PRODUCTION OF MICROBIAL CELLULASE AND XYLANASE ENZYMES AND THEIR ROLE IN WASTE PAPER RECYCLING

A THESIS

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

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CANDIDATE'S DECLARATION

I hereby certify that the work which is being presented in the thesis entitled **"PRODUCTION OF MICROBIAL CELLULASE AND XYLANASE ENZYMES AND THEIR ROLE IN WASTE PAPER RECYCLING"** in partial fulfilment of the requirements for the award of the **Degree of Doctor of Philosophy** and submitted in the **Department of Paper Technology** of the **Indian Institute of Technology Roorkee, Roorkee** is an authentic record of my own work carried out during the period from January, 2006 to October, 2011 under the supervision of Dr. Dharm Dutt, Associate Professor, Dr. C.H. Tyagi, Associate Professor, Department of Paper Technology and Dr. R.P. Singh, Professor, Department of Biotechnology, Indian Institute of Technology Roorkee, Roorkee.

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

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

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ABSTRACT

Pulp and paper worldwide is today looking at, to gain the reputation of being environmental friendly through innovative process technologies, state of art of modern plant & machinery and compliance of stringent government policies on corporate responsibilities of environmental problems. Water pollution through discharge of treated effluents from paper industry is a matter of great apprehension. The second burning issue is the scarcity of the raw material for pulp and paper making. The paper consumption is increasing day-by-day and available resources are declining every day which has made the pulp and paper industry to search for new avenues in order to bridge the gap. Since the wood is not much available for pulp and paper making so the future of Indian industry looks to be dependent on either agro-based raw materials or the waste paper recycling. Thirdly, the pulp and paper manufacturing, unfortunately, is energy intensive. Energy is therefore a vital subject affecting the economy of any pulp and paper operations. In integrated Indian pulp and paper mills energy cost accounts for up to 20 to 30% of the total cost of productions. This is an alarming situation and we should focus all of our attention toward these above discussed problems, otherwise it will be difficult for us to survive in the globally competitive market. The global concerns about energy, preservation of forests and elimination of pollution from pulp and paper making processes have led us towards exploration of alternate fibrous resources other than wood that are compassionate with the environment without sacrificing product quality.

Keeping in view the above problems in mind, the raw material selection for pulp and paper making becomes important. If we look back at the problems, the waste paper (secondary fibers) as a pulp and making raw material seems to be one of the promising raw materials for pulp and paper making process. Deinking is an important operation while using waste paper as a raw material for paper making. Toner particles produced during conventional repulping process with high cost chemicals are too large to be removed by the ink flotation and washing processes and are a major technical obstacle for greater use of these varieties. The use of micro-organisms and their enzymes to replace or reduce chemical consumption during deinking of office waste paper in the pulp and paper industry is gaining utmost interest.

The industries are using chemicals for this purpose and they are costly and have adverse effect on the environment. Therefore an effort has been to utilize. In past two decades, microbial enzymes have been used extensively in pulp and paper industry in various sections like pulp bleaching, pulp refining and bio-de-inking. Commercial enzyme preparations can replace conventional deinking chemicals to remove toner inks. The enzyme for deinking and the intensive research studies have been made to develop such enzymes in the laboratory to make the process economically viable and environmental friendly the pulp and paper industry.

As mentioned above and keeping upon requirements in today's industrial scenario, the major outline of the work were structured as given below:

Isolation screening and evaluation of fungal strains for cellulase and xylanase production.

A total of 22 fungal strains were isolated from different sites (lignocellulosic waste, decomposing manure, sugarcane dumping site, fruiting body and paper industry waste). On the basis of primary (CMC-agar and xylan-agar plate assays) and secondary screening (enzyme production under SSF), AT-2 and AT-3 were selected as the best cellulase producers while the strain AT-1 held prominent xylanase production ability among all the isolates. The test isolates AT-2 and AT-3 were identified as different strains of *Aspergillus* species i.e. *Aspergillus flavus* AT-2 and *Aspergillus niger* AT-3 respectively from Indian Agricultural Research Institute (IARI), New Delhi (India). The selected fungal isolate AT-1 was morphologically acknowledged as a wild mushroom from Indian Type Culture Collection (ITCC), Plant Pathology Division, IARI, New Delhi (India). For the identification of *wild mushroom AT-1* up to species level, molecular identification (ITS) was done at MTCC (IMTECH, Chandigarh). The NCBI-BLAST search analysis of the DNA sequence-data indicated that the fungus was a member of *Coprinus cinereus*. The Scanning electron microscopy (SEM) was carried out to study the finer structural of strains AT-1, AT-2 and AT-3.

Analysis of cellulase production under submerged (SmF) and solid-state fermentation (SSF) system and selection of fermentation system yielding higher levels of cellulase and evaluation of critical parameters for achieving maximum production levels

Production of cellulase by fungal isolates AT-2 and AT-3 was analyzed under SmF and SSF. Among the different carbon sources (rice straw, wheat bran, wheat straw, sorghum and bagasse) used during the SSF of strains AT-2 and AT-3, rice straw led to the maximum level of cellulase production. XRD and SEM studies valorized rice straw (pretreated) as the carbon substrate to be used for further enzyme (cellulases) optimization studies. SEM photomicrographs had exposed that untreated rice straw had compact and rigid structure compared to the treated rice straw, which was loose and fibrous. The maximum level of cellulase production by AT-2 and AT-3 occurred on 5^{th} day of incubation, pH 4.8 and 5.3 respectively, temperature 32^0C and a solid substrate: moisture content ratio of 1:3. Nitrogen sources $(NH_4)_2$ SO_4 (inorganic) and yeast extract (organic) were found to be the best for both the strains. Both the strains were biochemically characterized by determining their temperature and pH stabilities and based on their pH and thermal stabilities, AT-3 was chosen for the bio-deinking studies and SDS PAGE analysis shows molecular weight of AT-3 29kDa.

Analysis of xylanase production under SmF and SSF system and selection of fermentation system yielding higher levels of xylanase and evaluation of critical parameters for achieving maximum production levels

Production of xylanase by fungal isolate AT-1 was analyzed under SmF and SSF. SSF appeared to be a suitable and led to relatively higher production levels. Among the different carbon sources (rice straw, wheat bran, wheat straw, sorghum and bagasse) used during the SSF of strain AT-1, wheat bran led to the maximum level of xylanase production. The maximum level of xylanase production by AT-1 occurred on 7^{th} day of incubation. The maximum xylanase production by a strain AT-1 was obtained at optimum conditions like pH 6.4, temperature 37^0C and a solid substrate: moisture content ratio of 1:3. Among organic nitrogen sources tested, yeast extract facilitated the highest xylanase production in strain AT-1. The strain AT-1 was biochemically characterised for temperature and pH stabilities and based on its thermo-alkali tolerant behaviour, strain AT-1 was chosen for the bio-deinking studies. SDS PAGE analysis shows molecular weight of AT-1 29kDa.

Analysis and evaluation of the crude enzyme preparations for its application in bio-deinking of SOP

The crude enzyme (cellulase) preparation from AT-3 was used in enzymatic deinking trial of SOP. Enzymes were added before pulping, during pulping and after pulping. It was observed that out of three distinct addition points of enzyme and surfactant, excellent results were obtained in case when enzyme and surfactant were added after pulping Optimum conditions for SOP pulping were: pulping time 20 min, pulping temperature 65^0C, conditions for enzymatic treatments were: cellulase dose of 6 IU/g (O.D. pulp basis), temperature 55 ^{0}C, pH 5.3, surfactant dose 0.05%, pulp consistency 12% and reaction time 60 min and floatation deinking was carried out for 10 min. At these optimized conditions, maximum reductions in ERIC values and dirt counts and maximum improvement in brightness and deinkability factors were observed.

Deinking of SOP with different natural and commercial enzymes concoctions under optimized conditions

The enzyme concoctions used on SOP pulps improved deinking efficiency in the following ascending order of enzyme doses: Control<C (6IU/mL)<CX (3 and 3IU/mL) < CX (6 and 3IU/mL) < CX (6 and 6IU/mL) < CXA (6, 3 and 1.5 IU/mL) < CXA (6, 3 and 3 IU/mL) < CXA (6, 3 and 6 IU/mL) <CXAL (6, 3, 1.5 and 1.5 IU/mL) < CXAL (6, 3, 1.5 and 3 IU/mL) <CXAL (6, 3, 1.5 and 6 IU/mL) [C= cellulase, X=xylanase, A=amylase, L=lipase].

Evaluation and comparison of various deinking processes i.e. conventional, enzymatic and chemi-enzymatic deinking

Chemical deinking of SOP showed hike in brightness (10.35%), D_B (24.67%) and D_E (78.71%) along with reduction in ERIC value (64.27%) and dirt count (83.74%) compared to control. SOP pulp when treated with crude cellulase (*A. niger* AT-3) prior to ink flotation, mitigated ERIC value and dirt counts by 61.84 and 82.29% where as brightness, D_B and D_E improved by 8.13, 23.10 and 76.30% respectively compared to control. In chemi-enzymatic deinking trials, the chemical dosages were reduced from 100% to 75, 50 and 0% while keeping the dosage of cellulase constant. Brightness, D_B, and D_E increased with increasing chemical dosing and conversely, dirt count and ERIC values decreased accordingly during chemi-enzymatic deinking compared to enzymatic deinking. Similarly, all the strength properties mitigated as a result of increasing chemical dosing and total solids, COD and BOD of combined effluent increased in chemi-enzymatic deinking compared to enzymatic deinking.

Effect of repeated recycling on paper properties during enzymatic and conventional deinking processes

As a result of repeated recycling mechanical strength properties like burst index, tensile index and double fold numbers decreased whereas tear index increased up to 3rd recycling. The 4th recycling showed an insignificant increase in tear index. Bulk and opacity increased up to 3rd recycling and then remained almost constant.

Effect of enzymatic and conventional deinking on removal of micro-stickies

Chemical, cellulase, cellulase+xylanase, cellulase xylanase+amylase and cellulase xylanase+amylase+lipase deinking processes removed micro-stickies by 42.02, 46.01, 46.07, 46.24 and 48.59% respectively compared to control (3060 no./kg).

Effect of enzymatic and conventional deinking on ink particle size distributions

Ink particles size in terms of number of specks and number of specks/cm^3 in chemical and enzymatic deinking processes reduced top to bottom in the following order: chemical>cellulase>cellulase+xylanase>cellulase+xylanase+amylanase>cellulase+xylanase+amylase+lipase.

SEM, AFM and XRD, TGA and FTIR studies of different deinked pulps

AFM and SEM studies indicated various morphological changes in fiber surface brought about during conventional, enzymatic and chemi-enzymatic deinking trials. The surface roughness increased in the following descending order of enzymatic concoction: control< cellulase< cellulase+xylanase< cellulase+xylanase+amylase< cellulase+xylanase+amylase. The introduction of lipase to the mixture of cellulase, xylanase and amylase caused surface roughness increased by 159% compared to control SOP

FTIR studies of SOP pulp showed the appearance of band at 875 cm^{-1}. The disappearance of this band at 875.82 cm^{-1} showed the dissolution of xylan by crude enzymes during enzymatic deinking and by peeling reactions during chemical deinking. The peak cantered at 1770-1800 cm^{-1} corresponds to vinyl ester group, which comes under strong intensity region. The band at a wavelength of 1797.80 cm^{-1} was observed in FT-IR spectrum of SOP (control) which disappeared in chemical and enzymatic deinked pulp.

XRD analysis of deinked pulp showed that the crystalline behaviour increased with enzyme concoction and found to be maximum in case of deinking process having cellulase+xylanase+amylase+lipase. There was an insignificant increase in crystallinity of

pulps with increasing chemical dosing i.e. from 41.3 to 42% in chemi-enzymatic deinking process.

TGA analysis indicated the weight loss in SOP at a temperature between 27-100 ^{0}C was 5.40% and weight losses decreased with increasing crystallinity of deinked pulps in the following order: chemical deinked pulp>cellulase deinked pulp> cellulase+chemical (50%)>cellulase+xylanase deinked pulp>cellulase+chemical (100%) deinked pulp. In case of enzymatic and chemi-enzymatic deinking the weight losses decreased in the following order:

cellulase>cellulase+xylanase>cellulase+chemical (50%)>cellulase+ chemical (100%). At 500 ^{0}C, SOP, chemically and cellulase deinked pulps showed a residual mass of 11.86, 2.10 and 0.11% respectively. The fist DTG peak temperatures observed for SOP, cellulase, chemical, cellulase+xylanase, cellulase+chemical (100%) and cellulase+chemical (50%) deinked pulps were 344, 343, 342, 344, 341 and 345 ^{0}C and their degradation rate per min was 1.83, 2.29, 1.73, 1.16, 0.95 and 0.17 mg/min respectively. Whereas, second DTG peaks were observed at 476, 482, 475, 477, 450 and 496 ^{0}C with degradation rate per min as 0.11. 0.15, 0.13, 0.09, 0.08 and 0.17 mg/min respectively for the above mentioned pulps.

Deinking model equations and statistical analysis

All experiments were done in triplicates and standard deviation was given in each table. For deinking experimental runs, statistical analysis was done and empirical model equations developed with the help of mathematical polynomial regression analysis program for estimation of deinkability factors i.e. D_E and D_B respectively.

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ABBREVIATIONS

AFM	Atomic Force Microscopy
AOX	Adsorbable Organic Halide
BOD	Biological Oxygen Demand
BSA	Bovine Serum Albumin
bp	Base pair
cm	Centimètre
CBD	Cellulose binding domain
CBH	Cellobiohydrolase
CED	Cupriethylenediamine
CMC	Carboxymethylcellulose
COD	Chemical Oxygen Demand
^{0}C	Degrees Celsius
^{0}SR	Degrees Schopper Reigler
DP	Degree of polymerization
DTG	Differential thermo gravimetric
EDTA	Ethylenediaminetetraacetate
EG	Endoglucanase
g	Gram
GDP	Gross domestic products
h	Hours
IU	International unit
ISO	International organization of standardization
kDa	Kilo Dalton
kg	Kilograms
kJ	Kilo joule
kPa	Kilo pascal
kV	Kilovolt
L	Litres
LCW	Lignocellulosic waste

CHAPTER 1

INTRODUCTION

Today, we live in a highly competitive world. It applies to all walks of life whether you are in consumer industry service sector or in manufacturing sector. These seem to be an unprecedented urgency to stay ahead and excel. Everybody is in hurry as though there is no tomorrow. Paper industry is no exception. The scarcity of raw material in paper industry, the shaky global economy and adverse environmental impacts have compelled the manufacturer to think over towards devising for the technology which is cheap and environmental friendly. To achieve these targets we have to be positive while making the selection of cheapest raw material, optimum energy consumption and minimum hazards to the environment.

Any manufacturing operation brings along with it a distinct impact on the environment. Paper manufacturing also is no exception. The paper industry is power intensive as well as water intensive. The researchers are trying their best by finding a suitable and cheap raw materials (as a substitute of wood), innovative technology for pulp and paper making with no or minimum environmental impacts and this is the reason that small paper mill in india are going either by installing a recovery plant or switching over to waste paper (recycled fiber) to become environmental friendly. In addition to the adaptation of new technologies in pulping/ bleaching, attention is also being paid to the use of environment friendly enzymes to enhance the pulping/bleaching operation with reduced impacts of chemicals which in turn saves us from the environment hazards.

It will be unfortunate if we do not highlight and discuss the energy consumption in pulp and paper industry. The cost of energy for manufacturing of pulp and paper accounts for nearly 20% of total variable cost [6]. After the economy of the country was thrown open, the industry has to face tough competition against global players with respect to quality and cost. To add to this problem, the cost of power, fuel and raw materials are increasing day- by- day threatening the very existence of the industry under the globalization and liberalization scenario. So it becomes necessary to be more attentive towards these major problems and to take timely steps in this direction. Now we will like to know what is happening around the world.

The consumption of paper is an index of a nation's growth. Therefore, per capita consumption of paper may be considered as a measure for progress in areas pertaining to industrial, cultural and educational developments. The global market for paper was about 80 million metric tons per annum at the beginning of 1980s that is reached to 350 million metric tones by the year 2003 [44]. The consumption of paper may be directly correlated to population and literacy rate and 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 1.1).** GDP and paper demand per capita are allied with economic growth [41]. **Figure 1.2** reveals that India has a huge growth potential in concern with paper industry.

In **Table 1.1,** the average pulp and energy consumptions are shown for the different grades. Raw wood use, energy use and by-product fuels produced show high differences amongst the different paper products as shown in **Table 1.1.** Board production with a large recycled fiber share leads to very small raw wood and energy use. Newsprint paper production stands out with its electricity consumption and fine paper production has the largest wood and steam consumption. On the other hand, its fuel production reflects the amount of wood used [56].

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 insufficient to meet this growing demand for paper especially in the Asia-Pacific region and Eastern Europe [3]. The world consumption of paper and paper board in 2006 was 380.28 million tonnes [70] of which 36% were consumed in Asia and 64% in rest of the world **(Figure 1.3).** 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 [52, 26, 13]. 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 [42]. The consumption of paper in India is one of the lowest i.e. 8.5 kg/person and will be expected to increase 9 kg/person by 2010 **(Table 1.7)** [53, 24]. On the other hand, paper consumption in the world in 2004 amounted to an average 52.45 kg per person/ year which was 16.32% higher than that in 1991 [46].

The annual current production gap is 0.7 million tonnes and will become 1½ times greater during 2010-15 [32]. Demand for paper in India is projected to grow at a compounded annual growth rate of 6.1 % from 2004-05 to an estimated 7.4 million tonnes by 2008-09 and 11.87 million tonnes by 2020 [53, 51]. The projected demand for paper board and newsprint is expected to touch 8.3 million tonnes by the year 2010 [27]. The per capita consumption of paper and paper board of five selected countries of Asia was 11 kg/person, while in China; it was 49 kgs/ person in 2006 and will be expected to increase by 14 and 66 kg/person for five selected Asian countries and China **(Figure 1.4).**

At present, the Indian paper sector has maintained a growth rate of around 8% [24]. With such growth prospects, the Indian paper industry has a vital role to play in the socio-economic developments of the country. Indian pulp and paper industry has a number of issues and challenges, particularly in the following thrust areas, such as:

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

The Indian paper industry is primarily dependent on three major raw materials viz. forest, agricultural residues and secondary fibers [62]. Pulp and paper industry uses 39% of forest-based fiber, 31% agro-residue based fiber and 30% fiber is obtained from waste paper [31]. The proportion of non-wood raw materials and waste paper is increasing over the year and at present about 60.8% of non-wood raw materials and 39.2% is based on wood [49]. Forest cover in India is reported to be 67.8 million ha, or 20.6% of the country's surface area, which translates into a per capita forest area of only 0.8 ha/person, one of the lowest in the world [17]. Therefore, forest and woodlands occupy around 20.6%, agricultural land 47% while uncultivated, non-agricultural and barren land 32.4% respectively of a total land area of 328.8 million ha. **(Figure 1.5)** [41]. The country's fuel wood requirement alone is 280 million tonnes/yr, and this will rise to 356 million tonnes/yr by 2010 [38]. Total fiber consumption for the production of paper and paperboard in India will nearly be double between 2006 and 2016, growing from 7.4 million tones/yr to 13.7 million tonnes/yr. India's total wood fiber deficit as per forecast

is to increase at an 11.3% annual rate through 2016 [17]. In the year 2000, the final demand for the wood by Indian paper industry was 5.8 million tonnes. It is projected that this figure will grow over 9 and 13 million tonnes by the year 2010 and 2020 respectively **(Figure 1.6)** [41], as the literacy rate in India has been steadily growing (52% in 2000) [41]. Likely raw material supply, utilization and production of pulp and paper through 2010 are summarized in **Tables 1.2 and 1.3** [54].

Due to dearth of forest resources, the wood-based segment of the paper industry has considerably shrunk, as in 1970, forest based segment of Indian paper industry was 84% which had been reduced to 31% in 2000 [52]. Therefore, inadequate supply of wood fiber due to rapid depletion of forest wealth, forces the paper technocrats to search new alternative and hitherto unexploited sources of fibers such as non-woody plants, agricultural residues and waste paper which may cater the demand for pulp and paper industry. The share of agro based segment of the paper industry was only 9% in 1970 which has been increased up to 31% in 2000 [52].

The main agricultural residues utilized by the paper industry include sugarcane bagasse, cereal straws (wheat and rice), kenaf/mesta, jute sticks, grasses and cotton stalks. The annual potential of agro fibers in India is given in **Table 1.4** [41]. It is expected that even if only 20% of the total quantity can be made available to Indian pulp and paper industry, there should be no difficulty in meeting the targeted demand of paper and board products [15]. The bagasse based paper production estimated by Indian Agro paper Mills Association (IAPMA) is given in **Table 1.5** [55].Indian paper mills utilizing rice and wheat straws have capacities up to 24000 tonnes/annum, which is very small. According to an IAPMA estimate, 9.1 million tonnes of surplus straw will be available for the paper industry by 2010-2011, as shown **in Table 1.6** [18].

The pulp and paper industry is one of the oldest industries and is recognized as one of the seventeen industries causing extensive pollution and damage to the environment in India [29]. With the recognition of climate change as a consequence of anthropogenic carbon emission, every effort is being made to either mitigate environmental impact by reducing emission or working on adaptations of emerging technologies which suits the changing situations. Since, forests can be used as sinks for immobilizing large quantity of carbon dioxide [25]; the existing forest policies do not

allow the paper industry to use wood from any of the national forest reserves [28]. The pulp and paper industry is also under constant pressure to reduce and modify environmental emissions to air and water due to stringent rules of the government. Paper industry in India is on constant watch by the Ministry of Environment and Forest since it is an environment sensitive sector and falls under red category of industries [45].

In view of depleting forest resources, escalating energy costs, large water requirements and desirability of having full scale chemical recovery system; recycled paper forms an important raw material input to the paper-making process. Although recycling is both economically and ecologically sound but recovered paper cannot be efficiently used in all paper grades. In spite of this, paper recovery is expected to grow fast in the future in order to cope up with the increased demand. This may be most probably lead to a continuous price increase of recovered paper. The recovery of the fibre limits certain parameters like fibre length, fibre quality and then only its usability can be decided depending upon the grade of paper to be manufactured. Fiber shortens up every time when it is recycled and at some point, usually after 4–6 cycles, it is too short to be used in papermaking. Therefore a certain amount of virgin pulp blending will always be needed to meet the quality demand set for the products. In the mix of resources for paper making, recycled fiber typically replaces for mechanical pulp in newsprint and in some board grades. Because of its economic and environmental advantages, more and more recycled fiber is expected to be used as raw material in the paper making. The favorable inherent advantages of using waste papers for pulp and paper making are as follows:

- Shortage of raw material
- Save a tree i.e. environmental friendly
- Economical
- Low process utilities cost
- Reduce land fill problem

A paper mill uses 40% less energy to make paper from recycled paper than it is being spent in paper making from fresh lumber. However, a recycling mill may consume more fossil fuels than a paper mill. A tonne of paper made from recycled fibers instead of virgin fibers conserves 7000 gallons of water, 17-31 trees, 4000 Kwh of electricity, 60 pounds of air pollutants and reduce three cubic yards of landfill material. Therefore, it has

become an important environmental issue to recover and reuse secondary fibers as a source of raw material for paper production [35].

The paper industry has turned over to the use of fast growing wood species, alternative non-wood fibers and secondary fibers for paper production [31]. Recovered paper exploitation as raw material for paper making has augmented hugely over the last decade. The recovery of waste paper has increased from 65000 tonnes in 1995 to 850000 tonnes in 2000. About 40% of the total paper production in the world was based on the secondary fiber in 2006 [50]. Almost 200 million metric tonnes of global recovered paper consumption was in 2006, which would be 250 million metric tonnes by 2012. The proportion of non-wood raw materials and waste paper is escalating over the year and at present about 60.8% of paper production is based on non-wood raw materials and 39.2% is based on wood [36]. Waste paper based industry accounts for about one third of Indian paper capacity. Consumption of recovered paper (both imported and domestic) is estimated to be 4-5 million tonnes in India only [11]. 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 in comparison to International standards: Thailand (42%), China (33%), and Germany (71%) [40]. Vishwanathan [63] have stated that the recycling percentage of waste paper is over 40% in developed countries like US, Europe and Japan. It is said to be as low as 20% in developing countries like India. Recovered paper consumption in china is continuously growing, which suggest that china have the largest growth in the recovered paper consumption [14]. Globally, the paper industry uses approximately 50% recycled fibers. [8]. Tandon et al. [57] have indicated that France has recycling rate 84% and Japans has recycling rate up to 60% in domestic paper production. US paper industry uses approximately 40% recycled fibers to make paper products. AF-QPS and NLK associates [2] in their study reported that in the future, levels of recovery of secondary fiber should be close to 70% in Europe and 55% in North America. In most other (often developing) countries, where the recovery rate is still low (less than 40%) the recovery rate is likely to increase more slowly but steadily during the next 15 years. Lopez et al. [33] in their study reported that in 2000, more than 41 million tonnes of recycled paper were used as raw material by the paper industry in Western Europe, the recycling rate for the region being 49.8%. At present about 50% of the fibers used in Europe derived from

recycled paper. In Spain the utilization rate is as high as 79.7% and in Denmark it is around 62%.

Deinking is no longer just a laboratory success story. Today it is a commercially successful industry. Numerous plants have been set up in countries like Canada, United States, Germany, Sweden, etc. Extensive research is continuing at universities and other research centers to further improve the deinking process. The process is very chemistry-intensive and has been studied extensively.

Deinking of office waste paper is very difficult by conventional techniques such as dewashing, dispersion, washing and flotation. Despite of the greater availability of the mix office waste (MOW), the use of these recovered papers in recycling industry to produce higher grade pulp is very much restricted because of bigger visible specks and contaminants in the end product. Toner particles produced during conventional repulping process with high cost chemicals are too large to be removed by the flotation and washing processes and are a major technical obstacle for greater use of these varieties. Most of deinking chemicals increase energy dispersion steps employed in current deinking technologies which are tedious, cost prohibitive, environmentally hazardous and sometimes may lead to loss in pulp yield. The recycling industry is in search of new technologies which can improve the product quality, reduce the production cost and can be accommodated easily in to the existing process design. An alternative to this problem is enzyme-assisted deinking.

The possibilities for employing biotechnology in the pulp and paper industries are numerous. It has the potential to increase the product quality and supply of feed stocks for pulp and paper, reduce manufacturing cost, and produce novel high-value paper products. New enzyme technologies can reduce environmental problems and alter fiber properties.

Enzymes are proteins that catalyze chemical reactions in living organisms. Enzymes work by lowering the activation energy for reactions, thus increasing the rate of the reaction Enzymes are usually very specific to the catalysis reactions and the substrates that are involved in these reactions [30, 39]. Xylanase and cellulase from microbial sources have received a lot of attention, particularly those being derived from fungi. At present, the enzymes are commonly used in many industrial applications and

the demand for more stable, highly active and specific enzymes is growing rapidly. The estimated world sale of industrial enzymes in 1995 was 1.0 billion US dollars which was expected to reach 1.7 and 2.0 billion US dollars by the year 2005 [20]. According to a recent publication, the industrial enzymes have already reached a market of 1.6 billion US dollars [12]. Interestingly, 60% of the total world supply of industrial enzymes comes from Europe, and the remaining 40% from USA and Japan. Also, approximately 75% of the industrial enzymes are hydrolases, with carbohydrolases forming the second largest group. Biotechnology of cellulases and hemicellulases began in early 1980s, first in animal feed followed by food applications [10, 59, 65, 66]. Subsequently, these enzymes were used in the textile, laundry as well as in the pulp and paper industries [19, 68, 69]. During the last two decades, the use of cellulases, hemicellulases and pectinases has increased considerably, especially in textile, food, brewery and wine as well as in pulp and paper industries [21, 22, 48, 60]. Today, these enzymes account for approximately 20% of the world enzyme market [34], mostly from *Trichoderma* and *Aspergillus* [21, 60].

The use of cellulases and hemicellulases is most extensively reported in literature. De-inking, using enzymes at acidic pH, also prevents the alkaline yellowing, simplifies the de-inking process, changes the ink particle size distribution and reduces the environmental pollution. In addition, the enzymatic de-inking improves the fiber brightness, strength properties, pulp freeness and cleanliness as well as reduces fine particles in the pulp. Xylanase treatment has been reported to increase the strength properties, while cellulase treatment improved the brightness and freeness of the pulp [43]. Various hydrolytic enzymes such as cellulase, glucanase, amylase, lipase, and xylanase of bacterial or fungal origins [37, 61, 71] and more recently oxidative enzyme laccase [16] have been used, individually or in combination for deinking of office waste paper [36]. Cellulases and hemicellulases have been demonstrated to dislodge inks by peeling off fibers or fines on paper surfaces [67, 5]. Lipases have shown some direct action on ink particles either degrading oil carriers or breaking down pigments [37]. Lignin-degrading enzymes, such as laccase, also hold some potential for deinking old newsprint, as they may selectively remove surface lignin, and hence facilitate ink removal [64]. So far, cellulolytic enzymes have shown the most promising results for

deinking of mixed office waste (MOW). Different kinds of cellulases are used to facilitate ink detachment, essential in the deinking process for the removal of ink later [9, 7]. Both pilot plant trials and mill applications have demonstrated that cellulase deinking can reduce chemical cost, enhance ink and stickies removal, improve drainage and runnability, and decrease COD and BOD in process waters and effluent [23, 4, 47].

Consequently, the use of micro-organisms and their enzymes to replace or reduce chemical consumption during deinking of office waste paper in the pulp and paper industry is gaining utmost interest. Keeping above perspectives in view, the present research aims at developing environmental benign technology for deinking of mix office waste paper.

The major outlines of the work are defined as below:

- Isolation of fungal strains from lignocellulosic wastes, screening and evaluation of strains for higher cellulase and xylanase production.
- Analysis of cellulase and xylanase production under submerged (SmF) and solid-state fermentation (SSF) systems. Selection of fermentation system in order to get efficient cellulase and xylanase production and optimization of various operating parameters for achieving maximum cellulase and xylanase production.
- Biochemical characterization of enzymes using SDS-PAGE and Zymogram analysis.
- Optimization of various operating parameters for enzymatic deinking.
- Deinking of sorted office waste paper with different natural and commercialized enzyme combinations under optimized conditions.
- Comparison of conventional, enzymatic and chemi-enzymatic deinking processes.
- Effect of repeated recycling on paper properties.
- Micro-stickies removal and ink particle size distribution in different deinking processes.
- Evaluation of deinking efficiency by monitoring optical parameters (brightness, ERIC, deinkablity factor (D_B and D_E) and dirt specks), effluent properties (COD, BOD and total solid) and mechanical strength properties.

- Evaluation of deinking by various advanced instrumental analysis i.e., scanning electron microscopy (SEM), atomic force microscopy (AFM), FTIR analysis, XRD analysis, and Thermal (TGA and DTG) analysis.

Table 1.1: Material and energy use in papermaking

Pulp Shares	News	Fine	Boards
Mechanical pulp	0.60	0.00	0.13
Chemical pulp	0.11	0.74	0.14
Recycled paper pulp	0.23	0.14	0.62
Raw wood use, m^3	2.3	3.9	1.1
Energy, mWh Electricity	2.2	1.4	1.4
Steam	1.6	4.6	2.4
Fuels produced	1.0	3.5	0.7

Table 1.2: Raw material supplies and production of pulp and paper (million tonnes) [58].

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

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

Table 1.3: Utilization of raw materials through 2010 [58].

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

*Bamboo not included

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

Table 1.4: Annual potential of agro based fibers in India in 2001 [41].

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

Table 1.5: Estimated bagasse based paper production: IAPMA 1996 (million tonnes per annum) [55].

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

Table 1.6: Estimated wheat straw based paper production: IAPMA 1996 (million tonnes/annum) [18].

Particulars	1995-1996	2000-01	2005-06	2010-11
Surplus straw available	7.9	8.3	8.7	9.1
Possible paper production	2.2	2.4	2.5	2.6

Table 1.7: Current status of pulp and paper in India [24].

Sl. No.	Particulars	Values	Time of Information
1	Population (million)	1148.0	2008
2	Total pulp/paper/paperboard production	8.5	2008
3	Import	1.38	2007
4	Export	0.3	2007
5	Per capita consumption (kg)	8.5	2008
6	No. of pulp/paper mills	500	2008
7	Main raw materials	Hardwoods, Bamboo, Agricultural residues, Recycled fiber	2008

Note: All production, import, export and net consumption numbers are in million metric tonnes (1,000,000,000 kg).

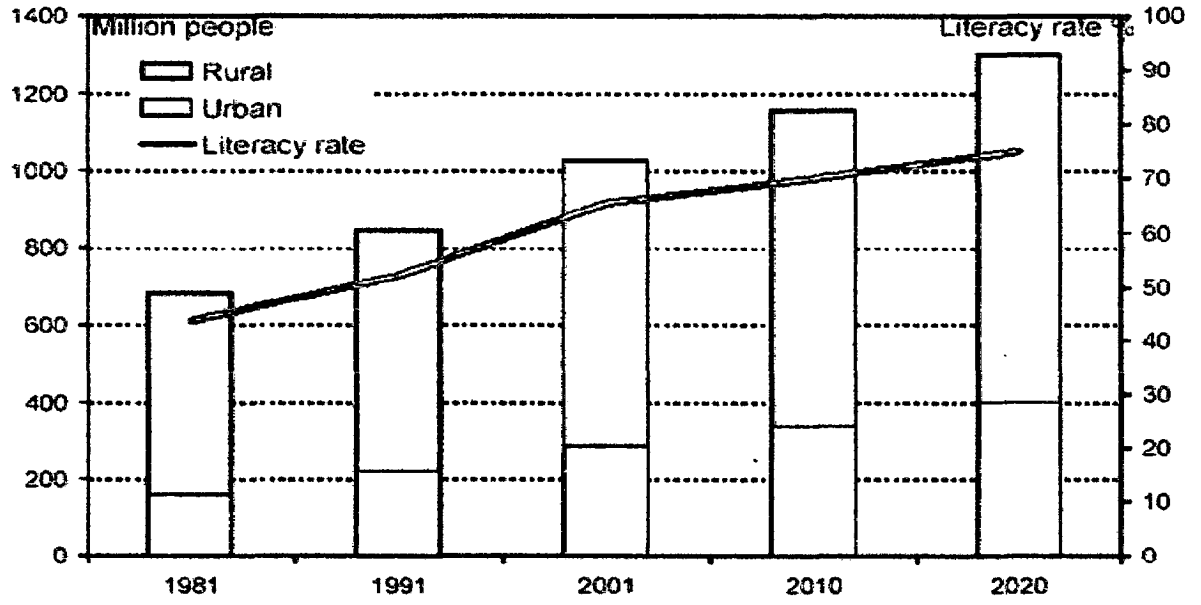


Figure 1.1: Indian population and literacy rate in both rural and urban areas [41].

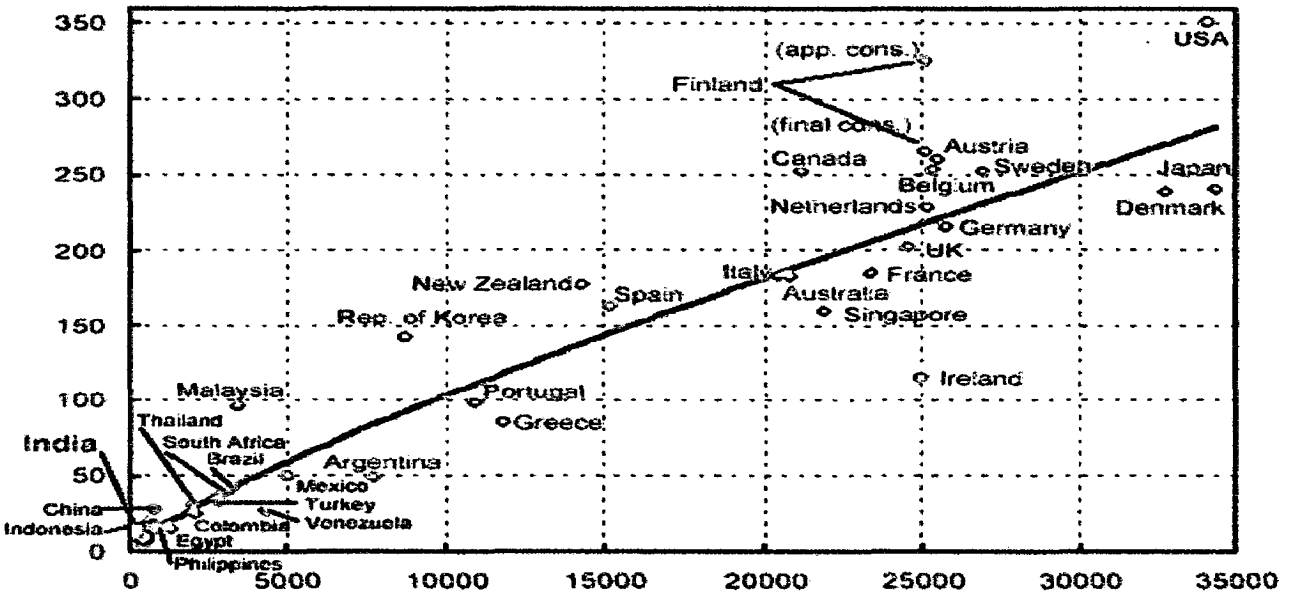


Figure 1.2: GDP and paper demand in the year 2000, kg per capita [41].

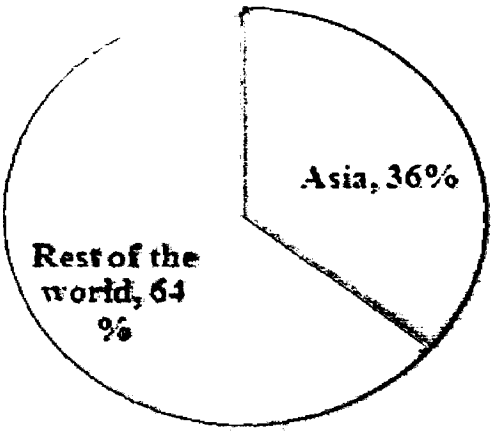


Figure 1.3: Total world paper and paperboard consumption in 2006 [53].

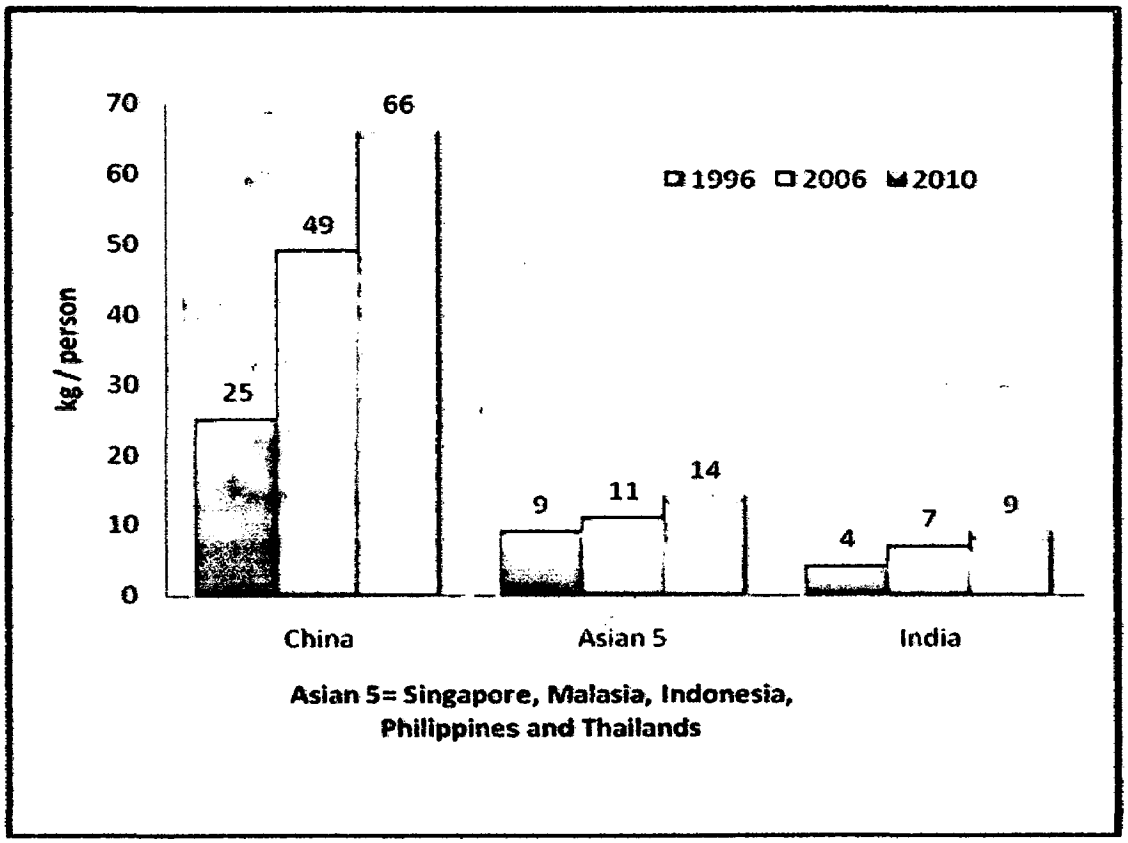


Figure 1.4: Per capita consumption of paper and paper board in Asia [1, 40].

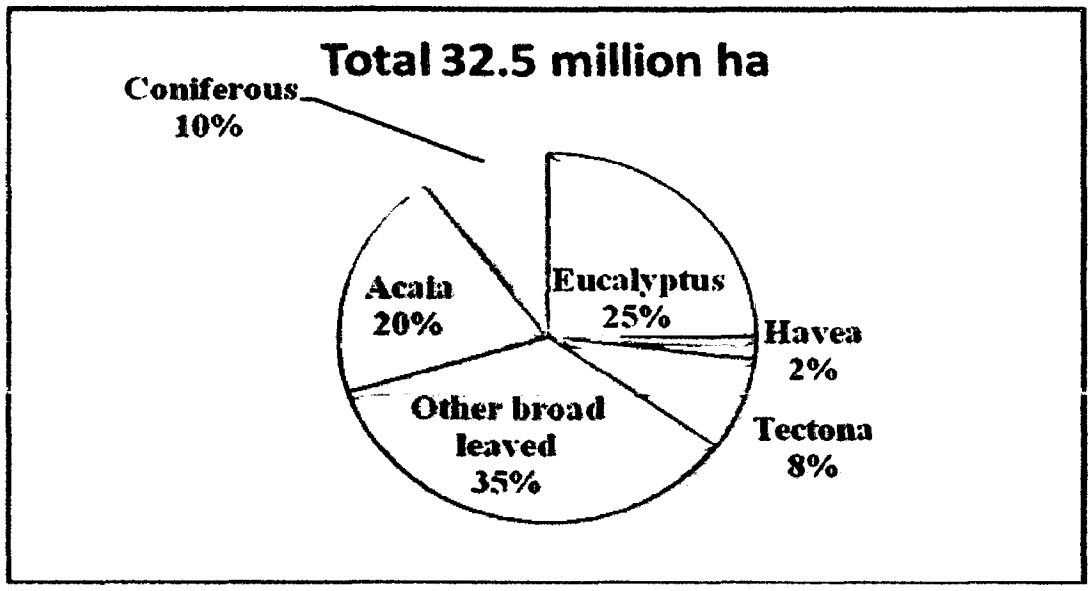


Figure 1.5: Forest plantation in India [41].

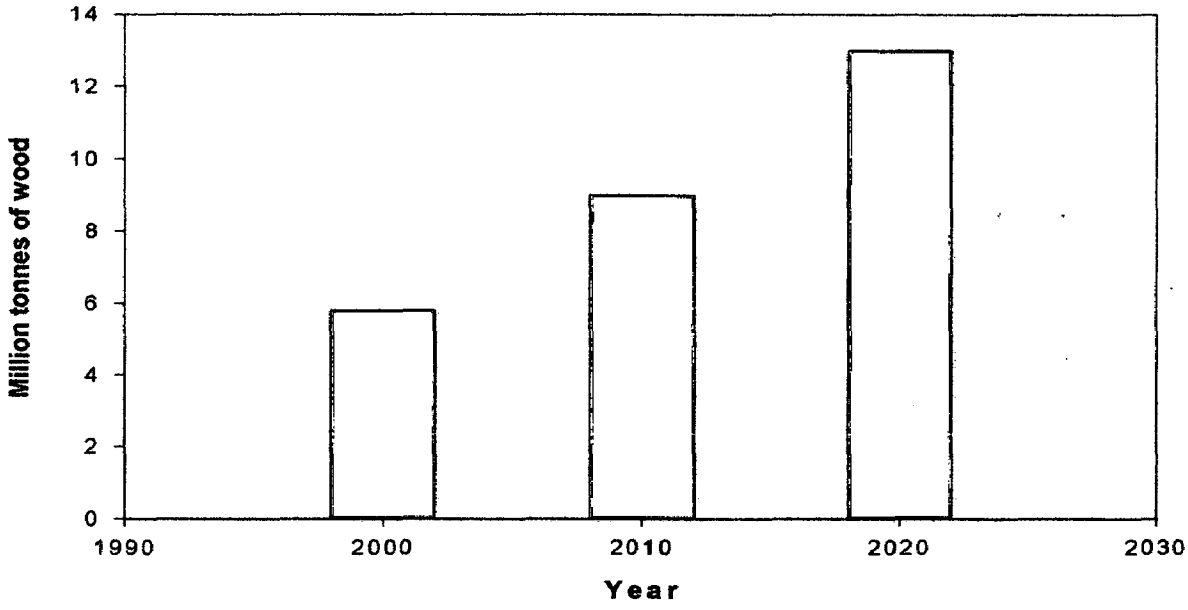


Figure 1.6: Industrial wood demand scenario for India [41].

REFERENCES

1. Anonymous, Yash papers sees demand growing at 6.1%, Business daily, The Hindu Group of Publications, Saturday, July 08 (2006).
2. AF-QPS and NLK Associates, U.K paper Mills: Review of current recycled paper usage secondary fiber study, The waste and resources action programme (WRAP) - secondary fiber study. **www.wrap.orq.uk**, March 2002.
3. Ashore, A., A potential source of raw material in paper making, *Polymer-Plastics Technology and Engineering*, 45(10):1133-1136 (2006).
4. Ausche J.G., Mill-scale benefits in enzymatic deinking, *In: Proceeding of Pira 7th International Recycling Technology Conference,* Brussels, Belgium, Paper7 (2002) pp.1-3.
5. Bajpai, P., Enzymatic deinking, *Adv. Appl. Microbiol.,* 45: 241-69(1997).
6. Bajpai, P. and Bajpai, P. K., Biotechnology in the Pulp and Paper Industry – A Rout to Energy Conservation, Published by PIRA International, U. K. (1998) pp.59.
7. Bennington, C.P.J., Sui, O.S. and Smith, J.D., The effect of mechanical action on waste paper defibering and ink removal in repulping operations, *J. Pulp Paper Sci.,* 24(11):341-8(1998).
8. Bill Moore, WWW.MARecycle.coms.
9. Carre, B., Galland, G. and Saint Amand, F.J., Third research forum on recycling (1995) pp.73-88.
10. Chesson, A., Supplementary enzymes to improve the utilization of pigs and poultry diets, In: *Recent advances in animal nutrition*, Haresign, W., and Cole, D.J.A. (Ed.), Butterworths (London) (1987) pp.71-89.
11. Chowdhary, P., What's available in deinking market, *IPPTA J.,* 17(3):111(2005).
12. Demain, A.L., Microbial biotechnology, *Trends Biotechnol.,* 18(12):26–31(2000).
13. Diesen, M., (Ed.), Economics of the pulp and paper industry, Tappi and the Finish Paper Engineer's Association (1998) pp.13.
14. Doshi, M.R. and Jong, R.L.D., Semiannual conference review, *Progress in Paper Recycling*, 15: 22-30(2005).

15. Dutt, D., Upadhyaya, J.S., Malik, R.S. and Tyagi, C.H., Studies on pulp and paper making characteristics of some Indian non woody fibrous raw material: part 1, *Journal of Scientific & Industrial Research.*, 63:48-57(2004).
16. Franks, N.E., Methods for deinking and decolorizing printed paper, US Patent No. 6, 241, and 849 (2001).
17. Flynn, B., Shape of things to come, *PPI,* 12:1-2 (2007).
18. Ganapathy, P.M., Asia-Pacific forestry sector outlook study: Sources of non wood fiber for paper, board and panels production status, trends and prospects for India, FAO Working Paper No: APFSOS/WP/10, August (1997).
19. Godfrey, T., Textiles, In: *Industrial Enzymology*, Godfrey, T. and West, S., (eds.), 2nd edn, Macmillan Press, London (U.K.) (1996) pp. 360–71.
20. Godfrey, T. and West, S., Introduction to Industrial Enzymology, In: *Industrial Enzymology*, Godfrey, T. and West, S. (ed.), 2nd edn, Macmillan Press, London (U.K.) (1996a) pp.1-8.
21. Godfrey, T. and West, S., Introduction to Industrial Enzymology, In: *Industrial Enzymology*, 2nd edn, Macmillan Press, London (U.K.) (1996b).
22. Harman, G.E. and Kubicek, C.P., *Trichoderma* and *Gliocladium*: Enzymes, biological control and commercial applications, Taylor & Francis Ltd., Vol. 2, London (U.K.) (1998) pp. 393.
23. Heise O.U., Unwin, J.P., Klungness J.H., Fineran, W.G., Sykes, M. and Abubakr, S., Industrial scale up of enzyme-enhanced deinking of non impact printed toners, *Tappi J.*, 79(3):207-12(1996).
24. http://www.indianpapertrader.com (Jan 2009).
25. Inter governmental panel on climate change, 4th Assessment Report, WMO, UNEP (2007).
26. Jaakko Poyry Ltd., World paper markets up to 2020, Know How Wire Magazine, January 2006, Jaakko Poyry Ltd., **http://forestindustry.poyry.com/linked/en/news/KnowHowWire_January2006_internet.pdf**.
27. Jain, R.K., About Cess Committee of Development Council for Pulp, Paper and Allied Industries, 2nd Workshop on Frontier Technologies Addressed Under Cess Project and Energy Norms for Pulp and Paper Industry (2005) pp.1-5.

28. Jain, R.K., Singh, K. and Roy, T.K., Indian paper industry raw material scenario, growth prospects and pathways, *IPPTA J.*, 19(3):129-137(2007).
29. KSPCB, Handbook on Environment Laws and Guidelines, *Karnataka State Pollution Control Board* (2000).
30. Kuby, S.A., A study of enzymes, enzyme catalysis, kinetics, and substrate binding, CRC Press, Inc., Boca Raton, Vol. 1(1991) pp.427.
31. Kulkarni, A. G., Subramanyam, S.V., Godiyal, R.D. and Sharma, A. K., Monograph of Indian paper making fibers, In: *Proc. 2nd Workshop on Frontier Technologies Addressed Under Cess Projects and Energy Norms for Pulp and Paper Industry*-Part-A (2005).
32. Leaders, The price of virtue: Recycling, *The Economist,* June 9 (2007).
33. Lopez, Daniel, Colom Jose, F., Vidal, Teresa, Pastor, J. and Torres Antonio L, Flotation deinking of xerographic- printed-paper: a study of the dispersant, collector and cellulases on handsheet visual appearance, *APPITA J.*, 56(6):44 (2003).
34. Mantyla, A., Paloheimo, M. and Suominen, P., Industrial mutants and recombinant strains of *Trichoderma reesei,* In: *Trichoderma and Gliocladium—Enzymes, biological control and commercial applications*, Harman, G.F. and Kubicek, C.P., (Ed), Taylor & Francis Ltd, London (U.K.), Vol. 2(1998) pp. 291-309.
35. McKinney, R.W.J., Environmental labeling of paper products, *Prog. Paper. Recycl.*, 15-23(1993).
36. Mohandas, C., Lata, C. and Kumar, R., Biological deinking of inkjet-printed paper using *Vibrio alginolyticus* and its enzymes, *J. Ind. Microbiol. Biotechnol.*, 32: 424-429(2005).
37. Mørkbak, A.L. and Zimmermann, W., Deinking of mixed office paper, old newspaper and vegetable oil-based ink printed paper using cellulases, xylanases and lipases, *Prog. Paper Recycl.*, 7:14-21(1998).
38. Mukherji, A. K., Finding the fiber that India needs for the future, *PPI,* 3:1(1998).
39. Pandey, A., Webb, C., Soccol, C.R. and Larroche, C., Enzyme technology, Asiatech Publishers, Inc., New Delhi (2005) pp.742.
40. Pekka, N., Pekka, K. and Harri, A., Global competitiveness of the Indian paper industry, Jaakko Poyry Consulting, Vaanta (Finland), (9):139(2002).

41. Pekka, N., Pekka, K. and Harri, A., Global competitiveness of the Indian paper industry, Jaakko Poyry consulting, Vaanta (Finland), (9):18-264(2002).
42. Prasad, D.Y., Heitmann, J.A. and Joyce, T.W., Enzymatic deinking of black and white letterpress printed newsprint waste, *Prog. Paper. Recycl.*, 21-30(1992).
43. Prasad, D.Y., Heitmann, J.A. and Joyce T.W., Enzymatic deinking of colored offset newsprint, *Nord. Pulp. Pap. Res. J.*, 8:284(1993).
44. Prasad, N.S.K., An overview of the paper chemicals industry, Part 2, Chemical Weekly Dec. 2 (2002) pp.169-176.
45. Raghuveer, S., Residues to resources" environment friendly fly ash utilization at ITC-Bhadrachalam, *IPPTA J.*, 12(4):5-12(2000).
46. Rodriguez, A., Moral, A., Serrano, L., Labidi, J. and Jimenez, L., Rice straw pulp obtained by using various methods, *Biores. Technol.*, 99: 2881-2886(2008).
47. Saari, J., New process engineering for deinking, *In: Proceeding of 8th PIRA International Conference*, Prague, Paper 9, (2004) pp.1-15.
48. Saddler, J.N., Bioconversion of forest and agricultural plant residues Biotechnol, Agriculture, No. 9, C.A.B. International, Wallingford (Oxon, U.K.) (1993) pp. 349.
49. Sarkar, V., V Share, 0607/4 June 26 -July 08, (2006) pp. 3-6.
50. Sixta, H., Introduction (Chapter 1), *In: Handbook of pulp,* Sixta, H. (ed.), Wiley - VCH Verlag GmbH & Co. KgaA, (2006) pp.2-19.
51. Stanley, G.L., Third year of record sales expected for U.S. paper and related industries in 1996, *Tappi J.*, 79:37-42(1996).
52. Statistics of the Indian paper industry, paper, paperboard & newsprint, A Report by CPPRI (2005) pp. 3-23.
53. Sood, S., India in the world paper markets and emerging Asia, *8th International Technical Conference on Pulp, Paper, Conversion & Allied Industry*, New Delhi (2007) pp.3-7.
54. Sources of non-wood fiber for paper, board and panels production: status, 3- the future of non-wood and recycled fiber: outlook 2010, FAO a corporate document repository, originated by: Forestry department (1997).

55. Sousa, F. De., Kolar, M-C., Kringstad, K.P., Swanson, S.E., Rappe, C. and Glas, B., Influence of chlorine ratio and oxygen bleaching on the formation of PCDFs and PCDDs in pulp bleaching: part 1- a laboratory study, *Tappi J.,* 72:147-153(1989).
56. Szabó, L., Soria, A., Forsström, J., Keränen, J.T. and Hytönen, E., A world model of the pulp and paper industry: Demand, energy consumption and emission scenarios to 2030, Environmental Science & Policy, 12:257-269(2009).
57. Tandon, R., Mathur, R.M. and Kulkarni, A.G., Practices Prevailing in collection, Sorting and Grading of Recovered Paper- A Comparison, *IPPTA J.,* 17(3): 93(2005).
58. The future of non wood and recycled fiber: outlook 2010, FAO, A corporate document repository, originated by forestry department (1997).
59. Thomke, S., Rundgreen, M. and Hesselman, K., The effect of feeding high-viscosity barley to pigs, In: *Proceedings of the 31st meeting of the European Association of Animal production,* Commission on Animal Production, Munich, Germany (1980) pp.5.
60. Uhlig, H., Industrial enzymes and their applications, New York, John Wiley & Sons, Inc. (1998) pp.435.
61. Viesturs, U. and Leite, M., Pulp and wastepaper bleaching using xylanase and other enzymatic complexes, In: *Latvian state institute of wood chemistry yearbook,* (1999) pp.49-50.
62. Vishwanathan, K.S.K., Utilization of bagasse for papermaking, *IPPTA J.,* 10(3):9-13(1998).
63. Viswanathan Kasi, K.S., Processing of waste paper and stock preparation, Presidential address, *IPPTA J.,* 17(3): 33(2005).
64. Von, H. P., Raven, A. and Leyerer, H., Application of lignolytic enzymes in the production of pulp and paper, *Das Papier,* 44(10A):33-41(1990).
65. Voragen, A.G.J., Wolters, H., Verdonschot-Kroef, T., Rombouts, F.M. and Pilnik, W., Effect of juice-releasing enzymes on juice quality. In: *International Fruit Juice Symposium,* The Hague (NL), May 1986. Zurich: Juris Druck Verlag (1986) pp. 453-62.
66. Voragen, A.G.J., Tailor-made enzymes in fruit juice processing, *Fruit Processing,* 7:98-102(1992).

67. Welt, T. and Dinus, R.J., Enzymatic deinking-a review, *Prog. Paper. Recycl.*, 4(2):36-47(1995).

68. Wong, K.K.Y. and Saddler, J.N., *Trichoderma* xylanases, their properties and applications, In: *Xylans and xylanases,* Visser, J., Beldman, G., Kusters-van Someren, M.A. and Voragen, A.G.J. (ed.), *Progress in Biotechnology,* 171-86(1992).

69. Wong, K.K.Y. and Saddler, J.N., Applications of hemicellulases in the food, feed, and pulp and paper industries, In: *Hemicellulose and hemicellulases,* Coughlan, M.P and Hazlewood, G.P., (eds.), Portland Press, London (U.K.) (1993) pp.127-43.

70. Woodward, J.L., Stephan, M., Koran, L.J. and Saddler, J.N., Enzymatic separation of high quality unlinked pulp fibers from recycled newspaper, *Biotechnology*, 12:905-908 (1994).

71. Zollner, H.K., Schroeder, L. and Leland, R., Enzymatic deinking of non-impact printed white office paper with α-amylase, *Tappi J.,* 81:166-170(1998).

CHAPTER 2

STUDIES ON CELLULASE PRODUCTION

STUDIES ON CELLULASE PRODUCTION

2.1: Introduction

Enzymes are the catalytic cornerstone of metabolism, and as such are the focus of intense worldwide research, not only in the biological community, but also with process designers/engineers, chemical engineers, and researchers working in other scientific fields. Since, ancient times, enzymes have played a central role in many manufacturing processes, such as in the production of wine, cheese, bread and modification of starch etc. The latter half of the twentieth century has seen an unprecedented expansion in our knowledge for the use of microorganisms, their metabolic products and enzymes in a broad area of basic research and their potential industrial applications. In past two decades, however, microbial enzymes have been used in pulp and paper industry [15].

Cellulase is a multi-component enzyme system that is well considered because of its potential industrial applications [201]. Till date, a variety of microorganisms those are capable of producing cellulases have been isolated [16]. Traditionally, cellulases obtained from these fungi have been utilized in the bioconversion of lignocellulosic materials into biofuels like ethanol and single cell proteins [207]. Cellulases are industrially important enzymes [166] with a current market value of about 190 million US $ [139]. Attempts are still being made to increase cellulase yields by mutation, protoplast fusion, and genetic engineering techniques [22, 26]. Cellulolytic enzymes are produced by variety of bacteria, fungi, actinomycetes, aerobes and anaerobes. They are detected in mesophiles and thermophiles [22]. Each of these microorganisms can produce different kinds of cellulases that differ in their mode of action as well as properties like activity towards crystalline cellulose, activity and stability in acidic or alkaline pH. The most privileged sources for research and production of cellulases are fungi because of their higher enzyme yields and capacities to produce complete cellulase complex. The cellulase systems of the aerobic fungi *Trichoderma reesei, T. viride, T. koningii Penicillium pinophilum, Phanerochaete chrysosporium* (*Sporotrichum pulverulentum*), *Fusarium solani, Talaromyces emersonii,* and *Rhizopus oryzae* [22, 132], *Sclerotium rolfsii* [102], *Humicola insolens* [165], *Aspergillus niger* [80,141] and *Melanocarpus albomyces* [8] are well characterized. Some thermophilic aerobic fungi and mesophilic anaerobic fungi (*Neocallimastix frontalis, Piromonas communis, Sphaeromonas communis*) [22] also produce cellulases. Thermophilic fungi have

In crystalline cellulose the chains adhere to each other by hydrogen bonding and Vander- walls forces to form highly insoluble structures. The individual crystal of cellulose contains tens of glucan chains in parallel orientation with their reducing ends at one terminus and non-reducing chain end at other. Although, highly crystalline, the structure of cellulose is not uniform. In addition to crystalline regions, native cellulose contains less-ordered amorphous or para crystalline regions [192]. Six polymorphs of cellulose (I, II, III-1, III-11, IV-1 and IV-11) have been documented [142]. Due to such a complex structure of cellulosic substrates, its efficient solubilization requires presence of different enzymes in a typical cellulolytic enzyme complex.

2.1.2: Typical cellulolytic system

Recently, 3D structures of the different cellulolytic enzymes have been solved [39, 186]. This has enhanced our understanding regarding structure and its functional relationships of enzyme with their substrates. Although, all cellulolytic enzymes share the same chemical specificity for β-1, 4-glycosidic bonds, they show difference in their specificities towards macroscopic properties of substrate. Generally, a typical cellulolytic complex includes a variety of hydrolytic and oxidative enzymes. Hydrolytic enzymes such as endoglucanase [1, 4-β-D glucan glucanohydrolase; (EC 3.2.1.4)], cellobiohydrolase (CBH, also called exoglucanases) [1, 4-β-D glucancellobiohydrolase; (EC 3.2.1.91)], 1, 4-β-D-glucan glucohydrolase (EC 3.2.1.74) and β-glucosidase [1, 4-β-D glucoside glucohydrolase, (EC 3.2.1.21)] are involved in degradation of crystalline cellulose to glucose. The native structure of cellulose is composed of crystalline and amorphous regions. The enzyme action is generally initiated by random acting endoglucanases to produce cello-oligosaccharides. Endoglucanases cleave bonds along the length of the cellulose chains in the middle of the amorphous regions, resulting in a decrease in the degree of polymerization (DP) of the substrate [192]. The cellobiohydrolases then act sequentially by removing cellobiose units from non-reducing as well as reducing ends of the cellulose chains. They attack the crystalline parts of the substrate, produce primarily cellobiose, and decrease the DP of the substrate very slowly. The hydrolysis of the glycosidic bonds occurs by general acid catalysis with the involvement of two carboxylic amino acids [98]. The cellobiohydrolases act synergistically with each other and with endoglucanases: *i.e.* mixtures have a higher activity than the sum of the activities of the individual enzyme acting alone. Cellulolytic fungi generally produce two different CBHs. Finally, β-glucosidase completes the hydrolysis by degrading cellobiose to glucose [49]. The organization of native cellulose and its hydrolysis by different endoglucanases and cellobiohydrolases is demonstrated schematically in (**Figure**

2.2). Oxidative enzymes, such as cellobiose dehydrogenase (EC 1.1.5.1) [206], cellobiose oxidase (EC 1.1.99.18) [128] have been characterized from cellulolytic fungus *Phanerochaete chrysosporium*. Oxidative enzymes are found to induce hydrolytic components of cellulase complex. Lactonases [(D-glucano-1, 5, lactonohydrolase (EC 3.1.1.17)] are responsible for hydrolysis of inhibitors of cellulase system [27].

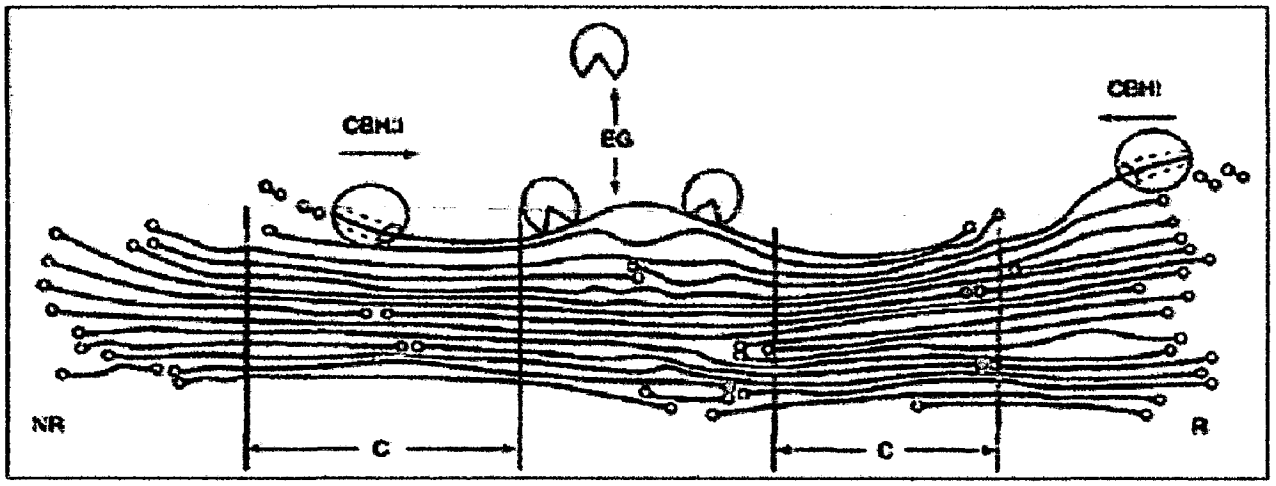


Figure 2.2: Schematic representation of the organization of crystalline cellulose and its hydrolysis with cellulolytic enzymes. Symbols: • R= reducing ends,° NR= non-reducing ends, C= crystalline regions, CBH I= cellobiohydrolase I, CBH II= cellobiohydrolase II, EG= endoglucanase. [192]

2.1.3: Factors affecting cellulose hydrolysis

Enzyme properties and factors such as substrate characteristics can limit the rate and degree of cellulose hydrolysis.

2.1.3.1: Substrate characteristics that affect cellulose hydrolysis

Anatomical and ultrastructural characteristics of cellulose fibers that can potentially limit hydrolysis of cellulose have been mentioned in **Table 2.1.** Each of these characteristics is briefly discussed as below:

2.1.3.1.1: Degree of polymerization

The degradation of insoluble cellulosic substrates finally results in generating soluble cello-oligosaccharides, i.e. cellulose molecules with DP < 6 units. The most accepted opinion suggests that the extent of enzymatic degradation is affected by DP of cellulosic substrate. Beyond a definite molecular weight range cellulose is recalcitrant to hydrolysis and its degradation is limited **(Table 2.1)** [31].

2.1.3.1.2: Crystallinity

Ghose *et al.*, proved that the amorphous part of cellulose is hydrolyzed first by the enzyme, leaving recalcitrant crystalline part unhydrolyzed [61]. This observation suggested

However, later studies on lignocellulosic substrates ruled out direct correlation between crystallinity of cellulosic substrates and its hydrolysis [149]. Now it is established that hydrolysis of crystalline cellulose is brought about by synergistic action of tightly binding cellobiohydrolases [192]. Thus, it depends mainly on properties of enzyme rather than the nature of substrate.

2.1.3.1.3: Accessible surface area

A different study indicates that the relative digestibility of cellulosic substrates is directly proportional to the accessibility of enzyme molecules [64]. It is also observed that the removal of hemicellulose and distribution of lignin during the pretreatment of *Pinus radiata* increases the surface area present in the form of pores, thus increasing the accessibility to enzymes [211].

2.1.3.1.4: Particle size

Adsorption of cellulases on cellulose surface is the key step in efficient hydrolysis of cellulosic substrates. It has been shown that the smaller sized fractions within pulp are hydrolyzed preferentially in the initial stages of the hydrolysis reaction [84, 116]. The studies demonstrate that pretreatment of cellulosic substrates results in a decreased particle size and improved hydrolysis yield [163].

2.1.3.1.5: Lignin distribution

Lignin content in lignocellulosic substrates adversely influences the hydrolysis reaction. Cellulases are irreversibly adsorbed on lignin which prevents their action on cellulose [104]. The removal of lignin improves hydrolysis of cellulosic material as it creates additional surface area for enzyme adsorption on the cellulosic substrates [64].

2.1.3.2: Enzyme characteristics important in cellulose hydrolysis

Various characteristics of cellulases such as their adsorption capacities, inhibition by end products, stability under different environmental conditions (pH, temperature etc.) and synergism between different enzymes components have shown to influence enzymatic hydrolysis of cellulose.

2.1.3.2.1A: Adsorption of cellulases on cellulose

This adsorption of cellulase is facilitated by cellulose binding domain (CBD) of the enzyme [208]. These CBDs are noncatalytic regions found in tightly binding cellulases and are rich in hydroxy amino acids [107]. Binding of cellulases on cellulose with CBD improves rate of enzymatic cellulose hydrolysis. The binding of enzyme on cellulose surface results in an increased concentration of enzyme at the surface. Furthermore, it liberates cellulose chains from the surface of crystalline cellulose by non-hydrolytic mechanism [191]. The removal of

CBD from enzyme reduces hydrolytic efficiency of enzyme on crystalline cellulose but not on the amorphous cellulose [195]. The adsorption of cellulases on cellulose is also influenced by substrate characterization such as crystallinity, DP and surface area [117].

2.1.3.2.2: End product inhibition

One of the key factors that affect cellulose hydrolysis includes inhibition of cellulolytic enzymes by their end products. Both cellobiose and glucose have shown to inhibit cellulose hydrolysis either competitively [181] and/or noncompetitively [79]. According to Ghose and Das [61] inhibition due to glucose is weaker than cellobiose. Halliwell and Griffin [67] have demonstrated that cellobiose inhibits *T. koningii* cellobiohydrolase competitively. Other workers suggested those family 7 cellulases from *Trichoderma reesei* [20] and EG I from *Humicola* sp [165] show such characteristic inhibition of endoglucanases by different cellobiose concentrations.

2.1.3.2.3: Stability under different conditions

It is observed that under industrial processing conditions such as high temperatures, pH, etc. enzymes get rapidly inactivated. Hence, the stability and activity of proteins under industrial operational conditions are the most important characteristics of enzyme that affect cellulose hydrolysis. Thermostable cellulases that are active and stable at temperatures 60°C and above are preferred for enzymatic cellulose hydrolysis [97]. Various extremophilic microorganisms are being explored to obtain such stable enzymes. The cellulase stabilities can also be improved by their immobilization and by protein engineering of cellulases [162; 166]. Other environmental factors such as the presence of heavy metals, surfactants can also affect stability and activity of cellulolytic enzymes.

2.1.3.2.4: Synergism between components of the cellulolytic system

Extensive investigations by various researchers indicated that synergistic action between the different cellulase components is required for efficient cellulose hydrolysis [210]. Different types of synergism shown by multiple forms of individual components are as follows: (a) between endoglucanase and CBH [209]; (b) exo-exo synergism between two distinct CBHs [73]; (c) endo-endo-type in the reactions between endoglucanases [118] and (d) between β -glucosidase and either endoglucanase or CBH [48]. The degree of synergism has shown to vary with the nature of cellulosic substrate used. It is demonstrated that with *Trichoderma* CBHs synergism is more when crystalline substrates are used [138]. At the same time synergism between CBH and EG1 is the highest when semi-crystalline substrates are used and almost zero with crystalline cellulose substrate such as valonia cellulose [154].

2.1.4: Classification of cellulases

Cellulases belong to the O-glycoside hydrolases (EC 3.2.1.), which is a widespread group of enzymes hydrolyzing the glycosidic bond between two or more carbohydrates or between a carbohydrate and a non-carbohydrate moiety. The IUB Enzyme Nomenclature (EC-number) is based on the type of reaction that enzymes catalyze and on their substrate specificity. According to the new classification, glycoside hydrolases (GHs) are classified in families based on amino acid sequence similarities [74, 75, 24]. There is a direct relationship between sequence and folding [74]. There are two major cleavage mechanisms for glycoside hydrolases, leading to an overall retention or inversion of the stereochemistry at the cleavage point, and the mechanism appears to be conserved within each family [75]. Thus, the catalytic domains of GHs in one family having the same three-dimensional fold and exhibit the same stereo specificity of hydrolysis; for example retaining in family 5 and inverting in family 6 [76]. There are currently (as in July 2004) 97 families of GHs (URLhttp://afmb.cnrs-mrs.fr/~cazy/CAZY/index.html), with cellulases being found in at least 13 of them. Some 450 cellulase genes (derived from all kinds of microorganisms) exist in public domain databases [166].

2.1.5: Mechanism of action of cellulases

The fundamental studies on catalytic mechanisms of cellulases have revealed that enzyme cleaves β, 1-4 glycosidic bonds using acid-base catalysis [39, 214]. Catalysis may be performed with either inversion or net retention of the anomeric configuration of the substrate **(Figure 2.3).** Inversion is a simple single displacement reaction. A catalytic acid gives protonic assistance to leaving group departure, whilst a catalytic base is required to deprotonate water for nucleophilic substitution at the anomeric centre. The acids and bases are typically located some 7-13 Å apart in order to accommodate the nucleophilic water below the pyranoside ring. On many cellulase systems, the identification, indeed the existence, of the catalytic base remains controversial. The retention mechanism is a double displacement essentially as described by Koshland [99]. A covalent glycosyl enzyme intermediate is formed and subsequently hydrolyzed, via oxacarbenium ion like transition states. This requires two essential residues, an enzymatic nucleophile and a catalytic acid/base. It first serves as a classical Bronsted acid, protonating the leaving group to assist departure and then functions as a base, deprotonating the incoming water nucleophile for the second step. The nucleophile and acid/base are always found some 5-6 Å apart on all systems studied so far. Since catalytic mechanism is dictated by the location of functional groups on the protein, the stereochemistry of catalysis is conserved within each family [166].

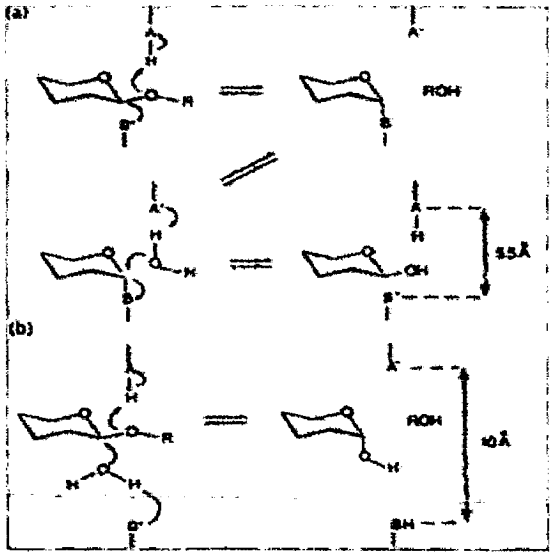


Figure 2.3: The two major mechanisms of enzymatic hydrolysis of glycosidic bonds (a) The retaining mechanism, in which the glycosidic oxygen is protonated by the acid catalyst (AH) and nucleophilic assistance to a glycon departure is provided by base (B-). The resulting glycosyl enzyme is hydrolyzed by water molecules and this second nucleophilic substitution at anomeric carbon generates a product with the same stereochemistry (b) The inverting mechanism, in which protonation of glycosidic oxygen and a glycon departure are accompanied by concomitant attack of a water molecule that is activated by the base residue (B^-). This single nucleophilic substitution yields a product with opposite stereochemistry to the substrate. [39]

2.1.6: Regulation of biosynthesis

The synergistic action of cellulase complex initially results in to cellobiose as the major end product [46]. This is the first soluble compound from cellulose and its appearance in the cell indicates the presence of extra cellular cellulase. It, therefore, could be the logical inducer of further cellulase biosynthesis. However, the regulation of cellulase biosynthesis may differ from species to species [160] and is not well defined. Borgia and Syphered [23] have reported that catabolite activator protein (CAP, also known as cAMP) not only inactivate the cellulase activity but also repress the synthesis of new enzyme. On the other hand, Robson and Chambliss [157] have discussed cAMP as an inductive regulator for endoglucanase synthesis. Similarly, Wood *et al.*, [210] and Shiang *et al.*, [171] have demonstrated higher cellulase yields with increasing levels of cAMP, Sorbose, L-glucose, 2-deoxy glucose, glucose-1-phosphate, sophorose and sugar alcohols enhance cellulase activity at smaller concentration [172, 157]. All these substances may function as a moderator of cellulase synthesis [171]. In a possible regulatory mechanism of cellulase synthesis, the repressor, inducer, cAMP and moderator may all be involved in regulating the rate and yield of enzyme production [157]. However, with respect to the ecology of cellulose breakdown is not known, if the enzymes alone are able to bind to cellulose during the initial phase of

contact. Some authors have reported the importance of adsorption on to cellulose for efficient hydrolysis [155].

2.1.7: Protein engineering of cellulases

Protein engineering of cellulases has predominantly been used to improve biochemical and catalytic activities of cellulases. It is also used as an efficient tool in the study of their catalytic mechanisms [166]. A number of examples of successful protein engineering of cellulases are summarized in **Table 2.3**

The genus *Aspergillus* comprises of a group of ascomycetes filamentous fungal genera that are widely used in industrial applications because of their ability to produce extra cellular lignocellulose-degrading hydrolase's in huge amount. *Aspergillus* species is one of the best known cellulolytic organisms, widely used in the fermentation industry. Species such as *A. niger* have a **GRAS** status (generally recognized as safe) from the food and drug administration **(FDA)** due to its extensive commercial and industrial uses. As a common member of the microbial communities found in soils, *A. niger* plays a significant role in the global carbon cycle. *A. niger* is also an important model organism for several important research areas including the study of eukaryotic protein secretion, molecular mechanisms critical to fermentation process development, and mechanisms involved in the control of fungal morphology. *A. flavus* is one of the most abundant and widely distributed soil-borne moulds in nature and can be found anywhere on earth. *Aspergillus flavus* is an imperfect filamentous fungus that is an opportunistic pathogen causing invasive and non-invasive aspergillosis in humans, animals, and insects. The availability of *A. flavus* genomic data marks a new era in research for fungal biology, medical mycology, and agricultural ecology.

Present study aims at isolating, screening and identifying the microorganisms (fungi) which are capable of producing the extracellular cellulases. The optimization of various operating physico-chemical parameters was done to achieve the higher cellulase activity from the screened strains. The cellulase was biochemically characterized to check its temperature and pH stability for its successful utilization in enzymatic deinking experiments.

2.2: Experimental methodology

2.2.1: Materials

Sodium salt of carboxymethylcellulose (CMC) of medium viscosity, birch wood xylan, p-nitro phenyl-β-D glucopyranoside (p-NPG), p-nitro phenyl-β-D-xylopyranoside, (p-NPX) were procured from Sigma Chemical Co (U.S.A.), Avicel PH101, 4-hydroxybenzoicacid hydrazide from Fluka AG (Switzerland), cellulose-123 and solkafloc from Carl Schleicher & Schüll Company (Germany). Tween-80, Tween-40 Triton-X-100

absorbent cotton, D-glucose and D-xylose (AR grade), dinitro salicylic acid (DNS), bovine serum albumin (BSA, AR grade), Whatmann No.1 filter paper, agar-agar and other media chemicals from High Media Chemicals (India) and standard protein markers from Bangalore-genei (India). Rice straw, rice bran, wheat bran, wheat straw, corncob, soya bean meal and sugarcane bagasse pith were collected from local market, Roorkee (India). Buffer salts and microbial media components of analytical grade were procured from other prestigious make.

2.2.2: Strain isolation

Different fungal strains were isolated from dead and decaying lignocellulosic wastes, decomposing droppings, sugarcane dumping site, and paper industry waste by enrichment technique. Samples were collected in the vicinity of Department of Paper Technology, I.I.T. Roorkee, Saharanpur campus (UP), Star Paper Mills Ltd. Saharanpur, local sugar units and main campus of I.I.T. Roorkee (UK) located in the foothills of Shivalik hills in Northern India. Microorganisms were isolated from dead and decaying woods and decomposing wastes using moist wheat bran, buried in petridishes and incubated at 37 ^{0}C. Growth of various fungal strains was observed between 1-10 days. 12 numbers of fungal colonies with different morphological features appeared in different petridishes were isolated. The moisture level was carefully controlled with sterile tap water, so as to provide a solid substrate for fungal growth, with no free water available. These fungal strains were further purified by sub-culturing. Purified cultures were transferred to PDA slants, incubated at 30 ^{0}C for 4-5 days and further stored at 4 ^{0}C for future usage. The cultures were maintained as a suspension of spores in 5 % (*v/v*) sterile glycerol at −20 ^{0}C for long term preservation.

2.2.3: Screening of cellulase producing strains

Primary screening of isolated strains for cellulase production was carried out on CMC-agar plates. The CMC-agar medium contained 1% CMC and 2% of agar dissolved in 1 L of double distilled water and autoclaved at 15 psi for 15 min. The crude enzyme extract (50 µL) of each fungal isolates, obtained under solid-state fermentation (SSF) was placed separately into 2-3 mm diameter well cut into the solidified medium in each petridish and the plates were incubated at 30^{0}C for 48 h. The plates were then stained with Congo red solution composed of 0.5% (*w/v*) Congo red and 5% (*v/v*) ethanol in distilled water for 15 min in order to enhance the visibility of hydrolyzed area and destained with 1 M NaCl as per Teather and Wood method [190]. The isolated strains were then analyzed for their cellulase production ability by measuring the hydrolyzed zone diameter encircled as red background [167]. Secondary screening of the selected strains was carried out based on high cellulase

activity of crude enzyme extract produced under SSF. Out of twelve, two fungal strains were selected for further investigations based on maximum hydrolyzed zone diameter and cellulase production.

2.2.4: Identification of strains

Two selected fungal strains with high cellulase activities were designated as AT-2 and AT-3 and were sent to Indian Agriculture Research Institute (IARI) New Delhi for further identification up to species level.

2.2.5: Preparation of lignocellulosic substrates

Different naturally available lignocellulosic waste (LCW) like rice straw, rice bran, sugarcane bagasse, wheat bran and wheat straw were washed thoroughly in hot and cold distilled water subsequently until the starch, residual sugar and rest of the undesirable material was completely removed. Washed lignocellulosic substrates were dried in sunlight, milled into powder in a laboratory Wiley mill and fraction retained on +100 mesh size was stored in sealed polythene bags for further use.

Above lignocellulosic substrates were further subjected to modification by high pressure steam treatment. In the present investigation the material was steam heated at a temperature of 180-190 ^{0}C and high pressure for 30-40 min. The steam treatment was a modified version of Jurasek [89] and Lipinsky's method [108]. Following this, pretreatment with ammonium hydroxide to expose cellulose in the lignin-hemicelluloses' matrix using the ammonia steeping method described by Cao *et al.* [28]. 20g of each powdered untreated lignocellulosic substrate was mixed with 100 mL of 2.9 M NH_4OH solution in a 250 mL Erlenmeyer flask on an orbital shaker at 30 ^{0}C for 24 hours which were then filtered, washed repeatedly with distilled water to remove excess of ammonia and dried at 80 ^{0}C.

2.2.6: X-Ray diffractographic analysis of naturally available lignocellulosic substrates

The crystallinity was measured by X-ray diffraction method [137]. Untreated and treated lignocellulosic substrates were dried in vacuum desiccators to remove the moisture content from the sample. The X-ray diffraction of each set of samples was recorded using Philips Analytical X-ray diffractometer (PW 1710). The wavelength of Cu radiation source was 0.1540 nm and spectra were obtained at 30 mA with an accelerating voltage of 40 kV. Samples were scanned in step scan mode on the automated diffractometer with the range of 10-80° of 2θ (Bragg angle) with data acquisition taken at intervals of 0.02 for 1s. Diffraction patterns were collected at a scan-speed of 1^0/min. A peak observed at 2θ shows the degree of crystallinity of the lignocellulosic substrates. A peak resolution program was used to calculate the crystallinity index of cellulose. [77]. The phase identification of samples was

conducted using the X-pert High score software with the support of the ICDD-PDF-2 database. Percent crystallinity was calculated from raw XRD spectra by comparing the area of the crystalline regions to the total area. The crystallinity index (CrI) of cellulose was determined by empirical method described by Segal et al. [168].

$$CrI = (I_{002} - I_{am})\ I_{002} \times 100$$

I_{002} means both crystalline and amorphous intensity (background) while I_{am} represents background intensity only (reflection attributed to amorphous regions of the sample).

