viscosity, cps	28.8±0.25	30.9±0.26 (+7.29)	31.0±0.23 (+7.63)
Total chlorine charged, % (as available Cl_2)	6.00	6.00	6.00
Chlorination (C) stage			
% Cl_2 applied as available Cl_2 (on o.d. pulp basis)	3.60	3.60	3.60
% Cl_2 consumed as available Cl_2 (on o.d. pulp basis)	3.58	3.43	3.40
Brightness, % (ISO)	44.4	51.6	52.9
Alkali extraction (E_2)			
% NaOH applied on o.d. pulp basis	1.80	1.80	1.80
Hypochlorite (H_1) stage			
% $Ca(OCl)_2$ applied as available Cl_2 (on o.d. pulp basis)	1.20	1.20	1.20
a% $Ca(OCl)_2$ consumed as available Cl_2 (on o.d. pulp basis)	1.18	1.15	1.12
Hypochlorite (H_2) stage			
% $Ca(OCl)_2$ applied as available Cl_2 (on o.d. pulp basis)	1.20	1.20	1.20
% $Ca(OCl)_2$ consumed as available Cl_2 (on o.d. pulp basis)	1.16	1.13	1.10
Final brightness, % (ISO)	79.9±0.24	86.7±0.27 (+9.63)	88.27±0.20 (+10.47)
Final viscosity, cps	8.2±0.05	7.4±0.06 (−9.76)	7.8±0.08 (−4.87)
Number of revolutions to achieve 35 ^{0}SR	900	550 (−38.89)	600 (−33.33)
Mechanical strength properties			
Tensile index, Nm/g	56.1	58.4 (+4.09)	61.2 (+9.09)
Burst index, kPa m^2/g	5.76	6.22 (+7.99)	6.42 (+11.45)
Tear index, mNm^2/g	6.4	5.5 (−14.06)	5.8 (−9.37)
Double fold, number	32	35 (+9.37)	39 (+21.87)
Combined bleach effluent characteristics			
AOX, kg/T	2.394	1.338 (-44.11)	1.114 (-53.46)
COD, mg/L	1199	1760 (+46.78)	1950 (+62.63)
Colour, PTU	1490	1980 (+32.88)	1760 (+18.12)

Bleaching conditions

Particulars	Temperature, ^{0}C	Reaction time, min	Initial pH	Consistency, %	Dose
C- stage	Ambient	45	2.5	3	3.6%
E_1, E_2 -stages	70±2	90	11.4	10	1.8%
H_1, H_2 -stages	70±2	90	11.3	10	1.2%
Enzyme-A	75±2	90	7.5	10	5 IU/g
Enzyme-B	65±2	180	8.0	10	10 IU/g

A = Enzyme extracted from strain MLK01, B= Enzyme extracted from strain MLK07
+/□ = % difference compared to control pulp
± Refers to standard deviation

Table 5.6: Results and conditions of kraft-AQ pulp of *A. cadamba* of 16 kappa number during CEHH, ^{A}XECEHH and ^{B}XECEHH bleaching sequences at different chlorine demands

Particulars	^{A}XECEHH	^{A}XECEHH	^{B}XECEHH	^{B}XECEHH
Unbleached kappa number	16±0.40			
Unbleached brightness, % (ISO)	35±0.32			
Unbleached viscosity, cps	27.8±0.26			
Enzyme treatment followed by alkali extraction with 2% NaOH (XE_1)				
Kappa number	10.8±0.12	10.8±0.12	10.5±0.10	10.5±0.10
Brightness, % (ISO)	36.5±0.23	36.5±0.23	36.2±0.28	36.2±0.28
Viscosity, cps	29.6±0.25	29.6±0.25	30.0±0.32	30.0±0.32
Total chlorine charged, % (as available Cl_2)	4.00	2.00	4.00	2.00
Chlorination (C) stage				
% Cl_2 applied as available Cl_2 (on o.d. pulp)	2.40	1.20	2.40	1.20
% Cl_2 consumed as available Cl_2 (on o.d. pulp)	2.12	1.19	2.11	1.20
Kappa number				
Brightness, % (ISO)	50.6	43.90	51.9	45.20
Alkali extraction (E_2) stage				
% NaOH applied on o.d. pulp basis	1.20	1.20	1.20	1.20
Hypochlorite (H_1) stage				
% $Ca(OCl)_2$ applied as available Cl_2 (on o.d. pulp)	0.80	0.40	0.80	0.40
% $Ca(OCl)_2$ consumed as available Cl_2 (on o.d. pulp)	0.78	0.40	0.72	0.40
Hypochlorite (H_2) stage				
% $Ca(OCl)_2$ applied as available Cl_2 (on o.d. pulp)	0.80	0.40	0.80	0.40
% $Ca(OCl)_2$ consumed as available Cl_2 (on o.d. pulp)	0.75	0.40	0.76	0.40
Final brightness, % (ISO)	84.9±0.23	77.57±0.20(−8.6)	85.27±0.26	79.6±0.22 (−6.6)
Final viscosity, cps	7.6 ±0.05	7.8±0.06	7.9±0.06	8.1 ±0.07
Number of revolutions to achieve 35 ^{0}SR	520	550	580	600
Mechanical strength properties				
Tensile index, Nm/g	59.2	59.8	62.0	62.5
Burst index, kPa m^2/g	6.38	6.50	6.58	6.67
Tear index, mNm^2/g	5.8	6.2	6.1	6.3
Double fold, number	40	42	43	43
Combined bleach effluent characteristics				
AOX, kg/T	1.460	0.820 (−43.83)	1.038	0.640 (−38.34)
COD, mg/L	2024	1234 (−39.03)	2110	1250 (−40.48)
Colour, PTU	1400	1012 (−27.71)	1430	990 (−30.77)

Bleaching conditions

Particulars	Temperature, ^{0}C	Reaction time, min	Initial pH	Consistency, %	Dose
C-stage	Ambient	45	2.5	3	2.4%
E- stages	70±2	90	11.4	10	1.2%
H_1& H_2 -stages	70±2	90	11.3	10	0.8%
Enzyme-A	75±2	90	7.5	10	5 IU/g
Enzyme-B	65±2	180	8.0	10	10 IU/g

A = Enzyme extracted from strain MLK01, B= Enzyme extracted from strain MLK07
+/□ = % difference compared to 4% chlorine demand
± Refers to standard deviation

Table 5.7: Results and conditions of kraft- AQ pulp of *A. cadamba* of 16 kappa number during CEHH and ^{A}XECEHH bleaching sequences at different pH levels

Particulars	^{A}XECEHH			
Unbleached kappa number	16±0.40			
Unbleached brightness, % (ISO)	35±0.32			
Unbleached viscosity, cps	27.8±0.26			
Enzymatic treatment at different pH levels followed by alkali extraction with 2% NaOH (XE_1)	**5.0**	**6.0**	**8.0**	**9.0**
Kappa number	10.4±0.42	10.5±0.41	10.8±0.38	11.0±0.34
Brightness, % (ISO)	37.2±0.34	36.0±0.38	35.4±0.30	35.2±0.35
Viscosity, cps	28.8±0.22	29.0±0.20	29.9±0.24	30.2±0.21
Total chlorine charged, % (as available Cl_2)	4.00	4.00	4.00	4.00
Chlorination (C) stage				
% Cl_2 applied as available Cl_2 (on o.d. pulp)	2.40	2.40	2.40	2.40
% Cl_2 consumed as available Cl_2 (on o.d. pulp)	2.11	2.09	2.08	2.09
Brightness, % (ISO)	52.1	52.0	50.2	48.3
Alkali extraction (E_2) stage				
% NaOH applied (on o.d. pulp)	1.20	1.20	1.20	1.20
Hypochlorite (H_1) stage				
% $Ca(OCl)_2$ applied as available Cl_2 (on o.d. pulp)	0.8	0.8	0.80	0.80
% $Ca(OCl)_2$ consumed as available Cl_2 (on o.d. pulp)	0.73	0.73	0.74	0.78
Hypochlorite (H_2) stage				
% $Ca(OCl)_2$ applied as available Cl_2 (on o.d. pulp)	0.8	0.8	0.8	0.80
% $Ca(OCl)_2$ consumed as available Cl_2 (on o.d. pulp)	0.68	0.70	0.71	0.68
Final brightness, % (ISO)	86.0±0.26	85.2±0.20	84.0±0.21	82.6±0.23
Final viscosity, cps	7.3±0.08	7.5±0.05	7.8±0.03	8.3±0.07
Number of revolutions to achieve 35 ^{0}SR	480	470	460	445
Mechanical strength properties				
Tensile index, Nm/g	55.67	56.80	62.14	65.48
Burst index, kPa m^2/g	4.20	5.08	5.18	5.20
Tear index, mNm^2/g	5.3	5.5	5.8	6.0
Double fold, number	25	30	35	40
Combined bleach effluent characteristics				
AOX, kg/T	1.621	1.532	1.446	1.315
COD, mg/L	1760	1710	1480	1430
Colour, PTU	1440	1370	1220	1160

Bleaching conditions

Particulars	Temperature, ^{0}C	Reaction time, min	Initial pH	Consistency, %	Dose
C- stage	Ambient	45	2.5	3	2.4%
E- stages	70±2	90	11.4	10	1.2%
H_1 & H_2 stages	70±2	90	11.3	10	0.8%
Enzyme-A	75±2	90	7.5	10	5 IU/g

A = Enzyme extracted from strain MLK01
± Refers to standard deviation

Table 5.8: Results and conditions of kraft- AQ pulp of *A. cadamba* of 16 kappa number at different pH levels during CEHH and ^{B}XECEHH bleaching sequences

Particulars	Results			
Unbleached kappa number	16±0.40			
Unbleached brightness, % (ISO)	35±0.32			
Unbleached viscosity, cps	27.8±0.26			
Enzymatic treatment at different pH levels followed by alkali extraction with 2% NaOH (XE_1)	**^{B}XECEHH**			
	5.0	**6.0**	**8.0**	**9.0**
Kappa number	10.2±0.33	10.4±0.34	10.5±0.42	10.8±0.35
Brightness, % (ISO)	36.4±0.30	35.8±0.36	35.2±0.31	34.8±0.32
Viscosity, cps	28.6±0.22	29.4±0.20	30.2±0.26	31.0±0.27
Total chlorine demand, % (as available Cl_2)	4.00	4.00	4.00	4.00
Chlorination (C) stage				
% Cl_2 applied as available Cl_2 (on o.d. pulp)	2.40	2.40	2.40	2.40
% Cl_2 consumed as available Cl_2 (on o.d. pulp)	2.10	2.06	2.04	2.06
Brightness, % (ISO)				
Alkali extraction (E_2) stage				
% NaOH applied (on o.d. pulp)	1.20	1.20	1.20	1.20
Hypochlorite (H_1) stage				
% $Ca(OCl)_2$ applied as available Cl_2 (on o.d. pulp)	0.8	0.8	0.80	0.80
% $Ca(OCl)_2$ consumed as available Cl_2 (on o.d. pulp)	0.71	0.73	0.75	0.77
Hypochlorite (H_2) stage				
% $Ca(OCl)_2$ applied as available Cl_2 (on o.d. pulp)	0.8	0.8	0.8	0.80
% $Ca(OCl)_2$ consumed as available Cl_2 (on o.d. pulp)	0.69	0.71	0.72	0.70
Final brightness, % (ISO)	85.6±0.20	85.4±0.26	84.2±0.22	82.8±0.18
Final viscosity, cps	7.8±0.06	8.0±0.05	8.3±0.07	8.4±0.03
Number of revolutions to achieve 35 ^{0}SR	450	470	495	525
Mechanical strength properties				
Tensile index, Nm/g	56.67	56.85	62.25	64.40
Burst index, kPa m^2/g	5.20	6.08	6.58	6.80
Tear index, mNm^2/g	5.9	5.6	5.4	5.3
Double fold, number	39	40	45	50
Combined bleach effluent characteristics				
AOX, kg/T	1.45	1.32	1.11	1.03
COD, mg/L	1575	1410	1380	1330
Colour, PTU	1350	1320	1225	1150

Bleaching conditions

Particulars	Temperature, ^{0}C	Reaction time, min	Initial pH	Consistency, %	Dose
C-stage	Ambient	45	2.5	3	2.4%
E_1 & E_2- stages	70±2	90	11.4	10	1.2%
H_1& H_2 stages	70±2	90	11.3	10	0.8%
Enzyme-B	65±2	180	8.0	10	10 IU/g

B = Enzyme extracted from strain MLK07
+/□ = % difference compared to control pulp
± Refers to standard deviation

Table 5.9: Results and conditions of kraft- AQ pulp of *A. cadamba* during ODED, O^AXEDED and O^BXEDED bleaching sequences

Particulars	ODED	O^AXEDED	O^BXEDED
O_2 delignification (O) stage			
O_2 pressure, kg/cm^2	5		
NaOH applied, % (o.d. pulp)	2.0		
$MgSO_4$ applied, % (o.d. pulp)	0.2		
Kappa number	9.9±0.30		
Brightness, % (ISO)	45.1±0.28		
Viscosity, cps	22.6±0.22		
Enzyme treatment followed by alkali extraction with 2% NaOH (OXE_1)			
Kappa number	9.9±0.30	8.25±0.23(–16.67)	8.36±0.20(–15.55)
Brightness, % (ISO)	45.1±0.38	48.35±0.34(+7.2)	47.80±0.35(+5.98)
Viscosity, cps	22.6±0.22	23.8±0.20(+5.31)	25.6±0.22(+13.27)
Chlorine dioxide (D_1) stage			
% ClO_2 applied as available Cl_2 (on o.d. pulp)	1.8	1.8	1.8
% ClO_2 consumed as available Cl_2 (on o.d. pulp)	1.8	1.57	1.66
Alkali extraction (E_2) stage			
NaOH applied, %	1.0	1.0	1.0
Chlorine dioxide (D_2) stage			
% ClO_2 applied as available Cl_2 (on o.d. pulp)	1.2	1.2	1.2
% ClO_2 consumed as available Cl_2 (on o.d. pulp)	1.16	1.15	1.14
Final brightness, % (ISO)	72.5±0.18	73.35±0.13(+1.17)	73.95±0.15(+2.00)
Final viscosity, cps	9.98±0.05	9.50±0.10(–4.80)	9.25±0.08(□7.44)
Number of revolutions to achieve 35 ^{0}SR	2600	2750(+5.76)	2900(+11.53)
Mechanical strength properties			
Tensile index, Nm/g	74.2	75.3(+1.48)	76.74(+3.42)
Burst index, kPa m^2/g	6.10	6.45(+5.73)	7.04(+15.40)
Tear index, mNm^2/g	6.7	6.1(–8.95)	6.5(–2.98)
Double fold, number	45	47(+4.44)	52(+15.55)
Combined bleach effluent characteristics			
AOX, kg/T	0.876	0.453 (–48.28)	0.580 (-33.78)
COD, mg/L	600	995(+65.83)	1060 (+76.66)
Colour, PTU	500	980(+96.00)	1030 (+106.0)

Bleaching conditions

Particulars	Enzyme-A	Enzyme-B	O	E	D_1&D_2
pH	7.5	8.0	11.0	11.4	2.5
Consistency	10	10	15	10	7
Retention time, min	90	180	90	90	120
Temperature, ^{0}C	75±2	65±2	110±2	70±2	70±2
Dose	5 IU/g	10 IU/g	5 kg/cm^2	1.0%	1.8 & 1.2%

Unbleached pulp kappa number = 16, Pulp viscosity = 27.8, Pulp brightness, % (ISO) = 35

A = Enzyme extracted from strain MLK01, B= Enzyme extracted from strain MLK07

+/□ = % difference compared to control pulp

± Refers to standard deviation

Table 5.10: Results and conditions of kraft- AQ pulp of *A. cadamba* during ODEP, O^AXEDEP and O^BXEDEP bleaching sequences

Particulars	ODEP	O^AXEDEP	O^BXEDEP
O_2 delignification (O) stage			
O_2 pressure, kg/cm^2	5.0		
NaOH applied, % on o.d. pulp basis	2.0		
$MgSO_4$ applied, % on o.d. pulp basis	0.2		
Kappa number	9.9±0.30		
Brightness, % ISO	45.1±0.28		
Viscosity, cps	22.6±0.22		
Enzyme treatment followed by alkali extraction with 2% NaOH (OXE)			
Kappa number	9.9±0.30	8.25±0.23(–16.67)	8.36±0.20(–15.55)
Brightness, % ISO	45.1±0.38	48.35±0.34(+7.2)	47.80±0.35(+5.98)
Viscosity, cps	22.6±0.22	23.8±0.20(+5.31)	25.6±0.22(+13.27)
Chlorine dioxide (D) stage			
% ClO_2 applied as available Cl_2 (on o.d. pulp)	2.0	2.0	2.0
% ClO_2 consumed as available Cl_2 (on o.d. pulp)	1.88	1.85	1.81
Alkali extraction (E_2)	1.0	1.0	1.0
Peroxide (P) stage			
%, Applied on o.d. pulp	1.50	1.50	1.50
% , Consumed on o.d. pulp	1.49	1.45	1.43
Final brightness, % (ISO)	81.3±0.22	83.36±0.20(+2.52)	83.83±0.18(+3.11)
Final viscosity, cps	10.1±0.05	9.6±0.02(–4.95)	9.4±0.03(□6.93)
Number of revolutions to achieve 35 ^{0}SR	1950	2100(+7.6)	2200(+12.82)
Mechanical strength properties			
Tensile index, Nm/g	75.36	76.79(+1.89)	77.70(+3.10)
Burst index, kPa m^2/g	6.35	6.64(+4.57)	6.76(+6.46)
Tear index, mNm^2/g	6.9	6.5(–5.79)	6.6(–4.37)
Double fold, number	68	72(+5.88)	80(+17.64)
Combined bleach effluent characteristics			
AOX, kg/T	0.790	0.358 (-54.68)	0.495 (-37.34)
COD, mg/L	550	990 (+44.44)	920(+40.21)
Colour, PTU	540	820 (+51.85)	650 (+20.37)

Bleaching conditions

Particulars	Enzyme-A	Enzyme-B	E	O	D	P
pH	7.5	8.0	10.6	11.0	2.5	11.2
Consistency, %	10	10	10	15	7	10
Retention time, min	90	180	90	90	120	120
Temperature, ^{0}C	75±2	65±2	70±2	110±2	70±2	80±2
Dose	5 IU/g	10 IU/g	1.0%	5 kg/cm^2	2%	1.5%

Unbleached pulp kappa number = 16, Pulp viscosity = 27.8, Pulp brightness, % (ISO) = 35,
A = Enzyme extracted from strain MLK01, B= Enzyme extracted from strain MLK07
+/□ = % difference compared to control pulp
± Refers to standard deviation

Table 5.11 Results and conditions of kraft- AQ pulp of *A. cadamba* during ODEDP, OAXEDEDP and $O^BXEDEDP$ bleaching sequence

Particulars	ODEDP	$O^AXEDEDP$	$O^BXEDEDP$
O_2 delignification (O stage)			
O_2 pressure, kg/cm^2		5	
NaOH applied, %		2.0	
$MgSO_4$ applied, %		0.2	
Kappa number		9.9±0.30	
Brightness, % (ISO)		45.1±0.28	
Viscosity, cps		22.6±0.22	
Enzyme treatment followed by alkali extraction with 2% NaOH (OXE_1)			
Kappa number	9.9±0.30	8.25±0.23(–16.67)	8.36±0.20(–15.55)
brightness, % (ISO)	45.1±0.38	48.35±0.34(+7.2)	47.80±0.35(+5.98)
Viscosity, cps	22.6±0.22	23.8±0.20(+5.31)	25.6±0.22(+13.27)
Chlorine dioxide (D_1) stage			
% ClO_2 applied as available Cl_2 (on o.d. pulp)	1.00	1.00	1.00
% ClO_2 consumed as available Cl_2 (on o.d. pulp)	1.0	0.94	0.95
Alkali extraction stage (E_2)			
% NaOH applied (on o.d. pulp)	1.0	1.0	1.0
Chlorine dioxide (D_2) stage			
% ClO_2 applied as available Cl_2 (on o.d. pulp)	1.00	1.00	1.00
% ClO_2 consumed as available Cl_2 (on o.d. pulp)	0.99	0.86	0.87
Peroxide (P) stage			
% Applied on o.d. pulp	1.50	1.50	1.50
% Consumed on o.d. pulp	1.35	1.28	1.25
Final brightness, % (ISO)	82.3±0.30	84.3±0.27(+2.43)	85.70±0.28(+4.13)
Final viscosity, cps	9.90±0.08	9.56±0.04(–3.43)	9.35±0.06(□5.56)
Number of revolutions to achieve 35 ^{0}SR	2700	2800(+3.7)	3000(+11.11)
Mechanical strength properties			
Tensile index, Nm/g	74.99	75.92(+1.24)	75.15(+0.21)
Burst index, kPa m^2/g	6.15	6.25(+1.62)	6.20(+0.81)
Tear index, mNm^2/g	6.65	6.45(–3.00)	6.50(–2.25)
Double fold, number	47	49(+4.25)	48(+2.13)
Combined bleach effluent characteristics			
AOX, kg/T	0.8370	0.3251 (-61.15)	0.5747 (-31.33)
COD, mg/L	540	1010 (+87.00)	870(+61.11)
Colour, PTU	460	850 (+84.78)	590 (+28.26)

Bleaching conditions

Particulars	Enzyme-A	Enzyme-B	E	O	D_1&D_2	P
pH	7.5	8.0	10.6	11.0	2.5	11.2
Consistency, %	10	10	10	15	7	10
Retention time, min.	90	180	90	90	120	120
Temperature, ^{0}C	75±2	65±2	70±2	110±2	70±2	80±2
Dose	5 IU/g	10 IU/g	1.0%	5 kg/cm^2	2%	1.5%

Unbleached pulp kappa number = 16, Pulp viscosity = 27.8, Pulp brightness, % (ISO) = 35,
A = Enzyme extracted from strain MLK01, B= Enzyme extracted from strain MLK07
+/□ = % difference compared to control pulp
± Refers to standard deviation

Table 5.12 Results and conditions of kraft- AQ pulp of *A. cadamba* during OQPP, O^AXEQPP and O^BXEQPP bleaching sequences

Particulars	OQPP	O^AXEQPP	O^BXEQPP
O_2 delignification (O) stage			
O_2 pressure, kg/cm^2	5		
NaOH applied, %	2.0		
$MgSO_4$ applied, %	0.2		
Kappa number	9.9±0.30		
Brightness, % (ISO)	45.1±0.28		
Viscosity, cps	22.6±0.22		
Enzyme treatment followed by alkali extraction with 2% NaOH (OXE_1)			
Kappa number	9.9±0.30	8.25±0.23(–16.67)	8.36±0.20(–15.55)
Brightness, % (ISO)	45.1±0.38	48.35±0.34(+7.2)	47.80±0.35(+5.98)
Viscosity, cps	22.6±0.22	23.8±0.20(+5.31)	25.6±0.22(+13.27)
Chelating stage (Q)			
EDTA charged, %	1.0	1.0	1.0
Total peroxide charge, %	3.0	3.0	3.0
Peroxide stage (P_1)			
% Applied on o.d. pulp	1.50	1.50	1.50
% Consumed on o.d. pulp	1.49	1.47	1.45
Peroxide stage (P_2)			
% Applied on o.d. pulp	1.50	1.50	1.50
% Consumed on o.d. pulp	1.46	1.18	1.18
Final brightness, % ISO	71.47±0.30	73.87±0.22(+3.36)	74.05±0.25(+3.61)
Final viscosity, cps	11.5±0.08	10.8±0.05(–6.08)	10.5±0.04(–8.69)
Number of revolutions to achieve 35 ^{0}SR	2500	3000(+20.00)	2800(+12.00)
Mechanical strength properties			
Tensile index, Nm/g	62.55	65.84(+5.25)	63.61(+1.69)
Burst index, kPa m^2/g	5.85	6.35(+8.54)	6.15(+5.12)
Tear index, mNm^2/g	7.55	7.20(–4.63)	7.25(–3.97)
Double fold, number	39	46(+17.9)	44(+12.82)
Combined bleach effluent characteristics			
COD, mg/L	650	1200 (+45.83)	1060 (+38.67)
Colour, PTU	1480	1374 (-7.16)	1250 (-15.54)

Bleaching conditions

Particulars	Enzyme-A	Enzyme-B	O	Q	P
pH	7.5	8.0	10	5.5	11
Consistency, %	10	10	15	3	10
Retention time, min	90	180	90	30	120
Temperature, ^{0}C	75±2	65±2	110±2	ambient	80±2
Dose	5 IU/g	10 IU/g	5 kg/cm^2	1.0%	2.0%

Unbleached pulp kappa number = 16, Pulp viscosity = 27.8, Pulp brightness, % (ISO) = 35,
A = Enzyme extracted from strain MLK01, B= Enzyme extracted from strain MLK07
+/□ = % difference compared to control pulp
± Refers to standard deviation

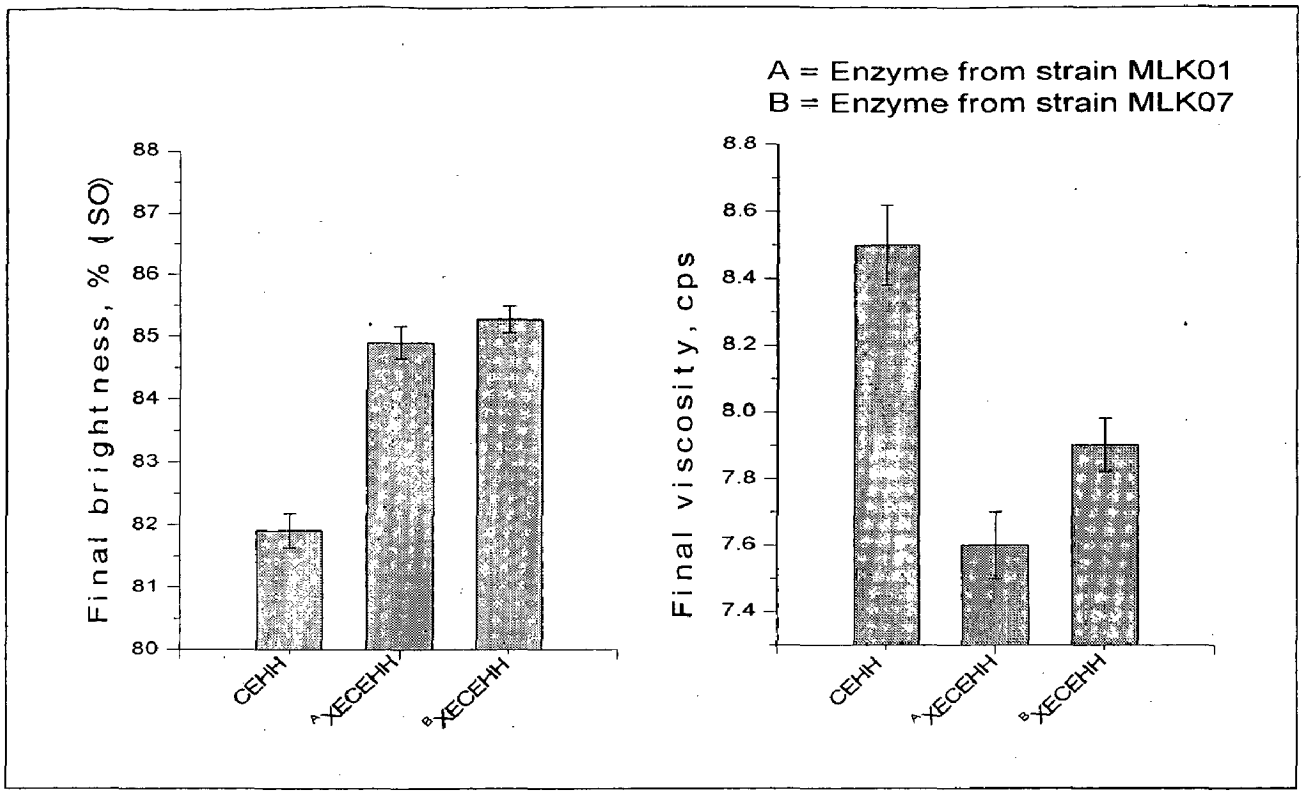

Figure 5.1: Effect of enzymatic prebleaching on final brightness and viscosity on kraft-AQ pulp of *A. cadamba* (kappa number 16) during CEHH, ^{A}XECEHH and ^{B}XECEHH bleaching sequences

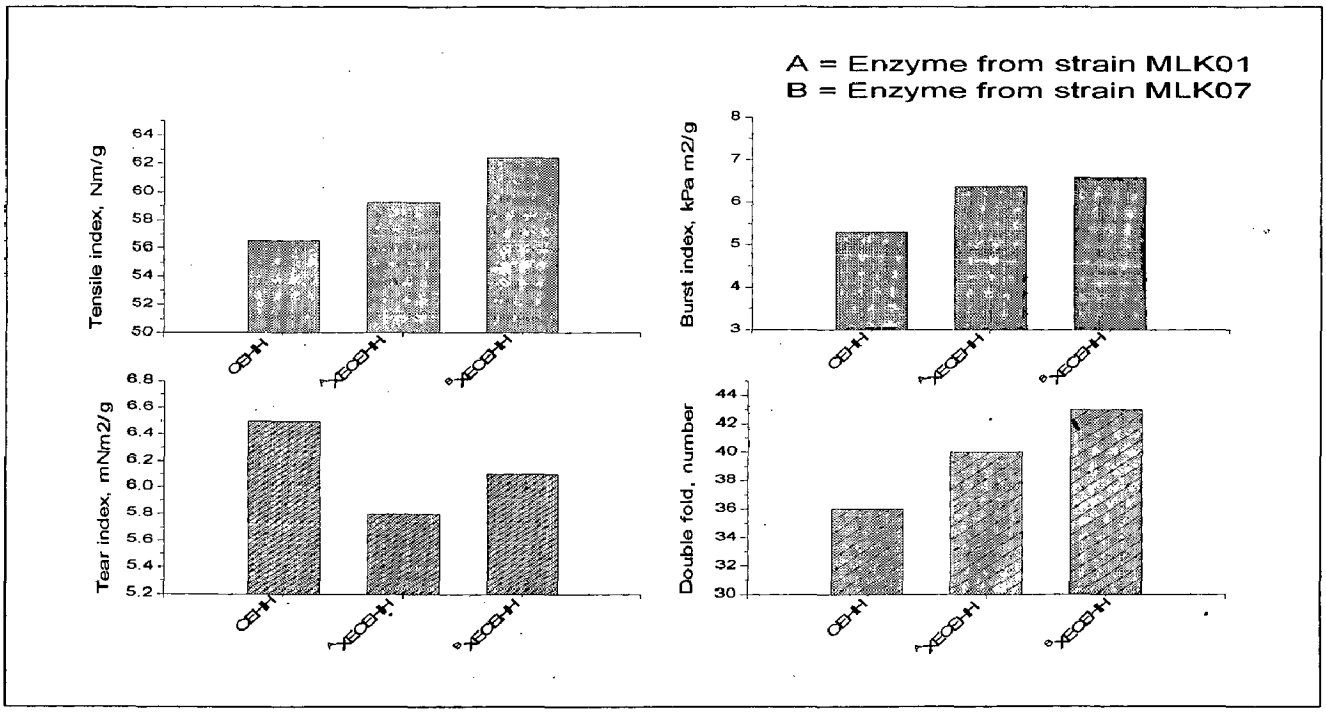

Figure 5.2: Effect of enzyme prebleaching on mechanical strength properties of kraft-AQ pulp of *A. cadamba* (kappa number 16) during CEHH, CEHH, ^{A}XECEHH and ^{B}XECEHH bleaching sequences

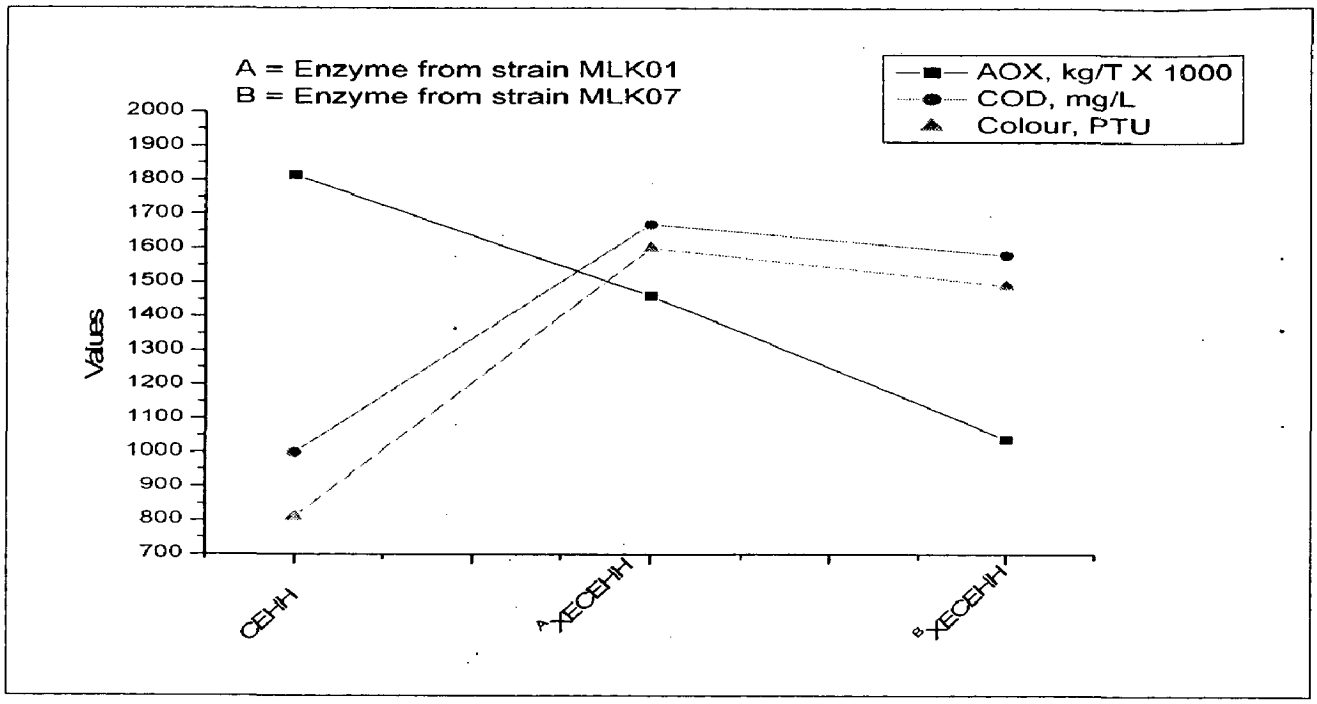

Figure 5.3: Effect of enzymatic prebleaching on COD, colour and AOX) of kraft-AQ pulp of *A. cadamba* (kappa number 16) during CEHH, ^{A}XECEHH and ^{B}XECEHH bleaching sequences