2.2.7: Scanning electron microscopy of fungal strains and treated and untreated rice straws

The detailed morphological study of the fungal strains was carried out using scanning electron microscopy (SEM, Leo 435 VP, England). Fungal mat was taken and subjected to for fixation using 3% glutaraldehyde (*v/v*) and 2% formaldehyde (4:1) (*v/v*) 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 absolute alcohol (99.9%) 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 absolute alcohol, samples were air dried and examined under SEM using gold shadowing technique [55]. Electron photomicrographs were taken at desired magnifications. Likewise, in another set of experiment, the detailed morphological studies of rice straws (lignocellulosic wastes) before and after treatment were carried out.

2.2.8: Preparation of inoculum

The culture of the PDA slant was first incubated at 30^0C for 24h. The activated spores were removed and suspended in Tween-80 (0.01%, *v/v*) and 2 mL of spore suspension (2×10^6 spores per mL) was transferred in to 50 mL of Mandels and Weber's modified pre-culture medium containing 10 g/L glucose at pH 4.8 [175]. Medium after inoculation was incubated at 30 ^{0}C for 24h with continuous shaking (120 rpm) in an orbital shaker.

2.2.9: Composition of the production medium

The Nutrient salt solution (NSS) prepared for the production of cellulases contained, as g/L, 2.1 $(NH_4)_2SO_4$, 2.0 KH_2PO_4, 0.3 $MgSO_4.7H_2O$, 0.3 $CaCl_2$, 0.00156 $MnSO_4.5H_2O$, 0.0014 $ZnSO_4.7H_2O$ and 0.0026 $CoCl_2.6H_2O$ with 2.0 mL/L Tween-80. 1% cellulosic substrate (synthetic or natural) (*w/v*) was added in NSS. The desired pH (Knick, Germany, Model-761 Calimatic) of the solution was adjusted with $NaOH/H_2SO_4$. The medium is called as Mandels and Weber's modified medium which was customized by Hataka and Prithonen later on and used for gearing up the cellulase production [114, 70].

2.2.10: Cellulase production by fungal strains

2.2.10.1: Submerged fermentation

SmF was carried out in 50 mL of basal medium, containing NSS with 1% cellulosic substrate (synthetic or lignocellulosic waste) as carbon source in Erlenmeyer flasks (250 mL). Medium was inoculated with 5% of the inoculum of fungal cultures followed by incubation at 30 ^{0}C for 5 days in an orbital incubator shaker (Sanyo, Orbi-safe, UK) with constant shaking (120 rpm). Samples collected at different incubation periods were centrifuged at 5000 rpm for 10 min and the supernatant (crude enzyme) was harvested and assayed for different cellulases; endoglucanase (CMCase), exoglucanase (FPase) [115], β-glucosidase [124] and xylanase [125] activities as per standard protocols.

2.2.10.2:Solid-state fermentation (SSF)

SSF was performed as described by Beg *et al.*, [14]. Slurry of the fermentation medium containing 5 g of treated rice straw and 15 mL of NSS was prepared in Erlenmeyer flasks (250 mL) and inoculated with fungal inoculum. The culture flasks were incubated at 30 ^{0}C for 5 days. The crude enzyme was harvested and assayed for different cellulases, endoglucanase (CMCase), exoglucanase (FPase,) [115], β- glucosidase [124] and xylanase [125] activities as per standard protocols.

2.2.11: Extraction of enzymes

In SmF process enzyme was directly filtered through four layered cheese cloth while in SSF enzyme was extracted using 15 mL of distilled water. The contents of the flask were crushed with the help of a glass rod and were shaken for 30 min to harvest the enzyme from the fungal cells. The whole contents were then filtered through four layered cheese cloth as in case of SmF. The filtrate obtained was centrifuged (Sigma centrifuge model 2K15) at 5000 rpm for 10 min at 4 ^{0}C. The clear brown coloured supernatant was used as crude enzyme and stored at –20 ^{0}C until used.

2.2.12: Analytical methods

2.2.12.1: Estimation of cellulase (CMCase) activity

CMCase activity was determined as described by [115]. The assay mixture, in a total volume of 2 mL, contained 0.5 mL of 1 mM of CMC in 50 mM citrate buffer (pH 4.8) and 0.5 mL of the supernatant obtained from fermentation broth as the source of enzyme. The same was repeated as control using buffer in place of crude enzyme preparation. The mixture was incubated at 50 ^{0}C for 30 min. The reducing sugars released were measured optically at 540 nm with a UV-Vis spectrophotometer (Cary 100 Bio, Varian-Australia) at 25 ^{0}C using 3,

5-dinitrosalicylic acid (DNS) reagent [125]. One unit of enzyme activity was expressed as one μmol of glucose liberated per min per mL of the reaction under the assay conditions.

2.2.12.2: Estimation of exoglucanase (FPase) activity

FPase activity was determined as described by Rajendran *et al.,* [151]. The assay mixture (total volume 2 mL) contained 50 mg of Whatman No.1 strip (1×6 cm) in 1 mL of 50 mM citrate buffer (pH 4.8) and 0.5 mL of the supernatant obtained from fermentation broth as the source of enzyme. The same was repeated as control using buffer in place of crude enzyme preparation. The reaction mixture was incubated at 50 ^{0}C for 30 min. The reducing sugars released were measured optically at 540 nm with a UV-Vis spectrophotometer (Cary 100 Bio, Varian-Australia) at 25^{0}C using DNS reagent [125]. One unit of enzyme activity was expressed as one μmol of glucose liberated per min per mL of the reaction under the assay conditions.

2.2.12.3: Estimation of β- glucosidase activity

β- glucosidase activity was estimated using p-nitro phenyl β-D glucopranoside (p-NPG) as the substrate [124]. The assay mixture, in a total volume of 1mL, contained 50 mM of substrate, 50 mM citrate buffer (pH 4.8) and 0.1 mL of supernatant obtained from fermentation broth as the source of enzyme. The same was repeated as control using buffer in place of crude enzyme preparation. The reaction mixture was incubated at 50 ^{0}C for 30 min. Following incubation, 2 mL of 4% sodium carbonate solution was added and p-nitro phenol released was measured at 410 nm with a UV-Vis spectrophotometer. The enzyme activity was expressed as one μmol of glucose liberated per min per mL of the reaction under the assay conditions.

2.2.12.4: Estimation of xylanase activity

Xylanase activity was estimated by analysis of the xylose released by DNS method [125]. DNS reagent was prepared by dissolving 1.0g DNS acid, 0.2g phenol, 0.05g sodium sulphite and 20.0g sodium potassium tartarate, sequentially in 100 mL of 1% sodium hydroxide solution. Mixture was shaken for 5 min and filtered through Whatman filter paper (No.1). The regent was stored in dark at 4^{0}C for future use. 0.4 mL of 1% birch wood xylan solution was mixed with 1.6 mL of suitably diluted culture filtrate in 50 mM potassium phosphate buffer (pH 6.4) and incubated at 55^{0}C for 15 min. 0.3 mL of solution was taken from the incubated mixture in a test tube and 0.9 mL of DNS reagent was then added, heated on boiling water bath for 5 min and the xylose released was estimated calorimetrically at 540 nm with a UV-Vis spectrophotometer (Cary 100 Bio Varian-Australia) at 25 ^{0}C. The same

was repeated as control using buffer in place of crude enzyme preparation. One unit of enzyme corresponded to one μmol of xylose released per min per mL under the assay conditions.

2.2.12.5: Estimation of protein concentration

Protein concentration was estimated according to Lowry et al., [109] with bovine serum albumin (BSA) as a standard and described as below:

Reagents

Lowry-A: 2% Na_2CO_3 in 0.1M NaOH

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

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

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

A total volume of 200 μL of a protein sample was taken and 1 mL of Lowry-C was added. The reaction mixture was put for 10 min at room temperature. After 10 min 100 μL of Lowry-D was added and incubated for 30 min at room temperature. Absorbance of the sample was measured at 750 nm with a UV-Vis spectrophotometer (Cary 100 Bio, Varian-Australia) at 25 ^{0}C. The concentration of protein was determined by comparing the absorbance of the sample protein with that of a standard (BSA) using a standard curve.

2.2.13: Optimization of various nutritional parameters under SSF

Various physiochemical parameters (incubation period, temperature, moisture level pH, carbon sources and nitrogen sources), biological (inoculum) as well as other significant factors (sugars, phosphorous, organic acids, and surfactants) were studied for achieving maximal production of cellulases. The bioconversion reactions were studied under SSF and supernatant obtained after completion of reaction was analyzed.

2.2.13.1: Optimization of inoculum

For the optimization of inoculum level, a set of Erlenmeyer flasks (250 mL) was prepared containing 5 g of treated rice straw and 15 mL of NSS (pH 4.8). These flasks were autoclaved at 15 psi for 15 min. Different doses of 24 h old fungal inoculum inoculated aseptically varying from 1 to 8%, incubated at 30 ^{0}C, pH 4.8 and harvested after 5 day of incubation. The cellulase activity in terms of CMCase [115] and FP_{ase} [151] was determined as per standard protocols.

2.2.13.2: Optimization of incubation period

For the optimization of incubation period, two sets of Erlenmeyer flasks (250 mL) was prepared containing 5 g of treated rice straw and 15 mL of NSS (pH 4.8). These flasks

were autoclaved at 15 psi for 15 min and inoculated with 5% fungal inoculum as described above. These were incubated at 30 ^{0}C and harvested after 1 to 11th day of incubation with an interval of one day. The cellulase activity in terms of CMCase [115] and FPase [151] was determined as per standard protocols.

2.2.13.3: Optimization of incubation temperature

Two sets of Erlenmeyer flasks (250 mL) containing 5g of treated rice straw and 15 mL of NSS (pH 4.8) was prepared and after autoclaving, inoculated with 5% fungal inoculum as mentioned above. The effect of temperature on the production of cellulase produced by both the strains was studied by incubating the inoculated flasks from 25 to 50 ^{0}C with an interval of 5^{0}C for 5 days. The cellulase activity in terms of CMCase [115] and FPase [151] was determined as per standard protocols.

2.2.13.4: Optimization of initial pH

For the optimization of pH, two sets of 250 mL Erlenmeyer flasks was prepared as described above and pH was adjusted at 4.3, 4.8, 5.3, 5.8, 6.3, 6.9, 7.5 and 8.0 with 1 N $NaOH/H_2SO_4$ separately. These flasks were autoclaved at 15 psi for 15 min, inoculated with 5% fungal inoculum aseptically, incubated at 30 for fungal strain AT-2 and 35^{0}C for fungal strain AT-3 and harvested after 5 days. The various buffer i.e. 50 mM citrate buffer (pH 3-6), potassium-phosphate (pH 6.0-7.4) and sodium phosphate buffer (pH 7- 8) were used to determined enzyme activity. The cellulase activity in terms of CMCase [115] and FPase [151] was determined as per standard protocols.

2.2.13.5: Optimization of moisture level

Two sets of 250 mL Erlenmeyer flasks was prepared as described above. The effect of moisture level on the cellulase titre was carried out by varying the treated rice straw to NSS ratio (*w/v*) i.e.1:2, 1:2.5, 1:3, 1:3.5 and 1:4.0. The substrates were autoclaved, inoculated and incubated at pH 4.8 (fungal strain AT-2) and 5.3 (fungal strain AT-3) and temperature 30^{0}C (fungal strain AT-2) and 35^{0}C (fungal strain AT-3) for an incubation period of 5 days. The cellulase activity in terms of CMCase [115] and FPase [151] was determined as per standard protocols.

2.2.13.6: Effect of carbon sources

Two sets of Erlenmeyer flasks of capacity 250 mL were prepared containing 5 g of milled each of various agricultural by-products/residues like rice straw and treated rice straw, wheat bran, rice bran, sugarcane bagasse, and wheat straw retained on +100 mesh size were used as substrates for cellulase production by both of the fungal strains. The substrates were moistened with NSS (substrate: NSS= 1:3), autoclaved, inoculated and incubated at pH and

temperature as mentioned in paragraph **2.2.13.5.** The crude enzyme was extracted on 5th day of incubation and assayed as per standard protocol mentioned for cellulase activity in terms of CMCase [115] and FPase [151] and xylanase activities [125].

2.2.13.7: Effect of nitrogen sources

Two sets of Erlenmeyer flasks of capacity 250 mL were prepared as described above. The effect of nitrogen sources i.e. both inorganic and organic was studied similarly by replacing ammonium sulphate from the medium with different nitrogen sources (yeast extract, malt extract, peptone, soyabean meal and beef extract). The treated rice straw moistened with NSS in the ratio of 1:3 was maintained at pH 4.8 and 5.3 for fungal strain AT-2 and AT-3 respectively. Fermentation was carried out at 30 ^{0}C for fungal strain AT-2 and 35^{0}C for fungal strain AT-3 for a period of 5 days. The crude enzyme was extracted on 5th day and assayed as per standard protocol mentioned for cellulase activity in terms of CMCase [115] and FPase [151].

2.2.13.8: Effect of phosphorous sources

Two sets of Erlenmeyer flasks of capacity 250 mL were prepared as described above. The effect of phosphorous sources (i.e.tetrasodium pyrophosphate, potassium dihydrogen phosphate, sodium β–glycerol phosphate and dipotassium hydrogen phosphate was studied similarly by replacing potassium dihydrogen phosphate from the medium while keeping other parameters constant. The treated rice straw moistened with NSS (1:3) was maintained at 4.8 and 5.3 for fungal strain AT-2 and AT-3 respectively. Fermentation was carried out at 30 ^{0}C for fungal strain AT-2 and 35 ^{0}C for fungal strain AT-3 for a period of 5 days and crude enzyme harvested on 5th day of incubation and assayed as per standard protocol mentioned for cellulase activity, in terms of CMCase [115] and FPase [151].

2.2.13.9: Effect of organic acids

Two sets of Erlenmeyer flasks of capacity 250 mL were prepared as described above. The effect of different organic acids (0.1%) i.e. acetic acid, ascorbic acid, citric acid, oxoglutaric acid, propionic acid and succinic acid were studied by keeping other parameters constant as mentioned above for fungal strain AT-2 and AT-3 respectively. Crude enzyme was harvested on 5th day of incubation and assayed as per standard protocol mentioned for cellulase activity in terms of CMCase [115] and FPase [151].

2.2.13.10: Effect of soluble sugars concentration on cellulase production

Two sets of Erlenmeyer flasks of capacity 250 mL were prepared as described above. The treated rice straw moistened with NSS (1:3) was incubated with various sugars (0.2%) i.e. glucose, lactose and cellobiose while keeping other parameters constant as above. The

cellulase activity in terms of CMCase [115], FPase [151] and β-glucosidase was determined as per standard protocols.

2.2.13.11: Effect of various surfactants on cellulase production

Two sets of Erlenmeyer flasks of capacity 250 mL were prepared as described above and the effect of various surfactants (0.1%) i.e. Tween-20, Tween-40, Tween-60, Tween-80 and Triton–x were observed while keeping other parameters constant as mentioned above. The cellulase activity in terms of CMCase [115], FPase [151] and β-glucosidase was determined as per standard protocols.

2.2.14: Bio-chemical characterization of crude cellulase

2.2.14.1: Optimum pH and pH stability

The pH stability of enzymes from fungal strains AT-2 and AT-3 respectively was checked in the pH range of 4.0-7.5 by incubating the enzyme with buffers of different pH *viz.*, citrate buffer (pH 3-6), potassium-phosphate (pH 6.0-7.4) and sodium phosphate buffer (pH 7- 8). After 30 min of incubation, the cellulase activity [115] of the crude enzyme samples was determined under standard assay conditions.

2.2.14.2: Optimum temperature and thermo-stability

The thermo-stability of the enzyme was checked by incubating the crude enzyme preparations at temperatures ranging from 50-80 ^{0}C for 30 min. Samples were withdrawn after 30 min and evaluated for cellulase activity [115] under standard assay conditions.

2.2.14.3: Sodium-dodecylsulphate polyacrylamide gel electrophoresis

SDS-PAGE of the samples was performed according to Laemmli [103]. Polyacrylamide gels (12%) of 1.5 mm thickness were prepared for electrophoretic analysis.

2.2.14.3.1: Reagents

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

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

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

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

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

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

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

2.2.14.3.2: Preparation of resolving and stacking gels

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

2.2.14.3.3: Casting of gel

A sandwich of glass plates (16 x 18 x 0.3 cm) was assembled by plastic spacers (1.5 mm) and the plates were held together by plastic clamps. The base of the gel mould was sealed. Resolving gel mixture was prepared by mixing all the components except ammonium per sulphate and TEMED. Mixture was degassed for 15 min; ammonium per sulphate and TEMED were added to it. The mixture was gently mixed and poured between the plates and leaving the required space at the top for pouring the stacking gel. After polymerization of the resolving gel, the assembly was tilted to pour off the over layer, washed with sterile distilled water and wiped gently to dry the empty space. Stacking gel mixture was then similarly degassed and layered on the resolving gel; comb was immediately inserted into the mixture to form the wells. After polymerization of stacking gel, comb was carefully removed, wells formed were rinsed with reservoir buffer and the polymerized gel was used for electrophoresis.

2.2.14.3.4: Sample preparation

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

2.2.14.3.5: Electrophoresis

Samples (100 μg of the protein) were gently loaded on the gel using a micropipette. Electrophoresis was carried out at constant voltage (stacking at 60 V, resolving at 90 V) until the tracking dye reached to about 1 cm above from the bottom of the gel. Gel after electrophoresis was removed and stained overnight with gentle shaking in 0.1% coomassie brilliant blue R-250 in methanol: glacial acetic acid: water (5:2:5 *v/v/v*) at room temperature. Gel was then destained by washing in 10% isopropanol and 7% acetic acid solution [203]. Destaining was done so as to get the stained bands over the clear background. Gel was stored in 7% acetic acid until photographed.

2.2.14.4: Zymogram analysis of cellulase components

Native polyacrylamide gel electrophoresis (PAGE) using 12% gel was performed for visualization of enzyme activities *in situ* as described by Reyes *et al.*, and Schwarz *et al.*, [156, 167]. Substrate i.e. CMC (for CMC_{ase} activity) to the final concentrations of 0.1% was incorporated into separating gel before adding the ammonium per sulphate and TEMED for polymerization. The enzyme samples were heated at 50 ^{0}C for 10 min and mixed with sample buffer (62.5 mM Tris–HCL buffer, pH 6.8 containing 5% (*v/v*) glycerol and 0.01% β-merceptoethanol) lacking SDS. One set of sample was loaded in each half of the gel along with other standard markers. After electrophoresis, the gel was sliced in to two halves, one half was stained with coomasie brilliant blue R -250. On the other hand, the second half was used for visualizing the enzymatic activities *in situ*. The second half of the gels was treated with 25% isopropanol for 25-30 min followed by incubation at 50 ^{0}C for 25 min. For CMCase activity, the gel was soaked with 0.1% (*w/v*) Congo red for 5 min with mild shaking. Excess of dye was decanted and gel was washed with 5% (*w/v*) NaCl until excess stain was totally removed and the background was clear.

2.2.15: Mass production of cellulase under SSF

The mass production of cellulase was carried out under SSF conditions. 40 g of treated rice straw was taken in a 2 L flask and 120 mL of NSS (substrate to NSS ratio 1: 3 and pH 5.3) was added. The actively growing culture of test strain AT-3 was used as inoculum (5%) for the biotransformation process. The flask was incubated at 35 ^{0}C using ammonium sulphate and yeast extract as a nitrogen source, KH_2PO_4 as a phosphorous source, succinic acid as organic acid, Tween-80 as a surfactant and cellobiose as a sugar. Crude enzyme was harvested after 5 days of incubation and evaluated for cellulase activity in terms

of CMCase [115], FPase [151], β-glucosidase, xylanase activity [125] and fungal protein concentration [109] as per standard protocols.

2.2.16: Statistical analysis

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

2.3: Results and discussion

2.3.1: Isolation, purification and screening of fungal strains

Table 2.4 reveals the morphological characteristics of 12 fungal strains isolated from different lignocellulosic sources. The fungal strains were isolated by enrichment culture technique using moist wheat bran, incubated at 37 ^{0}C to isolate microorganisms directly from the nature and colour of mycelia, spores and colony appearance was observed **(Photograph 2.1 A-I).** These strains were purified by single colony isolation in two distinct screening. The cellulase production ability of these strains on CMC-agar plate was observed after primary screening based on clear zone diameter following Congo red staining. Nine of these isolates resulted into zone formation onto CMC-agar plates, of which AT-2 and AT-3 resulted in to the maximum clear zone diameter **(Photograph 2.2 A-B).** These isolates were selected as cellulase producers. In secondary screening, these 9 isolates were subjected to extra cellular production of cellulases using Mandels and Weber's modified medium with CMC as the substrate under SSF conditions **(Table 2.5 and Figure 2.4)**. Out of nine, two fungal strains namely AT-2 and AT-3 produced maximal cellulase activities i.e. 7.8 and 10.3 IU/mL respectively. These two fungal strains were selected for further studies based on their higher cellulase activities.

2.3.2: Identification of selected isolates

The test isolates AT-2 and AT-3 were identified as different strains of *Aspergillus* species i.e. *Aspergillus flavus* AT-2 and *Aspergillus niger* AT-3 respectively from Indian Agricultural Research Institute (IARI), New Delhi (India). Both of these strains were deposited in the Indian Type Culture Collection, Plant Pathology Division, IARI, New Delhi, under ITCC Culture Nos. 6324 and 6325 respectively. Maintenance of these cultures was done by periodical transfer on potato dextrose agar (PDA) slants and these cultures were stored at 4^{0}C for further study.

2.3.3: Morphological characteristics of *A. flavus* AT-2 and *A. niger* AT-3

The characteristic features of *A. flavus* AT-2 and *A. niger* AT-3 are shown in **Photograph 2.1 A-B.** Conidiogenesis was prolific in both the strains and their characteristic conidia colour, type and size made them morphologically distinct from the other species of

genera *Aspergillus*. *A. Flavus* AT-2 colonies were yellowish-green, consisting of a dense felt of conidiophores or mature vesicles over their entire surface (**Photograph 2.1 A).** The finer structural details of the mycelium of *A. Flavus* AT-2 as observed through the scanning electron microscopy (SEM) are shown in **Photographs 2.3.** The hyphae of *A. Flavus* AT-2 were thin walled, white coloured, elongated and straight. *Aspergillus* section *flavi* historically includes species with conidial heads in shades from yellow-green to brown and dark sclerotia. Conidia are single-celled, smooth or rough-walled, hyaline or pigmented, pale green (sub) spherical, 3.5-6 μm in diameter. Some species may produce Hülle cells or sclerotia. *Aspergilli* are uniseriate or biseriate with lightly packed metulae and phialides The SEM of fungal strain *A. flavus* AT-2 showing their condiospores (**Photographs 2.4 A-D).** The conidia are typically globose and unicellular with extremely roughened walls [6]. The hyphae of fungal strain, *A. flavus* AT-2 were found to be well developed, profusely branched, septate, and hyaline; their cells were, as a rule, multinucleated [6].

In case of fungal strain *A. niger* AT-3, SEM analysis of mycelia clearly signified that mycelia were thick walled, usually inconspicuous, stipes smooth and white in colour **(Photograph 2.5).** Conidia are blackish in colour when observed with the naked eye and may be globose or subglobose **(Photograph 2.1B**). *Aspergilli* are biseriate with lightly packed metulae and phialides. The finer structures of spores (SEM) are given away in **Photograph 2.6 A-D.** Conidia were arranged in long chains as visible in **Photograph 2.6 B.** Individual conidia have been approximated in a diameter range of 3 to 4μm and are ornamented with irregular warts and ridges. Most of the species produce conidia in the size range of 2.5 -5 μm [161]. Based on morphological and microscopic investigation, it was found that the two strains differed with respect to their colony colour, conidia type uniseriate in *A. flavus AT-2* while biseriate in *A. niger* AT-3 while both had a white coloured mycelium, thin walled in *A. flavus AT-2* and thick walled in *A. niger* AT-3 and conidiogenesis was copious found in both the test isolates which is a characteristic feature of all the members of ascomycotina group.

2.3.4: Effect of commercial ligninocellulosic substrate for cellulase production

Table 2.6 reveals the effect of five different commercially available lignocellulosic substrates i.e. cellulose acetate, phosphocellulose, diethylaminomethylcellulose (DEAE-cellulose) carboxymethylcellulose (CMC) and acacia powder on cellulase production under SSF conditions using Mandels and Weber's modified medium by both the fungal strains i.e. *A. flavus* AT-2 and *A. niger* AT-3 respectively. Among these, *A. flavus* AT-2 showed maximum CMCase and FPase activity as 13.46 and 1.5 IU/mL while *A. niger* AT-3 showed its maximum CMCase and FPase as 18.00 and 2.33 IU/mL activity with CMC substrate. The

variation in enzyme production might be due to different crystallinity of the substrates. The nature and relative distribution of the subsequent groups e.g. methyl, ethyl, carboxymethyl and hydroxymethyl etc., could have accounted for the solubility and crystallinity of the cellulosic substrates [21, 37, 51]. Among the five different ligninocellulosic substrates used, since CMC was less crystalline, more amorphous in nature therefore, it was more susceptible to enzymatic attack on its surface as compared to other substrates. Besides crystallinity, the adsorption profile of cellulases on these substrates might equally affect the degree of hydrolysis of the substrates. A positive correlation between the adsorption of cellulase and enzymatic hydrolysis had been observed by many researchers [29, 96,]. Adsorption of cellulase was related to the adsorption sites and the surface area of the cellulose fibers [93].

2.3.5: Evaluation of various ligninocellulosic residues as substrates for cellulase production by X-ray diffraction analysis

The crystallinity of biomass was one of the potential factors affecting the enzymatic hydrolysis [185] and the crystallinity index (CrI) was strongly influenced by biomass compositions. The X-ray diffraction analysis of diverse naturally available ligninocellulosic substrates i.e., rice straw, wheat bran, rice bran, sugarcane bagasse and wheat straw before and after pretreatment were done to investigate the crystalline behavior and diffractograms were shown in **Micrographs 2.1 and 2.2.** All the lignocellulosic samples presented a major and the highest intensity peak at 2θ within the range of 22-24° related to their crystalline structure. The increasing order of CrI of different lignocellulosic substrates under untreated condition were: wheat bran (23.00%) < rice bran (25.60%) < wheat straw (29.00%) sugarcane bagasse < (32.00%) < rice straw (37.42%) **(Micrograph 2.1).** Among these, rice straw showed more crystalline behaviour. Rice straw had more natural cellulosic content and hence a well resolved peak was obtained, while X-ray peak of other substrates was less resolved due to lesser crystallinity. Further, CrI in wheat bran, rice bran, wheat straw, sugarcane bagasse and rice straw respectively increased by 25.11, 27.73, 31.30, 34.45, and 40.11% compared to untreated ligninocellulosic substrates as a result of alkali treatment followed by steaming at high temperature 121 ^{0}C for 60 min **(Micrograph 2.2).** From the XRD graphs prototype it was clear that the treated fibers could be evidenced for significant crystalline behavior while, untreated fibers were more amorphous with less crystallinity. **Micrograph 2.3** shows the XRD pattern of untreated rice straw with lesser crystallinity, which was evidenced as a peak at 2θ= 21°. In comparison, the XRD pattern of treated rice straw showed increased crystallinity as a relatively intense peak at 2θ =23°. The sharp peak in the X-ray diffraction pattern of the alkaline treated fibers exhibited a higher crystallinity

caused by the more efficient removal of noncellulosic polysaccharides and dissolution of amorphous zones.

Combined action of chemical and physical treatments played a significant role in dissolving hemicelluloses and alteration of lignin structures, providing improved accessibility of the cellulose (in amorphous regions) for hydrolytic enzymes [72]. The native structure of lignocellulosics simply was not adapted to enzymatic hydrolysis. The presence of lignin made them resistant to enzymatic hydrolysis. Hydrolysis took place preferentially in the amorphous region of cellulose. Therefore, in order to make the cellulose susceptible to enzymatic hydrolysis, the ligninocellulosic substrates were subjected to pre-treatment (steam treatment followed by alkali treatment) for breaking the ternary complex that would reduce the crystallinity of cellulose and increase the accessible surface area for enzyme attack which therefore, would convert carbohydrate polymers into fermentable sugars by enhancing the rate of saccharification [32, 72, 130, 185, 189]. A variety of pretreatment methods had been developed to facilitate the enzymatic hydrolysis of cellulosic fibres, such as steam explosion [106], alkali and organosolv pretreatments [130], liquid hot water pretreatment, diluted acid pretreatment, lime pretreatment, ammonia explosion (AFEX) and ammonia percolation recycle (ARP) [72].

Jiebing *et al.,* confirmed that from a chemistry point of view, the structural changes taking place in the steam explosion of biomass were similar to those occurring in steam treatment [86]. Generally, the steam explosion process resulted in the hydrolysis of glycosidic bonds in hemicelluloses and to a lesser extent in cellulose. It also led to a cleavage of hemicellulose-lignin bonds. The reactions resulted in increased water solubilization of hemicelluloses and in an increased solubility of lignin in alkaline or organic solvents, leaving the α-cellulose as the solid residue with a reduced degree of polymerization [81,148, 159]. Steam treatment resulted in an increase in the surface area, pore size and thus affecting the decrystallization of cellulose [5, 42]. The high pressure steam treatment followed by extraction of lignocellulosic material with aqueous alkaline solution (2.9 M NH_4OH) had led to a further disintegration and removal of hemicellulose and lignin component of the substrates. Steam and alkali treatments had widely been attempted earlier by several researchers [44, 174, 205] and were found to be highly effective for optimal cellulase hydrolysis. Evidently, the crystalline structure of cellulose affected physical and mechanical properties of cellulose fibres. Zhong *et al.,* [217] reported that the physical properties and cellulose microstructure were among the potential factors influencing hydrolysis. An increasing ratio of crystalline to amorphous regions results in the increases in the rigidity of

the cellulose fibres, but decreases their flexibility [82,129].In addition, the crystallinity of cellulose had an important role on the accessibility of both chemical reagents and enzymes, [10, 19, 89] suggesting that noncrystalline forms of cellulose would chemically be more reactive. Crystallinity index of cellulose was increased because merely imperfect hemicellulose was removed while crystalline plane was not changed [32, 92]. The ligninocellulosic substrates were characterized by a ternary complex composed of cellulose, hemicellulose and lignin. Hemicellulose and lignin were both amorphous substances, whereas cellulose had crystalline and amorphous regions [52, 218]. Similar studies conducted by Kim and Hotzapple [94] showed that the enzymatic digestibility of alkali treated corn stove was affected by the change of structural feature resulting from treatment. The XRD analysis indicated that rice straw among five different ligninocellulosic substrates showed a significant increase in crystallinity due to sufficient removal of lignin and other hemicellulosic contents thus offering an increase in the amorphosity in cellulose regions and was highly susceptible to enzyme attack which resulted into its bioconversion to reducing sugars.

2.3.6: SEM analysis of untreated and pretreated rice straw

The SEM analysis of rice straw fibres was carried out to find out the morphological variations before and after pretreatment at different magnifications **(Photomicrographs 2.7and 2.8).** The SEM studies exposed the sharp morphological variations after treatment of the rice straw. The surface of untreated rice straw fiber was marked by some boundary edges, rigid and compact structure with complex and hard epidermal layer known a cuticle and absence of any pores, trenches or surface cracks **(Photograph 2.7).** On the other hand, SEM of pretreated rice straw was demarcated by the presence of cracks and trenches on the surface i.e. epidermal peeling occurred **(Photograph 2.8).** These surface modifications might have occurred as a result of mild hydrolysis over the crystal surface, whereas cracks were seen, perhaps due to the removal of amorphous region (lignin and hemicellulose) after alkali treatment because alkali may react with the cementing materials of the fibre, particularly hemicellulose, and led to the destruction of the mesh structure of the fibre, splitting the fibres into finer filaments [153]. The breakdown of the fibre bundles would increase the effective surface area, exposing further hydroxyl and carboxyl groups and increasing the surface charge. It could be seen evidently that the steam exploded fibers had a rough surface which was very important if the substrate was subsequently to be used for enzymatic treatment. As a whole, pretrement resulted in breakage of the fibers and partial defibrillation and extraction. Post-treatment led to dissolution of hemicelluloses and lignin and a significant defibrillation.

SEM photomicrographs had exposed that untreated rice straw had compact and rigid structure **(Photograph 2.7)** compared to the treated rice straw, which was loose and fibrous **(Photograph 2.8).** Similar observations were reported by Khuad and Johri [101]. Some ultra-structure changes caused by the steam pre-treatment (e.g. the separation of the cell wall components and an increase in the porosity) were also observed [200, 204].

XRD studies and further SEM analysis valorized rice straw (pretreated) as the carbon substrate to be used for further enzyme (cellulases) optimization studies.

2.3.7: Comparative analysis of different fermentation conditions

Table 2.7 and Figure 2.6 reveals a comparison between SSF and SmF on cellulase production abilities of both the fungal strains i.e. *A. flavus* AT-2 and *A. niger* AT-3. Under SSF, CMC_{ase} activities of *A. flavus* AT-2 and *A. niger* AT-3 were found to be 8.9 and 11.35 IU/mL respectively while FPase activities were 1.00 and 1.33 IU/mL respectively. On the other hand, under SmF, the CMC_{ase} activities of *A. flavus* AT-2 and *A. niger* AT-3 were 5.1 and 6.8 IU/mL while FPase activities were 0.56 and 0.72 IU/mL respectively. The CMC_{ase} activities of *A. flavus* AT-2 and *A. niger* AT-3 under SSF conditions were 42.69 and 40.08 % respectively higher than that of SmF conditions. In the same way, FP_{ase} respectively of *A. flavus* AT-2 and *A. niger* AT-3 increased by 44 and 45.86 % respectively in SSF compared to SmF. SSF was a simple technique that was easy to apply on small scale and it had many advantages compared to the SmF [11,134] like simple technology, high volumetric productivity, thus reduced downstream processing costs [78], low water requirement and high enzyme concentration. The production cost of crude enzymes by SmF was about US$ 20/kg compared to US$ 0.2/kg in case of SSF [193]. SSF was predominantly useful for enzyme production by fungi. Most filamentous fungi were reported to perform much better in solid substrate than in liquid cultivation because they were adapted to growth on solid surfaces. An additional but less investigated advantage of SSF might be enhanced physiological processes in cell adhesion or biofilm formation that is characteristic for SSF [65].

2.3.8: Optimization of critical parameters for cellulase production

SSF was carried out for optimizing various operating parameters i.e. incubation period, temperature, pH, carbon and nitrogen sources and moisture contents using hyper-cellulase producing *A. flavus* AT-2 and *A. niger* AT-3. Using SSF conditions, critical physicochemical and biological factors were analyzed for deciphering the most favourable conditions for achieving enhanced levels of cellulase production.

2.3.8.1: Optimization of inoculum level for cellulase production

The effect of inoculum doses varying from 1 to 8% on enzyme production by *A. flavus* AT-2 and *A. niger* AT-3 was depicted in **Table 2.8. Figure 2.7** reveals that the CMC_{ase} and FP_{ase} activities were 8.9 and 0.85 IU/mL respectively for *A. flavus* AT-2 and 10.1 and 1.70 IU/mL respectively for *A. niger* AT-3 at an inoculum dose of 5% under SSF conditions. The CMC_{ase} and FP_{ase} activities of *A. flavus* AT-2 and *A. niger* AT-3 declined beyond an inoculum dose of 5%. The decrease in cellulase production was due to clumping of the cells that could reduce the sugar and oxygen uptake and also the enzyme release [179]. Larger inoculum size was detrimental to the fungal growth and cellulase production apart from adding to the fermentation cost [131]. Other researchers observed that an inoculum dose varying between 4-6% were optimum for maximum production of cellulase [66, 179].

2.3.8.2: Optimization of incubation period for cellulase production

The effect of incubation period on cellulase production by *A. flavus* AT-2 and *A. niger* AT-3 was shown in **Table 2.9.** The CMC_{ase} activities of *A. flavus* AT-2 and *A. niger* AT-3 increased steadily with increasing incubation period and attained maximal (9.20 and 10.14 IU/mL) on 5^{th} day of incubation which corresponded to mycelial biomass in terms of protein concentration. The FP_{ase} activities on 5^{th} day of incubation period were observed to be 1.00 and 1.18 IU/mL respectively for the strains *A. flavus* AT-2 and *A. niger* AT-3. **Figure 2.8** revealed that at the onset of enzyme activities by *A. flavus* AT-2 and *A. niger* AT-3 in the fermentation broth started on 2^{nd} day of incubation period and onwards. The enzyme production slightly decreased after 5^{th} day of incubation **(Figure 2.8).** The enzyme production was associated with a drop in pH as observed previously by different workers in other cellulolytic microorganisms. On longer incubation, enzyme activities of both the strains decreased while protein biomass continued to increase from 2^{nd} to 6^{th} day of incubation for both the strains and then nearly became constant. It suggests that the enzyme production is dependent on biomass but only during the exponential phase of growth of fungi. Because, cellulases and xylanases are a part of primary metabolites thus, these are produced during exponential phase of growth and at the onset of death phase, the enzyme secretion starts decreasing. Cellulase production was maximum at the end of exponential phase (5^{th} day) and then slowly started decreasing on the onset of death phase. It suggested that the enzyme production was dependent on fungal biomass but up to some extent [30].

Incubation time during the course of enzyme production played a critical role in enzyme synthesis. Incubation period varied among different microorganisms and also depended on the several physiological and cultural conditions. Usama *et al.*, reported that the

maximum yield of cellulases in *A. niger*, using *Eichhornia crassipes* as a substrate was found after 7th and 4th day incubation under static and shaked conditions respectively [198]. Abu-Shady *et al.*, found that cellulases of *Trichoderma longibrachiata* grown on corn stalks attained their maximal accumulation after 7th day of static cultivation [2]. Haq *et al.* also reported that the production of cellulase was maximum after 72 h of inoculation in case of *Trichoderma harzianum* KM07 using wheat bran as the carbon source under SSF [69]. Ojumu *et al.*, [140] found that the highest level of cellulase activity occurred at the 12th day of fermentation by *A. flavus*.

2.3.8. 3: Optimization of incubation temperature for cellulase production

Temperature greatly influenced the growth, development and in general metabolic activities of an organism [127] hence, it was essential to optimize temperature for maximum cellulase production in both of the fungal strains under SSF using treated rice straw as the carbon source. **Table 2.10 and Figure 2.9** shows that *A. flavus* AT-2 produced maximum CMC_{ase} (9.50 IU/mL) and FP_{ase} (1.17 IU/mL) activities at 30 ^{0}C whereas, *A. niger* AT-3 produced maximum CMC_{ase} (10.75 IU/mL) and FP_{ase} (1.30 IU/mL) activities at 35 ^{0}C, hence proving their mesophilic nature. However, a temperature of 30 ^{0}C was found to be suitable for biomass accumulation. On the other hand, fungal strain *A. niger* AT-3 was found to be comparatively more thermo-tolerant in comparison with *A. flavus* AT-2. The decrease in cellulase production levels might be possibly due to lower transport of substrate across the cells at lower temperature causing lower yield of the product. At higher temperature, the maintenance energy requirement of cellular growth was high due to thermal denaturation of enzymes of the metabolic pathway resulting in lower production of the metabolites [3]. The optimum pH and temperature for production of cellulases might vary to some extent depending on physiology of the cellulolytic microorganisms [121, 197]. Temperature was a cardinal factor affecting the amount and rate of growth of an organism [59] and the increasing temperature had a general effect of increasing enzyme activity [127] but, the enzyme began to suffer thermal inactivation at higher temperatures. The optimum temperature for cellulolytic activities of *A. niger* was 30 ^{0}C and *A. nidulans* was 35 ^{0}C as reported by Usama *et al.*, [198]. Ali *et al.*, reported maximum yield of cellulases from *A. terreus* QTC 828 at a temperature of 40 ^{0}C and pH 6.0 [7] while *A. niger* produced maximum cellulase activity at 28 ^{0}C using saw dust as a substrate [136].

2.3.8.4: Optimization of pH of the medium for the production of cellulase

Among the various operating factors, pH of the medium was correlated to the enzymatic adsorption thus, significantly affecting the saccharification process [93, 151].

Table 2.11 and Figure 2.10 reveals that cellulase activity increased with increasing pH from 4.0 to 4.8 for *A. flavus* AT-2 and from 4.0 to 5.3 for *A. niger* while keeping other fermentation conditions constant. The maximal CMC_{ase} activities of *A. flavus* AT-2 and *A. niger* AT-3 were 9.50 and 12.73 IU/mL at pH 4.8 and 5.3 respectively. FPase activities were also found to be maximum (1.4 and 1.73 IU/mL) at same pH values for both the strains *A. flavus* AT-2 and *A. niger* AT-3 respectively. A further increase in pH reduced the CMC_{ase} activity in both the fungal strains. The reason for pitiable production at higher pH was probably due to proteolytic inactivation of the cellulase. It hence suggested that slightly acidic pH values favoured the cellulase production and further on increasing pH, the cellulase activity decreased gradually, which was in agreement with earlier researchers. [47, 83, 115]. The H^+ concentration in the fermentation medium thereby had a profound effect on the enzyme production.

The initial pH influenced much enzymatic system and the transport of several species of enzymes across the cell membrane [9]. It was well known that pH of the culture medium affected the availability of certain metabolic ions and permeability of fungal cell membranes. Fermentation at higher pH proved to be detrimental perhaps because of the inactivation of the enzyme system. Eberhart *et al.,* [46] reported that induction and release of cellulase depended on the pH of the medium. His observation indicated that extracellular release of cellulase from *Neurospora crassa* occurred at pH 7, whereas the enzyme remained accumulated in the cell at pH 7.5. Similarly, pH 7 was suitable for extracellular production of cellulase from the *Humicola fuscoatra* [151]. The optimal pH for cellulase production from *A. niger* was reported between 6.0 and 7.0 by Akiba *et al.*,[4], while a pH range of 4.5-4.8 was favourable for cellulase production by *Trichoderma reesei* and *Aspergillus phoenicis* [45]. The pH range 4.6-5.0 was found suitable for CMC_{ase}, FP_{ase} and β-glucosidase production with *Aspergillus ornatus* and *Trichoderma reesei* ATCC-26921 [213] whereas, a broad pH range from 5 to 7 was observed suitable for extracellular as well as cell associated β-glucosidase production and the pH of 5.5 was found to yield maximum production [25]

2.3.8.5: Optimization of moisture contents for cellulase production

The moisture levels in SSF media influenced microbial growth and product biosynthesis [135, 150]. **Table 2.12** shows the effect of moisture content on cellulase production by *A. flavus* AT-2 and *A. niger* AT-3. **Figure 2.11** reveals that both the fungal strains produced maximum cellulase (CMC_{ase}) titre (*A. flavus* AT-2, 12.75 IU/mL and *A. niger* AT-3, 16.80 IU/mL) when treated rice straw was moistened with NSS in a ratio of 1:3 and it declined (11.0 and 14.2 IU/mL respectively) with further increase in substrate to

moisture ratio of 1:3.5. Also, a reduction in moisture content (1:2.5) led to depletion in cellulase production in terms of CMCase activity by the strains *A. flavus* AT-2 (11.85 IU/mL) and *A. niger* AT-3 (15.0 IU/mL). It was observed that the moisture enabled better utilization of the substrate by the microorganisms and the efficiency of mass transfer in the solid phase particles depended on the substrate characteristics and the appropriate moisture under SSF growth condition [53, 150]. But further increase in moisture influenced the enzyme production negatively. It reduced surface area of the particles and made the water film thicker, which affected the accessibility of the air to the particles. The moisture level below optimum led to reduced solubility of the nutrients of the solid substrate and lower degree of swelling of substrate [53]. The free water of the substrate determined the void space which was occupied by air. Since the transfer of oxygen affected the growth and metabolism, the substrate should contain suitable amount of water to enhance mass transfer. Production of cellulolytic enzymes under SSF using various substrates and mineral solution in 1:0.4 to 1:10 proportion was reported by several workers [41]. Shamla *et al.,* [169] reported that for maximal cellulase and D- xylanase activity the optimum level of rice straw and Toyamas mineral solution was 1:4 to 1:5 respectively whereas a proportion of 1:2 appeared to be more suitable for β-glucosidase elaboration.

2.3.8. 6: Effect of carbon sources on cellulase production

Generally, the production of cellulases was shown to be inducible and was affected by the nature of the substrate used in fermentation. Therefore, the choice of an appropriate inducing substrate was important for high titre of cellulase production [90]. Although, high crystallinity of rice straw was justified morphologically by XRD and SEM studies; Therefore, in this section, along with rice straw other cheaper lignocellulosic substrates (rice straw, rice bran, sugarcane bagasse and wheat straw in untreated conditions) were also tested for fungal cellulase production **(Table 2.13).** Further, rice straw, rice straw + wheat straw, rice straw + rice bran, rice straw + wheat bran and rice straw + sugarcane bagasse in treated conditions were also tested. **Figure 2.12** depicts that rice straw (untreated) was the most suitable substrate which induced the cellulase (*A. flavus* AT-2, 10.14 IU/mL and *A. niger* AT-3, 14.22 IU/mL) and FPase (*A. flavus* AT-2, 0.90 IU/mL and *A. niger* AT-3, 1.12 IU/mL) productions by both of the strains up to the highest levels followed by wheat bran, wheat straw, sugarcane bagasse and rice bran in untreated conditions. The CMC_{ase} (*A. flavus* AT-2, 13.00 IU/mL and *A. niger* AT-3, 17.27 IU/mL) and FP_{ase} (*A. flavus* AT-2, 1.40 IU/mL and *A. niger* AT-3, 2.18 IU/mL) production was further improved using treated rice straw as the carbon source in place of untreated rice straw. The descending order of cellulase activities on different carbon

sources (in treated conditions) were: rice straw>rice straw + wheat bran>rice straw + wheat straw> rice straw+ sugarcane bagasse> rice straw + rice bran. Rice straw was considered to be the best substrate for cellulase induction, as it contained sufficient nutrients and was able to remain loose in moist conditions, thereby providing good aeration and large surface area, which could be used by fungi for growth and metabolic activity. Rice straw contained 32-47% of cellulose and 19-27% of hemicellulose. The cellulose and hemicellulose content of rice straw could be hydrolyzed chemically or enzymatically. The cell wall of rice straw was made chiefly of cellulose, hemicellulose and lignin. The cellulose and hemicellulose were rather easily attacked by cellulolytic organisms and decomposed. However, it was the lignin-cellulose complex (LCC) that made straw difficult to digest. Digestibility of lignocellulosic material was generally inversely correlated to the amount of lignin present in the substrates. [13, 68]. More than half of the dry matter of rice straw consisted of cellulose and hemicelluloses; the rest was composed of lignin, nitrogenous compound and ash (mostly silica). Because of the nature of its constituents (i.e. more amount of cellulose and hemicellulose and less amount of lignin), rice straw was most useful lignocellulosic material used by cellulolytic microorganisms [34]. Cellulose in plants was composed of both crystalline and amorphous structure. The degree of crystallinity of cellulose was believed to affect the rate of its decomposition by cellulolytic organisms, greater the degree of crystallinity, the slower would be the rate of microbial cellulose degradation [12, 168]. The consensus was that the crystalline cellulose was difficult to penetrate by solvents, enzymes or chemical reagents, whereas the amorphous cellulose was easily penetrated and X-ray were used to estimate the ratio of crystalline to amorphous fraction e.g., cotton linter has 73% crystalline and rice straw 43%. Therefore, rice straw (treated) was selected as the solid substrate for further optimization studies under SSF. Shamala *et al.*, [169] concluded that rice straw was the best carbon source for the cellulase production as it contained more nutrients as compared to wheat bran. Toyama and Ogawa had also reported that rice straw was a better substrate for enzyme production compared to wheat bran [196]. Benkun *et al.*, examined the production of cellulases from *Trichoderma viride*; SSF was performed by using different ratios of rice straw (RS) and wheat bran (WB) as substrates. From the results, they concluded that rice straw cooperate a major role in cellulase production in terms of CMCase activity, as well as with an increase in RS content in the substrate mixture, a corresponding increased in extracellular protein concentration was observed [18]. Singh *et al.*, detailed efficient cellulase production from *Aspergillus niger* and *A. heteromorphus* using pretreated rice straw as the carbon source [176]. Jahromi *et al.*, had also been investigated the ability of

Aspergillus terreus for the production of cellulolytic enzymes by using rice straw as the carbon source in SSF on 8th days of fermentation period and found noteworthy cellulase production [85].

2.3.8.7: Effect of nitrogen sources on cellulase production

Table 2.14 and Figure 2.13 shows the effect of different inorganic nitrogen sources i.e. $(NH_4)_2SO_4$, $(NH_4)_2HPO_4$, KNO_3, $NaNO_3$, $(NH_4)NO_3$ and NH_4Cl on cellulase production by both of the strains i.e. *A. flavus* AT-2 and *A. niger* AT-3. The maximum CMC_{ase} (*A. flavus* AT-2, 13.46 IU/mL and *A. niger* AT-3, 17.65 IU/mL) and FP_{ase} (*A. flavus* AT-2, 1.49 IU/mL and *A. niger* AT-3, 2.23 IU/mL) activities were observed with $(NH_4)_2SO_4$. The second highest cellulase (*A. flavus* AT-2, 12.68 IU/mL and *A. niger* AT-3, 16.11 IU/mL) and FP_{ase} (*A. flavus* AT-2, 1.34 IU/mL and *A. niger* AT-3, 2.00 IU/mL) activities were observed with $(NH_4)_2HPO_4$. Gokhle *et al.,* observed that $(NH_4)_2SO_4$ and $(NH_4)_2HPO_4$ were the best inorganic nitrogen sources for the production of CMC_{ase}, β-glucosidase and xylanase activity by *A. niger* NCIM1207 strain [62].

Table 2.15 shows the effect of different organic nitrogen sources (yeast extract, peptone, urea, soya bean meal and beef extract) on xylanase production by *A. flavus* AT-2 and *A. niger* AT-3. The maximum CMC_{ase} (*A. flavus* AT-2, 13.40 IU/mL and *A. niger* AT-3, 17.10 5 IU/mL) and FP_{ase} (*A. flavus* AT-2, 1.42 IU/mL and *A. niger* AT-3, 1.98 IU/mL) activities were observed with yeast extract. Experiments regarding effect of various organic nitrogen sources (peptone, yeast extract, beef extract, soyabean meal and urea) on cellulase production demonstrated that there was substantial increase in the enzyme activity when the medium was supplemented with complex nitrogen sources like yeast extract and urea. These results were in agreement with a previous report by Gao *et al.*, [58] whereas yeast extract was found to be the best organic nitrogen source in enhancing endoglucanase production as compared to peptone and urea. The favourable effect of yeast extract on cellulase production by white-rot fungi was also observed by Johansson [87]. Showkat *et al.,* [170] also reported the favourable effect of both inorganic and organic nitrogen sources i.e. $(NH_4)_2SO_4$ and yeast extract respectively on cellulase production by *Humicola* species. Various other researchers had shown that different organic and inorganic nitrogen sources such, as yeast extract [57]; soya meal [63] and corn steep liquor [71] influenced the cellulase production. Studies on effect of nitrogen sources on the production of cellulases by *P. purpurogenum* indicated the use both inorganic and organic nitrogen sources for fungal growth and enzyme production [57].

2.3.8.8: Effect of phosphorous sources on cellulase production

Table 2.16 and Figure 2.15 shows the effect of different phosphorous sources i.e., tetra sodium pyrophosphate, potassium dihydrogen phosphate, sodium β–glycerol phosphate and dipotassium hydrogen phosphate on cellulase production. The maximum CMC_{ase} (*A. flavus* AT-2, 13.48 IU/mL and *A. niger* AT-3, 17.37 IU/mL) and FPase (*A. flavus* AT-2, 1.1IU/mL and *A. niger* AT-3, 1.21 IU/mL) activities were observed with potassium dihydrogen phosphate. Garg and Neelkantan [60], Macris [110] and Singh *et al.,* [175] had also demonstrated potassium dihydrogen phosphate to have an inductive effect on cellulase activity.

2.3.8.9: Effect of organic acids

Table 2.17 and Figure 2.16 shows the effect of different organic acids (0.1%) i.e. acetic acid, citric acid, oxoglutaric acid, propionic acid and succinic acid on the production of cellulase. The maximum CMC_{ase} (*A. flavus* AT-2, 13.50 IU/mL and *A. niger* AT-3, 17.41 IU/mL) and FPase (*A. flavus* AT-2, 0.97 IU/mL and *A. niger* AT-3, 1.39 IU/mL) activities were observed with succinic acid. The CMC_{ase} activity induced by acetic acid in the medium resembled with that of succinic acid. Singh *et al.,* [172] observed the increased production of CMC_{ase}, FP_{ase} and β-glucosidase with acetic acid. The pH of the system during cellulolytic activity increased slightly during incubation as observed by Doppelbauer *et al.,* [44] and might be regulated by addition of organic acids. Kumar also studied effect of organic acid during microbial production of cellulase with sugarcane baggase and *Eichornia crassipes* biomass as the substrate by *Aspergillus niger RK-3* [91].

2.3.8.10: Effect of surfactants on cellulase production

The effect of various surfactants (0.1%, *v/v*) on cellulase production by *A. flavus* AT-2 and *A. niger* AT-3 was presented as shown in **Table 2.18 and Figure 2.17**. The maximum CMC_{ase} (*A. flavus* AT-2, 13.65 IU/mL and *A. niger* AT-3, 17.89 IU/mL) and FP_{ase} (*A. flavus* AT-2, 1.46 IU/mL and *A. niger* AT-3, 2.05 IU/mL) activities were observed with surfactant Tween-80. According to a study carried out by Dominguez *et al.*, [43], Tween-80 influenced the morphology of *Trichoderma reesei Rut* C-30 as well as the enzyme production. Sukan *et al.*, [184] demonstrated that emulsification with Tween-80 led to higher cellulase activities presumably by causing increased permeability of cell membranes and/or by promoting the release of cell-bound enzymes. Takashi *et al.,* [188] also examined that Tween-80 stimulated the production of cellulase components by the strain Y-94 more effectively when it was added to the medium at an early stage of cultivation of the culture.

The effects of some non-ionic surfactants and fatty acids on the production/release of enzymes of the cellulase complex on the production of the cellulase complex enzymes from *Neurospora crassa* were examined by Yazdi *et al.*, [212]. They found the greatest improvement in the production of extracellular exoglucanase after addition of Tween-80. Similarly, Tween-80 and Brij-35 were found to enhance endoglucanase production. All the surfactants tested were observed to mitigate β-glucosidase production. The effect of Tween-80 gave results similar to that of fatty acids and also effected changes in the cell membrane. It appeared that the effects noted here were due to change in the growth patterns of the fungus, especially when there was no change in the overall growth rate of the mycelium on addition of any of these compounds. The negative effect of higher concentrations of Tween-80 and oleic acid became detectable at levels where the surfactant or fatty acid could well provide the major carbon source for the organism, thus providing a possible indirect mode of action *via* carbon catabolite repression [212].

2.3.8.11: Effect of soluble sugars concentration on cellulase production

Table 2.19 shows the effect of soluble sugars like, glucose, lactose and cellobiose on cellulase production by *A. flavus* AT-2 and *A. niger* AT-3. **Figure 2.18** shows that the addition of 0.2% cellobiose to the medium increased CMC_{ase} activities by 17.2 and 22.93 IU/mL respectively for *A. flavus* AT-2 and *A. niger* AT-3 while FP_{ase} activities by 1.85 and 2.30 IU/mL respectively. The induction to enhance CMC_{ase} activity by glucose and lactose was minimum in comparison to cellobiose. Presence of soluble sugars like glucose/cellobiose or any other soluble sugars along with cellulose might initially lead to cell growth for their necessary cell mass which was then induced by cellulose for enzyme production [173]. Waki *et al.*, [202] proposed a mechanism of cellulase biosynthesis and stated that cellulose degraded into soluble oligosaccharides and cellobiose. Cellobiose then entered in to the cell wall and degraded into glucose due to cellobiase (β-glucosidase). The glucose or its catabolite generated acted as catabolite repressor and thus cellobiose acted as inducer. Cellobiose might act as an effective inducer of cellulase in fungi and also induced aryl-β-glucosidase in *Neurospora* [46,113]. Shiang *et al.*, [171] proposed the possible regulatory mechanism of cellulase biosynthesis. According to him, sugar analogues i.e. cellobiose, glucose, sucrose, sorbose, xylose etc. at a particular concentration induced cellulase activator molecule (CAM), a cellulase regulatory protein. CAM might or might not be the same component as suggested by Stutzenberger [183] who described the regulation of cellulase synthesis using a modification of the arabinose operon [171]. The formation rate and yield of CAM was reportedly dependant on substrates, substrate concentration and some unknown factors

imparted by moderators. Several investigations so far had indicated that cellulases were inducible enzymes. Increased rate of endoglucanase biosynthesis in *Bacillus* species was reported in presence of cellobiose or glucose (0.2%) added to the culture medium [144]. Xylanase biosynthesis was also induced by xylose or cellobiose added to the culture medium during growth [144]. The catabolite repression of cellulase biosynthesis occurred at the translation level [100].

Based on above discussion, a fungal inoculum dose of 5%, incubation period 5 days, temperature and pH 30 ^{0}C and 4.8 for *A. flavus* AT-2 and 35 ^{0}C and 5.3 for *A. niger* AT-3 respectively, solid substrate: moisture content 1:3, rice straw (treated) as the carbon source, $(NH_4)_2$ SO_4 + yeast extract as the nitrogen source, KH_2PO_4 as the phosphorous source, succinic acid as the source of organic acid, Tween-80 as the surfactant and cellobiose as the source of soluble sugar might be taken as optimum parameters for cellulase production by both the fungal strains **(Table 2.20).**

The mass production of extracellular enzymes under SSF was carried out at optimum conditions as mentioned in **Table 2.20**. **Table 2.21** reveals that the optimum CMC_{ase} (17.24 IU/mL), FP_{ase} (1.92 IU/mL), β-glucosidase (0.69 IU/mL) and xylanase activities (5.73 IU/mL) and fungal protein concentration (2.90 mg/L) for *A. flavus* AT-2 and CMC_{ase} (24.32 IU/mL), FP_{ase} (2.47 IU/mL), β-glucosidase (0.82 IU/mL) and xylanase activities (4.80 IU/mL) and protein concentration (3.07 mg/L) for *A. niger* AT-3 were observed under optimum SSF conditions.

2.3.9: Biochemical characterization of cellulase

2.3.9.1: Effect of pH on the activity and stability of cellulase

Robustness of the cellulolytic enzymes was a key factor for industrial applications. **Table 2.22 and Figure 2.19** shows the effect of pH on the cellulase activity of *A. flavus* AT-2 and *A. niger* AT-3 at 50 ^{0}C for 30 min of reaction time. **Figure 2.20** shows that the crude cellulase produced by *A. flavus* AT-2 and *A. niger* AT-3 were active in the pH range of 4.0 to 7.5 and maximum cellulase activity was obtained at pH 5.0 for *A. flavus* AT-2 (17.31IU/mL) and at pH 5.5 for *A. niger* AT-3 (24.50 IU/mL). Cellulase activities of *A. flavus* AT-2 and *A. niger* AT-3 started decreasing above buffer pH 5.0 and 5.5 respectively. It was observed that at neutral pH (7.0), *A. flavus* AT-2 retained about 54% of its optimum cellulase activity while *A. niger* AT-3 retained only 65% of its optimum cellulase activity. At pH 4.0, *A. flavus* AT-2 retained 84% and *A. niger* AT-3 retained 90% of its activity compared to optimum. The cellulase produced by *A. niger* AT-3 maintained its high stability (more than 60% of its

optimum cellulase activity) over a pH range of 4.0 to 7.0. Therefore, cellulase produced by strain *A. niger* AT-3 was less acidic compared to *A. flavus* AT-2.

Enzymes are protein in nature. In the harsh conditions such as change in pH, high temperature or in presence of high concentration of metal ions, proteins tend to lose their basic structure (denaturation). Subsequently, lose of active site in turn resulted loss in enzyme activity. Besides this, the pH activity profiles of enzymes were highly dependent on the pKa value of the catalytic residues which were themselves dependent on the local environment and hence on the nature of the amino-acids in the vicinity of the catalytic residues. pH stability increased with decreasing the pKa value [88]. Paul *et al.*, [147] earlier reported in their study that the pH optimum for the enzyme produced by *A. niger* was pH 3.8-4.0, and it was stable at 25°C over the range pH 1-9; maximum activity (at pH 4.0) was obtained at 45°C while cellulase was more stable to heat treatment at pH 8.0 than at 4.0. They also found that kinetic studies gave pK values between 4.2 and 5.3 for groups involved in the enzyme-substrate complex [147]. Yazdi *et al.,* [212] investigated the combined pH and temperature eflects on enzyme stability of cellulase complex of *Neurospora crassa* and found that the endoglucanase stability was pH-dependent even at low temperature with optimum stability in the range pH 4.5-7-0 while glucosidase and exoglucanase activities at temperatures up to 37 ^{0}C was virtually independent of pH; the enzymes retained more than 90% of its activity in the pH range of 3.5-9.0. At temperatures at or above 45 ^{0}C, the stability of allenzymes in the cellulase complex was pH-dependent. The endoglucanase showed optimal activity at 50^{0}C and pH 5.0, but over the pH range 4.0-7-0, about 70% of the activity was retained and after 36 h, it resulted an enzyme activity of 40% only at pH 8.0 [212]. Ravindran *et al.,* found *Chaetomium* sp. was tolerable to alkaline pH up to 12. It was able to use agricultural and industrial wastes as carbon sources which would be economical for commercial production of cellulase [152]. Rosa *et al.*, compared the effect of pH on the stabilities of cellulases produced by two white-rot fungi i.e. *Bjerkandera adusta* and *Pycnoporus sanguineus* cultivated on wheat straw-agar medium. When the stability of enzymes checked from very acidic (2) to alkaline (8) conditions for 1 h, *B. adusta* enzymes retained more than 50% of its CMC_{ase} activity. *P. sanguineus* enzymes were almost inactive at pH 2, but retained 80% of its activity at pH 7 and shows more than 50% residual activity at pH levels of 3 and 8. On the other hand, enzyme activity was reduced by 84% at pH 10 [158]. Similar results obtained by Aboul-Enein and his coworkers in case of thermo active cellulase produced from thermophilic actinomycete. The enzyme was optimally active at 60^{0}C and pH 8 and was

stable from pH range of 6 to 9 retaining more than 80 % its activity after incubation at room temperature for 12 h [1].

2.3.9.2: Effect of temperature on the activity and stability of cellulase

Table 2.23 shows the effect of temperature on the activity of crude cellulase produced by *A. flavus* AT-2 and *A. niger* AT-3 at their optimum pH i.e. 5.0 and 5.5 respectively for 30 min of reaction time. **Figure 2.20** reveals that the optimum temperature for the crude cellulase activities of both the fungal strains (*A. flavus* AT-2, IU/mL and *A. niger* AT-3, IU/mL) were 50 ^{0}C and beyond that cellulase activities were found to decrease. At temperature 60 ^{0}C, cellulase produced from *A. flavus* AT-2 retained about 51% of its cellulase activity while that of *A. niger* AT-3 exhibited about 64% of its cellulase activity compared to that at optimum temperature i.e. 50 ^{0}C. When assayed at higher temperatures (70 and 80 ^{0}C), both of the strains lost a large amount of their cellulase activities; *A. flavus* AT-2 could maintain 19 and 4% while, *A. niger* AT-3 retained only 29 and 7% of its cellulase activities respectively, as compared to optimum cellulase activity at 50 ^{0}C. *A. niger* AT-3 was found to be slightly more thermo-tolerant as it showed more tolerance to higher temperature as compared to *A. flavus* AT-2. Thermo stability of enzymes appeared to be a property acquired by a protein through a combination of many small structural modifications that were achieved with the exchange of some amino acids. The variation of canonical forces e.g. hydrogen bonds, ion-pair interactions and hydrophobic interactions provided thermozymes resistance at high temperature [164]. The optimal temperature for the crude cellulase activity of both the strains was 50 ^{0}C. This result substantiated with the finding that the optimum temperature for assaying cellulase activities was generally within the range of 50-65 ^{0}C for a variety of microbial strains e.g. *Thielavia terrestris*-255B, *Myceliophthora fergussi*-246C, *Aspergillus wentii, Penicillum rubrum, Aspergillus niger, Aspergillus ornatus* [124, 174, 213]. The temperature required for measuring endoglucanase, cellobiohydrolases and β-glucosidase from *Neurospora crassa* was observed to be as 50, 55 and 60 ^{0}C respectively whereas growth temperature was found to be 25 ^{0}C [25, 111]. Similarly, a native strain of *Penicillum purpurogenum* showed a higher growth at 28 ^{0}C but maximum cellulase activities at 50^{0}C [180] and about 98, 59 and 76% of the CMC_{ase}, FP_{ase} and β-glucosidase activites retained after 48h at 40 ^{0}C respectively. Bronnenmeier and Staudenbauer [25] reported that extra cellular as well as cell bound β-glucosidase from *Clostridium sterocorarium* required an identical temperature of 65^{0}C for activity. His observation further indicated that crude enzyme was markedly thermostable compared to the purified extracellular enzyme.

The temperature and pH optima and the temperature and pH stability of crude and purified enzymes of the cellulase complex of the cellulolytic ascomycete fungus *Neurospora crussu* were investigated by Yazdi *et al.,* [212]. They found that the total N. *crassa* endoglucanase activity in crude preparations was fairly stable over 48 h at a temperature up to 50 ^{0}C. The exoglucanase activity was fairly stable at a temperature up to 45 ^{0}C for 48 h, but declined significantly at higher temperature in first 6 h, dropping to approximately 70% at 50 ^{0}C, 60% at 55 ^{0}C and 40% at 60 ^{0}C and beyond 60 ^{0}C, it remained fairly stable.

2.3.9.3: Molecular characterization by SDS PAGE analysis and cellulase activity detection by zymogram analysis

The extra cellular protein profile produced by *A. niger* AT-3 using concentrated crude culture filtrate and CMC as the substrate were analyzed by electrophoresis on SDS-polyacrylamide gel. Molecular weight analysis of the respective bands indicated that cellulase had a molecular weight of 29 kDa (**Photograph 2.10**). A wide range of proteins from 10-190 kDa was observed. The variation in molecular weight of protein among cellulase components was due to the variation in the conserved region and glycosylation [123, 178]. Bands of molecular masses equivalent to 91.2 kDa, 68 kDa and 52.4 kDa were detected in the native gel corresponding to the CMC_{ase}, FP_{ase} and β-glucosidase activities respectively. Cellulase complex components with a broad range of molecular mass i.e. CMC_{ase} (12.5-145 kDa), FP_{ase} (5.6-76 kDa) and β-glucosidase (47-400 kDa) were reported by other researchers [174, 194, 219].

2.3.10: Mass production of crude cellulase from the test strains, under optimized conditions of S.S.F

The mass production of crude cellulase was done in 1000 mL flasks under fermentation conditions as optimized above and the results are summarized in **Table 2.24.** *A. niger* AT-3 exhibited CMC_{ase} (25.12 IU/mL), FPase (2.23 IU/mL) and β-glucosidase (0.87 IU/mL) activities respectively. A minor xylanase activity of 5.77 IU/mL and protein concentration of 3.11mg/mL were associated with the crude enzyme preparations which explains that crude enzyme obtained from strain *Coprinus cinereus* AT-1 was a concentrated solution of diversity of proteins.

Table 2.1: Anatomical and ultra structural characteristics of cellulosic fibres that limits its enzymatic hydrolysis [119]

Structural level	Characteristic	Description	References
Micro fibril	Molecular orientation	Parallel Vs antiparallel cellulose	[33]
	Crystallinity (CrI)	Ratio of crystalline to amorphous cellulose	[50, 117]
	Degree of Polymerization	Molecular chain length of cellulose	[95]
Fibril	Lattice structure	Cellulose lattice (I, II, III, V or X)	[105]
	Composition	Structural moieties (carbohydrate and lignin	[64. 126]
	Particle size	Fibril dimensions	[56]
Fibre	Intrinsic strength	Tensile strength	[117, 145,146]
	Dimensions	Fibre length, cell wall thickness, coarseness	[36]
	Weight loss	Carbohydrate Solubilization	[117]
	Surface area	Total surface area	(35)
	Water retention	Fibre swelling	[182]
	Pore structure	Pore distribution on fibre surface	[17]
	Surface characteristics	Microscopic determination (SEM, TEM, AFM,CLSM)	[38]

Table 2.2: Properties of various endoglucanases of thermophilic fungi [112]

Optimal pH	Fungus species	Optimal temp., °C	Mol mass, kDa	Carbohydrate, %
5-6	*Chaetomium thermophile**	55-60	36-41	n.r
5	*Humicola grisea var. Thermoidea*	n.r.	63	n.r
5-5.6	*Humicola insolens*	50	45-57	16-39
4.8	*Myceliophthora thermophila***	65	100	n.r
5.5-5.8	*Talaromyces emersonii*	75-80	35	27.7-50.8
2.9-4.5	*Thermoascus aurantiacus*	65-76	32-34	1.7-1.8

* Other names *C. thermophilum, C. thermophilium*

** Other names *Sporotrichum thermophilum / thermophile, Chrysosporium thermophilum*

n.r. = not reported

Table 2.3: Protein engineering of cellulases

System	Objective	Strategy	Reference
Bacillus sp (NK1) cellulase, family 5 endoglucanase	Alteration in pH optima alkaline neutral	Mutation at Ser. 287-Asn Ala 296-Ser	[133]
T. fusca E 2 family 6 endocellulase	To study entry of substrate molecules in active site and to improve enzyme activity	Arg. 237-Ala show increased activity on CMCLys-259-His ear active site yields higher activity on CMC and acid swollen cellulose	[215,216]
C. fimic cellobiohydrolase family 6 Cel B	Alteration of substrate specificity crystalline amorphous	Deletion of C terminal proximal loop resulted in structure similar to endoglucanase that showed improved catalytic activity on CMC	[122]
Humicola insolens endoglucanase Cel 7B	Alteration in sub site to bind longer chain substrate	Mutation at Ser 37-Trp and Pro–39 Trp improved binding to longer chain substrates shows lowered K_m on such substrates	[40]
H. insolense Endoglucanase Cel 45	Improved stability in the presence of detergents	Mutation at argr-158-glu reduced affinity towards detergents and improved enzyme stability in the presence of detergents	[143]
H. insolense endoglucanase Cel 7B	Glycosyl hydrolases, Glycosynthases	Replacement of carboxylate nucleophile of retaining β glycosidases glu--- ser	[54, 120]

Table 2.4: Morphological characteristics of various fungal isolates

Sl. No.	Isolated strains nos.	Sources	Spore colour	Zone diameter, mm
1	AT-2	Decaying cotton cloths	Green	8
2	AT-3	Dead and decaying wood	Black	10
3	AT-4	Decomposing manure	Black	3.7
4	AT-5	Dead and decaying wood	White	2.8
5	AT-6	Paper industry waste	Yellowish green	7
6	AT-7	Paper industry waste	Whitish green	6
7	AT-8	Dead and decaying wood	Green	7
8	AT-9	Sugarcane dumping site	Light grey	—
9	AT-10	Dead and decaying wood	Brown	1.2
10	AT-11	Dead and decaying wood	Brown	-
11	AT-12	Decomposing manure	Black	
12	AT-13	Mango tree bark	Greenish	0.9

Table 2.5 Enzyme production from the fungal isolates under SSF conditions

Sl. No.	Fungal strain nos.	Cellulase activity, IU/mL
1	AT-2	7.8±0.69
2	AT-3	10.3±0.94
3	AT-4	4.12±0.37
4	AT-5	3.70±0.34
5	AT-6	6.50±0.58
6	AT-7	4.25±0.38
7	AT-8	5.73±0.49
8	AT-10	2.0±0.18
9	AT-13	1.3±0.12

± refers standard deviation

Fermentation conditions:

Rice straw, g : 5
Nutrient salt solution, mL : 15
pH : 4.8
Temperature, ^{0}C : 30
Incubation period, days : 5

Table 2.6: Effect of commercially available lignocellulosic substrates for cellulase production by *A. flavus* AT-2 and *A. niger* AT-3

Sl. No.	Substrates	*A. flavus* AT-2		*A. niger* AT-3	
		CMCase activity, IU/mL	FPase activity, IU/mL	CMCase activity, IU/mL	FPase activity, IU/mL
1	Cellulose acetate	11.40±0.75	1.17±0.8	15.43±1.00	1.86±0.16
2	Phosphocellulose	12.20 ±0.95	1.34±0.15	16.11±1.14	2.00±0.14
3	Diethylamino methylcellulose (DEAE-cellulose)	12.95 ±0.82	0.70±0.9	16.80±1.06	1.05±0.12
4	Carboxymethyl Cellulose (CMC)	13.46±1.01	1.49±0.18	18.00±1.12	2.33±0.23
5	Acacia powder	11.50±0.87	1.10±0.11	15.37±1.10	1.92±0.20

± refers standard deviation

Table 2.7: Comparison of solid-state and submerged fermentation conditions for cellulase production by *A. flavus* AT-2 and *A. niger* AT-3

Sl. No.	Fermentations	*A. flavus* AT-2		*A. niger* AT-3	
		CMCase activity, IU/ mL	FPase activity, IU/ mL	CMCase activity, IU/mL	FPase activity, IU/ mL
1	SmF	5.1±0.42	0.56±0.05	6.8±0.59	0.72±0.08
2	SSF	8.9±0.81	1.00±0.09	11.35±0.92	1.33±0.20

± refers standard deviation

Fermentation conditions:

Submerged fermentation:		**Solid-state fermentation:**	
Rice straw, g	: 5	Rice straw, g	: 5
Nutrient salt solution, mL	: 15	Nutrient salt solution, mL	: 15
Temperature, ^{0}C	: 30	Temperatures, ^{0}C	: 35
pH	:4.8	pH	: 5.3
Incubation period, days	: 5	Incubation period, days	: 5

Table 2.8: Optimization of inoculum level for cellulase production by *A. flavus* AT-2 and *A. niger* AT-3

Sl. No.	Inoculum, %	*A. flavus* AT-2			*A. niger* AT-3		
		CMCase activity, IU/ mL	FPase activity, IU/mL	Protein concentration, mg/L	CMCase activity, IU/ mL	FPase activity, IU/ mL	Protein concentration, mg/L
1	1	1.6 ±0.13	0.20±0.01	0.04±0.03	2.3 ±0.19	0.24±0.02	0.06±0.04
2	2	3.8 ±0.2.8	0.43±0.03	0.34±0.040	4.0±0.28	0.39±0.04	0.38±0.042
3	3	5.7±0.47	0.75±0.06	1.40±0.073	6.6 ±0.51	0.71±0.07	1.46±0.078
4	4	8.6±0.73	1.00±0.09	2.82±0.18	9.9±0.87	1.30±0.9	3.00±0.21
5	5	8.9±0.56	0.85±0.08	2.86±0.16	10.1±0.78	1.70±0.10	3.06±0.19
6	6	3.1±0.29	0.50±0.04	1.77±0.13	6.8±0.43	0.52 ±0.07	2.40±0.16
7	7	1.4 ±0.26	0.37±0.03	0.70±0.10	2.9±0.31	0.19 ±0.03	0.75±0.11
8	8	0.9 ±0.17	0.25 ±0.03	0.05±0.01	1.1±0.30	0.11 ±0.03	0.09±0.03

± refers standard deviation

Fermentation conditions:

Rice straw, g	: 5
Nutrient salt solution, mL	: 15
pH	: 4.8 and 5.3 for *A. flavus* AT-2 and *A. niger* AT-3 respectively
Temperature, °C	: 30 and 35 for *A. flavus* AT-2 and *A. niger* AT-3 respectively

Table 2.9: Optimization of incubation period for cellulase production by *A. flavus* AT-2 and *A. niger* AT-3

Sl. No.	Day	*A. flavus* AT-2			*A. niger* AT-3		
		CMCase activity, IU/ mL	FPase activity, IU/mL	Protein concentration, mg/L	CMCase activity, IU/ mL	FPase activity, IU/ mL	Protein concentration, mg/L
1	2	1.7±0.14	0.20±0.01	0.05±0.03	2.1±0.19	0.27±0.02	0.07±0.004
2	3	3.7±0.30	0.43±0.03	0.36±0.040	4.0±0.32	0.46±0.03	0.40±0.042
3	4	6.5±0.59	0.75±0.06	1.43±0.073	6.8±0.56	0.75±0.07	1.49±0.078
4	5	9.2±0.82	1.00±0.09	2.87±0.18	10.14±0.92	1.18±0.11	3.03±0.21
5	6	7.8±0.67	0.85±0.08	2.90±0.16	8.2±0.73	0.98±0.10	3.08±0.19
6	7	4.1±0.35	0.50±0.04	1.80±0.13	6.0±0.52	0.68±0.07	2.44±0.16
7	8	3.0±0.24	0.37±0.03	1.06±0.10	3.5±0.33	0.40±0.03	1.28±0.11
8	9	2.2±0.20	0.25±0.01	0.57±0.06	2.7±0.23	0.33±0.02	0.90±0.07
9	10	0.7±0.05	n.d.	n.d	1.1±0.10	0.09±0.005	0.11±0.02

± refers standard deviation; n.d.= not determined

Fermentation conditions:

Rice straw, g : 5
Nutrient salt solution, mL : 15
pH : 4.8 and 5.3 for *A. flavus* AT-2 and *A. niger* AT-3 respectively
Temperature, °C : 30 and 35 for *A. flavus* AT-2 and *A. niger* AT-3

Table 2.10: Optimization of incubation temperature for cellulase production by *A. flavus* AT-2 and *A. niger* AT-3

Sl. No.	Temperature, ^{0}C	*A. flavus* AT-2		*A. niger* AT-3	
		CMCase activity, IU/mL	FPase activity, IU/ mL	CMCase activity, IU/mL	FPase activity, IU/ mL
1	25	6.57±0.50	0.71±0.05	7.80±0.66	0.87±0.07
2	30	9.50±0.84	1.17±0.11	9.50 ±0.82	1.02±0.10
3	35	8.30±0.71	1.01±0.09	10.75± 0.94	1.30±0.12
4	40	7.80±0.64	0.90±0.08	9.90±0.89	1.13±0.09
5	45	7.00±0.58	0.81±0.06	8.2±0.68	0.93±0.08
6	50	5.80±0.43	0.50±0.04	6.95±0.56	0.7±0.05

± refers standard deviation

Fermentation conditions:

Rice straw, g : 5
Nutrient salt solution, mL : 15
pH : 4.8 and 5.3 for *A. flavus* AT-2 and *A. niger* AT-3 respectively
Temperature, °C : 30 and 35 for *A. flavus* AT-2 and *A. niger* AT-3

Table 2.11: Optimization of initial pH for cellulase production by *A. flavus* AT-2 and *A. niger* AT-3

Sl. No.	pH	*A. flavus* AT-2		*A. niger* AT-3	
		CMCase activity, IU/mL	FPase activity, IU/mL	CMCase activity, IU/ mL	FPase activity, IU/ mL
1	4.3	6.6±0.52	0.9±0.07	7.2±0.60	0.98± 0.08
2	4.8	9.5±0.83	1.4±0.13	10.70±0.88	1.40±0.23
3	5.3	9.1±0.80	1.2±0.11	12.73±1.01	1.73±0.25
4	5.8	8.2±0.76	1.0±0.09	11.00±0.95	1.34±0.21
5	6.3	7.09±0.67	0.9±0.082	9.2±0.78	1.27±0.19
6	6.9	5.33±0.40	0.75±0.067	6.7±0.58	0.87±0.16
7	7.5	1.75±0.15	0.11±0.01	3.12±0.29	0.50±0.04
8	8.0	0.72±0.06	n.d.	1.25±0.12	0.12±0.01

± refers standard deviation; n.d.= not determined

Fermentation conditions:

Rice straw, g : 5
Nutrient salt solution, mL : 15
pH : 4.8 and 5.3 for *A. flavus* AT-2 and *A. niger* AT-3 respectively
Temperature, °C : 30 and 35 for *A. flavus* AT-2 and *A. niger* AT-3 respectively

Table 2.12: Optimization of solid substrate: moisture content for cellulase production by *A. flavus* AT-2 and *A. niger* AT-3

Sl. No.	Solid substrate: moisture content	*A. flavus* AT-2		*A. niger* AT-3	
		CMCase activity, IU/mL	FPase activity, IU/mL	CMCase activity, IU/mL	FPase activity, IU/mL
1	1:2.0	10.26±0.77	1.42±0.14	13.0±1.02	1.80±0.24
2	1:2.5	11.85±0.90	1.57±0.20	15.0±1.17	2.01±0.32
3	1:3.0	12.75±0.97	1.91±0.27	16.80±1.22	2.34±0.40
4	1:3.5	11.0±0.85	1.46±0.16	14.2±1.12	1.95±0.28
5	1:4.0	10.11±0.67	1.2±0.12	12.8±0.99	1.6±0.20

± refers standard deviation

Fermentation conditions:

Rice straw, g : 5
Nutrient salt solution, mL : 15
pH : 4.8 and 5.3 for *A. flavus* AT-2 and *A. niger* AT-3 respectively
Temperature, °C : 30 and 35 for *A. flavus* AT-2 and *A. niger* AT-3 respectively

Table 2.13: Effect of carbon sources on cellulase production by *A. flavus* AT-2 and *A. niger* AT-3

Sl. No.	Carbon sources	*A. flavus* AT-2		*A. niger* AT-3	
		CMCase activity, IU/mL	FPase activity, IU/mL	CMCase activity, IU/mL	FPase activity, IU/ mL
1	*Sugarcane bagasse	6.00±0.57	0.53±0.04	8.9±0.71	0.77±0.05
2	*Wheat straw	6.80±0.64	0.59±0.05	9.5±0.75	0.86±0.06
3	*Rice bran	3.52±0.45	0.28±0.02	5.0±0.56	0.44±0.03
4	*Wheat bran	9.85±0.93	0.81±0.07	12.75±1.10	1.02±0.09
5	*Rice straw	10.14±0.98	0.90±0.08	14.22±1.15	1.12±0.10
6	**Rice straw	13.00± 1.12	1.40±0.16	17.27±1.25	2.18±0.37
7	**Rice straw +wheat bran (1:1 *w/w*)	12.12±1.08	1.28±0.13	15.75±1.17	1.90±0.27
8	**Rice straw+ rice bran (1:1 *w/w*)	8.57±0.87	0.75±0.04	11.39±0.87	0.95±0.07
9	**Rice straw+Sugarcane bagasse (1:1 *w/w*)	10.20±1.00	0.95±0.07	13.9±1.07	1.08±0.09
10	**Rice straw+wheat straw(1:1 *w/w*)	11.00±1.02	1.10±0.11	15.64±1.15	1.80±0.24

± refers standard deviation; *= untreated, **= pretreated

Fermentation conditions:

Rice straw, g : 5
Nutrient salt solution, mL : 15
pH : 4.8 and 5.3 for *A. flavus* AT-2 and *A. niger* AT-3 respectively
Temperature, °C : 30 and 35 for *A. flavus* AT-2 and *A. niger* AT-3 respectively

Table 2.14: Effect of inorganic nitrogen sources on cellulase production by *A. flavus* AT-2 and *A. niger* AT-3

Sl. No.	*Inorganic nitrogen sources	*A. flavus* AT-2		*A. niger* AT-3	
		CMCase activity, IU/mL	FPase activity, IU/mL	CMCase activity, IU/mL	FPase activity, IU/mL
1	$(NH4)_2$ SO4	13.46±1.01	1.49±0.18	17.65±1.35	2.23±0.33
2	$(NH4)_2$ HPO4	12.68±0.95	1.34±0.15	16.11±1.14	2.00±0.30
3	KNO3	10.90±0.82	1.00±0.9	14.00±1.06	1.65±0.16
4	NaNO3	10.40±0.75	0.90±0.8	13.43±1.00	1.46±0.13
5	(NH4) NO3	11.50±0.87	1.10±0.11	15.37±1.10	1.92±0.23

± refers standard deviation; *=2.1g/L

Fermentation conditions:

Rice straw, g : 5
Nutrient salt solution, mL : 15
pH : 4.8 and 5.3 for *A. flavus* AT-2 and *A. niger* AT-3 respectively
Temperature, °C : 30 and 35 for *A. flavus* AT-2 and *A. niger* AT-3 respectively

Table 2.15: Effect of organic nitrogen sources on cellulase production by *A. flavus* AT-2 and *A. niger* AT-3

Sl. No.	*Organic nitrogen sources	*A. niger* AT-2		*A. niger* AT-3	
		CMCase activity, IU/mL	FPase activity, IU/mL	CMCase activity, IU/mL	FPase activity, IU/mL
1	Yeast extract	13.40±0.98	1.42±0.15	17.10±1.15	1.98±0.26
2	Peptone	12.34±0.90	1.24±0.13	15.93±1.10	1.60±0.20
3	Urea	13.01±0.95	1.32±0.14	16.24±1.12	1.72±0.22
4	Soya bean meal	11.65±0.82	1.18±0.11	14.58±1.03	1.49±0.17
5	Beef extract	10.85±0.77	0.97±0.09	13.00±0.91	1.36±0.14