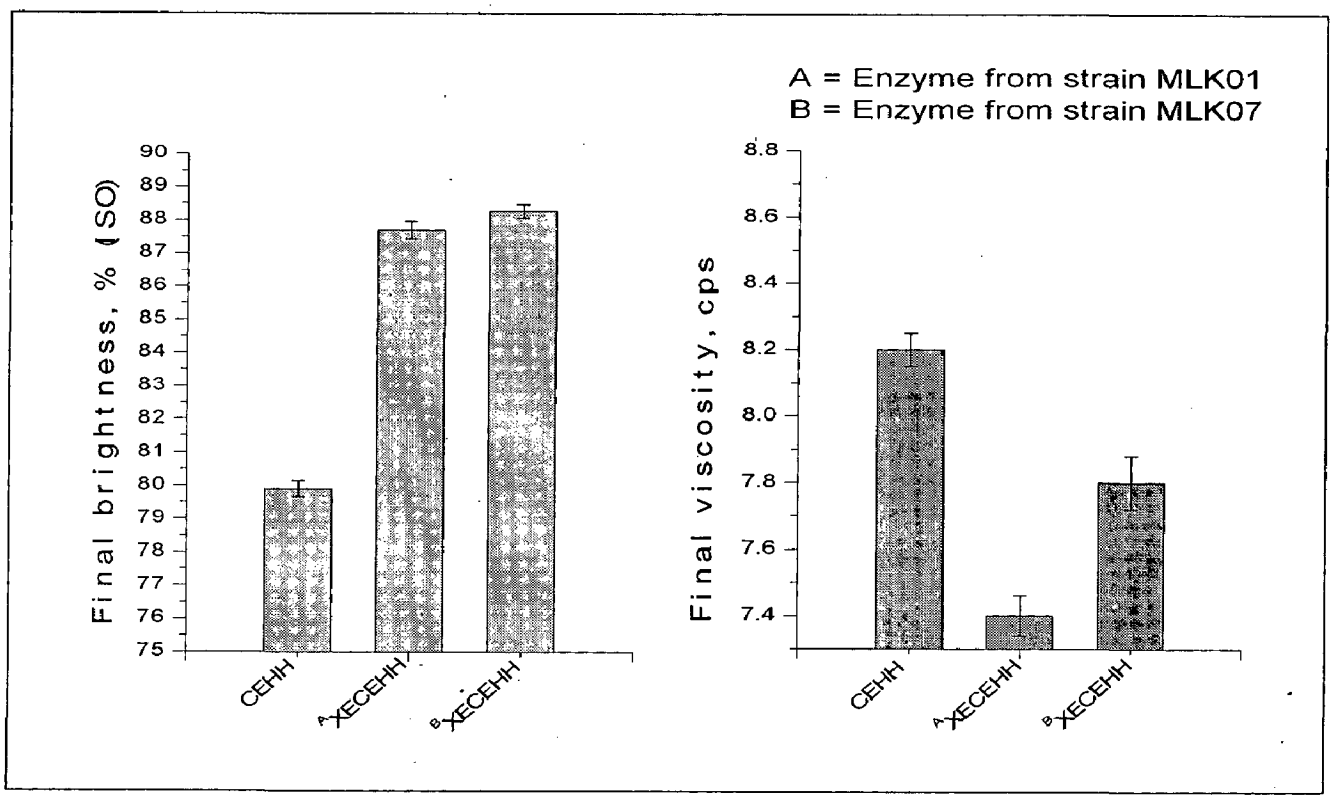

Figure 5.4: Effect of enzymatic prebleaching on final brightness and viscosity on kraft-AQ pulp of *A. cadamba* (kappa number 24) during CEHH, ^{A}XECEHH and ^{B}XECEHH bleaching sequences

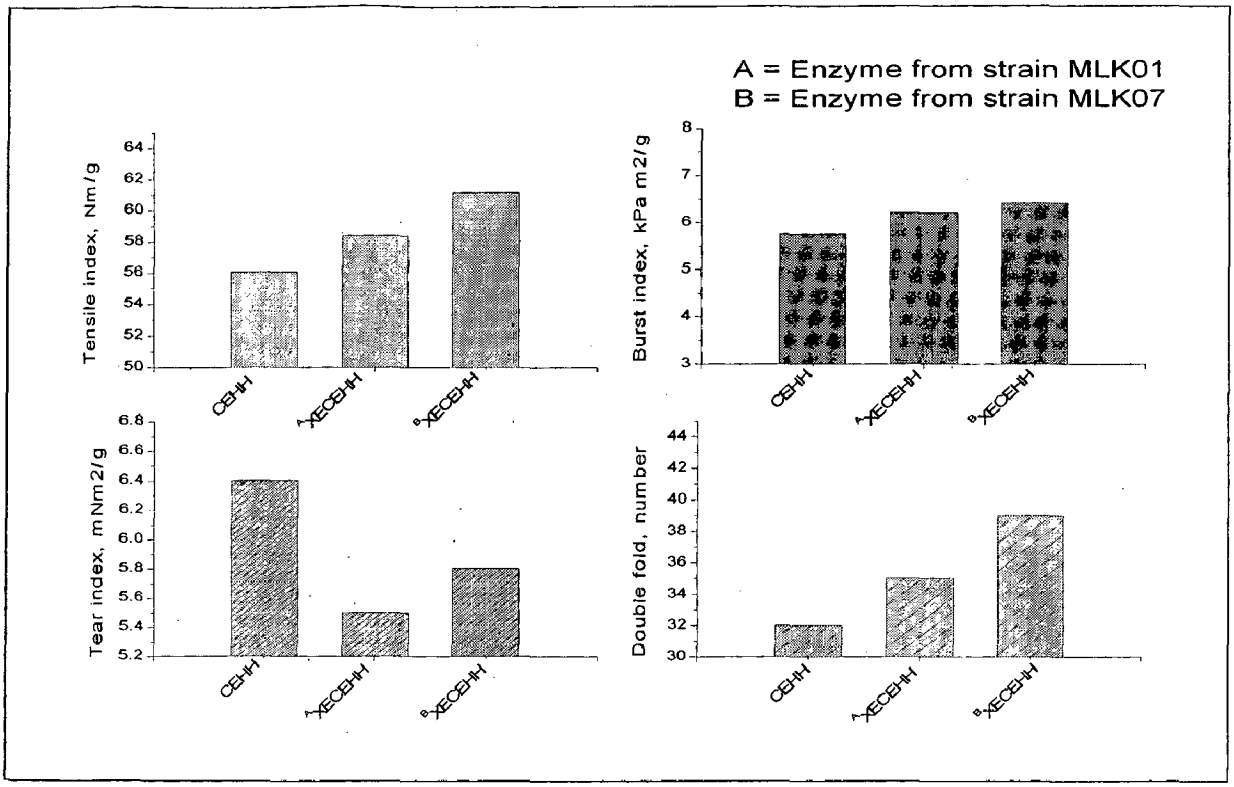

Figure 5.5: Effect on mechanical strength properties of enzyme prebleaching on kraft-AQ pulp of *A. cadamba* (kappa number 24) during CEHH, ^{A}XECEHH and ^{B}XECEHH bleaching sequences

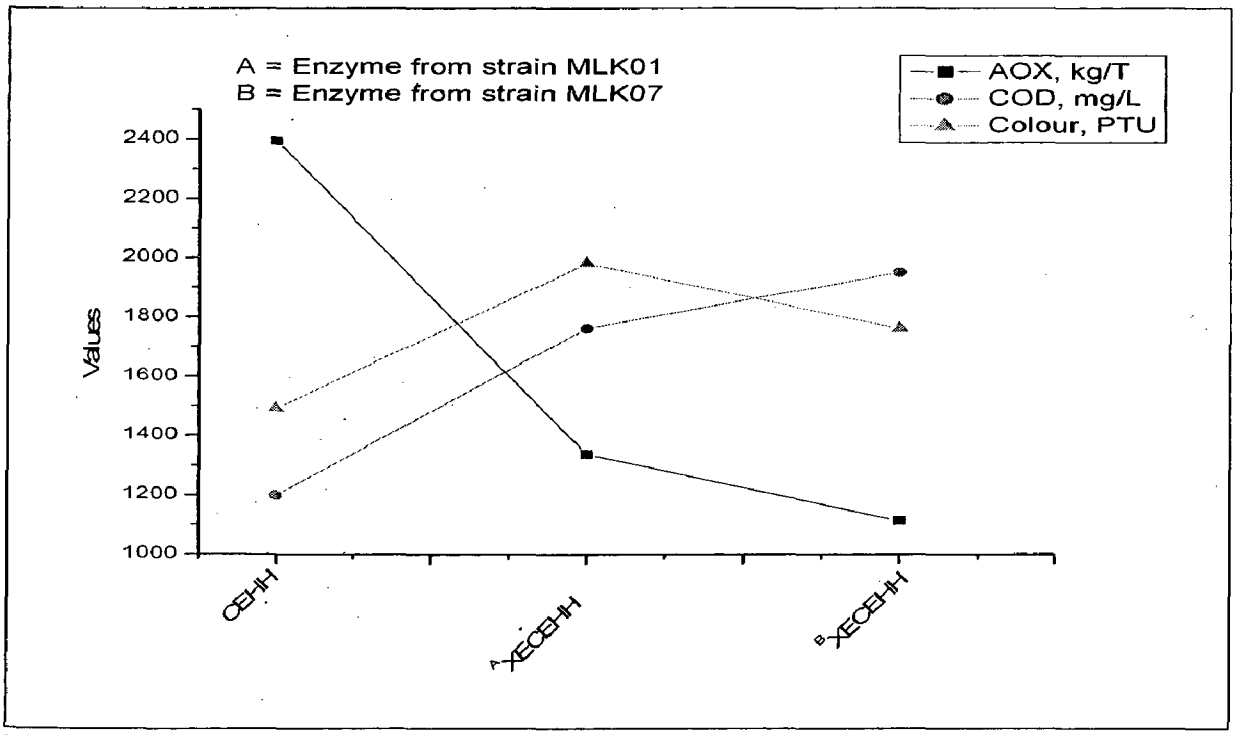

Figure 5.6: Effect of enzymatic prebleaching on COD, colour and AOX on kraft-AQ pulp of *A. cadamba* (kappa number 24) during CEHH, ^{A}XECEHH and ^{B}XECEHH bleaching sequences

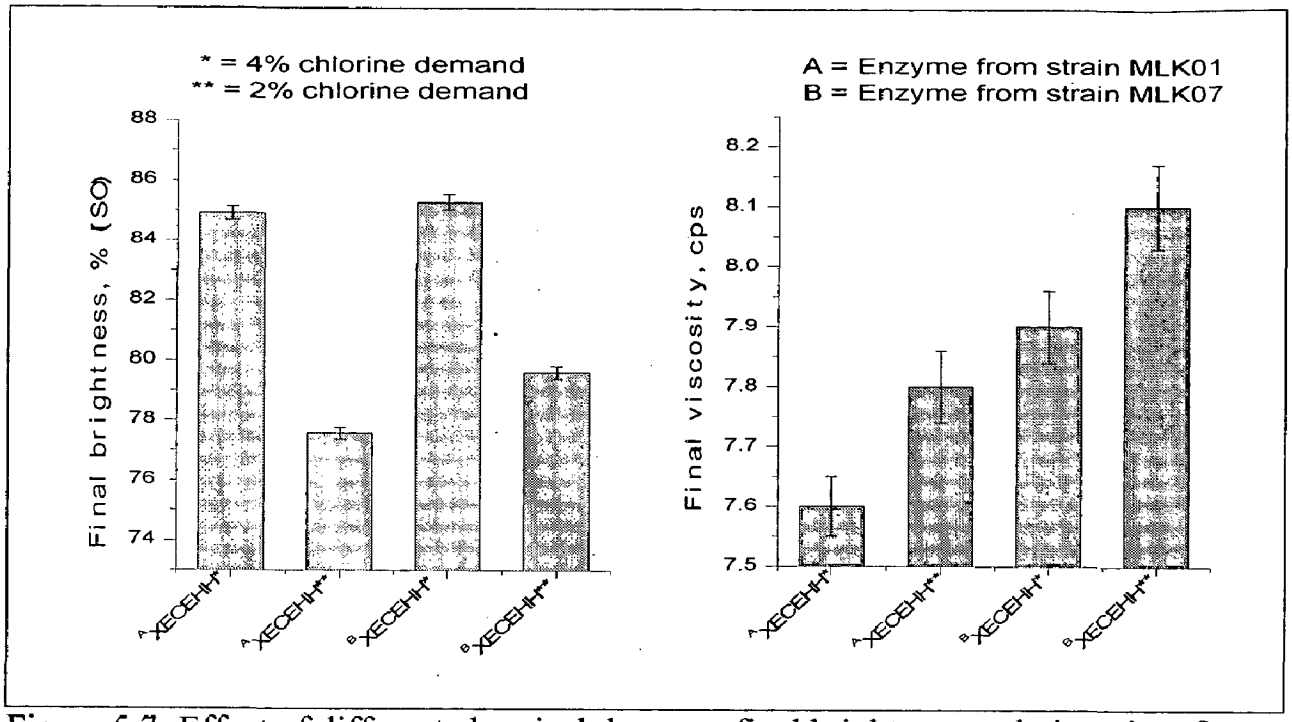

Figure 5.7: Effect of different chemical doses on final brightness and viscosity of enzymatic prebleached kraft-AQ pulp of *A. cadamba* during CEHH, ^{A}XECEHH and ^{B}XECEHH bleaching sequences

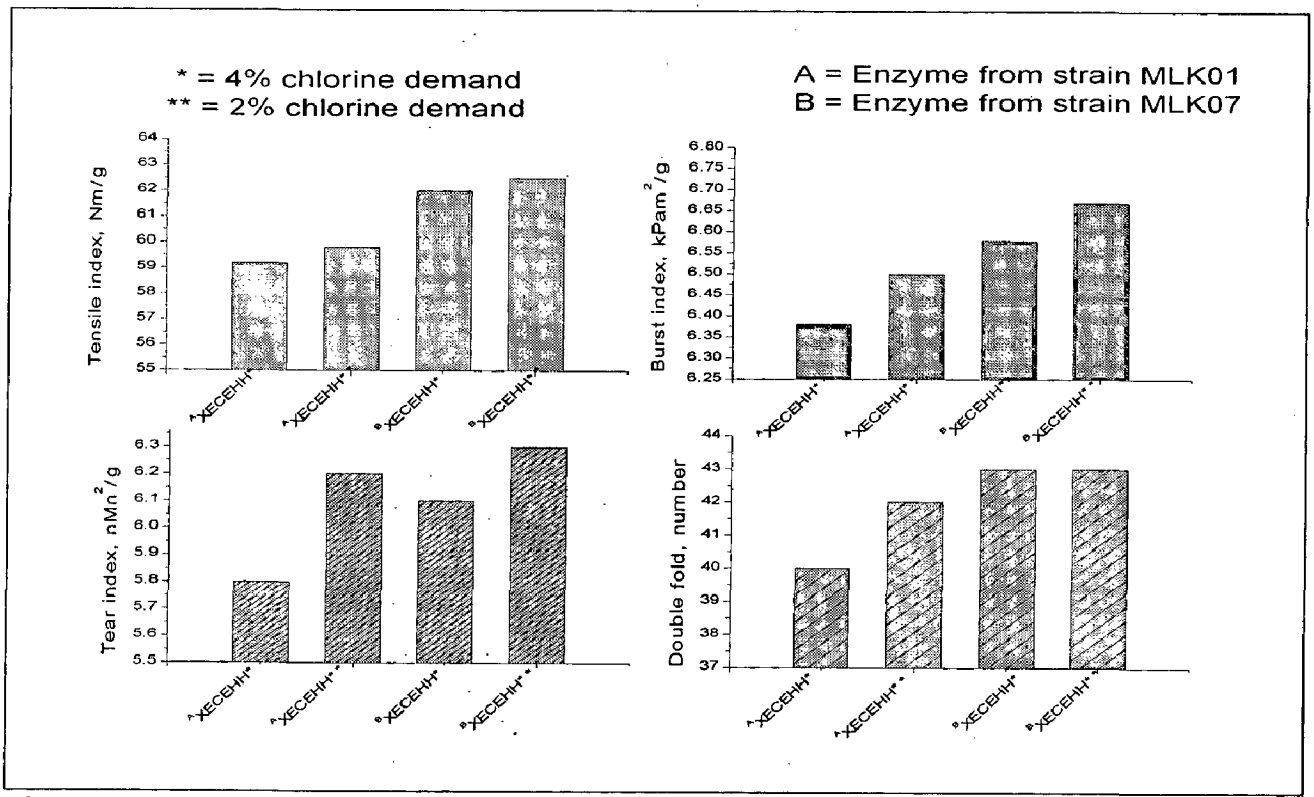

Figure 5.8: Effect of different chemical doses on mechanical strength properties of enzymatic prebleached kraft-AQ pulp of *A. cadamba* during CEHH, ^{A}XECEHH and ^{B}XECEHH bleaching sequences

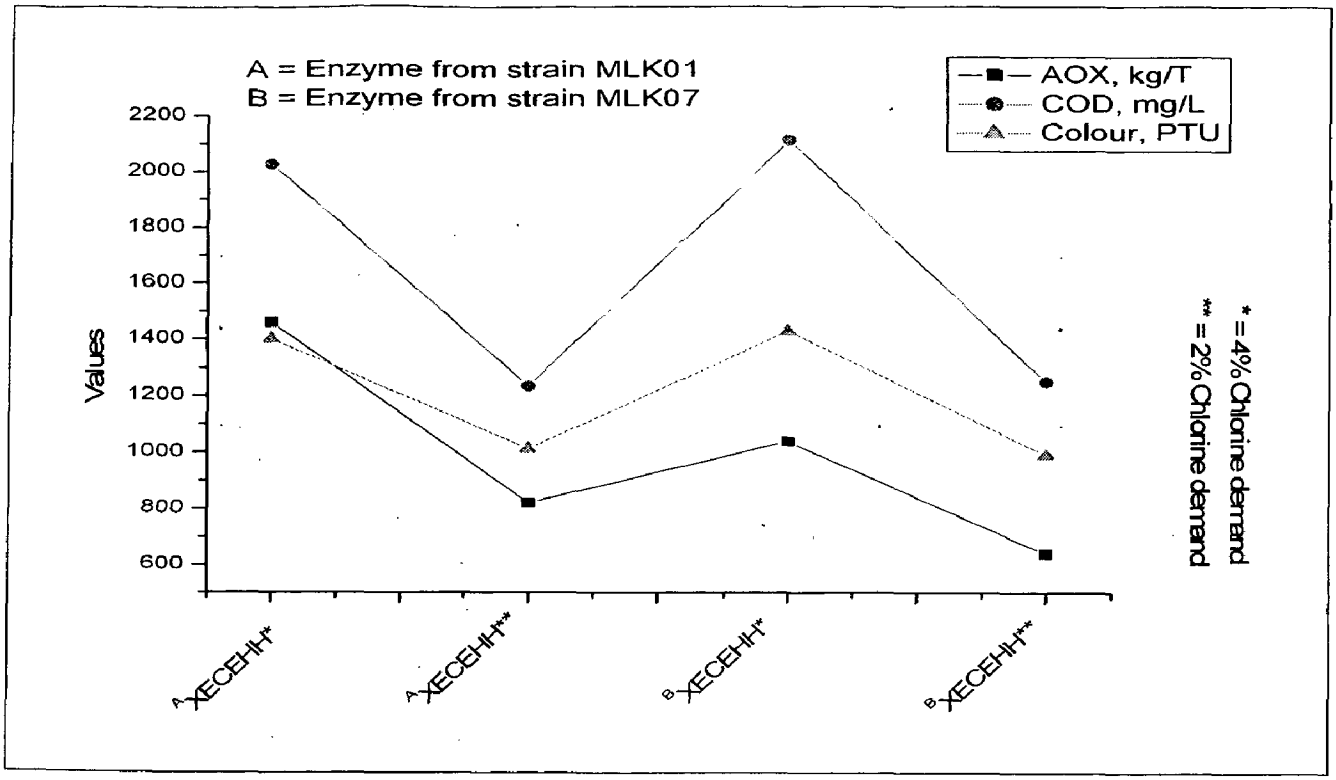

Figure 5.9: Effect of different chemical doses on COD, colour and AOX of enzymatic prebleached kraft-AQ pulp of *A. cadamba* during CEHH, ^{A}XECEHH and ^{B}XECEHH bleaching sequences