± refers standard deviation; *= 2.1 g/L

Fermentation conditions:

Rice straw, g : 5
Nutrient salt solution, mL : 15
pH : 4.8 and 5.3 for *A. flavus* AT-2 and *A. niger* AT-3 respectively.
Temperature, ^{0}C : 30 and 35 for *A. flavus* AT-2 and *A. niger* AT-3 respectively.
Incubation period, days : 5

Table 2.16: Effect of phosphorous on cellulase production by *A. flavus* AT-2 and *A. niger* AT-3

Sl. No.	*Phosphorous sources	*A. flavus* AT-2		*A. niger* AT-3	
		CMCase activity, IU/mL	FPase activity, IU/mL	CMCase activity, IU/mL	FPase activity, IU/mL
1	KH_2PO_4	13.48±0.98	1.10±0.10	17.37±1.19	1.21±0.17
2	K_2HPO_4	12.97±0.96	1.00±0.08	16.8±1.14	1.13±0.12
3	Sodium β-glycerophosphate	8.3±0.74	0.66±0.05	10.24±0.89	0.80±0.07
4	Tetra sodium pyrophosphate	7.1±0.67	0.47±0.03	9.8±0.80	0.71±0.06

± refers standard deviation;*=2.0 g/L

Fermentation conditions:

Rice straw, g : 5
Nutrient salt solution, mL : 15
pH : 4.8 and 5.3 for *A. flavus* AT-2 and *A. niger* AT-3 respectively
Temperature, °C : 30 and 35 for *A. flavus* AT-2 and *A. niger* AT-3 respectively

Table 2.17: Effect of organic acids on cellulase production by *A. flavus* AT-2 and *A. niger* AT-3

Sl. No.	*Organic acids	*A. flavus* AT-2		*A. niger* AT-3	
		CMCase activity, IU/mL	FPase activity, IU/mL	CMCase activity, IU/mL	FPase activity, IU/mL
1	Acetic acid	13.40±0.95	1.01±0.09	17.30±1.16	1.34±0.20
2	Citric acid	10.5±0.83	0.94±0.06	13.9±0.99	1.16±0.14
3	Prop ionic acid	8.0±0.7	0.65±0.05	11.11±0.88	0.87±0.06
4	Oxoglutaric acid	7.6±0.62	0.56±0.04	10.9±0.85	0.82±0.05
5	Succinic acid	13.5±0.98	0.97±0.07	17.41±1.21	1.39±0.22

± refers standard deviation; *=0.1%

Fermentation conditions:

Rice straw, g : 5
Nutrient salt solution, mL : 15
pH : 4.8 and 5.3 for *A. flavus* AT-2 and *A. niger* AT-3 respectively
Temperature, °C : 30 and 35 for *A. flavus* AT-2 and *A. niger* AT-3 respectively

Table 2.18: Effect of surfactant on cellulase production by *A. flavus* AT-2 and *A. niger* AT-3

Sl. No.	*Surfactants	*A. flavus* AT-2		*A. niger* AT-3	
		CMCase activity, IU/mL	FPase activity, IU/mL	CMCase activity, IU/mL	FPase activity, IU/ mL
1	Tween-20	10.80±0.8	1.11±0.10	13.61±1.00	1.28±0.18
2	Tween-40	10.39±0.87	1.00±0.09	13.90±1.17	1.40±0.21
3	Tween-60	11.87±0.92	1.24±0.17	14.70±1.23	1.48±0.22
4	Tween-80	13.65±0.96	1.46±0.20	17.89±1.40	2.05±0.30
5	Triton–x	09.70±0.75	0.93±0.06	12.32±0.91	1.18±0.16

± refers standard deviation; *=0.1%

Fermentation conditions:

Rice straw, g : 5
Nutrient salt solution, mL : 15
pH : 4.8 and 5.3 for *A. flavus* AT-2 and *A. niger* AT-3 respectively
Temperature, °C : 30 and 35 for *A. flavus* AT-2 and *A. niger* AT-3 respectively

Table 2.19: Effect of soluble sugars on cellulase production by *A. flavus* AT-2 and *A. niger* AT-3

Sl. No.	Soluble sugars*	*A. flavus* AT-2		*A. niger* AT-3	
		CMCase activity, IU/mL	FPase activity, IU/mL	CMCase activity, IU/mL	FPase activity, IU/mL
1	Glucose	14.8±0.98	1.53±0.18	19.2±1.5	2.01±0.26
2	Lactose	13.7±0.90	1.40±0.16	18.1±1.4	1.87±0.20
3	Cellobiose	17.2±1.25	1.85±0.19	22.93±1.7	2.30±0.31

± refers standard deviation; *=0.2%

Fermentation conditions:

Rice straw, g : 5
Nutrient salt solution, mL : 15
pH : 4.8 and 5.3 for *A. flavus* AT-2 and *A. niger* AT-3 respectively
Temperature, °C : 30 and 35 for *A. flavus* AT-2 and *A. niger* AT-3 respectively

Table 2.20: Derivation of various physico-chemical parameters for cellulase production by *A. flavus* AT-2 and *A. niger* AT-3

Sl. No.	Parameters	*A. flavus* AT-2	*A. niger* AT-3
1	Fungal inoculum, %	5	5
2	Incubation period, days	5	5
3	Incubation temperature, ^{0}C	35	40
4	pH	4.8	5.3
5	Solid substrate: moisture content	1:3	1:3
6	Carbon source	Rice straw	Rice straw
7	Nitrogen source	$(NH_4)_2 SO_4$+ yeast extract	$(NH_4)_2 SO_4$+/yeast extract
8	Phosphorous source	KH_2PO_4	KH_2PO_4
9	Organic acids	Succinic acid	Succinic acid
10	Surfactants	Tween-80	Tween-80
11	Soluble sugars	Cellobiose	Cellobiose

Table 2.21: Production of extra cellular enzymes by *A. flavus* AT-2 and *A. niger* AT-3 under optimized conditions

Strains	CMCase activity, IU/mL	FPase activity, IU/mL	β-glucosidase activity, IU/mL	Xylanase activity, IU/mL	Protein concentration, mg/L
***A. flavus* AT-2**	17.24±1.40	1.92±0.19	0.69±0.08	7.73±0.97	2.90±0.19
***A. niger* AT-3**	24.32±1.65	2.47±0.23	0.82 ±0.08	4.80±0.70	3.07±0.23

± refers standard deviation

Fermentation conditions:

Rice straw, g : 5
Nutrient salt solution, mL : 15
pH : 4.8 and 5.3 for *A. flavus* AT-2 and *A. niger* AT-3 respectively
Temperature, °C : 30 and 35 for *A. flavus* AT-2 and *A. niger* AT-3 respectively

Table 2.22: pH stabilization of cellulase produced by *A. flavus* AT-2 and *A. Niger* AT-3

Sl. No.	pH	*A. flavus* AT-2		*A. niger* AT-3	
		CMCase activity, IU/ mL	Relative cellulase activity, %	CMCase activity, IU/ mL	Relative cellulase activity, %
1	4.0	14.56	84.	22.03	90
2	4.5	16.81	98	22.92	94
3	5.0	17.31±1.1	100.00	23.78	97
4	5.5	14.29	83	24.50±1.1	100
5	6.0	13.27	77	23.35	95
6	6.5	12.33	71	21.23	87
7	7.0	9.31	54	15.89	65
8	7.5	4.98	29	9.19	38

± refers standard deviation

Assay conditions:

pH of buffer : 4.8 and 5.3 for *A. flavus* AT-2 and *A. niger* AT-3 respectively Substrate concentration for cellulase activity : 20 mg/mL citrate buffer

Table 2.23: Temperature stabilization of cellulase produced by *A. flavus* AT-2 and *A. niger* AT-3

Sl. No.	Temp, ^{0}C	Time, min	*A. flavus* AT-2		*A. niger* AT-3	
			CMCase activity, IU/mL	Relative cellulase activity, %	CMCase activity, IU/mL	Relative cellulase activity, %
1	50	30	17.44±0.81	100	24.73±0.81	100
2	60	30	8.92	51	15.72	64
3	70	30	3.36	19	7.13	29
4	80	30	0.73	4	1.62	7

± refers standard deviation

Assay conditions:

pH of buffer : 4.8 and 5.3 for *A. flavus* AT-2 and *A. niger* AT-3 respectively Substrate concentration for cellulase activity : 20 mg/mL citrate buffer

Table 2.24: Mass production of extracellular enzymes by *A. niger- AT-3* under optimized conditions

CMCase activity, IU/mL	FPase activity, IU/mL	β-glucosidase activity, IU/mL	Protein concentration, mg/mL	Xylanase activity, IU/mL
25.12±1.42	2.23±0.13	0.87±0.09	3.11±0.21	5.77±0.98

±refers standard deviation

Fermentation conditions:

Rice straw, g : 5
Nutrient salt solution, mL : 15
pH : 5.3
Temperature : 35°C

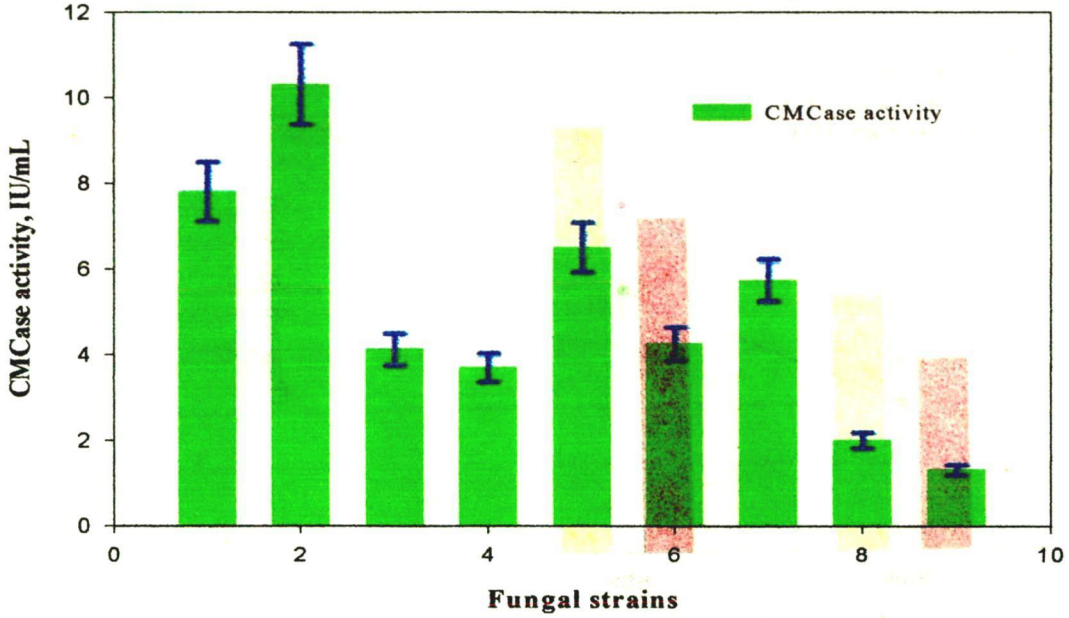


Figure 2.4: Enzyme production from the fungal isolates under SSF conditions (1=AT-2, 2=AT-3, 3=AT-4, 4=AT-5, 5=AT-6, 6=AT-7, 7=AT-8, 8=AT-10, 9=AT-13

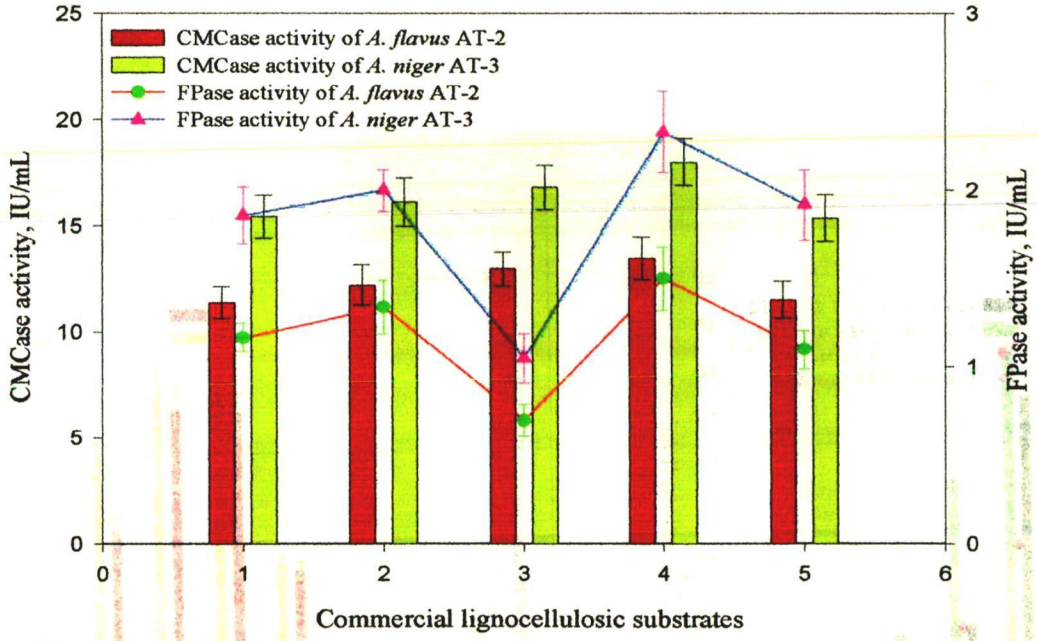


Figure 2.5: Optimization of commercial lignocellulosic substrates for cellulase production by *A. flavus* AT-2 and *A. niger* AT-3 (1=cellulose acetate 2=phosphocellulose, 3=diethylaminomethylcellulose, 4=carboxymethylcellulose and 5= acacia powder)

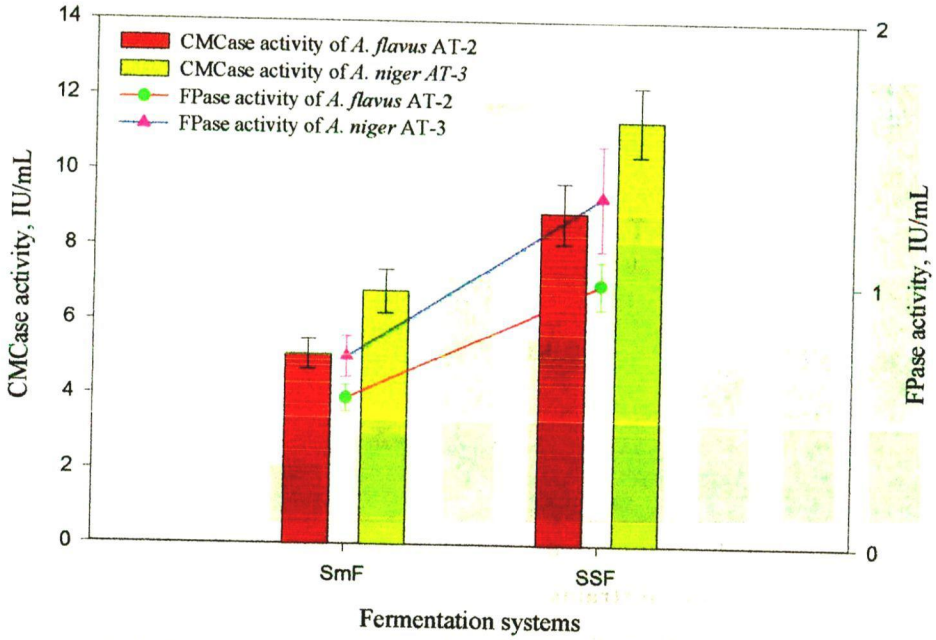


Figure 2.6: Comparison of fermentation systems for the enzyme production by *A. flavus* AT-2 and *A. niger* AT-3

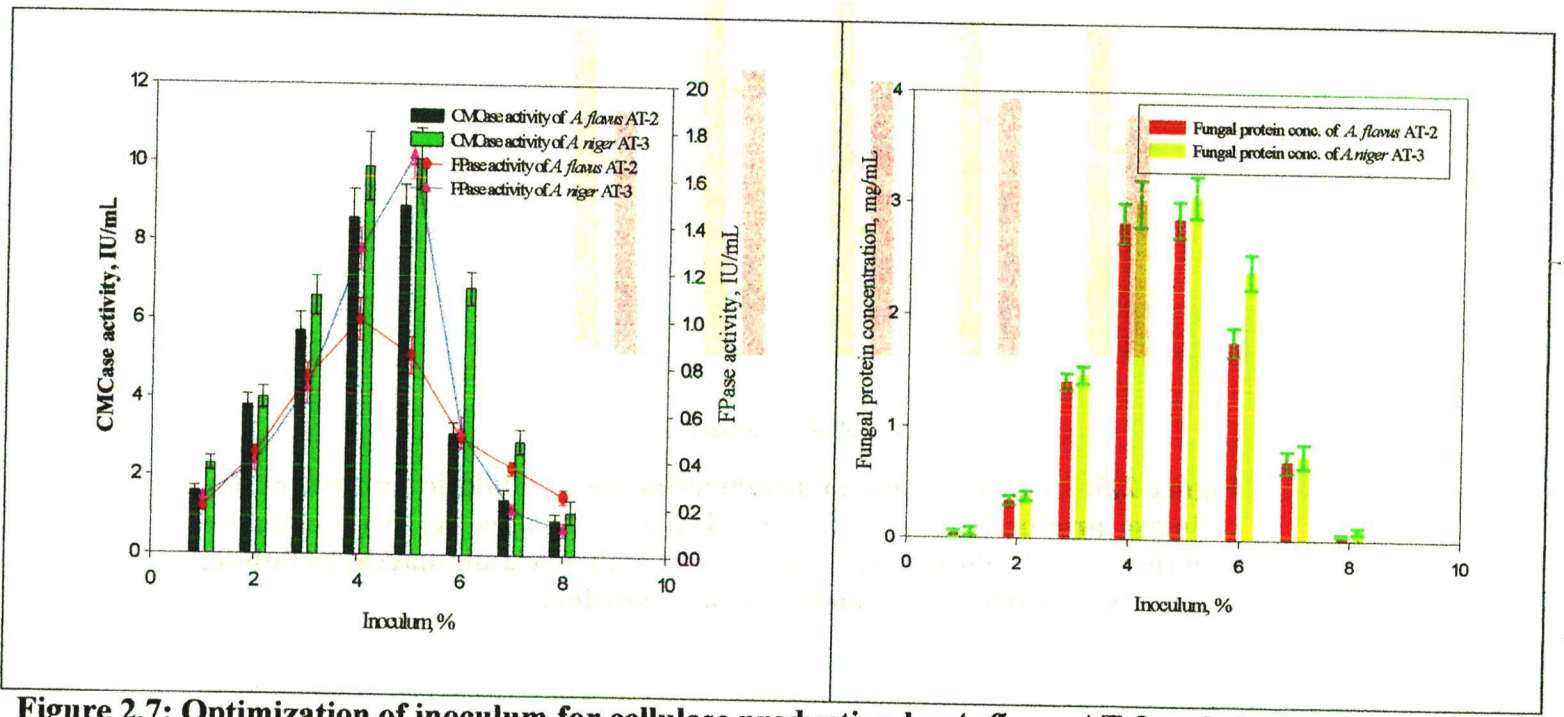


Figure 2.7: Optimization of inoculum for cellulase production by *A. flavus* AT-2 and *A. niger* AT-3

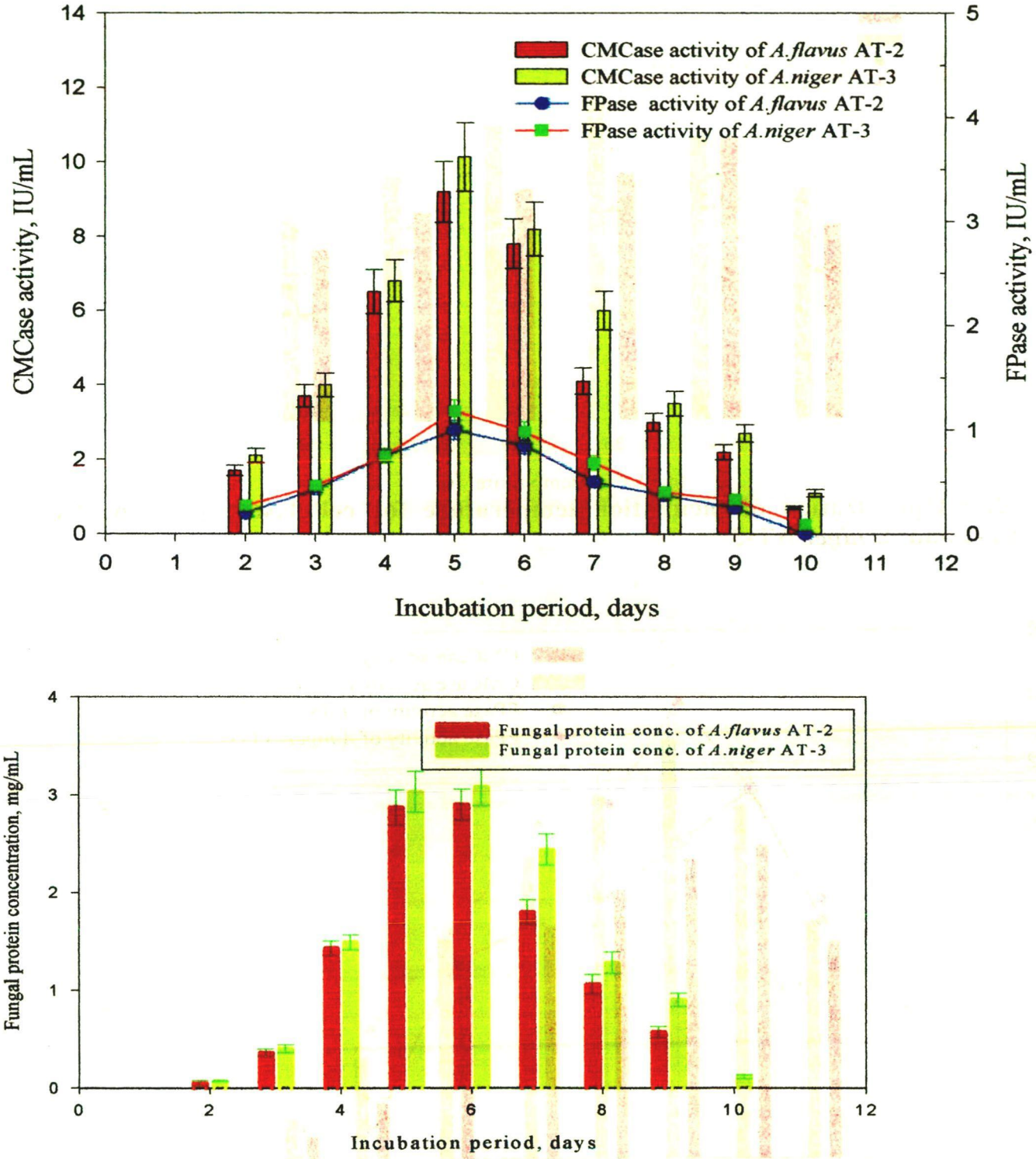


Figure 2.8: Optimization of incubation period for cellulase production by *A. flavus* AT-2 and *A. niger* AT-3

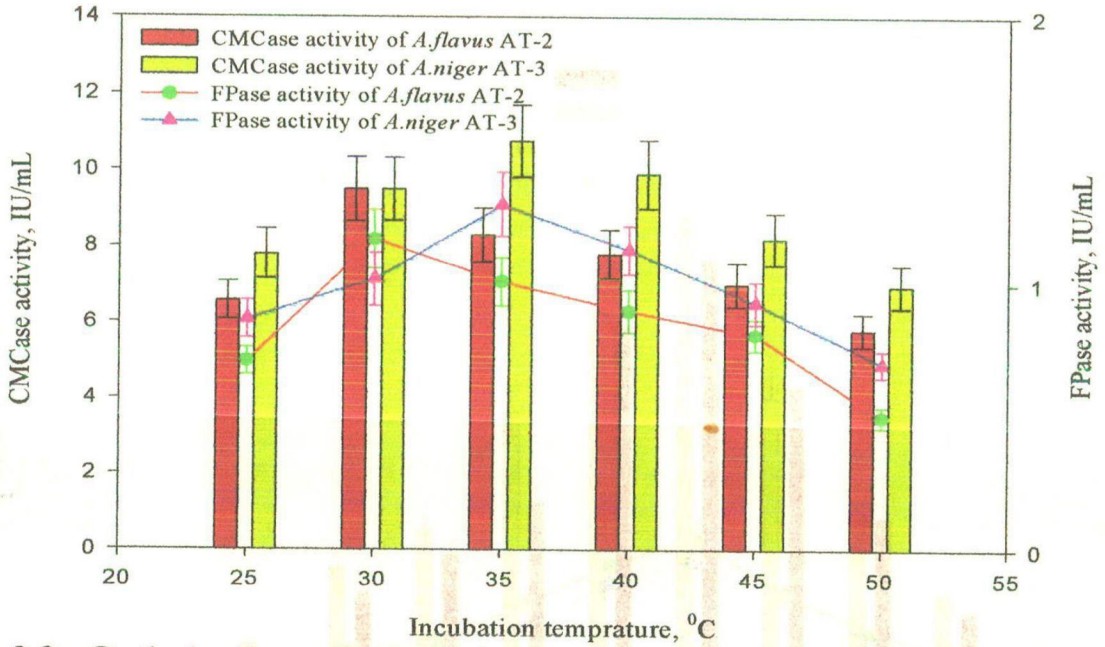


Figure 2.9: Optimization of incubation temperature for cellulase production by *A. flavus* AT-2 and *A. niger* AT-3

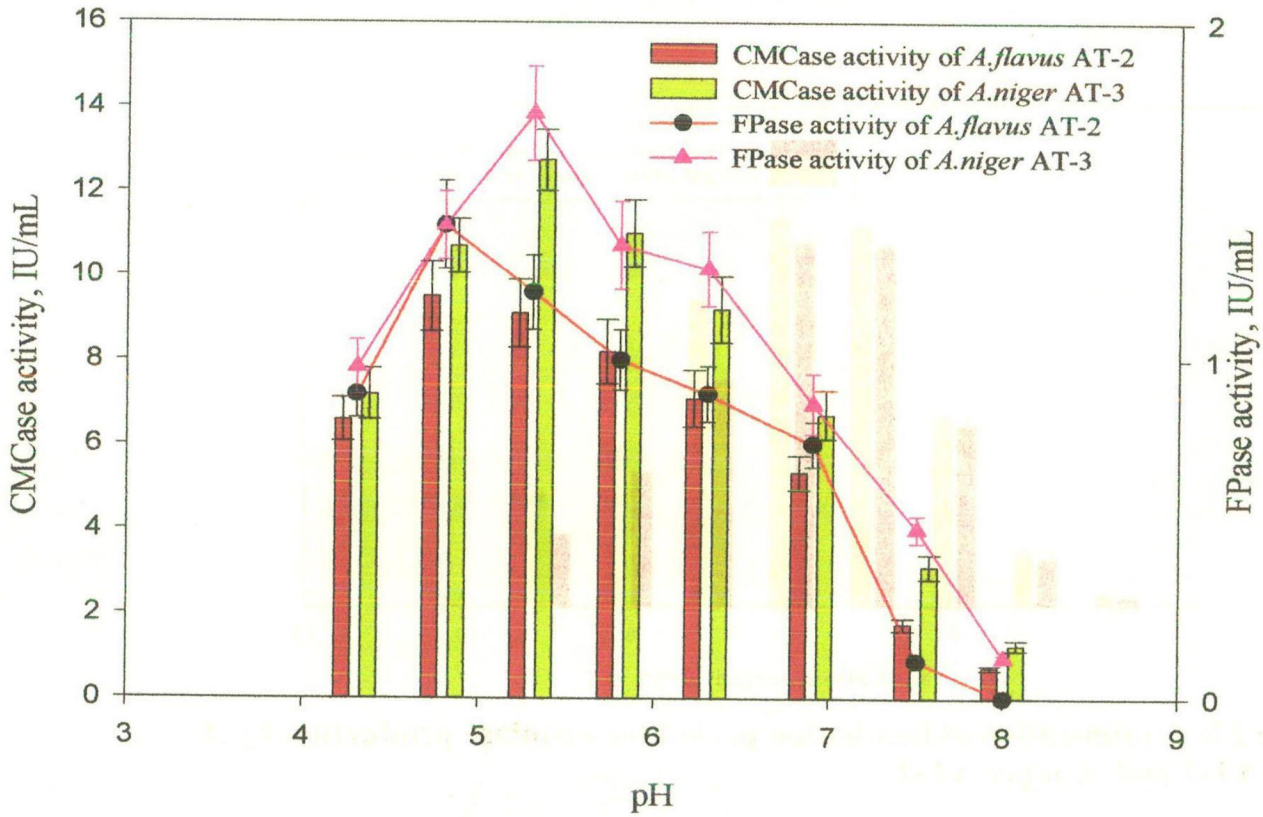


Figure 2.10: Optimization of initial pH for cellulase production by *A. flavus* AT-2 and *A. niger* AT-3

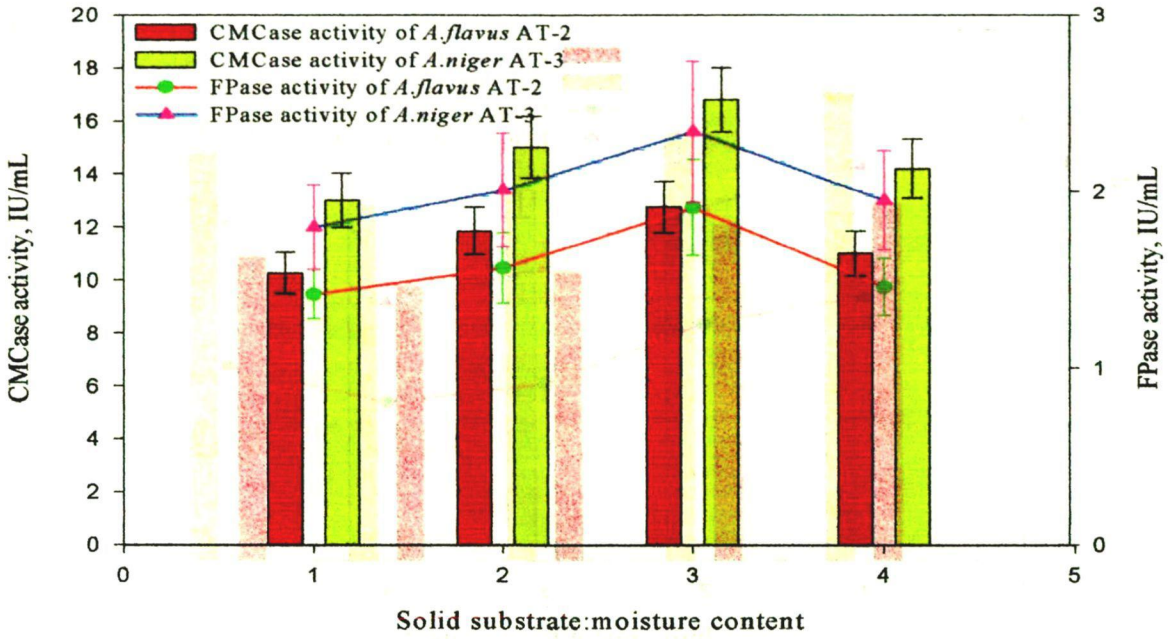


Figure 2.11: Optimization of moisture content for cellulase production by *A. flavus* AT-2 and *A. niger* AT-3(1=1:1, 2=1:2, 3=1:3, 4=1:4)

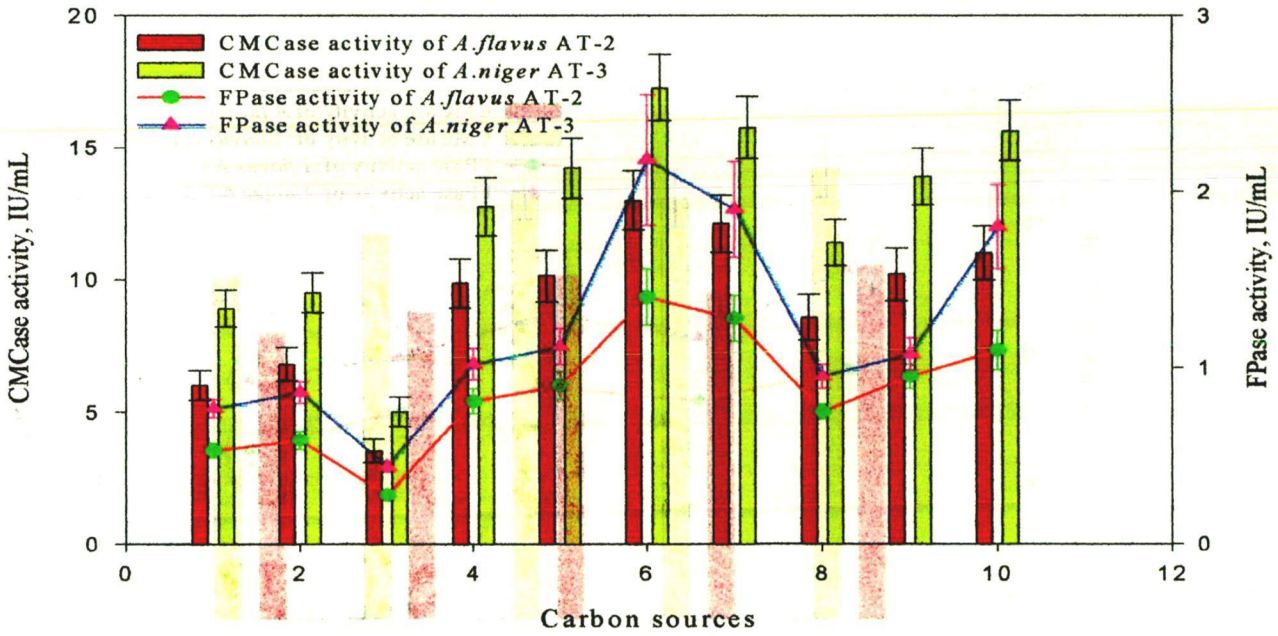


Figure 2.12: Effect of carbon sources on cellulase production by *A. flavus* AT-2 and *A. niger* AT-3 (1: sugarcane bagasse (untreated), 2: wheat straw (untreated), 3: rice bran (untreated), 4: wheat bran (untreated), 5: rice straw (untreated), 6: rice straw (pretreated),7:rice straw + wheat bran (pretreated), 8: rice straw+ rice bran (pretreated), 9: rice straw + sugarcane bagasse (pretreated), 10: rice straw+ wheat straw (pretreated)

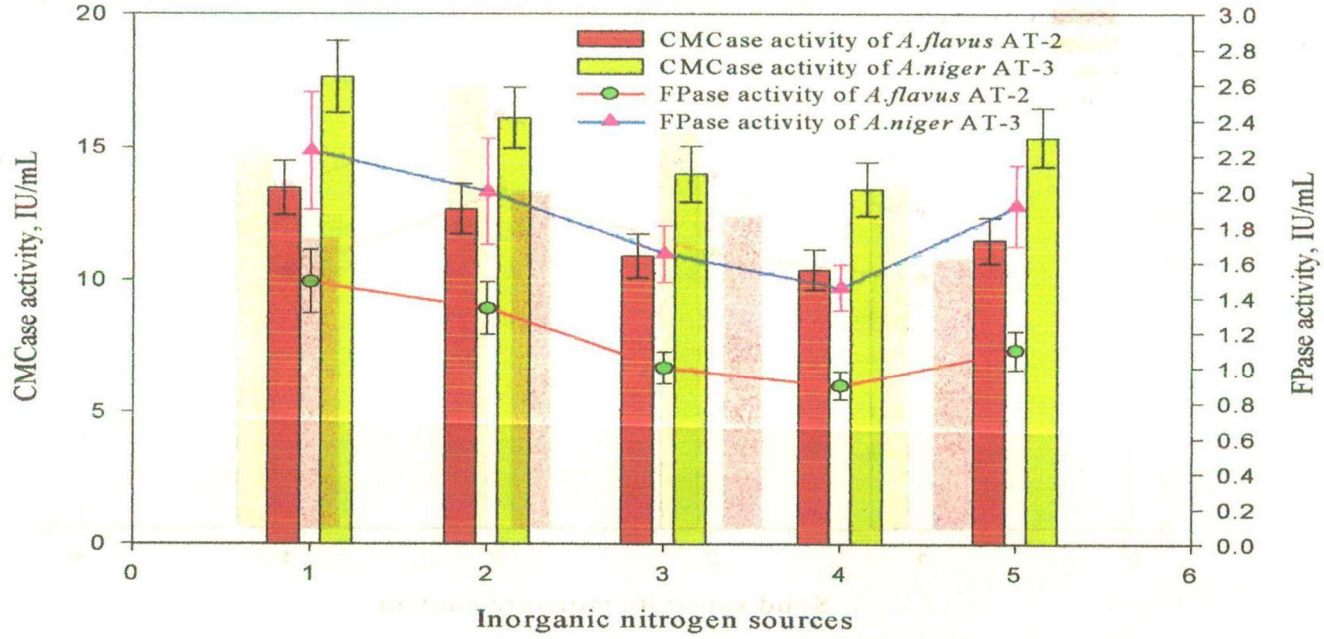


Figure 2.13: Effect of inorganic nitrogen sources on cellulase production by *A. flavus* AT-2 and *A. niger* AT-3 (1= $(NH_4)_2 SO_4$, 2=$(NH_4)_2HPO_4$, 3= KNO_3, 4=$NaNO_3$, 5=$(NH_4) NO_3$)

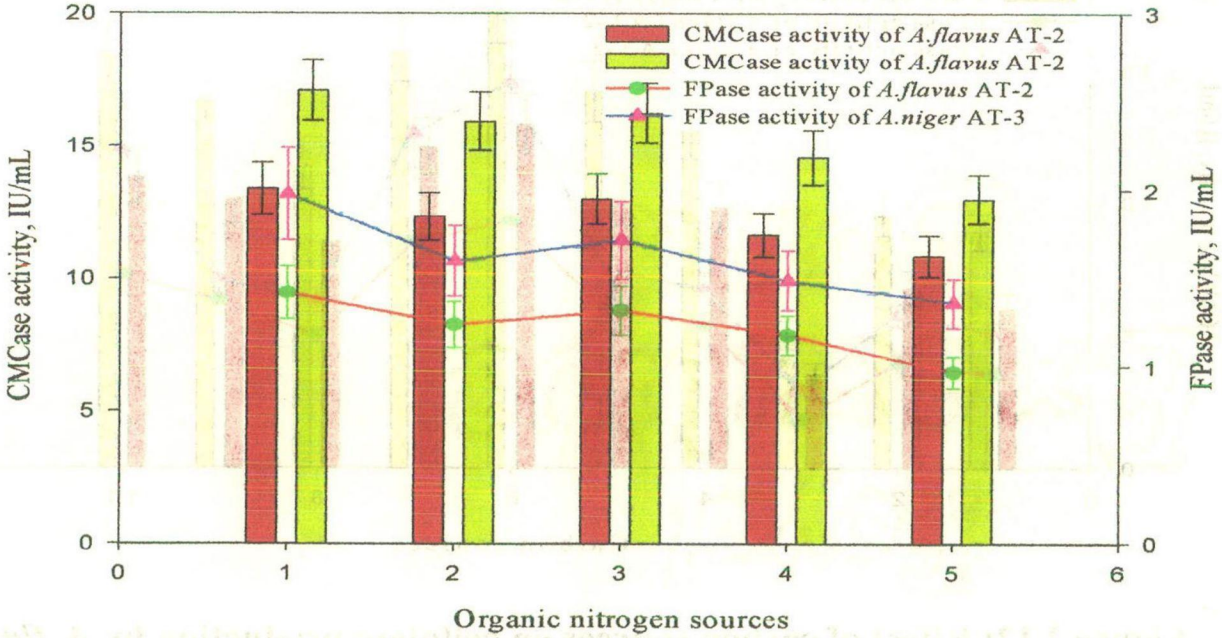


Figure 2.14: Effect of organic nitrogen sources on cellulase production by *A. flavus* AT-2 and *A. niger* AT-3 (1=Yeast extract, 2=Peptone, 3=Urea, 4=Soya bean meal, 5=Beef extract)

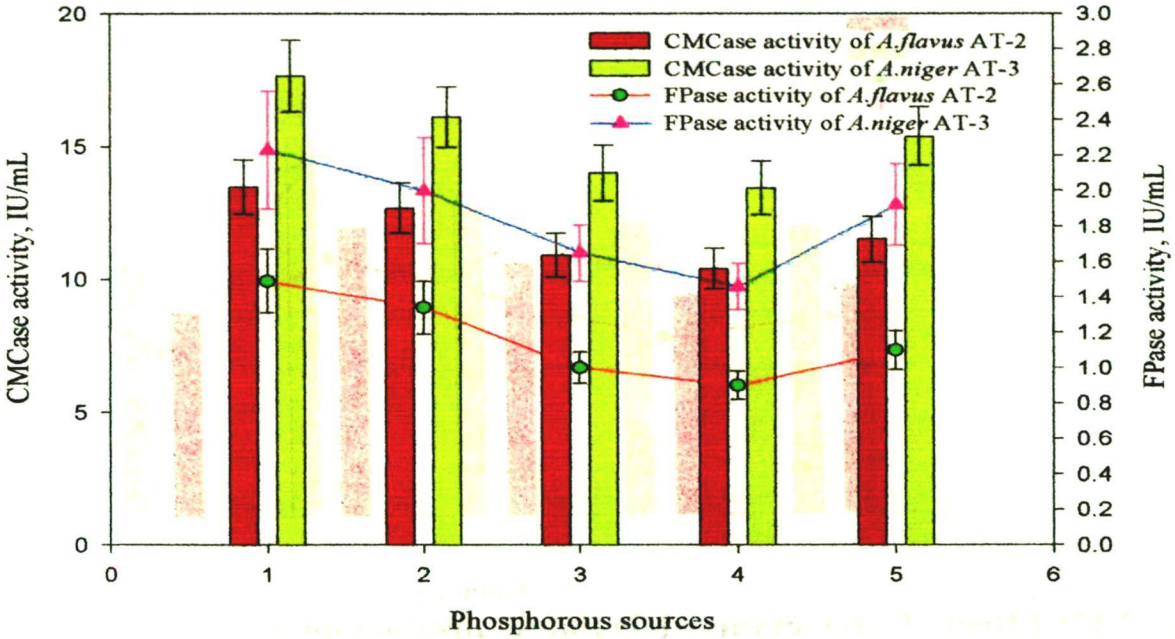


Figure 2.15: Effect of phosphorous sources on cellulase production by *A. flavus* AT-2 and *A. niger* AT-3(1=KH_2PO_4, 2=K_2HPO_4, 3=Sodium β-glycerophosphate, 4=Tetra sodium pyrophosphate)

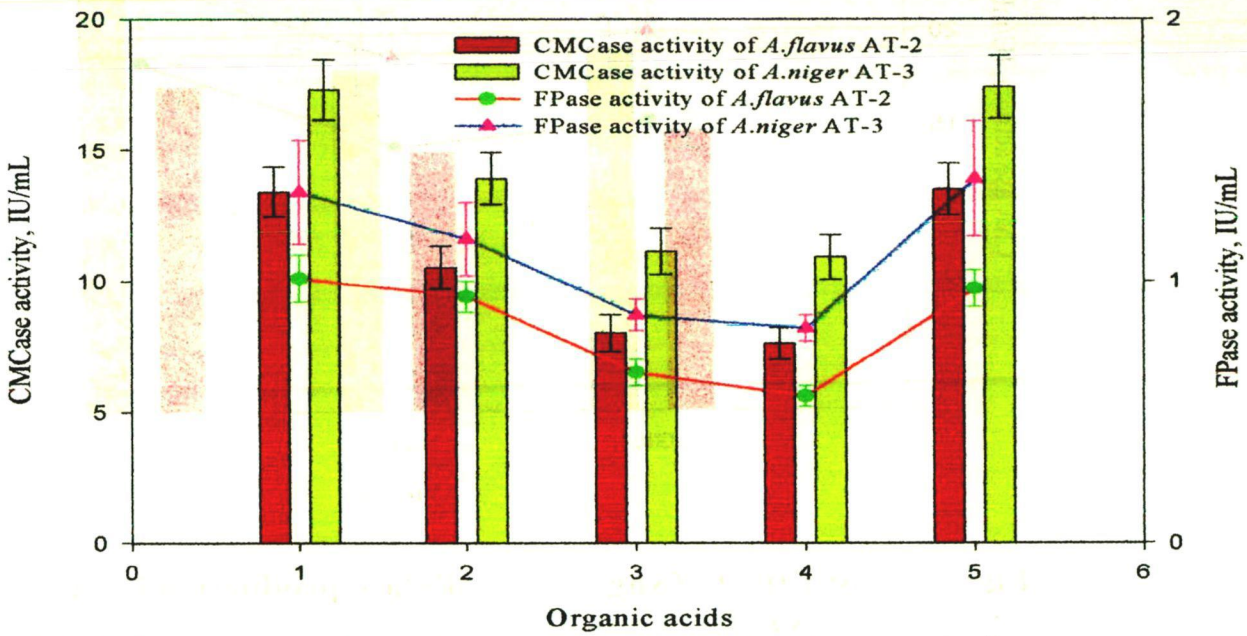


Figure 2.16: Effect of organic acids on cellulase production by *A. flavus* AT-2 and *A. niger* AT-3 (1=Acetic acid, 2= Citric acid, 3= Prop ionic acid, 4=Oxoglutaric acid, 5=Succinic acid)

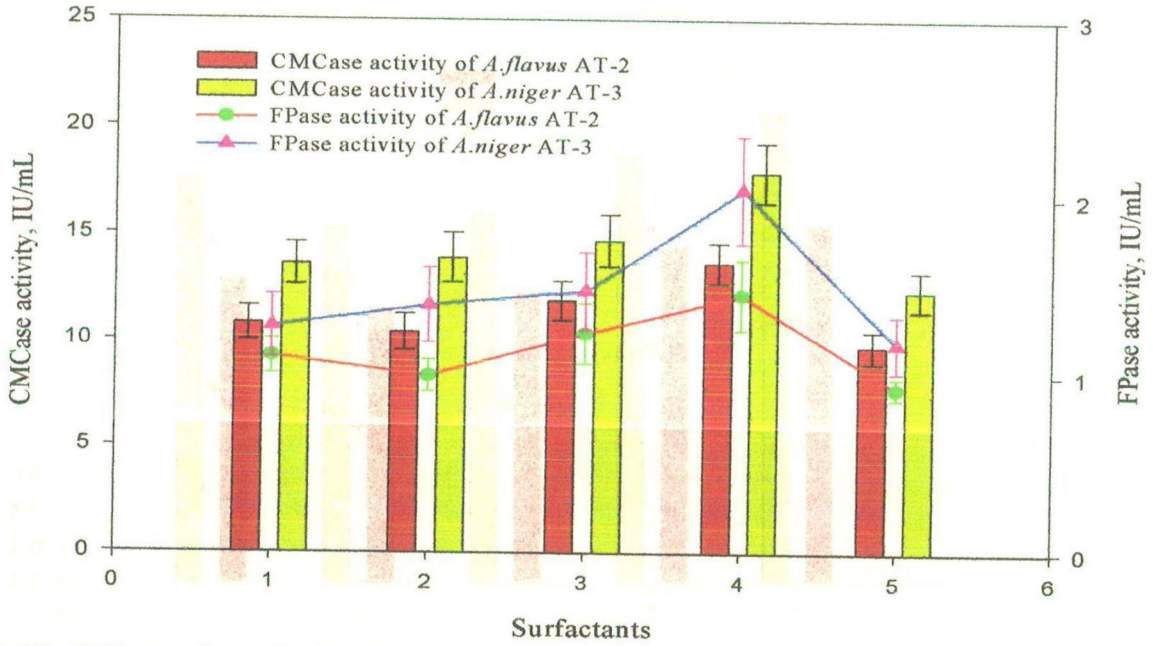


Figure 2.17: Effect of surfactants on cellulase production by *A. flavus* AT-2 and *A. niger* AT-3 (1=Tween-20, 2=Tween-40, 3=Tween-60, 4=Tween- 80, 5= Triton–x)

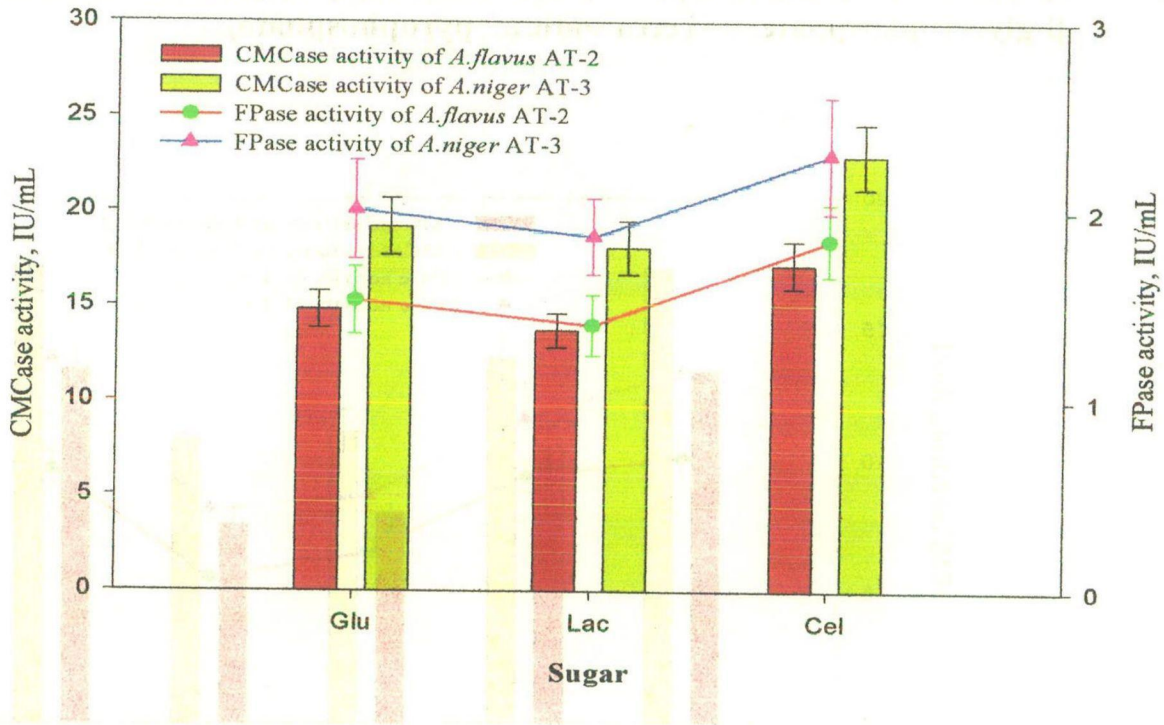


Figure 2.18: Effect of sugars on cellulase production by *A. flavus* AT-2 and *A. niger* AT-3

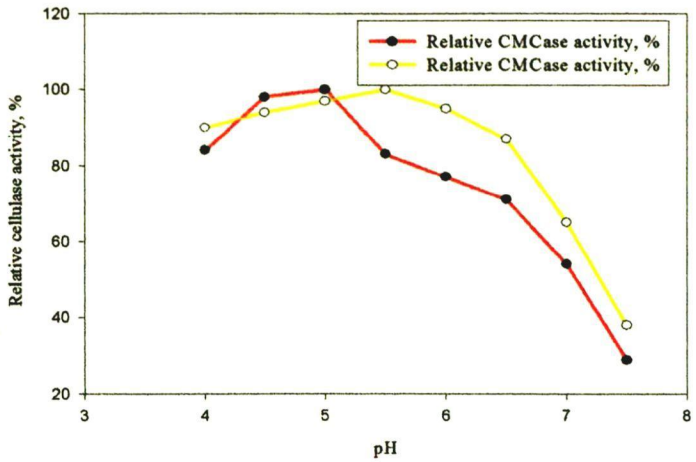


Figure 2.19: pH stability of cellulase produced by *A. flavus* AT-2 and *A. niger* AT-3

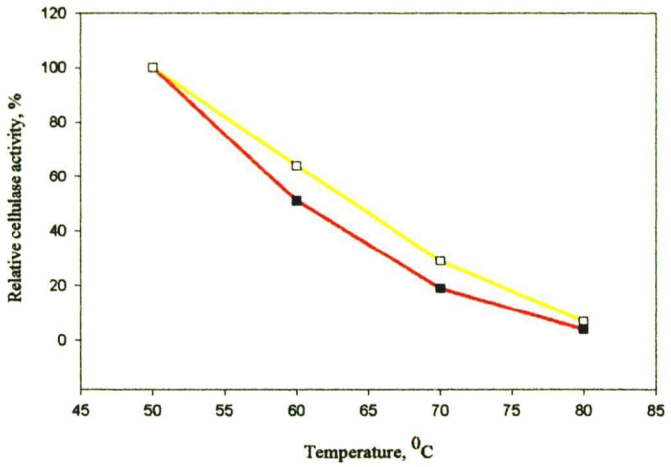
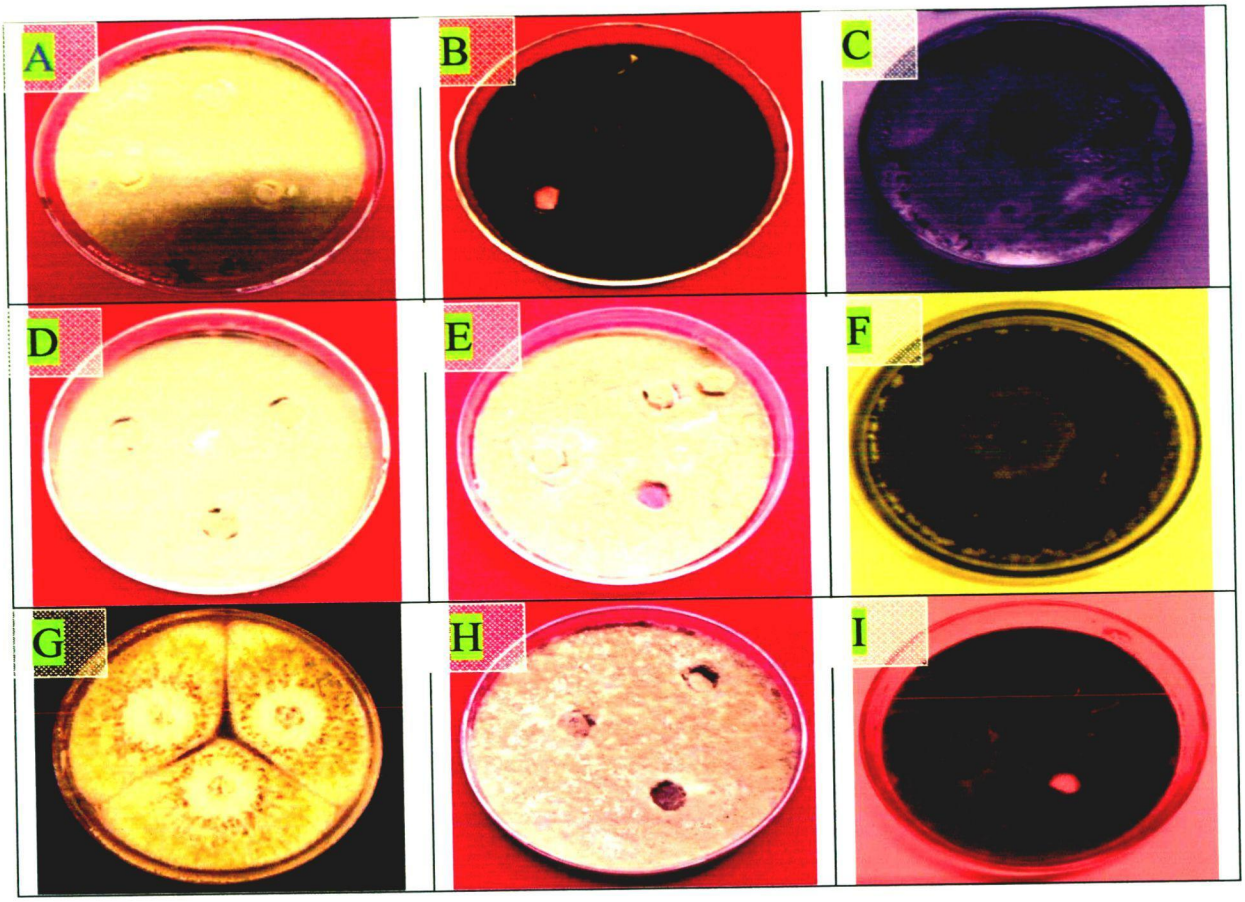
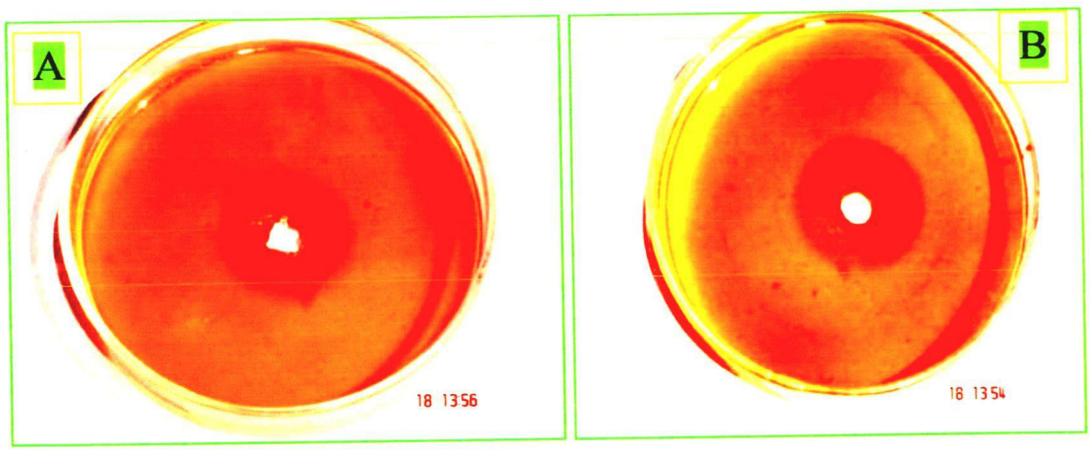


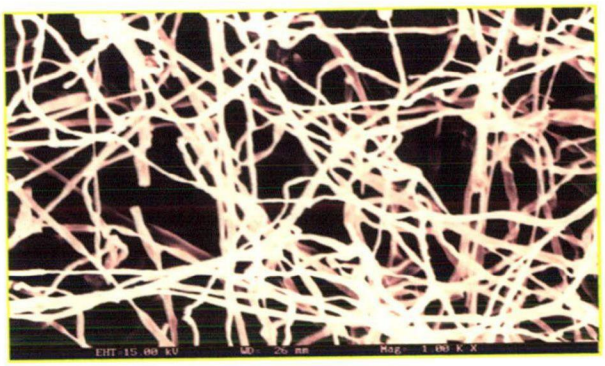
Figure 2.20: Temperature stability of cellulase produced by *A. flavus* AT-2 and *A. niger* AT-3



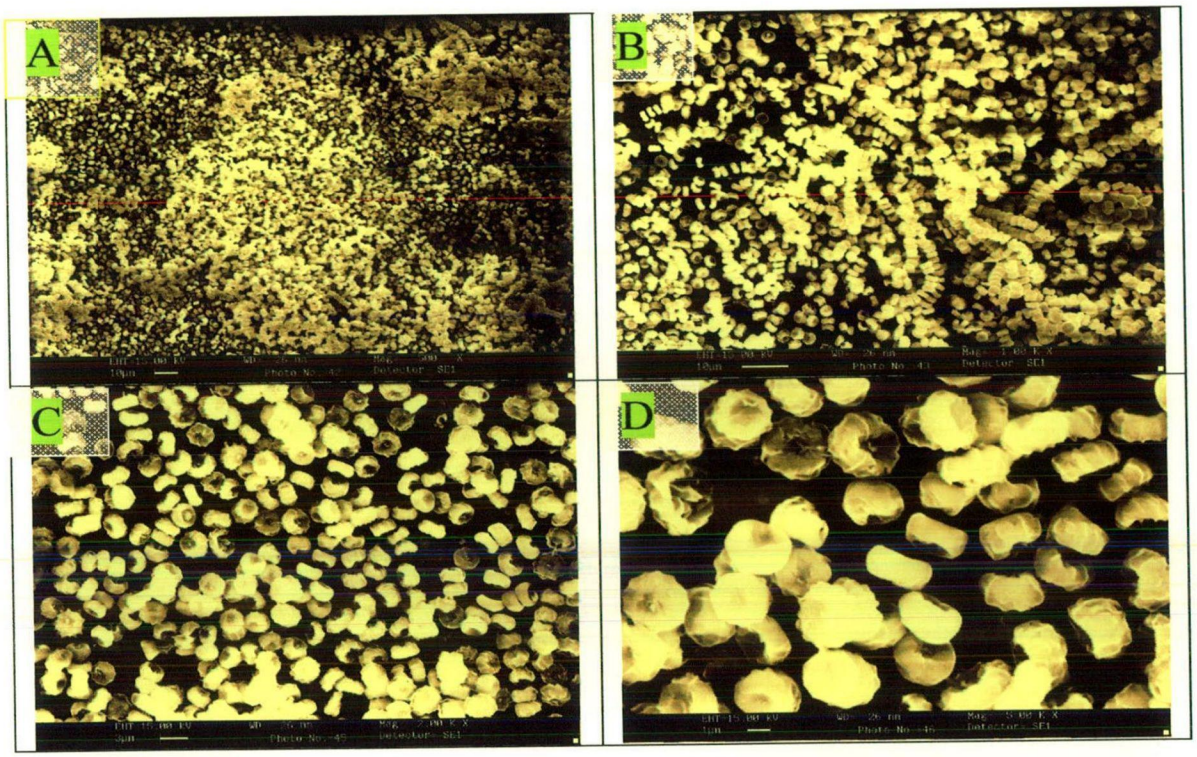
Photograph 2.1: Morphological features of different fungal strains(A=AT-2, B=AT-3, C=AT-4, D=AT-5, E=AT-6, F=AT-7, G=AT-8, H=AT-10, I=AT- 13)



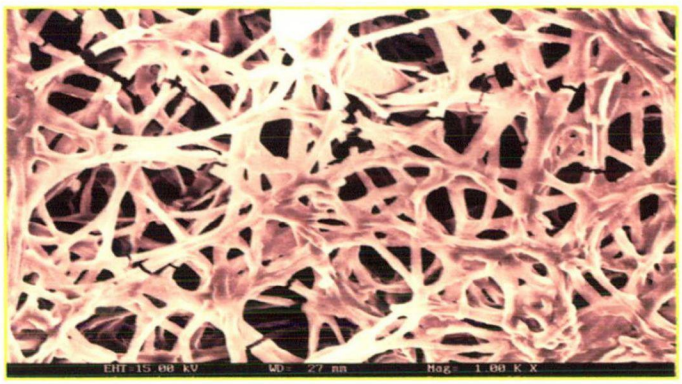
Photograph 2.2: Plate assay for cellulase, produced by *A. flavus* AT-2(A) and *A. niger* AT-3(B)



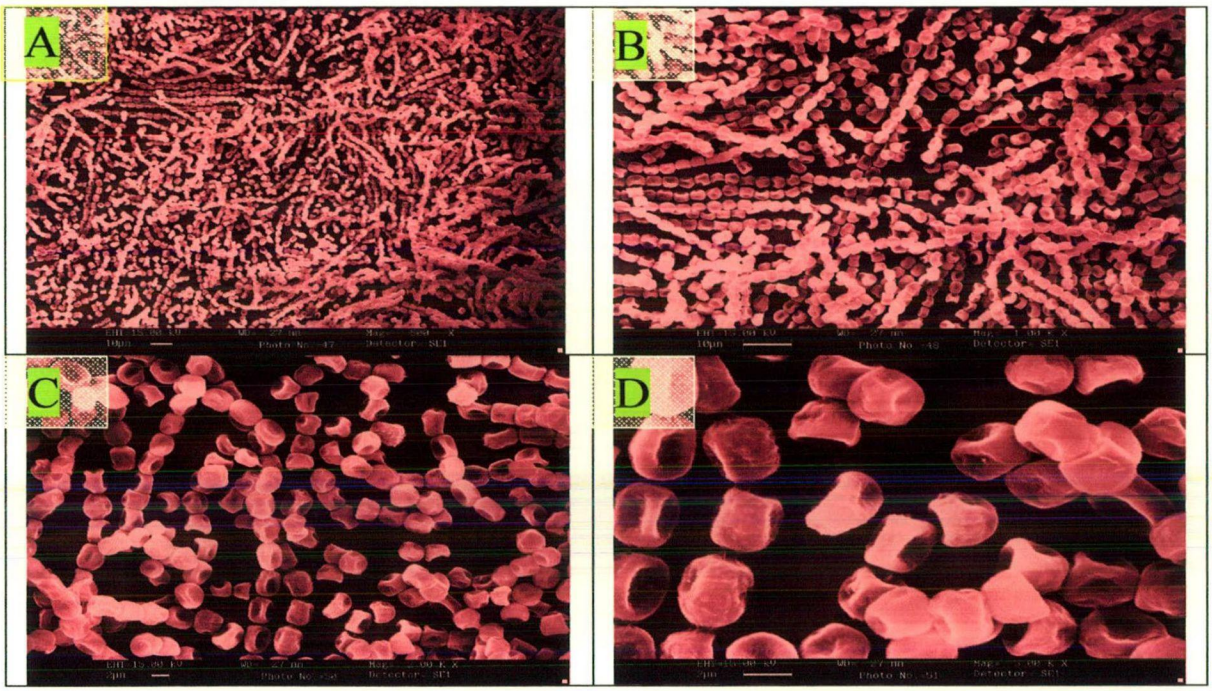
Photograph 2.3: Scanning electron microscopy of the mycelia of *A. flavus* AT- 2 at a magnification of 1.00KX



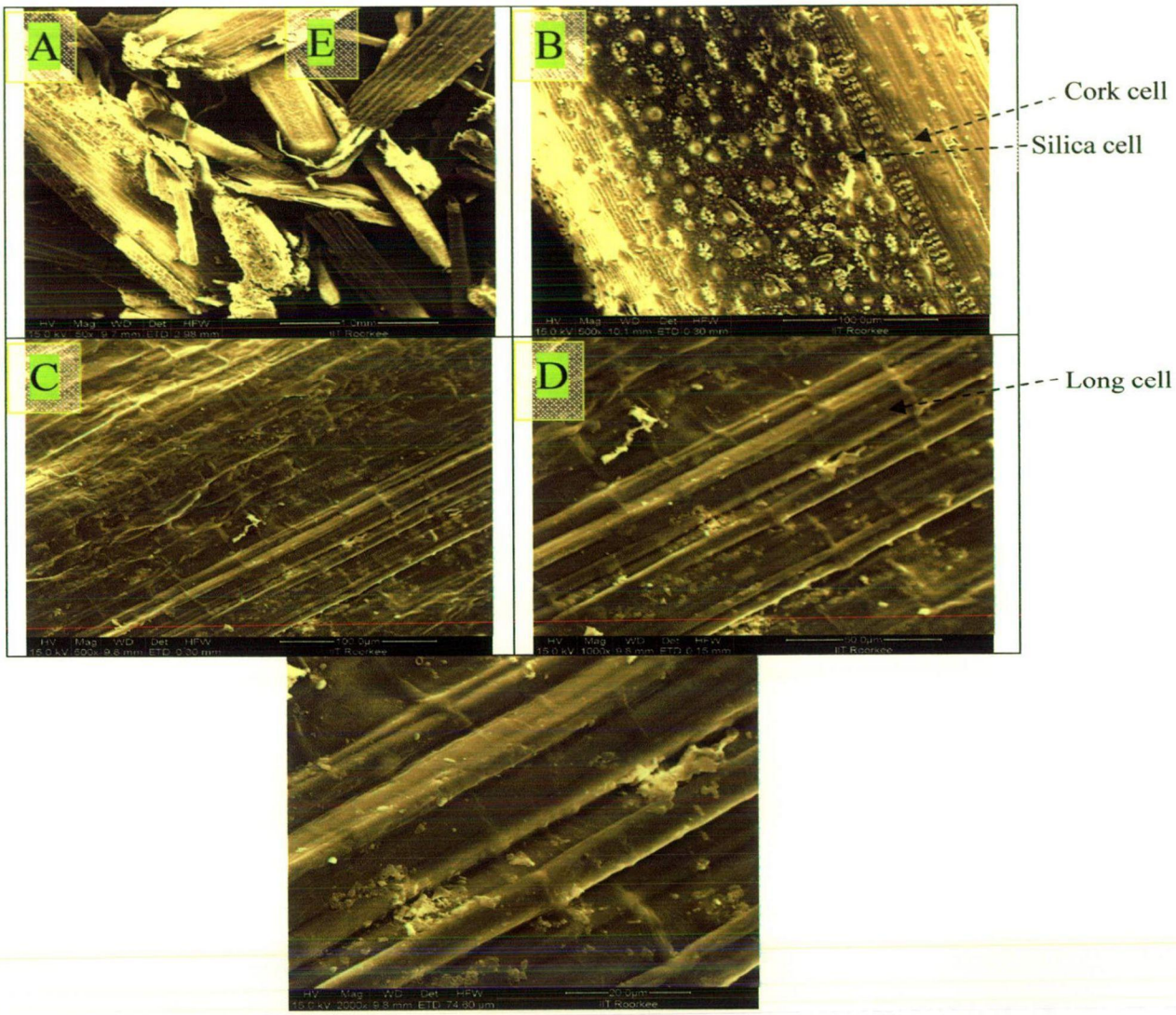
Photograph 2.4: Scanning electron microscopy of spores of *A. flavus* AT-2 at a magnification of (A) 500X, (B) 1.00KX, (C) 2.00KX and (D) 5.00 KX



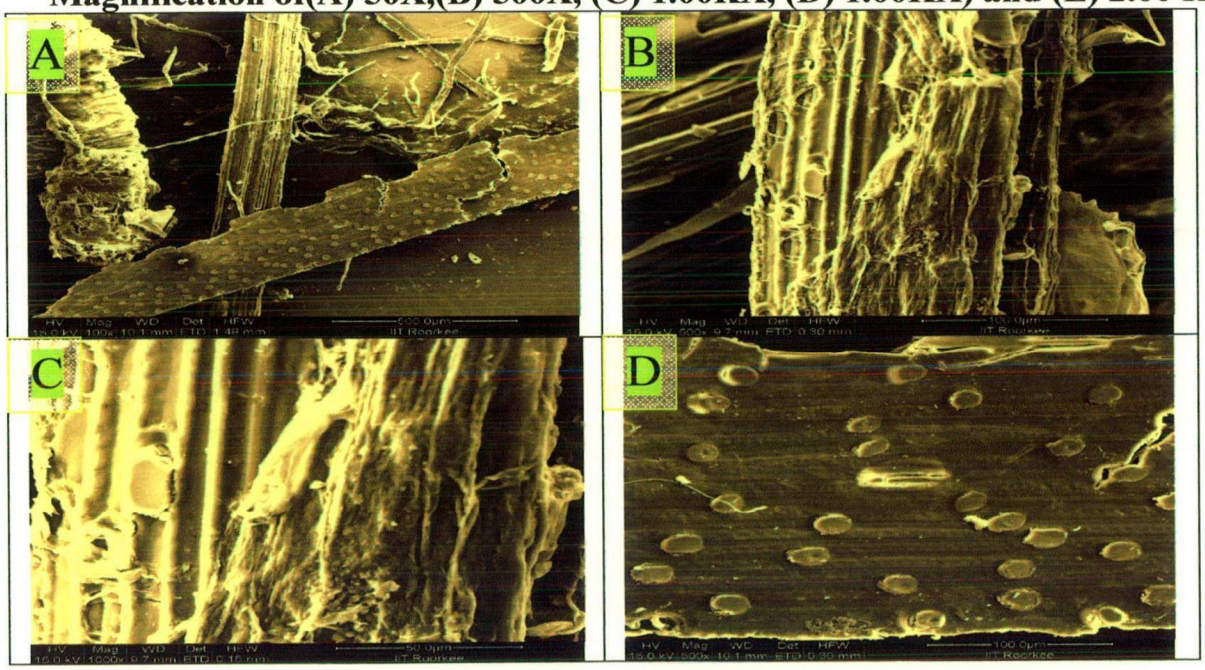
Photograph 2.5: Scanning electron microscopy of the mycelia of *A. niger* AT-3 at a magnification of 1.00KX

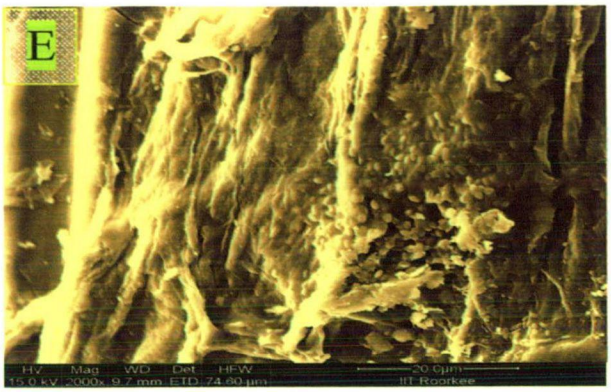


Photograph 2.6: Scanning electron microscopy of spores of *A. niger* AT-3 at a magnification of (A) 500X, (B) 1.00KX, (C) 2.00KX and (D) 5.00 KX

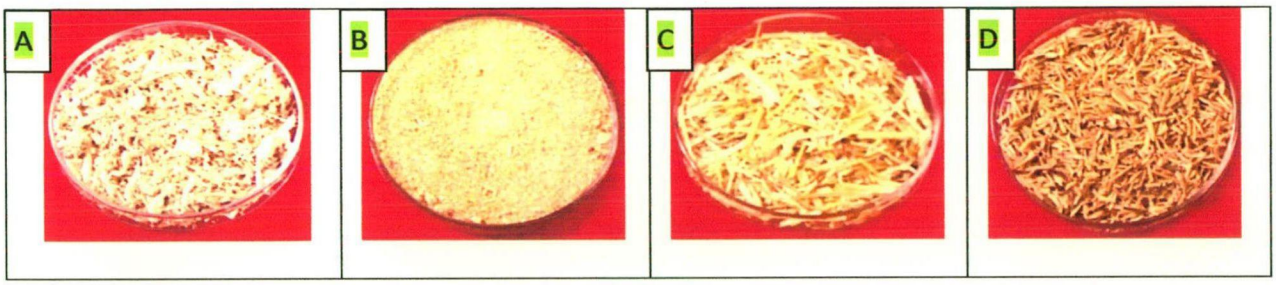


Photograph 2.7: Scanning electron microscopy of untreated rice straw at a Magnification of(A) 50X,(B) 500X, (C) 1.00KX, (D) 1.00KX) and (E) 2.00 KX

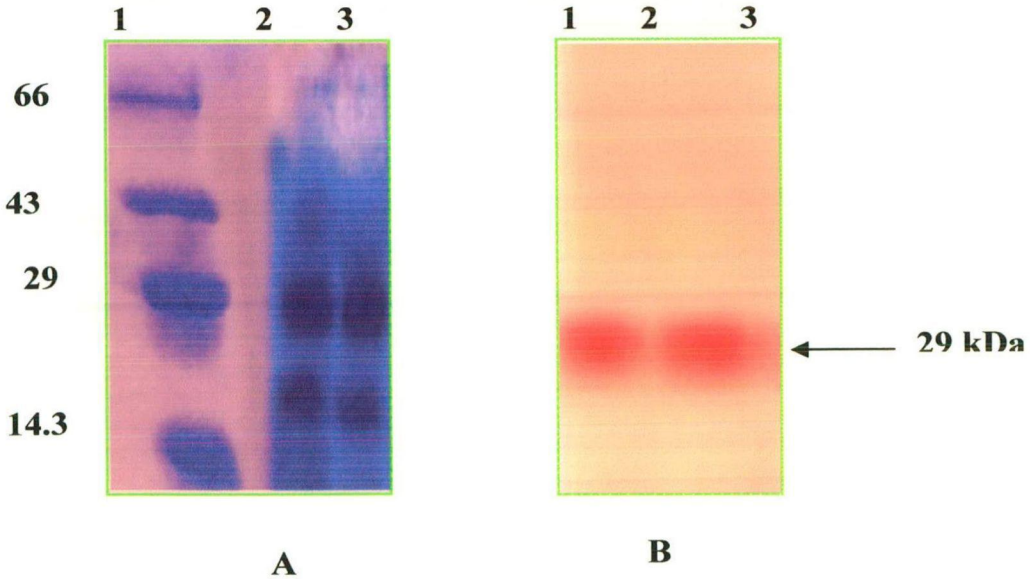




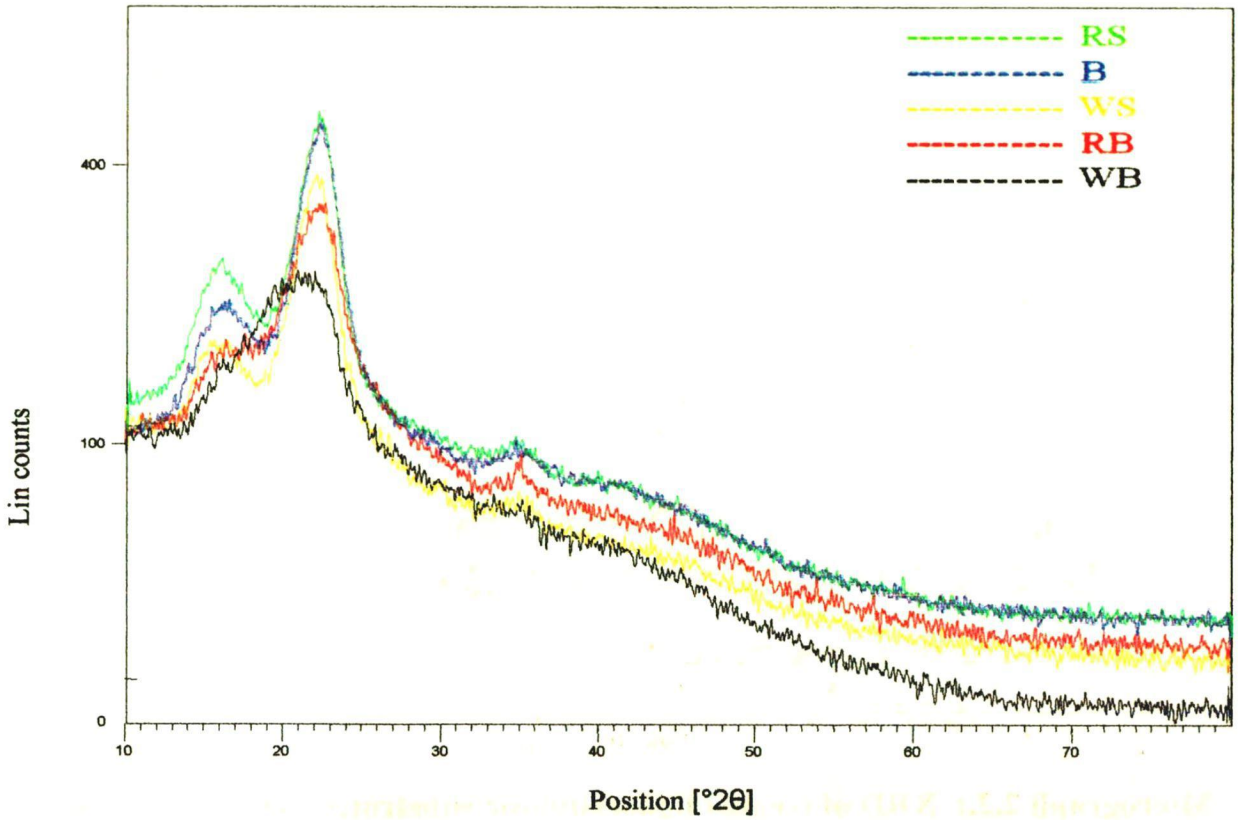
Photograph 2.8: Scanning electron microscopy of pretreated rice straw at a Magnification of (A) 50X, (B) 500X, (C) 1.00KX, (D) 1.00KX) and (E) 2.00 KX



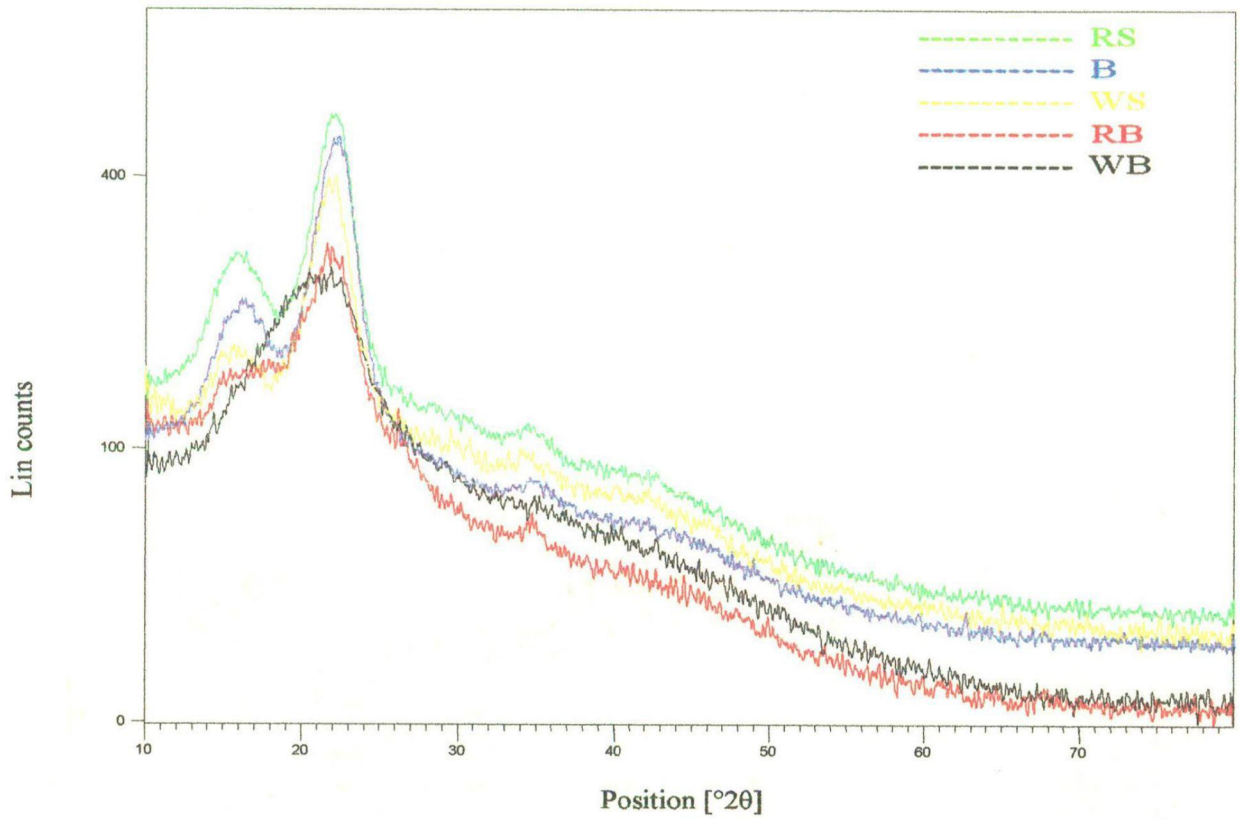
Photograph 2.9: Different carbon sources used during SSF (A= Sugarcane bagasse, B= Wheat bran, C= Wheat straw and D= Rice straw)



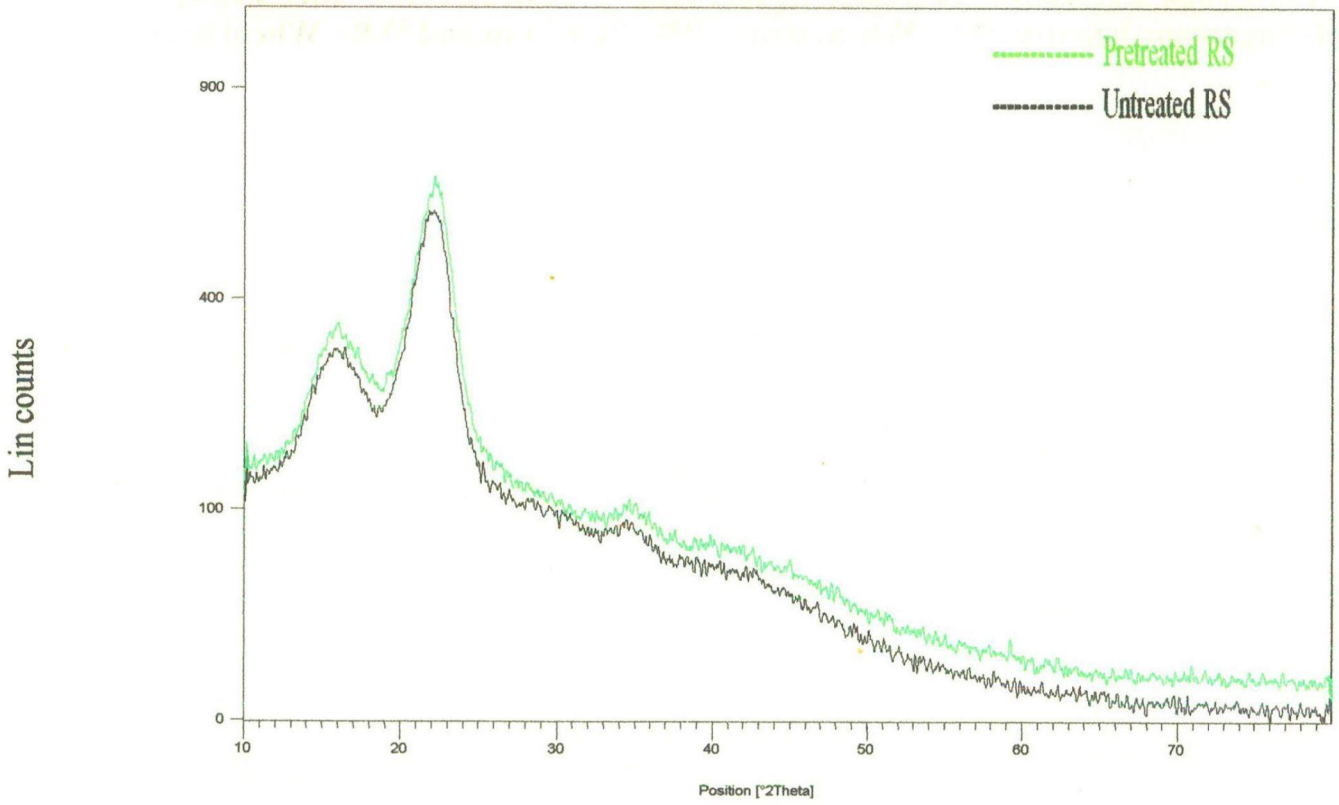
Photograph 2.10: SDS-PAGE (A) and Zymogram (B) analysis of cellulase produced by *A.niger* AT-3



Micrograph 2.1: XRD of untreated lignocellulosic substrates (RS= Rice straw, B=Sugarcane bagasse, WS= Wheat straw, RB= Rice bran and WB= Wheat bran)



Micrograph 2.2.: XRD of treated lignocellulosic substrates (RS= Rice straw, B=Sugarcane bagasse, WS= Wheat straw, RB= Rice bran and WB= Wheat bran)



Micrograph 2.3.: XRD of untreated and treated rice straw

References

1. Aboul-Enein, A., Abou elalla, F., Serour, E. and Hussien, T., Purification and characterization of a novel thermo-active cellulase from thermophilic actinomycetes isolated from soil samples of Egypt, *International Journal of Academic Research,* 2(1): 81-86(2010).
2. Abu-Shady, M.R., El-Gindy, A.A., Saad, R.R. and Fawzy, E.M., Optimization of the production of cellulase by *Trichoderma longibrachiatum* under solid substrate cultivation, *The African, Journal of Mycology and Biotechnology,* 9:5-15(2001).
3. Aiba, S., Humphrey, A.E. and Millis, N.F., Kinetics, *In: Biochemical engineering, Academic Press,* 2nd Edition, New York (1973) pp. 92-127.
4. Akiba, S., Kimura, Y., Yamamoto, K. and Kumagai, H., Purification and characterization of a protease-resistant cellulase from *Aspergillus niger, J. Ferment. Bioeng.,* 79:125-130(1995).
5. Alemdar, A. and Sain, M., Isolation and characterization of nanofibers from agricultural residues-wheat straw and soyahulls, *Biores. Technol.,* 99:1664-1671(2008).
6. Alexopoulos, C.J., Mims C.W. and Blackwell, M.I., *In: Introductory Mycology,* Alexopoulos, C.J., (ed.), (1996).
7. Ali, S., Sayed, A., Sarker, R.I. and Alam, R., Factors affecting cellulase production by *Aspergillus terreus* using water hyacinth, *World J. Microbiol. Biotechnol.,* 7:62-66(1991).
8. Arja, M.O., Londesborough, J., Joutsjoki, V., Lantto, R. and Vehmaanperä, J., Three cellulases from *Melanocarpus albomyces* for textile treatment at neutral pH, *Enzyme Microbial. Technol.,* 34:332-341(2004).
9. Asha Poorna, C. and Prema P., Production of cellulase-free endoxylanase from novel alkalophilic thermotolerant *Bacillus pumilus* by solid-state fermentation and its application in wastepaper recycling, *Biores. Technol.,* 98:485-490(2007).
10. Awadel-Karim, S., Nazhad, M.M. and Pazsner, L., Factors affecting crystalline structure of cellulose during solvent purification treatment, *Holzforschung,* 53:1-8(1999).
11. Babu, K.R. and Satyanarayana, T., α-amylase production by thermophilic *Bacillus coagulans* in solid-state fermentation, *Process Biochem.,* 30:305-309(1995).