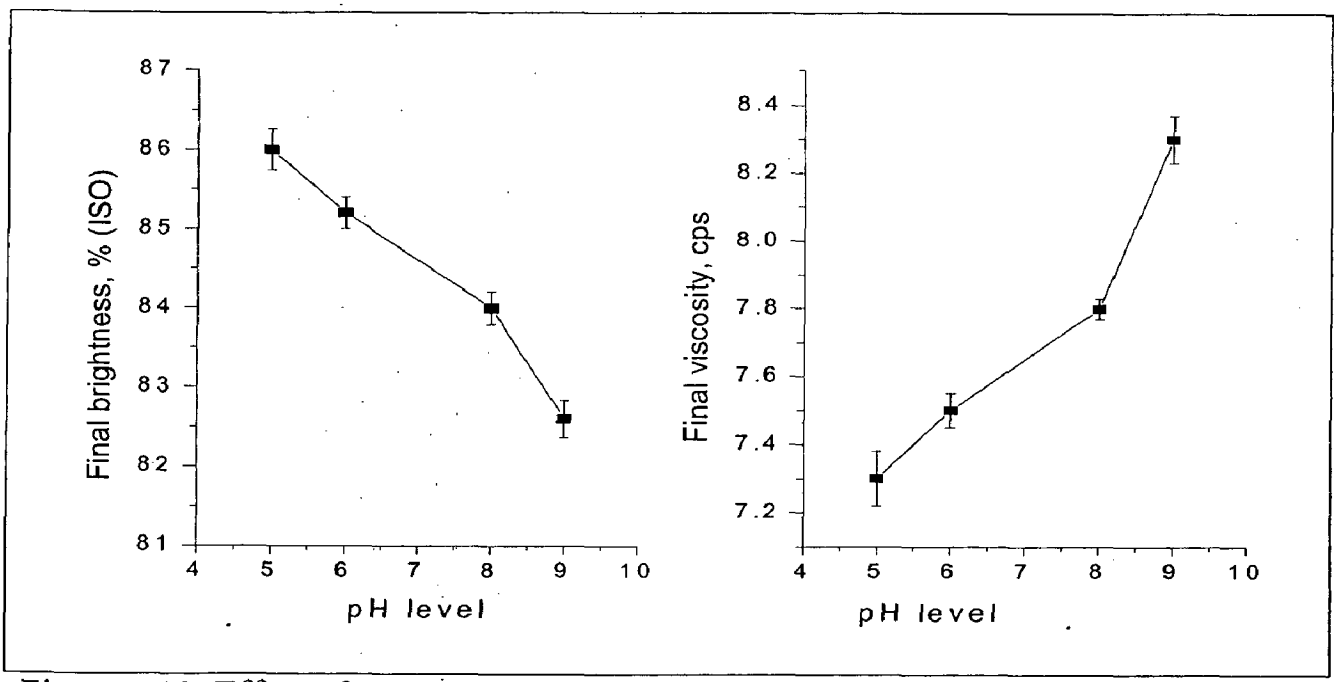

Figure 5.10: Effect of enzyme prebleaching (enzyme-A from strain MLK01) at different pH levels on final brightness and viscosity of kraft-AQ pulp of *A. cadamba* during ^{A}XECEHH bleaching sequences

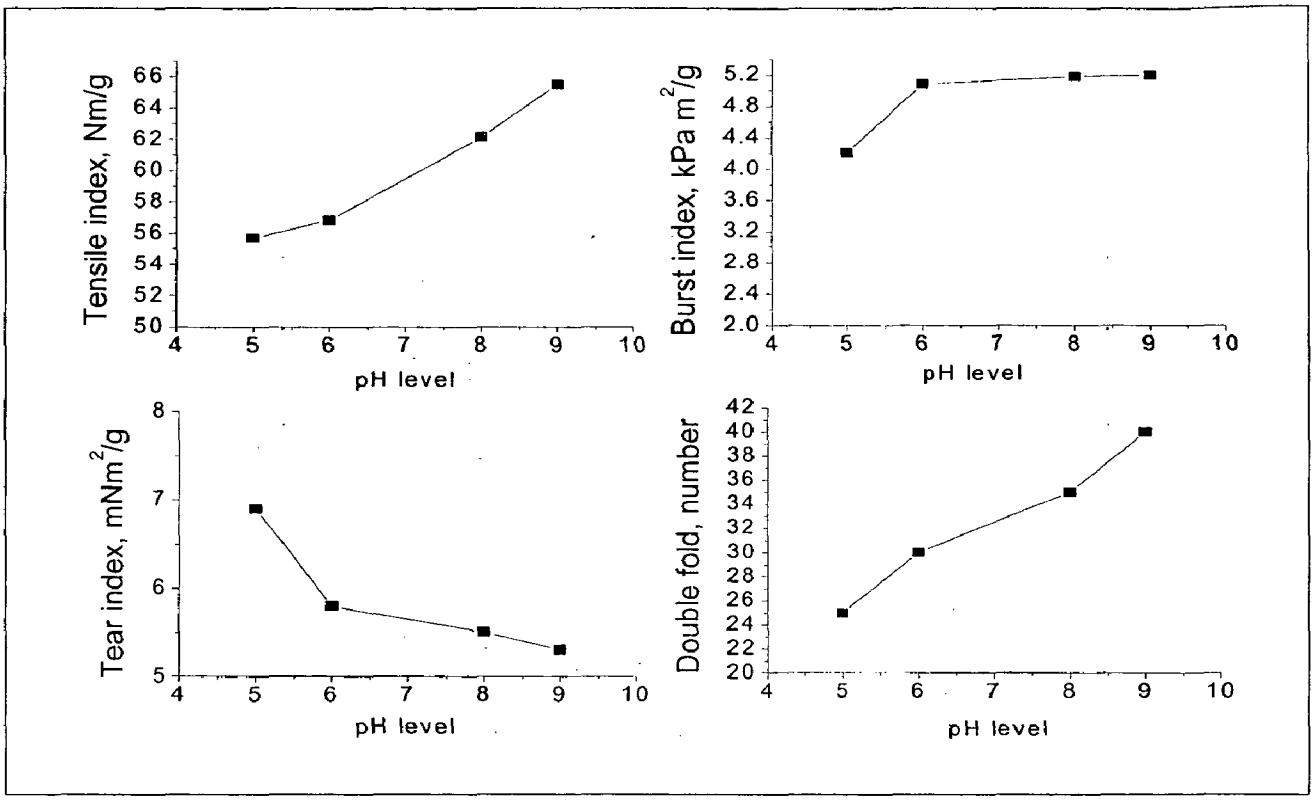

Figure 5.11: Effect of enzyme prebleaching (enzyme-A from strain MLK01) at different pH levels on mechanical strength properties of kraft-AQ pulp of *A. cadamba* during ^{A}XECEHH bleaching sequence

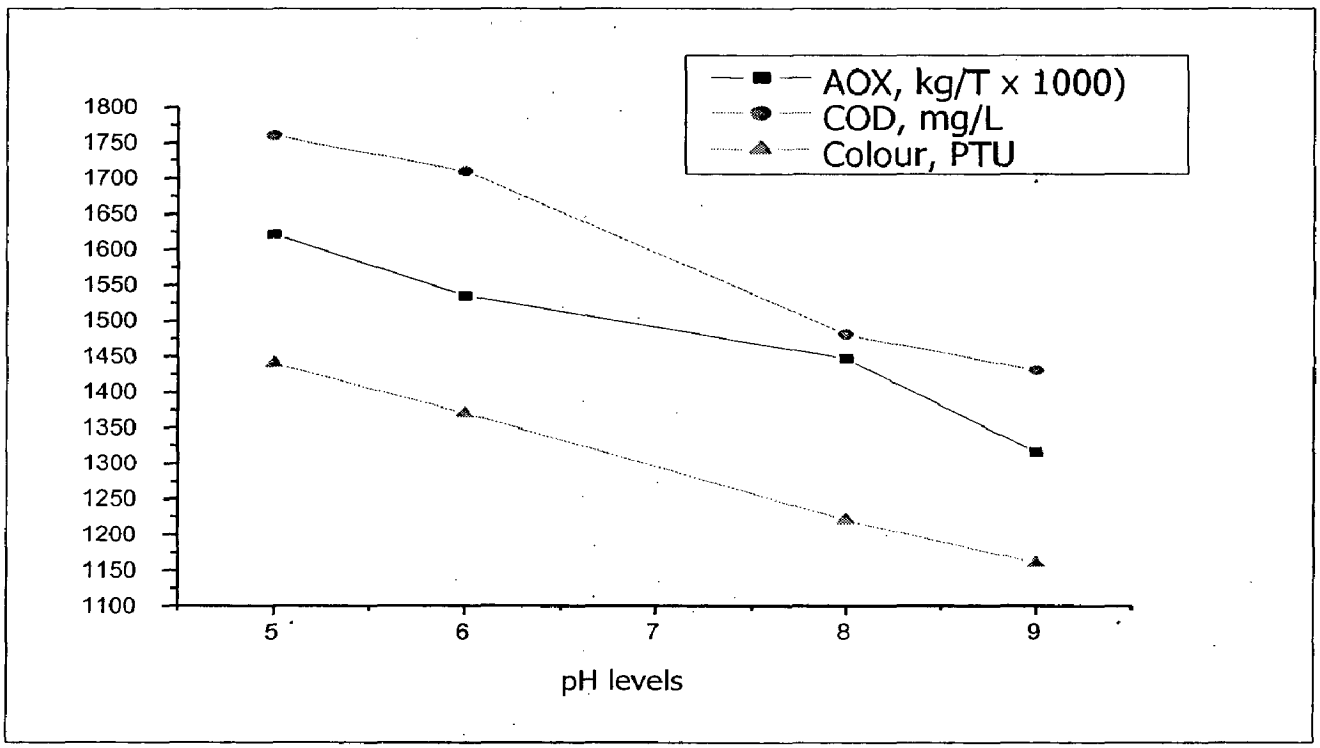

Figure 5.12: Effect of enzyme prebleaching (enzyme-A from strain MLK01) at different pH levels on AOX, COD, and colour of kraft- AQ pulp of *A. cadamba* during ^{A}XECEHH bleaching sequence

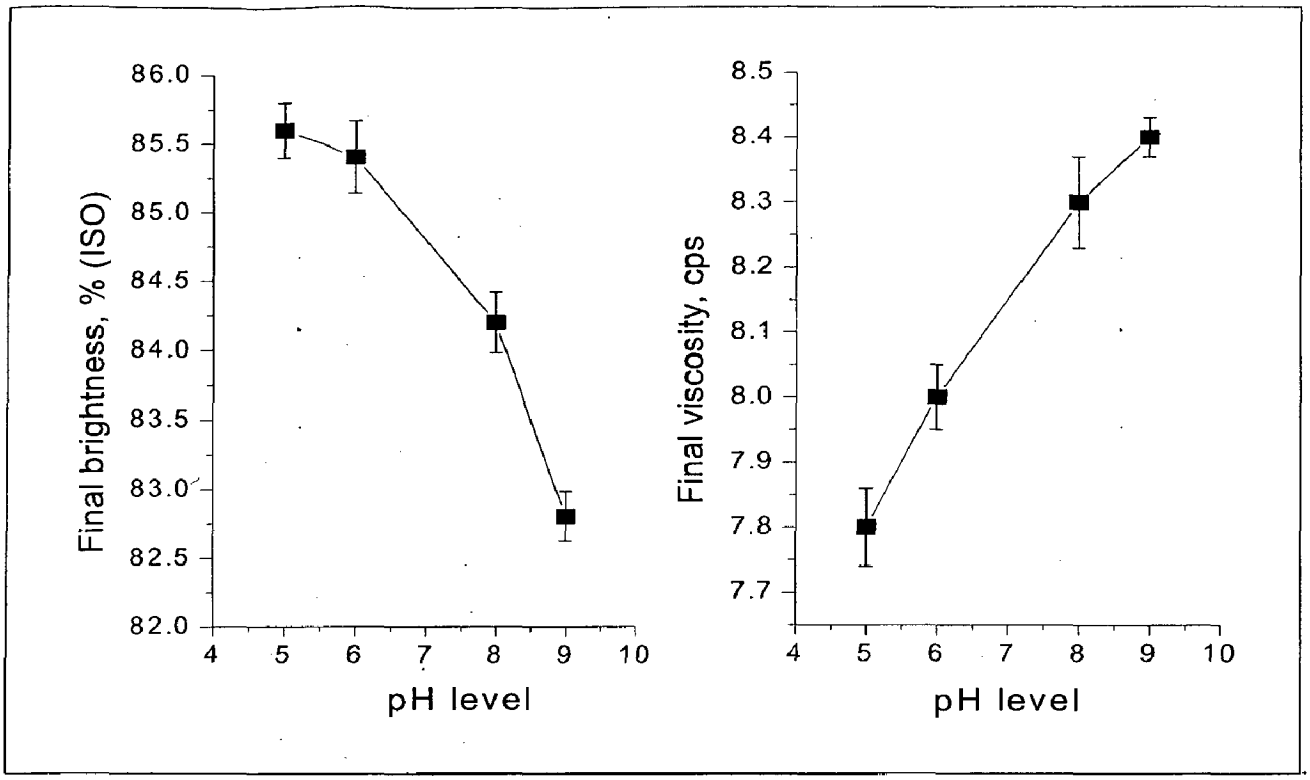

Figure 5.13: Effect of enzyme prebleaching (enzyme-B from strain MLK07) at different pH levels, on final brightness and viscosity of kraft-AQ pulp of *A. cadamba* during ^{B}XECEHH bleaching sequence

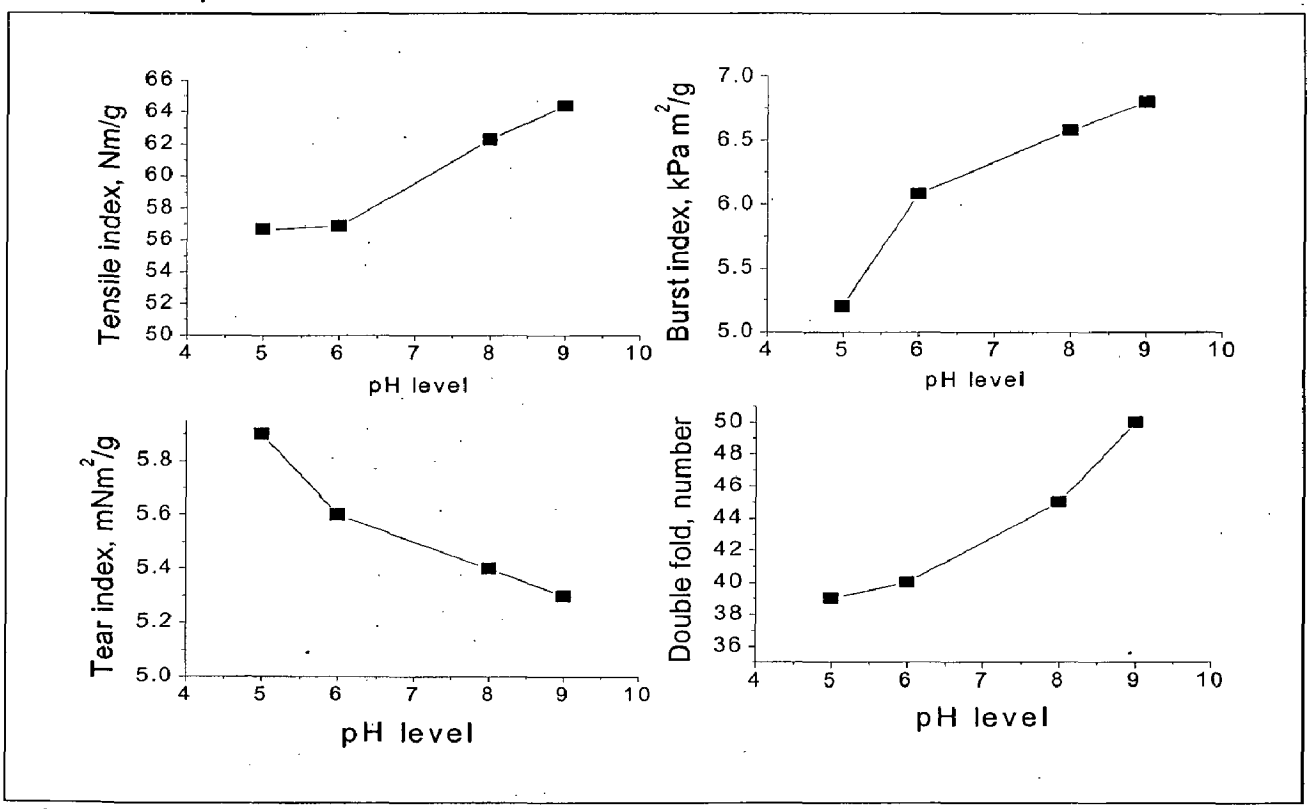

Figure 5.14: Effect of enzyme prebleaching (enzyme-B from strain MLK07)at different pH levels on mechanical strength properties of kraft-AQ pulp of *A. cadamba* during ^{B}XECEHH bleaching sequence

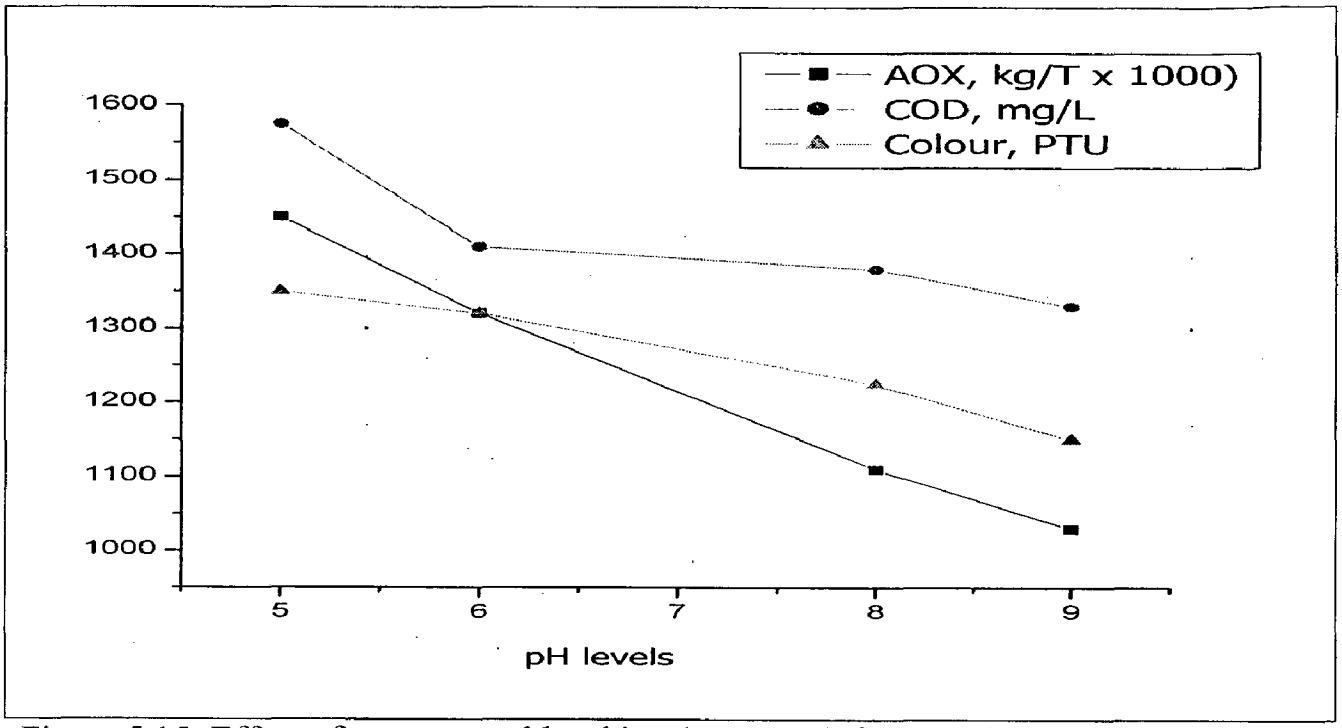

Figure 5.15: Effect of enzyme prebleaching (enzyme-B from strain MLK07) at different pH levels, on AOX, COD and colour of kraft-AQ pulp of *A. cadamba* during ^{B}XECEHH bleaching sequence.

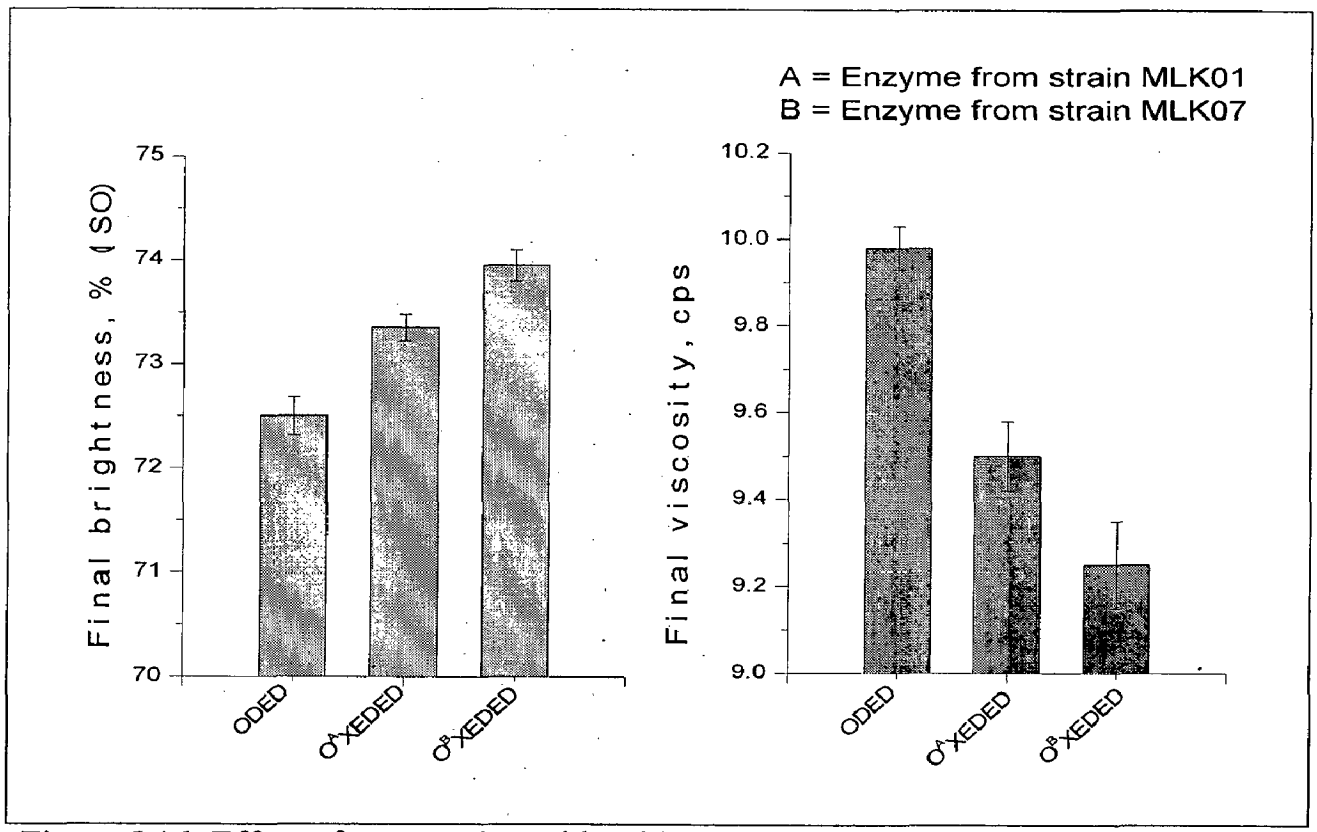

Figure 5.16: Effect of enzymatic prebleaching on final brightness and viscosity of kraft-AQ pulp of *A. cadamba* during ODED, O^{A}XEDED and O^{B}XEDED bleaching sequences

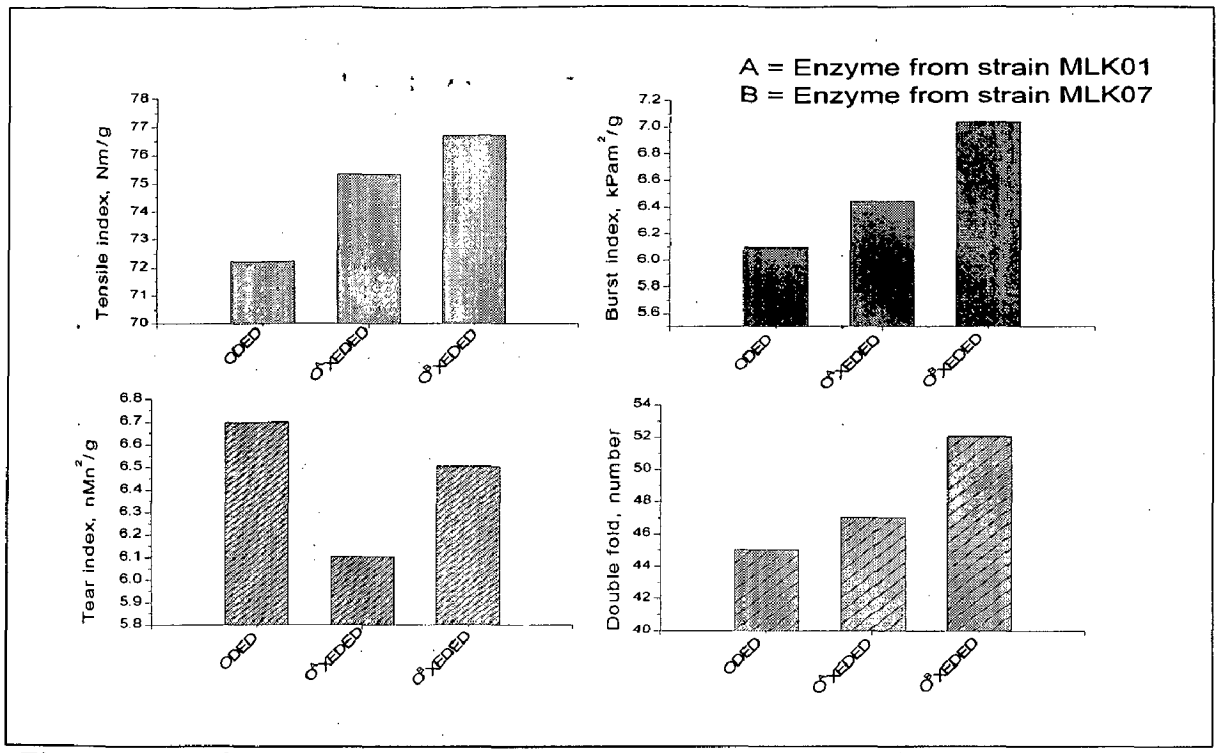

Figure 5.17: Effect of enzymatic prebleaching on mechanical strength properties of kraft-AQ pulp of *A. cadamba* during ODED, $O^{A}XEDED$ and $O^{B}XEDED$ bleaching sequences

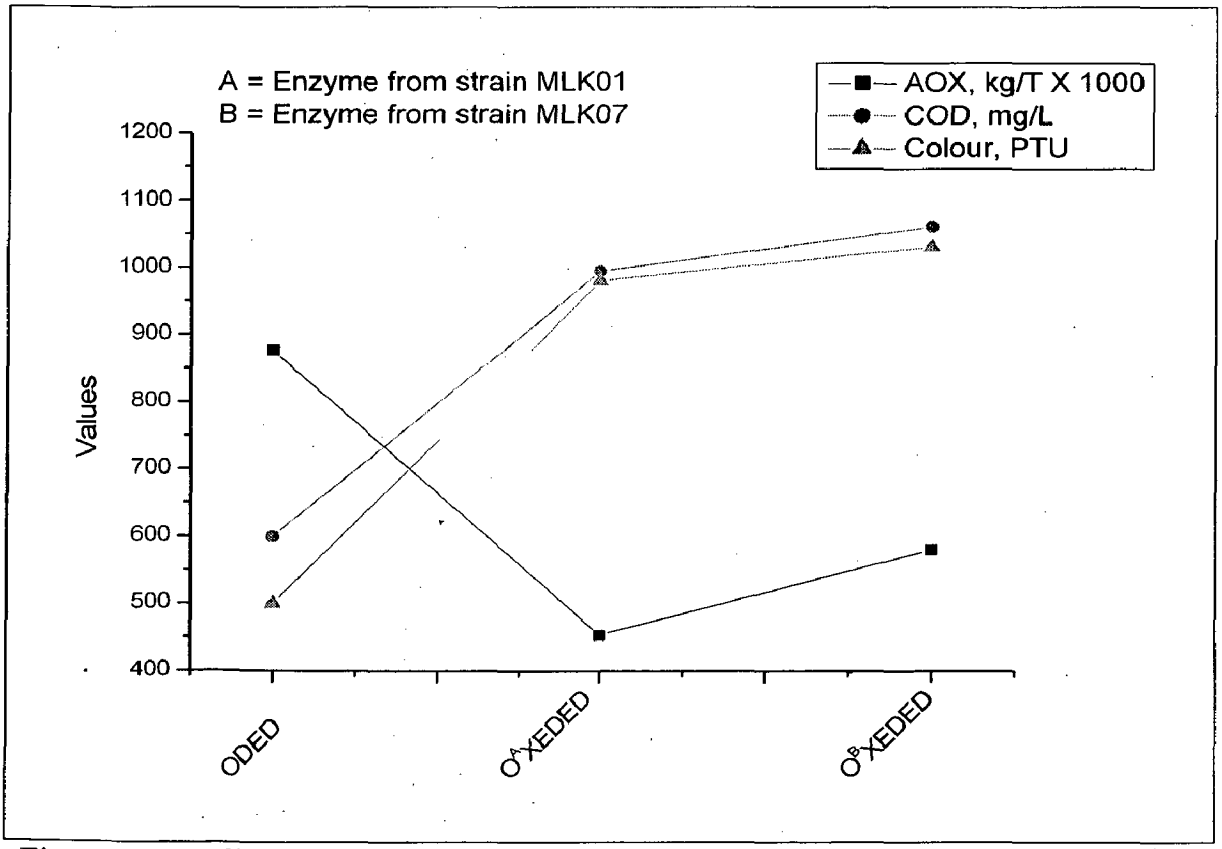

Figure 5.18: Effect of enzymatic prebleaching on COD, colour and AOX on kraft-AQ pulp of *A. cadamba* during ODED, $O^{A}XEDED$ and $O^{B}XEDED$ bleaching sequences

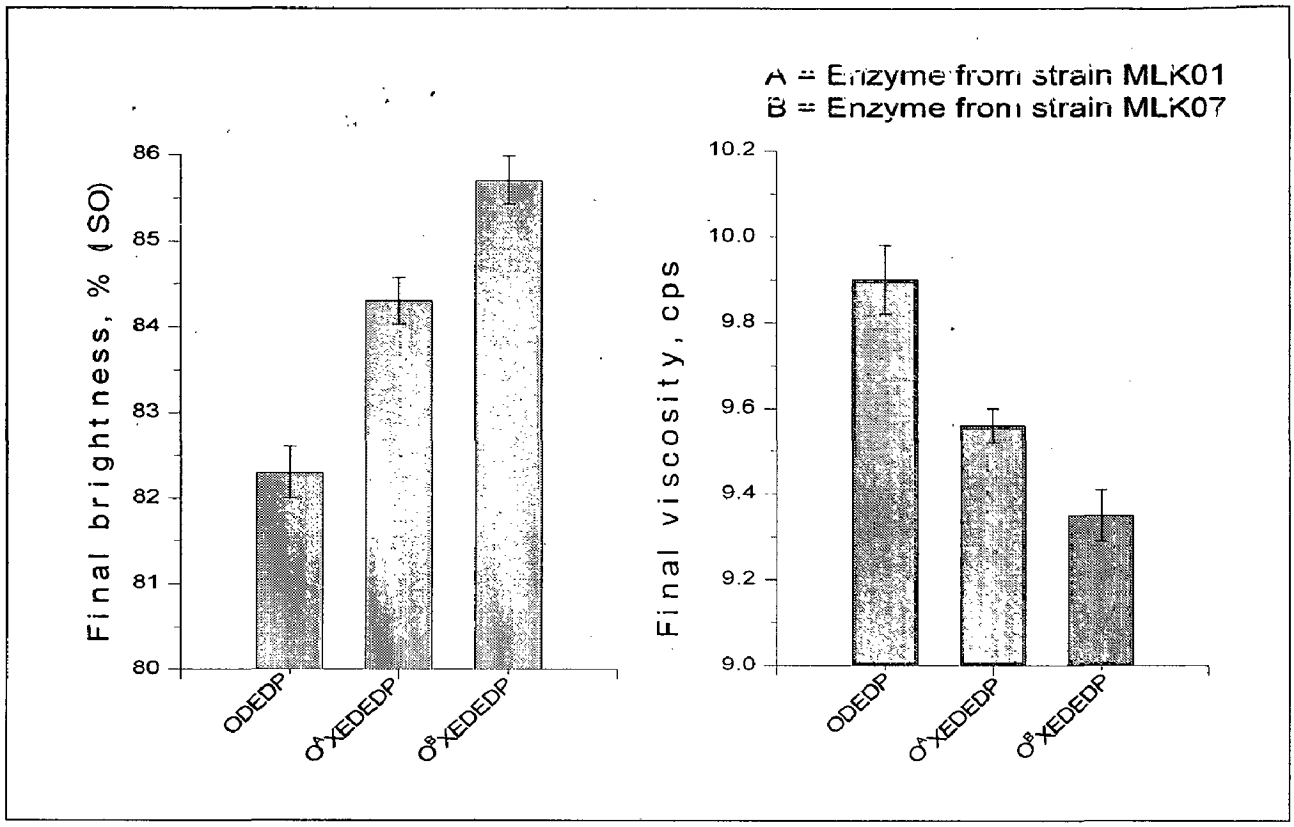

Figure 5.19: Effect of enzymatic prebleaching on final brightness and viscosity of kraft-AQ pulp of *A. cadamba* during ODEDP, $O^{A}XEDEDP$ and $O^{B}XEDEDP$ bleaching sequences