12. Baker, T. B., Quicke, G. V., Bentley, O.G., Johnson, R.R. and Moxon, A.L., The influence of certain physical properties of purified celluloses and forage celluloses on their digestibility by rumen microorganisms *in vitro*, *J. Anim. Sci.*, 18:655(1959).

13. Baker, A.J., Effect of lignin on the *in vitro* digestibility of wood pulp, *J. Anim. Sci.*, 36:768 (1973).

14. Beg, Q.K., Bhushan, B., Kapoor, M. and Hoondal, G.S., Enhanced production of a thermostable xylanase from *Streptomyces* sp. QG-11-3 and its application in bio-bleaching of eucalyptus kraft pulp, *Enzyme Microbial. Technol.*, 27:459-466(2000).

15. Beg, Q.K., Kapoor, M., Mahajan, L. and Hoondal, G.S., Microbial xylanases and their industrial applications: a review, *Appl. Microbiol. Biotechnol.*, 56:326-338(2001).

16. Beguin, P. and Aubert, J.P., The biological degradation of cellulose, *FEMS Microbiol Rev.*, 13: 25-58(1993).

17. Bendzalova, M., Pekarovicova, A., Kokta, B.V. and Chen, R.B., Accessibility of swollen cellulosic fibres, *Cellulose Chem. Technol.*, 30:19-32(1996).

18. Benkun Q., Risheng, Y., Ying, Y. and Yuan, C., Influence of different ratios of rice straw to wheat bran on production of cellulolytic enzymes by *Trichderma virde ZY-01* in solid state fermentation, *EJEAFChe*, 6 (9): 2341-2349(2007).

19. Bertran, M.S. and Dale, B.E., Determination of cellulose accessibility by differential scanning calorimetry, *Journal of Applied Polymer Science*, 32(3): 4241-4253(2003).

20. Biely, P., Vrsanska, M. and Claeyssens, M., The endo-1-4-β-D-glucanase-I from *Trichoderma reesei*- Action on β-1-4 oligomers and polymers derived from D-glucose and D-xylose, *Eur. J. Biochem.*, 200:157-163(1991).

21. Bisaria, V.S. and Mishra, S., *Crit. Rev. Biotechnol.* 9, 61(1989), *In: Bioconversion of waste materials to industrial products,* Martin, A. M., (ed.), *Pub. Blackie. Acad. Prof. London, SEI 8 HN.,* (1998) pp.197-246.

22. Bhat, M. and Bhat, S., Cellulose degrading enzymes and their potential industrial applications, *Biotechnol. Adv.*, 15: 583-620(1997).

23. Borgia, P. and Sypherd, P.S., Control of β-glucosidase synthesis in *Mucor racemosus, J. Bacteriol.*, 130(2): 812-817(1977).

24. Bourne, Y. and Henrissat, B., Glycoside hydrolases and glycosyl transferases: families and functional modules, *Curr. Opin. Struct. Biol.*, 11:593-600(2001).

25. Bronnenmeier, K. and Staudenbauer, W.L., Purification and properties of an extracellular β-glucosidase from the cellulolytic thermophilic *Clostridium stercorarium, Appl. Microbiol. Biotechnol,* 28:380-386 (1988).

26. Brown, A.J., Ogawa, K., and Wood, T.M., Studies on preparation and regeneration of protoplast from cellulolytic fungus, *P. pinophillum, Enzyme Microbial. Technol.*, 9: 527-532(1986).

27. Bruchmann, E.E., Schach, H.S. and Graf, H., Role and properties of lactonase in cellulase system, *Biotech. Appl. Biochem.*, 9:146-159(1987).

28. Cao, N.J, Krishnan, M.S., Du, J.X., Gong, Ho, N.W.Y., Chen, Z.D. and Tsao, G.T., Ethanol production from corncob pretreated by ammonia steeping process using genetically engineered yeast, *Biotechnol. Lett.*, 18: 1013 -1018(1996).

29. Castanon, M. and Wilk. C.R., Adsorption and recovery of cellulases during hydrolysis of news paper, *Biotechnol. Bioeng.*, 22:1037-1053(1980).

30. Chandra, S., Application of fungal hemicellulases for biobleach process for hardwood kraft pulp, *Ph.D. Thesis,* C.C.S. University Meerut, India, (2001) pp. 82.

31. Chang, M.M., Chou, T.Y.C. and Tsao, G.T., Structure, pre-treatment and hydrolysis of cellulose, *In: Adv Biochem Eng Bioenergy,* Fieshter, A., (ed.) *Springer-Verlag:* Berlin, Germany, Vol. 20 (1991) pp.15-42.

32. Chang, V.S. and Holtzapple, M.T., Fundamental factors affecting biomass enzymatic reactivity, *Appl. Biochem. Biotechnol.*, 84:3-17(2000).

33. Chanzy, H. and Henrissat, B., Unidirectional degradation of *Valonia* cellulose microcrystals subjected to cellulase action, *FEBS Lett,* 184:285-288(1985).

34. Clawson, W. J., Garrett, W.N. and Richards, S.., Rice straw utilization by livestock, A literature review, Univ. of California, Agr. Ext. Publ. MA-1(1970).

35. Converse, A.O., Ooshima, H. and Burns, D.S., Kinetics of enzymatic hydrolysis of lignocellulosic materials based on surface area of cellulose accessible to enzyme and enzyme adsorption on lignin and cellulose, *Appl. Biochem. Biotechnol.*, 24/25: 67-73(1990).

36. Converse, A.O., Substrate factors limiting enzymatic hydrolysis, *In: Conversion of Forest and Agricultural Plant Residues*; Saddler, J.N., (Ed.) C.A.B. International: Wallingford, U.K., (1993) pp 93-106.

37. Cowling, E.B., Physical and chemical constraints in the hydrolysis of cellulose and lignocellulosic materials, *Biotechnol. Bioeng. Symp.*, 5: 163-181(1975).

38. Daniel, G., Use of electron microscopy for aiding our understanding of wood biodegradation, *FEMS Microbiol Rev.*, 13: 199-233(1994).

39. Davies, G.J. and Henrissat, B., Structure and mechanism of glycosyl hydrolases structure, 3:853-859(1995).

40. Davies, G.J., Ducros, V., Lewis, R.J., Borchert, T.V. and Schülein M., Oligosaccharide specificity of family 7 endoglucanases: Insertion of sugar binding subsites, *J. Biotechnol.*,57: 91-100(1997).

41. Deschampus, F. and Huet, M.C., β- glucosidase production in agitated solid-state fermentation *Biotechnol. Lett.*, 6:55(1984).

42. De Souza, I. J., Bouchard, J., Methot, M., Berry, R. and Argyropoulos, D.S., Carbohydrate in oxygen delignification part I: Changes in cellulose crystallinity, *J. Pulp Pap. Sci.*, 28: 167-170(2002).

43. Dominguez, F.C., Queiroz, J.A., Cabral, J.M. and Fonseca, L.P., The influence of culture conditions on mycelial structure and cellulase production by *Trichoderma reesei* RutC-30, *Enzyme Microbial. Technol.*, 26: 394-40(2000).

44. Doppelbauer, P., Esterbauer, H., Steiner, W., Lafferty, R.M. and Steinmuller, H., The use of lignocellulosic wastes for production of cellulase by *Trichoderma reesei, Appl. Microbiol. Biotechnol.*, 26: 485-494 (1987).

45. Duff, J.B.S., Cooper, D.G. and Fuller, O.M., Evaluation of the hydrolytic potential of crude cellulase from mixed cultivation of *Trichoderma reesei* and *Aspergillus phoenicis, Enzyme Microbial. Technol., 8*(5): 305-308(1986).

46. Eberhart, B.M., Beek, R.S. and Goolsby, K.M., Cellulase of *Neurospora crassa, J. Microbiol.* 130: 181-186(1977).

47. Eriksson, K.E., Regulation of endo-l-4, β-glucanase production in *Sporotrichum pulverulentum, Eur. J. Biochem.*, 90:183-190 (1976).

48. Eriksson, K.E. and Wood, T.M., Biodegradation of cellulose, *In: Biosynthesis and biodegradation of wood components,* Higuchi, T., (ed.), Academic Press New York, (1985) pp.469-503.

49. Eveleigh, D.E., Cellulase: a perspective, Phil. Trans. Royal Society of London, 321:435-437(1987).

50. Fan, L.T., Lee, Y.H. and Beardmore, D.R., The influence of major structural features of cellulose on rate of enzymatic hydrolysis, *Biotechnol. Bioeng.*, 23: 419-424(1981).

51. Fan, L.T., Lee, Y.H. and Gharpuray, M.M., The nature of lignocellulosic and their pretreatment for enzymatic hydrolysis, *Adv. Biochem. Eng.*, 23:157-187(1982).

52. Fengel, D. and Wegener, G., Carboxymethylcellulose, *Wood chemistry Ultrastructure*, de Gruyter, W., (ed.), Berlin, New York (1984) pp.78.

53. Feniksova, R.V., Tikhomrova, A.S. and Rakhleeva, B.E., Conditions for forming amylase and proteinase in surface culture of *Bacillus subtilis*, *Mikrobiologica.*, 29:745-748(1960).

54. Fort, S., Boyer, V., Gree, L., Davies, G., Moroz, O., Christiansen, L., Schúlein, M., Cottaz, S. and Driguez, H., Highly efficient synthesis of β,1-4 Oligo and polysaccharides using mutant cellulase, *J. Am. Chem. Soc.*, 122: 5429-5437(2000).

55. Gabriel, B.L., In: *Biological scanning electron microscopy*, Von Nostrand Reinhold Company, New York, (1982) pp.186.

56. Gama, F.M., Carvalho, M.G., Figueiredo, M.M. and Mota, M., Comparative study of cellulose fragmentation by enzymes and ultrasound, *Enzyme Microbial. Technol.*, 20: 12-17(1997).

57. Ganguly, R. and Mukherjee, S., Effects of different pure and complex carbon and nitrogen sources on production of cellulases by an isolated strain *Penicillium purpurogenum, J. Microbiol. Biotechnol.*, 10: 47-58(1995).

58. Gao, J., Weng, H., Zhu, D., Yuan, M., Guan, F. and Xi, Y., Production and characterization of cellulolytic enzymes from the thermo-acidophilic fungal *Aspergillus terreus* M11 under solid-state cultivation of corn stover, *Biores. Technol.*, 99:7623-7629(2008).

59. Garg, A.P., Sudha, G., Mukerji, K.G. and Pugh, G.J.F., Ecology of keretenophilic fungi, *Proc. Ind. Acad. Sci. (Plant Sci.)*, 94:194-163(1985).

60. Garg, S.K. and Neelkantan, S., Effect of nutritional factors on cellulase enzyme and microbial protein production by *Aspergillus terreus* and its evaluation, *Biotechnol. Bioeng.*, 24:109-125(1982).

61. Ghose, T.K. and Das, K., A simplified kinetic approach to cellulose cellulase system, *In: Adv Biochem Eng.*, Ghose, T.K., Fiechter, A., (eds.), Berlin, Germany, *Springer-Verlag.*, 1:55-77(1971).

62. Gokhale, D.V., Patil, S.G. and Bastawde, K.B., Optimization of cellulase production by *Aspergillus Niger* NCIM 1207, *Applied Biochemistry and Biotechnology*, (1991).

63. Gomes, I., Gomes, J., Gomes, D.J. and Steiner, W., Simultaneous production of high activities of thermostable endoglucanase and β-glucosidase by the wild thermophilic fungus *Thermoascus aurantiacus, Appl. Microbiol. Biotechnol.*, 53:461-468(2000).

64. Grethlein, H.E., Allen, D.C. and Converse A.O., A comparative study of the enzymatic hydrolysis of acid-pretreated white pine and mixed hardwood, *Biotechnol. Bioeng.*, 26:1498-1505(1984).

65. Gretty, K., Villena, Marcel. and Gutiérrez-correa., Production of lignocellulolytic enzymes by *Aspergillus niger* biofilms at variable water activities, *Electronic Journal of Biotechnology.*, 10 (2007).

66. Gunju, R.K., Vithayuthil, P.J and Murthy, S.K., Factors influencing production of cellulases by *Chaetomium themophile Var. Coprophile, Indian. J. Exp. Biol.*, 12:259-264(1990).

67. Halliwel, G. and Griffin, M., The nature and mode of action of the cellulolytic component C1 of *Trichoderma koningii* on native cellulose, *Biochemical. J.*, 135: 587-594(1973).

68. Han, Y.W. and Callihan, C.D., Cellulose fermentation: Effect of substrate pretreatment on microbial growth, *Appl. Microbiol.*, 27:159-165(1974).

69. Haq, I., Hameed, M., Javed, M. and Qadeer, M.A., Biosynthesis of cellulase *Trichoderma harzanium* using shake flask technique, *Indus J. Biol. Sci.*,3:685-692(2006).

70. Hatakka, A.I. and Prithonen, T.I., Cultivation of wood rotting fungi on agricultural lignocellulosic materials for the production of crude protein, *Agri. Wastes.*, 12:81-97(1985).

71. Hayward, T.K., Hamilton, J., Tholudur, A. and McMillan, D., Improvements in titter productivity and yield using solka-floc for cellulase production, *Appl. Biochem. Biotechnol.*, 84-86: 859-887(2000).

72. Hendriks, A.T.W.M. and Zeeman, G., Pretreatments to enhance the digestibility of lignocellulosic biomass, *Biores. Technol.*, 100:10-18(2009).

73. Henrissat, B., Driguez, H., Viet, C. and Schülein, M., Synergism of cellulases from *Trichoderma reesei* in the degradation of cellulose, *Biotechnology*, 3: 722-726(1985).

74. Henrissat, B., A classification of glycosyl hydrolase based on the amino acid sequence similarities, *Biochem. J.*, 280: 305-316(1991).

75. Henrissat, B. and Bairoch, A., Updating the sequence-based classification of glycosyl hydrolases, *Biochem. J.*, 316:695-696 (1996).

76. Henrissat, B., Teeri, T. and Warren, R., A scheme for designating enzymes that hydrolyse the polysaccharides in the cell walls of plants, *FEBS Letters.*, 425:352-354(1998).

77. Hindeleh, A.M. and Johnson, D.J., Crystallinity and crystallite size measurements in polyamide and polyester fibers, *Polymer*, 19: 27-32(1978).

78. Hölker, U., Höfer, M. and Lenz, J., Biotechnological advantages of laboratory-scale solid-state fermentation with fungi, *Applied Microbiol. Biotechnol,* 64(12): 175-186(2004).

79. Howell, A. and Mangat, M., Enzyme deactivation during cellulose hydrolysis, *Biotechnol. Bioeng.,* 20:847-863(1978).

80. Hurst, P.L., Nielsen, J., Sullivan, P.A. and Shepherd, M.G., Purification and properties of cellulase from *Aspergillus niger, Biochem. J.,* 65: 33-41(1977).

81. Ibrahim, M. and Glaser, W.G., Steam-assisted biomass fractionation, Part III: a quantitative evaluation of the "clean fractionation" concept, *Biores. Technol.,* **70**(2): 181–192(1999).

82. Ishikawa A, Sugiyama J. and Okano, T., Fine structure and tensile properties of ramie fibers in the crystalline form of cellulose I, II and III, *Wood Research,* 81:16-18(1994).

83. Ismail, A.S., Hamdy, N.N. and El-Refai, A.H., Enzymatic saccharification of Egyptian sugarcane bagasse, *Agri. wastes,* 12: 99-109(1985).

84. Jackson, L.S., Heitmann, J. and Joyce, T., Enzymatic modifications of secondary fibre, *Tappi J.,* 76:147-154(1993).

85. Jahromi, M.F., Liang, J.B., Rosfarizan, M., Goh, Y.M., Shokryazdan, P. and Ho, Y.W., Efficiency of rice straw lignocelluloses degradability by *Aspergillus terreus* ATCC 74135 in solid state fermentation, *African Journal of Biotechnology,* 10(21): 4428-4435(2011).

86. Jiebing, Li., Gunnar, H. and Göran, G., Lignin depolymerization/repolymerization and its critical role for delignification of aspen wood by steam explosion, *Biores. Technol.*, 98: 3061–3068(2007).

87. Johansson, M., A comparison between the cellulolytic activity of white and brown rot fungi, The activity on insoluble cellulose, *Physiol. Plant,* 19:709-722(1966).

88. Joshi, M.D., Sidhu, G., Nielsen, J.E., Brayer, G.D., Withers, S.G. and McIntosh, L.P., Dissecting the electrostatic interactions and pH-dependent activity of a family 11 glycosidase, *Biochemistry,* 40:10115-10139(2001).

89. Jurasek, L., Enzymatic hydrolysis of aspen wood, *Dev. Ind. Microbiol.,* 20:177-183 (1978).

90. Kang, S.W., Park, Y.S., Lee, J.S., Hong, S.I. and Kim, S.W., Production of cellulases and hemicellulases by *Aspergillus niger* KK2 from lignocellulosic biomass, *Biores. Technol.*, 91: 153-156(2004).

91. Kumar, R., Microbial production of cellulase for bioconversion of lignocellulosic waste, *Ph.D. thesis*, Indian Institute of Technology Roorkee, Roorkee, (2000).

92. Kasahara, K., Sasaki, H., Donkai, N., Yoshihara, T. and Takagishi, T., Modification of tencel with treatment of ferric sodium tartrate complex solution, effect of treatment condition, *Cellulose,* 8: 23-28(2001).

93. Kim, D.W., Yang, J.H. and Jeong, Y.K., Adsorption of cellulase from *Trichoderma virde* on microcrystalline cellulose, *Appl. Microbiol. Biotechnol.,* 28:148-154 (1988).

94. Kim, S. and Holtzapple, M.T.., Effect on structural feature on digestibility of corn stover, *Biores. Technol.,* 97:583-591(2006).

95. Kleman-Leyer, K., Gilkes, N.R., Miller, R.C. Jr. and Kirk, T.K., Changes in molecular size distribution of insoluble celluloses by the action of recombinant *Cellulomonas fimi* cellulases, *Biochem. J.*, 302:463-469(1994).

96. Klyosov, A.A., Mitevich, D.V. and Sinitsyn, A.P., Role of the activity and adsorption of cellulases in the efficiency of the enzymatic hydrolysis of amorphous and crystalline cellulose, *Biochem.*, 25:540-542 (1986).

97. Klyosov, A.A., Cellulases of 3rd generation, *In: Biochemistry and genetics of cellulose degradation,* Aubert, J.P., Beguin, P. and Millete, J., (eds.,) Academic press, London, (1988) pp 87-99.

98. Koivula, A., Linder, M. and Teeri, T., Structure-function relationships in *Trichoderma* cellulolytic enzymes, *In: Trichoderma* and *Gliocladium*, Enzymes, biological control and commercial applications, Harman, G. and Kubicek, C., (eds.) 2: (1998) pp.3-24.

99. Koshland, D.E., Stereochemistry and mechanism of enzymatic reaction, Reviews of Cambridge Phil Soc., 28: 416-436(1953).

100. Kubicek, C. P., Eveleigh, D.E., Esterbauer, H., Steiner, W. and Kubicek-Pranz, E.M., *Trichoderma* cellulases: Biochemistry, physiology, genetics and applications, *Royal Soc. Chem.,* Cambridge, UK (1990).

101. Kuhad, R.C. and Johri, B.N., Fungal decomposition of paddy straw, light and scanning electron microscopic study, *Indian J. Microbiol.,* 32 (3):255-258 (1992).

102. Lachke, A.H. and Deshpande, M.V., *Sclerotium rofsii,* Status in cellulase research, *FEMS Microbiol. Rev.,* 54: 177-193(1988).

103. Laemmli, U.K., Cleavage of structural proteins during the assembly of the head of bacteriophage T_4, *Nature, 227*: 680-685 (1970).

104. Lee, D., Yu, A.H.C, Wong, K.K.Y. and Saddler, J.N., Evaluation of the enzymatic susceptibility of cellulosic substrates using specific hydrolysis rates and enzyme adsorption, *Appl. Biochem. Biotechnol.*, 45/46: 407-415(1994).

105. Lenz, J., Schurz, J. and Wrentschur, E., The length of the crystalline domains in fibres of regenerated cellulose, determination of the crystallite length of cellulose II by means of wide-angle X-ray diffraction and transmission electron microscopy, *Holzforschung,* 42:117-122(1988).

106. Li, J., Henriksson, G. and Gellerstedt, G., Lignin depolymerization/repolymerization and its critical role for delignification of aspen wood by steam explosion, *Biores.Technol.,* 98: 3061–3068(2007).

107. Linder, M. and Teeri, T., The roles and function of cellulose-binding domains, *J. Biotechnol.*, 57:15-28(1997).

108. Lipinsky, E.S., Perspective on preparation of cellulose for hydrolysis. In: *Hydrolysis of cellulose mechanisms of enzymatic and acid catalysis*, Brow, R.D. and Jurasek, C., (Eds.), *Adv. Chem. Ser.,* 181:1-23 (1979).

109. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J., Protein measurement with Folin Phenol reagents, *J. Biol. Chem.,* 193:265-275(1951).

110. Macris, B.J., Kekos, D., Evangelidou, X., Galiotou-Panayotou, M. and Sodis, P., Solid state fermentation of straw with *Neurospora crassa* for CMCase and β-glucosidase production, *Biotechnology Letters,* 9(9):661-664 (1987).

111. Macris, B.J., Kekos, D. and Evangelidou, E., A simple and inexpensive method for cellulase and β-glucosidase production by *Neurospora crassa, Appl. Microbial. Biotechnol.,* 31:150-151(1989).

112. Maheshwari, R., Bharadwaj, G. and Bhat, M., Thermophilic fungi: their physiology and enzymes, *Microbiol. Mol. Biol. Rev.*, 64:461-488 (2000).

113. Mandels, M. and Reese, T.M., Induction of cellulase in fungi by cellobiose, *J. Bacteriol.,* 78:816-826(1960).

114. Mandels, M. and Weber, J., The Production of cellulases, *Advances in Chemistry.,* 95:391-414 (1969).

115. Mandels, M., Microbial sources of cellulases, *Biotech. Bioengg. Symp.,* 5: 81-105(1975).

116. Mansfield, S.D., Wong, K.K.Y., de Jong, E. and Saddler, J.N, Modification of Douglas-fir mechanical and Kraft pulps by enzyme treatment, *Tappi J.*, 79:125-132(1996).

117. Mansfield, S.D., de Jong, E., Stephens, R.S. and Saddler, J.N., Physical characterization of enzymatically modified kraft pulp fibres, *J. Biotechnol.*, 57: 205-216(1997).

118. Mansfield, S.D., Saddler, J.N. and Gübitz, G.M., Characterization of endoglucanases from the brown rot fungi *Gloeophyllum sepiarium* and *Gloeophyllum trabeum, Enzyme Microbial. Technol.*, 23: 133-140(1998).

119. Mansfield, S.D., Mooney, C. and Saddler, J.N., Substrate and enzyme characteristics that limit cellulose hydrolysis, *Biotechnol. Prog.* 15: 804-816(1999).

120. Mayer, C., Zechel, D.L., Reid, S.P., Warren, R.A.J. and Withers, S.G., The E358S mutant of *Agro bacterium sp.* glucosidase is a greatly improved glycosynthase, *FEBS Lett.* 466:40-44(2000).

121. McCarthy, A.J., Lignocellulose degrading actinomycetes, *FEMS Microbiol., Rev.* 104, 145-163 (1987).

122. Meinke, A., Damude, H.G, Tomme, P., Kwan, E., Kilburn, D.G., Miller, R.C., Warren, R.A.J. and Gilkes, N.R., *J. Biol. Chem.*, 270: 4383-4386(1995).

123. Meldgaard, M. and Svendsen, I.B., Different effects of N-glycosylation on the thermo stability of highly homologous bacterial (1, 3-1, 4)-β-glucanases secreted from yeast, *Microbiol.*, 140:159-166 (1994).

124. Menon, K., Rao, K.K. and Pushalkar, S., Production of β-glucosidase by *Penicillium rubrum* O stall, *Indian. J. Exp. Biol.*, 32: 705-709(1994).

125. Miller, G.L., Use of dinitrosalicylic acid reagent for determination of reducing sugar, *Anal. Chem.*, 31:426-428(1959).

126. Mooney, C.A., Mansfield, S.D., Touhy, M.G. and Saddler, J.N., The effect of initial pore volume and lignin content on the enzymatic hydrolysis of softwoods, *Biores. Technol.*, 64:113-119(1998).

127. Moore-Landecker, E., In: Fundamentals of fungi, *Prenitice Hall, Englewood cliffs*, 3rd edition, New Jersey (1990) pp.296-298.

128. Morpeth, F.F., Some properties of cellobiose oxidase from white rot fungus *Sporotrichum Pulverulentum, Biochem. J.*, 228: 557-564(1985).

129. Mo, Z., Yang, B. Zhang, H., The degree of crystallinity of multi-component polymers by waxd, *Chinese J. Polym. Sci.*, 12(4):296–301(1994).

130. Mosier, N., Wyman, C., Dale, B., Elander, R., Lee, Y.Y., Holtzapple, M. and Ladisch, M., Features of promising technologies for pretreatment of lignocellulosic biomass, *Biores. Technol.*, 96:673-686(2005).

131. Muniswaran, P.K.A. and Charyulu, N.C.L.N., Solid substrate fermentation of coconut coir pith for cellulase production, *Enzyme Microbial.Technol.*, 16:436-440(1994).

132. Murashima, K., Nishimura, T., Nakamura, Y., Koga, J., Moriya, T., Sumida, N., Yaguchi, T. and Kono, T., Purification and characterization of new endo-1, 4-β-D-glucanases from *Rhizopus oryzae, Enzyme Microbial. Technol.*, 30: 319-326(2002).

133. Nakamura, A., Fukumori, F., Horinouchi, S., Masaki, H., Kudo, T., Uozumi, T., Horikoshi K. and Beppu, T., Construction and characterization of the chimeric enzymes between the *Bacillus subtilis* cellulase and an alkalophilic *Bacillus* cellulase, *J. Biol. Chem.*, 266:1579-1583(1991).

134. Nandakumar, M.P., Thakur, M.S., Raghavarao, K.S.M.S. and Ghildyal, N.P., *Enzyme Microbial. Technol.*, 18:121-125(1996).

135. Narahara, H., Koyama, Y., Yoshida, T., Pichangkura, S., Ueda, R. and Taguchi, H., Growth and enzyme production in a solid state culture of *Aspergillus oryzae, J. Ferment. Technol.*, 60:311-319(1982).

136. Narasimha, G., Sridevi, A., Buddolla, V., Subhosh, C.M., and Rajsekhar, R.B., Nutrient effect on production of cellulolytic enzymes by *Aspergillus niger, Afr. J Biotechnol.*, 5: 472-476(2006).

137. Nelson, N. and Oliver, D. W., Study of cellulose structure and its relation to reactivity, *J. Polym. Sci. Part* C., 36:305-320(1971).

138. Nidetzky, B. and Claeyssens, M., Specific quantification of *Trichoderma reesei* cellulases in reconstituted mixtures and its applications to cellulase-cellulose binding studies, *Biotechnol. Bioeng.*, 44:961-966(1994).

139. Nierstrasz, V. and Warmoeskerken, M., In: *Process engineering and industrial enzyme applications*, Cavaco-Paulo, A. and Gübitz, G. (Eds.), Textile processing with enzymes, Wood head publishing Ltd., England (2003) pp.120-157.

140. Ojumu, T.V., Solomon, B.O., Betiku, E., Layokun, S.K., and Amigun, B., Cellulase production by *Aspergillus flavus* Linn isolate NSPR 101fermented in sawdust, bagasse and corncob, *Afr. J. Biotechnol.*, 2(6):150-152(2003).

141. Okada, G., Purification and properties of cellulase from *Aspergillus niger, Agric. Biol. Chem.*, 49: 1257-1265(1985).

142. O'Sullivan, A., Cellulose: the structure slowly unravels, *Cellulose,* 4:173.207(1997).

143. Otzen, D.E., Christiansen, L. and Schülein, M., A comparative study of the unfolding of endoglucanase cel45 from *Humicola insolens* in denaturant and surfactant, *Protein Sci.* 8:1878-1887(1999).

144. Paul, J. and Varma, A., Influence of sugars on endoglucanase and β-xylanase activities of a *Bacillus* strain, *Biotech. Lett.*, 22: 61-64 (1990).

145. Pere, J., Siika-aho, M., Buchert, J. and Viikari, L., Effects of purified *Trichoderma reesei* cellulases on the fibre properties of kraft pulp, *Tappi J.* 78: 71-78(1995).

146. Pere, J., Ellmen, J., Honkasalo, J., Taipalus, P. and Tienvieri, T., Enhancement of TMP reject refining by enzymatic modification of pulp carbohydrates, A mill study, In: *Biotechnology in the Pulp and Paper Industry*, Viikari, L. & Lantto, R., (Eds.), 8th ICBPPI Meeting, Progress in Biotechnology, *Elsevier Science B*,21:281-290(2002).

147. Pual, L.H., Jan, N., Patrick, A.S. and Maxwell, S., Purification and properties of a cellulase from *Aspergillus niger*, *Biochem. J.*, 165: 33-41 (1977).

148. Puri, V.P. and Mamers, H., Explosive pretreatment of lingo-cellulosic residues with high pressure carbon dioxide for the production of fermentation substrates, *Biotechnol. Bioeng.*, 25: 3149–3162(1983).

149. Puri, V.P. and Pearce, G.R., Alkali explosion pretreatment of straw and bagasse for enzymatic hydrolysis, *Biotech. Bioeng.*, 28:480-485 (1986).

150. Raimbault, M. and Alazard, D., Culture method to study fungal growth in solid fermentation, *Eur. J. Appl. Microbiol. Biotechnol.*, 9:199-209(1980).

151. Rajendran, A., Gunasekaran, P. and Lakshmanan, M., Cellulase activity of *Humicola fuscoatra, Indian J. Microbiol.*, 34:289-295 (1994).

152. Ravindran, C., Naveenan, T. and Varatharajan, G., Optimization of alkaline cellulase production from marine derived fungi, *Chaetomium* sp., using agricultural and industrial wastes as substrates, *Bot. Mar.*, (3): 275-282(2010).

153. Ray, D., Sarkar, B.K, Rana, A.K. and Bose, N.R., The mechanical properties of vinyl ester resin matrix composites reinforced with alkali-treated jute fibres composites part A, *Applied Science and Manufacturing*, 32(1):119(2001).

154. Reidel, K., Ritter, J. and Bronnrnmeier, K., Synergistic interaction of the *Clostridium stercorarium* cellulases Avicelase I (Cel Z) and Avicelase II (CelY) in the degradation of microcrystalline cellulose, *FEMS Microbiol. Lett.*, 147: 239-243(1997).

155. Reinikainen, T., Telman, O. and Teeri, T.T., Effects of pH and high ionic strength on the adsorption and activity of native and mutated cellobiohydrolase I from *Trichoderma reesei, Proteins: Str. Func. Genet.*, 22: 392-403(1995).

156. Reyes, L.M. and Noyola, T.P., Isolation of a hyperxylanolytic *Cellulomonas flavigena* mutant growing on continuous culture on sugarcane bagasse, *Biotechnol. Lett.*, 20:443-446(1998).

157. Robson, L. and Chambliss, G.H., Cellulases of bacterial origin, *Enzyme Microbiol. Technol.*, 11:626-644 (1989).

158. Rosa E.Quiroz-Castañeda., Edgar Balcázar-López., Edgar Dantán-González., Alfredo Martinez., Jorge Folch-Mallol. and Claudia Martínez Anaya., Characterization of cellulolytic activities of *Bjerkandera adusta* and *Pycnoporus sanguineus* on solid wheat straw medium, *Electronic Journal of Biotechnology*, 12 (4): (2009).

159. Ruiz, E., Cara, C., Manzanares, P., Ballesteros, M. and Castro, E., Evaluation of steam explosion pre-treatment for enzymatic hydrolysis of sunflower stalks, *Enzyme Microbial. Technol., 42:* 160-166(2008).

160. Ryu, D.D.Y. and Mandels, M., Cellulases, biosynthesis and applications, *Enzyme microbial. Technol.*, 2: 91-102(1980).

161. Samson, R.A., Noonim, P., Meijer, M., Houbraken, J., Frisvad, J.C. and Varga, J., Diagnostic tools to identify black *Aspergilli, Studies in Mycology*, 59: 129-145(2007).

162. Sastry, M., Entrapment of proteins and DNA in thermally evaporated lipid films, *Trends in Biotechnol.*, 20:185-188(2002).

163. Sawada, T., Characterization of cellulose treated by the steam explosion method part 2: Effect of treatment conditions on changes in morphology, degree of polymerization, solubility in aqueous sodium hydroxide and super molecular structure of softwood pulp during steam explosion, *J. Brit. Polym.*, 22:121–128**(1990)**.

164. Scandurra, R., Consalvi, V., Chiaraluce, R., Politi, L. and Engel, P.C., Protein thermo-stability in extremophiles, *Biochimie.*, 80:933-941(1998).

165. Schülein, M., Enzymatic properties of cellulases from *Humicola insolens, J. Biotechnol.*, 57:71-81(1997).

166. Schülein, M., Protein engineering of cellulases, *Biochim. Biophys. Acta.*, 1543:239-252(2000).

167. Schwarz, W.H., Jausis, S. and Kouba, M., Cloning and expression of *Closlridium stercorarium* cellulase genes in *Escherichia coli, Biotechnol. Lett.*, 11:461-466(1989).

168. Segal, L., Creely, J.J., Martin, A.E. and Conrad, C.M., An empirical method for estimating the degree of crystallinity of native cellulose using X-ray diffractometer, *Textiles Res J.*, 29:786-793(1959).

169. Shamala, T.R. and Srikantaiah, K.R., Production of cellulases and D-xylanase by some selected fungal isolates, *Enzyme Microbial. Technol.*, 8:78-182(1986).

170. Showkat Ali, M., Akhand, A.A, Gomez, P.F. and Sarker, R.I., Cellulase from *Humicola sp, Journal of basic microbiology*, 33(3):155-159(2007).

171. Shiang, M., Linden, J.C., Mohagheghi, A., Grohmam, K. and Himmel, M.E., Regulation of cellulase synthesis in *Acidolhennus cellolyticus, Biotechnol. Prog.,* 7: 315-322(1991).

172. Singh, A., Abidi, A.B., Agarwal, A.K., Darmwal, N.S. and Srivastawa, S., Utilization of cellulosic substrate for the production of single cell protein and cellulase enzyme, *Indian J. Bio. Res.,* 6:1-6 (1988).

173. Singh, A., Abidi, A.B., Darmwal, N.S. and Agrawal, A.K., Production of protein and cellulase by *Aspergillus niger* ASI01 in solid state culture, *MIRCEN Journal.,* 5:451-456 (1989).

174. Singh, A., Abidi, A.B., Darmwal, A.S. and Agrawal, A.K., Saccharification of cellulosic substrates by *Aspergillus niger* cellulase, *World J. Microbiol. Biotechnol., 6:333-*336 (1990).

175. Singh, A., Abidi, A.B., Darmwal, N.S. and Agrawal, A.K., Influence of nutritional factors on cellulase production from natural cellulosic residues by *Aspergillus niger* AS101, *Agri. Biol. Res.,* 7: 19-27 (1991).

176. Singh, A., Singh, N. and Bishnoi, N., Enzymatic hydrolysis of chemically pretreated rice straw by two indigenous fungal strains: a comparative study, *Journal of scientific & Industrial Research,* 69:232-237(2010).

177. Sjöström, E., In: *Wood chemistry fundamentals and applications,* Academic Press New York (1981) pp.49.

178. Sprey, H. and Bochem, P., Formation of cross-fractures in cellulose microfibril structure by an endoglucanase-cellobiohydrolase complex from *Trichoderma reesei, FEMS Microbiol. Lett.,* 106:239-244(1991).

179. Srivastava, S.K., Gopalkrishnan, K.S and Ramachandran, K.B., The production of β-glucosidase in shake-flasks by *Aspergillus wentii, J. Ferment. Technol.,* 65(1): 95-99(1987).

180. Steiner, J., Saccha, C. and Enzyaguirre, J., Culture conditions for enhanced cellulase production by a native strain of *Penicillium purpurogenum, World. J. Microbiol. Biotechnol.,* 10:280-284 (1993).

181. Sternberg, D., β-glucosidase from *Trichoderma*: its biosynthesis and role in saccharification of cellulose, *Appl. Environ. Microbiol.,* 31:648-654(1976).

182. Stork, G., Pereira, H., Wood, T., Dusterhoft, E., Toft, A. and Puls, J., Upgrading recycled pulps using enzymatic treatment, *Tappi J.,* 78:79-88(1995).

183. Stutzenberger, F., Regulation of cellulolytic activity, *Annv. Rep. Ferment. Processes*, 8:111-154(1985).

184. Sukan, S., Guray, S., Ayse, V.S. and Fazilet., Effects of natural oils and surfactants on cellulase production and activity, *J. Chem. Technol. Biotechnol.*, 46:179-87(1989).

185. Sun, Y. and Cheng, J., Hydrolysis of lignocellulosic materials for ethanol production: a review, *Biores. Technol.*, 83:1-11(2002).

186. Sulzenbacher, G., Schülein, M. and Davies, G.J., Structure of Endoglucanase I from *Fusarium oxysporum*: native cellobiose and 3-4 epoxy butyl β-D cellobioside-inhibited forms with 2.3 Å resolution, *Biochemistry,* 36: 5902-5911(1997).

187. Suurnäkki, A., Niku-Paavola, M-L., Buchert, J. and Viikari, L., Enzymes in pulp and paper processing, In: *Enzymes in industry,* Aehle, W., (ed.), Weinheim, Wiley-VCH, 437-439(2004)

188. Takashi, Y., Yasushi, M. and Yoshiyuki, T., Isolation of a cellulolytic enzyme producing microorganism, culture conditions and some properties of the enzymes, *Agric. Biol. Chem.*, 51(1): 65-74(1987).

189. Tanahashi, M., Takada, S., Aoki, T., Goto, T., Higuchi, T. and Hanai, S., Characterization of explosion wood I. Structure and physical properties, *Wood Res.*, 69: 36–51(**1982**).

190. Teather, R.M. and Wood, P.J., Use of Congo red polysaccharide interactions in and enumeration characterization of cellulolytic bacteria from bovine rumen, *Appl. Environ. Microbiol.*, 43:777-780(1982).

191. Teeri, T.T., Reinikainen, T., Ruohonen, L. and Jones, T.A., Domain function in *Trichoderma reesei* cellobiohydrolases, *J. Biotechnol.*, 21:169-176(1992).

192. Teeri, T.T., Crystalline cellulose degradation: New insight into the function of cellobiohydrolases, *TIBTECH,* 15:160-167(1997).

193. Tengerdy, R.P., Cellulase production by solid substrate fermentation, *J. Sci. Ind. Res.*, 55:313-316(1996).

194. Teunissen, M.J., Lahaye, D.H.T.P., Veld, J.H.J.H and Vogels, G.D., Purification and characterization of an extracellular β,1-4-glucosidase from the anaerobic fungus *Piromyces sp. Strain E2, Arch. Microbiol.*, 158:(1992).

195. Tomme, P., Van Tilbeurgh, H., Pettersson, G., Van Damme, J., Vandekerckhove, J., Knowles, J., Teeri, T.T. and Claeyssens, M., Studies of the cellulolytic system of *Trichoderma reesei* QM 9414, Analysis of domain function in cellobiohydrolases by limited proteolysis, *Eur. J. Biochem.*, 170: 575-581(1988).

196. Toyama, N. and Ogowa, K., In: *International course on Biochemical Engineering and Bioconversion,* Ghose, T.K., (Ed.), Biochem. Eng. Research Centre, IIT, New Delhi, India (1977).

197. Tuncer, M., Ball, A.S., Rob, A. and Wilson, M.T., Optimization of extracellular lignocellulolytic enzyme production by a thermophilic actinomycete *Thermonospora fusca* BD 25, *Enzyme Microbial. Technol., 25*:38-47(1999).

198. Usama, F.A. and Hala, S. Saad El-Dein., Production and partial purification of cellulase complex by *Aspergillus niger* and *A. nidulans* grown on water hyacinth blend, *Journal of Applied Sciences Research,* 4(7): 875-891(2008).

199. Vehviläinen, M., Nousiainen, P., Struszczyk, H., Ciechanska, D., Wawro, D. and East, G., Celsol ., Biotransformation of cellulose for fibre spinning, In: *The chemistry and processing of wood and plant fibrous materials,* Kennedy, J.F., Phillips, G.O. and Williams, P.A. (Eds.), Wood head Publishing Limited, Cambridge (1996) pp.197-204.

200. Vignon, M.R., Dupeyre, D. and Garcia-Jaldon, C., Morphological characterization of steam-exploded hemp fibers and their utilization in polypropylene-based composites, *Biores.Technol,* 20(2):203-215(1996.).

201. Vyas, S., Sainkar, S.R. and Lachke, A., Fabrication of flotation cell device for enzymatic deinking of waste paper, *Indian J. Chem. Technol.*, 10: 593-597(2003).

202. Waki, T., Suga, K. and Ichikowa, K., Production of cellulase in fed-batch culture, In: Proceeding II international symposium on bioconversion and fed back control on cellulase production, *Biochemicals engineering, BERC,* IIT Delhi India, 359-364(1976).

203. Walker, J.M., SDS-Polyacarylamide gel electrophoresis of proteins, In: *The protein protocols handbook,* Walker, J.M., (Ed.), Humana *Press Inc.999,* Totowa, New Jersey (1996) pp.55-61.

204. Wang, K., Wang, F., Jiang, J.X., Zhu, L.W and Fan, H.Z., Structure, composition and enzymatic hydrolysis of steam-exploded Lespedeza stalks, *For. Stud. China.,* 9(2):137-41(2007).

205. Weil, J., Westgate, P., Kohlman, K. and Ladish, M.R., Cellulose pretreatment of lignocellulosic substrates, Enzyme *Microbial. Technol.,* 16: 1002-1004(1994).

206. Westermark, U. and Eriksson, K.E., Celllobiose: Quinone oxido reductase- a new wood-degrading enzymes from white rot fungi, *Acta Chem. Scand.,* 28:209-214(1974).

207. Wheals, A.E., Basso, L.C., Denise, M.G. and Amorim, H.V., Fuel ethanol after 25 years, *Trends in Biotechnol.*, 17: 482-487(1999).

208. Wilson, D.B., Spezio, M., Irwin, D., Karplus, A. and Taylor, J., Comparison of enzymes catalyzing the hydrolysis of insoluble polysaccharides, In: *Enzymatic degradation of insoluble carbohydrates,* Saddler, J.N., Penner, M., (eds.), *American Chemical Society,* Washington, D.C, USA (1995).

209. Wood, T.M, McCrae, S.I. and Mac Farlane, C.C., The isolation purification and properties of the cellobiohydrolase component of *Penicillium pinophillum* cellulase, *Biochem. J.,* 189:51-65(1980).

210. Wood, W.E., Neubauer, D.G. and Stutzenberger, F., *Biotechnol. Bioengg,* 160:1017-1054 (1984), In: *Cellulases of bacterial origin,* Robson, L.M. and Chambliss, G.H., (eds.), *Enzyme Microbial. Technol.,* 11:626-644 (1989).

211. Wong, K.K.Y., Deverell, K.F., Mackie, K.L., Clark, T.A. and Donaldson, L.A., The relationship between fiber porosity and cellulose digestibility in steam-exploded *Pinus radiate, Biotechnol Bioeng.*, 31: 447-456(1988).

212. Yazdi, M.T., Woodward, J.R. and Radford, A., The cellulase complex of *Neurospora crassa:* activity, stability and release, *Journal of General Microbiology,* 136: 1313-1319(1990).

213. Yeoh, H.H, Tan, T.K. and Koh, S.K., Kinetic properties of β-glucosidase from *Aspergillus ornatus,* 25: 25-28 (1986).

214. Zechel, D.L. and Withers, S.G., Glycoside mechanisms: Anatomy of a finely tuned catalyst, *Acc.Chem. Res.,* 33: 11-18(2000).

215. Zhang, S. and Wilson, D.B., Surface residue mutations which change the substrate specificity of *T. fusca* endoglucanase E-2, *J. Biotechnol.,* 57:101-113(1997).

216. Zhang, S., Barr, B.K. and Wilson, D.B., Effect of non-catalytic residue mutations on substrate specificity and ligand binding of *T. Fusca* endocellulase Cel 6A, *Eur. J. Biochem.,* 267:244-252(2000).

217. Zhong, X.W., Qunhui, H.J., Zhao, Y., Xui-xin and Yongzhen, J., Enzymatic hydrolysis of pretreated soyabean straw, *Biomass Bioenergy.,* 31:162-167(2007).

218. Zickler, G.A., Wagermaier, W., Funari, S.S., Burghammer and Paris, O., In situ: X-ray diffraction investigation of thermal decomposition of wood cellulose, *J. Analytical Applied pyrolysis,* 80(1):134-140(2007).

219. Zurbriggen, B., Bailey, M.J., Pentilla., M.E., Poutanen, K. and Linko, M., Pilot scale production of a heterologous *Trichoderma reesei* cellulase *Saccharomyces cerevisiae, J. Biotechnol., 13:267*-278 (1990).

CHAPTER 3

STUDIES ON XYLANASE PRODUCTION

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3.1: Introduction

In the last decade, production of xylanase enzymes has attracted the attention of many researchers as these enzymes are essential for the degradation of plant biomass. Xylanases have potential applications in the pulp and paper, food, feed and beverage industries [22, 145,166]. For commercial applications, xylanases should ideally be produced quickly and in large quantities from simple and inexpensive substrates. Natural xylan sources such as agricultural and forestry wastes, paper industry wastes and various fruit wastes are potential raw materials for xylanase production. Among these, food industry wastes are the major sources contain high amount of xylan, as it is one of the main polymer in the plant cell wall.

Lignocelluloses are the most abundant renewable natural material in the biosphere, contributing approximately 50% of the total biomass generated every year (10-50 x 10^9 metric tonnes) and occur mainly as the agricultural, forestry, fruit and vegetable processing wastes. According to Kuhad and Singh [74] lignocellulosic biomass comprises of cellulose (30-55%), hemicellulose (10-39%) and lignin (11-29%). The use of microbial enzymes for the industrial hydrolysis of lignocellulose is advantageous because of the high specificity of enzyme reactions, the mildness of the reaction conditions and the absence of substrate loss due to chemical modifications. The abundance of xylan clearly indicates that xylanolytic enzymes can play an important role in bioconversion. Although xylanases from eubacteria and archaebacteria have considerable higher temperature optima and stability than those of fungi, but the amount of enzyme produced by these bacteria is comparatively lower than that of produced by fungi. In general, the level of xylanase production in fungal culture is typically much higher than those from yeasts or bacteria [131]. Filamentous fungi are particularly interesting producers of xylanases from an industrial point of view, due to the fact that they excrete xylan–degrading enzymes in to the medium thus eliminating the need for cell disruption.

3.1.1: Hemicellulose

Schulze [124] introduced the term hemicellulose for the fractions isolated or extracted from plant materials with dilute alkali. The classification of these hemicellulosic fractions

depends on the types of sugar moieties present. Various microorganisms are actively involved in the degradation of hemicellulose. Hemicellulose are composed of both hexoses and pentoses, mainly D-xylose, D-mannose, D-glucose, D-galactose, 4-O-methyl-D-glucuronic acid and to lesser extent L-rhamnose, L-fucose and various O-methylated sugars. D-xylose and L-arabinose are the major constituents of the pentosans (xylans), while D-glucose, D-galactose and D-mannose are the constituents of the hexosans (mannans) [11, 43, 75]. However, many combinations of these residues in nature with or without branched chains give a multiplicity of different chemical structures. The major hemicellulosic components present in softwood are mannan-based and that in hardwood are xylan-based.

The plant cell wall is a composite material in which cellulose, hemicellulose (mainly xylan), and lignin are closely associated. In plants, xylans or hemicelluloses are situated between the lignin and the cellulosic fibers present underneath. Due to substitutions, xylan appears to be interspersed, intertwined and covalently linked at various points with the overlying lignin layer, while producing a covering around the cellulose via hydrogen bonding [15, 159]. It also provides a physical barrier to the oxidizing chemicals or to enzymatic attack during their usage to strip off the lignin layer.

3.1.2: Structure of xylan

Xylan is the second most abundant polysaccharide on the earth, accounting for approximately one-third of all renewable organic carbon on earth [110]. Xylan, a major structural component of plant cell walls and the most abundant renewable hemicellulose, constitutes 20–40% of total plant biomass. Xylan is found in large quantities in angiosperms (15–30% of the cell wall content) and gymnosperms (7–10%), as well as in annual plants (<30%) [131]. It is typically located in the secondary cell wall of plants but is also found in the primary cell wall, particularly in monocots [165]. Hydrolysis of xylan becomes an important step towards proper utilization of abundantly available lignocellulosic material in nature [15, 74, 75, 108]. The basic structure of xylan consists of a core chain containing 1, 4-linked β-D-xylopyranosyl residues. Xylan is a heteropolysaccharide containing the substituents groups of acetyl, 4-O-methyl-D-glucuronosyl and α-arabinofuranosyl residues linked to backbone of β-1-4-linked xylopyranose units. Lignin is bound to xylans by an ester linkage to 4-O-methyl-D-glucuronic residues. Due to structural heterogeneity of the xylans, xylan degrading enzyme systems include several hydrolytic enzymes.

3.1.3: Enzymatic degradation of xylan

The enzymatic hydrolysis of hemicellulose is brought about by 'hemicellulases' which are well defined and classified according to the substrate on which they act and are collectively grouped as glycan hydrolases (E.C. 3.2.1. **) [11]. Xylanases are fastly growing as a major group of industrial enzymes, finding significant application in the pulp and paper industry. The main enzyme needed to enhance the delignification of pulp is endo-β-1, 4-xylanase but other hemicellulolytic components have been shown to improve the effect of enzymatic treatment. Xylanases by attacking the bonds existing between xylan and lignin, lead to the release of the lignin, which can then diffuse more easily into the bleaching liquor.

3.1.4: Xylanolytic system

Xylanases are widespread in nature and are reported from marine and terrestrial bacteria, fungi, marine algae, protozoa, snails, crustaceans, insects and seeds of terrestrial plants [11, 15, 32, 35,153]. The xylanolytic system represent the repertoire of hydrolytic enzymes mainly composed of endo-β-1,4-xylanases (β-1,4-D-xylanxylanohydrolase; E.C. 3.2.1.8) and β-1,4-xylosidases (β-1,4-D-xyloside xylohydrolase; E.C. 3.2.1.37); along with α-glucuronidase, acetylxylan esterase and phenolic acid esterase that act co-operatively to convert xylan to its constituent sugars as shown in **Figure 3.1** [146].

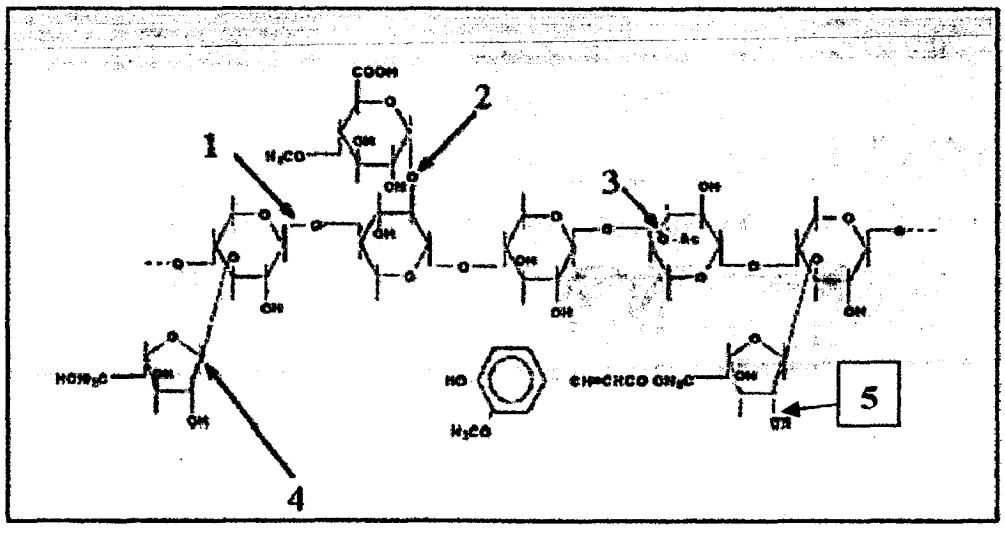


Fig. 3.1: Enzymes involved in xylan degradation :1, Endo-β-1,4-xylanase (EC 3.2.1.8); 2, α-glucuronidase (EC 3.2.1.1);3, Acetylesterase (EC 3.1.1.6); 4, α-L-arabinofuranosidase (EC 3.2.1.55); 5, β-xylosidase (EC 3.2.1.37)

3.1.4.1: Endoxylanase (β-1,4-D-xylanxylanohydrolase; E.C. 3.2.1.8)

The endoxylanases are the most widely studied and characterized hemicellulolytic enzyme. Endoxylanases cleave the internal glycosidic linkages of the heteroxylans backbone resulting in a decreased degree of polymerization of the substrate. Attack on the substrate is not random and the bonds hydrolyzed depend on nature of the substrate or presence of the substituents. The endo-acting xylanases have been differentiated according to the end products released from the hydrolysis of xylan (e.g. xylose, xylobiose and xylotriose and/or arabinose) as non-debranching (arabinose non-liberating) or debranching (arabinose liberating) enzymes [35, 165]. Wong *et al.* [165] suggested that endoxylanases can be grouped into those that are low molecular weight (below 30 kD_a) basic protein with high pI values and high molecular weight (above 30 kD_a) acidic protein with low pI values. However, several exceptions to this pattern have been found [146] and approximately 30% of presently identified xylanases, in particular fungal xylanases, cannot be classified by this system [32]. Endoxylanases have been identified in *Aspergillus* species [51,135], *Bacillus* species [58], *Cellulomonas* NCIM 2353 [28], *Streptomyces* species [14], *Staphylococcus* species SG-13 [58], *Trichoderma reesei* [79] and many other microorganisms.

3.1.4.2: β-D-xylosidase (xylobiase, β-1, 4-D-xyloside xylohydrolase, EC 3.2.1.37)

β-D-xylosidases are exoglycosidases that hydrolyze smaller xylo oligosaccharides and xylobiose from the non-reducing ends to liberate monomeric xylose [165]. Molecular weight lies between 60 to 360 kD_a and may be monomeric or dimeric proteins. β-xylosidase appears to be mainly cell associated in bacteria and yeast but is extra-cellular in moulds [18]. Among the xylo-oligomers, xylobiose is usually the best substrate. β-xylosidase have been reported in bacteria and fungi that include, *Bacillus thermantarcticus* [78], *Melanocarpus albomyces* IIS 68[122], *Trichoderma koningii* G-39 [82], *Aspergillus phoenicis* [118].

3.1.4.3: α-L-Arabinofuranosidase (EC 3.2.1.55)

Arabinosidases (EC 3.2.1.55) are exo-acting glycoside hydrolases which generally catalyze the hydrolysis of α-L-arabinofuranosidic moieties or α(1→2) or/and α-(1→3) bonds linking a L-arabinofuranose to a D-xylose in polymers such as arabinoxylan respectively. Arabinosidases belongs to family 51 of the glycoside hydrolases [115].

3.1.4.4: α-D-Glucuronidase (EC 3.2.1.131)

α-glucuronidases (EC 3.2.1.131) are enzymes, which are able to hydrolyze the α-1, 2-linkage between 4-*O*-methylglucuronic acid and xylose. α-glucuronidase activity has been detected in the culture filtrates of both fungi and bacteria [21, 40]. However, most of organisms secrete only low levels of α-glucuronidases. The enzymes of *P. chrysosporium*, *Thermoanaerobacterium* sp. and *Aspergillus tubingensis* are also reported to exhibit low activity towards polymeric xylan [39].

3.1.4.5: Acetyl xylan esterase (EC 3.2.1.72)

Acetyl xylan esterases are responsible for removal of acetate residues from the main chain of xylan, which is a complex heteropolysaccharide and requires the concerted action of a number of glycanases and esterases [29]. Acetyl xylan esterases mainly present in *Trichoderma reesei*, *Aspergillus niger* and *Schizophyllum commune*. Acetyl xylan esterases can preferably be used in processes wherein xylan has to be degraded. As a consequence of the deacetylate reaction, xylan becomes better accessible for xylanases.

3.1.4.6: Ferulic acid esterase (EC 3.1.1.73)

Feruloyl esterases or ferulic acid esterase (FAE, E.C.3.1.1.73) responsible for cleaving the ester-linkage between the main chain of xylan and monomeric or dimeric ferulic acid have been purified and partially characterized [156, 157,164]. These enzymes act synergistically with xylanases to hydrolyze ester-linked ferulic acid (FA) from cell wall material [156, 157]. Reports have suggested that microorganisms, such as *Aspergillus*, *Penicillium*, *Fusarium* and *Talaromyces*, produce several types of feruloyl esterases that differ in affinity for 5-O- and 2-O-feruloylated α-L-arabinofuranosyl residues [156, 157, 163, 164]. Carbohydrate esters of ferulic acid can also be involved in ether linkages with lignin components providing thus the connection between lignin and hemicellulose. An understanding of the co-production of xylanases and esterases by microorganisms and synergistic interactions between the enzymes is of applied interest to pulp and paper industry [114] and food industry [87]

3.1.5: Sources of xylanase

Xylanases occur in both prokaryotes and eukaryotes [35]. Intracellular and extracellular xylanases from various bacterial and fungal sources have been studied extensively. Intracellular xylanases occur in rumen bacteria and protozoa [35]. Xylanase and β-xylosidase are widely

distributed in nature. These enzymes are secreted by cells into the surrounding medium [17] but some xylanases are cell bound [35]. In almost, all bacteria and yeast, β-xylosidase is cell associated [56], however, in some fungi; it is secreted in the medium (extracellular xylanase) [166]. Filamentous fungi on the other hand are particularly useful producers since they secrete the enzyme into the medium and their enzyme levels are much higher than those of yeast and bacteria.

3.1.5.1: Xylanase from fungal origin

Most important xylanolytic enzyme producers include certain fungal strains like *Aspergillus awamori, A. phoenicis, A. niger, A. fumigatus, A. foetidus,* Basidiomyces species *Coprinellus disseminatus* SW-1 NTCC 1165, *Sporotrichum pulverulentum, Trichoderma reesei, T. harzianum* and *T. viride Fusarium oxysporum, Neurospora crassa, Penicillium janthinellum, P. wortmanni, P. capsulatum,* C. di [3, 18, 127, 136, 165]. The white-rot fungus *P. chrysosporium* has been shown to produce multiple endoxylanases [70]. Thermophilic fungi include *Humicola lanuginosa, Thermoascus aurantiacus, Talaromyces byssochlamydoides* and *Sporotrichium thermophile* [138].

3.1.5.2: Xylanase from bacterial origin

Among aerobic or facultative anaerobic bactria, xylanolytic activity has been reported in *Bacillus subtilis, B. circulans, B. pumilus, B. polymyxa* and *B. coagulans* [161,165, 170]. *Streptomyces* species includes *Streptomyces exfoliates, S. flavogriseus, S. lividans, S. xylophagus* and *S. halstedii* JM8 [41, 165]. Strictly anaerobic, fermenting microbes, which grow under mesophilic conditions, have also been reported, such as *Clostridium acetobutylicum, Clostridium stercorarium* and *Clostridium papyrosolvens* C7 [98,165]. The gram negative, aerobic, non-spore forming soil microbe, *Pseudomonas fluorescens* subspecies *cellulosa* has also been shown to degrade xylans [159]. Thermophilic bacterial strains include *Bacillus stearothermophilus, Clostridium thermocellum, Clostridium thermohydrosulfuricum* and *Clostridium thermosaccharolyticum* [18]. Hyperthermophilic bacteria include *Caldocellum saccharolyticum, Dictyoglomus* species, *Thermotoga maritima Fj*SB8, *Rhodothermus marinus* and *Thermotoga* sp strain FjSS3-B.1 [86, 144]. Among the actinomycetes, *Streptomyces raseiscleroticus, Saccharomonospora viridis,* thermotolerant *Streptomyces* sp. T7 and *Nocardiopsis dassonvillei,* were reported to secrete xylanase [141]. *Trichosporon beigelii* [143] and *Aurobasidium pullulans* [80] have also been reported to produce extra-cellular endoxylanase.

3.1.6: Heterogeneity of xylanases

A greater extent of variation is observed among xylanases, which although perform similar function but differ in their physiochemical properties, such as molecular weight, isoelectric point, etc. The varying xylosidic linkages in lignocelluloses are not equally accessible to xylanolytic enzymes. Moreover, there are changes in the accessibility of some of the linkages during hydrolysis. Different hemicellulolytic microorganisms produce varying levels of the xylanolytic enzymes, many of which have different degrees of specificity. For instance, the zymogram analysis has detected various xylanases in the culture filtrate of *Aspergillus niger* [18] and *Butyrivibrio fibrisolvens* H17c [84]. However, the detection of multiple bands on zymogram may be due to post-translational modification such as glycosylation or proteolysis or both leading to a greater degree of heterogeneity of these enzymes. The detection of minor bands with xylanolytic activity may be due to distinct growth and/or purification conditions, or these components may be involved in specialized functions such as the hydrolysis of linkages, which are not found frequently [165].

The importance of xylanases is not bound to the paper industry alone but these are equally employed for other industries as well **(Table 3.1).** A considerable amount of worldwide research has focused on the search for novel microbial isolates having the ability to produce thermotolerant and alkalophilic xylanases [97, 130 , 132,152], mainly because of their use in the pulp and paper industry [15,145]. Some of the thermophilic fungi, *Chaetomium thermophile, Humicola insolens, Thermomyces lanuginosus* and *Thermoascus aurantiacus* have been reported to produce biotechnologically important, thermostable xylanases [54]. The temperature optima of most xylanases ranges from 55-65 ^{0}C. Xylanases of some strains of *T. aurantiacus* and *T. lanuginosus* are optimally active at 70 to 80 ^{0}C [88]. The purified xylanase produced from *T. lanuginosus* CBS 288.54 exhibits its optimal activity at 70-75 ^{0}C. It is stable up to 65 ^{0}C for 30 min and retains more than 50% of its activity after heating at 85 ^{0}C for 30 min [83]. The optimum temperature for xylanase production by *Penicillium oxalicum* under SSF is 45 ^{0}C [94] which is similar to *A. fumigates* and *A. nidulans* [26]. A very thermostable xylanase described is that of an extremely thermophilic *Thermotoga* sp. with a half life of 20 min at 105 ^{0}C [20]. It has been reported that *Aspergillus sydowii* MG 49 produces two xylanases with optimal activity at 60 ^{0}C and a stability in the range of 40 to 70 ^{0}C. The enzyme activity is found declining sharply around temperature at 70 ^{0}C [55].

The majority of xylanases have pH optima ranging from 4.5 to 6.5 [88]. The optimum pH for xylan hydrolysis is around 5 for most fungal xylanases, and they are normally stable between pH values of 2 to 9. The pH optima of bacterial xylanases are generally slightly higher than that of the fungal xylanases [22]. The pH stability of the xylanase produced by *Thermomyces lanuginosus* strains DSM 5826 and ATCC 46882 is in the pH range of 5.0 to 9.0 [16]. The xylanase produced by *Bacillus* sp. Sam-3 is found to be most stable at pH 8, retaining 80% of its activity. At pH 9 and 6 the enzyme retaines 57 and 52% of its activity [134]. The crude xylanase obtained from *Aspergillus carneus* M34 is stable in the pH range of 3-10 (>70% relative activity) [45] while the partially purified xylanase from *A. nidulans* KK-99 is optimally active at pH 8.0 and is stable over a broad pH range (4.0-9.5) at 55 ^{0}C [150].

The use of crude xylanase in pulp processing should be cost effective, hence purification of xylanase is not necessary in pulp processing [6]. Xylanases have been studied using a wide variety of carbon sources including pure xylan [153] The use of purified xylan is uneconomical for industrial use while agricultural residues such as sawdust [169], corn cob [16, 57], wheat bran [168] and sugarcane bagasse [27, 109] offer cost effective alternatives for xylanase production.

Solid-state fermentation (SSF) which is closer to natural system is an attractive alternative process to produce microbial enzymes and metabolites due to its lower capital investment and lower operating cost [66,72, 103,]. SSF is generally defined as the growth of microorganisms on solid materials in the absence or nearly in the absence of free water. The substrate however must contain enough moisture, which exists in the absorbed form within the solid matrix [30]. SSF holds tremendous potential for the production of xylanases [154] and can be of special interest in those processes where the crude fermented product may be used directly as enzyme source [103]. SSF is considered as the most appropriate method for filamentous fungi [126,140] and actinomycetes [99] cultivation and lignocellulosic enzyme production. As they grow under conditions close to their natural habitats i.e. moist substrates with less moisture content due to which they may be more capable of producing certain enzymes and metabolites, which usually will not be produced or will be produced only at low yield in submerged cultures [102]. Bacteria need high moisture content to grow [8] and there are only fewer reports related to successful utilization of bacteria for SSF [8, 161]. SSF has been evaluated successfully using *Trichoderma reesei* [155], *T. harzianum* [117], *Fusarium oxysporum* [101], *Streptomyces* sp.

QG-11-3 [14], *Aspergillus oryzae* [149], *Thermomyces lanuginosus* [30] and *T. aurantiacus* [92] for xylanase production. The use of SSF as a production method of xylanase can offer some apparent economic and engineering advantages over the classical submerged fermentation (SmF). These include high concentration of the product (greater yields), simple fermentation equipments as well as low effluent generation and low requirements for aeration and agitation (energy saving) during enzyme production [92,103,104,].

Xylanases have been isolated from basidiomycetes [64,73], but relatively little is known about patterns of proteins of white and brown-rot fungi. Brown-rot fungi depolymerize cellulose and degrade all polysaccharides early in the decay process [19], hemicellulose being apparently degraded prior to cellulose [60]. White-rot fungi, in contrast, show different patterns of cell wall attack, depending on their species [100]. Like most fungi, white-rot fungi exist primarily as branching threads termed hyphae, usually 1 to 2 μm in diameter, which grow from the tips [3], originating from spores or from nearly colonies, hyphae rapidly invade wood cells and lie along the lumen walls. From that, they secrete the battery of enzymes and metabolites that bring about the depolymerization of the hemicelluloses and cellulose and fragmentation of lignin. The white-rot fungi exhibit two gross patterns of decay [1]: a simultaneous decay, in which the cellulose, hemicellulose, and lignin are removed more or less simultaneously and [2] delignification, in which the lignin and hemicelluloses are removed ahead of the cellulose [167].

Nature has plentiful variety of micro-flora and fauna with various vital functions. A stable microbe having distinctive production ability of the compound of interest is a precondition for any triumphant fermentation process. Screening and selection of potent microbial strain producing the compound of interest is a critical and exhaustive step for accomplishing the production of a particular compound. A common practice among the researchers is to look for and select the microorganism, which secretes the required enzymatic component for a specific process. Many workers have employed the stepwise screening approaches for selecting the xylanase producing microorganism. The xylanase production is thought to be carried out by various bacterial and fungal organisms but fungal system is found to be effective due to multiple enzyme system, which enhances the degradation of the hemicelluloses [68].

Present study aims at isolating, screening and identifying the white-rot basidiomycetes which are capable of producing the extracellular xylanases. The optimization of various

operating physico-chemical parameters is done to achieve the higher xylanase activity from the screened strains. The xylanase was biochemically characterized to check its temperature and pH stability for its successful utilization in enzymatic deinking and biobleaching experiments.

3.2: Experimental methodology

3.2.1: Materials

Birchwood xylan and ABTS (2, 2'-azino-bis-3-ethylbenz-thiazoline-6-sulphonic acid) were purchased from Sigma Chemical Company (USA); D-glucose and D-xylose (AR grade) from Qualigens Chemicals (India); di nitro salicylic acid and bovine serum albumin (AR grade) from Loba Chemie (India); agar-agar from High Media Chemicals (USA); standard protein markers used for electrophoresis from Bangalore genei (India) and wheat bran, rice bran and soybean meal from the local market of Roorkee, U.P. (India). All other chemicals were of analytical grade and purchased from standard commercial manufacturers.

3.2.2: Strain isolation

Different fungal strains were isolated from lignocellulosic wastes (dead and decaying woods), decomposing manure, sugarcane dumping site, fruiting body and paper industry waste by enrichment technique. Samples were collected from different sites at Department of Paper Technology, I.I.T. Roorkee, Saharanpur campus (U.P), Star Paper Mills Ltd. Saharanpur, local sugar units, Haridwar, Dehradun and main campus of I.I.T. Roorkee (U.K) located in the foothills of Shivalik hills in Northern India. Isolation of microorganism using dead, decaying woods and decomposing waste buried in the moist wheat bran in Petri dishes and incubated at 37 ^{0}C. The plates were observed for the appearance of fungal growth and those exhibiting fruit bodies (indicative of growth of basidiomycetes) were isolated from these plates. The moisture level was carefully controlled with sterile tap water, so as to provide a solid substrate for fungal growth, with no free water available. A number of fungal colonies with different morphological features appeared in different Petri dishes. These plates were checked for fungal growth after 2-3 days and were further purified by sub-culturing. Purified cultures were transferred to potato dextrose agar (PDA) slants, incubated at 37 ^{0}C for 5 days and further stored at 4 ^{0}C for future usage. The cultures were maintained as a suspension of spores and hyphal fragments in 15% (v/v) sterile glycerol at -20°C for long term preservation.

3.2.3: Screening of isolates for potent xylanase producing strains

A set of highly discriminatory procedures were used to select the fungal strains which were found the best among the isolates in terms of higher xylanase activity. The basidiomycetous isolates among the prevalent fungi recovered in the present work were screened for their abilities to produce extracellular xylanases during growth on xylan-agar medium. Primary screening of all the 10 isolated strains for xylanase production was carried out on xylan agar plates as described [151]. The xylan agar medium contained 1% xylan and 2% of agar dissolved in 1 L of double distilled water and autoclaved at 15 psi for 15 min. The crude enzyme extract (50 μL), obtained by SSF of each fungal isolates, was placed separately into 2-3 mm diameter well cut into the solidified medium in each Petri dish and the plates were incubated at 30 ^{0}C for 48 h. The plates were then stained with Congo red solution composed of 0.5% (w/v) Congo red and 5% (v/v) ethanol in distilled water for 15 min and destained with 1 M NaCl. The xylanase producing micro-organisms were selected by observing yellow zones around the colonies against the red background. Enzymatic hydrolysis of the surrounding xylan resulted into clear zones in the medium. Controls with heat killed (140 ^{0}C, 20min) supernatant did not produce any clear zones. The strains were primarily screened for their xylanase production ability on the basis of clear zone diameter obtained on the xylan-agar (XA) plate. The isolates exhibiting areas of clear zones when grown on xylan-agar medium were further subjected to SSF conditions to determine their actual xylanase activities. The enzymes from all the isolates were harvested on 6th day of incubation and the xylanase and cellulase activities of enzyme samples from each isolate were determined. The supernatant protein concentrations of the enzyme samples were also determined. The aim of primary screening was to select the fungal strains exhibiting the higher xylanase activities. Secondary screening was carried out on the basis of higher xylanase [93] and lower cellulase [90] activity of crude enzyme extract produced by SSF of selected 7 fungal strains and finally the best strain was selected for further studies.

3.2.4: Scanning electron microscopy

The detailed morphological study of the fungal strains was carried out using scanning electron microscopy (SEM, Leo 435 VP, England). Fungal mat was taken and subjected to 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 [49]. Electron photomicrographs were taken at desired magnifications.

3.2.5: Identification of strains

Finally, the selected fungal strains was sent to Indian Agriculture research Institute, New Delhi for morphological identification and further sent to Microbial Type Culture Collection & Gene Bank (Institute of Microbial Technology, Chandigarh) for molecular identification up to species level.

3.2.6: Fermentation medium

3.2.6.1: Nutrient salt solution

For production of extracellular enzymes, the nutrient salt solution (N.S.S.), prepared according to Vishniac and Santer [162] and as standardized by Singh and Garg [129], was used. The medium contained KH_2PO_4, 1.5 g/L; NH_4Cl, 4.0 g/L; $MgSO_4.7H_2O$, 0.5 g/L; KCl, 0.5 g/L and yeast extract, 1.0 g/L in distilled water with 0.04 mL/L trace elements solution having $FeSO_4.7H_2O$, 200 µg/L; $ZnSO_4.7H_2O$, 180 µg/L and $MnSO_4.7H_2O$, 20 µg/L. The desired pH (Knick, Germany, Model-761 Calimatic) of the solution was adjusted with $NaOH/H_2SO_4$.

3.2.6.2: Pretreatment of solid substrate

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

3.2.6.3: Inoculum

Fungal cultures were transferred to Petriplates (wheat bran-agar medium) from PDA slants and incubated at 37 ^{0}C for 3 days. From this actively growing culture, 2 disks of size 5 mm were cut with the help of a cork borer and used as the inoculum for the biotransformation process.

3.2.7: Xylanase production by fungal strains

3.2.7.1: Submerged fermentation

SmF was carried out in 40 mL of NSS with 2% of wheat bran as a carbon source in Erlenmeyer flasks (250 mL) [129]. Medium was inoculated with fungal cultures following incubation at 37 ^{0}C for 8 days in an orbital incubator shaker (Sanyo, Orbi-safe, UK) with constant shaking (120 rpm). The crude enzyme was harvested and assayed for xylanase [93], and cellulase [90] activities as per standard protocols.

3.2.7.2: Solid-state fermentation

SSF was performed as described by Beg *et al.* [14]. Slurry of the fermentation medium containing 5 g of wheat bran and 15 mL of NSS was added thereby maintaining a solid to liquid ratio of 1:3 [69] in Erlenmeyer flasks (250 mL) and inoculated with fungal culture. The culture flasks were incubated at 37 ^{0}C for 8 days. The crude enzyme was harvested and assayed for xylanase [93], and cellulase [90] activities as per standard protocols.

3.2.8: Extraction of enzymes

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

3.2.9: Analytical methods

3.2.9.1: Estimation of xylanase activity

Xylanase activity was estimated by analysis of the xylose released by DNS method [93]. DNS reagent was prepared by dissolving 1g DNS acid, 0.2 g phenol, 0.05g sodium sulphite and 20.0g sodium potassium tartarate sequentially in 100 mL of 1% sodium hydroxide solution. Mixture was shaken for 5 min and filtered through Whatman filter paper (No.1). The reagent was stored in dark at 4 ^{0}C for future use. 0.4 mL of 1% Birchwood xylan solution was mixed with 1.6 mL of suitably diluted culture filtrate in 50 mM potassium phosphate buffer (pH 6.4) and incubated at 55 ^{0}C for 15 min. 0.3 mL of solution was taken from the incubated mixture in a

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

3.2.9.2: Estimation of cellulase (CMCase) activity

Carboxymethyl cellulase (CMCase) activity was determined as described by Mandels [90]. The assay mixture, in a total volume of 2 mL, contained 0.5 mL of 1 mM of carboxymethyl cellulose (CMC) in 50 mM citrate buffer (pH 4.8) and 0.5 mL of the supernatant obtained from fermentation broth as the source of enzyme. Controls were routinely included in which enzyme or substrate were omitted and treated similarly. The mixture was incubated at 50 ^{0}C for 30 min. The reducing sugars released were measured optically at 575 nm using UV-Vis spectrophotometer (Cary 100 Bio, Varian-Australia) at 25 ^{0}C using DNS reagent [93]. One unit of enzyme activity was expressed as one μmol of glucose liberated per min per mL of the reaction under above defined conditions.

3.2.9.3: Estimation of protein concentrations

Protein concentration was estimated according to Lowery et al., [85] with bovine serum albumin (BSA) as a standard as described in Chapter 2 section 2.2.12.5.

3.2.9.4: Estimation of laccase activity

Laccase assay [38] was performed using ABTS (2, 2'-azino-bis-3-ethylbenz-thiazoline-6-sulphonic acid) as substrate. Reaction was carried out by taking 100 mM citric acid buffer at pH 5.0, enzyme extract and 1.0 mM of ABTS. Reaction was monitored at 420 nm using UV-Vis spectrophotometer (Cary 100 Bio, Varian-Australia) at 25 ^{0}C. The enzyme activity was expressed as the amount of enzyme which produced an increase of 1.0 absorbance unit per 30 seconds.

3.2.10: Optimization of various physicochemical parameters under SSF

SSF was carried out to study the effect of different ecological and nutritional factors on xylanase production. This was done to determine the most favorable conditions for achieving enhanced levels of enzyme production conditions by the test isolates. Wheat bran was used as the

substrate unless mentioned otherwise. The culture conditions were optimized by changing one independent variable at a time while keeping the other variables constant.

3.2.10.1: Optimization of incubation period

For the optimization of incubation period, a set of Erlenmeyer flasks (250 mL) was prepared containing 5 g of wheat bran and 15 mL of NSS (pH 6.0). These flasks were autoclaved at 15 Psi for 15 min and inoculated aseptically with fungal cultures as described above. These were incubated at 37^{0}C and were harvested daily starting from 2nd to 11th day. The estimation of the protein concentration [85], xylanase [93] and cellulase activities [90] was done as per standard protocols.

3.2.10.2: Optimization of incubation temperature

A set of Erlenmeyer flasks (250 mL) containing 10 g of wheat bran and 15 mL of NSS (pH 6) was prepared and after autoclaving inoculated with fungal cultures. The effect of temperature on the production of xylanases produced by both the strains was studied by incubating the inoculated flasks at an interval of 5 ^{0}C starting from 27 to 52 ^{0}C for 7 days. The xylanase [93] and cellulase activities [90] were determined as per the standard protocol.

3.2.10.3: Optimization of initial pH

For the optimization of pH, a set of 250 mL Erlenmeyer flasks was prepared as described above and pH was adjusted between 4.6 to 11.2 after an interval of every 0.5 pH with 1 N $NaOH/H_2SO_4$ separately. These flasks were autoclaved at 15 Psi for 15 min, inoculated with fungal cultures aseptically and incubated at 37 ^{0}C. Flasks were harvested after 7 days and the xylanase [93] and cellulase activities [90] were measured as per the standard protocol.

3.2.10.4: Effect of carbon source

Erlenmeyer flasks (250 mL) containing 5 g each of various agricultural by-products/residues like wheat bran, sugarcane bagasse, wheat straw and rice straw (grinded up to +100 mesh size) were used as substrates for xylanase production by both of the strains. The substrates were moistened with NSS, autoclaved, inoculated and incubated at 37 ^{0}C. The crude enzyme was extracted on 7th day and assayed as per standard protocol mentioned for xylanase [93] and cellulase activities [90].

3.2.10.5: Effect of nitrogen source

A set of 5 Erlenmeyer flasks (250 mL) was prepared as described above and each flask was supplemented with five different nitrogen sources including yeast extract, malt extract,

peptone, soya bean meal and beef extract. The pH of NSS was set at 6.4. Fermentation was carried out for 7 days at 37 ^{0}C. Crude enzyme was harvested and xylanase [93] and cellulase activities [90] were estimated as per the standard protocols described.

3.2.10.6: Optimization of moisture level

The influence of moisture level on the xylanase titer was evaluated by varying the ratio (w/v) of wheat bran to NSS (1:2, 1:2.5, 1:3, 1:3.5 and 1:4.0). The fermentation was carried out for 7 days at 37 ^{0}C. The xylanase [93] and cellulase activities [90] in crude enzyme extract were checked as per standard protocols.