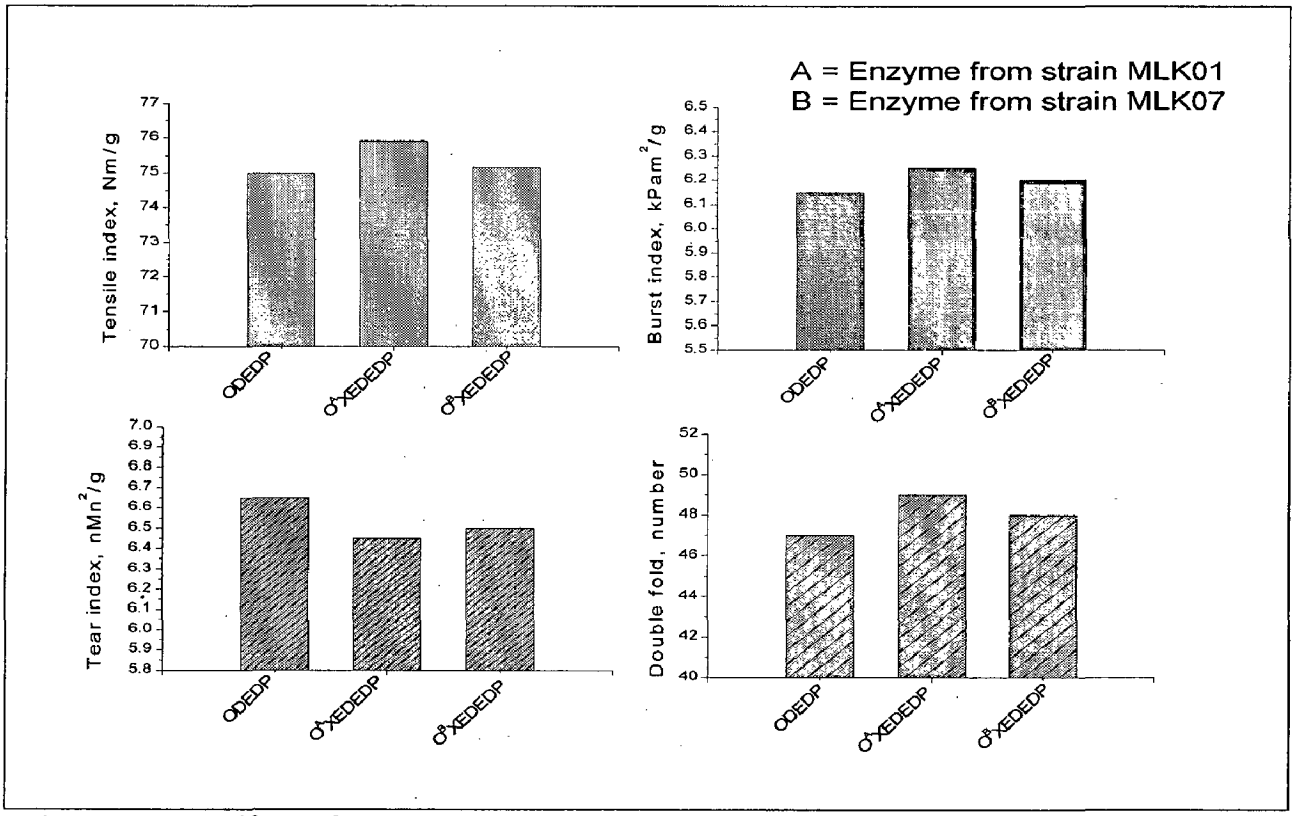

Figure 5.20: Effect of enzymatic prebleaching on mechanical strength properties of kraft-AQ pulp of *A. cadamba* during ODEDP, $O^{A}XEDEDP$ and $O^{B}XEDEDP$ bleaching sequences

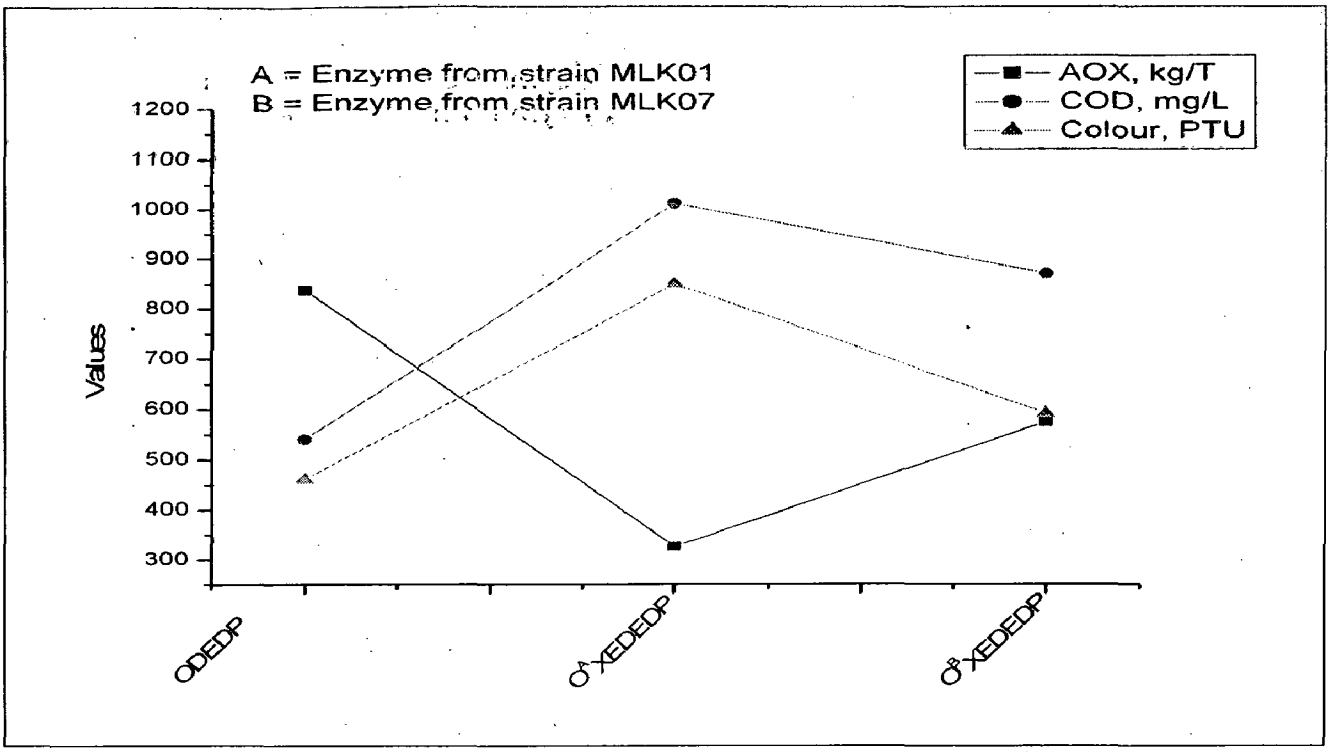

Figure 5.21: Effect of enzymatic prebleaching on COD, Colour and AOX of kraft-AQ pulp of *A. cadamba* during ODEDP, O^{A}XEDEDP and O^{B}XEDEDP bleaching sequences

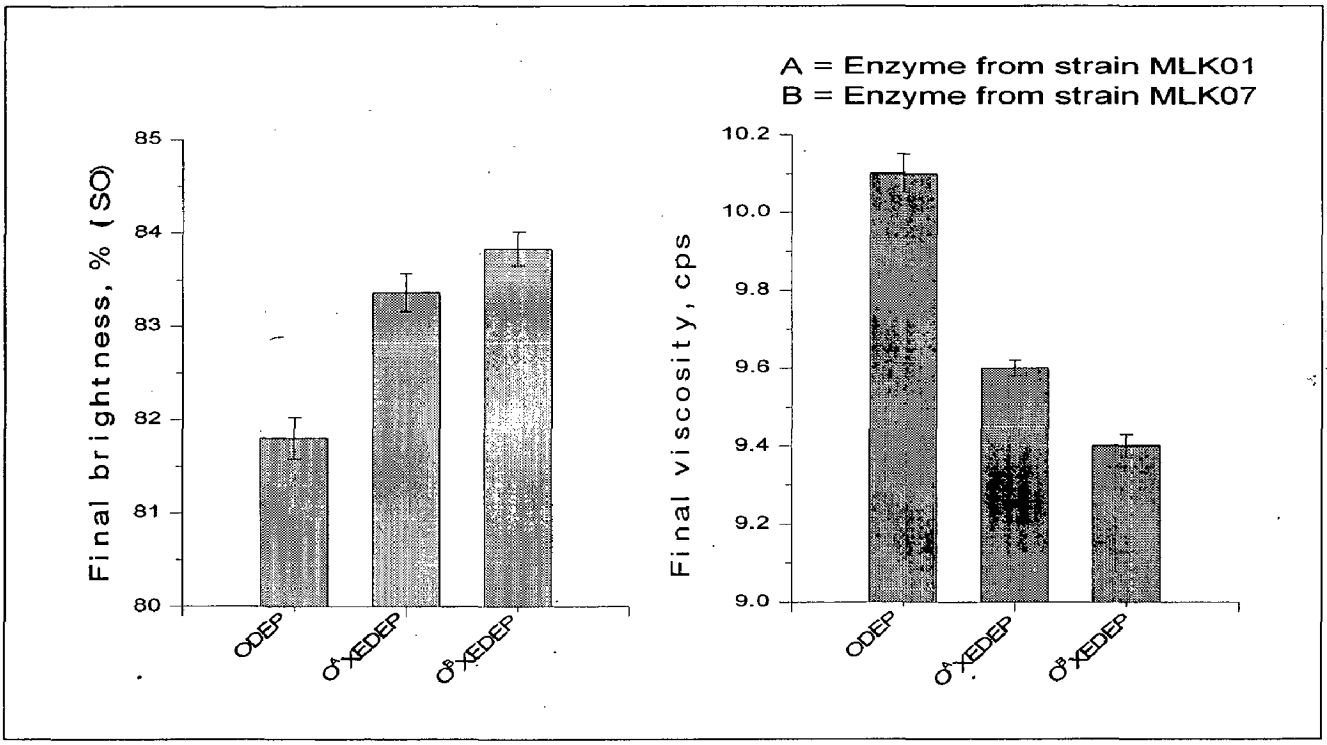

Figure 5.22: Effect of enzymatic prebleaching on final brightness and viscosity of kraft-AQ pulp of *A. cadamba* during ODEP, O^{A}XEDEP and O^{B}XEDEP bleaching sequences

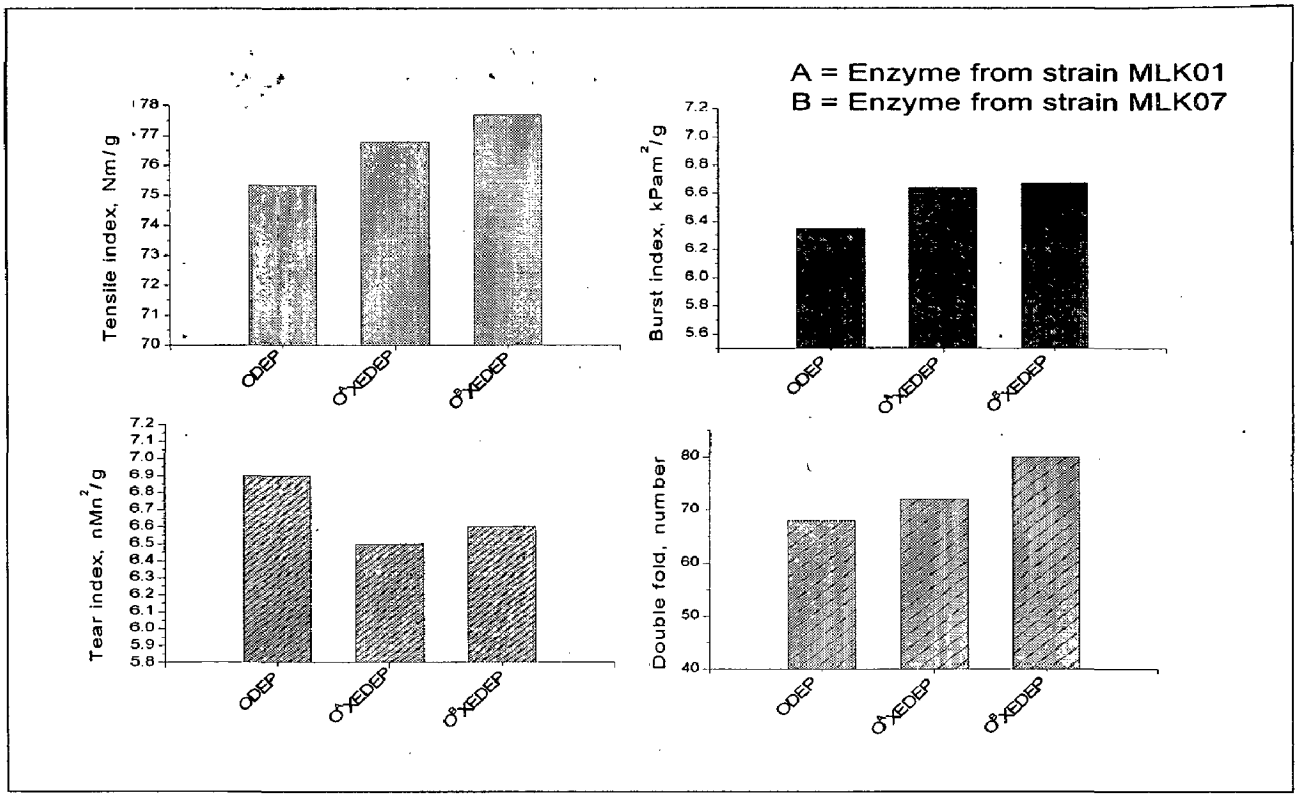

Figure 5.23: Effect of enzymatic prebleaching on mechanical strength properties of kraft-AQ pulp of *A. cadamba* during ODEP, $O^{A}XEDEP$ and $O^{B}XEDEP$ bleaching sequences

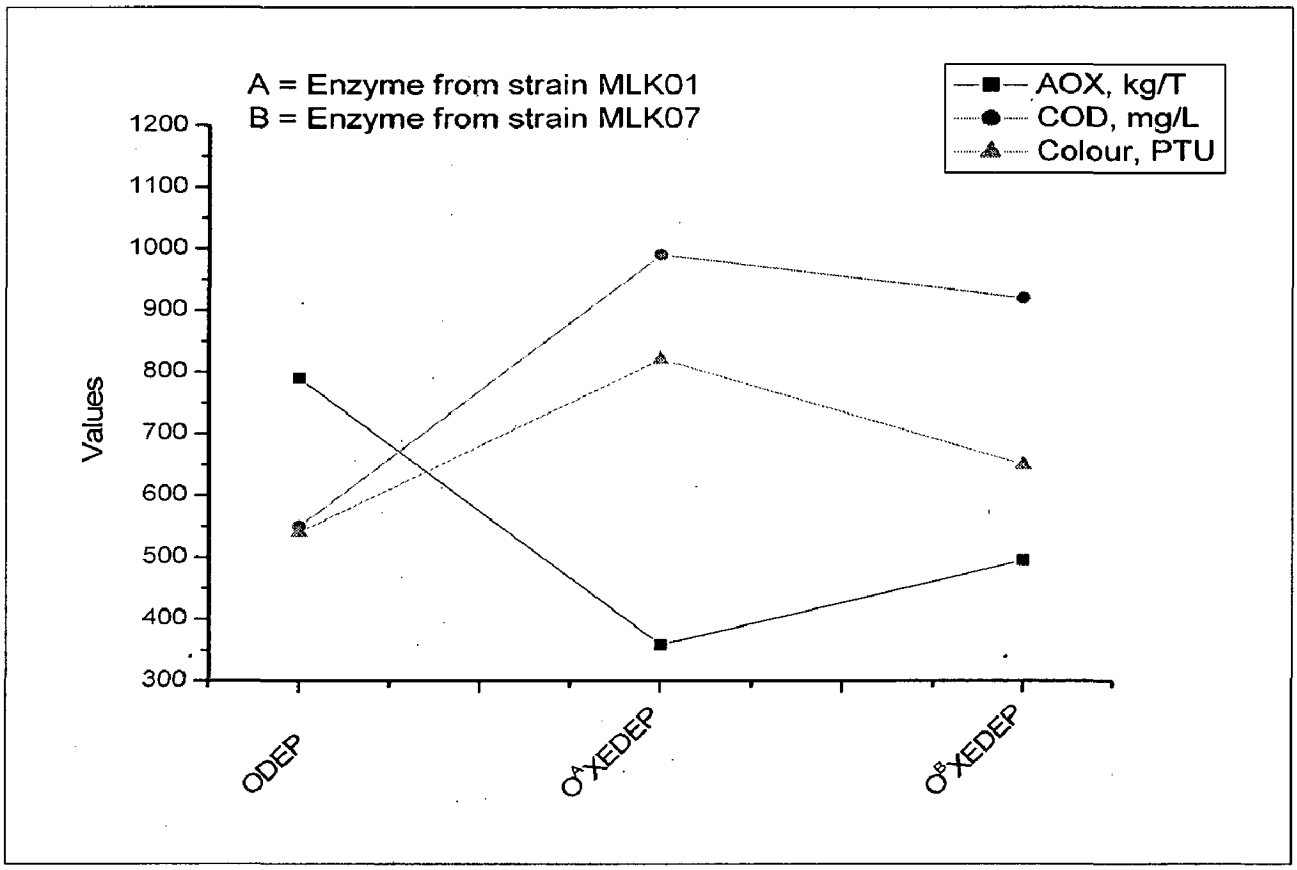

Figure 5.24: Effect of enzymatic prebleaching on COD, colour, AOX of kraft- AQ pulp of *A. cadamba* during ODEP, $O^{A}XEDEP$ and $O^{B}XEDEP$ bleaching sequences

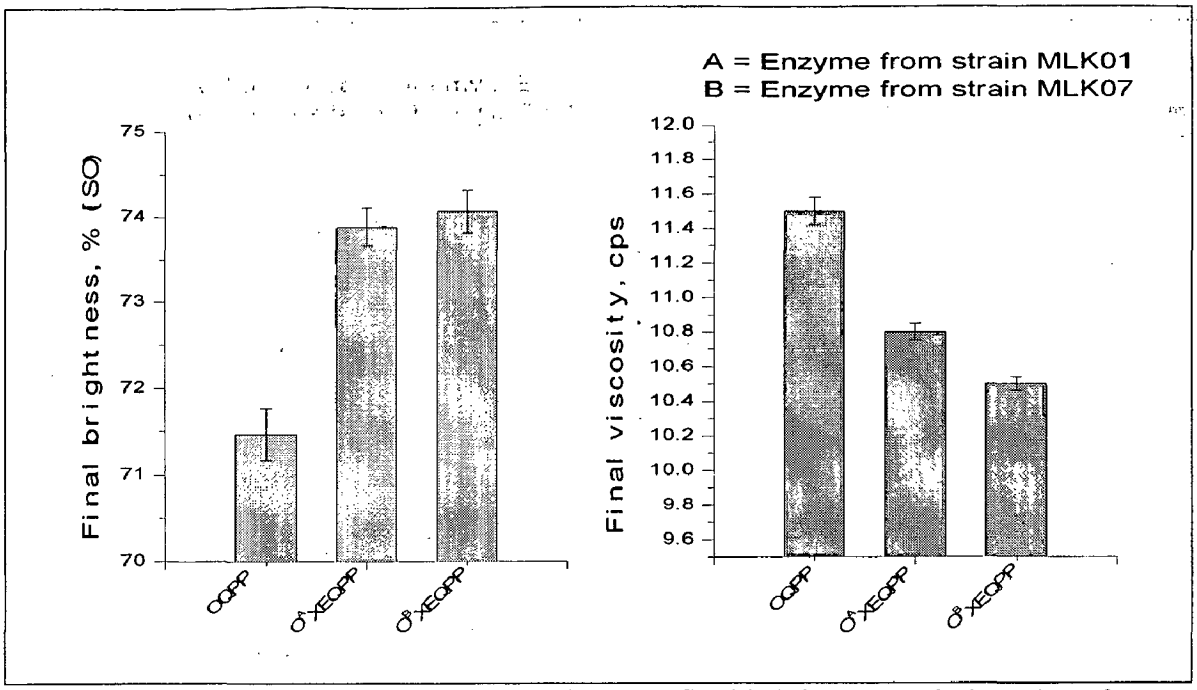

Figure 5.25: Effect of enzymatic prebleaching on final brightness and viscosity of kraft-AQ pulp of *A. cadamba* during OQPP, O^{A}XEQPP and O^{B}XEQPP bleaching sequences.

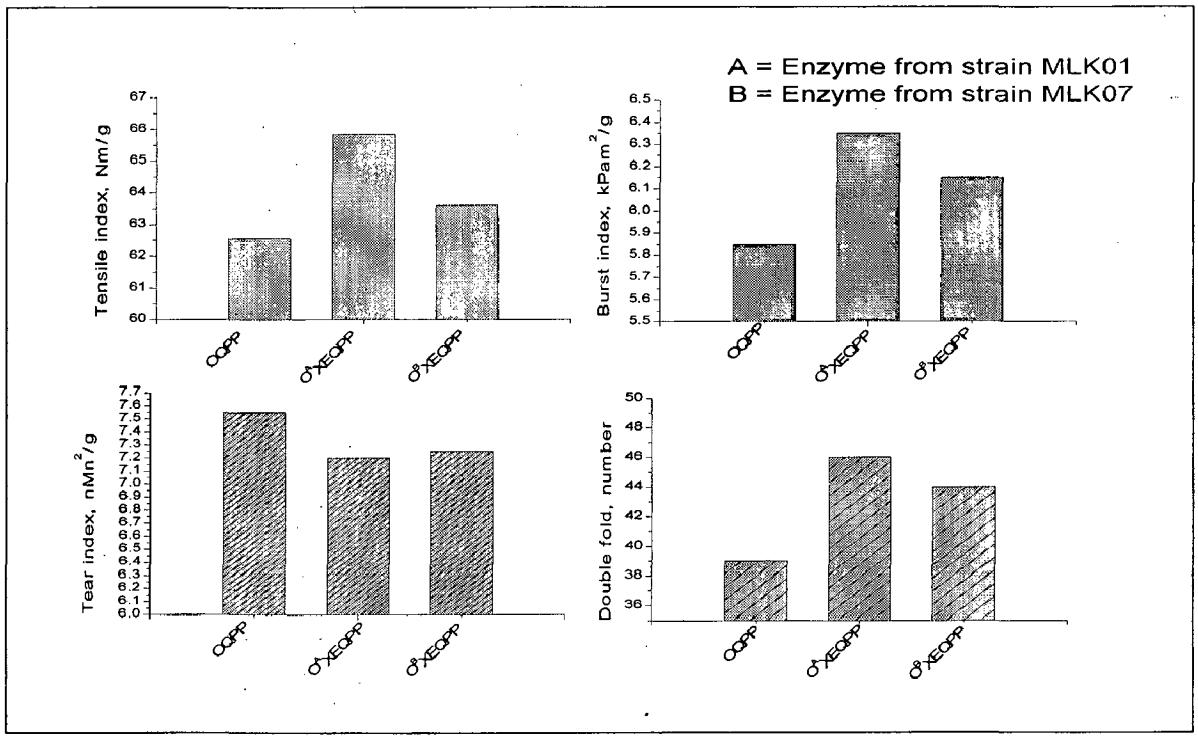

Figure 5.26: Effect of enzymatic prebleaching on mechanical strength properties of kraft-AQ pulp of *A. cadamba* during OQPP, O^{A}XEQPP and O^{B}XEQPP bleaching sequences.

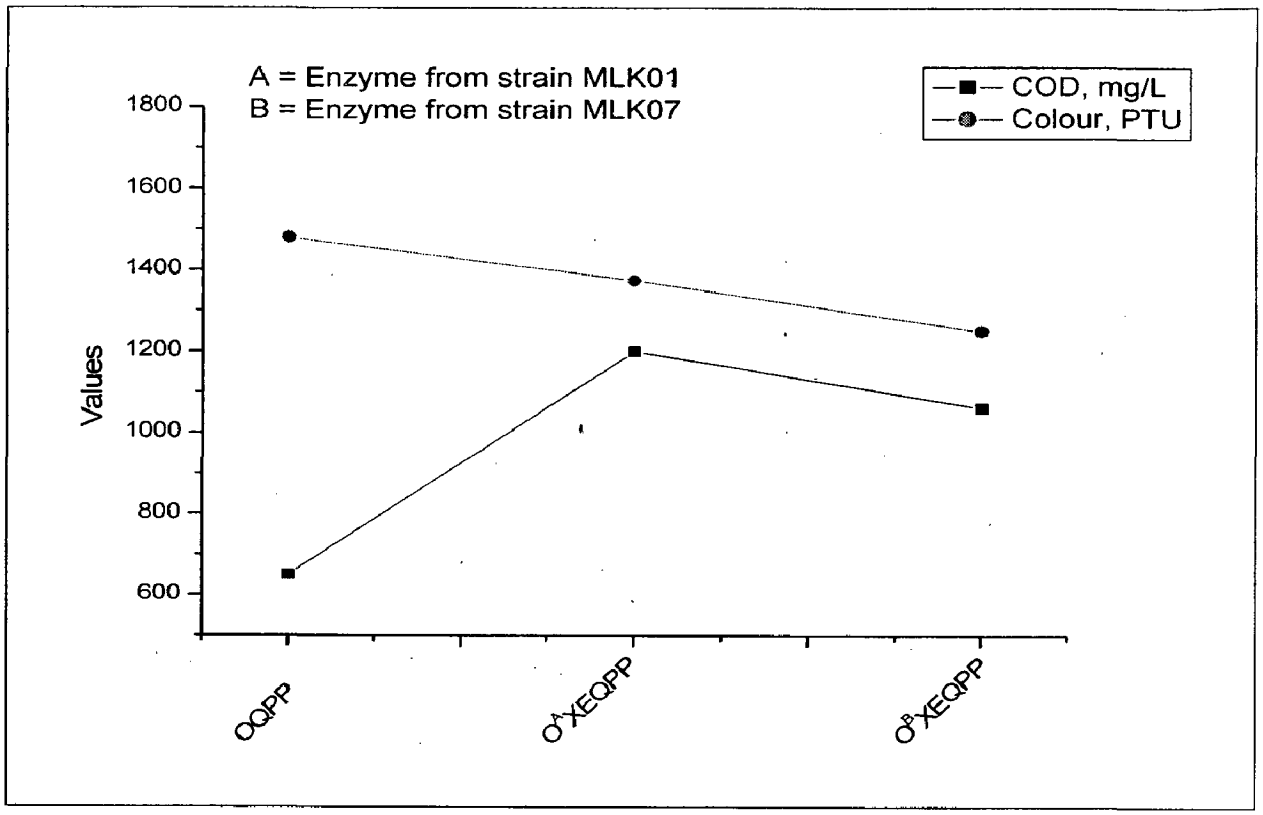

Figure 5.27: Effect of enzymatic prebleaching on COD, Colour, AOX of kraft-AQ pulp of *A. cadamba* during OQPP, O^{A}XEQPP and O^{B}XEQPP bleaching sequences.

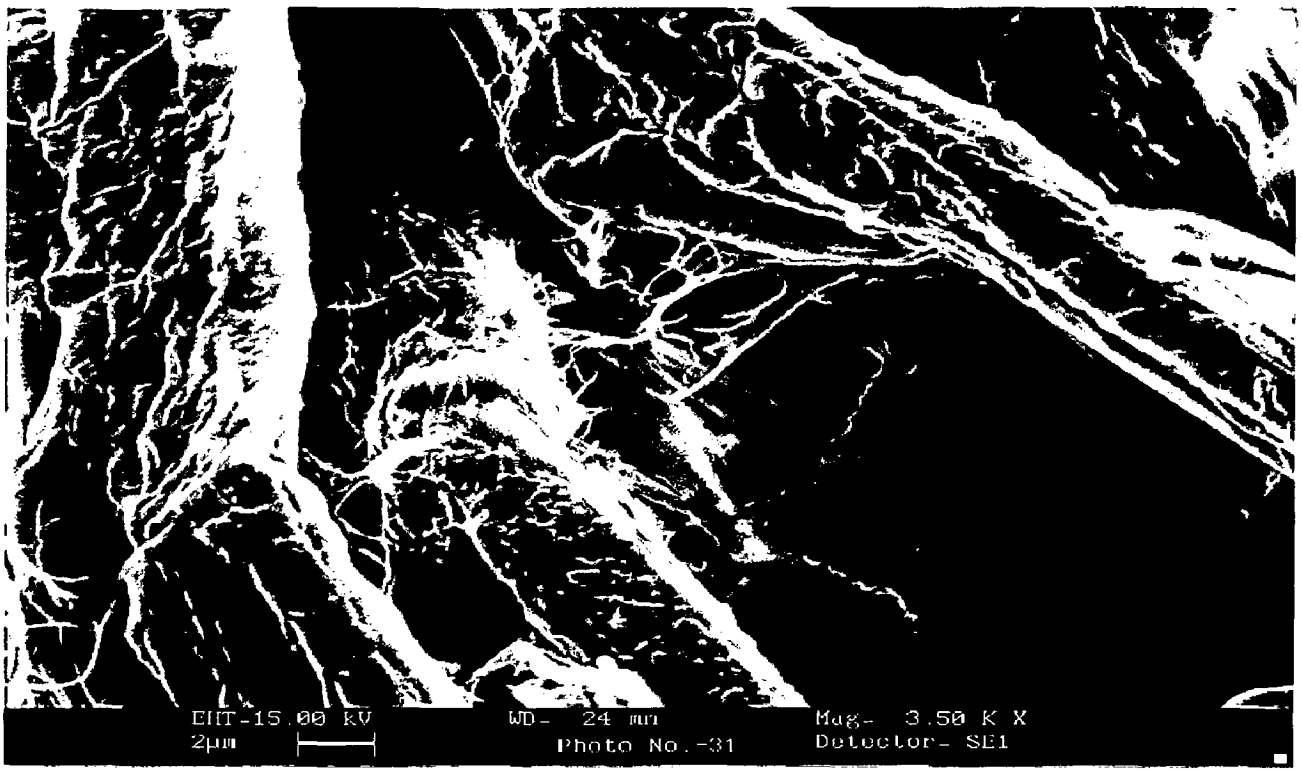

Plate 5.1: Scanning electron microphotograph (SEM) of fully bleached kraft pulp of *A. cadamba* after beating in PFI mill at 35 ^{0}SR and magnification of 3.5 KX showing poorer fibrillation.

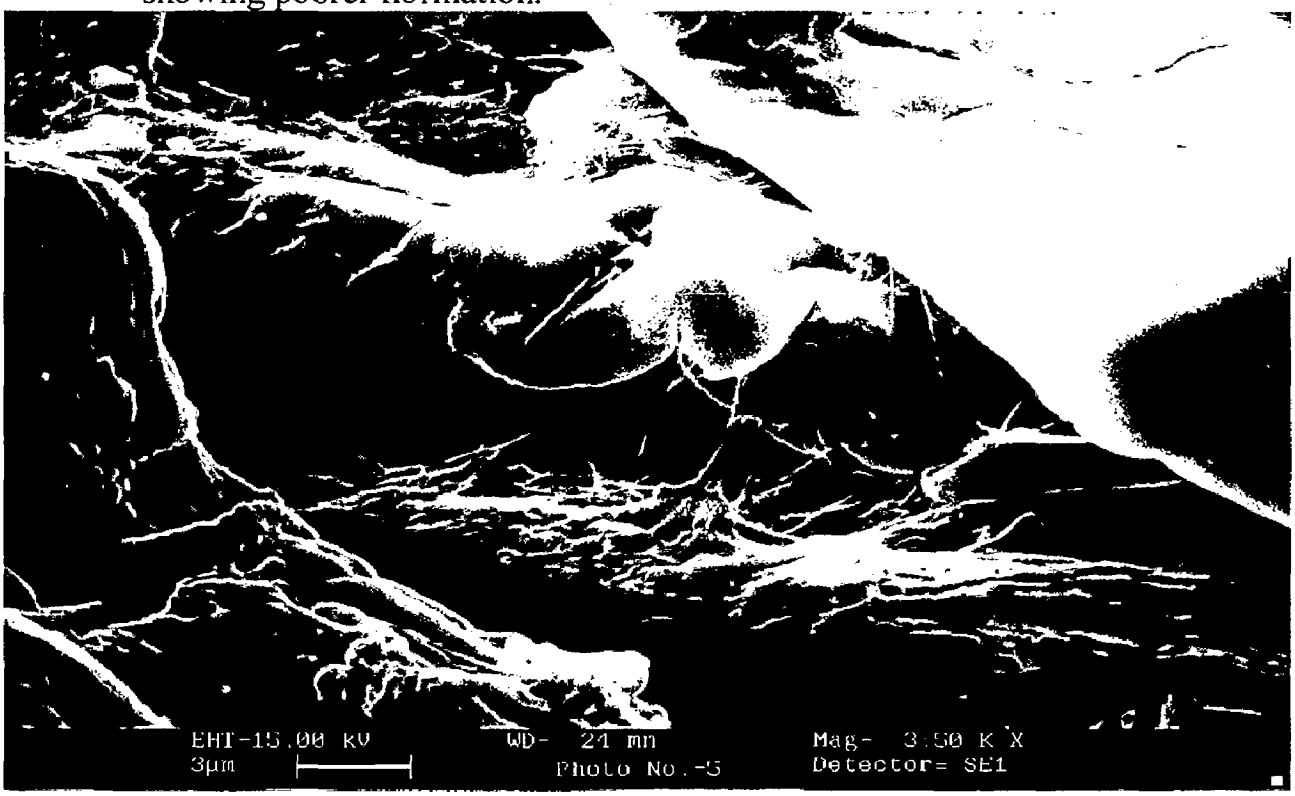

Plate 5.2: Scanning electron microphotograph (SEM) of fully bleached enzyme treated kraft pulp of *A. cadamba* after beating in PFI mill at 35 ^{0}SR at a magnification of 3.5 KX showing more fibrillation and swelling.

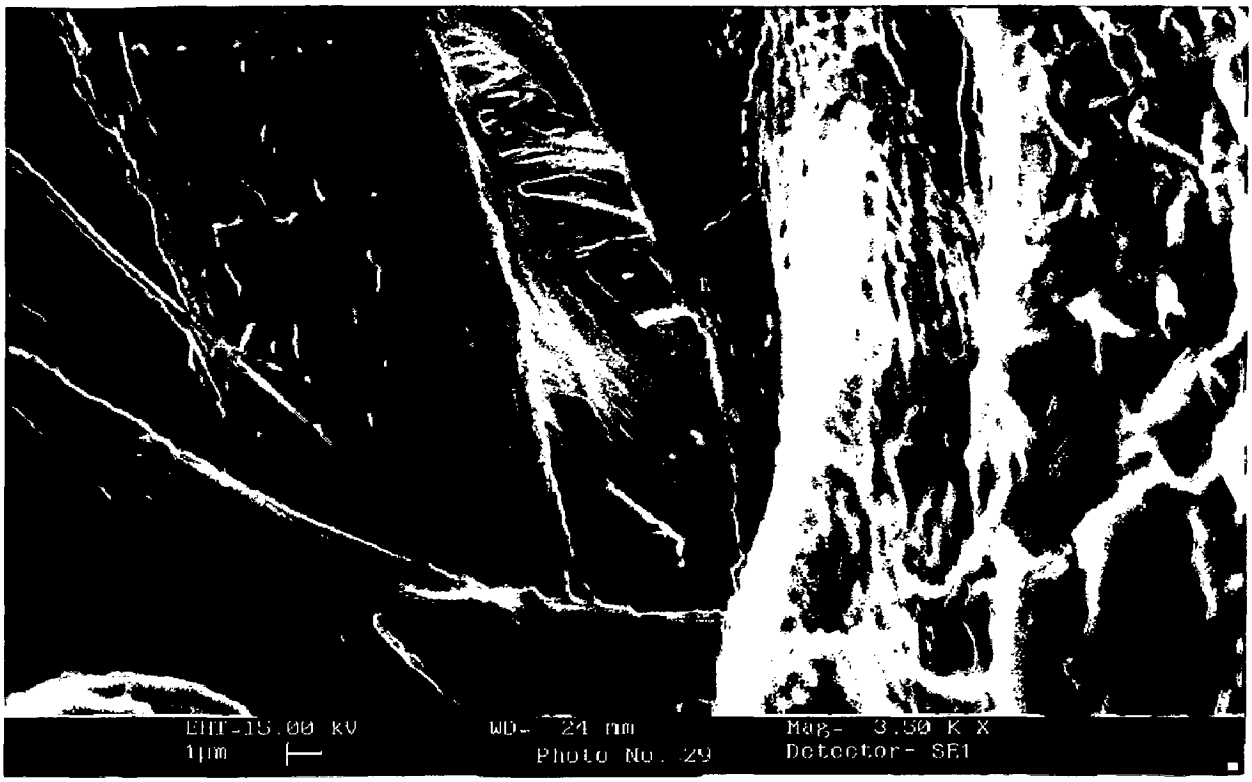

Plate 5.3: Scanning electron microphotograph (SEM) of oxygen delignified enzyme treated kraft pulp of *A. cadamba* without beating at magnifications of 3.5 KX showing smooth surface.