3.2.10.7: Effect of glucose and lactose concentration on xylanase production

Different levels of glucose/lactose (1-5 g/L) were incorporated into wheat bran moistened with NSS in a ratio of 1:3 and its effect on xylanase titer of both the strains was studied. Fermentation was carried out under optimum conditions mentioned in Table 4.12 and 4.13. Xylanase activity [93] was determined as per standard protocol.

3.2.11: Characterization of crude xylanase

3.2.11.1: Optimum pH and pH stability

The pH stability was determined in the pH range of 6.0-9.0 by incubating the enzyme in buffers of different pH (potassium-phosphate; pH range: 6.0-7.4 and borax-boric acid; pH range: 7.6-9.0). After 15 min incubation, the residual xylanase activity [93] of the crude enzyme samples was determined, under standard assay conditions.

3.2.11.2: Optimum temperature and thermostability

The thermostability of the enzyme was determined by incubating the crude enzyme preparations at temperatures ranging between 55-85 ^{0}C for up to 15 min. Samples were withdrawn after 15 min and analyzed for residual xylanase activity [93] under standard assay conditions.

3.2.11.3: Sodium-dodecylsulphate Polyacrylamide Gel Electrophoresis

SDS-PAGE of the samples was performed according to Laemmli [77] as previously mentioned in materials and methods section of chapter 2 and section 2.2.14.3.

3.2.11.4: Zymogram analysis of xylanase components

Native polyacrylamide gel electrophoresis (PAGE) using 12% gel was performed for visualization of enzyme activities in situ as described [116, 125]. Substrate i.e. xylan to the final concentrations of 0.1% was incorporated into separating gel before adding the ammonium

persulphate and tetramethylethylenediamine (TEMED) for polymerization. The enzyme samples were mixed with sample buffer lacking SDS and β-merceptoethanol. After electrophoresis, the gel was soaked with 0.1% (w/v) Congo red for 5 min with mild shaking. Excess dye was decanted and gel was washed with 5% (w/v) NaCl until excess stain was totally removed and the background was clear.

3.2.12: Mass production of xylanase

After optimizing fermentation conditions, mass production of xylanase was carried out by SSF process for their use in biodeinking and biobleaching experiments. 40 g of wheat bran was taken in a 2 L flask and 120 mL of NSS (pH 6.0) was added to it. From the actively growing culture of strain AT-1, 14 disks of size 5 mm were cut with the help of a cork borer and used as the inoculum for the biotransformation process. The flask was incubated at 37 ^{0}C and harvested after 7 days of incubation. The crude enzyme extract obtained was used for the determination of xylanase [93], cellulase [90] and laccase activities [38] and protein estimation [85] as per standard protocols.

3.2.13: Statistical analysis

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

3.3: Results and discussion

3.3.1: Isolation, purification and screening of fungal strains

Table 3.2 reveals the morphological characteristics of 10 fungal strains collected from different lignocellulosic sources. The fungal strains were isolated on wheat bran agar plates. In present investigation, moist wheat bran was used in the medium as a sole carbon source, as it has already been shown to be a promising carbon source for xylanase production [3,42]. The formation of fruiting bodies and white thread like mycelial network on the decaying wood and aerial fruit bodies, after successive degradation indicates the growth of basidiomycetes **(Photograph 3.1).** When decaying wood was buried in moist wheat bran and incubated at 37 ^{0}C, it showed mycelial growth, spores and the appearance of colony **(Photographs 3.2A-I).** The xylanase production ability of the fungal strain on xylan-agar (XA) plate was observed after primary screening. Out of ten, seven isolates resulted into zone formation onto xylan-agar (XA) plates, of which AT-1 resulted in to maximum clear zone diameter when stained with Congo red **(Photograph 3.3).** These isolates were selected as xylanase producers. These seven strains were

further subjected to secondary screening for their xylanase production ability in SSF condition **(Table 3.3 and Figure 3.2).** Out of seven, two fungal strains namely, AT-1 and AT-13 produced maximal of xylanase activity i.e. 467.00 and 360.80IU/mL with minimum cellulase activities i.e. 0.94 and 0.80 IU/mL respectively. Fungal isolate (AT-1) was selected for further studies based on its higher xylanase activity.

3.3.2: Identification of selected isolate

3.3.2.1: Morphological identification

The selected fungal isolate AT-1 was morphologically identified as a wild mushroom from Indian Type Culture Collection (ITCC), Plant Pathology Division, IARI, New Delhi (India).

3.3.2.2: Molecular identification

For the identification of fungal isolate wild mushroom AT-1 up to a species level, molecular identification was done at MTCC (IMTECH, Chandigarh). The NCBI-BLAST search analysis of the DNA sequence-data indicated that the fungus is a member of *Coprinus cinereus.* The fungal isolate was deposited in MTCC with an accession number 9695. The DNA sequence data of the fungal isolate *Coprinus cinereus* AT-1 is shown as under:

Coprinus cinereus isolate AT-1, 28S large subunit ribosomal RNA gene, partial sequence

GGATTCCCCTAGTAACTGCGAGTGAAGCGGGAAAAGCTCAAATTTAAAAT
CTGGCAGTCTTTGGCTGTCCGAGTTGTAAACTAGAGAAGTGTTACCCGCG
TCGGACCGTGTACAAGTCTCCTGGAATGGAGCATCATAGAGGGTGAGAAT
CCCGTCTTTGACATGGACTACCGAGGCTTTTGTGGTGCGCTCTCAACGAG
TCGAGTTGTTTGGGAATGCAGCTCAAAATGGGTGGTAAATTCCATCTAAA
GCTAAATATTGGCGAGAGACCGATAGCGAACAAGTACCGTGAGGGAAAGA
TGAAAAGAACTTTGGAAAGAGAGTTAAACAGTACGTGAAATTGCTGAAAG
GGAAACGCTTGAAGTCAGTCGCGTTGGCCGGAAATCAACCCTGCTTCTGT
TGGGTGTACTTTCTGGTTGACGGGCCAGCATCAGTTTTGACCGGTGGAGA
AAGTCCAAGGGAATGTGGCATCTCCGGATGTGTTATAGCCCTTGGTCGTA
TACATCGGTTGGGACTGAGGAATTCAGCACGCCGCAAGGCCGGGTTTCGA
CCACGTTCGTGCTTAGGATGCTGGCATAATGGCTTTAATCGACCCGTCTT
GAAACACGGACCAAGGAGTCTAACATACCTGCGAGTGTTTGGGTGGAAAA
CCCGGACGCGTAATGAAAGTGAAAGTCAGGATCCCTGTCATGGGGAGCAC
TGGCGCCCGCACCTGAAGTTTACGGACGGATGTGAGGTAAAGCATGTATG
CTAGGACCCGAAAGATGGTGAACTATGCCTGAATAGGGTGAAGCCAGAGG
AAACTCTGGTGGAGGCTCGTAGCGATTCTGACGTGCAAATCGATCGTCGA
ATTTGGGTATAGGGGCGAAAGACTAATCG

Coprinus cinereus isolate AT-1, 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1 and 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence

GATATGCTTAAGTTCAGCGGGTAGTCTTGCCTGATTTGAGGTCAAAATTG
TCAAAAGTTGTCCAAGGGACGGTTAGAAGCAGGTCTTCAAGTAACCAACC
CAATCCACGGCGTAGATAATTATCACACCAATAGATTAGGGGCACAACCC
ACTAATACATTTCAGAGGAGCAGACCACGAGAGTGGACCTGCAACCCCCA
CATCCAAGCCTGCACACAACAAAGTTGGTGAGGTTGAGAATTTAATGACA
CTCAAACAGGCATGCTCCTCGGAATACCAAGGAGCGCAAGGTGCGTTCAA
AGATTCGATGATTCACTGAATTCTGCAATTCACATTACTTATCGCATTTC
GCTGCGTTCTTCATCGATGCGAGAGCCAAGAGATCCGTTGCTGAAAGTTG
TATAGGTTTATAGGCACAAGGCCTTGAGATGACATTCCATAACATTCAGT
TTGGGGTGTGTAAGAAATCATAGACCTGGAAATTCATGGCAAGCCGGCCT
TCTTTCGAAAGCGACAGCAAGCCACGCATCCGCCTTGCGACGAGAGGTAT
CCAGGCCTACAGTGTGTGCACAGGTGGAGAAATAAAGGTGACGGGCGTGC
ACATGCTCCCGGGGGAGCCAGCGACAACCACGTCAGATTTATTCAATA

3.3.3: Morphological characteristics of *C. cinereus* AT-1

The characteristic features of the different fungal isolates namely (A=AT-13, B=AT-14, C=AT-17, D=AT-18, E=AT-19, F=AT-20, G= AT-21, H= AT-1, I= AT-22) were shown in **Photographs 3.2A-I**. The mycelia of the fungal strain AT-1 which was identified as *Coprinus cinereus* AT-1 were white in color while the basidiopores produced were black. **(Photographs 3.4A).** Fungal strain *C. cinereus* AT-1 showed smooth colony without any distinct growth rings. The fruiting bodies indicated that the fungi were related to basidiomycetes family which ultimately lead to the spore formation on 11^{th} day of incubation. The finer structural details of the mycelia and spores as observed through the scanning electron microscopy (SEM) were shown in **Photographs 3.5-3.6**. The hyphae of *C. cinereus* AT-1 were thin, elongated and straight. The presence of basidium, basidiospores, pileocystidea and hymenial layers was demarcated in **Photograph 3.6.** The basidiospores produced by *C. cinereus* AT-1 were club-shaped which is a characteristic feature of all the members of basidiomycotina group. In natural habitat a cap is present in wild mushroom, initially white in colour but soon begins to turn grayish brown, with a brownish center **(Photograph 3.7).** It was easily recognized as a wild mushroom by its cap shape and grayish black gills. It is an extremely fragile mushroom, and the caps quickly crumble when handled [27].

3.3.4: Comparative analysis of different fermentation conditions

Table 3.4 and Figure 3.3 reveals a comparison between solid-state and submerged fermentations on xylanase production ability of the fungal strain *C. cinereus* AT-1. The xylanase activity of fungal strain AT-1 was **475.50 IU/mL** and cellulase activity was **0.84 IU/mL** under SSF. On the other hand during SmF, the xylanase activity of *C. cinereus* AT-1 was observed to

be **224.10 IU/mL** and cellulase activity was **0.48 IU/mL**. Results showed that xylanase activity of strain *C. cinereus* AT-1 under SSF conditions was **54.55 %** higher than that of SmF conditions. Hence, the xylanase production in SSF was much higher than that of SmF. In the same way, cellulase activity of the fungal strain *C. cinereus* AT-1 increased accordingly in SSF compared to SmF.

Filamentous fungi could grow to significant extent in the absence of free water [50]. Considering that submerged free floating fungi growth was not natural, growth on and within solid substrate was fundamentally related to cell adhesion [160]. The heterogeneous nature of the susbtrate used in SSF process [137], the concentraion gradient of sugar and mineral salts [139] and the localized drop in substrate concentraion [113] allowed enhanced metabolic avtivity in microorganisms growing in SSF and significantly minimized catabolite repression [137, 113]. The aim of SSF was to bring the cultivated fungi or bacteria into tight contact with the insoluble substrate and thus to achieve the highest substrate concentrations for fermentation so the increased yield of xylanase produced by the fungal strain *C. cinereus* AT-1 observed in SSF might be attributed to close contact between mycelium and substrate [61] which was not possible during SmF. Malarvizhi *et al.* observed 30-fold enhancement of xylanase production in SSF than liquid culture when wheat bran was used as the substrate for a culture of *Ganoderma lucidum* [89]. Agnihotri et al, achieved similar results with *Coprinellus disseminatus* SW-1 NTCC 1165 using wheat bran under SSF [3] Archana and Satyanaryana described a SSF process for the production of thermostable xylanase by thermophilic *Bacilus licheniformis*, enzyme production was 22 fold higher in SSF system than in SmF [7]. Enzyme production in SSF system was more thermostable than in SmF system. Cai *et al.* also reported production of thermostable xylanase in SSF system [23]. Therefore, SSF was chosen for further optimization studies for crude xylanase production by the fungal strain *Coprinus cinereus* AT-1

3.3.5: Analysis of critical parameters for improving the xylanase production by the strains AT-1 of *Coprinus cinerus*

SSF was carried out for optimizing various operating parameters i.e. incubation period, temperature, pH, carbon source, nitrogen source and moisture content using hyper-xylanase producing fungal strain *C. cinereus* AT-1 (**Photograph 3.8**). Using SSF conditions, significant physicochemical and biological factors were analyzed for deciphering the most favorable conditions for achieving enhanced levels of enzymatic production.

3.3.5.1: Optimization of incubation period for xylanase production

The influence of incubation period on enzymatic production by *C. cinereus* AT-1 in a batch process was depicted in **Table 3.5.** The xylanase activity of fungal strain *C. cinereus* AT-1 increased steadily with increasing incubation period and attained maximum level on 7th day of incubation **i.e. 615.05 IU/mL** which corresponded to mycelial biomass in terms of protein concentration **(Figure 3.4).** Further, on increasing incubation period beyond 7th day the xylanase activity of the fungal strain i.e. *C. cinereus* AT-1 decreased. Cellulase production also increased to its maximum level after 7 days of incubation i.e. **0.92 IU/mL.** On longer incubation, enzyme activities of the fungal strain decreased while protein biomass continued to increase from day 1st to 7th and then nearly became constant with a small decrease after 10th day. It suggested that the enzyme production was dependent on biomass but only during the exponential phase of fungal growth. For the reason that, cellulase and xylanase were a part of primary metabolites thus, these were produced during exponential phase of growth and at the onset of death phase, the enzyme secretion started decreasing. Xylanase production was maximal at the end of exponential phase (7th day) and then slowly started decreasing on the onset of death phase. It suggested that the enzyme production was dependent on fungal biomass but up to some extent [26]. A similar trend was reported for production of xylanase by *Thermoactinomyces thalophilus* [71], *Aspergillus carneus* M34 [45] and *Trichoderma reesei* SAF3 (149). Basidiomycetes were slow growing fungi, so xylanase production was maximum after 7 days in *Pleurotus ostreatus* [111], *Coprinellus disseminatus* SW-1 NTCC 1165 [3] and *Trichoderma harzianum* 1073 D3 [128], 6 days in *Phanerochaete chrysosporium* [148] and 8 days in *Volvariella diplasia* [107]. Other fungi such as *Thermomyces lanuginosus* and *Thermomyces auranticus* had produced maximum xylanase on 7th day of incubation [5] while maximum xylanase production was observed on 6th day in *Penicillium oxalicum* in SSF [94].

3.3.5.2: Optimization of incubation temperature for xylanase production

Temperature regulation is one of the decisive factors for SSF. **Table 3.6 and Figure 3.5** shows the effect of incubation temperature on xylanase production by *C. cinereus* AT-1 and shows the highest xylanase activity i.e. **658.57 IU/mL** at 37 ^{0}C while the lowest activity i.e. **33.60IU/mL** at 27 ^{0}C. While moving towards the extreme ends of the optima, the activities decrease since the fungal growth ceases. The xylanase activity of strain *C. cinereus* AT-1 at temperatures 42 and 47 ^{0}C was **331.08 and 198.65 IU/mL** respectively. It was found that *C.*

cinereus AT-1 could maintain productivity even at temperature above 50 ^{0}C with a xylanase activity of **39.41IU/mL.** The maximum xylanase activity at 37 ^{0}C for the fungal strain *C. cinereus* AT-1 suggests that the test isolate is mesophilic in nature. The literature also supports our results as it has been reported that most of the white-rot fungi are mesophiles [37].

The decrease in xylanase production level was possibly due to lower transport of substrate across the cells at lower temperature that might be the reason for the lower yield of the product. At higher temperature, the maintenance energy requirement of cellular growth was high due to thermal denaturation of enzymes of the metabolic pathway resulting in lower production of the metabolites [4]. The productivity declination observed at high temperature could be due to the reversible denaturation of enzyme formed on optimized-medium as described previously [33]. The cellulase activity of fungal strain also increased with increasing incubation temperature up to 37 ^{0}C and then declined. Stoichiometric global equation and respiration was highly exothermic and, heat generation due to higher levels of fungal activity within the solids lead to thermal gradients because of the limited heat transfer in the solid substrates. The thermal gradation could be controlled by maintaining the desired level of humidity [68]. Similarly, a thermostable and cellulase free xylanase was produced by *Streptomyces* sp. QG-11-3 in the SSF using wheat bran as the carbon source at 37 ^{0}C [14]. Xylanase production was found to be optimum at 37 ^{0}C under SSF for *Bacillus pumilus* ASH using wheat bran [12] while the highest xylanase production by *B. licheniformis* A99 was observed at 50 ^{0}C under SSF using wheat bran as the carbon source [7].

3.3.5.3: Optimization of pH of the medium for the production of xylanases

The pH of the medium is an important parameter for enzyme production. **Table 3.7** reveals that xylanase activity of the fungal strain *C. cinereus* AT-1 increased with increasing pH from 4.6 to 6.4 while keeping other fermentation conditions constant and further on increasing pH the xylanase activity decreased steadily. Three distinct phases as a function of pH were illustrated in **Figure 3.6**; at low pH (4.6 to 5.8), there was an increase in enzyme activity, at high pH (above 7.6) there was a decrease and at an intermediate (optimal) pH range (6.4-7.0), the activity was maximal, therby leading to a characteristic bell-shaped curve. The maximal xylanase activity **(664.74 IU/mL)** for the fungal strain *C. cinereus* AT-1 was attained at pH 6.4, however, further increase in pH steadily reduced the xylanase titer. The strtain AT-1 could maintaine 54.29% of its maximal xylanase activity even at an alkaline pH of 8.2, which was

quite significant. The maximum cellulase activity of strain AT-1 **(0.87 IU/mL)** was observed at pH 6.4 while minimum at pH 10 **(0.41 IU/mL).**

The H^+ concentration in the fermentation medium had profound effect on the xylanase production. A regulatory influence of pH shift on the production of both primary [158] and secondary metabolites [65] was reported. The pH of the culture medium was one of several parameters which affected morphology and productivity. Dramatic changes in cell size or shape were a consequence of the responses of microbes to change in pH. The initial pH influenced much enzymatic system and the transport of several species of enzymes across the cell membrane [8]. Structures such as, cell membrane in contact with the external environment could also undergo chemical changes in response to pH. Microorganisms might need to adapt their function in order to cope up with a change in hydrogen ion concentration. If this change was too abrupt, the response of microbes might lag behind or overshoot. Apart from affecting cell membrane permeability, pH might also determine the solubility of some components of the medium. Thus, a modification in the pH might also cause some micronutrients to precipitate and become impossible to be assimilated [120]. Fermentation at lower and higher pH proved to be detrimental perhaps because of the inactivation of the enzyme system. Since, enzymes are proteins, the ionic character of the amino and carboxylic acid groups on the protein surface are likely to be affected by pH changes and the catalytic property of the enzyme is markedly influenced. The reason for poor production at higher pH was probably due to proteolytic inactivation of the xylanase. It suggested that slightly acidic pH values favor the xylanase production. The fungal strain AT-1 could hence be considered as alkali-tolerant according to the definitions of Nagai *et al.* [95]. These results are in agreement with those reported for other fungal species. The optimum pH for the xylanase production by *A. versicolor* was 6.5 [24] under SmF at 30 ^{0}C and pH 6.0 by aquatic hypomycetes under SmF at 20-25 ^{0}C [1] while *A. carneus* M34 grew well in the pH range of 4.5 to 5.5 in the temperature range of 33-36 ^{0}C under response surface methodology (RSM) [45]. The alkali tolerant property makes the enzyme accessible for direct application on the alkaline pulp without any intervening pH adjustment. Thus, our enzyme preparation fulfills the current paper industry requirements.

3.3.5.4: Effect of carbon sources on xylanase production

The effect of different lignocellulosic substrates (cheap agricultural wastes including sugarcane bagasse, wheat straw, rice straw and wheat bran) as the sole carbon source on

xylanase production by *C. cinereus* AT-1 was shown in **Table 3.8** and graphically represented in **Figure 3.7.** The highest xylanase activity **(677.85 IU/mL)** was found when wheat bran was used as the sole carbon source. The enzyme activity in other carbon sources was found to be decreased in descending order like rice straw **(404.03 IU/mL)**, wheat straw **(117.86IU/mL)** and sugarcane bagasse **(43.36 IU/mL)** respectively. The addition of wheat bran to rice straw in the ratio of 1:1 improved xylanase production **(461.00 IU/mL)** by *C. cinereus* AT-1 up to a good extent among other mixtures of carbon sources and xylanase production reduced in the following descending order: bagasse+wheat bran>wheat straw+wheat bran>wheat straw+rice straw>bagasse+rice straw>bagasse+wheat straw. In the same way the cellulase activity of the fungal strain was the highest with wheat bran **(0.95 IU/mL)** and decreasaed in the following descending order: rice straw>wheat straw>bagasse while in mixture it decreased in the following descending order: rice straw +wheat bran> wheat straw+wheat bran> sugarcane bagasse +wheat bran> sugarcane bagasse+rice straw> wheat straw+ rice straw> sugar cane bagasse +wheat straw. Hence, based on results, wheat bran was selected as a solid substrate for enzyme production under SSF. Wheat bran was found to be the best substrate for xylanase induction, as it contains sufficient nutrients and remained loose in moist conditions, thereby providing good aeration and large surface area [105, 7] which could be used by microbes for growth and metabolic activity [103]. The biochemical composition of wheat bran [81] indicated that wheat bran contained substantial amount of soluble sugars like glucose (42.5% dry wt.), xylose (15.4% dry wt.), arabinose (3.1% dry wt.) and galactose (2.7% dry wt.) requisite for the initiation of growth and replication of the microorganism. The degree of substitution of the main xylan chains by arabinose was higher in wheat bran [81]. It contains 45% hemicellulose (40% xylans), which may fulfill the role of inducers, and organic nitrogen sources (23%), essential for protein synthesis [9]. In addition, it contains about 28% proteins which might serve as the sources of carbon and nitrogen for the microorganisms. Further, its lignin and silica contents were also very low [121].

Xylanases was shown to be inducible enzymes with rare examples of constitutive xylanase expression [142]. Hydrolytic enzymes were generally induced by xylan, xylobiose, xylose and also by lignocellulosic residues that contained xylan [7, 48]. Xylan was a high molecular mass polymer and therefore could not penetrate the cell wall. The low molecular

mass fragments of xylan (including xylose, xylobiose, xylooligosaccharides, heterodisaccharides of xylose and glucose) that were liberated from xylan by the action of small amounts of constitutively produced enzyme, played a key role in the regulation of xylanase biosynthesis. Cellulose was shown to act as an inducer of the xylanase in a few cases, but it was not clear whether the inducing effect lied with cellulose or the contaminating xylan fraction. The low molecular mass substances that were identified as xylanase inducers needed transferase enzymes for their translocation into the cytoplasm. Hence, the level of inducers and/or the required enzymes in the culture filtrate also affected the xylanase synthesis [76]. In general, the xylanase induction was a complex phenomenon and the level of response to an individual inducer varied with the organisms [62]. It was also dependent upon the nature and the concentration of the carbon sources. Generally, the slow release of the inducer molecules and the possibility of the culture filtrate converting the inducer to its non-metabolizable derivative were believed to boost up the level of xylanase activity [76].

Wheat bran was described as a potent substrate for the xylanase production under SSF by *Streptomyces cyaneus* SN32 [99], *Aspergillus fischeri* [25], *Bacillus pumilus* [8], *Bacillus subtilis* [121] *Bacillus pumilus* ASH [13] and *Coprinellus disseminatus* NTCC 1165 [3].

3.3.5.5: Effect of nitrogen sources on xylanase production

Table 3.9 shows the effect of different organic nitrogen sources (urea, yeast extract, peptone, malt extract, soya bean meal and beef extract) on xylanase production by the fungal strain *C. cinereus* AT-1. **Figure 3.8** shows that all the tested nitrogen sources stimulated the production of xylanase from *C. cinereus* AT-1. Yeast extract was the best nitrogen sources for both xylanase **(691.40 IU/mL)** and **cellulase (0.97 IU/mL)** production. In the presence of more available nitrogen, the mycelium grew better and its activity also increased [63]. Earlier studies had proved that both the nature and concentration of nitrogen sources were powerful nutrition factors regulating lignocellulolytic enzyme production by wood-rotting basidiomycetes [147]. The mechanisms that govern the formation of extracellular enzymes were influenced by the availability of precursors for protein synthesis [76] and the differences in enzyme activity obtained for media containing various complex nitrogen sources that were used in the present study could be caused by their varying contents of amino acids, peptides, vitamin, trace elements and /or mineral salts [106]. The highest xylanase production observed with yeast extract by the

fungal strain *C. cinereus* AT-1 might also be attributed to a better absorption of amino acids of yeast extract directly through mycelia of the test strains [111]. Singh *et al.,* obtained maximum xylanse production for the fungal strain *Coprinellus disseminatus* SH-1 NTCC-1163 and SH-2 NTCC-1164 [133] with yeast extract as nitrogen source while Agnihotri *et al.,* quoted soyabean meal as the most favorable nitrogen source for the basidiomycetes strain *Coprinellus disseminatus* SW-1 NTCC-1165 [3]

3.3.5.6: Optimization of moisture content for xylanase production

Table 3.10 shows the effect of moisture content on xylanase production by *C. cinereus* AT-1. **Figure 3.9** shows that maximum xylanase titer **(697.90 IU/mL)** was obtained when wheat bran was moistened with NSS in a ratio of 1:3 which declined with further increase in substrate to moisture ratio. Similarly, maximum cellulase activity **(0.92 IU/mL)** was also found at solid substrate to moisture content ratio of 1:3. The moisture content in SSF was one of the crucial factors affecting enzyme activity [34]. Its influence on microbial growth and product biosynthesis might be attributed to its impact on the physical properties of the solid substrate [47, 96,112,]. Higher moisture content resulted in a decreased porosity, alteration in wheat bran particle structure, gummy texture, lower oxygen transfer (as void space of the solid support was filled with moisture) and enhancement of the formation of the aerial mycelia [47, 51,52, 112]. Likewise, moisture level below optimum lead to a reduced solubility of the nutrients of the solid substrate and lower degree of swelling of substrate [47]. In SSF using wheat bran and eucalyptus Kraft pulp as the primary solid substrates, *Streptomyces sp.* QG-11-3 [14] produced maximum xylanase yield at substrate-to-moisture ratio of 1:2.5 and 1:3, respectively. In contrast, a lower solid substrate to moisture level of 1:1 was reported for maximum xylanase production by *Bacillus* sp. A-009 [53].

Based on above discussion, the following were the optimum physico-chemical parameters for crude xylanase production by *C. cinereus* AT-1: incubation period 7 days, incubation temperature 37 ^{0}C, pH 6.4, solid substrate to moisture ratio 1:3, carbon source wheat bran and nitrogen source yeast extract **(Table 3.11). Table 3.12** shows the enzyme production by *C. cinereus* AT-1 under optimized conditions. The xylanase, cellulase and laccase activities of *C. cinereus* AT-1 were **698.75 IU/mL, 1.01 IU/mL** and **25.6 IU/mL** respectively while protein biomass was **5.7 mg/mL.**

3.3.5.7: Effect of glucose and lactose on xylanase production

Table 3.13 shows the effect of glucose and lactose concentration on xylanase production by fungal strain *C. cinereus* AT-1. **Figures 3.10** and **3.11** showed that xylanase production by *C. cinereus* AT-1 decreased with increasing glucose and lactose concentration from 1 to 5 g/L. **Figures 3.10** showed that in absence of glucose; *C. cinereus* AT-1 produced **699.88 IU/mL** of xylanase activity which decreased later on with increasing the concentration of glucose. Similar trend was obtained by addition of lactose in the medium during SSF **(Figure 3.11).** The addition of glucose and lactose had repressed the xylanase production, which might be possibly due to catabolite repression [161]. As mentioned earlier, xylanases had shown to be inducible enzymes, with rare examples of constitutive xylanase expression [44]. The xylanase induction, in general, was a complex phenomenon and the level of response to an individual inducer varies with the organisms [62]. The substrate derivatives and the enzymatic end products might often play a key positive role in the induction of xylanases; they could also act as the end product inhibitors, possibly at much higher concentrations. The exact mechanism by which carbon catabolite repression mediated glucose repression in filamentous fungi is not wholly understood [119].

Catabolite repression of xylanase production by glucose was also observed by Sanghi *et al.* [121] in *Bacillus subtilis* ASH and Hamzah *et al.* [59] in *Bacillus pumilus*. While Srivastava observed that xylanase produced from the bacterium *Thermomonospora* was resistant to catabolite repression by glucose and xylose [142].

3.3.6: Biochemical characterization of xylanase

3.3.6.1: Effect of pH on the activity and stability of xylanase

Table 3.14 shows the effect of pH on the xylanase activity of *C. cinereus* AT-1 at a temperature of 55 ^{0}C for 15 min of reaction time. **Figure 3.12** shows that the crude xylanase produced by *C. cinereus* AT-1 was active in the pH range of 6.0 to 9.0 with the maximum xylanase activity at pH 6.4 **(699.60 IU/mL).** At pH 6.0, 67% of the maximal activity was maintained. Above pH 6.4, a decrease in xylanase activity was recorded. At neutral pH (7.0), xylanase retained about 64% of its optimum activity while at a pH of 7.5, 56% of the optimum activity was retained. Even at pH 8.0, the enzyme could retain 30% of its optimum activity which showed its alkali-tolerant nature.

Enzymes are protein in nature. In the harsh conditions such as change in pH, high temperature or in presence of high concentration of metal ions, proteins tended to lose their basic structure (denaturation), subsequently, losing active site that in turn resulted in a loss of enzyme activity. Besides this, the pH activity profiles of enzymes were highly dependent on the pKa value of the catalytic residues which were themselves dependent on the local environment and hence on the nature of the amino-acids in the vicinity of the catalytic residues. pH stability increased with decreasing the pKa value [67]. The optimum pH for the xylanase produced by *Aspergillus fumigatus* AR1 was 6.0-6.5 [6]. The xylanase produced by white-rot fungus *Pleurotus ostreatus* was active over a broad range of pH (3.0-7.0) and showing optimum activity at 6.0 [111]. Studies carried out with other fungal species [31,134] also concluded that the most suitable pH value for xylanase activity was within acidic region which supported our findings.

3.3.6.2: Effect of temperature on the activity and stability of xylanase

Table 3.15 shows the effect of temperature on the activity of crude xylanase produced by *C. cinereus* AT-1 at optimum pH 6.4 for 15 min of reaction time. **Figure 3.13** depicts that the optimum temperature for the crude xylanase activity of the fungal strain was 55 ^{0}C **(702.00 IU/mL)** and beyond that xylanase activity went on decreasing. At a temperature of 65 ^{0}C, xylanase from *C. cinereus* AT-1 retained about 74% of its optimum xylanase activity. When assayed at higher temperatures i.e. 75 and 85 ^{0}C, the xylanase lost most of its activity by retaining just 17 and 11% of its optimum activity respectively. The experimental results clearly indicated thermo-tolerant nature of xylanase produced by *C. cinereus* AT-1. Thermostability of enzymes seem to be a property acquired by a protein through a combination of many small structural modifications that were achieved with the exchange of some amino acids. The variation of the canonical forces e.g. hydrogen bonds, ion-pair interactions and hydrophobic interactions provided thermozymes resistance at high temperature [123]. The optimal temperature for the crude xylanase activity of the fungal strain was 55 ^{0}C. This result substantiated with the finding that the optimum temperature for the xylanase produced by most of the fungi was in the range of 40-60 ^{0}C [76]. 55 ^{0}C was considered as the optimum temperature for xylanase activity of *Aspergillus nidulans* KK-99 [150] while xylanase from *Aspergillus fumigatus* AR1 exhibited maximum xylanase activity at temperature of 60-65 ^{0}C. The crude xylanase produced from *Aspergillus oryzae* had the temperature optima between 60

to 65 ^{0}C. After 10 and 30 min of incubation at 60 ^{0}C, the residual activity was 40% and 10% respectively of the optimum activity for *Aspergillus oryzae* [149]. The pulp produced after brown stock washing or bleaching had temperature about 65±5 ^{0}C and pH about 8. The crude xylanase produced by strain *C. cinereus* AT-1 was active in the pH range of 6 to 9 and temperature from 55 to 85 ^{0}C whereas, high xylanase stability was observed in pH range of 6-7.6 and temperature from 55 to 65 ^{0}C. Looking at alkali-thermo-tolerant nature of crude xylanase produced by the strain *C. cinereus* AT-1 has potential in biodeinking and biobleaching processes.

The mass production of crude xylanases was done in 1000 mL flasks under fermentaion conditions as optimized above and the results are summarized in **Table 3.16** *Coprinus cinereus* AT-1 exhibited **742.47 IU/mL of xylanase activity, and 25.9 IU/mL of laccase activity.** A minor **cellulase activity of 0.98 IU/mL and protein concentration of 5.8 mg/mL** were associated with the crude enzyme preparations which explains that crude enzyme obtained from strain *Coprinus cinereus* AT-1 was a concentrated solution of diversity of proteins.

3.3.6.3: Molecular characterization by SDS PAGE analysis

Photograph 3.10 A-B be evidence for the SDS-PAGE profile of the crude enzyme preparations obtained from the *C. cinereus* AT-1 using concentrated crude culture filtrate as samples. The concentration of some proteins in these extracts was low which caused weak staining of some proteins and difficulty in detection of some other proteins by polyacrylamide gel electrophoresis. It was noted that only an insight to the protein profile of the crude enzymes could be gained, as there was a high possibility that a number of proteins would exhibit similar molecular weights which might appear as overlapping bands in the gel. Multiplicity of proteins in the crude extracts was demonstrated, but not their exact number. Thus, zymogram analysis of the crude enzyme sample obtained from the *C. cinereus* AT-1 was also done using birch wood xylan in the gel for detection of xylanase activity after PAGE. The xylanase activity was indicated by appearance of clear zones against dark blue background. The crude enzyme preparation was subjected to SDS-PAGE to determine the homogeneity and molecular weight of the enzyme. During the electrophoresis of the enzyme, two bands showing xylanolytic activity were detected as the hydrolysis zones in the zymogram **(Photograph 3.10B).** The molecular weights of these proteins were found to be about 50 kDa and 86kDa respectively for fungal strain *C. cinereus* AT-1. It was reported that these proteins might be isoenzymes or the

different subunits of the same enzyme proteins. Thus, zymogram analysis showing clearing zone corresponding to protein bands indicated xylanase activity.

According to the classification given by Wong *et al.* [165], the xylanase isolated from strain *C. cinereus* AT-1 belongs to the high molecular weight category. The white-rot fungus *Phanerochaete chrysosporium* produced three xylanases with molecular weights of 52, 30 and 50 kDa respectively [36] while molecular weight of xylanase produced by *Ceriporiopsis subvermispora* was 29 kDa [91]. *Chaetomium cellulolyticum* produced three xylanases with molecular weights of 25, 47 and 57 kDa and pIs of 8.9, 8.4 and 5.0 respectively [10]. The molecular weight of xylanase obtained from fungal strain *C. cinereus* AT-1 also supported by the literature, as microbial xylanases had been reported to be single subunit proteins with molecular masses in the range of 8-145 kDa [46].

Table 3.1 : Applications of xylanases

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

Table 3.2: Morphological characteristics of different fungal isolates

Sl. No.	Isolated strains	Sources	Mycelia color	Spore color	Zone diameter, mm
1	AT-01	Dead and decaying wood	White	Black	16.8
2	AT-13	Dead and decaying wood	White	Brown	12.2
3	AT-14	Decomposing manure	White	Black	10.5
4	AT-15	Dead and decaying wood	Green	Green	—
5	AT-17	Paper industry waste	White	Brown	10.5
6	AT-18	Paper industry waste	Black	Black	—
7	AT-19	Fruiting body	White	No spore formation	6.5
8	AT-20	Sugarcane dumping site	Yellowish white	No spore formation	—
9	AT-21	Decomposing manure	Dirty white	Black	3.7
10	AT-22	Mango tree bark	Creamish white	No spore formation	1.4

Table 3.3: Enzyme activities of different fungal isolates under SSF conditions

Sl. No.	Fungal strains	Xylanase activity, IU/mL	Cellulase activity, IU/mL
1	AT-01	467.00±9.1	0.94±0.12
2	AT-13	360.80±8.7	0.80±0.11
3	AT-14	166.50±8.2	1.49±0.14
4	AT-17	138.20±9.1	1.41±0.16
5	AT-19	85.96±6.2	1.82±0.18
6	AT-21	57.37±5.0	0.97±0.13
7	AT-22	23.55.±3.5	1.03±0.15

± refers standard deviation

Fermentation conditions:

Wheat bran, g : 5 Temperature, ^{0}C : 35

NSS, mL :15 Incubation period, days : 8

pH : 6

Table 3.4: Comparison of solid-state and submerged fermentation conditions for enzyme production by *Coprinus cinereus* AT-1

Sl. No.	Fermentation conditions	*Coprinus cinereus* AT-1	
		Xylanase activity, IU/mL	Cellulase activity, IU/mL
1	SmF	224.1 ±7.1	0.48±0.13
2	SSF	475.5 ±10.5	0.84±0.16

± refers standard deviation

Fermentation conditions:

Submerged fermentation

Wheat bran, g : 0.8

NSS, mL :40

Temperature, ^{0}C : 37

Incubation period, days :8

pH : 6

Solid state fermentation

Wheat bran, g : 5

NSS, mL :15

Temperature, ^{0}C : 37

Incubation period, days :8

pH : 6

Table 3.5: Optimization of incubation period for enzyme production by *Coprinus cinereus* AT-1

Sl. No.	Day	Xylanase activity, IU/mL	Cellulase activity, IU/mL	Protein concentration, mg/mL
1	0	0.0±0.0	0.0	0.10±0.01
2	1	11.00±0.80	0.10±0.05	0.38±0.03
3	2	25.70±1.5	0.31±0.11	0.94±0.05
4	3	47.00 ±3.9	0.39±0.12	1.60±0.08
5	4	99.50±4.2	0.53 ±0.15	2.70±0.14
6	5	219.75±6.5	0.66±0.13	3.40±0.26
7	6	523.22±9.2	0.72 ±0.17	3.56±0.15
8	7	615.05 ±10.8	0. 92±0.15	3.65±0.20
9	8	180.20±6.1	0.89 ±0.12	3.71±0.10
10	9	65.85±4.1	0.54 ±0.11	3.67±0.17
11	10	43.50±3.8	0.38±0.09	3.60±0.17

± refers standard deviation

Fermentation conditions:

Wheat bran, g : 5 Temperature, ^{0}C : 35

NSS, mL :15 pH : 6

Table 3.6: Optimization of incubation temperature for enzyme production by *Coprinus cinereus* AT-1

Sl. No.	Temperature, ^{0}C	*Coprinus cinereus* AT-1	
		Xylanase activity, IU/mL	Cellulase activity, IU/mL
1	27	33.60±2.7	0.48±0.13
2	32	562.91±9.8	0.59±0.11
3	37	658.57±11.4	0.83 ±0.18
4	42	331.08±6.9	0.67 ±0.23
5	47	198.65±5.4	0.54±0.14
6	52	39.41±3.0	0.31 ±0.09

±refers standard deviation

Fermentation conditions:

Wheat bran, g : 5 Incubation period, days: 7

NSS, mL :15 pH : 6

Table 3.7: Optimization of initial pH for enzyme production by *Coprinus cinereus* AT-1

Sl. No.	pH	*Coprinus cinereus* AT-1	
		Xylanase activity, IU/mL	Cellulase activity, IU/mL
1	4.6	176.80±4.3	0.67±0.10
2	5.2	285.53±5.0	0.66±0.08
3	5.8	457.13±6.4	0.75±0.12
4	6.4	664.74±10.0	0.87 ±0.14
5	7.0	531.01±8.5	0.82±0.13
6	7.6	446.87±6.3	0.67±0.10
7	8.2	392.88±5.9	0.60±0.16
8	8.8	361.20±5.6	0.58±0.11
9	9.4	242.72±5.1	0.54 ±0.13
10	10.0	217.70±4.8	0.41±0.09

± refers standard deviation
Fermentation conditions:
Wheat bran, g : 5 Incubation period, days: 7
NSS, mL :15 Temperature, ^{0}C : 37

Table 3.8: Effect of carbon sources on enzyme production by *Coprinus cinereus* AT-1

Sl. No.	Carbon sources	*Coprinus cinereus* AT-1	
		Xylanase activity, IU/mL	Cellulase activity, IU/mL
1	Sugar cane bagasse	43.36±2.9	0.19±0.05
2	Wheat straw	117.86±3.5	0.33 ±0.07
3	Rice straw	404.03±9.7	0.52±0.11
4	Wheat bran	677.85±10.8	0.95±0.15
5	Sugar cane bagasse +wheat straw	71.11±3.0	0.26±0.06
6	Sugarcane bagasse+rice straw	197.83±3.5	0.35±0.07
7	Sugarcane bagasse +wheat bran	232.50 ±4.1	0.42±0.03
8	Wheat straw+ rice straw	317.63±4.6	0.34±0.06
9	Wheat straw+wheat bran	461.00±5.4	0.49 ±0.08
10	Rice straw +wheat bran	162.34±8.2	0.57±0.010

± refers standard deviation
Fermentation conditions:
pH :6.4 Incubation period, days: 7
NSS, mL :15 Temperature, ^{0}C : 37

Table 3.9: Effect of nitrogen sources for xylanase production by *Coprinus cinereus* AT-1

Sl. No.	Nitrogen sources	*Coprinus cinereus* AT-1	
		Xylanase activity, IU/mL	Cellulase activity, IU/mL
1	Urea	670.50±10.8	0.82±0.09
2	Peptone	634.88±10.3	0.67±0.07
3	Malt extract	617.22±9.9	0.55 ±0.05
4	Yeast extract	691.40 ±9.5	0.97±0.12
5	Beef extract	660.01 ±9.4	0.740.08
6	Soya bean meal	652.53±9.3	0.75±0.08

± refers standard deviation
Fermentation conditions:
Wheat bran, g : 5 Temperature, ^{0}C : 37
NSS, mL :15 Incubation period, days: 7
pH : 6.4

Table 3.10: Optimization of solid substrate: moisture content for enzyme production by *Coprinus cinereus AT-1*

Sl. No.	Solid substrate: moisture ratio	*Coprinus cinereus* AT-1	
		Xylanase activity, IU/mL	Cellulase ctivity, IU/mL
1	1:2.0	367.50±6.8	0.60±0.09
2	1:2.5	542.00±8.2	0.85±0.12
3	1:3.0	697.90±10.5	0.92 ±0.13
4	1:3.5	490.08±9.6	0.70±0.10
5	1:4.0	315.22±7.7	0.52±0.08

± refers standard deviation
Fermentation conditions:
pH :6.4 Incubation period, days: 7
Wheat bran, g : 5 Temperature, ^{0}C : 37

Table 3.11: Derivation of various physico-chemical parameters for enzyme production by *Coprinus cinereus* AT-1

Sl. No.	Parameters	*Coprinus cinereus* AT-1
1	Incubation period, days	7
2	Incubation temperature, ^{0}C	37
3	Carbon source	Wheat bran
4	Nitrogen source	Yeast extract
5	pH	6.4
6	Solid substrate: moisture content	1:3

Table 3.12: Production of extracellular enzymes by *Coprinus cinereus* AT-1 under optimized conditions

Fungal strain	Xylanase activity, IU/mL	Cellulase activity, IU/mL	Laccase activity, IU/mL	Protein concentration, mg/mL
Coprinus cinereus AT-1	698.75±11.7	1.01±0.15	25.6±3.2	5.7±1.1

± refers standard deviation

Table 3.13: Effect of glucose and lactose on xylanase production by *Coprinus cinereus* AT-1

Sl. No.	Glucose concentration, g/L	Xylanase activity, IU/mL	Lactose concentration, g/L	Xylanase activity, IU/mL
1	0	699.88±11.8	0	697.00±11.5
2	1	611±11.2	1	612.58±11.0
3	2	568.87±114	2	585.10±10.8
4	3	491.44±9.6	3	543.68±8.9
5	4	415.05±8.9	4	430.50±8.7
6	5	344.20±8.0	5	387.12±8.5

± refers standard deviation

Fermentation conditions:

Wheat bran, g : 5 Temperature, ^{0}C : 37

NSS, mL :15 Incubation period, days: 7

pH : 6.4

Table 3.14: pH stabilization of xylanase produced by *Coprinus cinereus* AT-1

Sl. No.	pH	Xylanase activity, IU/mL	Relative xylanase activity, %
1	6.0	468.73±10.6	67
2	6.4	699.60±11.9	100
3	7.0	447.74±10.4	64
4	7.5	391.77±9.3	56
5	8.0	209.88±7.7	30
6	8.5	90.94±4.2	13
7	9.0	41.97±2.1	6

± refers standard deviation

Assay conditions:

pH :6.4 Reaction time, min :30

Substrate concentration for xylanase activity : 10 mg/mL potassium phosphate buffer

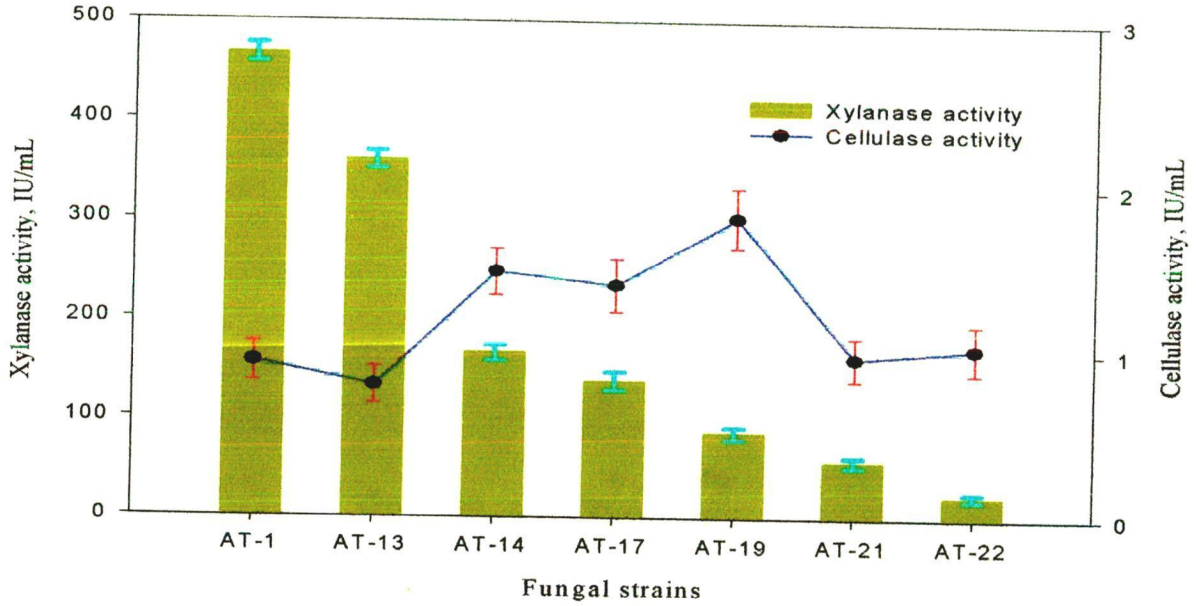


Figure 3.2: Enzyme production from different fungal isolates under SSF condition

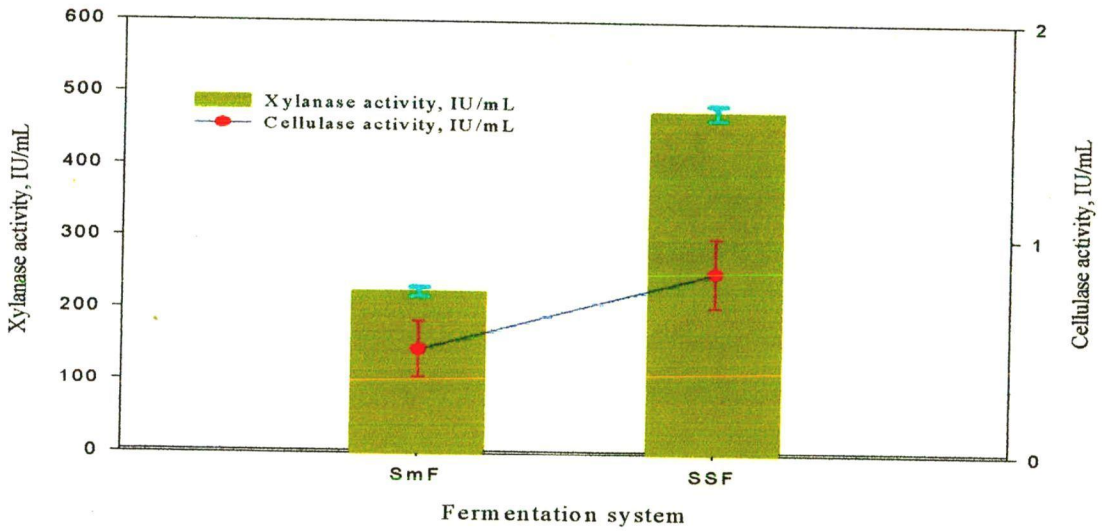


Figure 3.3: Comparison of fermentation systems for enzyme production by *Coprinus cinereus* AT-1

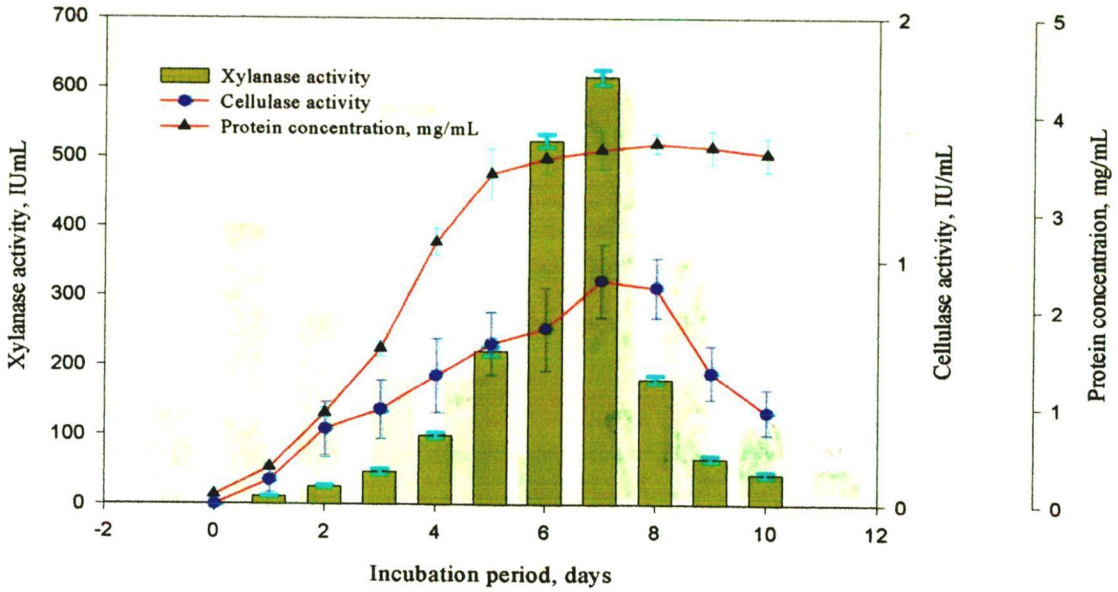


Figure 3.4: Optimization of incubation period for enzyme production by *Coprinus cinereus* AT-1

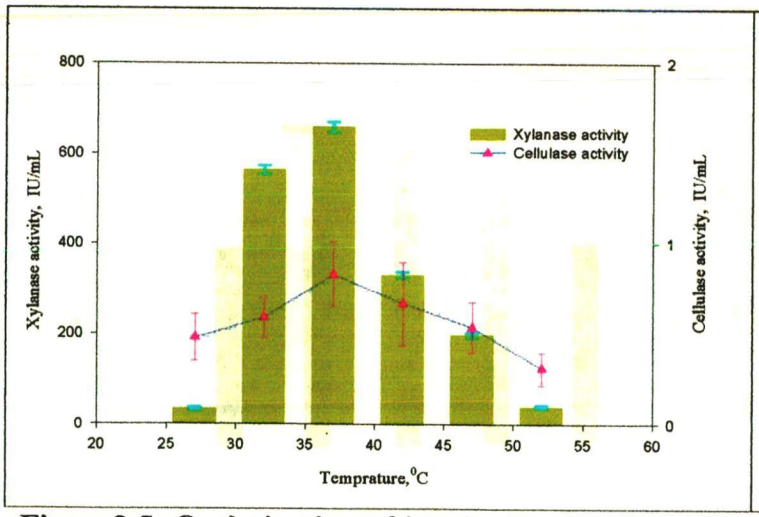


Figure3.5: Optimization of incubation temperature for enzyme production by *Coprinus cinereus* AT-1

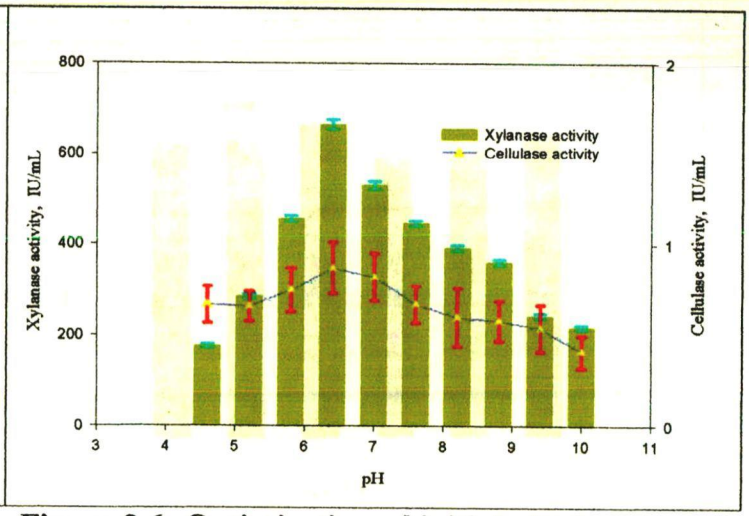


Figure 3.6: Optimization of initial pH for enzyme production by *Coprinus cinereus* AT-1

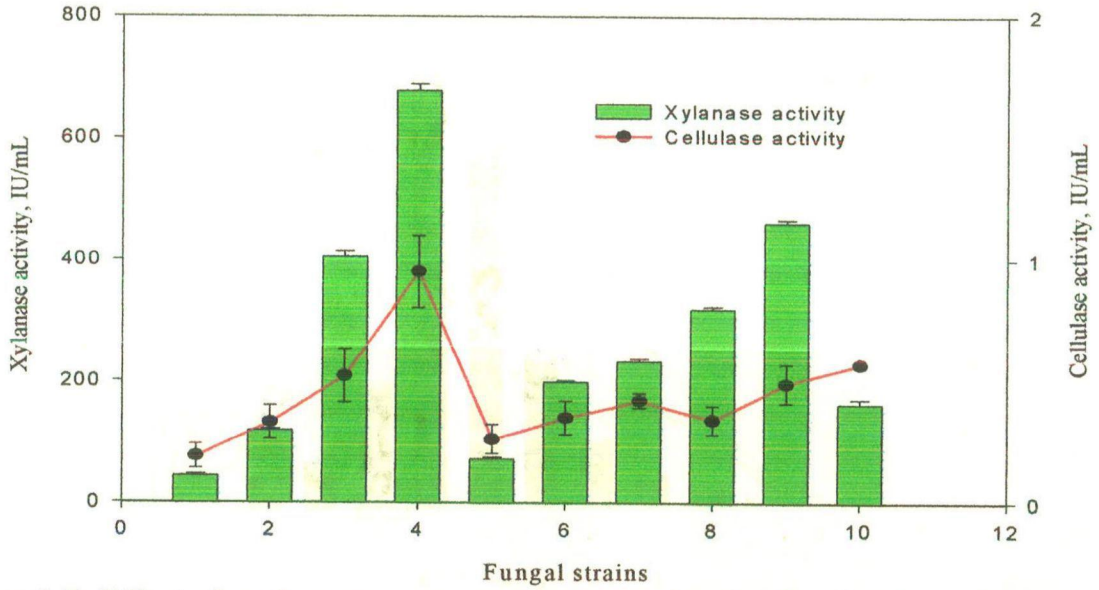


Figure 3.7: Effect of carbon sources on enzyme production by *Coprinus cinereus* AT-1 (1=Bagasse, 2=Wheat straw, 3=Rice bran, 4= Wheat bran, 5= Sugarcane bagasse+wheat straw, 6= Sugarcane bagasse + rice straw, 7= Sugarcane bagasse+wheat bran, 8= Wheat straw+ rice straw, 9= Wheat straw+wheat bran, 10= Rice straw+wheat bran)

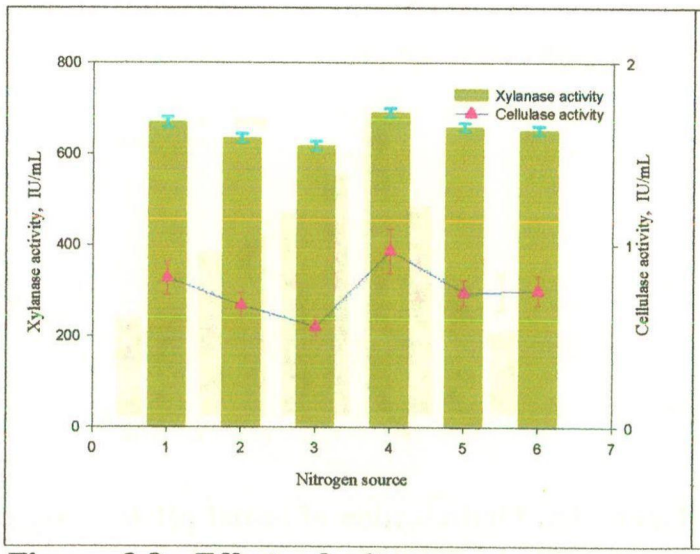


Figure 3.8: Effect of nitrogen sources on enzyme production by *Coprinus cinereus* AT-1(1= Urea, 2= Peptone, 3= Malt extract, 4= Yeast extract, 5= Beef extract, 6= Soya bean meal

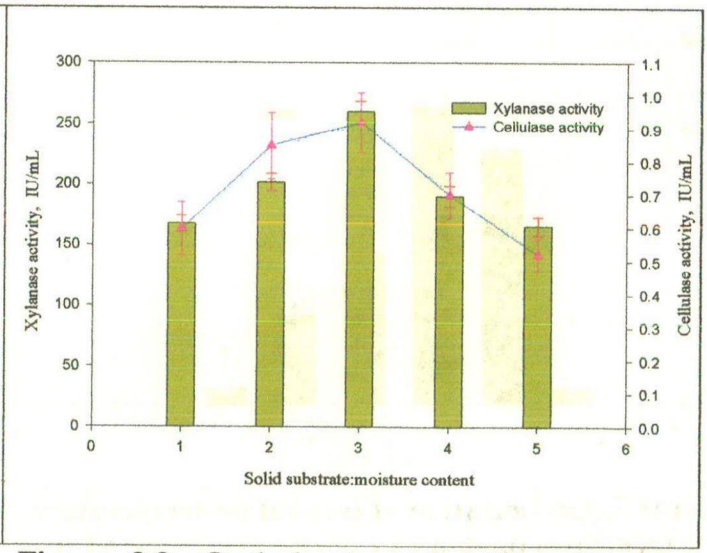


Figure 3.9: Optimization of solid substrate: moisture content for cellulase production by *Coprinus cinereus* AT-1 (1=1:2.0; 2=1:2.5; 3=1:3.0, 4=1:4.0)

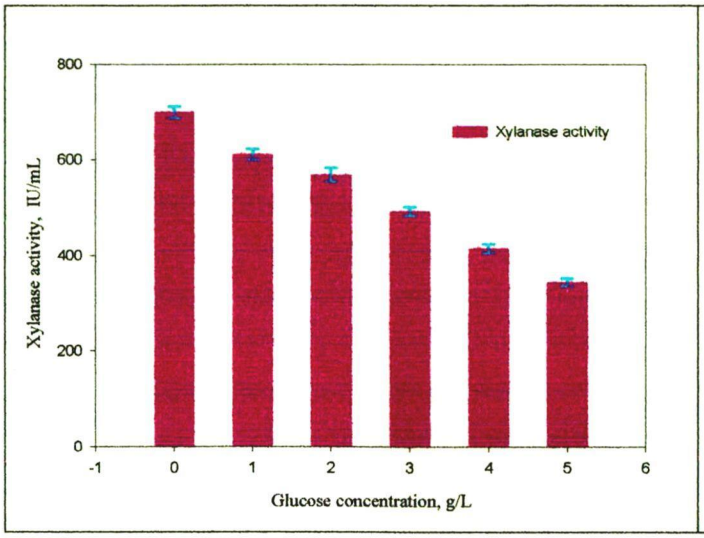


Figure 3.10: Effect of glucose concentration on crude xylanase production by *Coprinus cinereus* AT-1

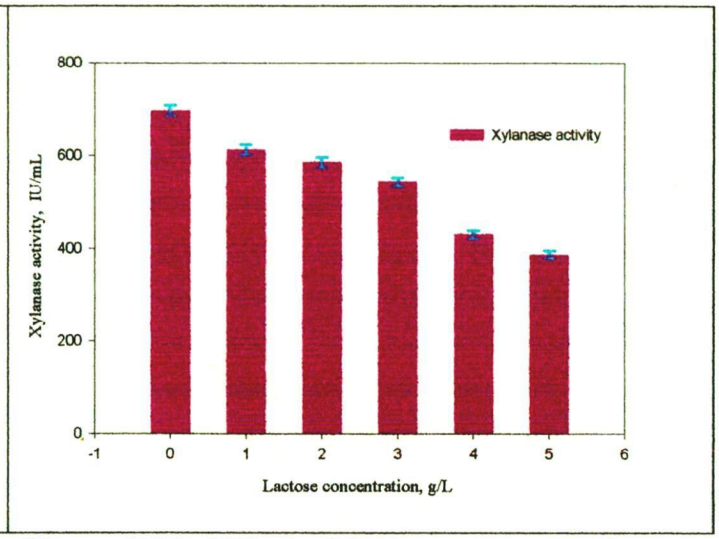


Figure 3.11: Effect of lactose concentration on crude xylanase production by *Coprinus cinereus* AT-1

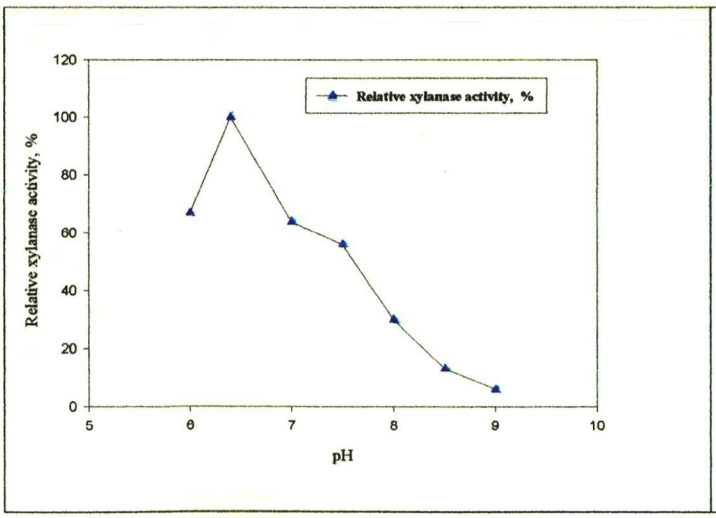


Figure 3.12: pH stability of crude xylanase produced by *Coprinus cinereus* AT-1

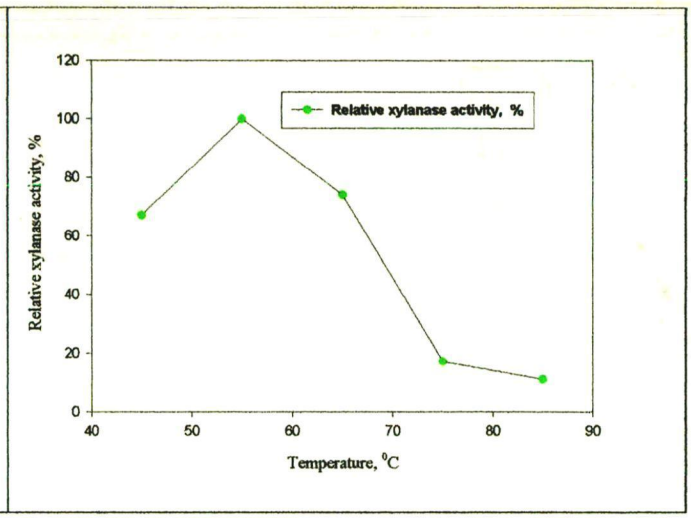
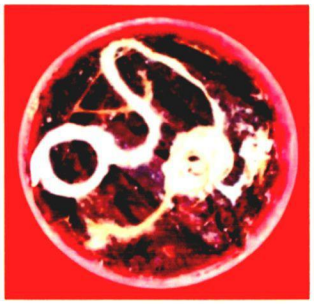
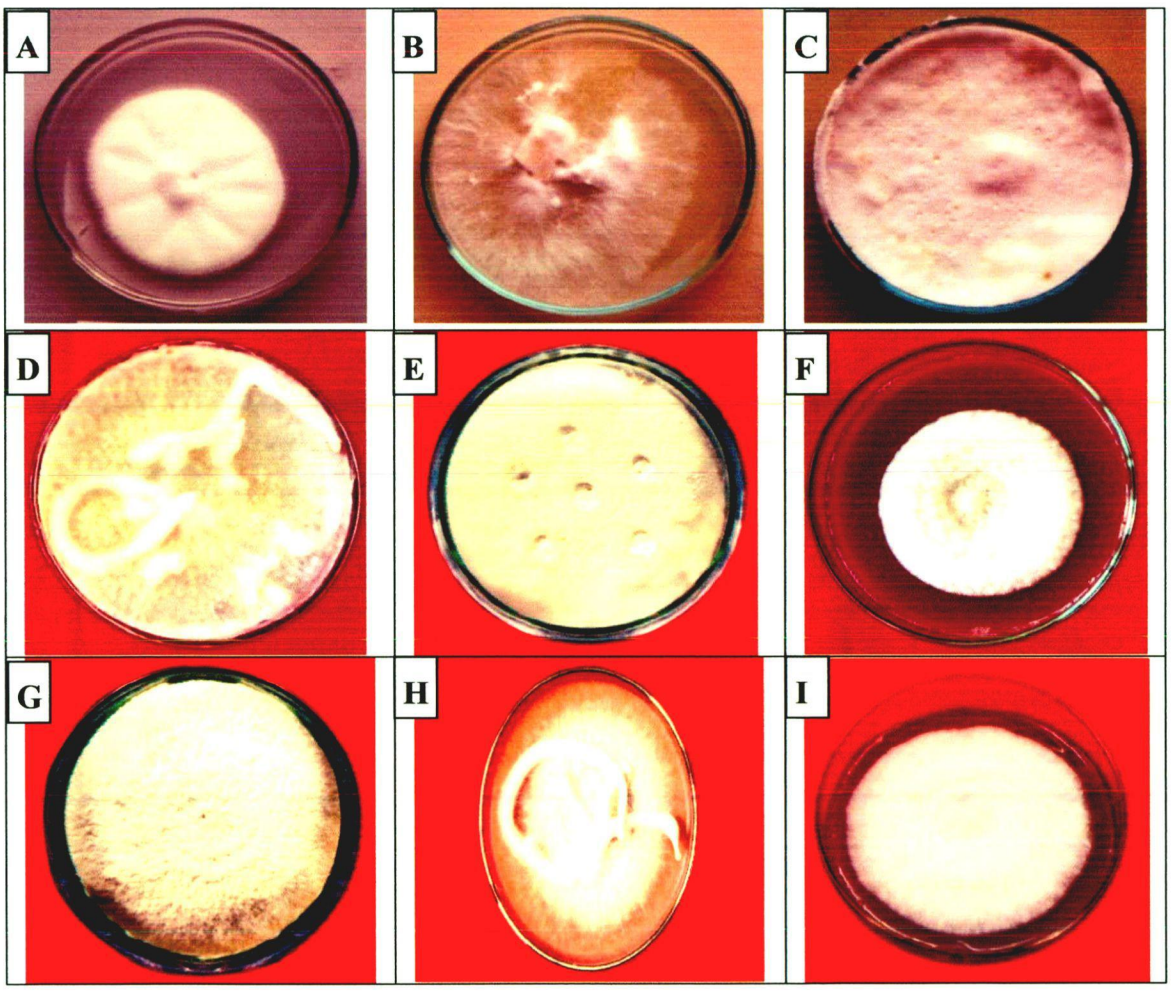


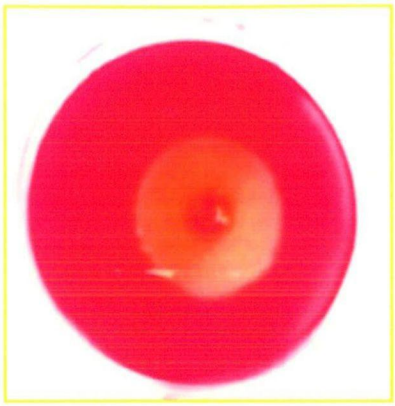
Figure 3.13: Temperature stability of xylanase produced by *Coprinus cinereus* AT-1



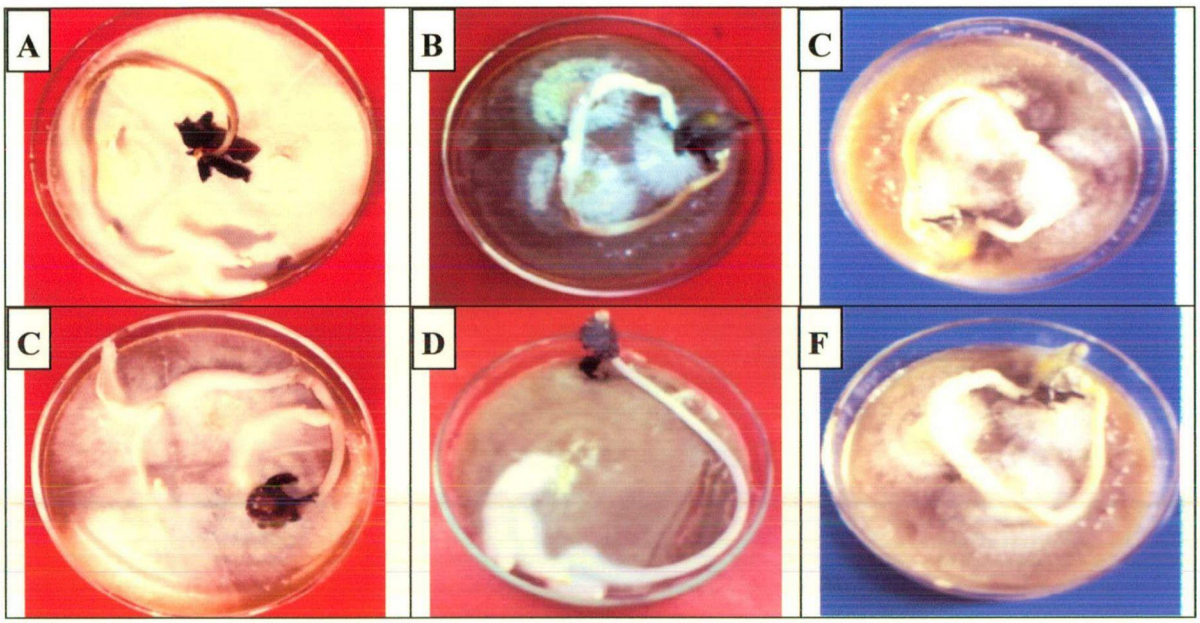
Photograph 3.1: The dead and decaying woods buried in moist wheat bran for isolation of fungal cultures



Photograph 3.2: Morphological features of different isolated fungal strains (A=AT-1, B=AT-13, C=AT-14, D=AT-17, E=AT-18, F=AT-19, G= AT-20, H= AT-21, I= AT-22)

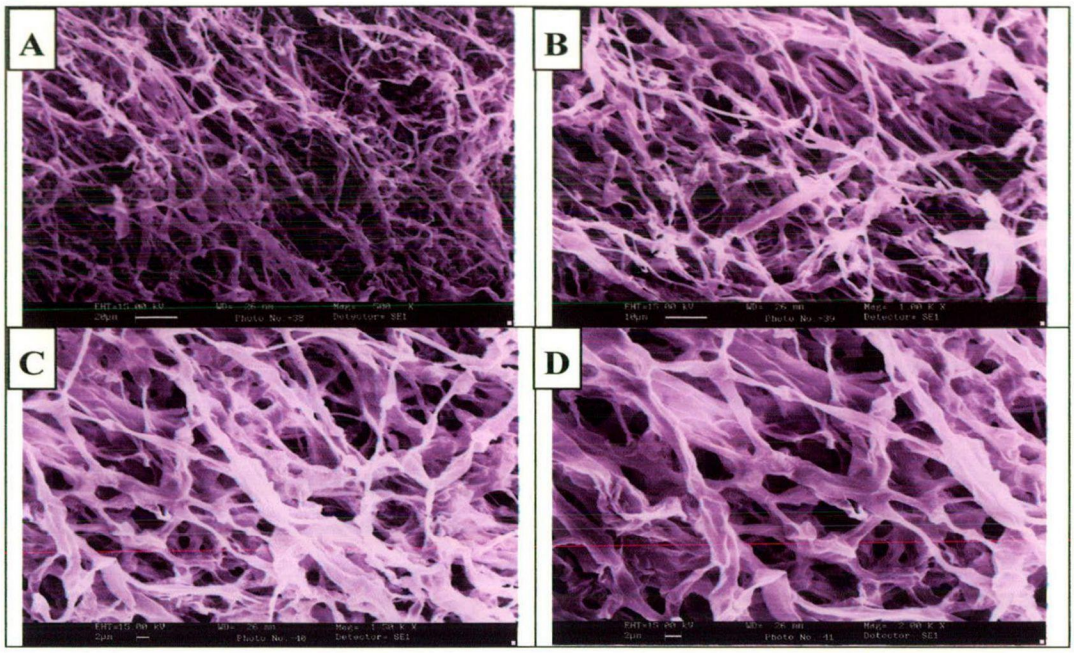


Photograph 3.3 : Plate assay for xylanase produced by *Coprinus cinereus* AT-1

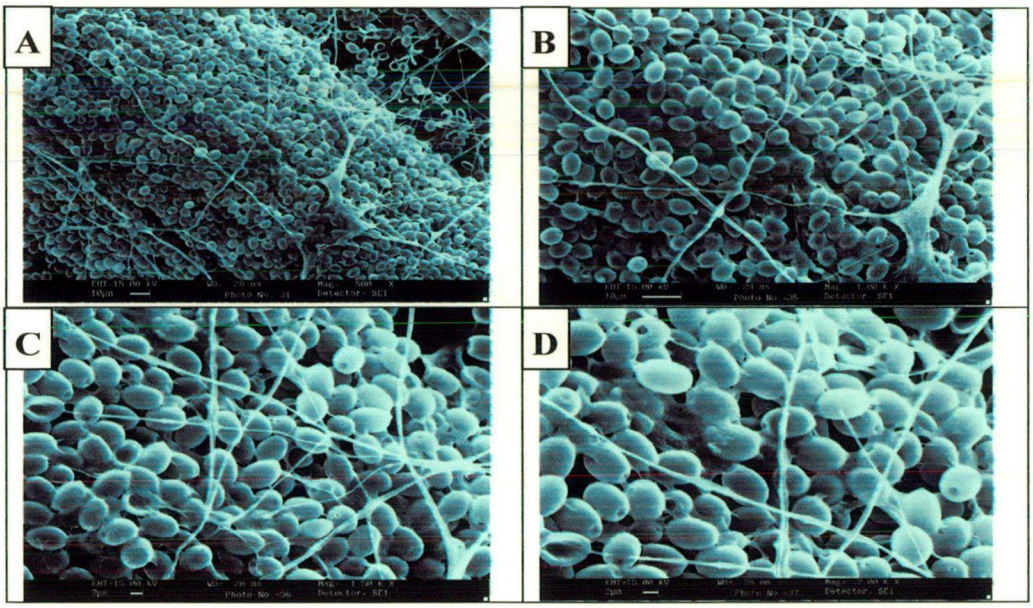


Photograph 3.4: Basidiospore formation among differentfungal isolates (A=AT-1, B=AT-13, C=AT-17, D=AT-19, E=AT-21, F=AT-22)

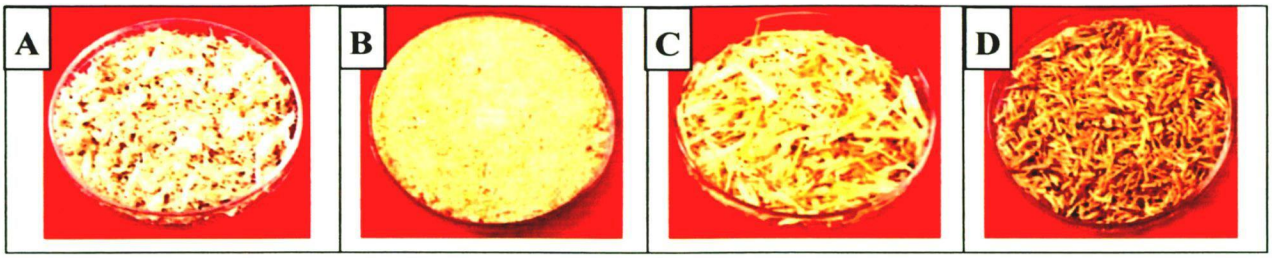
Photograph 3.5: Basidiospore formation of fungal culture *Coprinus cinereus* AT-1



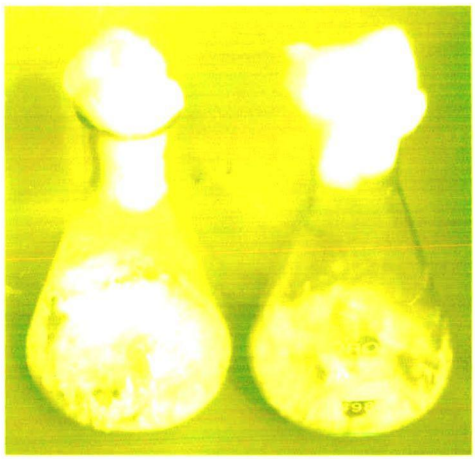
Photograph 3.6: SEM of hyphae of *Coprinus cinereus* AT-1 at a magnification of A= 500X, B= 1.00KX, C= 1.5KX and D= 2.00 KX



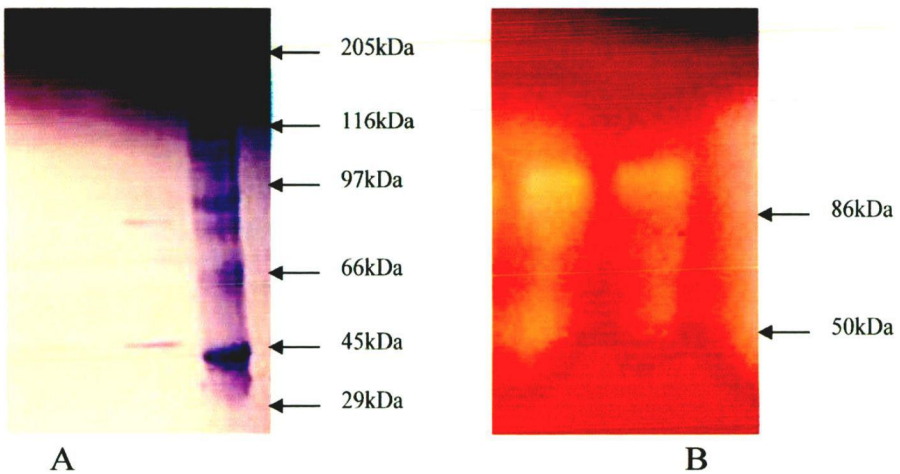
Photograph 3.7: SEM of club-shaped basidiospores of *Coprinus cinere* AT-1 at a magnification of A= 500X, B= 1.00KX, C= 1.5KX and D= 2.00 KX



Photograph 3.8: Different carbon sources used during SSF (A= Sugarcane bagasse, B= Wheat bran, C= Wheat straw and D= Rice straw)



Photograph 3.9: *Coprinus cinereus* AT-1 showing mass enzyme production under SSF



Photograph 3.10: SDS-PAGE (A) and Zymogram (B) of xylanase produced *Coprinus cinereus* AT-1

References

1. Abdel-Raheem, A.M. and Badran, R., Xylanolytic activity of some river Nile aquatic hyphomycetes species, *Egypt. J. Microbiol.*, 33:109-124(1998).
2. Abdel-Sater, M.A. and El-Said, A.H.M., Xylan-decomposing fungi and xylanolytic activity in agricultural and industrial wastes, *Int. Biodeter. Biodegr.*, 47:15-21(2001).
3. Agnihotri, S., Dutt, D., Tyagi, C.H., Kumar, A. and Upadhyaya, J.S., Production and biochemical characterization of a novel cellulase-poor alakali-thermo-tolerant xylanase from *Coprinellus disseminatus* SW-1 NTCC 1165, *World J. Microbiol Biotechnol.*, 26;1349-1359(2010).
4. Aiba, S., Humphrey, A.E. and Millis, N.F., Kinetics, In: *Biochemical engineering*, 2nd Edition *Academic Press*, New York (1973) pp. 92-127.
5. Alam, M., Gomes, I., Mohiuddin, G. and Hoq, M.M., Production and characterization of thermostable xylanase by *Thermomyces lanuginosus* and *Thermoascus auranticus* grown on lignocelluloses, *Enzyme Microbial. Technol.*, 16:298-302(1994).
6. Anthony, T., Chandra Raj, K., Rajendran, A. and Gunasekaran, P., High molecular weight cellulase-free xylanase from alkali-tolerant *Aspergillus fumigatus* AR1, *Enzyme Microbial. Technol.*, 32:647-654(2003).
7. Archana, A. and Satyanarayana, T., Xylanase production by thermophilic *Bacillus licheniformis* A99 in solid-state fermentation, *Enzyme Microbial. Technol.*, 21:12-17(1997).
8. Asha Poorna, C. and Prema P., Production of cellulase-free endoxylanase from novel alkalophilic thermotolerant *Bacillus pumilus* by solid-state fermentation and its application in wastepaper recycling, *Biores. Technol.*, 98:485-490(2007).
9. Babu, K.R. and Satyanarayana, T., Production of bacterial enzymes by solid-state fermentation, *JSIR*, 55:464-467(1996).
10. Baraznenok, V.A., Becker, E.G., Ankudimova, N.V. and Okunev, N.N., Characterization of neutral xylanases from *Chaetomeum cellulolyticum* and their biobleaching effect on eucalyptus pulp, *Enzyme Microbial. Technol.*, 25:651-659(1999).
11. Bastawde, K.B., Xylan structure, Microbiol xylanases and their mode of action, *World J. Microbiol. Biotechnol.*, 8:353-368(1992).

12. Battan, B., Sharma, J. and Kuhad, R.C., High-level xylanase production by alkaliphilic *Bacillus pumilus* ASH under solid-state fermentation, *World J. Microbiol. Biotechnol.*, 22:1281-1287(2006).
13. Battan, B., Sharma, J., Dhiman, S.S. and Kuhad, R.C., Enhanced production of cellulase-free thermostable xylanase by *Bacillus pumilus* ASH and its potential application in paper industry, *Enzyme Microbial. Technol.*, 41:733-739(2007).
14. Beg, Q.K., Bhushan, B., Kapoor, M. and Hoondal, G.S., Enhanced production of a thermostable xylanase from *Streptomyces* sp. QG-11-3 and its application in biobleaching of eucalyptus kraft pulp, *Enzyme Microbial. Technol.*, 27:459-466(2000).
15. Beg, Q.K., Kapoor, M., Mahajan, L. and Hoondal, G.S., Microbial xylanases and their industrial applications: a review, *Appl. Microbiol. Biotechnol.*, 56:326-338(2001).
16. Bennett, N.A., Ryan, J., Biely, P., Vrsanska, M., Kremnicky, L., Macris, B.J., Kekos, D., Christakopoulos, P., Katapodis, P., Claeyssens, M., Nerinckx, W., Ntauma, P. and Bhat, M.K., Biochemical and catalytic properties of an endoxylanase purified from the culture filtrate of *Thermomyces lanuginosus* ATCC 46882, *Carbohydr. Res.*, 306:445-455(1998).
17. Biely, P. and Petrakova, E., Glcosidic bond rearrangement in isomeric xylobioses by yeastxylan- degrading enzymes, *FEBS Lett.*, 178:323-326(1984).
18. Biely, P., Microbial xylanolyic systems, *Trends Biotechnol.*, 3:286-290(1985).
19. Blanchette, R.A. and Abad, A.R., Ultrastructural localization of hemicellulose in birch wood (*Betula papyfera*) decayed by brown and white-rot fungi, *Holzforschung*, 42:393-398(1988).
20. Bragger, J.M., Daniel, R.M., Coolbear, T. and Morgan, H.W., Very stable enzymes from extremely thermophilic archaebacteria and eubacteria, *Appl. Microbiol. Biotechnol.*, 31:556-561(1989).
21. Bronnenmeier, K., Meissner, H., Stocker, S. and Staudenbauer, W.L., α-D-glucuronidases from the xylanolytic thermophiles *Clostridium stercorarium* and *Thermoanaerobacterium saccharolyticum. Microbiol.*, 141:2033-2040(1995).
22. Buchert, J., Tenkanen, M., Kantelinen, A. and Viikari, L., Application of the xylanases in the pulp and paper industry, *Biores. Technol.*, 50:65-72(1994).

23. Cai, J., Wu, K., Zhang, J., He, X. and Pang, R., Production of xylanase by *Aspergillus niger* A3 using solid-state fermentation, *Ind. Microbiol.*, 27:1-4(1997).
24. Carmona, E.C., Pizzironi-Kleiner, A.A., Monteiro, R.T.R., Jorge, J.A., Xylanase production by *Aspergillus versicolor*, *J. Basic Microbiol.*, 38(6):387-394(1997).
25. Chandra, R.K. and Chandra, T.S., Production of cellulase-free xylanase from an alkalo-tolerant *Aspergillus fischeri* Fxn1, *Biotechnol. Lett.*, 17:309-314(1995).
26. Chandra, S., Application of fungal hemicellulases for biobleach process for hardwood kraft pulp, *Ph.D. Thesis,* C.C.S. University Meerut, India (2001) pp.82.
27. Chaudhary, S., Thakur, I.S., Goel, R. and Johri, B.N., Purification and characterization of two thermostable xylanases from *Melanocarpus albomyces*, *Biochem. Int.*, 17:563-575(1988).
28. Chaudhary, P. and Deobagker, D., Purification and characterization of xylanase from *Cellulomonas sp.* N. C. I. M. 2353, *Biotechnol. Appl. Biochem.*, 25:127-33(1997).
29. Chavez, R., Schachter, K., Navarro, C., Peirano, A., Bull, P. and Eyzaguirre, J., The acetyl xylan esterase II gene from *Penicillium purpurogenum* is differently expressed in several carbon sources, and tightly regulated by pH, *Biol Res.*, 37:107-113(2004).
30. Christopher, L., Bisoon, S., Singh, S., Szendefy, J. and Szakacs, G., Bleach-enhancing abilities of *Thermomyces lanuginosus* xylanase produced by solid state fermentation, *Process Biochem.*, 40:3230-3235(2005).
31. Coelho, G.D. and Carmona, E.C., Xylanolytic complex from *Aspergillus giganteus*: production and characterization, *J. Basic Microbiol.*, 43:269-277(2003).
32. Collins, T., Gerday, C. and Feller, G., Xylanases, xylanase families and extremophilic xylanases, *FEMS Microbiol. Rev.*, 29:3-23(2005).
33. Converti, A. and Dominguez, J.M., Influence of temperature and pH on xylitol production from xylose by *Debarryomyces hansenii*, *Biotechnol. Bioeng.*, 75(1):39-45(2001).
34. Daneault, C., Leduc, C. and Valade, J.L., The use of xylanases in kraft pulp bleaching: a review, *TAPPI J.*, 77(6):125-131(1994).
35. Dekker, R.F.H. and Richards, G.N, Hemicellulases: their occurrence, purification, properties and mode of action, *Adv. Carbohydr. Chem. Biochem.*, 32:277-352(1976).
36. Decelle, B., Tsang, A. and Storms, R.K., Cloning, functional expression and

characterization of three *Phanerochaete chrysosporium* endo-1,4-β-xylanases, *Curr. Genet.*, 46:166-175(2004).

37. de la Rosa, A., Ferrús, R., Colom, J.F. and Vidal, T., Effect of metal ions on TCF bleaching of kenaf chemical pulp, In: *Proc. 7th European workshop on Lignocellulosics and Pulp,* Turku, Finland (2002)pp. 36-366
38. de Souza-Cruz, P.B., Freer, J., Siika-Aho, M. and Ferraz, A., Extraction and determination of enzymes produced by *Ceriporiopsis subvermispora* during biopulping of *Pinus taeda* wood chips, *Enzyme Microbial. Technol.*, 34:228-234(2004).
39. de vries, R.P., Poulsen, C.H., Madrid, S. and Visser, J., *aguA*, the gene encoding an extra cellular α-glucuronidase from *Aspergillus tubingensis* is especially induced on xylose and not on glucuronic acid, *J. Bacteriol.*, 180:243-249(1998).
40. de Vries, R.P., Kester, H.C.M., Poulsen, C.H., Bene, J. A. E. and Vissar, J., Synergy between enzymes from *Aspergillus* involved in the degradation of plant cell wall polysaccharide, *Carbohydr. Res.*, 327:401-410(2000).
41. Díaz, M., Rodriguez, S., Fernández-Abalos, J.M., Rivas,.J.D.L., Ruiz-Arribas, A., Shnyrov, V. L. and Santamaría, R.I., Single mutations of residues outside the active center of the xylanase Xys1Δ from *Streptomyces halstedii* JM8 affect its activity, *FEMS Microbiol. Lett.*, 240, 237-243(2004).
42. Dhillon, A., Gupta, J.K., Jauhari, B.M. and Khanna, S., A cellulase poor, thermostable, alkalitolerant xylanase produced by *Bacillus circulans* AB 16 grown on rice straw and its application in biobleaching of eucalyptus pulp, *Biores. Technol.*, 73:273-277(2000).
43. Erikson, K.E.L., Blanchette, R.A. and Ander, P., Microbiol and enzymatic degradation of wood and wood components, First edition, *Springer*, Berlin(1990).
44. Fahn, A. and Leshem, B., Wood fibers with living protoplast, *New Phytol.*, 62:91-98(1963).
45. Fang, H.Y., Chang, S.M., Hsieh, M.C. and Fang, T.J., Production, optimization growth conditions and properties of the xylanase from *Aspergillus carneus* M34, *J. Mol. Catal. B-Enzym.*, 49:36-42(2007).
46. Fengel, D. and Wegener, G., Reactions in alkaline medium, In: Fengel, D. & Wegener, G. (Eds.), Wood chemistry, ultrastructure, reactions, *Walter de Gruyter, Berlin/New York,* pp. 296-318(1984).

47. Feniksova, R.V., Tikhomrova, A.S. and Rakhleeva, B.E., Conditions for forming amylase and proteinase in surface culture of *Bacillus subtilis*, *Mikrobiologica.*, 29:745-748(1960).

48. Flores, M.E., Perea, M., Rodriguez, O., Malvaez, A. and Huitron, C., Physiological studies on induction and catabolite repression of β-xylosidase and endoxylanase in *Streptomyces* sp. CH-M-1035, *J. Biotechnol.*, 49: 179-187(1996).

49. Gabriel, B.L., *In: Biological scanning electron microscopy,* Von Nostrand Reinhold Company, New York, (1982) pp.186

50. Gao, J., Weng, H., Zhu, D.,Yuan, M., Guan, F.and Xi, Y., Production and characterization of cellulolytic enzymes from the thermoacidophillic fungal *Aspergillus terreus M11* under solid state cultivation of corn stover, *Biores. Technol.*, 99: 7623(2009).

51. Gawande, P.V. and Kamat, M.Y., Poduction of *Aspergillus* xylanase by lignocellulosic waste fermentation and its application, *J. Appl. Microbiol.*, 87:511-519. (1999).

52. Gervais, P., Abadie, C. and Molin, P., Fungal turger pressure is directly involved in the hyphal growth rate, *Microbial. Res.*, 154:81-87(1999).

53. Gessesse, A. and Mamo, G., High level xylanase production by an alkalophilic *Bacillus* sp. by using solid-state fermentation, *Enzyme Microbial. Technol.*, 25:68-72(1999).

54. Ghatora, S.K., Chadha, B.S., Badhan, A.K., Saini, H.S. and Bhat, M.K., Identification and characterizarion of diverse xylanases from thermophilic and thermototerant fungi, *Biores. Technol.*, 1(1):18-33(2006).

55. Ghosh, M., Das, A., Mishra, A.K. and Nanda, G., *Aspergillus sydowii* MG 49 is a strong producer of thermostable xylanolytic enzymes, *Enzyme Microbial. Technol.*, 15:703-709 (1993).

56. Godden, D., Legon, T., Helvenstein, P. and Penninckx, M., Regulation of the production of hemicellulolytic and cellulolytic enzymes by a *Streptomyces* sp. growing on lignocellulose, *J. Gen. Microbiol.*, 135:285-292. (1989).

57. Gomes, J., Purkarthofer, H., Hayn, M., Kapplmuller, J., Sinner, M. **and** Steiner, W., Production of a high level cellulase-free xylanase by the thermophilic fungus *Thermomyceslanuginosus* in laboratory and pilot scales using lignocellulosic materials, *Appl. Microbiol. Biotechnol.*, 39:700-707(1993).

58. Gupta, N., Reddy, V.S., Maiti, S. and Ghosh, A., Cloning expression and sequence analyses of thegene encoding the alkali stable, thermostable endoxylanase from alkalophilic, mesophilic *Bacillus* sp strain NG 27, *Appl. Environ Microbiol.*, 66:26-31(2000).

59. Hamzah, A. and Abdulrashid, M., Characterization of xylanase produced by *Bacillus pumilus* strain PJ19, *World J. Microbiol. Biotechnol.*, 9:157-162(1999).

60. Highly, T.L., Changes in chemical components of hardwood by brown-rot fungi, *Mater. Org.*, 22:39-45(1987).

61. Hölker, U., Höfer, M. and Lenz, J., Biotechnological advantages of laboratory-scale solid state fermentation with fungi, *Appl. Microbiol. Biotechnol.*, 64:175-186(2004).

62. Hrmova, M., Beily, P. and Vrsanka, M., Cellulose and xylan degradation, *Enzyme Microbiol. Technol.*, 11: 610-616(1989).

63. Ikram-ul-Haq, Khan, A., Butt, W.A., Ali, S. and Qadeer, M.A., Effect of carbon and nitrogen sources on xylanase production by mutant strain of *Aspergillus niger* GCBMX-45, *Online J. Biol. Sci.*, 2(2):143-144(2002).

64. Inglis, G.D., Popp, A.P., Selinger, L.B., Kawchuk, L.M., Gaudet, D.A. and McAllister, T. A., Production of cellulases and xylanases by low-temperature basidiomycetes, *Can. J. Microbiol.*, 46(9):860-865(2000).

65. James, P.D.A., Edwerds, C. and Dwason, M., The effects of temperature, pH and growth rate on secondary metabolism in *Streptomyces thermoviolaceus* growth in a chemostat., *J. Gen. Micobiol.*, 137:1715-1720(1991).

66. Jecu, L., Solid state fermentation of agricultural wastes for endoglucanase production, *Ind. Crops Prod.*, 11:1-5(2000).

67. Joshi, M.D., Sidhu, G., Nielsen, J.E., Brayer, G.D., Withers, S.G. and McIntosh, L.P., Dissecting the electrostatic interactions and pH-dependent activity of a family 11 glycosidase, *Biochemistry*, 40:10115-10139(2001).

68. Kapoor, N., Studies on the microbial production of xylanase by *Aspergillus niger*, *Ph.D.thesis*, IndianInstitute of Roorkee, Roorkee, pp.97(2005).

69. Khandeparker, R.D.S. and Bhosle, N.B., Isolation, purification and characterization of thexylanase produced by *Arthrobacter* sp. MTCC 5214 when grown in solid-state fermentation, *Enzyme Microbial Technol.*, 39:732-742(2006).

70. Kirk, K. and Cullen, D., Enzymology and molecular genetics of wood degradation by white rot fungi, In: Young, R.A. & Akhtar, M. (Eds), Environmental friendly technologies for pulp and paper industry, *Wiley, New York*, (1998) pp.273-307.

71. Kohli, U., Nigam, P., Singh, D. and Chaudhary, K., Thermostable, alkalophilic and cellulose-free xylanase production by *Thermoactinomyces thalophilus* subgroup C, *Enzyme Microbial. Technol.*, 28:606-610(2001).

72. Krishna, C., Solid-state fermentation systems – an overview, *Crit. Rev. Biotechnol.*, 25:1-30(2005).

73. Kubacková, M. and Váradi, S.K., Studies on xylanase from basidiomycetes, *Folia Microbiol.*, 20:29-37(1975).

74. Kuhad, R.C. and Singh, A., A lignocellulose biotechnology: current and future prospects, *Crit. Rev. Biotechnol.*, 13:151-172(1993).

75. Kuhad, R.C., Singh, A. and Eriksson, K.E.L., Microorganisms and enzymes involved in the degradation of plant fibre cell wall, *Adv. Biochem. Engg/Biotechnol.*, 57:47-125(1997).

76. Kulkarni, N., Shendye, A. and Raom., Molecular and biotechnological aspects of xylanases. *FEMS Microbiol. Rev.*, 23:411-456(1999).

77. Laemmli, U.K., Cleavage of structural proteins during the assembly of the head of bacteriophage T4, *Nature.*, 227:680-685(1970).

78. Lama, L., Calandrelli, V., Gambacorta, A. and Nicolaus, B., Purification and characterization of thermostable xylanase and β-xylosidase by the thermophilic bacterium *Bacillus thermantarcticus. Res. Microbiol.* ,155:283-289(2004)

79. Lappalainen, A., Sikka-Aho, M., Kalkleinen, N., Fagerstorm, R. and Tenkenen, M., Endoxylanase II from *Trichoderma reesei* has several isoforms with different isoelectric points, *Biotechnol. Appl. Biochem.*, 31:61-68. (2000).

80. Leathers, T.D., Kurtzman, C.P. and Detroy, R.W., Overproduction and regulation of xylanase in *Aureobasidium pullulans* and *Cryptococcus albidus, Biotechnol Bioeng.*, 14:225-250(1984).

81. Lequart, C., Nuzillard, J.M., Kurek, B. and Debeire, P., Hydrolysis of wheat bran and strawby an endoxylanase: production and structural characterization of cinnamoyl-

oligosaccharides, *Carbohydr. Res.*, 319:102-111(1999).

82. Li, K., Azadi, P., Collins, R., Tolan, J., Kim, J. and Eriksson, K., Relationships between activities of xylanases and xylan structures, *Enzyme Microbial Technol.*, 27:89-94(2000).
83. Li, X.T., Jiang, Z.Q., Li, L.T., Yang, S.Q., Feng, W.Y., Fan, J.Y. and Kusakabe, I., Characterization of a cellulase-free, neutral xylanase from *Thermomyces lanuginosus* CBS 288.54 and its biobleaching effect on wheat straw pulp, *Biores. Technol.*, 96:1370-1379(2005).
84. Lin, L.L. and Thomson, J. A., An analysis of the extracellular xylanases and cellulases of *Butyrivibrio fibrisolvens* H17c, *FEMS Microbiol. Lett.*, 84:197-204. (1991).
85. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J., Protein measurement with the Folin-Phenol reagents, *J. Biol. Chem.*, 193:265-275(1951).
86. Luthi, E., Jasmat,N.B. and Berquist, P.L., Xylanase from the extremely thermophilic bacterium *Caldocellum saccharolyticum* over expression of the gene in *Escherichia coli* and characterization of the gene product. *Appl. Environ. Microbiol.*, 56:2677-2683(1990).
87. Maat, J., Roja, M., Verbaker, J., Stam, H., Bosse, M. and Rotterdam, S., Xylanases and their application in bakery. In : *Xylans and Xylanases*, Vissa, J., Beldman, G., Kusters-van M. A. and Voragen A. G. J. (editors), Elsevier Amsterdam, 346-360(1992).
88. Maheshwari, R., Bharadwaj, J. and Bhat, M.K., Thermophilic fungi: their physiology and enzymes, *Microbiol. Mol. Biol. Rev.*, 64:461-488(2000b).
89. Malarvizhi, K., Murugesan, K. and Kalaichelvan, P.T., Xylanase production by *Ganoderma lucidum* on liquid and solid state fermentation, *Ind. J. Exp. Biol.*, 41: 620-626(2003).
90. Mandels, M., Microbial sources of cellulases, *Biotechnol. Bioengg. Symp.*, 5:81-105(1975).
91. Milagres, A.M.F., Santos, E., Piovan, T. and Roberto, I.C., Production of xylanase by *Thermoascus aurantiacus* from sugarcane bagasse in an aerated growth fermentor, *Process Biochem.*, 39:1387-1391(2004).
92. Milagres, A.M.F., Maghalhães, P.O. and Ferraz, A., Purification and properties of a xylanase from *Ceriporiopsis subvermispora* cultivated on *Pinus taeda*, *FEMS Microbiol.*

Lett., 253:267-272(2005).

93. Miller, G.L., Use of dinitrosalicylic acid reagent for determination of reducing sugar, *Anal. Chem.*, 31:426–428(1959).
94. Muthezhilan, R., Ashok, R. and Jayalakshmi, S., Production and optimization of thermostable alkaline xylanase by *Penicillium oxalicum* in solid state fermentation, *Afr. J. Microbiol. Res.*, 3(12): 20-28(2007).
95. Nagai, K., Sakai, T., Rantiatmodjo, R.M., Suzuki, K., Gams, W. and Okada, G., Studies on the distribution of alkalophilic and alkali-tolerant soil fungi 1, *Mycoscience,* 36:247-256(1995).
96. Narahara, H., Koyama, Y., Yoshida, T., Pichangkura, S., Ueda, R. and Taguchi, H., Growth and enzyme production in a solid-state culture of *Aspergillus oryzae*, *J. Ferment. Technol.*, 60:311-319(1982).
97. Nigam, K.P., Singh, D. and Chaudhary, K., Thermostable alkalophilic and cellulase free xylanase production by *Thermoactinomyces thalpophilus* subgroup C, *Enzyme Microbial. Technol.*, 28:606-610(2001).
98. Nielsen, P., Mathrani, I.M. and Ahring, B.K., *Thermoanaerobium acetigenum* spec. nov., a new bacterium isolated from an icelandic hot spring. *Arch. Microbiol.*, 159:460-464. (1993).
99. Ninawe, S. and Kuhad, R.C., Use of xylan-rich cost effective agro-residues in the production of xylanase by *Streptomyces cyaneus* SN32, *J. Appl. Microbiol.*, 99:1141-1148(2005).
100. Otjen, L., Blanchette, R.A., Effland, M.G. and Leatham, G., Assessment of 30 white-rot basidiomycetes for selective lignin degradation, *Holzforschung*, 41:343-349(1987).
101. Panagiotou, G., Kekos, D., Macris, B.J. and Christakopoulos, P., Production of cellulolytic and xylanolytic enzymes by *Fusarium oxysporum* grown on corn stover in solid state fermentation, *Ind. Crops Prod.*, 18:37-45(2003).
102. Pandey, A., Selvakumar, P., Soccol, C.R. and Nigam, P., Solid state fermentation for the production of industrial enzymes, *Curr. Sci.*, 77:149-162(1999).
103. Pandey, A., Soccol, C.R. and Mitchell, D., New developments in solid state fermentation: part 1- bioprocess and products, *Process Biochem.*, 35:1153-1169(2000).
104. Pandey A., Soccol, C.R., Rodriguez-Leon J.A. and Nigam, P., Production of organic

acids by solid state fermentation, In: *Solid state fermentation in biotechnology – fundamentals and applications*, Pandey, A., Soccol, C.R., Rodriguez-Leon J.A. & Nigam, P. (Eds.),*Asiatech publishers Inc.*, New Delhi (2001) pp.113-126.

105. Park, Y.S., Kang, S.W., Lee, J.S., Hong, S.I. and Kim, S.W., Xylanase production in solid state fermentation by *Aspergillus niger* mutant using statistical experimental designs, *Appl. Microbiol. Biotechnol.*, 58:761-766(2002).
106. Pham, L.P., Taillandier, P., Delmas, M. and Strehaiano, P., Production of xylanases by *Bacillus polymyxa* using lignocellulosic wastes, *Ind. Crops Prod.*, 7: 195-203 (1998).
107. Phutel, R.P., Bhadauria, A., Sodhi, H.S. and Kapoor, S., Screening of Chinese mushroom (*Volvariella* sp.) strains for cellulases and xylanases production, *Indian J. Microbiol.*, 36(3):125-128(1996).
108. Polizeli, M.L.T.M., Rizzatti, A.C.S., Monti, R., Terenzi, H.F., Jorge, J.A. and Amorim, D.S., Xylanases from fungi: properties and industrial applications, *Appl. Microbiol. Biotechnol.*, 67:577-591(2005).
109. Prabhu, K.A. and Maheshwari, R., Biochemical properties of xylanases from a thermophilic fungus, *Melanocarpus albomyces* and their action on plant cell walls, *J. Biosci.*, 24:461-470(1999).
110. Prade, R.A., Xylanases: from biology to biotechnology, *Biotech. Genet. Eng. Rev.*, 13:100-131(1995).
111. Qinnghe, C., Xiaoyu, Y., Tiangui, N., Cheng, J. and Qiugang, M., The screening of culture condition and properties of xylanase by white-rot fungi *Pleurotus ostreatus*, *Process Biochem.*,39:1561-1566(2004).
112. Raimbault, M. and Alazard, D., Culture method to study fungal growth in solid fermentation, *Eur. J. Appl. Microbiol. Biotechnol.*, 9:199-209(1980).
113. Ramesh, M.V. and Lonsane, B.K., Regulation of alpha-amylase production in *Bacillus licheniformis* M27 by enzyme-end products in submerged fermentaion an dits overcoming in solid state fermentation system, *Biotechnol. Lett.*, 13:355-360(1991).
114. Record, E., Asther, M., Sigoillot, C., Pages, S., Punt, P.J, Haon, M., van den Hondel, C.A., Sigoillot, J.C., Lesage-Meessen, L. and Asther, M., Overproduction of the *Aspergillus niger* feruloyl esterase for pulp bleaching applications, *Appl. Microbiol.*

Biotechnol., 62:349-355(2003).

115. Remond, C., Plantier-Royon, R., Aubry, N., Maes, E., Bliard, C. and O'Donohue, M. J., Synthesis of pentose containing disaccharides using a thermostable α-L-arabinofuranosidase. *Carbohydr. Res.*, 339(11):2019-2025(2004).
116. Reyes, L.M. and Noyola, T.P., Isolation of hyperxylanolytic *Cellulomonas flavigena* mutant growing on continuous culture on sugarcane bagasse, *Biotechnol. Lett.*, 20:443-446(1998).
117. Rezende, M.I., Barbosa, A.M., Vasconcelos, A.F.D. and Endo, A.S., Xylanase production by *Trichoderma harzianum rifai* by solid state fermentation on sugarcane bagasse, *Braz. J. Microbiol.*, 33:1-11(2002).
118. Rizzatti, A.C.S., Jorge, J.A., Terenzi, H.F., Rechia, C.G.G.V. and Polizeli, M.L.T.M., Purification and properties of a thermostable β-D-xylosidase produced by a thermotolerant *Aspergillus phoenicis*, *J. Ind. Microbiol. Biotechnol.*, 25:156-160(2001).
119. Rujtier, G.J.G. and Visser, J., Carbon repression in *Aspergilli, FEMS Microbiol. Lett.*, 151:103-114(1997).
120. Sanchez, S., Bravo,V., Castro, E., Moya, A.J.and Camacho, F., The influene of pH and areation rate on the fermentation of D-xylose by *Candida shehatae, Enzyme Microbial. Technol.*, 21:355-360(1997).
121. Sanghi, A., Garg, N., Sharma, J., Kuhar, K., Kuhad, R.C. and Gupta, V.K., Optimization ofxylanase production using inexpensive agro-residues by alkalophilic *Bacillus subtilis* ASH in solid state fermentation, *World J. Microbiol. Biotechnol.*, 24:633-640(2008).
122. Saraswat,V. and Bisaria,V.S., Biosynthesis of xylanolytic and xylan debranching enzymes in *Melanocarpus albomyces* IIS 68, *J. Ferment. Bioengg,* 83, 352-57(1997).
123. Scandurra, R., Consalvi, V., Chiaraluce, R., Politi, L. and Engel, P.C., Protein thermostability in extremophiles, *Biochimie.*, 80:933-941(1998).
124. Schulze, E., Information regarding chemical composition of plant cell membrane, *Berichte der Deutschen Chemischem Gesellschoft.*, 24:2277-2287(1891).
125. Schwarz, W.H., Jausis, S. and Kouba, M., Cloning and expression of *Clostridium stercorarium* cellulase genes in *Escherichia coli, Biotechnol. Lett.*, 461-466(1989).
126. Senthilkumar, S.R., Ashok kumar, B., Raj, B.C. and Gunasekaran, P.,Optimization of

medium composition for alkali-stable xylanase production by *Aspergillus fischeri* Fxn 1 in solid-state fermentation using central composite rotary design, *Biores. Technol.* 96:1380-1386(2005).

127. Seyis, I. and Aksoz, N., Xylanase production from *Trichoderma harzianum* 1073 D3 with alternative carbon and nitrogen source, *Food Technol. Biotechnol.*, 43(1):37-40(2005a).
128. Seyis, I. and Aksoz, N., Effect of carbon and nitrogen sources on xylanase production by *Trichoderma harzianum* 1073 D3. *International Biodeterioration and Biodegradation.*, 55:115-119(2005b).
129. Singh, J. and Garg, A.P., Production of cellulases by *Gliocladium virens* on *Eichhorinia* under solid-state fermentation condition, *J. Indian bot. Soc.*, 74:305-309(1995).
130. Singh, S., du Preez, J.C. Pillay, B. and Prior, B.A., The production of hemicellulases by *Thermomyces lenuginosum* strain SSBP: influence of agitation and dissolved oxygen tension, *Appl. Microbiol. Biotechnol.*, 54:698-704(2000).
131. Singh, S., Madlala, A.M. and Prior, B.A., *Thermomyces lanuginosus*: properties of strains and their hemicellulases, *FEMS Microbiol. Rev.*, 27:3-16(2003).
132. Singh, S., Pillay, B. and Prior, B.A., Thermal stability of β- xylanases produced by different *hermomyces anuginosus* strains, *Enzyme Microbial. Technol.*, 26:502-508(2003).
133. Singh, S., Tyagi, C.H., Dutt, D. and Upadhyaya, J.S., Production of high level of cellulase poor xylanases by wild strains of white rot fungus *Coprinellus disseminates* in solid-state fermentation, *New Biotechnol.*, 26:165-170(2009).
134. Shah, A.K., Sidid, S.S., Ahmad, A. and Rele, M.V., Treatment of bagasse pulp with cellulase-free xylanase from an alkalophilic *Bacillus* sp. Sam-3, *Biores. Technol.*, 68:133-140(1999).
135. Shah, A.K., Cooper, D., Adolphson, R. and Eriksson, K-E.L., Xylanase treatment of oxygen-bleached hardwood kraft pulp at high temperature and alkaline pH levels gives substantial savings in bleaching chemicals, *JPPS,* 26(1):8-11(2000).
136. Shah, A.R. and Madamwar, D., Xylanase production by a newly isolated *Aspergillus foetidus* strain and its characterization, *Process Biochem.* 40:1763-1771(2005).

137. Shankaranand, V.S. and Lonsane, B.K., Ability of *Aspergillus niger* to tolerate metal ions andminerals in solid state fermentation system for the production of citric acid, *Process Biochem.*,29:29-37(1994).

138. Shao, W., De Blois, S. and Wiegel, J.A., high molecular weight, cell-associated xylanase isolated from exponentially growing *Thermoanaerobacterium* sp. strain JW/SI-YS485, *Appl. Environ. Microbiol.* 61, 937-940(1995).

139. Solis-Pereira, S., Favela-Torres, E., Viniegra-Gon-zalez, G. and Gutirrez-Rojas, M., Effect of different carbon sources on th esynthesis of pectinases by *Aspergillus niger* in submerged and absorbed substrate fermentation, *Appl. Microbiol.Biotechnol.*, 39:36-41(1993).

140. Souza, D.F., Souza, C.G.M. and Peralta, R.M., Effect of easily metabolizable sugars in the production of xylanase by *Aspergillus tamari* in solid-state fermentation, *Process Biochem.*, 36:835-838(2001).

141. Srinivasan, M. C. and Rele, M. V., Microbial xylanase for paper industry, *Curr. Sci.77*, 137-142(1999)..

142. Srivastava, R. and Sriyastava, A.K., Characterization of a bacterial xylanaseresistant to repression by glucose and xylose, *Biotechnol. Lett.*, 15: 847-852(1993).

143. Stevens, B. J. H. and Payne, J., Cellulose and xylanase production by yeast of the genus *Trichosporon, J. Gen. Microbiol.*, 100:381-393(1977).

144. Subramaniyan, S. and Prema, P.,Cellulase free xylanases from Bacillus and other microorganisms, *FEMS Micobiol. Lett.* 183, 1-7. (2000).

145. Subramaniyam, S. and Prema, P., Biotechnology of microbial xylanases: enzymology, molecular biology and application, *Crit. Rev. Biotechnol.*, 22:33-64(2002).

146. Sunna, A., Antranikian, G., Xylanolytic enzymes from fungi and bacteria, *Crit. Rev. Biotechnol.*, 17:39-67. (1997).

147. Sun, X., Zhang, R. and Zhang, Y., Production of lignocellulolytic enzymes by*Trametes gallica* and detection of polysaccharide hydrolase and laccaseactivities in polyacrylamide gels, *Journal of Basic Microbiology*, 44: 220-231(2004).

148. Szakacs Dobozi, M., Szakacs, G. and Bruschi, C.V., Xylanase activity of *Phanerochaete chrysosporium, Appl. Environ. Microbiol.*, 3466-3471(1992).

149. Szendefy, J., Szakacs, G. and Christopher, L., Potential of solid-state fermentation enzymes of *Aspergillus oryzae* in biobleaching of paper pulp, *Enzyme Microbial. Technol.*, 39:1354-1360(2006).

150. Taneja, K., Gupta, S. and Kuhad, R.C., Properties and application of a partially purifies alkaline xylanase from an alkalophilic fungus *Aspergillus nidulans* KK-99, *Biores. Technol.*, 85:39-42(2002).

151. Teater, R.M. and Wood, P.J., Use of Congo red-polysaccharide interactions in enumeration and characterization of cellulolytic bacteria from the bovine rumen, *Appl. Environ. Microbiol.*, 43:777-780(1982).

152. Techapun, C., Charoenrat, T., Watanabe, M., Sasaki, K. and Poosaran, N., Optimization of thermostable and alkaline-tolerant cellulase-free xylanase production from agricultural waste by thermotolerant *Streptomyces* sp. Ab106, using the central composite experimental design, *Biochem. Eng. J.*, 12:99-105(2002).

153. Techapun, C., Pooraran, N., Watanabe, M. and Sasaki, K., Thermostable and alkaline-tolerant microbial cellulase free xylanases produced from agricultural wastes and the properties required for use in pulp bleaching bioprocesses: a review, *Process Biochem.*, 38: 1327-1340(2003).

154. Tengerdy, R.P., Solid state fermentation for enzyme production, In: *Advances in biotechnology*, Pandey, A. (Ed.), Educational publishers and distributions, New Delhi, (1998) pp. 13-16.

155. Tenkanen, M., Puls, J. and Poutanen, K., Two major xylanases of *Trichoderma reesei, Enzyme. Microbiol Technol.*, 14:566-574(1992).

156. Topakas, E., Stamatis, H., Biely, P., Kekas, D., Macris, B.J. and Christakopoulos, P., (2003a). Purification and characterization of a feruloyl esterase from *Fusarium oxysporium* catalyzing esterification of phenolic acids in ternary water-organic solvent mixtures, *J. Biotechnol.*, 102: 33-44.

157. Topakas, E., Stamatis, H., Biely, P., Kekas, D., Macris, B. J. and Christakopoulos, P., Purification and characterization of a *Fusarium oxysporium* feruloyl esterase (Fo FAE-I) catalyzing transesterification of phenolic acid esters, *Enzyme Microbial. Technol.*,

33:729-737(2003b).

158. Tseng, C.-P. and Montville, T.J., Enzymatic regulation of glucose catabolism by Lactobacillus plantarum in response to pH shifts in a chemostat, *Appl. Microbiol. Biotechnol.*, 36:777-787(1992).

159. Uffen R.L., Xylan degradation: a glimpse at Microbiol diversity, *J. Ind. Microbiol. Biotechnol.*, 19:1-6(1997).

160. Villena, G.K.and Gutierrez-Correa,M., Morphological patterns of *Aspergillus niger* biofilmsand pellets related to lignocelluolytic enzyme productivities, *Lett. Appl. Microbiol.*, ISSN0266- 8254(2007).

161. Virupakshi, S., Babu, K.G., Gaikwad, S.R. and Naik, G.R., Production of xylanolytic enzyme bythermoalkaliphilic *Bacillus* sp. JB-99 in solid state fermentation, *Process Biochem.*, 40: 43135(2005).

162. Vishniac, W. and Santer, M., The Thiobacilli, *Bacteriol Rev.*, 21(3):195-213(1957).

163. Williamson, G., Faulds, C.B. and Kroon, P.A., Hairy plant polysaccharides: a close shave with Microbiol esterases, *Microbiol.* 144, 2011-2023. (1998a).

164. Williamson, G., Faulds, C.B. and Kroon, P.A., Specificity of ferulic and esterases. *Biochem. Soc. Trans.* 26, 205-209. (1998b).

165. Wong, K.K.Y., Tan, L.U.L. and Saddler, J.N., Multiplicity of β-1,4-xylanases in microorganisms: functions and applications, *Microbiol. Rev.*, 52:305-317(1988).

166. Wong, K.K.Y. and Saddler, J.N., *Trichoderma* xylanases, their properties and application, *Crit. Rev Biotechnol.*, 12:413-435(1992).

167. Young, R.A. and Akhtar, M., Enzymology and molecular genetics of wood degradation by white-rot fungi, In: *Environmentally friendly technologies for the pulp and paper industry*, Young, R.A. & Akhtar, M. (Eds.), John Wiley & Sons, Inc., New York (1998) pp. 273-308.

168. Yoshioka, H., Nagato, N., Chavanich, S., Nilubol, N. and Hayashida, S., Purification and properties of thermostable xylanase by *Talaromyces byssochlamydoides* YH-50, *Agric. Biol. Chem. J.*, 45:2425- 2432(1981).

169. Yu, E.K.C., Tan, L.U.L., Chan, M.K.-H., Deschatelets, L. and Saddler, J.N., Production of a thermostable xylanase by a thermophilic fungus, *Thermoascus aurantiacus*, *Enzyme*

CHAPTER 4

DEINKING STUDIES ON SORTED OFFICE WASTE PAPER

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4.1: Introduction

With the swift development of office automation and information; the consumption of office paper has been rising day by day. The incessantly growing paper manufacturing industry imposes a severe demand on green plants that forms the basic raw material. The galloping rise of fibrous raw materials and waste disposal problems are leading all over the world, to augment the use of recycled fibers for the paper industry. Most of the paper manufacturing steps utilize hazardous chemicals (like alkali, chlorinated bleaching agents, reducing agents, surfactants, defoamers, deinking agents, sizing agents, binders, colorants and different additives), resulting harmful impact on the environment. Pulp and paper industry is heavily exploiting natural resources like timber, water and energy. Every metric tonne of recycled fiber saves an average of 17 tree plus related energy. For every metric tonne of paper used for recycling, the savings are at least 30,000 liters of water, 3000-4000 kWh of electricity and 95% of air pollution. It also saves around 2.3m^3 landfill volumes. Papermakers are focusing on recycling as an economic obligation [85]. Worldwide more than one third of the paper is made from recycled fibers [206].

Paper recycling offers several advantages over virgin paper making process. Paper made from recycled paper requires 28 to 70% less energy consumption than virgin paper and less use of water during processing. Higher grade paper can be recycled several times, providing environmental saving every time. Waste paper pulp requires less refining energy than virgin pulp and may also be co-refined with hardwood pulp or mixed hardwood and softwood pulp without any significant damage. The paper made from waste paper has improved opacity, less curling tendency, less fuzziness and better formation as compared to paper made from wood pulp [13]. Deinking is one of the most fundamental steps in waste paper recycling. Waste paper has turned out to be a significant source of fibers in pulp and paper industry throughout the world [215]. The three major sources of raw material for such recycling are newsprint, photocopying paper and inkjet printed papers. Consequently, the technologists are looking forward to develop the novel, eco-friendly way of recycling the waste paper [174]. The research of bio-deinking technology has opened up a new way for paper de-inking [57, 89,127, 158,159 215]. Preliminary work on sorted office waste paper (SOP) indicated that enzyme- enhanced deinking is

economically and technically feasible on heterogeneous furnishes. Many researchers have reported the use of diverse enzymes during the eco-friendly deinking process, such as cellulases, hemicellulases, pectinases, amylases and ligninases [10, 75]

4.1.1: Processing of recycled paper

For manufacturing writing and printing grades, efficient deinking of the waste paper is an indispensable operation of the paper making process. Unutilized mixed office waste (MOW) is an inexpensive source of high quality bleached chemical fiber [84]. Despite of the greater availability of the MOW, their use in recycling industry to produce higher grade pulps is very much restricted because of bigger visible specks and contaminants in end products hence, it is considered to be one of the most difficult raw materials for deinking [71,179].

Various prevailing techniques such as conventional froth flotation and washing are available for deinking of secondary fibers. Flotation is one of the most momentous steps in deinking and controlled by the physiochemical and hydrodynamic properties of the pulp. The choice of method depends on type of the ink to be removed and the desired quality of pulp. The mechanical dispersion (steam explosion inclusive) and chemical agglomeration of ink particles, followed by screening and forward cleaning techniques, such as pressure screens and centrifugal cleaners, are the main approaches to improve the deinking effectiveness of toners-printed office papers [32,154]. Other methods like heat decolourization [59], irradiation [124], organic solvent-based deinking [60], ultrasound [189] and magnetic deinking [71] are still under experimental conditions. Most of the conventional deinking techniques require large amount of chemical agents [179, 224], resulting in a costly wastewater treatment to meet the environmental regulations [177]. Recycling generates large quantity of sludge and effluents with high oxygen demanding material [9].

Further, in order to solve problems pertaining to adhesion of foreign matter (stickies), fiber damage, effluent load, there is a need for expansion of an environmentally friendly deinking method that improves the current deinking technology which generate a heavy environmental burden. Enzyme assisted deinking (Bio-deinking) has been shown to represent a potential eco- friendly option to conventional alkaline deinking process [10, 11, 12, 89, 109, and 118]. Enzyme based deinking offers a potential way for reducing chemicals used in conventional process thus reducing the load on waste water treatment system [136]. Drainage enhancement is acknowledged to be a secondary benefit of enzymatic deinking [87,175,176 186]. In addition, it

is also found to reduce the deinking effluent toxicity and chemical oxygen demand load [107]. The most promising implication of high deinking efficiency from enzyme –enhanced deinking is that the dewatering and dispersion steps as well as subsequent reflotation and washing may not be essential. As a result this save capital expenses in construction of deinking plants while also reducing consumption of electrical energy used in dewatering and dispersion [10].

4.1.2: Deinking process

Deinking is the process of removing ink and other contaminants to imprinted-paper. According to Marchildon *et al.,* [126] deinking has improved since the description filed in first patent by Koops in 1800. De-inking efficiency depends on the technique and printing conditions, kind of ink and printing substrate [1, 23,168]. Deinking is a two-step process. In the first step, the ink particles are detached from the fibers (difiberization), using thermochemi-mechanical action (at high temperature) with the presence of chemical in the pulper. In the second step, the ink particles detached from fibers are removed from the pulp suspension in flotation cell by froth flotation. For the detachment of ink particles from the fibers, the presence of shearing forces is necessary. The four mechanical stages of deinking are detachment, redposition, suspension, and froth flotation. Detachment occurs mostly during pulping, when shear forces, caustic swelling, and elevated temperatures cause the bound ink to break in to fragments and to separate from the fibers. Redposition, a highly undesirable effect, can also occur during pulping. Ink may be readsorbed onto the fiber surface, or it may enter the fiber lumens during alkaline swelling. Ink redposition during pulping is largely irreversible, and tends to increase with increasing pulping time and consistency [63,64, 68]

4.1.3: Pulping operation

Pulping is decisive operation in deinking because in this stage, ink is removed from fibers and the particles size is controlled. A pulper is operated under alkaline conditions (pH 7 -11) and at temperature (45-75 ^{0}C). The strong agitation in the pulper creates friction among the fibers and generates hydrodynamic forces, both of which promote separation of ink particles from the fiber surface. Bennington *et al.,* [17] have stated that pulping may be achieved by batch and continuous process. Shear forces are created when the two ends of paper are caught in stock flows moving in different directions. The flow pattern creates a vortex around the rotor and baffles are used to improve mixing. Lassus [114] indicated that when ink particles have detached from the fibers and dispersed into the aqueous phase, a risk of redposition on the fibers surface

exists if the colloidal stability of the ink particles is not sufficiently high. Holik [81] Bennington *et al.,* [17] and Carre *et al.,* [34] had described pulping operation in detail.

4.1.4: Flotation operation

In the flotation operation, the ink particles detached from fibers are removed from the suspension by froth flotation. During flotation, hydrophobic or hydrophilic particles are converted into hydrophobic by the action of surface active agents [100, 196]. The flotation cell operations are greatly affected by the various operating conditions including, types of raw material, consistency, flotation time, types and size distribution of inks present, mineral filler types and concentration of ink collection chemicals. The chemistry and theory of flotation deinking is well covered in the given literatures [42, 55, 191, 196, 234] have indicated that flotation technique is generally used for removal of ink particles and more than 65% of the industries follow this practice for deinking flotation process. According to Catsburg [36], consistency is the most significant parameter in controlling the efficiency of a flotation cell. The optimal consistency varies with the design of individual units, but is usually in the range 0.8 - 1.2%. Air is introduced in to a diluted fiber suspension of 0.8-1.2% consistency. The water repelling ink particles attach to the air bubbles and rise to the surface. J. Saint *et al.,* [98] and Johansson *et al.,* [94] reported in their studies that the pH is another key parameter in flotation. They obtained maximum flotation yield in a pH range of 7-11, the reason being that agglomeration was favored by a high pH, while the contact between air bubbles and the ink agglomerates is favored by a low pH.

4.1.5: Flotation mechanism

In order to explain how ink particles agglomerate and attach to the air bubbles, Beneventi *et al.,* [16] proposed several models for flotation deinking. Olson *et al.,* [153], Larsson *et al.,* [113] and Liphard *et al.,* [119] have reported in their studies that two of the most important features for particle flotation are their size and the hydrophobicity of the particle surface. J. saint *et al.,* [100] have described that the principles of flotation and deinking are similar to those of conventional mineral flotation. Turvey [211] indicated that ink particles are more hydrophobic than paper fibers, which is the basis of separation by flotation. However, some fibers can be hydrophobic and hydrophilic. Galland *et al.,* [63] have reported that the flotation deinking processes are primarily involved in the removal of small ink particles for improving brightness, but particles in the visible range such as those produced by laser print and varnished papers are

also eliminated by flotation. According to J. Saint *et al.,* [99], considering the flotation mechanisms of particle-bubble collision attachment and detachment, consistency increase is assumed to increase the detachment probability of the large particles in relation to the turbulence level and bubble size distribution and to have almost no effect on small particles integrated in the bubble surface. Serres [192] and Bender *et al.,* [15] have reported in their studies about a patented process for deinking paper to optimize the flow of air bubbles introduced into the pulp slurry. The flotation stage is based upon a stream of air bubbles re-circulated through the pulp ink particles, which are carried along on the bubbles. A counter current flow of water also is re-circulated and eventually carries away ink-laden bubbles, separating the clean fibers and fillers. The combination of low and high-intensive flotation stages offer to improve the removal of stickies and ink particles during recycling

4.1.6: Role of ink particle size in deinking

Many researchers [81, 95,111] have indicated that the ink particle size is important in determining ink removal efficiency by flotation cell. Flotation removes particles that are too small to be removed by screening and cleaning and yet are too big to be removed by washing. Results presented by McCool [131] show that each technique (including washing and flotation) efficient for the removal of contaminant is in a defined size range. Washing efficiency is the best at a particle size range of 1-10 μm, flotation efficiency at 10-150 μm, cleaning equipment at 100-1000 μm and screening at 1,000 μm and above. As in general, the printing ink particles can be easily agglomerated (50-150μm) during pulping operation by the addition of proper chemicals, their removal is easier by flotation method. Borchardt [26], Beneventi *et al.,* [16] and Azevedo *et al.,* [8] have reported in their studies that in the flotation process, if the particle size is too small, the particles inertia is negligible and it tends to follow streamlines around the air bubble. This leads to a smaller probability of collision with an air bubble and therefore, the particle has less of a chance to be captured. Pundir *et al.,* [180] have indicated that in the flotation deinking operations, progression involves the agglomeration of small ink particles. Very small particles (<10 μm) tend to diffuse into the fibers resulting in a gray finished sheet hence; they are not preferred for flotation. Ink which is not properly broken up during pulping may produce particles greater than 200μm which are too large to be removed efficiently by flotation. Lassus [114] have stated that originally the basic size of ink particles, carbon black and pigments was 0.02- 0.1μm. Johansson *et al.,* [95] have reported that some ink particles remain as primary

particles throughout the entire deinking process. These ink particles are not removed because they are, too small to float without being agglomerated.

4.1.7: ERIC (Effective residual ink concentration)

Many researchers [132, 133, 162] have reported in their studies that ERIC values have been determined for repulped pulp (from hydrapulper) and deinked pulp obtained after flotation. The basic purpose of ERIC calculation is to determine how much residual ink remains in the sample of deinked paper. The unit of ERIC values is in parts per million (ppm). This is accomplished by appraise the reflectance's in the infra red region of spectrum (950nm) and manipulating reflectance's via Kubelka Munk analysis until the ERIC is computed. Technibrite Micro ERIC 950 from *Technidyne Corporation, New Albany, Indian, USA* fitted with an IR filter uses this principle to endow with values of ERIC in sheets of paper. Reflectance measurements of paper sheets at about 950 nanometer (nm) can provide a useful assessment of ERIC particles in the paper sheet. ERIC also depends on the kind of ink particles, size of the ink particles, the dispersion or agglomeration of the ink and the distribution of the ink in the sheet of paper. In spite of these limitations, ERIC shows a better correlation with ink removal in a deinking operation than the brightness.

4.1.8: Deinkability factor

Several researchers [41, 42, 45] have reported in their studies that the deinking ability of printed paper depends mainly on three factors: ink formulation, printing conditions and paper surface. Many researchers [34, 40] in their studies have stated that the efficiency of a deinking operation may be defined as the ratio of amount of ink removed by flotation cell, to the amount of ink present in the pulp before deinking by the flotation cell. Deinkability factor based on ERIC values (D_E,) for any process where ink particles have been removed, is defined as:

$$D_E, \% = \frac{E_P - E_F}{E_P - E_B} \times 100 \qquad \text{..... (1)}$$

Where:

E_B = ERIC value in the absence of ink particles (blank),
E_F = ERIC value after flotation deinking
E_P = ERIC value of the sample sheet before ink removal (after pulping)
D_E = Deinkability factor based on ERIC value, %.

Deinkability factor based on hand-sheet brightness after pulping and after flotation is used to evaluate process efficiency and establishes the optimum pulping and flotation deinking conditions. The ISO brightness (special reflectance factor R_{457}) of the unprinted paper subjected to the same disintegration and flotation conditions is considered as a reference value. Therefore, global efficiency of the deinking process would be defined by the following deinkability factor based on ISO brightness:

$$D_B, \% = \frac{B_F - B_P}{B_B - B_P} \times 100 \quad \text{.....} \quad (2)$$

Where:
B_P = Brightness after pulping (ISO %),
B_F = Brightness after flotation (ISO %)
B_B = Brightness of the sample paper without the presence of ink particles (blank) (ISO %)
D_B = Deinkability factor based on ISO brightness, %.

4.1.9: Enzymes for pulp and paper industry

It is well known fact that microbial systems manage their chemistry rather more efficiently than man-made chemical plants. Thus, the selected enzymes are more advantageous than chemicals for the desired modification of the pulp and very prominent in papermaking process. Enzymes are extensively used in deinking technology for increasing the recyclability of secondary fibre and in pulp treatment to improve paper properties and facilitate papermaking through enhanced pitch control [221]. Several microbial enzymes are useful for developing eco-friendly technologies for paper and pulp manufacturing. These enzymes includes cellulases for enzymatic deinking [72 89, 91 215], xylanases [214], mannanases [152] for biobleaching of pulp, and enhanced drainage rates [200], laccases and ligninases for delignification of pulp [30], lipases, esterases for pith removal in the pulp and deinking of waste papers [140] and pectinases for various fiber handling operations and enzymatic retting of flax fibers [2, 51]. The increased demand for microbial lipases can be owed to their specificity of action, mild conditions required for reaction, stereospecificity, and less energy consumption [97]. Commercial enzyme preparations can replace conventional deinking chemicals to remove toner inks [89]

4.10: Enzymatic deinking mechanism

As a matter of fact, the problem of understanding enzymatic deinking of waste paper is that the enzyme treatment is preceded by disintegration and followed by a cleaning operation. Therefore, it is difficult to determine the exact role of the enzyme. Enzymatic approaches of

deinking involve attacking either the ink or fiber surfaces. Deinking involves dislodging ink particles from fiber surfaces and then separating the dispersed ink from the fiber suspension by washing or flotation. Zeyer *et al.,* tried to explain fully the fiber- ink-enzyme interaction and how enzyme enhances the deinking operation and under what condition enzymes work best when performing the specific task [232]. Korean researchers pointed out that enzyme partially hydrolyze and depolymerize cellulose between fibers, making them free from one another [107]. Pommier *et al.,* [175] described the enzymatic action as a "peeling effect". These authors suggested that enzymes defibrillate fibers by removing molecules with high water affinity but with a small contribution to the overall hydrogen bonding potential of the fibers. This reduction in pulp-water interactions allows better drainage of the pulp without affecting the final mechanical properties of the paper. Enzyme specificity must also be considered. As shown by Pere *et al.,* [170], endo and exoglucanases affect paper technical properties differently. Nine different possible mechanisms for enzymatic deinking have been reported by Welt and Dinus [219]. Enzymes can be active in one way or in combination of the following ways (i) they hydrolyze cellulose to water soluble sugars (ii) they partly hydrolyze and depolymerize the cellulose molecules on the fiber surface by which the bonds among the fibers weakened because of the removal of surface layers (iii) cellulose causes peeling on the fiber surface (iv) hemicellulose destroys the complex lignin-cellulose and (v) cellulose fibers are removed by the impact of enzymes on hairy particles which increases the hydrophobic ability of toner. Cellulases bind and alter the fiber surface or bonds of the fiber present in the vicinity of the ink particles, resulting in the removal of small fibers from the surface of ink particles [197]. The differences in enzyme activity are attributed to the different modes of action. Endoglucanases are more active on amorphous cellulose and randomly attack the inner part of the cellulosic chain, whereas exoglucanases can hydrolyze both crystalline and amorphous cellulose by removing cellobiose from the terminal part of the cellulose chains [78]. Xia *et al.,* [226] reported that cellulases are mainly confined to fiber surface rather than extending into fiber walls without affecting fiber length distribution and individual fiber strength. Hemicellulases facilitate the deinking by the action on lignin-carbohydrate complex thereby releasing the ink particles from fiber surfaces. Endoglucanase initiate their action by splitting the cellulose fibers into several amorphous sites and generates innumerous reducing ends, which supports the loosening and releasing the ink particles from the recycled papers [72,215, 219]. Lipases are enzymes having surfactant

properties, catalyzes the hydrolysis of acyl glycerols at the oil-water interface, thereby facilitate the deinking of recovered papers. Lipases and esterases can degrade vegetable-oil based inks. Except that α-amylases influence the coatings on paper. Pectinases, hemicellulases, cellulases, and lignolytic enzymes alter the fiber surface or bonds in the vicinity of the ink particles, thereby facilitating the ink for removal by washing or flotation [10]. On the other hand laccase acts on lignin component of the fibers [10,141]

4.1.11: Enzymes application in deinking

Many patents for the use of enzymes in deinking have been recommended or applied [219]. Commercial use of enzymes for deinking has started recently in the United States [50]. The choice of enzymes to be used depends on the type of the ink to be removed as well as the type of the pulp. Microbial cellulases, [89, 215) hemicellulases [137, 138], amylases [46, 239] and lipases [140] have shown promising results in deinking.

Cellulases and hemicellulases are the principal enzymes used for fiber modification. The first reported use of cellulases for deinking of secondary fibers was with newsprint and white ledger in 1991[49]. Researchers at the Forest Products Laboratory have demonstrated that cellulase used in combination with surfactants and the mechanical action on medium consistency repulping effectively remove toners from laser and xerographic papers and reduce the residual particle counts with increasing pulp brightness and freeness [88, 89, 90]. Several researchers have proposed the application of cellulases to facilitate eco-friendly deinking process [46, 89, 177, 178, 187, 198]. Even though, some researchers [56, 76, 107] have shown that cellulases are the key to toner ink removal, but Klungness and Sykes, found that some of the most effective commercial deinking enzyme preparations which include combinations of cellulases and hemicellulases [110]. Cellulases [202] and hemicellulases are used in fiber modification, for example to improve beating, enhance drainage, improve certain strength properties and remove the ink from fibers during deinking [46, 127]. Deinking with hemicellulases has benefit of improved brightness of pulp due to its bio-bleaching effect. Hemicellulases have proven useful in deinking of newsprint wastepaper. Amylases are also important in bleaching and brightening processes [46, 239]. Prasad *et al.,* [177] carried out an enzyme treatment on newsprint at 3% consistency, pH 5.5 and observed a marked increase in brightness. Woodward *et al.,* [224] demonstrated a similar effect using cellulases with newsprint in a recycling system. Oltus *et al.,* [155] investigated enzymatic hydrolysis of various types of waste papers to reverse hornification

and regain the fiber surface activity of the original fibers. Pommier *et al.*, [175] have provided the most definitive evidence of strength enhancement of secondary fibers by enzymatic treatment. It has been demonstrated [89, 91] that commercial acidic or alkaline cellulase preparations can effectively remove and disperse laser and xerographic toners and enzymes appear to be promising for practical application in office paper deinking. Using a neutral cellulase as a post-treatment to a standard alkaline chemical treatment, additional brightness and greater ink removal are achieved [57]. Treatment with a pure alkaline cellulase significantly improves brightness level of photocopier and laser printed papers relative to pulping in water without enzymes [178]. Low-pH cellulase and hemicellulase mixtures have been evaluated for deinking of letterpress and color offset-printed newsprint at pH 5.5 [177, 178]. Paik [158] reported that the hemicellulases from *Aspergillus niger* and cellulases from *Trichoderma virdae* increases pulp brightness with increasing enzyme dosage and reaction time. An optimal blend of cellulase and hemicellulase give higher brightness gains than conventional deinking. Enzymatically deinked letter press-printed newspaper pulp has lower initial brightness values than conventionally deinked pulps However, subsequent bleaching with hydrogen peroxide produces similar brightness values, with lowest peroxide usage for the enzymatic process [185]. Similar observations were reported by Putz *et al.*, for enzymatically deinked offset-printed newspaper [181]. Nakano has reported and claimed that an alkaline lipase efficiently removed offset-printing inks [147]. Enzymes that catalyze the removal of surface lignin may hold promise for deinking of newsprint that contains a proportion of lignin-rich mechanical pulp. This approach has been evaluated using white-rot fungi *Phanerochaete chrysosporium* and with lignin-degrading enzymes, which shows that the enzyme-treated pulps showed higher brightness and were easier to bleach as compared to conventional deinked pulp [29].

The present work aims at applying extracellular enzymatic extracts (crude xylanase and cellulases) produced from *Coprinus cinereus* AT-1 and *A. niger* AT-3 respectively in deinking trials of SOP. The present chapter deals with conventional, enzymatic and chemi-enzymatic deinking on a comparative basis while evaluating several parameters studied during deinking of SOP. Applications of diverse enzyme combinations are also tried during deinking of SOP for recycling purpose.

4.2: Experimental methodology

4.2.1: Selection of raw materials

Imported sorted office paper (SOP) was collected from a recycled based M/s Khatema Fiber Ltd., Khatema, District Udham Singh Nagar (U.K., India). SOP consists of waste paper, as typically generated by offices, containing primarily white and colored ground wood free paper; free of unbleached fiber may include a small percentage of ground-wood computer printout and facsimile papers coated with toner and laser printing and industrial papers. The total prohibitive material and out throws should be within 2 to 5% in SOP [190]. Ash content of the SOP (20.72%) was estimated (TAPPI T 211 om-02 "Ash in wood, pulp, paper and paperboard: combustion at 525°C") [5]. The moisture content (TAPPI T 208 wd-98 "Moisture in wood, pulp, paper and paperboard by toluene distillation") [5] in SOP was 10%.

4.2.2: Deinking chemicals

In flotation deinking operation, the deinking chemicals were prepared in laboratory and the quantities of the chemicals charged in the deinking formulations were calculated as a percentage of the oven dry weight of paper fed to the pulper. Sodium hydroxide (minimum assay 97%), hydrogen peroxide (minimum assay 59%), sodium silicate (minimum assay 18.7- 23.4%), diethylene triamine penta acetic acid (DTPA, as chelating agent with minimum assay 8%), active deinking surfactants viz., oleic acid (minimum assay 50-70%) and Tween 80 (Polyoxy ethylene sorbittan mono-oleate) were purchased from High Media Chemicals, India.

4.2.3: Enzyme production and enzyme assays

The crude fungal cellulase enzyme was extracted from *A. niger* AT-3 and xylanase from *C. cinereus* AT-1 and their respective endoglucanase (CMC_{ase}), exoglucanase (FP_{ase}) and xylanase activities were measured as described in **Section 2.2.12: Analytical methods of chapter 2.**

4.2.4: Effect of enzyme and surfactant's addition points

The effect of addition points of enzyme and surfactant dosages on deinking efficiency during SOP deinking were studied at three distinct stages i.e. before pulping, during pulping and after pulping. SOP was pulped in a hydrapulper at 65 ^{0}C for 30 min, consistency 10% and at a surfactant dose of 0.05%. In order to study the effect of enzyme and surfactant doses before pulping, SOP was soaked in enzyme buffer solution (enzyme dose 6 IU/mL) at pH 5.3, temperature 55 ^{0}C and retention time 30 min with a surfactant dose of 0.05%. In another set of

deinking experiments, enzyme (6 IU/mL) and surfactant (0.05%) were added during pulping into the pulper at conditions: reaction time 30 min, pH 7.2, temperature 65 ^{0}C and consistency 10%. In 3rd set of experiments, an enzyme dose of 6 IU/mL and surfactant dose of 0.05% were added during enzyme treatment stage after pulping at pH 5.3, temperature 55 ^{0}C and retention time 60 min. Pulp brightness (TAPPI T 218 sp-02 "Forming handsheets for reflectance testing of pulp [Büchner funnel procedure]" and (TAPPI T452 om-02 "Brightness of pulp, paper and paperboard [Directional reflectance at 457 nm]") and ERIC (TAPPI T567 pm-97 "Determination of effective residual ink concentration (ERIC) by infrared reflectance measurement") were determined after pulping as well as ink floatation. Similarly, laboratory made handsheets were evaluated for dirt count (TAPPI T 213 om-01 "Dirt in pulp") and deinkability factors i.e. D_B and D_E respectively after ink floatation. Pulps after ink floatation were defibered in a disintegrator and evaluated for CSF (TAPPI T 227 om-99 "Freeness of pulp [Canadian standard method]"). Laboratory hand sheets (60 g/m^2) were prepared on British sheet former (TAPPI T 205 sp-02 "Forming handsheets for physical tests of pulp"), conditioned at relative humidity 65%±2 and temperature 27±1°C and evaluated for burst index (TAPPI T 403 om-97 "Bursting strength of paper"), tensile index (TAPPI T 494 om-01 "Tensile properties of paper and paperboard [using constant rate of elongation apparatus]"), double fold (TAPPI T 423 cm-98 "Folding endurance of paper [Schopper type tester]") and tear index (TAPPI T 414 om-98 "Internal tearing resistance of paper [Elmendorf-type method]") [5].

4.2.5: Impacts of enzyme/ surfactant synergy and surfactant doses

The pulp obtained at conditions mentioned in **Table 4.2** was treated with enzyme alone and enzyme with surfactant at an enzyme dose of 6IU/mL, surfactant dose 0.05%, reaction temperature 55 ^{0}C, pH 5.3 and retention time 120 min and it was subjected to ink floatation carried out at 1% consistency for 10 min in order to study the effect of enzyme and enzyme + surfactant on deinking efficiency. On the other hand, the effect of surfactant doses on SOP deinking efficiency was studied by varying its doses from 0 to 0.2% while keeping other conditions constant. The pulps produced after pulping, enzyme treatment stage and ink floatation were evaluated for various parameters as described in **Section 4.2.4**.

4.2.6: Effects of pulping time, temperature and consistency

The waste paper used in this study (SOP) was torn into small pieces and soaked in warm water at 50 ^{0}C for 30 min. Pulping was carried out in hydra-pulper (capacity 500g), having

provisions for controlling motor speed and temperature. SOP (250g oven dry basis) at different conditions like pulping time 20 min, temperature 65^0C, pulp consistency 12% and in presence of surfactant (oleic acid). pH was adjusted according to requirement with 1N NaOH/ H_2SO_4. The rotor speed of hydra-pulper was adjusted at 650 rpm. The pulp samples after hydra-pulping were evaluated for brightness and ERIC value.

In order to optimize pulping conditions of SOP, the effects of pulping time, temperature, consistency and enzyme dose were studied. In first set of experiments, the pulping time was varied from 10-30 min with an interval of five min while keeping other conditions constant as in **Table 4.4**. In 2nd set of experiments, pulping temperature was varied from 45-65 ^{0}C with a temperature difference of 5^0C and maintaining other conditions constant as in **Table 4.5.** Likewise, maintaining the other pulping conditions same as in **Table 4.6** except pulping consistency, the SOP was pulped at different consistencies keeping a difference of 2% i.e. from 6 to 12%.

The pulps produced were subjected to enzyme treatment and ink floatation stages as per conditions mentioned in their respective tables. The pulps produced after pulping, enzyme treatment stage and ink floatation were evaluated for various parameters as described in **Section 4.2.4**

4.2.7: Effects of enzyme dose and retention time

The SOP pulp produced at optimum pulping conditions was subjected to enzymatic treatment to study deinking efficiency of SOP pulp. In first set, enzyme doses were varied from 0 to 10 IU/g with an interval of 2 units while in second set, retention time was varied from 30 to 150 min with a difference of 30 min and keeping other reaction conditions constant as in **Tables 4.7 and 4.8** respectively. The enzymatic deinking trial was carried out with varying doses of cellulase (0-10 IU/g) while keeping other variables constant. The filtrate after each enzymatic stage was collected and analyzed for reducing sugars released during treatment. The pulps produced after enzyme treatment stage was subjected to ink floatation and was evaluated for various parameters as described in **Section 4.2.4**.

4.2.8: Effect of flotation time

The SOP pulp produced after pulping followed by enzymatic treatment at optimum conditions was subjected to ink floatation keeping temperature, consistency and pH constant as

in **Table 4.9** except ink floatation time varied from 5 to 15 min. The pulps produced after ink floatation were evaluated for various parameters as described in **Section 4.2.4.**

4.2.9: Effect of different enzyme combinations during enzymatic deinking of SOP

The pulp produced at optimum pulping conditions (**Table 4.2.10**) was treated with different doses of cellulases and xylanases produced under optimum conditions as described in **Table 2.24 of Chapter 2** and **Table 3.12 of Chapter 3** as well as commercial enzymes in combinations at conditions mentioned in **Table 4.2.10.** After that the pulps washed with tap water and were subjected to ink floatation maintaining conditions as in **Table 4.2.10.** The pulps produced after ink floatation were evaluated for various parameters as described in **Section 4.2.4** except pulp viscosity (TAPPI T 230 om-04 "Viscosity of pulp [capillary viscometer method") [5] and compared with control. The filtrates collected from each stage of the enzymatic treatment and ink floatation were separately mixed in equal amounts and the combined effluents were analyzed for COD (IS 3025: Part 58, 2006: COD- Closed reflux titrimetric method using Thermo reactor CR2010)[209, 225], BOD (IS 3025: Part 44: 2006- and colour) and total solids (IS 3025: Part 15, 2003- Total residue (total solids – dissolved and suspended) as per Bureau of Indian Standards for methods of sampling and test (Physical and Chemical) for water and wastewater.

4.2.10: Comparison of chemical, chemi-enzymatic and enzymatic deinking

Chemical deinking of SOP was conducted in a hydra-pulper using 2% NaOH (as such), 0.05% Surfactant (Tween 80), 1% H_2O_2, 2.5% sodium meta silicate and 0.5% diethylene triamine penta acetic acid (DTPA) at 20 min pulping time maintaining a temperature of 65±2 ^{0}C and pH 7.2±0.2. Various authors reported and applied these chemical dosing and conditions during chemical deinking [33, 167].

In a 2nd series of experiments, all the chemical dosing (as mentioned above) during pulping were taken 100, 50 and 25% respectively. These were followed by ink floatation using 6 IU/mL cellulase and 0.05% non ionic surfactant (Tween-80) at pH 5.3±2, consistency 12% and temperature 55±2 ^{0}C for 60 min.

In a 3rd set of experiments, the pulp produced as per conditions mentioned in **Table 4.2** was subjected to enzymatic treatment.

These pulps were washed with a tap water through a 200-mesh wire prior to enzymatic treatment after pulping. An enzyme of dose 6 IU/g was mixed with a buffer solution to get a pH level of 5.3 and this solution was added to the pulp slurry in a plastic bag maintaining a

consistency of 12%. The pulp was mixed vigorously by hand to ensure a uniform distribution of the enzyme. The plastic bags were kept in a water bath held at temperature 55 ^{0}C for 2 h retention time with a continuous slow mixing on water bath. The cellulose degradation was determined in terms of the release of reducing sugars using the DNS method [135]. After washing the pulp, ink flotation was carried out in a 25-L laboratory Voith flotation cell. The flotation aid (non-ionic surfactant, Tween -80 as frothing agent) was added to the pulp in the beginning. Nitrogen gas was blown into the pulp suspension at a rate of 14±0.075 standard liters/min (SLPM) through an air filter at the bottom of the flotation cell. The air flow rate was measured with a flow meter (Omega FMA 1700/1800). The pulp was diluted to 1% consistency with tap water as it contained salts of calcium and magnesium which helped in flotation process, as reported by Kanhekar *et al.,* [101]. One percent consistency during ink floatation was reported by Acosta *et al* [1]. A flotation time of 10 min was taken during SOP deinking. Many researchers [33, 95] reported in their studies that adequate flotation time should be provided so that the foam had sufficient time to float out. Since the flow of the air is proportional to the speed [55] hence adequate flow rate was maintained for a good flotation. At the end of flotation, the deinked fibers were washed on a laboratory mesh having slot size of 0.2mm from the drain valve of the flotation cell. Control runs were taken under same conditions. The pulps produced after ink floatation were evaluated for various parameters as described in **Section 4.2.9**.

4.2.11: Repeated recycling experiments of conventional and enzymatic deinking

In order to investigate the effect of repeated recycling on the various properties of the fiber and the resultant sheets, a series of recycling experiments were carried out using SOP chemically and enzymatically deinked pulps as the fibrous material. The procedure used was to defiber the pulps in a laboratory pulp disintegrator without fiber cutting (No load). After defibration, a quantified stock was removed for a British sheet former. The balance of the stock was also converted in to handsheets on a mold equipped with a circulating back water system and then dried on an electrically heated drier. The handsheets from the first cycle, corresponding to one recycling becomes now the raw material for the second recycling cycle. This procedure was repeated until four recycling cycles were completed on the same lot of fibers by dispersing the sheet from the previous cycle in water without any fiber damage. The handsheets were not wet pressed, so as to eliminate the effect of that variable. No additives (size, fillers etc) were used. Since these could have obscured effect of the fiber characteristics on the sheets.

4.2.12: Determination of stickies and ink particle size distribution

Micro-stickies were determined in control, chemically deinked and enzymatically (at optimum dosage of various enzymes concoctions obtained from section 4.2.9) deinked pulps by a Pulmac master screen and image analyzer (Paprican Microscanner). Pulps were passed through a Pulmac master screen, (a customized equipment for collection of micro-stickies) using 0.15 mm slot screen and quantified by using an image analysis. Total micro-stickies counts were calculated (TAPPI T 563 om-03 "Equivalent black area [EBA] and count of visible dirt in pulp, paper and paperboard by image analysis") [5]. Residual ink analysis (Ink particle size distribution) was determined by Paprican Ink Scanner as per Tappi test methods mentioned above.

4.2.13: Techniques applied for the evaluation of SOP pulp fibers

4.2.13.1: Scanning electron microscopy

The detailed morphological studies of pulp samples pertaining to changes in the fibers' surface appearance before and after enzymatic treatment were investigated by SEM (Leo 435 VP, England). Pulp samples were subjected for fixation and dehydration as per method stated by Gabriel [62] and described in **Section 2.2.7 of Chapter 2**. SEM **photomicrographs** were taken at desired magnifications. Results of SEM are shown in **Photomicrographs 3.1 A-F.**

4.2.13.2: Atomic force microscopy (AFM)

Atomic force microscopy was used to study the morphology of cellulose and lignin substrates after enzymatic modification and to obtain information on the changes in fiber surface properties [21]. AFM measurements were made with a Nanoscope IIIa multimode scanning probe microscope (Digital Instruments Inc., Santa Barbara, CA, USA). The images were scanned in tapping mode [128, 237] in air using silicon cantilevers (Point probes, type=NCH, delivered by Nanosensors, Neuchald, Switzerland). No image processing except flattening was done and at least three areas on each sample were measured. The root mean square (rms) roughness of all samples was determined from the 1 μm^2 AFM topography images.

4.2.13.3: Fourier transform infrared (FTIR) spectroscopy

Dried samples were ground into powder and mixed with crushed KBr for homogenization. The KBr to polymer ratio was kept between 1:20 and 1:50. A Nicolet economy sample press was used to obtain optically clear pellets. Pellets were analyzed using a transmission FTIR (Thermo Nicolet Avatar 370 FT-IR Spectrometer System). Dry air was used

as the chamber purge stream for all samples. The scanning resolution was set to 1 nm with a total of 1024 scans per sample. The FTIR spectra were obtained at room temperature over a spectral frequency range of 400-4000 cm^{-1}. IR bands were expressed in terms of frequency (cm^{-1}). The background was obtained against a pure KBr pellet and the data was analyzed by Omnic software. Functional groups present and thus the structure of cellulose samples (SOP, chemical and enzymatically deinked pulp) can be determined from FTIR.

4.2.13.4: X-Ray Diffraction (XRD) studies

X-ray powder diffraction (XRD) was a rapid analytical technique primarily used for phase identification of a crystalline material and could provide information on unit cell dimensions. The pulp samples were finely ground and homogenized in a laboratory Wiley mill. X-ray diffraction was used to determine the cellulose crystallinity of control and deinked pulp fiber samples. X-ray powder diffraction studies were carried out by a Bruker AXS D8 Advance (Germany) using Nickel filtered Cu/Kα radiation and Copper as target at wavelength of 1.54 Å. Goniometer speed was kept at 2^0/min. Wide angle X-ray scattering (WAXS) pattern of the samples were obtained using the DIFFRAC Plus XRD Commander software and analysis was done by DIFFRAC Plus (Version 8.0) software. The range of scanning angle for the sample was kept in the range of 2θ=10-120^0 (Bragg angle). The wavelength of the Cu/Kα radiation source was 0.154 nm. A peak resolution program was used to calculate the crystallinity index (CrI). As proposed by Hindeleh and Johnson [79], CrI was calculated as the ratio of the resolved peak area to the total area under the unresolved peak profile.

4.2.13.5: Thermal studies

Thermogravimetry, (TG) was used to measure the mass or change in mass of a sample as a function of temperature or time or both. Changes of mass occurred during sublimation, evaporation, decomposition and chemical reaction, magnetic or electrical transformations. TGA and DTG were carried out simultaneously by using a PYRIS Diamond TG/DTA thermal analyzer, supplied by Perkin Elmer and the data was processed and analyzed by PYRIS Muse Measure and standard analysis software (v. 3.3U;. #. 2002 Seiko Instruments Inc.). The sample was kept in alumina pan, the reference material was alumina powder and study was carried out at various heating rates such as 10, 20, 30, 40, 50^0C/min under 200 mL/min flow rate of air or nitrogen atmosphere. Indium and gallium were used as standards for temperature calibration. The

measurements were run from room temperature to 600^0C. Standards covering TG include ISO 11358 [143], ASTME 1131 [145] and DIN 51006 [144].

4.2.14: Statistical analysis

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

4.3: Results and discussion:

4.3.1: Effect of enzyme and surfactant's addition points on deinking of SOP

The addition of enzyme or enzyme + surfactant should be applied at the suitable point in the process because enzyme dosing point has been of great significance for achieving utmost enzyme efficiency. **Table 4.1** revealed the effect of addition point of enzyme and enzyme + surfactant on deinking efficiency of SOP. The studies were carried out by adding enzymes before pulping [107, 158], after pulping and [177, 178] during pulping [89]. When enzyme + surfactant treatment was given as a separate and independent stage between pulping and ink floatation stages (i.e. post pulping enzyme treatment), ERIC values and dirt counts mitigated by 67.5% and 92.70% whereas D_B, D_E and brightness improved by 25.52%, 82.36% and 11.46% respectively compared to control. Likewise, when enzyme and surfactant were added during pulping stage, ERIC values and dirt counts after ink floatation were found to reduce by 57.3 and 91.92%. On the other hand, D_B, D_E and brightness improved by 17.60, 69.40 and 8.23% respectively as compared to control. The results were highly disappointing when enzyme and surfactant were added prior to pulping (soaking stage/prepulping stage). In this case the reduction in ERIC values and dirt counts (37.0 and 91.09 %) were found to be the minimum. Also the improvement in D_B (7.43%), D_E (44.01%) and brightness (3.71%) compared to control was minimum.

It was observed that out of three distinct addition points of enzyme and surfactant, excellent results were obtained in case when enzyme and surfactant were added after pulping **(Figures 4.1 and 4.2)**. Deinking efficiency in case where enzyme and surfactant were added before pulping was poor because of availability of lesser surface area for the reaction of an enzyme due to non-shredding of SOP, enveloping of cellulose surface due to surface sizing or coating and minimum reaction time left for the proper hydrolysis of cellulosic substrates. The major role of cellulases in deinking might be the weakening of bonds between fibrous materials. Ink particles, especially larger ones, then might be dislodged and/or broken into smaller particles

as fibrous masses broken up in response to mechanical action because of shear forces and hydrodynamic forces during disintegration. The mechanism explained the removal of ink by enzymes was suggested by other authors as well [48, 224]. Also, a correlation between fiber separation and ink detachment was expected to occur in chemical deinking [25]. According to a recent review [219], cellulase activity released ink particles into suspension from fibers and/or fines and reduced ink areas by one or a combination of mechanisms: (a) enzyme attack fostered disaggregation of ink-fiber complexes during pulping [48], thereby reducing the number and size of residual ink spots, (b) enzymes attack at sites where ink was bonded to fibers, thereby making ink particles loosened and free from individual fibers [231]. The improper interaction of enzyme on substrate (paper surface) caused the poorest deinking efficiency. Suurnäkki reported that the action of enzymes in pulp was affected by the accessibility of substrates in the fiber matrix [201]. Deinking efficiency was found to be better in case where enzyme and surfactant were added during pulping. Removal of coating layer and more exposed fiber surface area due to shredding for enzyme interaction were the main reasons for improved deinking efficiency. However, excellent results were obtained by adding enzyme and surfactant after disintegration (post pulping). During pulping, deinking efficiency was adversely influenced by mechanical factors like shear forces which were shown to deactivate an enzyme under certain conditions such as agitation, flow, and pumping [37]. The lower the shear factor during pulping, the slower were both ink fragmentation and ink redposition and the more ink removed in flotation [52]. During agitation, the cellulase was believed to be deactivated when it was exposed to an air-liquid interface which caused unfolding of the protein. In the absence of agitation, the amount of protein at the liquid surface was small compared to the total protein and hence, these effects were negligible [106]. Agitation continually renewed the surface, thus subjecting much more of the protein to the unfolding process, with a consequent increased in denaturation [106]. Cellulase deactivation due to the interfacial effect combined with the shear effect could have been far more severe and extensive than that due to the shear effect alone [102,106]. Charm and Wong [37] investigated the influence of shear on catalase, carboxypeptidase, and a number of other proteins. Their findings indicated that turbulent flow resulted in greater loss of enzyme activity than does laminar flow. Increased shear rate reduced the reaction rate of the enzyme. Kim *et al.*, [106] asserted that deactivation, generally, accompanied a change in structural conformation and subsequent collapse of the binding domains of the enzyme. Apparently, mild mixing did not

denature the enzyme, but rather dispersed the enzyme better into the pulp slurry as in case of addition of enzyme and surfactant after pulping. However, further increased in shear rate caused a reduction in the binding probably due to excessive shear which could have resulted in an enzyme denaturation [106].

All the mechanical strength properties along with pulp freeness (tensile index, burst index and tear index) except double fold numbers increased after ink flotation in the following ascending order: control pulp<enzyme+ surfactant treatment before pulping< enzyme+ surfactant treatment during pulping< enzyme+ surfactant treatment between pulping.

4.3.2: Impact of enzyme and surfactant synergy

The SOP when pulped at a consistency of 10%, pH 7.2, pulping time 30 min and temperature 65 ^{0}C shows a pulp brightness of 61.22% (ISO) and ERIC value of 287.50 (ppm) **(Table 4.2).** Control pulp after ink flotation shows deinkability based on brightness (D_B) 3.57% and deinkability based on ERIC value (D_E) 5.0% with a dirt count of 20181 mm^2/m^2.

Objectives of pulping could be considered as defibering as well as detaching ink and other impurities of waste papers. The mechanism that promoted defibering also facilitated ink detachment i.e. acceleration, viscosity and clinging effects [53, 81]. Acceleration effect was clarified to acceleration of flakes due to inertia produced by falling pulp or rotor in the pulper. Viscosity effects could be considered to varying velocities of suspension and flakes. The clinging effects related to rotor and flakes interaction applying only to low consistency vat pulping. The shear was able to detach ink and the influence of fiber swelling into ink detachment was assumed to be due to the nature of ink that was not able to expand to the same extent as fibers swell [96]. Surface active agents had the ability to disperse and emulsify the oil vehicle. The surfactant in the flotation deinking process (most commonly fatty acid soaps) reacted with calcium ions in the system to form calcium soaps which could absorb onto the ink surface and provided the collector action [68]. In the study of Frank and coworkers [57], brightness, burst and tear strength improvement were observed when cellulase was employed.

Pulping of SOP followed by enzyme treatment and ink flotation shows a reduction in ERIC value by 61.72% and dirt count by 92.36% whereas, D_B, D_E and brightness improved by 85.45, 93.86 and 10.71% respectively compared to control **(Figures 4.3 and 4.4).** According to Woodward *et al.,* [224], cellulase binding on pulp fiber may result in fiber surface alteration, sufficient to favour ink detachment during repulping. Nevertheless, other authors reported that

the main effect was the hydrolysis and superficial degradation of cellulose that implied ink removal from fibers [48, 107]. It was also reported that enzymatic and mechanical actions were basic in the process [89]. The use of cellulases/ hemicellulases released the ink particles into suspension which was generally attributed to the cellulose hydrolysis on the fiber/ink inter-bonding regions, which facilitated ink detachment [107]. Additionally, these enzymes could remove small fibrils from the surface of the ink particles thus altering the relative hydrophobicity of the particles, which facilitated their separation in the flotation/washing step [89].

Pulping of SOP followed by enzyme + surfactant treatment and ink flotation shows a reduction in ERIC value and dirt count by 11.4 and 4.41% respectively whereas, D_B, D_E and brightness improved by 19.09, 7.81 and 2.9% respectively compared to enzymatically deinked pulp. On the other hand, SOP pulp followed by enzyme + surfactant treatments and ink floatation shows a reduction in ERIC value and dirt count by 67.50 and 92.70% respectively whereas, D_B, D_E and brightness improved by 88.22, 94.34 and 15.3% respectively compared to control **(Figures 4.3 and 4.4).** Enzymatic deinking of SOP indicated that maximum deinking efficiency in terms of both visible dirt counts and effective residual ink contents was achieved when enzymes were used along with surfactant and surfactant addition was also found to improve pulp brightness and dirt removal.

Surface active agents had the ability to disperse and emulsify the oil vehicle. Thus, they could improve ink detachment from fibers, but at the same time they improved ink fragmentation that could also be detrimental, if in excess [69, 96]. Surfactants made the cellulose more accessible to cellulase enzyme and facilitated enzyme dispersion thus making the enzyme available to attach to cellulose sites. The proposed mechanism for the enhancement of hydrolysis by cellulase in the presence of surfactants was that the surfactants hinder the immobilization of the enzymes on the substrate by reducing the binding strength. Thus, the enzyme could more easily desorb from the binding site after reaction and the enzyme could easily move to other binding sites on the substrate [35, 77, 63]. Ooshima *et al.,* [156] revealed that non-ionic, amphoteric, and cationic surfactants enhanced saccharification by cellulases while anionic surfactants did not. However, both cationic and anionic surfactants denatured cellulase in relatively low concentrations. They also concluded that the non-ionic surfactant Tween20 enhanced the hydrolysis of crystalline cellulose by reducing the binding of endoglucanase on cellulose and by varying the binding equilibrium of endoglucanases and exoglucanases.

Castanon and Wilke [35] observed that a non-ionic surfactant, Tween 80, increased the rate and extent of cellulose saccharification. The findings indicated that surfactants competed with the protein for the free surface area, thereby reducing the amount of protein exposed to the surface and preventing denaturation [106]. Park *et al.,* [163] and Helle *et al.,* [77] indicated that surfactants did not affect the hydrolysis of cellulase when water-soluble substrates were used. The increase in enzyme activity due to interaction with some non-ionic and cationic surfactants might be related to the fact that enzymes were dispersed well during the initial incubation period by the effect of surfactants. The logic behind this may be because cellulase enzymes were reported to form aggregates in the absence of surfactants, depending on the enzyme concentration in solution [65]. Most of the researchers observed that non-ionic surfactants generally improved the enzyme activity while cationic and anionic surfactants inhibited the enzyme activity, especially at high addition levels [35, 156, 163].

The improvement in freeness level (CSF), tensile index, tear index, burst index and double fold numbers occurred in the increasing order: control pulp> enzymatically deinked pulp> enzyme + surfactant treated deinked pulp. Addition of enzyme and enzyme + surfactant showed an improvement in freeness level of SOP deinked pulp. SOP contained contaminants like internal sizing agents (rosin or alkyl ketene dimer or alkyl succinic anhydride) and fillers with other non-functional additives (biocides, drainage aids, retention aids, pitch control agents and defoamers etc) which were known to mitigate paper strength. In addition to ink removal, a significant improvement of the pulp and paper physical properties was observed, which was probably due to the removal of fillers and fines [159]. These modifications might rendered the fibers less difficult to deink, because the dislodged ink particles redposition on the fibers surface and its penetration in the porous structure of the fibers was avoided [107], and also because, in the absence of additives, the fibers become more accessible/susceptible to the deinking aids action. Removal of contaminants increased the fiber-fiber contact and improved the hydrogen bonding but broken microfibrillar structure during recycling of secondary fibers decreased the strength of paper which acted as filler and adversely affect the bonding between the fibers. Crude enzyme preparation removed the lower DP xylan from the pulp, leaving stuffy, crystalline cellulose fiber in the pulp. Secondly, xylan acted as lubricant during beating of pulp [3]. Xylanase systems were developed to ensure selective hydrolysis of the hemicelluloses without loss of fiber strength [105]. Cellulase/hemicellulases formulation enhanced and restored fiber

strength, reduced refining energy requirement and increased inter-fiber bonding due to fibrillation, while increasing drainage rate and avoiding fiber breakage [108]. Frank and his coworkers observed that cellulase treatment improved the pulp brightness (due to significant removal of ink particles) burst and tear strengths [57]. Compared to conventional deinking, enzymatic deinking improves most of the physical strength properties. Especially, freeness, whiteness and tensile strength improved due to mild treatment conditions [151]. Enzymatic treatments of recovered paper enhanced brightness, cleanliness, drainage, and mechanical properties of the resulting pulp [177].