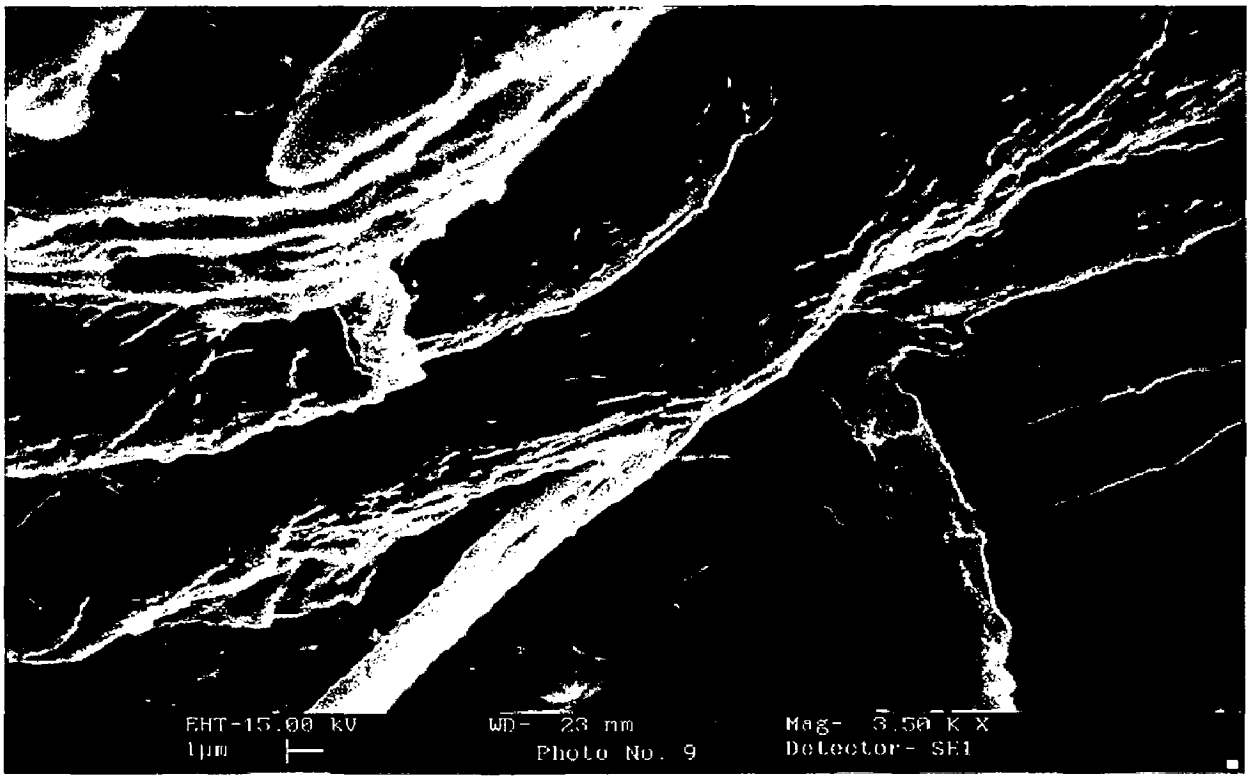

Plate 5.4: Scanning electron microphotograph (SEM) of enzyme treated brown stock kraft pulp of *A. cadamba* without beating at magnifications of 3.5 KX showing smooth surface.

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CHAPTER: 6

CONCLUSIONS AND RECOMMENDATIONS

6.1 CONCLUSIONS

The present study aims at isolating of alkali-tolerant white rot fungi for biobleaching of kraft-AQ pulp of *A. cadamba* and their effect on viscosity, optical and mechanical strength properties of paper and pollution load generated in terms of COD, colour and AOX were studied. The followings are the major conclusions drawn from the present investigations:

1. The fungal culture isolated from decaying wood samples are white rot basidiomycetes. Out of 15 isolates two are the alkali-tolerant fungi. Appearance of fruiting bodies, dark spores with apical germ pore, bipolar mating and pileocystidia with cylindrical neck and rounded apex validates that both the isolates are the different strains of *Coprinellus disseminatus*. Both of the fungal strains are xylanase, cellulase, mannanase, laccase, lignin peroxidase and amylase producers. Wheat bran is selected as a core substrate for further studies. Both the strains are designated as Enzyme-A (MLK01) and enzyme-B (MLK07).

2. Optimum incubation period for xylanase induction in case of both the strains of *C. disseminatus* i.e. MLK01 and MLK07 is 8 and 9 day respectively under LSF conditions. In case of strain MLK01 cellulase activity decreases after 9^{th} day of incubation while lignin peroxidase activity increases up to 11^{th} day of incubation. In the similar way, the cellulase activity of strain MLK07 decreases after 7^{th} day of incubation and lignin peroxidase activity increases up to 13^{th} day of incubation. Similarly, both the fungal strains show maximum xylanase production and mycelial growth at alkaline pH of 10.0. While cellulase and lignin peroxidase activities are found maximum between pH 4-5. All the sugars (xylan, glucose, xylose, and galactose) are found to repress the xylanase as well

as cellulase activity. The lignin peroxidase activity increases by 4 times in presence of xylose while, lignin peroxidase activity in presence of glucose and xylan separately increases by 2 and 3 times respectively. Wheat bran is found to be the best substrate for mycelial growth and xylanase production and the maximum xylanase activity is observed 30.32 IU/mL for strain MLK01 and 36.87 IU/mL for strain MLK07. The wood dust was found to be the poorest substrate for enzyme production.

3. The presence of urea in wheat bran medium is found to repress the xylanase as well as lignin peroxidase production while it enhances the mycelial growth and cellulases secretion. The crude xylanase from MLK01 shows the maximum xylanase activity at pH 7.5 and temperature of 75 ^{0}C while, xylanase from MLK07 shows maximum xylanase activity at pH 8.0 and temperature 65 ^{0}C. The xylanase activity in case of both the fungal strains is inhibited by the presence of metal ions like, $HgCl_2$ and $CuSO_4$ at 1.0 mM concentration whereas; metal ions like, $ZnSO_4$ and $FeSO_4$ are found to enhance the relative xylanase activity up to 200% at the same concentration.

4. Both the strains of white rot fungi (MLK01 and MLK07) are considered as alkali-tolerant strains of *Coprinellus disseminatus*. Both the strains produce thermo-alkaline xylanases on cheaper wheat bran substrate. The pulp produced after brown stock washing in a pulp and paper industry at high temperature is alkaline in nature. The xylanases produced from both the strains of *C. disseminatus* are cost effective and able to serve the purpose due to their thermo-alkaline nature.

5. Proximate chemical analysis indicates that *A. cadamba* is having higher holocellulose (76.2%) and α-cellulose (44.3%) contents which are directly related to strong and high pulp yield. *A. cadamba* contains 20.6% lignin content and wood is diffuse porous. It has been concluded that *A. cadamba* requires lower cooking chemicals and milder pulping conditions in order to produce the bleachable grade pulp of low kappa number. The

average fiber dimensions of *A. cadamba* are: fiber length 1.43 mm, fiber width 38.12 μm, lumen width 26.10 μm and cell wall thickness 5.51μm.

6. *A. cadamba* is fast growing hardwood. The bulk density and screened pulp yield increases with increasing plant age up to 12 years. The optimum cooking conditions of *A. cadamba* of age 4 years are: 16% alkali (as $N_{a2}O$), maximum cooking temperature 165 ^{0}C , time at maximum temperature 90 min, sulphidity 20% and liquor to wood ratio of 3.5:1. *A. cadamba* produces optimum screened pulp yield of 48.74% at kappa number 22.5. Addition of AQ at 0.1% increases pulp yield by 0.38% and kappa number reduces from 22.5 to 16. A beating level of 44 ^{0}SR is found optimum to produce optimal mechanical strength properties like tensile index, burst index and double fold except tear index which declines after 35 ^{0}SR.

7. The optimum conditions for prebleaching of kraft-AQ pulp of *A. cadamba* are: enzyme dose 5 IU/g (o.d. pulp basis), consistency10%, reaction time 90 min and temperature 75 ^{0}C for enzyme-A and enzyme dose 10 IU/g (o.d. pulp basis), consistency 10%, reaction time 120 min and temperature 65 ^{0}C for enzyme-B . Under optimized conditions enzyme-A reduces kappa number by 32.6% after prebleaching of kraft-AQ pulp followed alkali extraction (2% on o.d. pulp basis) with improvement in viscosity by 6.47% and marginal improvement in pulp brightness. In the same way, enzyme-B reduces kappa number by 34.4% with an increase in viscosity by 7.91% and marginal improvement in pulp brightness.

8. In conventional (CEHH) bleaching sequence, it has been observed that the higher residual lignin pulp has more effect of enzyme action than lower residual lignin pulp. The effect of enzymatic prebleaching during CEHH bleaching of kraft-AQ pulp of kappa number 16 (total chlorine demand 4%) and kraft-AQ pulp of kappa number 24 (total chlorine demand 6%) on viscosity, pulp brightness, optical and mechanical

strength properties and pollution load was studied. The brightness of 16 kappa number pulp increases by 3.66% and 4.11% in case of ^{A}XECEHH and ^{B}XECEHH bleaching sequences respectively compared to control. Similarly, the brightness of 24 kappa number pulp increases 7.84 and 9.48% in case of ^{A}XECEHH and ^{B}XECEHH bleaching sequences respectively. Viscosity of 16 kappa number pulp reduces by 0.9 and 0.6 cps in case of ^{A}XECEHH and ^{B}XECEHH bleaching sequences respectively compared to control. In the same way, pulp of kappa number 24 shows a reduction in viscosity by 0.8 and 0.4 cps in case of ^{A}XECEHH and ^{B}XECEHH bleaching sequences respectively. Both the bleached pulps of kappa number 16 and 24 require 45.26 and 38.89% lesser PFI revolutions to achieve a beating level of 35 ^{0}SR in case of ^{A}XECEHH bleaching sequence whereas; 38.95 and 33.33% lesser PFI revolutions are required for pulps of kappa number 16 and 24 respectively to achieve a same beating level in case of ^{B}XECEHH bleaching sequence compared to control. Both the enzymes i.e. A and B improve tensile index, burst index and double fold and reduces tear index of ^{A}XECEHH and ^{B}XECEHH bleached pulps of kappa numbers 16 and 24. Enzyme-A reduces AOX in combined effluent of pulps (kappa number of 16 and 24) bleached by ^{A}XECEHH bleaching sequence separately by 19.51 and 44.11% respectively compared to control while enzyme-B reduces AOX of pulps (kappa number of 16 and 24) bleached by ^{B}XECEHH bleaching sequence separately by 42.77 and 53.46% respectively compared to control. Both the enzymes i.e. A and B increase the COD and colour of combined effluent generated during bleaching of pulps of kappa numbers 16 and 24 by ^{A}XECEHH and ^{B}XECEHH bleaching sequences.

9. The brightness of ^{A}XECEHH and ^{B}XECEHH bleached pulps at 2% total chlorine demand reduces by 8.63 and 6.64 % respectively compared to ^{A}XECEHH and ^{B}XECEHH bleached pulps at 4% total chlorine demand. On the other hand, viscosity of ^{A}XECEHH and ^{B}XECEHH bleached pulps at 2% total chlorine demand improves

marginally compared to ^{A}XECEHH and ^{B}XECEHH bleached pulps at 4% total chlorine demand. The PFI revolutions to get a beating level of 35 ^{0}SR at chlorine demand of 2% are slightly more than that of pulp bleached at chlorine demand of 4%. Similarly, the mechanical strength properties such tensile index, burst index, double fold and tear index of ^{A}XECEHH and ^{B}XECEHH bleached pulps at 2% chlorine demand improve marginally compared to ^{A}XECEHH and ^{B}XECEHH bleached pulps at 4% chlorine demand. The AOX, colour and COD of combined effluent of ^{A}XECEHH and ^{B}XECEHH pulps at total chlorine demand of 4% reduces compared to ^{A}XECEHH and ^{B}XECEHH bleaching sequence bleached at 2% total chlorine demand.

10. The brightness of ^{A}XECEHH and ^{B}XECEHH bleached pulps decreases with increasing pH from 5 to 9 during enzymatic prebleaching with enzyme-A and B. While, viscosity of pulps in both the bleaching sequences increases with increasing pH (5 to 9) during enzymatic prebleaching with enzyme-A and B. All the mechanical properties such as tensile index, burst index, double fold and tear index of ^{A}XECEHH and ^{B}XECEHH bleached pulps of kraft-AQ pulp of *A. cadamba* improve with varying pH from 5 to 9 during enzymatic prebleaching with enzyme-A and B. In both the bleaching sequences, AOX in combined effluent of ^{A}XECEHH and ^{B}XECEHH sequences increases with increasing pH from 5 to 9. On the other hand, COD and colour of combined effluent of both the bleaching sequences decreases with increasing pH.

11. An oxygen pressure of 5 kg/cm^2 reduces the kappa number by 43.75%, viscosity by 18.70 and improves the pulp brightness 10.10 % of the unbleached pulp. The oxygen delignified pulp when treated with enzyme-A and B separately and followed by alkali extraction shows a reduction in kappa number (16.67 and 15.55% for enzyme-A and B respectively) and improvement in pulp brightness (7.2% for enzyme-A and 5.98% for enzyme-B) and viscosity (5.31% for enzyme-A and 5.98% for enzyme-B).

12. The $O^{A}XEDED$ and $O^{B}XEDED$ bleached pulps show an improvement in final brightness and viscosity compared to control. The PFI revolutions to get a beating level of 35 ^{0}SR, both the $O^{A}XEDED$ and $O^{B}XEDED$ bleached pulps require 5.76 and 11.53% respectively more revolutions compared to ODED bleached pulp. Similarly, both the bleaching sequences i.e. $O^{A}XEDED$ and $O^{B}XEDED$ show an improvement in tensile index, burst index and double fold and reduction in tear index over ODED bleaching sequence. Enzyme A and B reduces AOX load and increases COD and colour in the combined effluent of $O^{A}XEDED$ and $O^{B}XEDED$ bleaching sequences compared to ODED bleaching sequence.

13. The final brightness improves by 1.89 and 3.10% respectively in case of $O^{A}XEDEP$ and $O^{B}XEDEP$ bleached kraft-AQ pulps of *A. cadamba* whereas; viscosity improves marginally in both the bleaching sequences compared to ODEP bleaching sequence. The PFI revolutions to achieve a beating level of 35 ^{0}SR, both the $O^{A}XEDEP$ and $O^{B}XEDEP$ bleached pulps respectively require 7.6 and 12.82% more revolutions compared to ODEP bleached pulp. The mechanical strength properties and effluent characteristics of $O^{A}XEDEP$ and $O^{B}XEDEP$ bleaching sequences follow the same pattern as in case of $O^{A}XEDED$ and $O^{B}XEDED$ bleaching sequences but the gain in brightness and viscosity and reduction AOX is more in ODEP, $O^{A}XEDEP$ and $O^{B}XEDEP$ bleaching sequences than that of ODED, $O^{A}XEDED$ and $O^{B}XEDED$ bleaching sequences. Further, ODEDP, $O^{A}XEDEDP$ and $O^{B}XEDEDP$ bleaching sequences show an improvement in brightness and viscosity over ODEP, $O^{A}XEDEP$ and $O^{B}XEDEP$ bleaching sequences. Rest of the properties follows the same pattern.

14. The introduction of enzyme-A and B in $O^{A}XEQPP$ and $O^{B}XEQPP$ bleaching sequences of kraft-AQ pulp of *A. cadamba* improves the pulp viscosity and increases PFI revolutions to get a beating level of 35 ^{0}SR over OQPP bleaching sequence. All the

mechanical strength properties except tear index improve in $O^{A}XEQPP$ and $O^{B}XEQPP$ bleaching sequences while COD shows an increase except colour in both the bleaching sequences compare to OQPP bleaching sequence.

15. It is remarkable that the mechanical strength properties of test pulp are found to increase after enzyme treatment while both enzyme preparations reduce the final viscosity of test pulp. It might be due to the positive effect of cellulase on pulp fiber which increases the swelling of fiber this result in more surface area of fiber with spongy primary cell wall and require lesser beating time for get same beating level compared to untreated pulp.

6.2 Recommendations

With reference to the present work done and targets achieved; the following suggestions have been recommended:

1. The isolated fungal strains need more study for enhanced production of alkaline-thermostable xylanase from alkali-tolerant strains of *Coprinellus disseminatus* by employing strain improvement techniques such as mutation, protoplasm fusion, or by genetic engineering.
2. The sources of alkalinity and thermo-stability can be identified at gene level (genomics) by molecular techniques and their expression in commercial fungal strains such as *Trichoderma reesii* for commercial purpose can be done.
3. The thermo-alkaline xylanase can also be studied at protein level (proteomics) by identifying the particular amino acids which might be helpful to construct more thermostable and alkaline tolerant proteins (enzymes).
4. Similarly, the cellulase activity can be minimized at threshold level without affecting the ligninolytic activity by mutation or by altering the substrate composition, environmental parameters and other nutrient requirements.

5. The negative effect of endo-cellulase on pulp viscosity can also be minimized by optimizing the environmental parameters such as, enzyme dose, reaction time, reaction temperature and pH during enzymatic prebleaching of pulp.
6. The pollution load (COD, colour and AOX) can be minimized by lowering the chemical dose after enzyme prebleaching of pulp.

LIST OF PUBLICATIONS

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