4.3.3: Optimization of surfactant doses

When water was dropped on to the toner surfaces, it gave a large contact angle of 109^0, indicating poor wetting interaction between them [66, 67]. In order to obtain maximum wetting capability, the de-inking agent was mixed with a different concentration of surfactant until the surface tension of the solution became equal or less than 34 dynes/cm [120]. Results shows that **(Table 4.3)** SOP pulp (ERIC value and dirt counts 109.75 ppm and 1610 mm^2/m^2 respectively) when treated with a fixed dose of enzyme with varying doses of surfactant from 0.0 to 0.2%, ERIC value and dirt counts decreased up to a dose of 0.2% and beyond that reduction was insignificant. Similarly, brightness of SOP pulp was improved up to a surfactant dose of 0.2% **(Figure 4.5).** In the similar way, both D_B and D_E were also improved up to a surfactant dose of 0.2% and further increase in surfactant dose did not alter D_B and D_E significantly **(Figure 4.6).** The surfactant reduced the surface tension forces up to a critical value of a substrate and beyond this limit surfactant was not effective to improve deinking efficiency. Sykes and Klungness reported that 0.05% surfactant (non-ionic) was the appropriate addition level with the enzyme in laboratory and 0.13% surfactant dose was recommended for the industrial trial [203]. The addition of surfactants to the water solution could lower the surface tensions effectively. It was found that complete wetting could occur only when the surface tension of a liquid was reduced to a critical value γ_c, characteristic of the substrate [184]. It was observed that presence of surface active agents of non-ionic nature (Tween 80, Polyoxy ethylene sorbittan mono-oleate) could substantially increase hydrolase enzyme activity on cellulosic fiber, finally improving the ink-fiber detachment. Tween- 80 shows a behavior where perhaps the surfactant not only helped the ink agglomeration but also formed intermediate size agglomerate with dust or fines. The size of

agglomerate with dust, fines or ink was such that it went out on the air bubbles forming foam and led to a relatively cleaner pulp resulting in an increased deinkability factor.

Dispersants could solubilize the detached ink particles and create a stable emulsion that did not readily redeposit onto the fiber. Addition of surfactant to the mixed solvent was essential for its formation of a stable emulsion in water. When the surfactant was applied at a critical micelle concentration level or higher, the binder formed a tiny and stable emulsion of 537 nm in the pulp slurry that could be washed away easily through washing [183]. Ooshima *et al.*, [156] also concluded that the non-ionic surfactant Tween 20 enhanced the hydrolysis of crystalline cellulose by reducing the binding of endoglucanase on cellulose and by varying the binding equilibrium of endoglucanases and exoglucanases. The hydrolysis enhancement by non-ionic surfactants was also reported by Park *et al.*, [163]. However, one report also indicated that surfactants enhanced the hydrolysis of cellulose regardless of the surfactant charge [77]. It was also reported that surfactants could prevent the denaturation of cellulases in agitated systems [106].

Deinking model equations and statistical analysis

The following empirical equations were obtained by using the experimental data from the nonlinear polynomial regression analysis program to predict the D_B and D_E

$$y(D_B) = -70.15x^3-574.8x^2+191.2x+14.91 \quad \text{.....[1]}$$

$$y(D_E) = -2873x^3+316.1x^2+118x+78.71 \quad \text{.....[2]}$$

Process variables:

x = surfactant dose

y = D_B and D_E

In case of D_B,

Standard error of estimate = 1.0248, Constant variance test: Passed (P = 0.0600), R = 0.99379060, R^2 = 0.98761975, Adjustable R^2 = 0.96904938, Durbin-Watson statistics = 2.8078, Power of performed test with α = 0.0500: 0.9988.

In case of D_E,

Standard error of estimate = 0.4839, Constant variance test: Passed (P = 0.0600), R= 0.99837023, R^2 = 0.99674312, Adjustable R^2 = 0.99185779, Durbin-Watson statistics = 2.7856, Power of performed test with α = 0.0500: 1.0000

Where, R is regression coefficient.

Above results indicated that the value of R^2 was above 0.80 and less than 1.0, i.e. 0.98 and 0.99 for D_B and D_E respectively. It means that the predicted values of D_B and D_E showed positive results and gave minimum regression error up to 3rd order of polynomial regression analysis. Normality test was also carried out and it justified affirmative results for both D_B and D_E as shown below:

Normality test for D_B

Surfactant doses:	W-statistics = 0.925,	P = 0.539	(Passed)
D_B:	W-statistics = 0.941,	P = 0.671	(Passed)

Normality test for D_E

Surfactant doses:	W-statistics = 0.925,	P = 0.539	(Passed)
D_E:	W-statistics = 0.940,	P = 0.657	(Passed)

Where: P indicates normal distribution coefficient.

The predicted values of D_B and D_E plotted against experimental values of D_B and D_E respectively (**Figure 4.7**) also shows a reasonable fit for the data. Graphs plotted between experimental value of D_B versus predicted value of D_B and experimental value of D_E versus predicted value of D_E shows a linear relationship with a minimum deviation from the tangible values **(Figures 4.8 and 4.9).**

4.3.4: Optimization of pulping time

Table 4.4 revealed that pulp brightness and ERIC values both shows reverse trend on increasing brightness on account of increasing pulping time during SOP pulping. Both the curves remained almost constant beyond a pulping time of 20 min **(Figure 4.10).** The effect of pulping time followed by enzyme + surfactant treatments on deinking efficiency of SOP shows that dirt counts and ERIC values were found to be decreased with increasing pulping time up to 20 min and beyond that decrease in dirt counts and ERIC values were insignificant. Similarly, a pulp brightness of 72.22% (ISO) was achieved at the same pulping time. Further increase in pulping time did not contribute to brightness notably **(Figure 4.10).** Significant improvement in D_B (27.01%) and D_E (86.46%) was obtained at a pulping time of 20 min and beyond that increase in D_B and D_E was insignificant **(Figure 4.11).**

Too long pulping time could result into reduction of the ink particles size and decrease in the flotation removal efficiency. Pulping time should be limited to the time required to get acceptable defiberation of the processed raw material to minimize ink fragmentation and

redposition. Higher values of repulping time might be detrimental for complete ink removal, either due to an excessive fractionation that leads to lower-sized particles or by redeposition into the lumen fibers. Increase in the pulping time, the residual ink area gradually decreased, reaching the minimum after 20 min [213]. Many other researchers observed that a time 15 min for pulping of waste papers gave good results [14, 33, 122, 167]. By increasing the pulping time to 20 min, the ink removal efficiency increased to a certain extent, but after 20 min the efficiency increase was insignificant because if the ink particle size further reduced below 0.15mm (which was the proper ink size to get removed by flotation), the ink removal efficiency would be affected adversely [151]. As far as the energy savings as concerned by increasing the consistency, there will be decrease in the repulping time which in turn enhance savings. Moreover, the effect of a higher consistency on the ink detachment was very positive because of the increased effect of shear forces [168].

All the mechanical strength properties along with freeness (CSF) were found to increase with increase in pulping time except double fold numbers. Park *et al.*, [164] observed that conventional as well as bio-deinking (1% modified cellulose) conducted at a pulping temperature of 55 ^{0}C and pulping time of 30 min shows an improvement in physical properties of white ledger mixed office waste paper printed by laser and xerographic processes. Based on above observation pulping times of 20 min have be taken as an optimum time.

Deinking model equation and statistical analysis

The following empirical equations were obtained by using the experimental data from the nonlinear polynomial regression analysis program to predict the D_B and D_E

$$y\ (D_B) = -0.003x^3+0.141x^2-0.771x+12.13 \quad \text{.....[1]}$$

$$y\ (D_E) = -0.001x^3-0.162x^2+4.933x+38.01 \quad \text{.....[2]}$$

Where:

x= pulping time in min

y = D_B and D_E

In case of D_B,

R = 0.99995165, R^2 = 0.99990330, Adjustable R^2 = 0.99961321, Standard error of estimate = 0.1410, Durbin-Watson statistics = 3.5135, Constant variance test: Passed (P = 0.0500), Power of performed test with α= 0.0500: 1.0000

In case of D_E,

R = 0.99649355, R^2 = 0.99299940, Adjustable R^2 = 0.97199759, Standard error of estimate = 1.9611, Durbin-Watson statistics = 3.5135, Constant variance test: Passed (P = 0.0500), Power of performed test with α = 0.0500: 0.9942

Where, R is the regression coefficient

The value of R^2 was above 0.80 and less than 1.0, i.e. 0.99 and 0.99 for D_B and D_E respectively. It means the predicted values of D_B and D_E gave minimum regression errors up to 3rd order polynomial regression analysis.

The experimental results were validated statistically. In order to justify the results for both D_B and D_E, normality test was also performed.

Normality test for D_B

Pulping time:	W-Statistics = 0.979	P = 0.928	Passed
D_B:	W-Statistics = 0.910	P = 0.465	Passed

Normality test for D_E

Pulping time:	W-Statistics = 0.898	P = 0.928	Passed
D_E:	W-Statistics = 0.880	P = 0.308	Passed

Where, P is the normal distribution coefficient.

The predicted value of DB and DE were plotted in Figure 4.12 and shows a reasonable fit for the data. Curves were also plotted between experimental value of D_B against the predicted value of D_B **(Figure 4.13)** and experimental value of D_E against the predicted value of D_E **(Figure 4.14)** which exhibits a linear relationship with a minimum deviation from the tangible values.

4.3.5: Optimization of pulping temperature

When SOP was pulped at different temperatures i.e. 45 to 65 ^{0}C while keeping other variables constant, the maximum pulp brightness (63.29%) with minimum ERIC value (267.50 ppm) were obtained at a temperature of 60 ^{0}C. The different SOP pulp samples obtained were subjected to enzyme and surfactant treatment, followed by ink flotation as per conditions described in **Table 4.5. Figure 4.15** shows that dirt counts and ERIC value decreased with increasing pulping temperatures up to 60 ^{0}C and then remained practically constant. Similarly, brightness gain was also found to be insignificant beyond this temperature range. A remarkable improvement in D_B and D_E was obtained at 60 ^{0}C **(Figure 4.16).**

Defiberation was one of the important steps in deinking during pulping. Defiberation rate could be boosted up by raising the pulping temperature, while the influence of raising temperature on the amount of flakes was pronounced with lower temperatures. Temperatures over 40 ^{0}C had only a moderate effect on the required pulping time with a given flakes content. Magnin [125] stated that pulping temperature beyond a limit of 65 ^{0}C affected the deinkability, and the ink dissociation from the pulp started reversing back in to the system. Marchildon *et al.*, [126] concluded that an adequate combination of pulping temperature and percentage of active deinking chemicals favored the formation of big flocks of ink which were more easily removed by flotation and reduced the ink surface. Basta *et al.*, [14], Lopez *et al.*, [122] also indicated in their studies that pulping temperatures between 60-65 ^{0}C gave good deinking results. Ali *et al.*, [4] concluded that no practical improvement in pulping could be achieved by increasing temperature above 60 °C with ONP or OMG. Mechanistically, the rise in temperature decreased the water viscosity and thus decreased friction. In addition, the rise in temperature facilitated water penetration into the fibers, thus accelerating fiber bond breakage [53].

Deinking model equations and statistical analysis:

In order to predict D_B and D_E, the following empirical equations were obtained by using the experimental data from the nonlinear polynomial regression analysis program:

$$y\ (D_B) = -0.004x^3+0.731x^2-39.06x+709.2 \quad \text{.....[1]}$$

$$y\ (D_E) = -0.003x3+0.622x^2-31.54x+590.2 \quad \text{.....[2]}$$

Where:

x = pulping temperature,

y = D_B and D_E

In case of D_B,

R = 0.99949787, R^2 = 0.99899599, Adjustable R^2 = 0.99598395, Standard error of estimate = 0.1877, Durbin-Watson statistics = 3.5714, Constant variance test: Passed (P= 0.0500), Power of performed test with α = 0.0500: 1.0000

In case of D_E,

R = 0.99994238, R^2 = 0.99988476, Adjustable R^2 = 0.99953902, Standard error of estimate = 0.1554, Durbin-Watson statistics = 3.5714, Constant variance test: Passed (P = 0.0500), Power of performed test with α = 0.0500: 1.0000

Where, R is the regression coefficient.

The predicted values of D_B and D_E gave minimum regression errors up to 3rd order polynomial regression analysis.

Results were further justified by normality test.

Normality test (Shapiro-Wilk) for D_B

Pulping temperature:	W-Statistics = 0.987	P = 0.967	Passed
D_B:	W-Statistics = 0.901	P = 0.417	Passed

Normality test (Shapiro-Wilk) for D_E

Pulping temperature:	W-Statistics = 0.987	P = 0.967	Passed
D_E:	W-Statistics = 0.913	P = 0.483	Passed

Where, P is the normal distribution coefficient.

It passes the normality test if the value of P is less than the value of W- statistics.

The predicted values of D_B and D_E were plotted in **Figure 4.17** which shows a reasonable fit for the data. The curves plotted between experimental values of D_B against the predicted values of D_B and experimental values of D_E against the predicted values of D_E showed a linear relationship with a minimum deviation from the tangible values **(Figures 4.18, 4.19).**

4.3.6: Optimization of pulp consistency

SOP was pulped at diverse consistencies i.e. 6-14% while keeping other conditions constant as mentioned in **Table 4.6** and it was then followed by enzyme + surfactant treatment at the same consistencies while ink flotation was carried out at 1% consistency. **Figure 4.20** reveals that maximum reduction in dirt counts and ERIC values were obtained at a consistency of 12% where as brightness beyond a consistency of 12% shows an insignificant increase. Maximum improvement in D_B and D_E were also obtained at a consistency level of 12% **(Figure 4.21).**

As the consistency of pulp was increased the mobile layer was progressively eliminated leaving only the thin immobile layer enveloping the fiber, thus decreasing considerably the diffusion path length of reactant to the fiber [39, 74]. Water layer thickness now becomes the rate-determining step. However, the higher pulp consistency provided a close contact between enzymes and pulp fibers [39, 74] probably because of the reduced volume of the liquid phase, thus facilitating enzyme adsorption to pulp and the sequential hydrolysis of cellulose and hemicellulose [39]. Carre *et al.,* [34] stated that increasing pulp consistency up to an optimum induced an increase of the shearing forces, which dispersed ink favorably but further increase could result in ink redposition and reduction in the ink flotation removal efficiency. Sridhar *et*

al., [199] also described that with high consistency pulping and deinking, effects in terms of detachment and dispersion were lower than low/ medium consistency batch pulping. Enzyme preparations, when combined with inter-fiber friction of 14% consistency pulping, helped to separate detached ink particles from the pulp slurry by clipping and smoothing pulp fibrils from the fiber surface [204]. Jeffries *et al.,* [88, 89] observed that medium consistency (12%) pulping was advantageous for removing toner through the combined effect of enzyme and mechanical attrition. Mixing the pulp at medium consistency (11-16%) dislodged toner particles from the fibers, and the presence of a surfactant in the pulper helped the enzyme penetrate through paper additives. This increased cellulose availability for cellulase attachment. Increasing the consistency and decreasing the repulping time enhanced savings and therefore sustainability [166]. The main advantages of high consistency pulping compared to low consistency pulping were: better defibering at a higher consistency, lower energy consumption (between 15 and 30 kWh/t), lower pulping time, lower contaminant fragmentation, which enabled a more efficient removal, higher deinking chemicals/enzyme concentration, which improved ink detachment from fibres and fibre bleaching [134].

Good mixing was a critical requirement for effective interaction of enzymes with fibers. For the insignificant decreased in dirt counts, ERIC values, and improvement in D_B and D_E above pulp consistency of 12%, the pulp needed to be finely shredded to separate fiber aggregates to the greatest extent possible before contacting the fiber with reactant. Followed by enzyme and surfactant treatments, ink flotation of SOP pulps was carried out at 1% consistency because the deinking efficiency dropped with increasing pulping consistency. Lee *et al.,* [118] observed maximum deinking efficiency (62%) during flotation at a pulp consistency of 1–2%. Likewise, all the mechanical strength properties except double fold number improved up to a consistency level of 12%. It might be due to better fiber-enzyme interaction and removal of fillers. Hence, relatively high consistency of 12% was chosen because it prompted fibre/fibre attrition, favoring ink detachment; additionally, it would be advantageous for industrial usage.

Deinking model equations and statistical analysis:

The following empirical equations were obtained by using the experimental data from the nonlinear polynomial regression analysis program to predict the D_B and D_E

$$y(D_B) = -0.072x^3+2.010x^2-15.38x+53.13 \ldots\ldots\ldots\ldots [1]$$

$$y(D_E) = -0.060x^3+1.159x^2+-7.843x+68.78 \ldots\ldots\ldots\ldots [2]$$

Where,

x = consistency change,

y = D_B and D_E

In case of D_B,

R = 0.99938378, R^2 = 0.99876793, Adjustable R^2 = 0.99507173, Standard error of estimate = 0.4661, Durbin-Watson statistics = 3.5714, Constant variance test: Passed (P=0.0500), Power of performed test with α = 0.0500: 0.9999

In case of D_E,

R = 0.99763844, R^2 = 0.99528246, Adjustable R^2 = 0.98112984, Standard error of estimate = 1.5574, Constant Variance Test: Passed (P= 0.0500), Durbin-Watson statistics = 3.5714, Power of performed test with α = 0.0500: 0.9975

Where, R is regression coefficient

Since the value of R^2 was above 0.80 and below 1.0 (0.998 and 0.995 for D_B and D_E respectively), this indicated minimum regression errors up to 3rd order of polynomial regression analysis.

The results for both D_B and D_E were further revalidated by Normality test.

Normality test for D_B

Consistency:	W-statistics = 0.987	P = 0.967	Passed
D_B:	W-statistics = 0.907	P = 0.451	Passed

Normality test for D_E

Consistency:	W-statistics = 0.987	P = 0.967	Passed
D_E:	W-statistics = 0.918	P = 0.518	Passed

Where, P indicates normal distribution coefficient.

If the value of P was less than the value of W- statistics, it passed the normality test.

The predicted values of D_B and D_E were plotted in **Figure 4.22** which were reasonably fit for the data. The curves plotted between experimental values of D_B against the predicted values of D_B and experimental values of D_E against the predicted values of D_E shows a linear relationship with a minimum deviation from the tangible values **(Figures 4.23 and 4.24).**

4.3.7: Optimization of enzyme dose

Enzyme dose was one of the critical parameters which affected the yield, brightness and strength of the pulp. **Table 4.7** reveals the effect of varying enzyme doses (before ink flotation)

during enzymatic deinking of SOP on over all deinking efficiency. **Figure 4.25** shows that pulp brightness (**73.63%, ISO**) increased with increasing enzyme dose up to 6 IU/mL and beyond that the curve remained almost constant. Conversely, dirt counts (2329 mm^2/m^2) and ERIC values (86.75 ppm) decreased with increasing enzyme dose (6 IU/mL) and further increase in enzyme dose showed an insignificant decrease. **Figure 4.26** reveals the effect of enzyme dose on D_B and D_E respectively. A significant improvement in D_B (31.07%) and D_E (89.52%) respectively was achieved at an enzyme dose of 6 IU/mL. However, the improvement in D_B and D_E with respect to enzyme dose (above 6 IU/mL) was not encouraging.

The binding of the enzyme was found to be associated with various process variables like intensity of agitation, reaction time, pulp consistency, pH, temperature, and concentration of enzyme. The binding of enzyme onto fiber increased with increasing concentration of enzyme [103]. The selection of the optimal enzyme concentration was important since excessive enzymes might be detrimental to the fibers and could affect the strength of the paper and its quality [118]. There was no significant improvement in D_B and D_E beyond 6IU/mL which could be justified by the fact that the pulp being an insoluble substrate, the rate of reaction would therefore, not be directly proportional to the enzyme amount or concentration. Jeffries *et al.,* [89] reported that high enzyme loading led to a reduction in brightness as a result of accumulation of enzyme particles on the surfaces of the fibers. Gübitz *et al.,* [72] have shown that an enzyme concentration of about 25IU/g pulp resulted in a deinking efficiency of about 75%, while, Morkbak and Zimmermann [139] have shown that the enzyme concentration of about 3IU/g pulp was adequate to deink the mixed office waste papers, old newspapers and vegetable oil based ink printed papers. On the other hand, Prasad *et al.,* [178] and Vyas and Lachke [215] used a wider range of enzyme concentration of 0.1–15IU/g to achieve the maximum deinking efficiency of about 75%. These observations suggested that the variations in enzyme concentration needed for enzymatic hydrolysis depends on the type of enzymes used, source and the reaction conditions employed. The results obtained in the present work indicated that the enzyme concentration needed to give the maximum deinking efficiency were comparable to the other work reported under similar conditions implied.

All the mechanical strength properties except double fold numbers increased with increasing enzyme dose (6IU/mL). The higher enzyme dosage led to an increase in fines contents. This could be attributed to cellulase-induced disintegration of fibers [155]. Moreover,

owing to the high specific surface area of the fines, the attack of cellulases was specific towards this fraction of the pulp. Other authors pointed out that the fiber surface was stripped through the enzymatic hydrolysis of subsequent layers or fibrils. Therefore, the mechanism was called as "peeling effect" [87]. An intensive enzymatic reaction led to both intrinsic fiber strength and fiber length reduction, and excessive fines production. As a consequence, the paper strength could be drastically affected. Jackson *et al.,* [87] suggested that enzymes could either flocculated or hydrolyzed fines and removed fibrils from the surface of large fibers. According to these authors, the enzyme-aided flocculation occurred when a low enzyme dosage was used. In this case, fines and small fiber particles aggregated with each other or with the larger fibers, decreasing the amount of small particles in the pulp and consequently improved pulp drainage. For higher enzyme concentration, flocculation becomes less significant, and fragmentation of the fibers begins to dominate

Deinking model equations and statistical analysis

In order to validate the repeatability of results, the following empirical equations were obtained by using the experimental data from the nonlinear polynomial regression analysis program to predict the D_B and D_E

$$y\ (D_B) = -0.015x^3-0.569x^2+6.692x+7.262.....[1]$$

$$y\ (D_E) = 0.030x^3-1.605x^2+20.37x+16.06..... [2]$$

Variables,

x = Enzyme dose

y = D_B and D_E

In case of D_B,

R = 0.99807567, R^2= 0.99615503, Adjustable R^2 = 0.99038758, Standard error of estimate = 0.9697, Durbin-Watson statistics = 3.6567, Constant variance test: Passed (P = 0.0600) and Power of performed test with α = 0.0500: 1.0000

In case of D_E,

R = 0.99883228, R^2= 0.99766593, Adjustable R^2 = 0.99416483, Standard error of estimate = 2.2838, Durbin-Watson statistics = 3.4097, Constant variance test: Passed (P = 0.0600) and Power of performed test with α = 0.0500: 1.0000

Where, R is the regression coefficient

The above results indicated that the value of R^2 lied between 0.80-1.0, i.e. 0.996 and 0.997 for D_B and D_E respectively which gave minimum regression errors up to 3rd order of polynomial regression analysis.

A normality test was then performed in order to revalidate D_B and D_E values.

Normality test (Shapiro-Wilk) for D_B

Enzyme dose: W-statistics = 0.982 P = 0.961 Passed

D_B: W-statistics = 0.848 P = 0.153 Passed

Normality Test (Shapiro-Wilk) for D_E

Enzyme dose W-statistics = 0.982 P = 0.961 Passed

D_E: W-statistics = 0.815 P = 0.079 Passed

Where, P is the normal distribution coefficient.

The lesser value of P than W- statistics passed the normality test. The predicted value of DB and DE plotted in Figure 4.27 also shows a reasonable fit for the data. The curves plotted between experimental values of D_B against the predicted values of D_B and experimental values of D_E against the predicted values of D_E showed a linear relationship with a minimum deviation from the tangible values **(Figures 4.28 and 4.29).**

4.3.8: Optimization of retention time

The optimum retention time was very important factor for the improvement of fiber recovery and brightness. SOP pulp was subjected to enzyme + surfactant treatment under different enzyme retention time i.e. from 0 to 120 min and then followed by ink floatation to observe its effect on deinking efficiency (**Table 4.8**). **Figure 4.30** shows that dirt counts and ERIC values decreased sharply up to a reaction time of 60 min and beyond that the decrease was insignificant. Alike, brightness improved with retention time of 60 min and beyond that it was almost constant. The curve could be approximated by two straight lines at each reaction time investigated. In **Figure 4.31**, the curve with steeper slope pertain to a rapid improvement in D_B, whereas the part of curve with gentler slope pertain to slow improvement in D_B,. In the similar manner, the curve plotted between reaction time and D_E could be approximated into two straight lines. The curve plotted up to a reaction time of 60 min was steep showing significant improvement in D_E while, after a reaction time of 60 min, the curve shows a slow improvement. It indicated that 60 min time was enough for the attack of enzymes at sites where ink was bonded to fibers. The reaction time above 60 min might be damaging to the fibers and thus it could affect

the strength of the paper and its quality. It was validated by evaluation of mechanical strength properties at different reaction times. **Table 4.8** shows that tear, burst and tensile indexes except double fold numbers increased with increasing reaction time up to 60 min and beyond that an insignificant decrease was noticed.

Proper enzyme dosage and reaction time varied with enzyme, paper, and ink type. Pommier *et al.*, [175] reported that higher concentration of enzyme and prolonged reaction times affected the fibers, leading to a reduction in the average fiber length. Pélach *et al.*, [168] observed that old news paper showed a higher deinking efficiency at low consistency (6%) and reasonable contact time (30 min) as compared to conventional deinking. For this same consistency (6%) and 120 min of contact time, the efficacy was not found to improve. Further, for medium consistency (10%) and 30 min of contact time, the efficacy decreased although, the absolute detachment values improved. This behavior was similar for a contact time of 120 min. Too much enzyme or overly long reaction time could hence damage fibers [218].

Deinking model equations and statistical analysis

The following empirical equations were obtained by using the experimental data from the nonlinear polynomial regression analysis program to predict the D_B and D_E:

$$y\ (D_B) = -5E\text{-}06x^3-0.002x^2+0.530x+6.125 \quad \text{.....[1]}$$

$$y\ (D_E) = -5E\text{-}06x^3-0.008x^2+1.542x+21.04 \quad \text{.....[2]}$$

Where: x =Retention time and y = D_B and D_E

In case of D_B,

R = 0.98259569, R^2 = 0.96549429, Adjustable R^2 = 0.86197715, Standard error of estimate = 3.7196 Durbin-Watson statistics = 3.5714, Constant variance test: Passed (P = 0.0500), Power of performed test with α = 0.0500: 0.9175.

In case of D_E,

R = 0.98120093, R^2 = 0.96275527, Adjustable R^2 = 0.85102109, Standard error of estimate = 10.8503 Durbin-Watson statistics = 3.5714, Constant variance test: Passed (P = 0.0500), Power of performed test with α = 0.0500: 0.9088.

Where, R is the regression coefficient

Predicted values of D_B and D_E closely resembled with the experimental results up to 3rd order of polynomial regression analysis as the value of R^2 was above 0.80 and less than 1.0, i.e. 0.965 and 0.962 for D_B and D_E respectively and it gave minimum regression errors.

Results were further revalidated by normality test.

Normality test for D_B

Retention time: W-statistics = 0.987 P = 0.967 Passed

D_B: W-statistics = 0.877 P = 0.295 Passed

Normality test for D_E

Retention time: W-statistics = 0.987 P = 0.967 Passed

D_E : W-statistics = 0.870 P = 0.266 Passed

Where P indicates normal distribution coefficient.

The value of P was lesser than value of W- statistics so it passed the normality test.

The predicted values of D_B and D_E were plotted in **Figure 4.32** which shows a reasonable fit for the data. Curves plotted between D_B (experimental) versus D_B (predicted) and D_E (experimental) versus D_E (predicted) shows a linear relationship with a minimum deviation from the tangible values **(Figures 4.33 and 4.34).**

4.3.9: Optimization of flotation time

SOP subjected to pulping and enzyme + surfactant treatments (as per conditions mentioned in **Table 4.9**) was deinked at different flotation time (2-12 min). A significant decrease in dirt count (1790 mm^2/m^2) and ERIC value (81.50 ppm) was achieved at a flotation time of 10 min. A brightness level of 73.54% (ISO) was obtained at the same flotation time. However, a flotation time of 12 min neither improved brightness nor mitigated dirt count and ERIC value so efficiently **(Figure 4.35).** Therefore a flotation time of 10 might be considered to be optimum. Similarly, the effect of ink floatation time on D_B and D_E was investigated. Both D_B and D_E improved almost linearly up to an ink flotation time of 6 min and further improved slowly up to an ink flotation time of 10 min and then curves remained almost constant **(Figure 4.36).**

Both the rate of ink detachment from fiber and the rate of ink redposition during pulping were first-order processes [18]. Statistically designed experiments indicated that the process variables having the greatest effect on yield loss were flotation time and the interacting variables of pulping time and water hardness, and that of pulping temperature and flotation time [27].

Catsburg [36] indicated that increasing flotation time did not result in a higher removal efficiency of ink specks (50- 150 ppm). Borchardt [25] described that the increase in flotation time more than 10 min caused in yield loss and the brightness increase was also not substantial. Carrasco et al. [33] quoted a flotation time of 10 min to achieve an asymptotic value of 88% for deinkability factor. Further increase in flotation time produced minimal variation in deinking efficiency. Mathur [130] also reported that a 10 min flotation time gave good results. Prolonged flotation time might effect on ink particle size and also decrease in pulp yield.

Deinking model equations and statistical analysis

The nonlinear polynomial regression analysis program was used to predict the D_B and D_E for validation of experimental data.

$$y\ (D_B) = -0.023x^3+0.366x^2+1.403x+3.661 \quad [1]$$

$$y\ (D_E) = 0.033x^3-0.043x^2+11.90x+0.747 \quad [2]$$

Where:

x =Flotation time,

y = D_B and D_E

In case of D_B,

R = 0.99739597, R^2 = 0.99479872, Adjustable R^2 = 0.97919489, Standard error of estimate = 1.4953 Durbin-Watson statistics = 3.5238, Constant variance test: Passed (P = 0.0500), Power of performed test with α = 0.0500: 0.9969

In case of D_E,

R = 0.99984127, R^2 = 0.99968258, Adjustable R^2 = 0.99873030, Standard error of estimate = 1.0311, Durbin-Watson statistics = 3.5238, Constant variance test: Passed (P = 0.0500), Power of performed test with α = 0.0500: 1.0000

Where R is the regression coefficient.

The values of R^2 in above equations were 0.994 and 0.999 which stand at 0.80 and 1.0, i.e. for D_B and D_E respectively validating minimum regression errors up to 3rd order of polynomial regression analysis.

In order to revalidate the result for both D_B and D_E a normality test was also performed.

Normality test for D_B

Flotation time: W-statistics = 0.976 P = 0.911 Passed

D_B: W-statistics = 0.904 P = 0.435 Passed

Normality test for D_E

Flotation time: W-statistics = 0.976 P = 0.911 Passed

D_E: W-statistics = 0.860 P = 0.228 Passed

Where P indicates normal distribution coefficient.

The results of D_B and D_E passed the normality test as it gave lesser value of P than value of W-statistics.

The predicted value of DB and DE were plotted in Figure 4.37 and also shows a reasonable fit for the data. Curves were also plotted between experimental values of D_B against the predicted value of D_B and experimental values of D_E against the predicted value of D_E and show a linear relationship with a minimum deviation from the tangible values, shown in **Figures 4.38 and 4.39** respectively.

4.3.10: Deinking of sorted office waste paper (SOP) with different natural and commercialized enzyme concoctions under optimized conditions

Table 4.10 reveals the effect of different enzyme combinations on deinking efficiency compared to control during enzymatic deinking of SOP. SOP pulp when treated with crude cellulase (*A. niger* AT-3) prior to ink flotation, mitigated ERIC value and dirt counts by 61.84 and 82.29% where as brightness, D_B and D_E improved by 8.13, 23.10 and 76.30% respectively compared to control. Woodward *et al.,* [224], explained that cellulase binding on pulp fiber might result in a surface fiber alteration, sufficient to favour ink detachment during repulping. Nevertheless, other authors reported that the main effect was the hydrolysis and superficial degradation of cellulose that implied ink removal from fibers [48, 107,116]. Non-ionic surfactants were found to interact with cellulases to improve their action; they were often used to enhance enzyme assisted deinking [93,103]. Pélach *et al.,* [168] reported that cellulase improved ink detachment from old newspapers giving similar or better results when cellulase was used in place of classical chemicals. Bajpai and Bajpai [10] observed that increased ink removal was achieved, using an enzyme combined with a flotation deinking surfactant.

The introduction of cellulase treatment improved all the mechanical strength properties with freeness level and pulp viscosity except double fold numbers compared to control **(Table 4.10).** The increase in viscosity was due to the high specific surface area of the tertiary fines (generated during waste paper pulping) and the attack of cellulases was much specific towards this fraction of the pulp. Improvement in strength properties might be due to peeling effect

Jackson *et al.*, [87]. The cellulase present in crude enzyme preparation play an important role in reduction of refining energy as well as improvement in mechanical strength properties of enzymatically treated pulps. Pulp fibrillation by cellulase was recognized as means to enhance strength properties by Bolaski and his co-workers [24]. The combined effluent generated after completion of deinking trial at optimum conditions shows an increase in total solids, BOD and COD **(Table 4.10).** It might be due to hydrolysis of fibrils attached with ink particles and removal of additives /contaminants added during stock preparation of SOP. Magnin *et al.*, [125] has also experienced an increase in COD as a result of enzymatic treatments which might be due to the hydrolytic property of the enzymes. The enzyme releases soluble sugars from the pulp to the process water thus increasing COD.

Effects of multi-component enzyme in deinking of mix office waste was also investigated by Tandon *et al.*, [207] and their results proved that enzyme in double or triple component had much better impact than the enzyme having single component in terms of brightness and ink removal.

Table 4.10 shows the effect of cellulase (*A. niger* AT-3) and xylanase (*C. cinereus* AT-1) at different doses on the deinking efficiency of the SOP. At equivalent dose of cellulase and xylanase (6IU/mL each) during enzymatic treatment in presence of surfactant, pulp brightness, D_B and D_E improved by 11.20, 31.83 and 81.20% compared to control and 5.06, 8.73 and 4.9% respectively compared to cellulase treatment alone. Conversely, ERIC values and dirt counts reduced by 65.95 and 83.00% compared to control while 4.11 and 0.71 % respectively when compared to cellulase treatment alone. On the other hand, all the mechanical strength properties (burst index, tensile index and double fold numbers) decreased while tear index increased as a result of cellulase and xylanase treatment. Total solids, COD and BOD of combined effluent increased when compared to cellulase treatment alone. At the same time, tensile and burst indices showed an improvement compared to control while double fold numbers and tear index slightly decreased. Enzyme treatment hydrolyzed xylan (low molecular weight) from the pulp and resulted in an increase in the average molecular weight of the polymer system. Therefore, tear index slightly improved whereas other properties like, burst index and tensile index depending upon hydrogen bonding decreased due to depolymerization of xylan [194]

At half dosing of cellulase and xylanase treatments (3 IU/mL each), pulp brightness, D_B and D_E were found to increase by 8.23, 23.39 and 74.3% respectively compared to control. ERIC

value and dirt count decreased by 60.48 and 82.05% respectively. At the same time, all these parameters were found to be depleted in comparison to enzyme dosing in combination of 6 IU/mL each. Similarly, it shows a reduction in all the studied mechanical strength properties. It might be due to incomplete removal of ink particles and less hydrolysis of fines. It was validated by reduction in total solids, COD and BOD which were lower compared to full dosing of cellulase and xylanase during deinking.

Yet another enzymatic deinking trial was carried out using a cellulase and xylanase combination as 6 and 3 IU/mL respectively. An increase in the values of ERIC, dirt count, pulp brightness, D_B and D_E, freeness, viscosity, studied mechanical strength properties, total solids, COD and BOD of combined effluent generated during deinking was observed in comparison to the results obtained with half dosing of enzymes. The results were, however, the best with full enzyme dosing (i.e. 6 IU/mL for cellulase and xylanase each). The enzyme-combining (cellulase + hemicellulase) deinked pulp gave higher brightness and improved physical properties, and lower ERIC than pulps deinked with each individual enzyme [228]. The highest deinking efficiency of 62% was obtained using the cellulase–hemicellulase system during enzymatic deinking of laser printed office waste papers [118]. Paik and Park [158] observed that hemicellulases from *Aspergillus niger* and cellulases from *Trichoderma viridae* increased the brightness and deinking efficiency with increasing enzyme dosage and reaction time. Taleb and Maximino [206] investigated the effect of pergalase A-40 (a mixture of cellulase and hemicellulase) treatment on cellulosic fibers and observed that treated pulp showed higher tensile value with reduced tear index. A gain of 34% tensile index of the whole pulp was observed with 0.1% enzyme dose at 90 min reaction time. Gu *et al.*, [70] reported that a blend of cellulases and xylanases (50/50 ratio) was much effective and efficient for deinking of newsprint. Prasad *et al.*, [177] reported that cellulases and hemicellulases could also be effective in deinking and improving the brightness of letterpress and color offset printed newsprint. They found that the best brightness response on offset printed paper was obtained with a mixture of cellulase and hemicellulases. Pommeiar *et al.*, [175] and Bhat *et al.*, [19] reported that cellulase and mixtures of cellulase and hemicellulases, at low concentration, could be used to increase pulp freeness without affecting physical properties.

Since starch was widely present in mixed office waste, being used both as surface-sizing agent and wet-end additive, its degradation was very likely to aid cellulase assisted deinking.

Amylase enzyme influenced the degradation of the starch layer on the surface of the papers. The toner particles adhering to the paper surface released by the enzymatic treatment was subjected to subsequent separation from the pulp suspension *via* flotation. With this concept, SOP pulp was treated with different doses of cellulase, xylanase and amylase and its effect on deinking efficiency was studied. Cellulase, xylanase and amylase charged at different doses i.e. 6, 3 and 6 IU/mL respectively during enzymatic treatment improved brightness, D_B and D_E by 11.6, 33.25 and 82.5% respectively compared to control and these parameters improved by 1.90, 5.69 and 4.40% respectively compared to cellulase (6 IU/mL) and xylanase (3IU/mL) treatments **(Table 4.10)**. Alike, ERIC values and dirt counts mitigated by 67.05 and 86.81% respectively compared to control and 5.21 and 4.52% respectively compared to cellulase+xylanase (6+3IU/mL) treatments respectively. All the mechanical strength properties except tear index and effluent characteristics (total solids, COD and BOD) decreased compared to control as well cellulase and xylanase treatments.

In another set of experiments, the dosage of cellulase and xylanase was kept constant as above while amylase dosage was reduced from 6 IU/mL to 3 IU/mL; it shows insignificant reductions in brightness, D_B and D_E, ERIC value, dirt count, effluent characteristics and physical strength properties. A further reduction in amylase dosage from 6 IU/mL to 1.5 IU/mL shows reductions in all the parameters. All the three combinations where cellulase and xylanase dosage were kept constant while varying the amylase dosage at 6, 3 and 1.5 IU/mL respectively, brightness, D_B and D_E showed improvement and ERIC value and dirt count showed reduction compared to results obtained under a combination of cellulase and xylanase treatment (6IU/mL+3IU/mL).

Cellulase and hemicellulase, alone or in combination, were already routinely applied in many mill practices [75], but the strength properties of the deinked pulp were actually affected to some extent. Zollner and Schroeder [239] reported the use of a α-amylase to deink white office papers. They obtained a substantial increase in toner removal and showed that new enzymatic approaches could be useful. Mixed office waste often contained starch and therefore, the α-amylase increased the efficiency of the deinking treatment by degrading the starch layer on the surface of the paper. The results achieved by us indicated that by adding amylase enzyme to a cellulase assisted deinking process, it was possible to improve significantly the dirt removal by flotation. Using a selected surfactant along with a cellulase/amylase mixture, the area of

coverage of the residual toner particles measured by image analysis, was found to reduce up to 96% [46]. Zhenying *et al.*, [236] show based on their studies that combined enzymes of cellulase and amylase (1:1.5) had the best de-inking efficiency with 12% increment of brightness under optimal conditions of deinking: reaction temperature 40 °C and pH 9-10.

Further deinking trials involved a concoction of cellulase, xylanase, amylase and lipase. Printing inks containing vegetable oil based ink binders could only be degraded by lipase [137]. Lipases (triacylglycerol acyl hydrolases, E.C. 3.1.1.3) were enzymes catalyzing the hydrolysis of acyl glycerols at the interface of oil and water [95]. The deinking of other types of paper was also found to increase by treatment with lipases and esterases [61, 147], owing to an enzymatic hydrolysis of the oil-based binder or the resins in the ink. Furthermore, lipases had a surfactant effect due to their amphoteric properties and thereby facilitated the deinking of recovered paper. With this objective, SOP pulp was treated with varying dosages of lipase while keeping the dosage of cellulase, xylanase and amylase constant at 6, 3 and 1.5 IU/mL respectively (**Table 4.10**). Cellulase, xylanase, amylase and lipase at a dosing of 6, 3, 1.5 and 6 IU/mL respectively increased the pulp brightness, D_B and D_E by 13.3, 37.79 and 83.00% respectively compared to control and 2.58, 7.10 and 0.7% respectively compared to the concoction of cellulase, xylanase and amylase in the ratio of 6, 3 and 1.5 IU/mL. On the contrary, ERIC values and dirt counts mitigated by 68.17 and 88.03% compared to control and 1.95 and 1.39 % respectively compared to a mixture of cellulase, xylanase and amylase having concentrations 6, 3 and 1.5 IU/mL respectively.

Introduction of lipase in to the mixture mitigated all the mechanical strength properties except tear index and on contrary to that total solids, COD and BOD increased compared to control as well as the concoction of cellulase, xylanase and amylase (6, 3 and 1.5 IU/mL). Further, lipase dosages were reduced to 3 and 1.5 IU/mL respectively while keeping other enzyme concentrations constant. Minimum deinking efficiency was found when lipase concentration was decreased to 1.5 IU/mL.

Previous studies shows that a treatment of paper printed with soy bean oil based ink (VOI) with enzyme preparations containing cellulases, xylanases and lipases in addition to a neutral surfactant resulted in decreased dirt counts and residual ink areas [137]. The deinking effect of the lipases due to partial degradation of the binder of the soy bean oil based inks thereby released the ink particles from the paper [140]. Cellulase–hemicellulase–lipase gave a deinking

efficiency of 55–56% [118]. The addition of lipase from *Pseudomonas* species (KWI-56) to a deinking composition for ethylene oxide–propylene oxide adducts stearate improved whiteness of paper and reduces residual ink spots [61]. Ping qui *et al.*, [172] carried out enzymatic deinking using mixture of lipase, cellulase and xylanases (in which lipase was mixed with the 50/50 assembled cellulase/xylanase in a ratio of 40/60). The mixture displayed the best synergistic performance was observed by the increased breaking length, burst index and tear index values of the deinked pulp when compared with the cellulase/xylanase deinked pulp. The increasing deinking efficiency of our enzyme concoctions used on SOP pulps is summarized as under:

Control<C(6IU/mL)<CX(3 and 3IU/mL)< CX(6 and 3IU/mL)<CX(6 and 6IU/mL)<CXA(6,3 and 1.5 IU/mL)< CXA(6,3 and 3 IU/mL)< CXA(6,3 and 6 IU/mL)<CXAL(6,3,1.5 and 1.5 IU/mL)< CXAL(6,3,1.5 and 3 IU/mL)<CXAL(6,3,1.5 and 6 IU/mL) **(Figures 4.40-4.41).**

Deinking model equations and statistical analysis

The following empirical equations were obtained by using the experimental data from the nonlinear polynomial regression analysis program to predict the D_B and D_E

$$y\ (D_B) = 0.141x^3-2.974x^2+20.33x-4.866.....[1]$$

$$y\ (D_E) = 0.417\ x^3-8.903x^2+58.64x-29.32.....[2]$$

Variables,

x =Enzyme combinations, y = D_B and D_E

In case of D_B,

R = 0.94306469, R^2 = 0.88937100, Adjustable R^2 = 0.84195858, Standard error of estimate = 4.1088, Durbin-Watson statistics = 2.3920, Constant variance test: (P = 0.0290), Power of performed test with α= 0.0500: 0.9988

In case of D_E,

R = 0.89247699, R^2 = 0.79651519, Adjustable R^2 = 0.70930741, Standard error of estimate = 13.1889, Durbin-Watson statistics = 2.0739, Constant variance Test: Passed (P = 0.3535), Power of performed test with α = 0.0500: 0.9820

Where R is the regression coefficient

As the value of R^2 was above 0.80 and less than 1.0, i.e. 0.889 and 0.796 for D_B and D_E respectively, it means the predicted values of D_B and D_E gave minimum regression errors up to a 3rd order of polynomial regression analysis.

Further, normality test was performed for the validation of results.

Normality test for D_B

Enzyme combinations: W-statistics = 0.968 P = 0.870 Passed

D_B: W-statistics = 0.757 P = 0.632 Passed

Normality test for D_E

Enzyme combinations: W-statistics = 0.968 P = 0.870 Passed

D_E: W-statistics = 0.442 P = 0.312 Passed

Where P indicates normal distribution coefficient.

The lesser values of P compared to W-statistics pass the normality test.

The predicted values of D_B and D_E were plotted in **Figure 4.42** which shows a reasonable fit for the data. Curves were plotted between experimental values of D_B against the predicted value of D_B and experimental values of D_E against the predicted value of D_E and illustrated a linear relationship with a minimum deviation from the tangible values **(Figures 4.43 and 4.44 respectively).**

4.3.11: Comparison of conventional enzymatic and chemi-enzymatic deinking.

Table 4.11 shows the comparison among chemical, chemi-enzymatic and enzymatic deinking of SOP pulp. In conventional deinking, alkalis such as sodium hydroxide and sodium silicate, oxidative bleaching agents such as hydrogen peroxide, chelating agents and surfactants were added to the waste paper to accelerate the release of ink from the pulp fiber. Thereafter, a washing and/or flotation were used to carry out deinking to separate the pulp and ink. Chemical deinking showed hike in brightness (10.35%), D_B (24.67%) and D_E (78.71%) along with reduction in ERIC value (64.27%) and dirt count (83.74%) compared to control **(Figure 4.45).** During deinking of SOP paper, because the objective of effective deinking involved detachment and saponification of printing ink and pulp swelling, alkaline chemical agents were used. NaOH favored swelling and consequently, increased the fibers bonding potential by enhancing their flexibility, conformability and surface area; as the fiber/water interactions were modified (fibers become more hydrophilic), the fibers water intake increased and drainage was hindered [19, 129]. It was reported that sodium hydroxide (NaOH), a conventional deinking agent, turned the reprocessed paper yellow, and reduced strength of the reprocessed paper sufficiently so that an additional treatment was necessary [232].

Likewise, there was an improvement in all the mechanical strength properties and increment in total solids, COD and BOD compared to control. Ink removal, washing and

flotation also modified the pulp and paper properties, as they altered the amount of fines and mineral fillers in the pulps [159]. When fillers were removed, the inter-fiber interactions increased and the paper strength improved; on the contrary, when fines were removed, the paper sheet becomes more permeable and less dense, and the paper strength decreased. Due to their size (<2μm), washing was more efficient than flotation in the removal of both fines and fillers [44]. Further, as pulp was normally treated by being suspended in water of a volume that was equivalent to several dozen times the weight of the dry pulp; a vast amount of alkaline effluent was generated. The removal of fines, fillers and other functional additives (starch, resins, sizing agents and other specialty chemicals added as per requirement) and degradation of cellulose due to alkaline peeling reactions increased the effluent load.

In conventional chemical deinking technology, the pulp pH was 13–14. Toner particles were easily removed from fibers at the strong alkali condition. But the waste liquid of high pH was harmful to environment [236]. The chemical agents were used in the treatment to neutralize the effluent; the recovery treatment was one that involved a heavy environmental burden. In SOP, toners were very difficult to be removed by the application of current methodologies because they contained thermoplastic binders that polymerized and fused onto the paper fibers during the high-temperature printing process; when these fibers were chemically treated, the toner particles usually remained as large, flat, rigid particles that were separated very poorly from fibers during the fiber/ink separation stages [182]. Regarding polymeric inks, it was generally assumed that the chemical treatment did not induce fragmentation [8,151,157,222]

The SOP pulp having pulp brightness 61.80% (ISO) and ERIC values 283.20 ppm on treatment with cellulase before ink flotation showed a change in brightness, D_B, D_E, dirt counts and ERIC values compared to chemical deinking. However, there was an insignificant reduction in mechanical strength properties as well as in total solids, COD and BOD compared to chemical deinking. On the other hand, brightness, D_B and D_E improved with a reduction in dirt counts and ERIC values relative to pulping in water without chemicals and without cellulase treatment stage. The same findings were observed by Prasad [179] and Jeffries *et al.*, [89]. Elegir and Bussini [47] reported that enzymatic deinking showed a clear enhancement in ink removal and brightness with reduced specks contamination of pulp, in comparison to conventional deinking. Kim *et al.*, [107] reported that newspaper pulps bleached after being deinked by enzymatic and conventional means had similar brightness values. A similar study [185] with letterpress

newspaper produced enzymatically deinked pulps with lower initial brightness values than those for conventionally deinked pulps. They further added that the enzymatically deinked pulps were thus easier to bleach and required half the hydrogen peroxide. Subsequent bleaching with hydrogen peroxide, however, produced similar brightness values, with peroxide usage lowest for the enzymatic process.

Enzymatic deinking of mixed waste paper (laser/xerographic printed and UV-coated paper) and old magazines (ONP/OMG) eliminated or substantially reduced the use of chemicals in the deinking process. Kim *et al.,* [107] reported that waste water effluent from enzymatic deinking was 20-30% lower in COD than conventional deinking. Putz *et al.,* also observed that COD load after enzyme treatment was 50% lower than for conventional deinking [181]. Reduction in COD after enzymatic deinking was also reported by Xu *et al.,* [227] and Pala *et al.,* [160]. Elegir and Bussini [47] also evaluated the enzyme assisted deinking of wood containing paper and observed reduction in COD by 20 and 50% for offset and flexographic print respectively. Tandon *et al.,* [208] reported that the amount of sludge generation was 27% lower than the conventional process due to the less fiber loss. The properties, especially the physical properties of the deinked pulp were not as good as those of pulp deinked chemically [233].

Further in chemi-enzymatic deinking trials, the dosages of chemicals were reduced to from 100% to 75, 50 and 0% while keeping the dosage of cellulase constant. **Figures 4.45** and **4.46** shows that brightness, D_B, and D_E increased with increasing chemical dosing and conversely, dirt count and ERIC value decreased accordingly during chemi-enzymatic deinking compared to enzymatic deinking. Similarly, all the strength properties mitigated as a result of increasing chemical dosing and total solids, COD and BOD of combined effluent increased in chemi-enzymatic deinking compared to enzymatic deinking.

From ecological point of view, chemi-enzymatic deinking hence, have come up as one of the most promising deinking technology based on our studies carried out.

Deinking model equations and statistical analysis

The following empirical equations were obtained by using the experimental results from the nonlinear polynomial regression analysis program to predict the D_B and D_E

$$y\ (D_B) = -0.984\ x^3 - 10.86x^2 + 34.27x + 8.99 \quad \ldots\ldots\ldots\ldots\ldots\ (1)$$

$$y\ (D_E) = 1.649\ x^3 - 17.79\ x^2 + 55.02x + 45.24 \quad \ldots\ldots\ldots\ldots\ (2)$$

Variables,

x = enzymatic/chemi-enzymatic treatment, y = D_B and D_E

In case of D_B,

R = 0.94306469, R^2 = 0.88937100, Adjustable R^2 = 0.84195858, Standard error of estimate = 4.1088, Durbin-Watson statistics = 2.3920, Constant variance test: (P = 0.0290), Power of performed test with α= 0.0500: 0.9988

In case of D_E,

R = 0.89247699, R^2 = 0.79651519, Adjustable R^2 = 0.70930741, Standard error of estimate = 13.1889, Durbin-Watson statistics = 2.0739, Constant variance Test: Passed (P = 0.3535), Power of performed test with α = 0.0500: 0.9820

Where R is the regression coefficient

As the value of R^2 was above 0.80 and less than 1.0, i.e. 0.889 and 0.796 for D_B and D_E respectively, it means the predicted values of D_B and D_E gave minimum regression errors up to a 3rd order of polynomial regression analysis.

Further, results were reconfirmed by normality test.

Normality test for D_B

Enzyme combinations:	W-statistics = 0.968	P = 0.870	Passed
D_B:	W-statistics = 0.757	P = 0.632	Passed

Normality test for D_E

Enzyme combinations:	W-statistics = 0.968	P = 0.870	Passed
D_E:	W-statistics = 0.442	P = 0.312	Passed

Where P indicates normal distribution coefficient.

The lesser values of P compared with W-statistics passed the normality test.

The predicted values of D_B and D_E were plotted in **Figure 4.47** which also shows a reasonable fit for the data. Curves were plotted between experimental values of D_B against the predicted values of D_B and experimental values of D_E against the predicted value of D_E and showed a linear relationship with a minimum deviation from the tangible values, shown in **Figures 4.48 and 4.49** respectively.

4.3.12: Effect of repeated recycling on paper properties

Mechanical strength properties like burst index, tensile index and double fold number decreased as a result of repeated recycling whereas tear index increased up to 3rd recycling with

further recycling (4^{th}) produced an insignificant increase in tear index. Bulk and opacity increased up to 3^{rd} recycling and then remained almost constant. In case of enzymatic deinked pulp, strength properties follow the same pattern but they were inferior compared to chemical deinking due to the hydrolysis of cellulose by cellulases **(Table 4.12).** The deterioration of the burst index, tensile index and double fold properties of recycled fibers was mainly due to the irreversible structural changes in the fiber wall caused by drying [112]. When the fibers are dried they lose their conformability and swelling capacity, which could not be recovered by rewetting the fibers. The structural change in the fiber cell wall leads to the changes in pulp properties, i.e. hornification, was a typical feature of chemical pulps. It was suggested that hornification was caused by irreversible hydrogen bonding between cellulose microfibrils [112], resulting in inferior strength properties and bulky sheet. Surface deactivation addressed loss in the fiber surface bonding agent. The effect of surface bonding compared with fiber swelling on strength properties of recycled fibers was recently given attention [171]. It was reported that the surface treatment was more effective than fiber swelling if the strength properties of the recycled fibers were considered. The necessary work that had to be done to pull the fibers loose depends on the length of the fibers in tension as well as the bond strength. The increase in tear index may be explained as repeated recycling caused the fibers to become stiffer due to the effect of drying and inter fiber bond strength would be lesser due to removal of microfibrils. More energy would be required to pull the fibers from the mesh. When sheet was prepared from stiffer fibers, they tended to retain its tubular structure instead of forming double walled ribbon structure. They provide less surface contact area for bonding. The sheet would be bulky and opaque in nature.

4.3.13: Micro-stickies removal in different deinking processes

Stickies originate from adhesives, ink binders and coating binders. Stickies able to pass through a sieve of mesh size 100 or 150μm (depending on standard) were called micro-stickies, whereas, the particles retained on the screen were called macro-stickies. Dissolved and colloidal stickies were called secondary stickies [188]. Stickies were tacky, hydrophobic, pliable organic material found in recycled paper systems. Stickies were composed of a variety of materials including adhesives, styrene-butadiene latex, rubber, vinyl acetate and hot melts etc. They affect both the process efficiency (breaks, formation of deposits on various equipments of the paper machine, reduction of the drying section efficiency due to felts clogging, etc.), and the quality of the final product (presence of spots, holes and other defects) [22,43]. However, flotation was

very effective in stickies removal but various enzymes applications were also found effective to remove stickies in waste paper. Chemicals, cellulase, cellulase+xylanase, cellulase xylanase+amylase and cellulase xylanase+amylase+lipase deinking processes removed micro-stickies by 42.02, 46.01, 46.07, 46.24 and 48.59% respectively compared to control (3060 no./kg) **(Table 4.13).** Cellulase alone improved the stickies removal by 4% as compared to control. Further, xylanase and amylase introduced in the mixture did not bring a significant improvement (0.06 and 0.23% respectively) in stickies removal. Lipase addition was also significant in improving the stickies removal. It means xylanase with cellulase acted synergistically for ink removal and amylase effectively degraded starch size and released ink particles from the fiber surface instead of removing stickies. Different from amylases and xylanases, cellulases were also found to work as surface cleaning agent during deinking. Lipase attack the ester constituents of the tonners and sizing material in the paper furnish and released the ink particles from the fiber. As a possibility, the cellulase mixtures could have released sticky particles from fiber surfaces by the same mechanism used to describe enzymatic toner ink removal [87, 89]. Lipase might work on synthetic stickies substrate in a very different way when stickies become intermingled with printing inks and papermaking additives [205]. Park *et al.*, observed reduction in stickies by using cellulase in deinking of mixed office waste and old news print [165]. Sykes and John *et al.*, [205] also observed reduction in stickies in mixed office waste paper using lipase and cellulase alone and mixture of both during deinking.

4.3.14: Ink particle size distribution in different deinking processes

Proper ink particle size and air bubble ratio was important for good flotation. Care *et al.*, [34] proposed that the optimum bubble size was approximately five times than those of ink particles to be removed. Ink particle sizes (specks) in terms of speck numbers and specks/cm^2 were determined in pulps after chemical, enzymatic and chemi-enzymatic deinking processes and were found in the particle size range of 8-20, 20-80, 80-224 and 8-2000 μm respectively. The average speck diameter in the above particle size ranges for the chemical, cellulase, cellulase+xylanase, cellulase xylanase+amylase and cellulase xylanase+amylase+lipase, chemical (100%) +cellulase, chemical (50%) +cellulase chemical (25%) +cellulase deinking processes were found with standard deviations 12.34±0.46, 35.62±1.32, 122.19±3.6 and 44.64±1.52. Ink particles size in terms of number of specks and number of specks/cm^3 in chemical and enzymatic deinking processes reduced top to down **(Table 4.14)** in the following order:

chemical>cellulase>cellulase+xylanase>cellulase+xylanase+amylanase>cellulase+xylanase + amylase+lipase. It means that mixture of enzymes was capable of removing all sizes of ink particles from the deinked pulp. Number of specks in cellulase xylanase+amylase+lipase deinked pulp reduced by 55.71% in 8-20 μm size range, 37.29% in 20-80 μm size range, 42.42% in 80-224 μm size range and 48.70% in 8-2000 μm size range compared to chemical deinking. Similarly, number of specks/cm^3 reduced by 56.84% in 8-20 μm size range, 38.01% in 20-80 μm size range, 42.47% in 80-224 μm size range and 48.99% in 8-2000 μm size range compared to chemical deinking **(Table 4.14).** Regardless of ink type or printing process, enzymatic treatment was also helpful in reducing ink particle size. Kim *et al.,* [107] reported that reductions in particle size varied with pulping time in the presence of cellulases for standard newsprint and that overall reduction was greater than those observed with conventional deinking. Prasad *et al.,* [177] and Rushing *et al.,* [185] reported reductions varying from 16 to 37% depending on ink type. On contrary to this, in chemi-enzymatic deinking processes, ink particle sizes observed in the range of 8-20, 20-80 and 80-224 μm increased in the following order: chemical (100%)+ enzyme< chemical (50%)+ enzyme< chemical (25%)+ enzyme. Whereas ink particle sizes observed in the range of 8-2000 μm shows a decreasing trend for the above mentioned chemi-deinking processes (**Table 4.14**). Smaller ink particles had a greater tendency to redeposit on to the fibers than larger ones. The very small ink particles produced by pulping of paper printed with water-based (flexographic) inks, were extremely difficult to remove and could deposit inside the fiber lumen [173]. The equal amount of ink present in deinked pulp in the form of very small particles had a more adverse effect on brightness than that observed in case of larger ink particles [217]. Ink particles less than 2.5 mm in diameter had the most adverse effect on brightness [173]. Enzyme treatment resulted in the reduction of ink particle size. It was reported that reduction in particle size varied with pulping time in the presence of cellulases; overall reduction was greater than that observed in conventional deinking [107]. Prasad *et al.,* [177] and Rushing *et al.,* [185] reported reductions in particle size from 16% to 37%, depending on ink type. Reducing the pulping pH to near-neutral values can reduce newspaper ink dispersion [167,169, 173]. Image analysis indicates that increasing the sodium hydroxide concentration (and thus pH) increases the formation of very small ink particles [28]. Up to now, there is no clear cut justification for this reduction in the size of ink particles could be formed.

4.3.15: Evaluation of deinked pulp fibers of SOP by various techniques

4.3.15.1: Atomic force microscopy (AFM) and SEM

AFM was capable of measuring the surface structure at atomic resolution. The structural changes in the cellulose surface concomitant with the action of different enzyme combinations were analyzed. One of the features obtained from AFM measurements was the degree of roughness, which was used to analyze changes in the surface properties (handsheets prepared from deinked SOP pulp) brought about by friction, adhesion, and bio-catalytic activity. Ink particles, rosin and resins being hydrophobic in nature, were marked as dark areas in the phase imaging, while carbohydrate i.e. cellulose, starch and hemicelluloses being hydrophilic were marked as bright **(Microphotograph 4.1A).** On the surface of SOP fibers, irregular particles or granules were seen which might be of tertiary fines (broken microfibrils), ink particles or rosin or resins **(Microphotograph 4.1B)** which were further validated by SEM that shows the presence of small particles deposited on fiber surface **(Microphotograph 4.1D).** The surface of untreated SOP (control) fibers shows the presence of swollen fissures, surface cracks or trenches. **Microphotograph 4.1C** illustrates that the surface roughness of SOP fibers was found to lie in the range from 0-50 nm.

Chemically deinked SOP pulp shows surface irregularities that appeared as a result of dislodging of ink films from the fiber surface which were efficiently removed from the suspension during the flotation step **(Microphotograph 4.2A).** While acting on paper fibers (making them swell), NaOH contributed to ink removal as it favored the detachment and fragmentation of the adhered ink [222]. Additionally, it may also act directly on the printed ink film and weaken its structure, leading to fragmentation [193]. The topographical structure of cellulose fibers shows that removal of paper additives (rosin, resin, polymeric ink binders etc.) increased the hydrophilic area on fiber surface i.e. reduction in dark areas in the phase imaging as a result of chemical deinking **(Microphotograph 4.2B).** On the other hand the surface of chemically deinked fibers became grainy compared to control SOP fiber which might be due to deposition of hemicelluloses, celluloses and starches (added during stock preparation as dry strength additives) as a result of peeling reactions that occurred in presence of NaOH [80, 133]. SEM also revealed irregular particles or granules present on fiber surface **(Microphotograph 4.2D).** The surface roughness of paper increased by 10 nm compared to SOP pulp **(Microphotograph 4.2C).**

The surface roughness of cellulose fibers begins to increase after adding cellulase during enzymatic deinking of SOP **(Microphotograph 4.3A).**These observed changes were brought about by cellulase action which might constitute the first direct visualization, supporting the fact that the exocellulase selectively hydrolyzed the hydrophobic faces of cellulose. The limited accessibility of the hydrophobic faces in native cellulose might contribute significantly to the rate-limiting slowness of cellulose hydrolysis. Natural cellulose is a bundle of linear -1, 4-linked glucan chains held tightly in a crystalline structure by the cumulative effect of many inter- and intra chain hydrogen bonds. SEM **Microphotograph 4.3D** clearly demonstrates as to how the cellulase treatment modified the fiber surface by introducing external fibrillation, cracks, swelling and peeling and thereby making the fiber surface more rough and heterogeneous, with small microfibrils on surface **(Microphotograph 4.3B).** Instead, control pulps **(Microphotographs 4.1A-D)** were smoother and cleaner with no sign of fibrillation. Non-fibrous additives, which were deposited on the surface of fibers, constituted a physical barrier for the penetration of bleaching agents after deinking. Cellulase treatment was effective in opening closed cell-wall pores of pulps, as a result of the cellulose hydrolysis that caused hydrolysis of glycosidic linkages anywhere in cellulose chain (micro-fibrils), affected their bonding with non-fibrous additives and, their elimination facilitated the flow of bleaching agents. Commercial cellulase mixtures usually contained one or more exoglucanases such as cellobiohydrolase (CBH), which would proceed from either the reducing end or non-reducing end of the cellulose chain and produced a shortened chain and cellobiose. The cellulase mixture might also contain several endoglucanases (EG-I, EG-II, etc), which cleaved randomly the internal β-1, 4-glycosidic bonds of the cellulose chain along its length to produce free chain ends that would be acted upon by exoglucanases [229]. Treatment with cellobiohydrolase resulted in the appearance of distinct pathways or tracks along the length of the macro-fibril. Treatment with endoglucanases appeared to cause peeling and smoothening of the fiber surface [117]. The surface roughness (70 nm) of enzymatically (cellulase) deinked fibers was 20 nm more compared to SOP fibers **(Microphotograph 4.3C).** Presumably, the hydrophobic faces consisted of more than one cellulose chain, thus the roughness change might be indicative of the fact that the cellulose chains were hydrolyzed individually.

Microphotograph 4.4 A-C showed the AFM images of fiber surface obtained as a result of enzymatic (cellulase+xylanase) deinking of SOP pulp. Endoxylanases cleaved the internal

glycosidic linkages of the heteroxylans backbone, resulting in a decreased degree of polymerization of the substrate while β-D-xylosidases were exoglycosidases that hydrolyzed smaller xylo-oligosaccharides and xylobiose from the non-reducing ends to liberate monomeric xylose [223]. Release of xylan-stock preparation additives complexes increased hydrophilic character of fiber surface and the topographical structure shows much changes on fiber surface in terms of surface roughness **(Microphotograph 4.4 A)** which might be due to appearance of microfibrils on fiber surface. SEM studies also highlights the fibrillar structure of cellulase+xylanase treated fibers and appearance of grooves and ridges, a few cracks and considerable damage to the fiber **(Microphotograph 4.4 D).** The fiber surface was less grainy **(Microphotograph 4.4 B).** Cellulase+xylanase treatment enhanced surface roughness up to 90 nm **(Microphotograph 4.4 C).**

Besides cellulase and xylanase, the introduction of amylase during enzymatic deinking further led to an increase in paper roughness. Since starch was widely present in SOP being used both as surface-sizing agent (for improving surface properties) and wet-end additive (for improving dry strength of paper, its degradation was very likely to aid cellulase assisted deinking. Starch also formed bonds with binders present in ink, wet strength resins, rosin, fillers, cellulose and hemicelluloses. The fiber surface was attacked by amylase to release ink particles from their surface due to solubilization of starch. α-Amylases (E.C.3.2.1.1) were endo-amylases catalyzing the hydrolysis of internal α-1, 4-glycosidic linkages in starch in a random manner [148, 149]. AFM of cellulase, xylanase and amylase treated fibers shows that fiber surface was rough with more surface irregularities **(Microphotograph 4.5A),** grainy fiber surface **(Microphotograph 4.5B)** and surface roughness increased by 120 nm **(Microphotograph 4.5C).** SEM shows that the fibers were fibrillated with deposition of irregular particles on their surface **(Microphotograph 4.5D).** It indicates that amylase acts in a different way than cellulase, facilitating a greater removal by flotation of smaller ink particles and showing a great deal of synergism with cellulase. Using a selected surfactant along with a cellulase/amylase mixture the area of coverage of the residual toner particles measured by image analysis was reduced up to 96% [46].

The introduction of lipase to the mixture of cellulase, xylanase and amylase caused the hydrolysis of oil-based binders or of the resins in the ink thereby facilitating the deinking of recovered paper. Lipases (triacylglycerol acylhydrolases, E.C. 3.1.1.3) were enzymes catalyzing

the hydrolysis of acyl glycerols at the interface of oil and water [92]. **Microphotograph 4.6A** shows much rougher surface containing more granular particles. It may be due to removal of additives which increased the hydrophilic area on fiber surface (**Microphotograph 4.6B**). The surface roughness increased by 159% compared to control SOP **(Microphotograph 4.6C).** SEM confirmed the deposition of granules on fiber surface and broken microfibrils **(Microphotograph 4.6D)**

4.3.15.2: Fourier transform infrared (FTIR) spectroscopy

FTIR spectroscopy had been used to identify polysaccharides, elucidate structure and investigate complex and inter-molecular interactions. The chemical structure of SOP and deinked pulps (chemical and enzymatic) was confirmed by FTIR spectroscopy and shown in **Micrc graph 4.1.** The major variations were observed at wavelengths of 3430 cm^{-1}, 3424.89 cm^{-1}, 3415.06 cm^{-1}, 2909 cm^{-1}, 2901.20 cm^{-1}, 2900.16 cm^{-1}, 875 cm-1 and 1797.8 cm^{-1} during chemical and enzymatic deinking of SOP. The bands near 3400 cm^{-1} were representative of OH vibrations. The band of OH vibrations was wide and moved to higher wave number (3430 cm^{-1}) in case of SOP because of inter and intra molecular hydrogen bonds. After the chemical and enzymatic deinking, the bands near 3400 cm^{-1} became narrow and move to low wave numbers i.e. 3424.89 cm^{-1} and 3415.06 cm^{-1} respectively compared to SOP (control). There was good evidence that a portion of the hydrogen bonds were broken during chemical and enzymatic hydrolysis. The sharp band at 2909 cm^{-1} indicated the valence vibrations of the $-CH_3$ and $-CH_2$ groups i.e. aliphatic C-H stretching [104] corresponding to the aliphatic moieties in cellulose and hemicelluloses. After the chemical and enzymatic deinking, the bands near 2909 cm^{-1} were reduced to wavelength i.e. 2901.20 cm^{-1} and 2900.16 cm^{-1} indicating C-H bond stretching [54]. SOP pulp shows the appearance of band at 875 cm^{-1}. Hua *et al.,* [82] reported that mannose and glucose units were assigned characteristic peaks at about 814 and 873 cm^{-1}, respectively, which were attributed to C-H band vibration. Widjanarko *et al.,* [220] shows that mannose and glucose units of glucomannan were assigned the β- pyranose form with characteristic peaks at 808.12 and 875.62 cm^{-1}, respectively. These vibrations originate from the equatorially aligned hydrogen at C (2) in the mannose residues and they were thus orthogonally directed towards the backbone of the glucomannan [6]. The disappearance of this band at 875.82 cm^{-1} shows the dissolution of xylan by crude enzymes during enzymatic deinking and by peeling reactions during chemical deinking [80, 133]. The peak centered at 1770-1800 cm^{-1} corresponded to vinyl ester group,

which came under strong intensity region [38]. The band at a wavelength of 1797.80 cm^{-1} was observed in FTIR spectrum of SOP (control) which disappeared in chemical and enzymatic deinked pulp. Binders in ink served to bind the pigments and provided adhesion to the substrate (cellulose or hemicelluloses or starch) and it might be vinyl ester resin. On the other hand, SOP contained filler (mineral pigments) which were used to fill up the voids in paper. The four sharp absorptions at 2524 cm^{-1}, 1797 cm^{-1}, 875 cm^{-1} and 712 cm^{-1} and the strong but broad absorption between 1400 cm-^{1} and 1500 cm^{-1} were characteristic of chalk ($CaCO_3$), used as a filler in the paper. SOP pulp when subjected to chemical and enzymatic deinking caused the removal of $CaCO_3$. Similar findings were observed by Learner [115]. Verma *et al.,* [212] assigned the band at 1797 cm^{-1} to CO stretching of carboxylate groups corresponding to aragonite.

4.3.15.3: X-ray diffraction (XRD) studies

Micrograph 4.2 represents the X-ray diffractogram of SOP, enzymatically and chemically deinked pulps and showed major and the highest intensity peak at a 2θ value in the range of 20–28°. The percentage crystallinity of SOP was observed to be 36.90. The crystallinity of chemically deinked pulp was improved by 1.2%. The degree of crystallinity of cellulose was found to be higher in pulp samples than in control. Since peeling and hydrolysis reactions were known for cellulose under alkaline pulping conditions, less ordered cellulose fractions and impurities (pectins, waxes etc.) were removed by these reactions. Thus, the amount of crystalline cellulose increased in pulp samples [195]. Further, enzymatically deinked pulp shows an improvement in crystallinity by 4.4% compared to control. It was known that there were three processes that occurred before onset of the enzymatic hydrolysis of cellulose: (a) the diffusion of cellulase in the liquid; (b) the transfer of cellulase from the liquid to the surface of substrate and, (c) the enzyme absorption onto the cellulose with the formation of an enzyme–cellulose complex. The increase in the crystalline index was good evidence that the amorphous portion of the cellulose was more readily hydrolyzed than the crystalline. The hydrogen bonds of the OH groups were partly broken in the enzyme-hydrolyzed cellulose as estimated by FTIR spectroscopy also. The similar observations were obtained by Cao and Tan [31]. Vyas and Lachke [215] suggested that the amorphous portion of cellulose was more susceptible to the hydrolytic action of Endo-A and Endo-B than crystalline portion of the substrate. They also observed that the role of endoglucanase in overall process of cellulose degradation was to split the cellulose fibers at several amorphous sites and generate innumerous non-reducing ends of the

chain. SEM and FTIR studies also supported this action of loosening the fibers which in turn helped in releasing ink particles from SOP paper during flotation deinking process in presence of surfactants. Secondly, cellulases act at the frazzled surfaces of cellulose fibers and released short fibers from it. It could be interpreted that this short fiber forming activity also removed residual fibers from the toner surfaces. Finally, enzymes released fibers from surface of hairy toners that in turn enhances the flotation efficiencies by increasing hydrophobicity of ink particles **(Microphotograph 4.3)**. Therefore, the crystalline structure of cellulose affects physical and mechanical properties of cellulose fibres. The degree of crystallinity of cellulose was one of the most important crystalline structure parameters and the rigidity of cellulose fibres increased, but flexibility decreased with increasing ratio of crystalline to amorphous regions [86, 210]. In addition, the crystallinity of cellulose was an important role on accessibility [7].

Micrograph 4.3 shows the X-ray diffractogram of cellulase and combinations of other enzymes with cellulase like xylanase, amylase and lipase deinked pulps. The introduction of xylanase during cellulase deinking enhanced the crystallinity of cellulase+xylanase deinked pulp by 0.90% compared to cellulase deinked pulp. Hemicellulose had a random, amorphous structure, low molecular polysaccharides with little strength. It was easily hydrolyzed by dilute acid or base as well as myriad hemicellulases enzymes. β-1, 4-Xylan was amorphous, β-1, 3-xylan was crystalline [20]. The depolymerization of low molecular weight xylan by xylanase causes the increase in crystallinity of cellulase+xylanase deinked pulp. The cellulose and hemicellulose hydrolysis on the surface of the fibers led to a removal of small fibrils, a phenomenon known as ''peeling-off fibers'', which facilitated ink detachment from the surface.

Further, combination of enzymes (cellulase xylanase+amylase) improved the crystallinity of enzymatically deinked pulp by 1.7%. Native starch was composed of almost linear amylose, a α-1, 4 polymer, and amylopectin, a branched polymer consisting of short linear α-1, 4 polymer chains linked to each other by α-1, 6 linkages [238]. These two components were amenable to form a semi-crystalline structure in the starch granules, which consists of crystalline lamellae (ordered, tightly packed of parallel glucan chains) and amorphous lamellae (less-ordered regions, predominantly branch points) [150]. α-amylases (E.C.3.2.1.1) were endoamylases catalyzing the hydrolysis of internal α-1, 4-glycosidic linkages in starch in a random manner [148, 149,161]. Elegir *et al.*, [46] observed that amylase acts in a different way than cellulase facilitating a greater removal by flotation of smaller ink particles and showing a great deal of synergism with

cellulase. The introduction of lipase in the mixture of cellulase, xylanase and amylase improved crystallinity of enzymatically deinked pulp by 2.16% compared to cellulase deinked pulp. The waste paper pretreated with 1% lipase changed cellulose structure with the formation of carboxyl groups and increasing index of crystallinity during enzymatic deinking [123].

Micrograph 4.4 shows X-ray diffractogram of chemi-enzymatic deinked SOP pulp where the dosing of cellulase was kept constant at 6 IU/mL and chemical dosing (NaOH, H_2O_2, sodium meta silicate and DTPA were fixed at 0, 25, 50 and 100% respectively keeping other conditions constant as per **Table 4.11**). There was an insignificant increase in crystallinity of pulps with increasing chemical dosing i.e. from 41.3 to 42%.

It could be observed from **Micrograph 4.4,** the degree of crystallinity increased by increasing the chemical doses. **Table 4.11** reveals an indirectly proportional relation between pulp yields and crystallinity of cellulose in SOP pulp. Pulp yield reduced due to removal of some low molecular weight carbohydrate fractions and impurities included in SOP pulp during pulping. If the amorphous domains of cellulose were attacked, hydrolysis and peeling reactions occurred which reduced the total amount of amorphous cellulose and therefore, increased the relative degree of crystallinity. Gümüskaya *et al.,* [73] observed that the crystalline structure of cellulose in cotton linters was improved during soda pulping compared to control. Likewise, Hubbe *et al.,* [83] observed increase in crystallinity due to recycling.

4.3.15.4: Thermal studies

The information regarding thermal stability of a material was necessary to determine its thermo-mechanical properties. The TGA and DTG curves of SOP and pulps deinked by chemical, cellulase, cellulase+xylanase, cellulase+ chemical (100%) and cellulase+chemical (50%) were shown in **Thermograms 4.1-4.3.** The weight loss in SOP at a temperature between 27-100 ^{0}C was 5.40% and weight losses decreased with increasing crystallinity of deinked pulps in the following order: chemical deinked pulp>cellulase deinked pulp> cellulase+chemical (50%)>cellulase+xylanase deinked pulp>cellulase+chemical (100%) deinked pulp. Water contents in paper existed in two forms i.e. free and bound water contents. Free water content was the one which was present in voids (inter fiber spaces), and bound water content was found to be dependent on the degree of crystallinity of cellulose; the higher the crystallinity, the lower the bound water content [216]. It was well known that the moisture content of cellulose depends on the molecular structure of the amorphous part of cellulose [58]. Water molecules could only diffuse through amorphous regions of cellulose samples [121]. Nakamura *et al.,* [146] described

the relation between water molecules and crystallinity of various cellulose samples. Cellulose hydrolysis by microbial cellulases was often limited by the degree of crystallinity of the substrates. Hydrogen bonds between cellulose microfibrils in crystalline regions were so strong that no further hydrogen bonds with water molecules could be formed. However, the amorphous regions of the cellulose could form hydrogen bonds with water molecules, and hence, the water molecules in these regions remains as bound water. According to their studies, only amorphous regions in cellulose molecules could form hydrogen bonds with water molecules and could be regarded as the site of water molecule adsorption. Owing to lower crystallinity index (36.90%), of SOP, the weight loss (5.40%) in the evaporation phase was quite significant. The possible reason could be that SOP had internal sizing (alum-rosin or AKD or ASA sizing) and cross linking formed by wet strength resins. The free valances were satisfied by sizing agents and wet strength resins. Different fillers such as calcium carbonates and starch were used to improve brightness and strength properties of high-quality writing-grade paper. The higher amounts of bound water content in these samples might be attributed to the capacity of these fillers to bind water molecules tightly [216]. Thus, thermo-gravimetric studies to assess bound water in cellulose were found to be valuable in understanding the enzymatic hydrolysis of cellulosic substrates of different crystallinity.

The weight loss occurring at a temperature of 220-315 ^{0}C was probably due to thermal degradation of hemicelluloses [216, 230] and other low molecular weight carbohydrates. In case of SOP weight loss at this temperature was only 16.99% while weight loss in chemical deinking was 20.78%. The minimum weight loss in SOP was basically due to higher filler contents (inorganic pigments, alum and other additives). In case of enzymatic and chemi-enzymatic deinking the weight losses decreased in the following order: cellulase>cellulase+xylanase>cellulase+chemical (50%)>cellulase+chemical (100%). Cellulase and xylanase mainly attacked in amorphous regions of cellulose and hemicelluloses and the peeling reactions that occurred in alkaline medium caused the depolymerization of carbohydrates and hence an increase in crystalline portions.

The thermal decomposition of SOP, chemical, cellulase, cellulase+xylanase, cellulase+chemical (100%) and cellulase and chemical (50%) at a temperature range of 315 to 500 ^{0}C could be divided into three stages. A significant weight loss was observed at a temperature range of 315-360 ^{0}C. The decomposition was slow at a temperature range of 360-500 ^{0}C. Cellulose degraded quickly with the major loss mainly occurring at 355 ^{0}C [230]. At 500

^{0}C, SOP, chemically and cellulase deinked pulps show a residual mass of 11.86, 2.10 and 0.11% respectively which might be due to filler components. There were two obvious peaks observed in DTG curves of studied SOP and deinked pulps. The TGA and DTG curves of SOP, cellulase, chemical, cellulase+xylanase, cellulase+chemical (100%) and cellulase+chemical (50%) deinked pulps were found to be very similar to that of kraft pulp and alkali-PAA pulp reported by Zhao *et al.,* [235] and pure cellulose by Yang *et al.,* [230]. The first DTG peak temperatures observed for SOP, cellulase, chemical, cellulase+xylanase, cellulase+chemical (100%) and cellulase+chemical (50%) deinked pulps were 344, 343, 342, 344, 341 and 345 ^{0}C and their degradation rates per min were 1.83, 2.29, 1.73, 1.16, 0.95 and 0.17 mg/min respectively. Whereas, second DTG peaks were observed at 476, 482, 475, 477, 450 and 496 ^{0}C with degradation rate per min as 0.11. 0.15, 0.13, 0.09, 0.08 and 0.17 mg/min respectively for the above mentioned pulps. Owing to homogeneous nature of the carbohydrate polymers, it was noticed that the one peak was sharp, which confirmed the faster rate of cellulose carbohydrate degradation as compared to hemicellulose degrading slowly. Normally, DTG curves from biomass exhibited a peak at high temperatures that was mainly due to the pyrolysis of the cellulose and a shoulder at lower temperatures that could be attributed to the pyrolysis of the hemicelluloses [142].

Table 4.1: Effect of enzyme, surfactant and point of addition on pulp yield, optical and strength properties during deinking of sorted office paper

Particulars	Control*,[a]	Enzyme (X) + surfactant (S) dosing[e]		
		Before pulping*,c,**	During pulping*,d	After pulping**,b,*
Results after pulping				
Pulp brightness, % (ISO)	60.85±0.7	62.00 ±0.8 (+1.8)$^{\Phi}$	63.22±0.9 (+3.8)$^{\Phi}$	62.12±1.1(+5.1)$^{\Phi}$
ERIC, ppm	287.56±6.7	281.33±5.1(-2.1)$^{\Phi}$	275.00±4.7(-4.3)$^{\Phi}$	278.33±4.0(-6.6)$^{\Phi}$
Results after ink flotation				
Pulp yield, %	83.5±1.4	81.1±1.2	80.5±1.1	80.2±1.0
Pulp brightness, % (ISO)	62.11±1.0	65.82±1.1(+3.71)$^{\Phi}$	70.34±1.3(+8.23)$^{\Phi}$	73.57±1.4(+18.5)$^{\Phi}$
Deinkability (D_B), %	3.48±0.10	10.91±0.14(+7.43)	21.08±0.27(+17.60)	29.00±0.33(+25.52)
ERIC, ppm	276.3±8.7	174.7 ±5.8(-37.0)$^{\Phi}$	117.8±5.1(-57.3)$^{\Phi}$	89.61±4.5(-67.5)$^{\Phi}$
Deinkability (D_E), %	5.0±0.13	49.01±0.53(+44.01)	74.4±0.80(+69.40)	87.36±0.90(+82.36)
Dirt count, mm^2/m^2	20181±65	1798±20(-91.09)	1630±18(-91.92)	1474±15(-92.70)
CSF, mL	510±5	535±4	554±2	565±3
Tensile index, Nm/g	24.8±1.6	25.10±1.1	25.50±1.5	26.72±1.1
Tear index ,mNm^2/g	5.73±0.27	5.82±0.29	6.02±0.17	6.15±0.19
Burst index, $kPam^2/g$	1.23±0.11	1.27 ±0.12	1.30±0.14	1.38±0.09
Double fold, numbers	8	7	6	6

± refers standard deviation, −/+ shows % change in results compared to control (Φ)

***Pulping conditions:**

Temperature, ^0C = 65±2
Surfactant (Oleic acid) dose, % = 0.05
Consistency, % = 12
pH = 7.2±2

****Enzymatic treatment:**

Cellulase, IU/mL = 6
Surfactant (Tween 80) dose, % = 0.05
pH = 5.3±2
Consistency, % = 12
Temperature, ^0C = 55 ±2

Total time during pulping and enzyme treatment, min (e = enzyme + surfactant treatment time)

a = 30 (pulping time)
b = 90 (30 min pulping time + 60 min enzyme treatment)
c = 60 min (30 min for enzyme treatment during presoaking + 30 min for pulping)
d = 30 min (for both pulping as well as enzyme treatment with 6 IU/mL cellulase dosing)

Flotation conditions:

Flotation time, min = 10
Consistency, % = 1.0
Temperature, ^0C = 35±2
pH = 7.2±2

Table 4.2: Effect of enzyme/ surfactant synergy on pulp yield, optical and strength properties during deinking of sorted office paper

Particulars	Control*	Enzyme treatment (X)[*,b]	Enzyme (X) + surfactant (S) dosing[*, b, c]
Results after pulping*			
Pulp brightness, % (ISO)	61.22±1.0		
ERIC, ppm	287.50±6.7		
Results after ink flotation**			
Pulp yield %	83.5±1.5	79.8±1.2	80.2±1.3 (-3.9)$^{\Phi}$(+0.5)$^{¥}$
Pulp brightness, % (ISO)	62.50±1.1	70.00±1.2(+7.50)$^{\Phi}$	72.07±1.4(+9.57)$^{\Phi}$(+2.07)$^{¥}$
Deinkability (D_B), %	3.57±0.25	24.54±0.51(+20.97)$^{\Phi}$	30.33±0.55(+26.76)$^{\Phi}$ (+5.79)$^{¥}$
ERIC, ppm	276.3±8.7	105.75±4.7 (-61.72)$^{\Phi}$	89.61±4.5(-67.5)$^{\Phi}$(-11.4)$^{¥}$
Deinkability (D_E), %	5.0±0.15	81.5±1.0 (+76.5)$^{\Phi}$	88.4±1.2(+83.4)$^{\Phi}$ (+6.9)$^{¥}$
Dirt count, mm^2/m^2	20181±65	1542±16 (-92.36)$^{\Phi}$	1474±15 (-92.70)$^{\Phi}$ (-4.41)$^{¥}$
CSF, mL	510±5	562±3	565±3
Tensile index, Nm/g	24.8±1.6	25.60±1.3	26.72±1.1
Tear index, mNm^2/g	5.73±0.27	6.12±0.32	6.45±0.19
Burst index, $kPam^2/g$	1.23±0.11	1.35±0.10	1.38±0.09
Double fold, numbers	8	6	6

± refers standard deviation, −/+ shows % change in results compared to control (Φ) and enzyme treatment [¥],
[*, b, ***=] Pulping followed by enzyme treatment and ink floatation, [*, b, c, **=] Pulping followed by enzyme, surfactant treatment and ink floatation

Pulping conditions:

Pulping time, min	: 15	pH	: 7.2±2
Consistency, %	: 10	Temperature, ^{0}C	: 65±2

[b]Enzymatic treatment:

Cellulase, IU/mL	: 6	pH	: 5.3±2
[c]Surfactant (Tween 80) dose, %	; 0.1	Temperature, ^{0}C	: 55 ±2
Consistency, %			

****Flotation conditions:**

Flotation time, min	: 10	pH	: 7.2±2
Consistency, %	: 1	Temperature, ^{0}C	: 35±2

Table 4.3: Optimization of surfactant doses during enzymatic deinking of sorted office paper

Particulars	**Results after pulping***					
Pulp brightness, %(ISO)	61.92±0.90					
ERIC, ppm	278.11±7.6					
	Enzyme (X) + surfactant (S) treatment**					
Surfactant dose , %	**0.0**	**0.02**	**0.05**	**0.08**	**0.1**	**0.2**
	Results after ink flotation***					
Pulp yield, %	76.8±1.2	75.0±1.0	74.2±1.3	74.0±1.6	73.5±1.3	73.1±1.1
Pulp brightness, % (ISO)	67.00±0.49	68.72±0.56	69.91±0.53	70.93±0.61	72.05±0.58	72.30±0.61
Deinkability (D_B), %	14.48±0.20	19.38±0.25	22.78±0.28	25.69±0.30	28.88±0.33	29.59±0.35
ERIC, ppm	109.75±4.7	103.00±4.2	96.11±4.9	88.75±4.7	82.77±4.4	81.0±4.0
Deinkability (D_E), %	78.5±0.80	81.6±0.85	84.9±0.90	88.34±0.93	91.1±0.95	91.96±0.91
Dirt count, mm^2/m^2	1610±16	1575±13	1557±10	1540±16	1510±10	1500±12
CSF, mL	550±4	551±5.0	553±4.0	556±4.0	557±3	556±2
Tensile index, Nm/g	23.00±1.3	24.10±1.4	24.75±1.3	24.91±1.5	24.93±1.7	24.90±1.6
Tear index, mNm^2/g	5.52±0.29	5.61±0.27	5.79±0.25	5.97±0.22	6.16±0.23	6.20 ±0.21
Burst index, $kPam^2/g$	1.30±0.10	1.33±0.11	1.35±0.13	1.36±0.14	1.38±0.16	1.38±0.15
Double fold, number	5	6	6	7	6	6

± refers standard deviation

***Pulping conditions:**

Pulping time, min	= 20
Surfactant (Oleic acid) dose, %	= 0.05
Temperature, ^0C	= 65±2
Consistency, %	= 10
pH	= 7.2±2

****Enzymatic treatment:**

Cellulase, IU/mL	= 6
Surfactant (Tween 80) dose, %	= 0.1
pH	= 5.3±2
Consistency, %	= 10
Temperature, ^0C	= 55±2
Reaction time, min	= 60

*****Flotation conditions:**

Flotation time	= 10
Consistency, %	= 1
Temp, ^0C	= 5±2
pH	= 7.2±2

Table 4.4: Optimization of pulping time during enzymatic deinking of sorted office paper

Particulars	Results after pulping*				
Pulping time, min	**5**	**10**	**15**	**20**	**30**
Pulp brightness,%(ISO)	58.50±0.75	60.20±0.80	61.23±0.85	63.05±0.9	63.16±0.92
ERIC, ppm	288.70±7.5	284.20±7.3	280.00±7.1	273.67±7.0	273.00±6.9
Enzyme (X) + surfactant (S) treatment followed by flotation					
Pulp yield, %	70.50±1.0	71.2±1.2	71.7±1.4	73.0±1.7	71.9±1.3
Pulp brightness, %(ISO)	62.90±0.80	65.80±0.85	68.90±0.92	72.22±0.95	72.54±1.0
Deinkability (D_B), %	11.43±0.20	15.22±0.26	21.44±0.30	27.01±0.35	27.72±0.36
ERIC, ppm	157.00±5.0	121.20±4.2	107.12±3.8	91.50±3.0	90.80±3.5
Deinkability (D_E), %	58.55±0.68	73.94±0.80	79.95±0.85	86.46±0.90	87.08±0.93
Dirt count, mm^2/m^2	1770±21	1710±17	1660±15	1484±12	1480±10
CSF, mL	545±2.0	550±3.0	552±3.0	557±3	555±3.0
Tensile index, Nm/g	22.00±0.9	23.10±1.0	23.90±1.2	24.93±1.7	24.18±1.1
Tear index, mNm^2/g	5.54±0.23	5.58±0.20	5.84±0.18	6.16±0.23	6.20±0.18
Burst index, $kPam^2/g$	1.20±0.02	1.27±0.04	1.30±0.06	1.38±0.16	1.32±0.11
Double fold, numbers	7	6	5	5	5

± refers standard deviation

***Pulping conditions:**

Temperature, ^0C	= 65±2
Surfactant (Oleic acid) dose, %	= 0.05
Consistency, %	= 10
pH	= 7.2±2

****Enzymatic treatment:**

Cellulase, IU/mL	= 6
Surfactant (Tween 80) dose, %	= 0.1
pH	= 5.3±2
Consistency, %	= 10
Temperature, ^0C	= 55±2
Reaction time, min	= 60

*****Flotation conditions:**

Flotation time	= 10
Consistency, %	= 1
Temp, ^0C	= 35±2
pH	= 7.2±2

Table 4.5: Optimization of pulping temperature during enzymatic deinking of sorted office paper

Particulars	Results after pulping*				
Pulping temperature, ^{0}C	**45**	**50**	**55**	**60**	**65**
Pulp brightness, %(ISO)	62.00±0.80	62.55±0.85	63.00±0.90	63.29±0.92	62.60±0.88
ERIC, ppm	278.50±7.0	276.56±6.7	272.28±7.4	267.50±6.5	266.30±7.0
Enzyme (X) + surfactant (S) treatment followed by flotation					
Pulp yield, %	73.11±1.4	72.50±1.2	72.00±1.1	71.25±1.3	69.50±1.5
Pulp brightness, %(ISO)	70.55±0.90	71.12±1.0	72.50±1.1	73.75±1.3	72.92±1.2
Deinkability (D_B), %	24.43±0.25	24.88±0.30	27.94±0.28	31.03±0.35	30.00±0.32
ERIC, ppm	122.11±5.0	111.67±4.2	97.22±3.1	86.60±2.5	85.00±2.4
Deinkability (D_E), %	72.8±0.75	77.4±0.80	83.9±0.85	88.7±0.90	89.5±0.92
Dirt count, mm^2/m^2	1615±13	1590±9	1565±12	1480±14	1510±18
CSF, mL	540±2	545±2	550±2	553±2	556±3
Tensile index, Nm/g	22.90±1.0	23.78±1.3	24.00±1.5	24.93±1.6	23.11±1.1
Tear index, mNm^2/g	5.60±0.27	5.76±0.25	5.94±0.23	6.18±0.20	6.20±0.21
Burst index, $kPam^2/g$	1.28±0.14	1.31±0.16	1.35±0.18	1.37±0.20	1.33±0.17
Double fold, numbers	7	6	6	6	5

± refers standard deviation

***Pulping conditions:**

Pulping time, min	= 20
Surfactant (Oleic acid) dose, %	= 0.05
Consistency, %	= 10
pH	= 7.2±2

****Enzymatic treatment:**

Cellulase, IU/mL	= 6
Surfactant (Tween 80) dose, %	= 0.1
pH	= 5.3±2
Consistency, %	= 10
Temperature, ^{0}C	= 55±2
Reaction time, min	= 60

*****Flotation conditions:**

Flotation time	= 10
Consistency, %	= 1
Temp, ^{0}C	= 35±2
pH	= 7.2±2

Table 4.6: Effect of different pulp consistency during enzymatic deinking of sorted office paper

Particulars	Results after pulping*				
Consistency,%	**6**	**8**	**10**	**12**	**14**
Pulp brightness ,%(ISO)	62.00±0.70	62.75±0.75	63.37±0.80	64.11±0.86	64.70±0.90
ERIC, ppm	286.89±6.6	284.25±6.2	281.00±5.9	274.30±5.7	272.13±5.5
	Results after ink flotation* **				
Pulp yield, %	82.00±1.7	82.30±1.6	82.55±1.8	83.00±1.3	82.60±1.2
Pulp brightness ,%(ISO)	68.11±0.80	70.20±0.85	72.56±0.9	74.85±0.93	75.13±0.98
Deinkability (D_B), %	17.46±0.15	21.75±0.18	27.33±0.23	32.66±0.30	32.30±0.26
ERIC, ppm	145.98±5.5	123.48±5.0	106.29±4.7	86.41±3.8	84.87±3.5
Deinkability (D_E), %	63.1±0.60	72.9±0.65	80.4±0.74	89.2±0.85	89.87±0.90
Dirt count, mm^2/m^2	4434±24	2970±19	1856±15	1495±12	1450±10
CSF, mL	540±3.0	550±5.0	557±4.0	562±2.0	560±3
Tensile index, Nm/g	23.80±1.3	24.05±1.3	24.95±1.4	25.01±1.5	24.98±1.4
Tear index ,mNm^2/g	5.56±0.24	5.83±0.22	5.99±0.19	6.23±0.19	6.28 ±0.21
Burst index, $kPam^2/g$	1.32±0.16	1.36±0.19	1.38±0.21	1.40±0.21	1.38±0.17
Double fold, number	7	6	6	6	5

± refers standard deviation

***Pulping conditions:**

Pulping time, min	= 20
Surfactant (Oleic acid) dose, %	= 0.05
Temperature, ^0C	= 65±2
pH	= 7.2±2

****Enzymatic treatment:**

Cellulase, IU/mL	= 6
Surfactant (Tween 80) dose, %	= 0.1
pH	= 5.3±2
Consistency, %	= 10
Temperature, ^0C	= 55±2
Reaction time, min	= 60

*****Flotation conditions:**

Flotation time	= 10
Consistency, %	= 1
Temp, ^0C	= 35±2
pH	= 7.2±2

Table 4.7: Effect of enzyme dose during enzymatic deinking of sorted office paper

Particulars	**Results after pulping***					
Pulp brightness, % (ISO)	63.10±1.0					
ERIC, ppm	283.20±6.9					
Enzyme treatment						
Enzyme dose , IU/mL	**0**	**2**	**4**	**6**	**8**	**10**
Results after ink flotation* **						
Pulp yield, %	83.00±1.1	81.65±1.6	81.00±1.5	79.60±1.3	77.90±1.4	76.11±1.2
Pulp brightness ,%(ISO)	65.54±0.94	69.50±0.98	71.59±1.00	73.63±1.10	73.82±1.15	74.11±1.20
Deinkability (D_B), %	7.19±0.10	18.88±0.24	25.05±0.32	31.07±0.40	31.63±0.42	32.48±0.45
ERIC, ppm	247.50±6.0	172.59±5.4	123.20±3.9	86.75±3.5	85.40±3.2	84.64±3.0
Deinkability (D_E), %	16.26±0.22	50.40±0.60	72.91±0.80	89.52±0.95	90.14±0.97	90.48±1.00
Dirt count, mm^2/m^2	5780±27	3612±25	2759±19	2329±15	2290±18	2200±12
CSF, mL	510±3.0	525±3.0	535±5.0	555±4.0	557±4.0	555±5.0
Tensile index, Nm/g	22.92±1.0	23.40±1.3	24.35±1.3	24.99±1.4	24.90±1.3	24.12±1.3
Tear index ,mNm^2/g	5.63±0.26	5.80±0.24	5.96±0.22	6.20±0.19	6.22±0.15	6.26±0.18
Burst index, $kPam^2/g$	1.30±0.14	1.32±0.16	1.37±0.19	1.41±0.21	1.38±0.20	1.33±0.17
Double fold, number	7	6	6	6	5	5

± refers standard deviation

***Pulping conditions:**

Pulping time, min	= 20
Temperature, ^0C	= 65±2
Surfactant (Oleic acid) dose, %	= 0.05
Temperature, ^0C	= 65±2
pH	= 7.2±2

****Enzymatic treatment:**

Cellulase, IU/mL	varied
Surfactant (Tween 80) dose, %	= 0.1
pH	= 5.3±2
Consistency, %	= 10
Temperature, ^0C	= 55±2
Reaction time, min	= 60

*****Flotation conditions:**

Flotation time	= 10
Consistency, %	= 1
Temp, ^0C	= 35±2
pH	= 7.2±2

Table 4.8: Effect of retention time during enzymatic deinking of sorted office paper

Particulars	Results after pulping*				
Pulp brightness ,% (ISO)	62.45±0.85				
ERIC, ppm	282.50±7.3				
	Retention time, min				
Retention time, min	**0**	**30**	**60**	**90**	**120**
	Results after ink flotation* **				
Pulp yield, %	81.70±1.6	81.20±1.4	80.10±1.2	78.00±1.4	76.80±1.3
Pulp brightness ,% (ISO)	64.72±0.90	68.65±0.95	73.10±0.99	73.41±1.1	73.67±1.2
Deinkability (D_B), %	6.57±0.10	17.95±0.25	30.83±0.38	31.73±0.32	32.48±0.30
ERIC, ppm	234.41±5.0	163.64±4.6	84.50±4.0	83.55±3.8	82.83±3.6
Deinkability (D_E), %	22.34±0.30	54.54±0.60	90.50±1.0	90.95±0.95	91.28±0.88
Dirt count, mm^2/m^2	6420±24	3615±21	1816±12	1835±14	1860±15
CSF, mL	515±5.0	530±4.0	553±5.0	560±2.0	563±3.0
Tensile index, Nm/g	22.95±1.0	24.00±1.2	24.92±1.3	24.94±1.4	23.80±1.3
Tear index ,mNm^2/g	5.70±0.26	5.82±0.24	6.11±0.21	6..05±0.20	5.98±0.24
Burst index, $kPam^2/g$	1.30±0.14	1.37±0.18	1.42±0.21	1.43±0.22	1.35±0.16
Double fold, number	6	6	6	5	5

± refers standard deviation

***Pulping conditions:**

Pulping time, min	= 20
Temperature, ^0C	= 65±2
Surfactant (Oleic acid) dose, %	= 0.05
Temperature, ^0C	= 65±2
pH	= 7.2±2

****Enzymatic treatment:**

Cellulase, IU/mL	= 6
Surfactant (Tween 80) dose, %	= 0.1
pH	= 5.3±2
Consistency, %	= 10
Temperature, ^0C	= 55±2
Reaction time, min	varied

*****Flotation conditions:**

Flotation time	= 10
Consistency, %	= 1
Temp, ^0C	= 35±2
pH	= 7.2±2

Table 4.9: Effect of flotation time during enzymatic deinking of sorted office paper

Particulars	**Results after pulping***				
Pulp brightness ,% (ISO)	62.70 ±0.90				
ERIC, ppm	280.20±7.0				
	Flotation time, min				
Flotation time, min	**2**	**5**	**8**	**10**	**12**
	Results after ink flotation* **				
Pulp yield, %	81.00±1.6	80.40±1.4	79.10±1.2	77.76±1.4	74.80±1.3
Pulp brightness, % (ISO)	65.32±0.98	68.65±1.0	71.34±1.10	73.54±1.30	73.72±1.4
Deinkability (D_B), %	7.64±0.15	17.35±0.25	25.19±0.55	31.61±0.62	32.13±0.65
ERIC value, ppm	227.41±5.1	155.64±4.0	104.53±3.6	81.50±3.1	79.55±2.9
Deinkability (D_E), %	24.39±0.40	57.55±0.65	81.16±0.85	91.80±0.95	92.70±0.98
Dirt count, mm^2/m^2	6390±28	3585±25	2106±21	1790±14	1750±11
CSF, mL	513±5.0	528±4.0	545±5.0	556±2.0	558±3.0
Tensile index, Nm/g	22.45±1.0	23.90±1.2	24.54±1.3	25.00±1.4	24.30±1.1
Tear index ,mNm^2/g	5.52±0.26	5.80±0.24	5.93±0.21	6.12±0.20	5.85 ±0.25
Burst index, $kPam^2/g$	1.28±0.14	1.38±0.18	1.41±0.20	1.44±0.22	1.42±0.15
Double fold, number	6	6	6	5	5

± refers standard deviation

***Pulping conditions:**

Pulping time, min	= 20
Temperature, ^0C	= 65±2
Surfactant (Oleic acid) dose, %	= 0.05
Temperature, ^0C	= 65±2
pH	= 7.2±2

****Enzymatic treatment:**

Cellulase, IU/mL	= 6
Surfactant (Tween 80) dose, %	= 0.1
pH	= 5.3±2
Consistency, %	= 10
Temperature, ^0C	= 55±2
Reaction time, min	= 60

*****Flotation conditions:**

Flotation time	varied
Consistency, %	= 1
Temp, ^0C	= 35±2
pH	= 7.2±2

Table 4.10: Effect of different enzyme combinations during enzymatic deinking of sorted office paper

Particulars	Results after pulping*										
Brightness, %	61.80±0.85										
ERIC, ppm	283.20±6.9										
Enzyme treatment stage	**Control**	**Cellulase**	**Cellulase + Xylanase**			**Cellulase + Xylanase + Amylase**			**Cellulase +Xylanase + Amylase +Lipase**		
		100 (6 IU/mL)	100+50 (6+3 IU/mL)	50+50 (3+3 IU/mL)	100+100 (6+6 IU/mL)	100+50+25 (6+3+1.5 IU/mL)	100+50+50 (6+3+3 IU/mL)	100+50+100 (6+3+6 IU/mL)	100+50+25+25 (6+3+1.5+1.5 IU/mL)	100+50+25 +50 (6+3+1.5+3 IU/mL)	100+50+25+100 (6+3+1.5+6 IU/mL)
	Results after ink flotation* **										
Total pulp yield, %	82.80±1.4	80.10±2.0	79.25±1.7	79.15±1.2	79.00±1.4	78.80±1.0	78.10±1.3	77.79±1.4	78.25±1.4	78.00±1.2	77.75±1.3
Brightness, %	64.80±0.90	72.93±0.96	74.50±1.0	73.03±0.98	76.00±1.4	75.52±1.1	75.60±1.2	76.4±1.3	76.93±1.2	77.03±1.4	78.10±1.5
Deinkability (D_B), %	8.52±0.15	31.62±0.40	36.08±0.44	31.91±0.39	40.35±0.48	38.98±0.47	39.21±0.46	41.77±0.50	42.99±0.52	43.27±0.53	46.31±0.55
ERIC, ppm	276.23±8.74	105.39±6.5	100.80±4.8	109.15±5.6	94.05±5.0	91.75±4.2	91.60±4.4	91.00±4.0	90.17±2.9	88.65±3.1	87.90±3.0
Deinkability (D_E), %	5.0±0.10	81.3±0.85	83.1±0.87	79.3±0.84	86.2±0.89	87.2±0.90	87.3±0.91	87.5±0.92	87.6±0.94	87.8±0.96	88±0.99
Dirt count, mm^2/m^2	20181±65	3573±27	3360±28	3622±24	3130±26	2808±16	2714±15	2660±19	2610±20	2505±23	2414±14
CSF, mL	510±2.0	560±3.0	556±3.0	550±4.0	565±3.0	558±3	560±4.0	563±2	560±3.0	563±4	566±4
Pulp viscosity, cm^3/g	422.30±5.9	450.20±3.9	480.00	471.4±4.6	500.68	528±5.0	529.2±5.1	530±5.3	532.7±6.2	533±4.7	536±4.9
Characteristics of effluent											
Total solid, mg/L	1.42	1.60	1.62	1.62	1.65	1.72	1.73	1.77	1.80	1.81	1.83
COD, kg/Tonne	23.4	65.10	65.70	65.50	66.10	65.80	66.06	66.20	66.40	66.50	67.22
BOD, kg/ Tonne	8.0	25.66	25.85	25.10	26.30	26.10	26.25	26.33	26.30	26.35	26.40
Strength properties											
Tensile index, Nm/g	22.44±1.1	24.75±1.6	24.40±1.2	23.10±1.4	24.12±1.3	23.50±1.8	23.13±1.9	23.00±1.6	23.45±1.5	23.40±1.3	23.05±1.5
Tear index, mNm^2/g	6.73±0.27	6.00±0.23	6.15±0.19	6.25±0.16	6.35±0.18	6.85±0.40	7.05±0.44	7.12±0.37	7.25±0.32	6.88±0.30	6.86±0.33
Burst index, $kPam^2/g$	0.87±0.09	1.31±0.13	1.26±0.12	1.17±0.09	1.2 1±0.10	1.22±0.14	1.20±0.12	1.18±0.12	1.21 ±0.14	1.20±0.13	1.18±0.10
Double fold, number	7	6	5	6	7	5	8	6	7	7	6

± refers standard deviation,

Pulping conditions:

Pulping time, min = 20
Surfactant (Oleic acid) dose, % = 0.05
Temperature, ^0C = 65±2
Consistency, % = 12%
pH = 7.2±2

Enzymatic treatment:

Flotation time, min = 10
Surfactant (Tween 80) dose, % = 0.1
pH = 5.3±2
Consistency, % = 12
Temperature, ^0C = 55±2
Reaction time, min = 60

Flotation conditions:

Consistency, % = 1
Temperature, ^0C = 35±2
pH = 7.2±2

Table 4.11: Comparison of chemical, chemi-enzymatic and enzymatic deinking

Particulars	Deinking processes					
	Blank	(C)	(C100%+E)	(C50%+E)	(C25%+E)	(E)
Results after pulping*						
Characteristic of pulp						
Pulp brightness ,% (ISO)	61.80±0.85	64.30±0.80	65.62±0.90	64.50±0.88	63.46±0.95	61.80±0.75
ERIC, ppm	283.20±6.9	278.11±6.6	270.00±6.2	275.30±6.5	279.00±6.7	283.20±6.9
Results after ink flotation**						
Pulp yield, %	82.80±1.4	78.50±2.1	77.53±1.9	78.80±2.3	79.90±1.7	81.10±2.2
Pulp brightness ,%(ISO)	64.80±0.90	75.15±1.1	79.03±1.6	77.32±1.4	75.55±1.2	72.93±1.0
Deinkability (D_B), %	8.52±0.15	33.19±0.40	42.74±0.55	39.45±0.50	36.05±0.45	31.62±0.35
ERIC, ppm	276.23±8.74	98.67±6.2	65.89±3.4	80.58±5.7	89.79±6.0	105.39±6.5
Deinkability (D_E), %	5.0±0.10	83.71±0.88	99.01±1.1	92.20±0.95	87.91±0.90	81.30±0.85
Dirt count, mm^2/m^2	20181±65	3281±15	2252±10	2899±13	3160±16	3373±14
CSF, mL	510±2.0	546±3.0	575±4.0	568±4.0	565±3.0	560±3.0
Pulp viscosity,cm^3/g	422.30±5.9	590.90±4.8	424.40±4.4	430.70±5.0	432.50±4.6	450.20±3.9
Effluent characteristics						
Total solid ,	1.42	1.65	1.82	1.75	1.67	1.60
COD, kg/Tonne	23.4	58.70	55.90	53.50	51.90	50.10
BOD_{5days}, kg/Tonne	8.0	25.11	23.14	22.22	21.50	20.66
Mechanical strength properties						
Tensile index, Nm/g	22.44±1.1	23.72±1.2	23.00	23.10±1.1	23.25±1.6	23.50±1.6
Tear index, mNm^2/g	6.73±0.27	7.52±0.31	6.85	6.93±0.23	7.02±0.41	7.15±0.22
Burst index, $kPam^2/g$	0.87±0.09	1.17±0.10	1.06	1.07±0.10	1.09 ±0.13	1.10±0.13
Double fold, number	7	7	7	6	6	7

± refers standard deviation, all chemical and enzyme doses based on oven dry raw material basis, Blank= no chemicals are added during pulping, C= chemical deinking, E= enzymatic deinking.

Chemical deinking (C)		**Enzymatic deinking (E)**		**Chemical +Enzyme deinking (C+E)**	
***Pulping conditions:**		***Pulping conditions:**		***Pulping conditions**	
Pulping time, min	= 20	Pulping time, min	= 20	Pulping time, min	= 20
Surfactant (Tween 80), %	= 0.05	Surfactant (Tween 80), %	= 0.05	Surfactant (Tween 80), %	= 0.05
NaOH, % (as such)	= 2	**Enzymatic treatment (E+T):**		NaOH dosing	
				100%	=2
				50%	= 1
				25%	= 0.5
H_2O_2, %	= 1	Cellulase, IU/mL	= 6	H_2O_2 dosing	
				100%	=1
				50%	= 0.5
				25%	= 0.25
Sodium meta silicate	= 2.5	Surfactant (Tween 80), %	= 0.05	Sodium meta silicate dosing	
				100%	= 2.5
				50%	= 1.25
				25%	= 0.63
DTPA,%	= 0.5	pH	= 5.3±2	DTPA dosing	
				100%	= 0.5
				50%	= 0.25
				25%	= 0.13
Temperature, ^0C	= 65±2	Consistency, %	= 12	Temperature, ^0C	= 65±2
pH	= 7.2 ±2	Temperature, ^0C	= 55±2	pH	= 7.2 ±2
		Reaction time, min	= 60	**Enzyme conditions is same as mentioned in (E+T)**	

****Flotation conditions:** Flotation time, min = 10, consistency, % = 1, temperature, ^0C = 35±2 and pH =7.2±2

Table 4.12: Effect of repeated recycling on paper properties of chemical and enzymatic deinked of sorted office paper

Particulars	Number of recycling			
	1	2	3	4
Chemical deinked pulp				
Bulk, cm^3/g	1.81	1.84	1.87	1.87
Opacity, %	87.2	90.6	91.8	91.7
Tensile index, Nm/g	23.72±1.2	23.45±1.3	22.67±1.1	21.93±1.0
Tear index, mNm^2/g	7.52±0.31	8.60±0.46	9.08±0.31	9.51±0.38
Burst index, $kPam^2/g$	1.17±0.10	1.10±0.18	0.80±0.09	0.73±0.12
Double fold, numbers	7	6	4	5
Enzymatic deinked pulp				
Bulk, cm^3/g	1.80	1.85	1.87	1.90
Opacity, %	87.0	91.2	92.0	92.1
Tensile index, Nm/g	23.50±1.6	23.10±1.3	22.45±1.2	21.67±1.1
Tear index, mNm^2/g	7.15±0.22	8.14 ±0.45	8.99±0.29	9.08±0.31
Burst index, $kPam^2/g$	1.10±0.13 (+)	1.04±0.13	0.77 ±0.11	0.7 0±0.09
Double fold, numbers	7	5	4	4

± refers standard deviation, all chemical and enzyme doses based on oven dry raw material basis, No refining is carried and all the fines retained during washing

Chemical deinking (C)		Enzymatic deinking (E)	
Pulping conditions:			
Pulping time, min	= 20		
Surfactant (Tween 80), %	= 0.05		
NaOH, %	= 2	**Enzymatic treatment (E+T):**	
H_2O_2, %	= 1	Cellulase, IU/mL	= 6
Sodium meta silicate	= 2.5	Surfactant (Tween 80), %	= 0.05
DTPA,%	= 0.5	pH	= 5.3±2
Temperature, °C	= 65±2	Consistency, %	= 12
pH	= 7.2 ±2	Temperature, °C	= 55±2
		Reaction time, min	= 60
Flotation conditions:			
Flotation time, min	= 10		
consistency, %	= 1		
Temperature, °C	= 35±2		
pH	= 7.2±2		

Table 4.13: Reduction in stickies in different deinking processes of sorted office paper

Particulars	Stickies no/kg	% Stickies removal
Control	3060	–
Chemical deinking	1774	42.02
Enzymatic deinking		
Cellulase	1652	46.01
Cellulase + Xylanase	1650	46.07
Cellulase + Xylanase + Amylase	1645	46.24
Cellulase +Xylanase + Amylase +Lipase	1573	48.59

Table4.14: Ink particle size distribution in different deinking processes of sorted office paper

Particulars	Ink particles size range in μm			
	8–20	20–80	80–224	8–2000
		Conventional deinking		
Chemical deinking				
Number of specks	411	303	33	347
Number of specks/cm^2	1409	1039	113	1190
Average speck diameter, μm	12.96	38.26	124.28	47.29
		Enzymatic deinking		
Cellulase				
No. of specks	383(-6.81)	243(-19.14)	31(-6.06)	270(-22.19)
No. of specks/cm^2	1313(-6.81)	833(-19.82)	106(-6.19)	926(22.18)
Average speck diameter (μm)	12.65	36.08	123.67	45.73
Cellulase +xylanase				
No. of specks	366(-10.94)	248(-18.15)	28(-15.15)	276(-20.46)
No. of specks/cm^2	1255(-10.92)	850(-18.19)	95(-15.92)	947(-20.42)
Average speck diameter (μm)	12.60	35.29	119.81	44.42
Cellulase + xylanase+amylase				
No. of specks	331(-19.46)	201(-33.66)	24(-27.27)	238(-31.41)
No. of specks/cm^2	1135(-19.44)	689(-33.68)	83(-27.43)	816(-31.42)
Average speck diameter (μm)	12.17	35.25	119.47	43.26
Cellulase + xylanase+amylase+lipase				
No. of specks	182(-55.71)	190(-37.29)	19(-42.42)	178(-48.70)
No. of specks/cm^2	608(-56.84)	644(-38.01)	65 (-42.47)	607(-48.99)
Average speck diameter (μm)	12.01	34.44	116.45	42.94
		Chemi-enzymatic deinking		
(C100%+E)				
No. of specks	111(-72.99)	67(-77.88)	7(-78.87)	93(-73.19)
No. of specks/cm^2	377(-73.24)	224((-78.44	23(-79.64)	319(-73.19)
Average speck diameter (μm)	12.16	35.29	125.87	44.15
(C50%+E)				
No. of specks	146(-64.47)	85(-71.94)	12(-63.63)	113(-67.43)
No. of specks/cm^2	500(-64.51)	291(-71.99)	41(-63.71)	377(-68.31)
Average speck diameter (μm)	12.11	35.26	124.28	44.26
(C25%+E)				
No. of specks	190(-53.77)	127(-58.08)	19(-42.42)	145(-58.21)
No. of specks/cm^2	656(-53.58)	435(-58.13)	65(-42.47)	486(-59.15)
Average speck diameter (μm)	12.09	35.12	123.72	45.11
Average	12.34±0.46	35.62±1.32	122.19±3.6	44.64±1.52

-sign shows % reduction compared to chemical deinking, ± shows standard deviation.

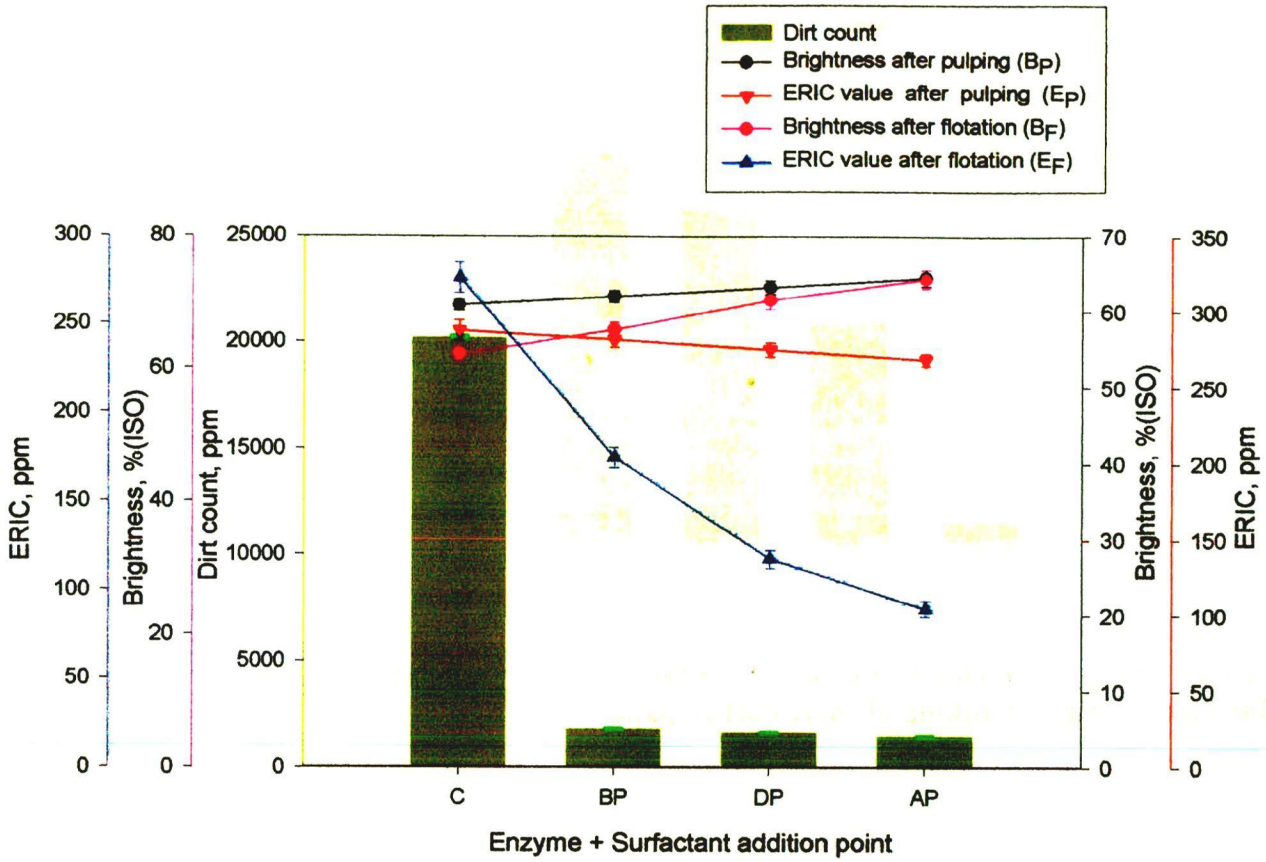


Figure 4.1: Effect of enzyme + surfactant's addition point on dirt count, brightness and ERIC value during enzymatic deinking of sorted office paper

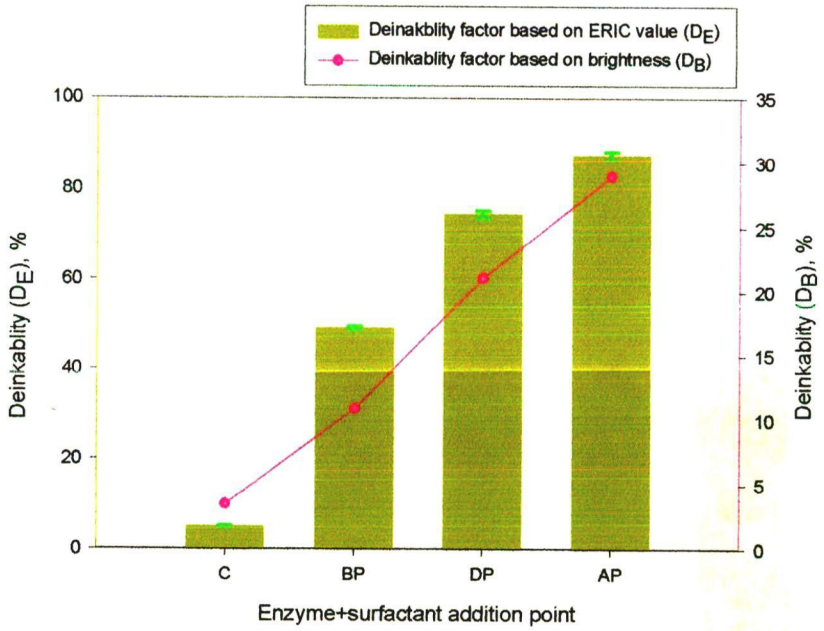


Figure 4.2: Effect of enzyme + surfactant's addition point on deinkability factor (D_E and D_B) during enzymatic deinking of sorted office paper

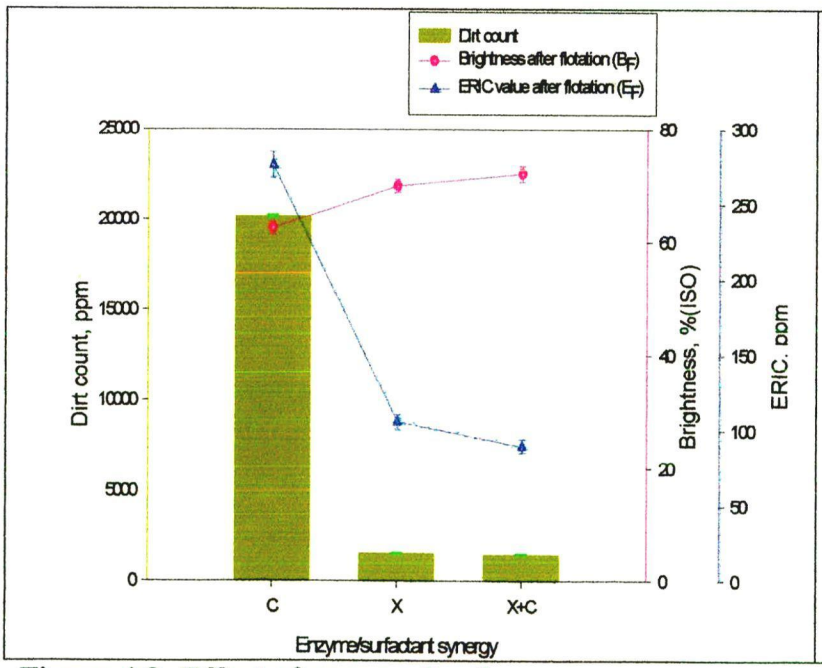


Figure 4.3: Effect of enzyme /surfactant synergy on dirt count, brightness and ERIC value during enzymatic deinking of sorted office paper

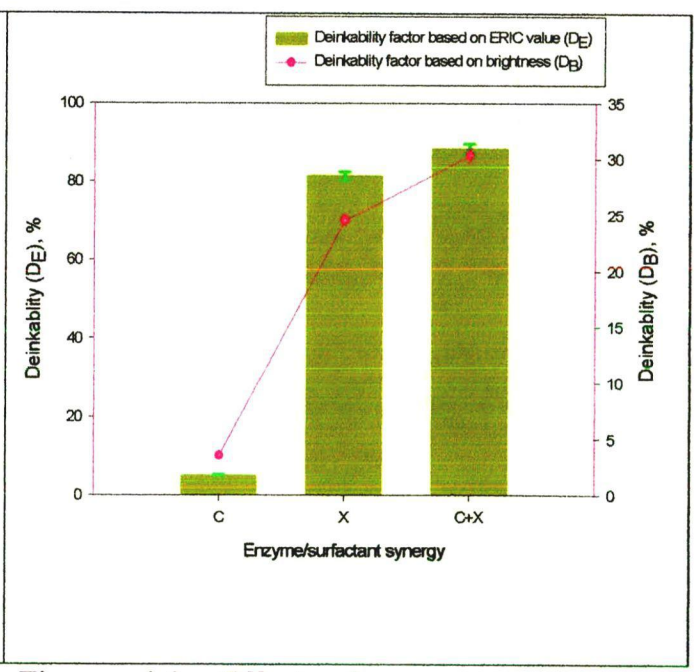


Figure 4.4: Effect of enzyme /surfactant synergy on deinkability factor (D_E and D_B) during enzymatic deinking of sorted office paper

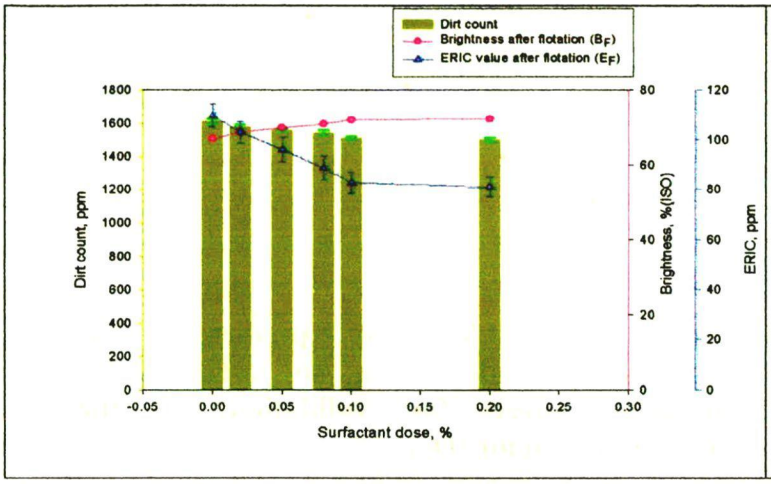


Figure 4.5: Effect of surfactant dose on dirt count, brightness and ERIC value during enzymatic deinking of sorted office paper

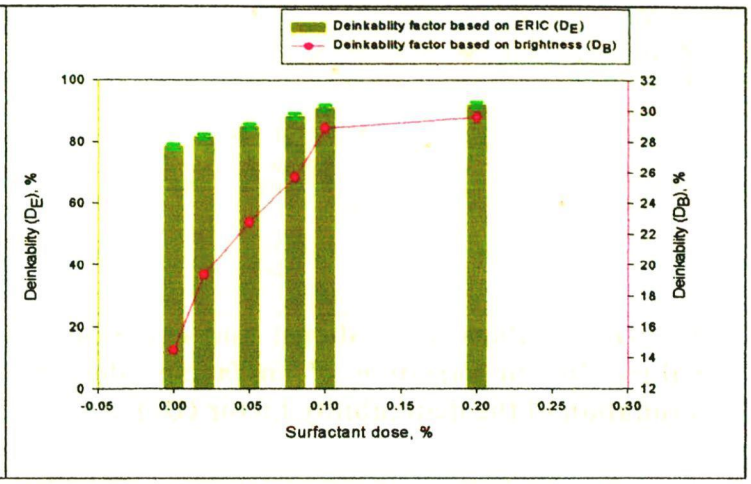


Figure 4.6: Effect of surfactant dose on deinkability factors (D_E and D_B) during enzymatic deinking of sorted office paper

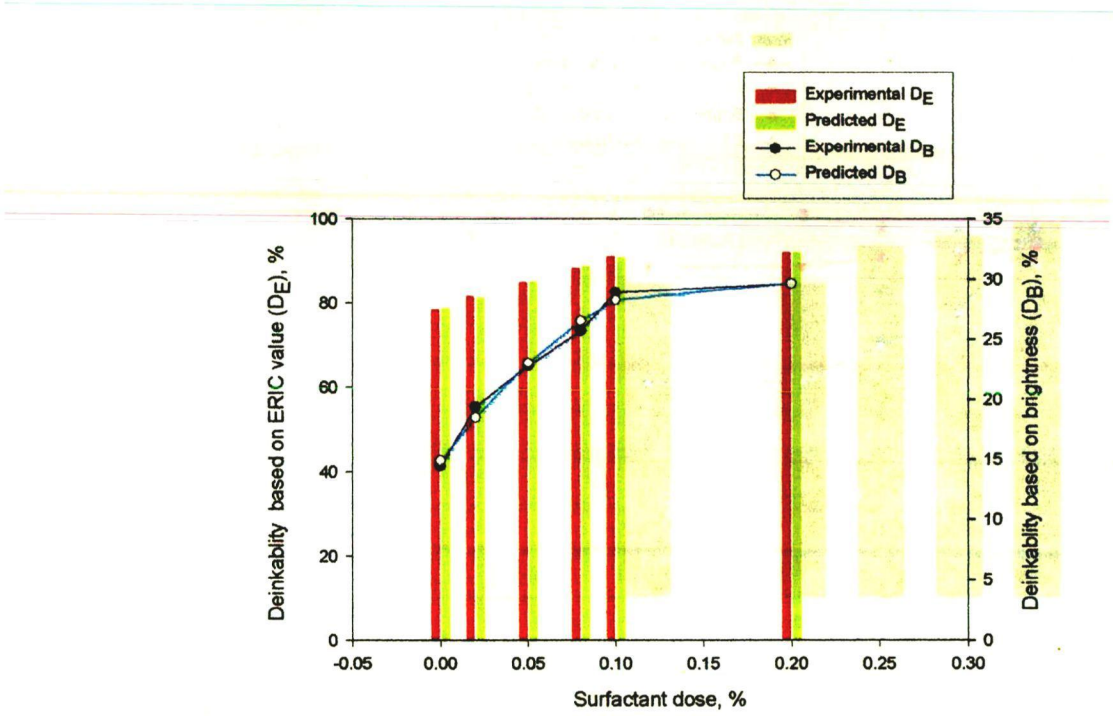


Figure 4.7: Effect of surfactant dose on experimental and predicted deinkability factors ($D_{E\ and}$ D_B) during enzymatic deinking of sorted office paper

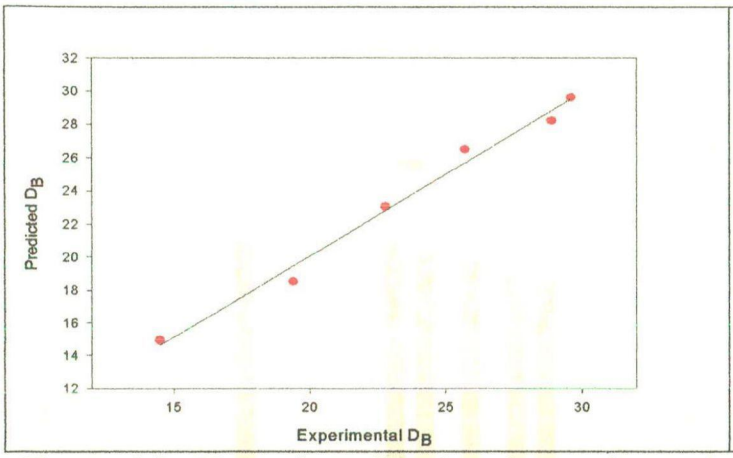


Figure 4.8: Plots of predicted and experimental values due to variation of surfactant dose for validation of the deinkability factor (D_B)

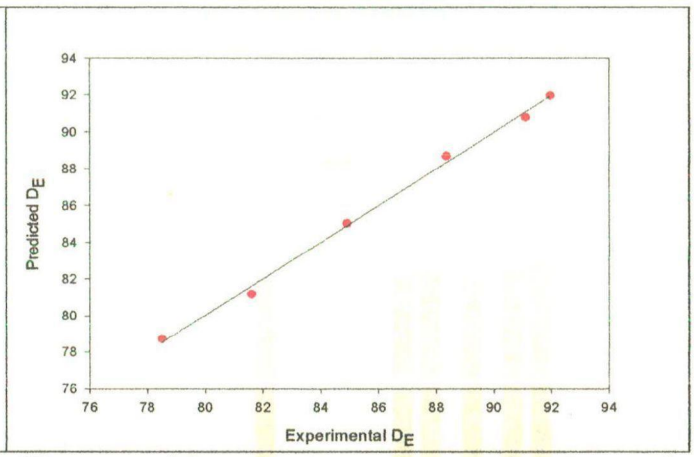


Figure 4.9: Plots of predicted and experimental values due to variation of surfactant dose for validation of the deinkability factor (D_E)

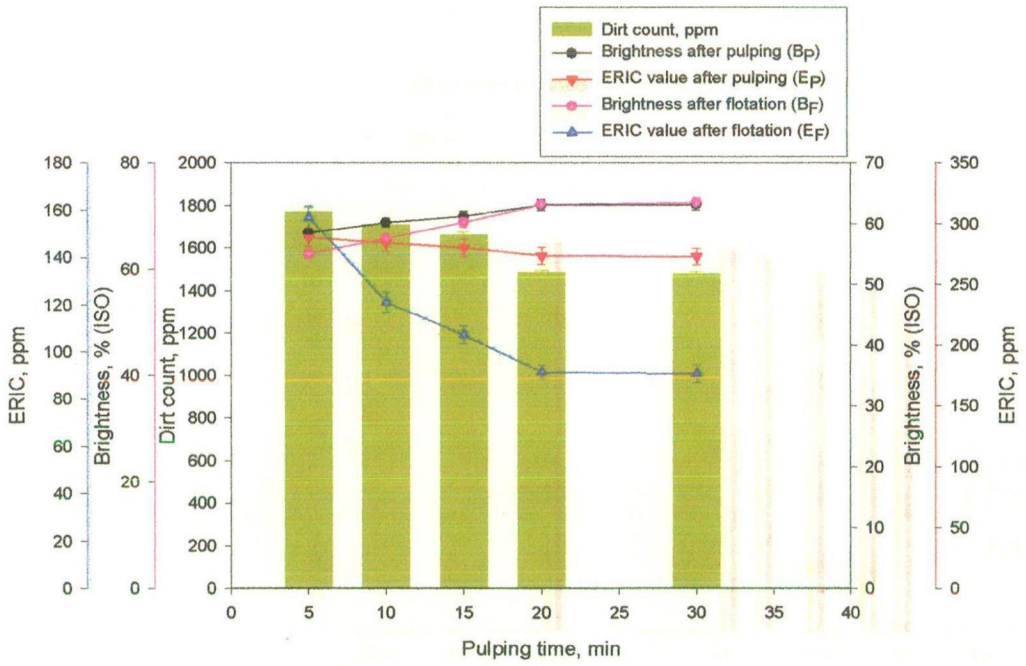


Figure 4.10: Effect of pulping time on dirt count, brightness and ERIC value during enzymatic deinking of sorted office paper

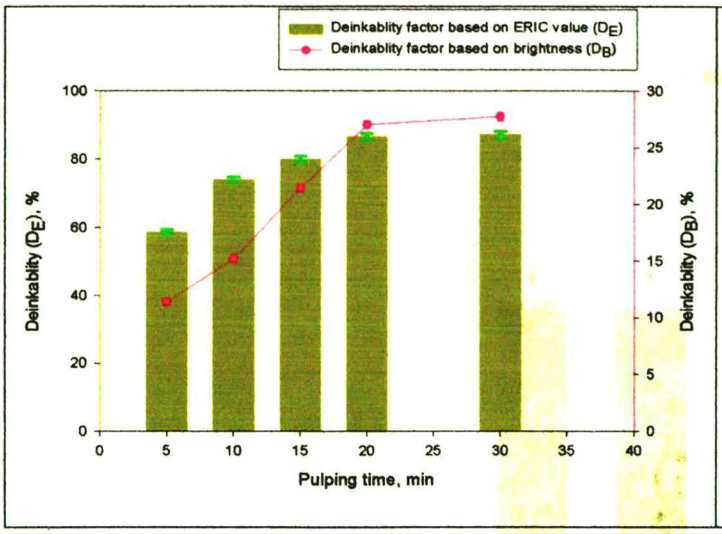


Figure 4.11: Effect of pulping time on deinkablity factors (D_E and D_B) during enzymatic deinking of sorted office paper

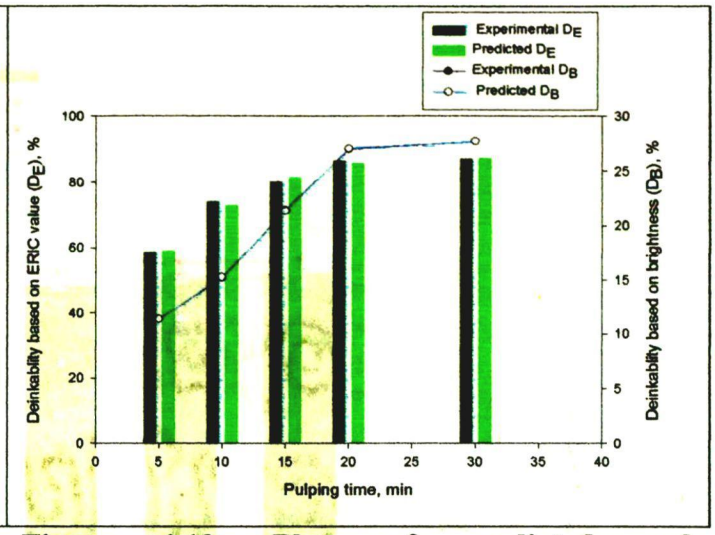


Figure 4.12: Plots of predicted and experimental deinkability factors (D_E and D_B) due to variation of pulping time during enzymatic deinking of sorted office paper

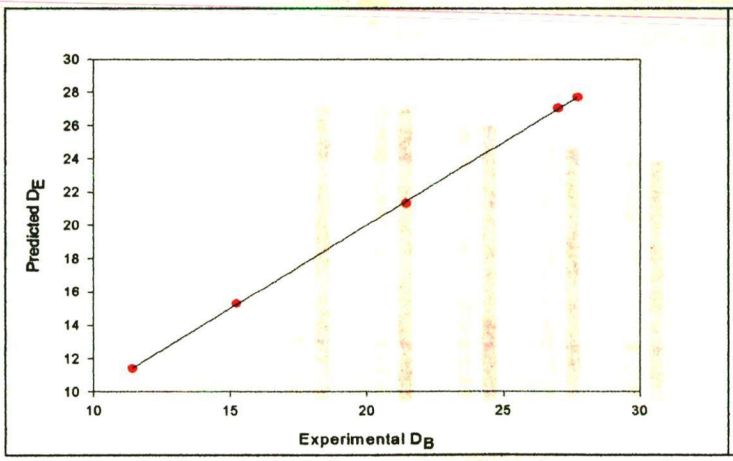


Figure 4.13: Plots of predicted values against experimental values due to variation in pulping time for the validation of deinkability factor (D_B)

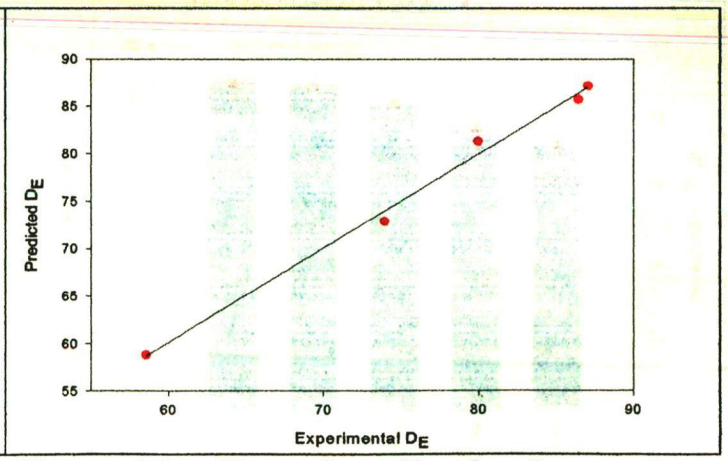


Figure 4.14: Plots of predicted values against experimental values due to variation in pulping time for the validation of deinkability factor (D_E)

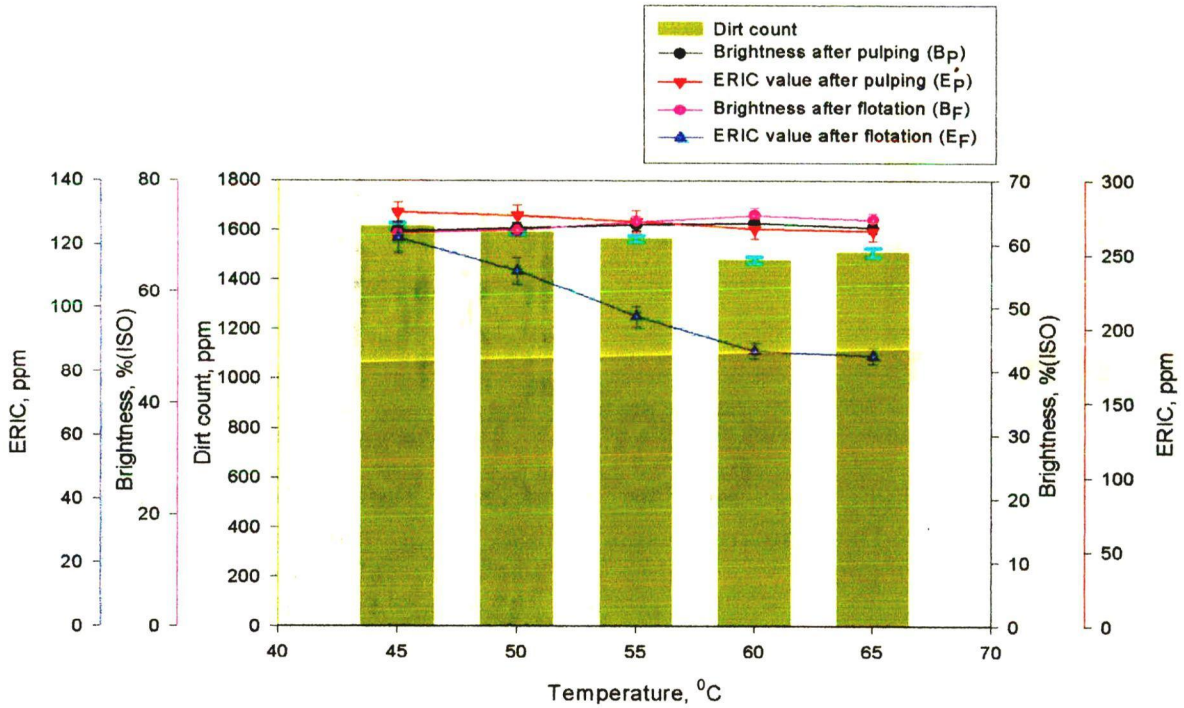


Figure 4.15: Effect of pulping temperature on dirt count, brightness and ERIC value during enzymatic deinking of sorted office paper

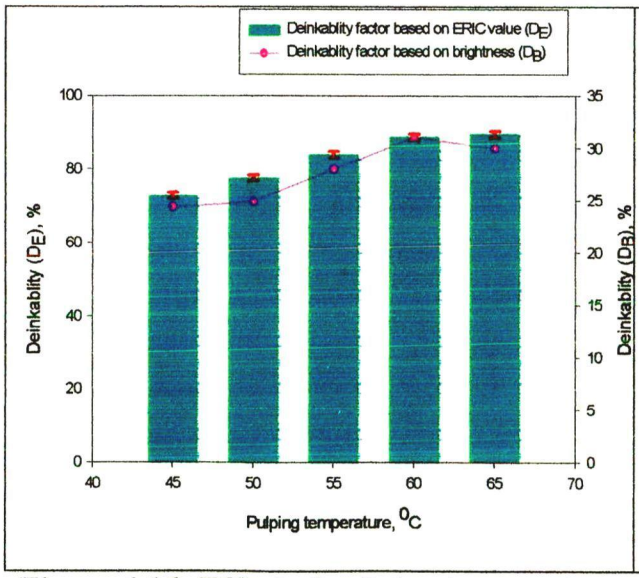


Figure 4.16: Effect of pulping temperature on deinkability factors (D_E and D_B) during enzymatic deinking of sorted office paper

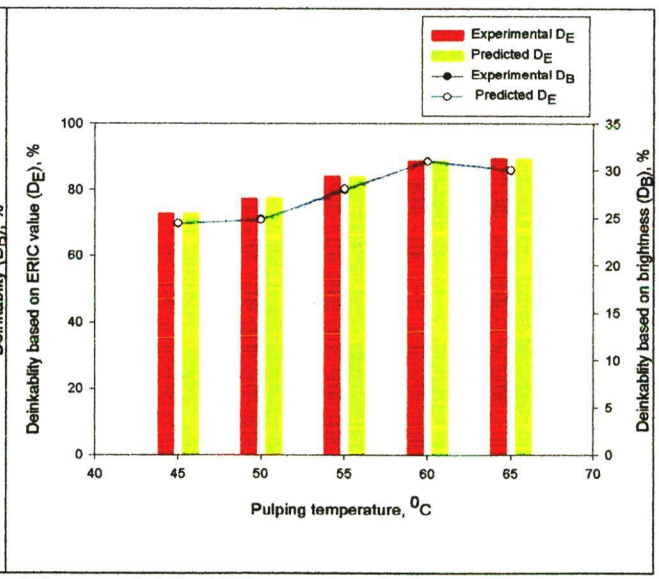


Figure 4.17: Effect of pulping temperature on experimental and predicted deinkability Factors (D_E and D_B) during enzymatic deinking of sorted office paper

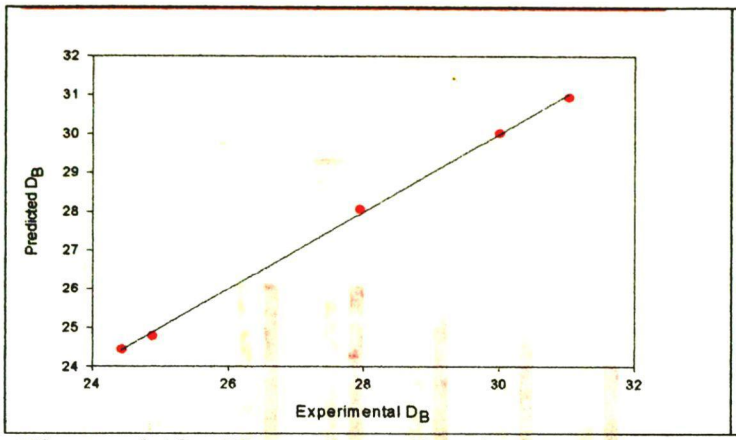


Figure 4.18: Plots of predicted values against Experimental values due to variation in pulping temperature for the validation of deinkability factor (D_B)

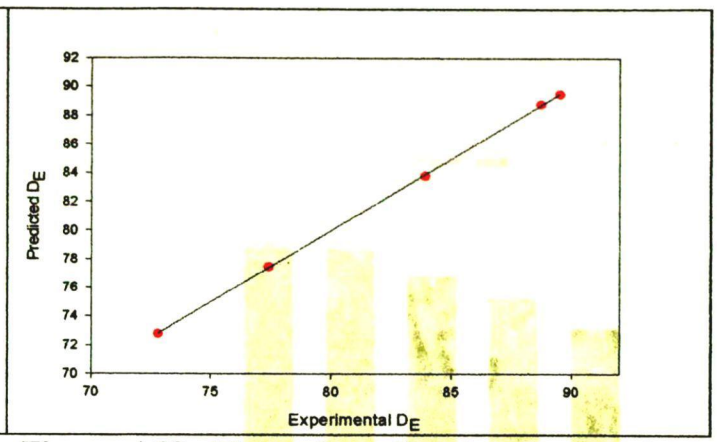


Figure 4.19: Plots of predicted values against experimental values due to variation in pulping temperature for the validation of deinkability factor (D_E)

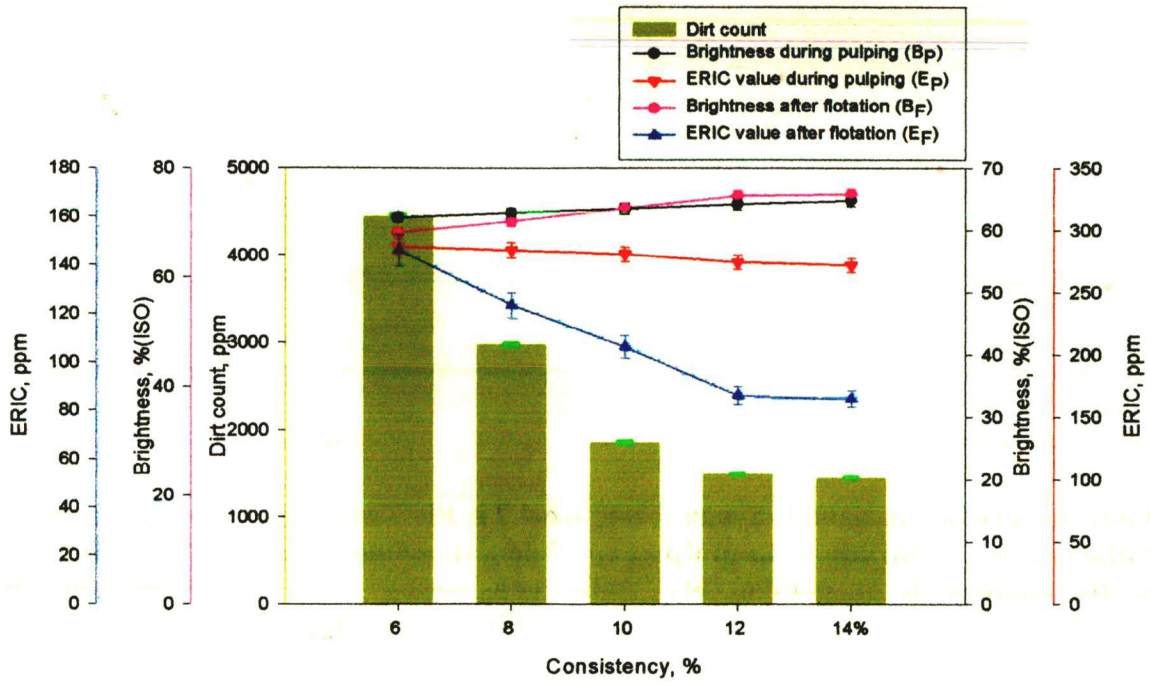


Figure 4.20: Effect of pulp consistency on dirt count, brightness and ERIC value during enzymatic deinking of sorted office paper

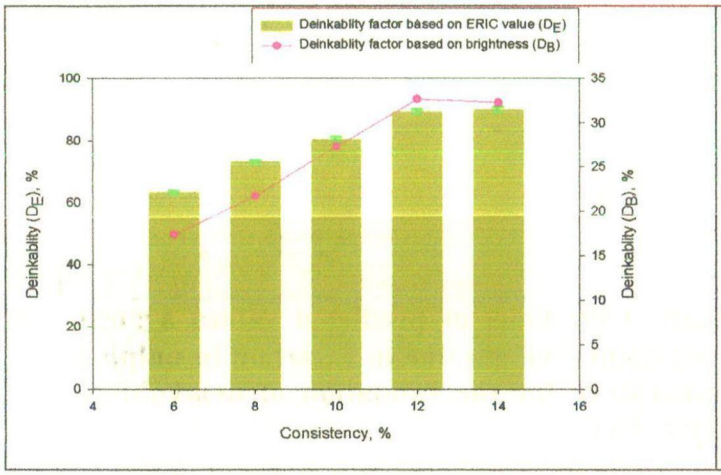


Figure 4.21: Effect of pulp consistency on deinkability factors (D_E and D_B) during enzymatic deinking of sorted office paper

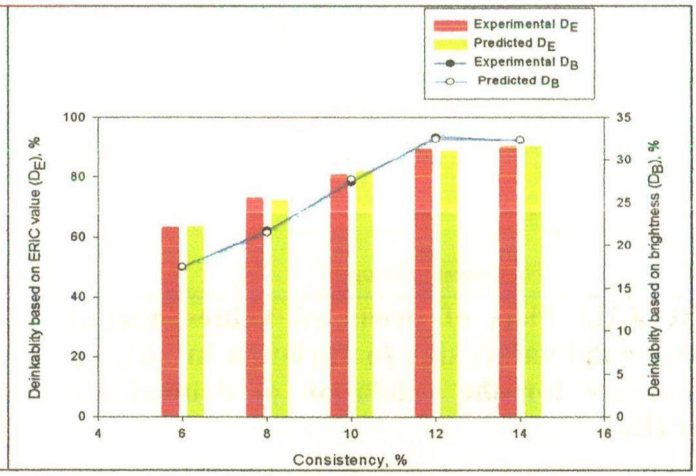


Figure 4.22: Effect of pulp consistency on experimental and predicted deinkability factors (D_E and D_B) during enzymatic deinking of sorted office paper

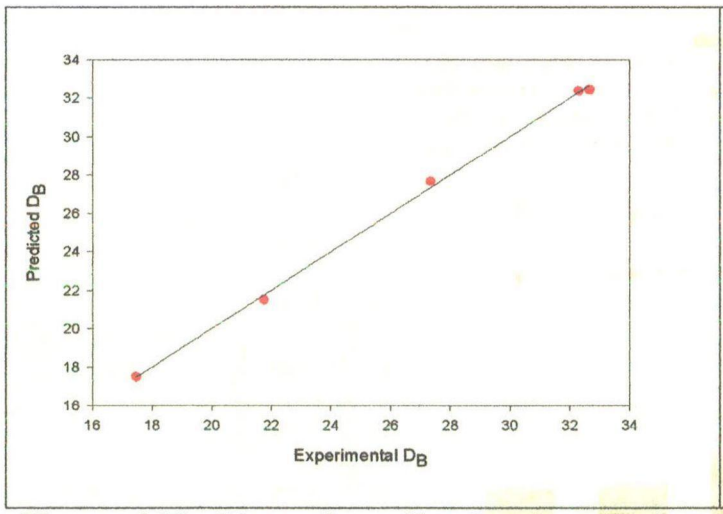


Figure 4.23: Plots of predicted values against experimental values due to variation in pulp consistency for the validation of deinkability factor (D_B)

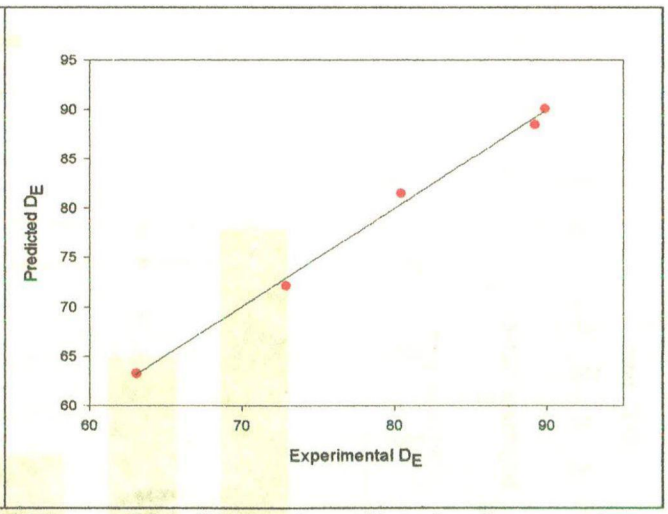


Figure 4.24: Plots of predicted values against experimental values due to variation in pulp consistency for the validation of deinkability factor (D_E)

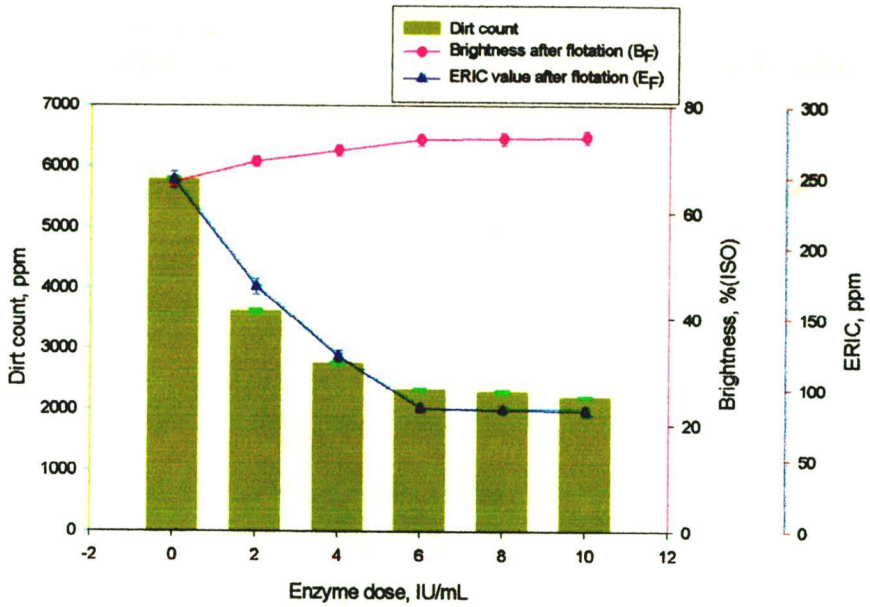


Figure 4.25: Effect of enzyme dose on dirt count, brightness and ERIC value during enzymatic deinking of sorted office paper

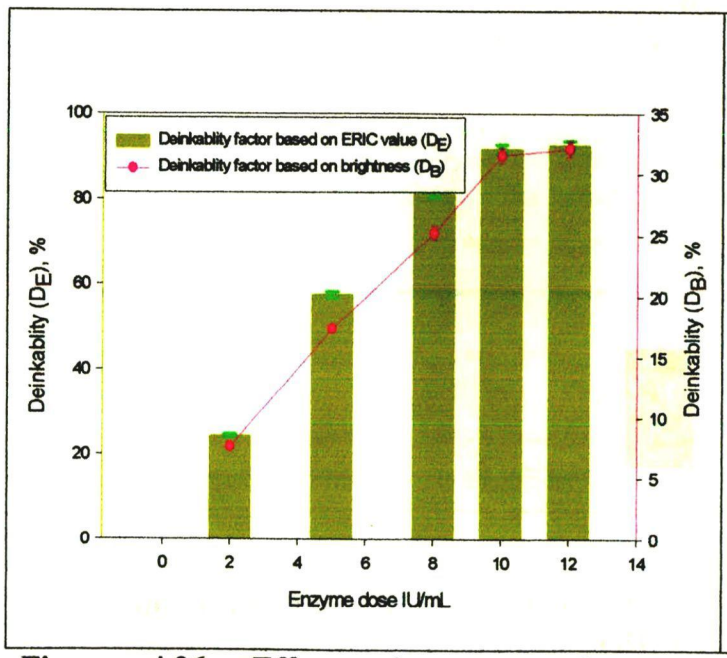


Figure 4.26: Effect of enzyme dose on

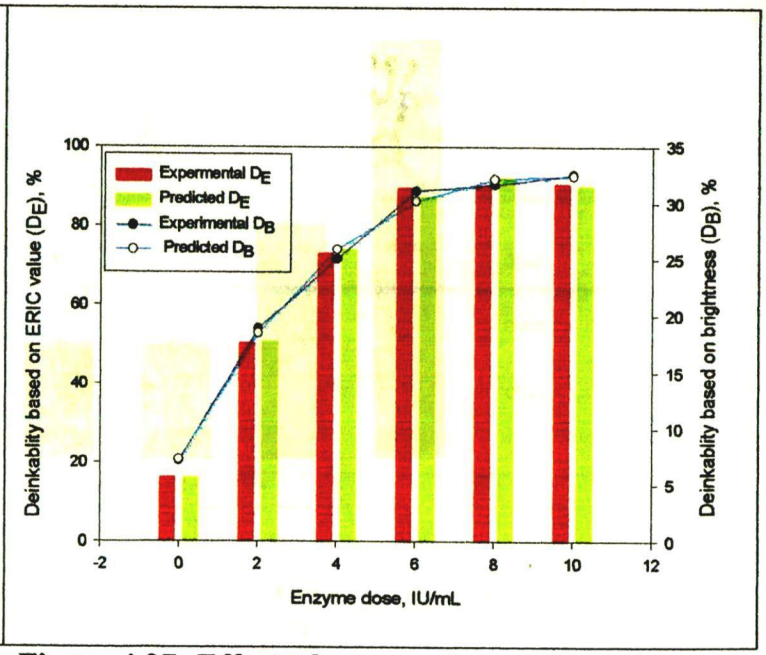


Figure 4.27: Effect of enzyme dose on experimental

deinkability factors (D_E and D_B) during enzymatic deinking of sorted office paper

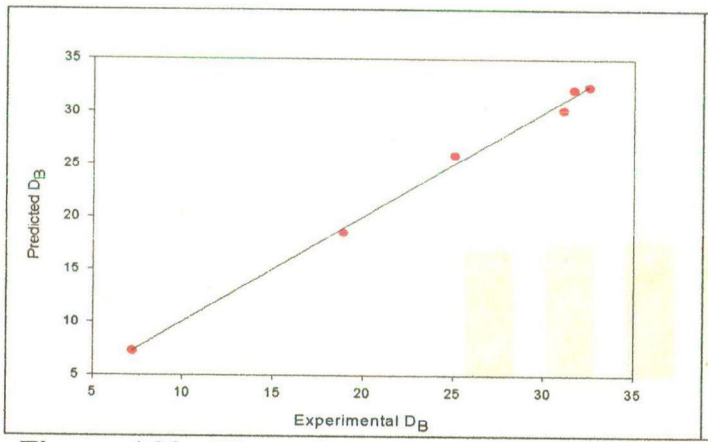


Figure 4.28: Plots of predicted values against experimental values due to variation in enzyme dose for the validation of deinkability factor (D_B

and predicted deinkability factors (D_E and D_B) during enzymatic deinking of sorted office paper

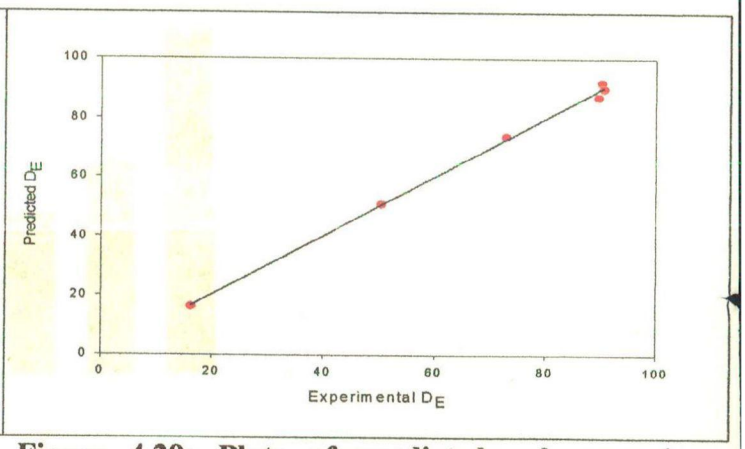


Figure 4.29: Plots of predicted values against experimental values due to variation in enzyme dose for the validation of deinkability factor (D_E)

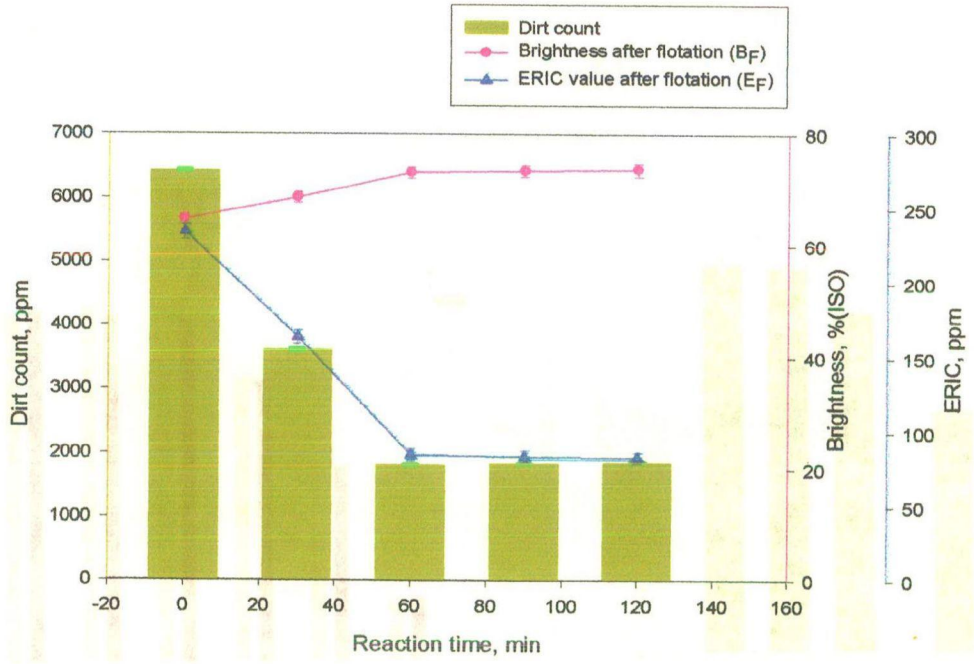


Figure 4.30: Plots of predicted values against experimental values due to variation in enzyme dose for the validation of deinkablity factor (D_E)

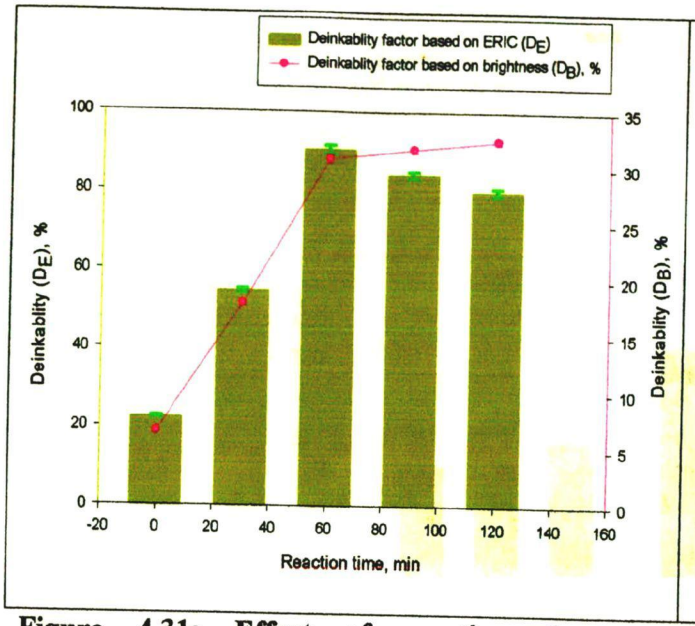


Figure 4.31: Effect of reaction time on deinkability factors (D_E and D_B) during enzymatic deinking of sorted office paper

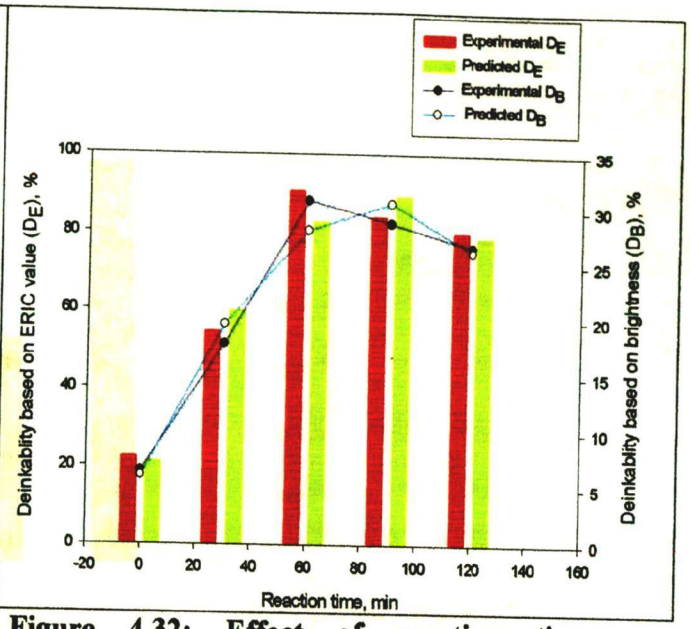


Figure 4.32: Effect of reaction time on experimental and predicted deinkability factors (D_E and D_B) during enzymatic deinking of sorted office paper

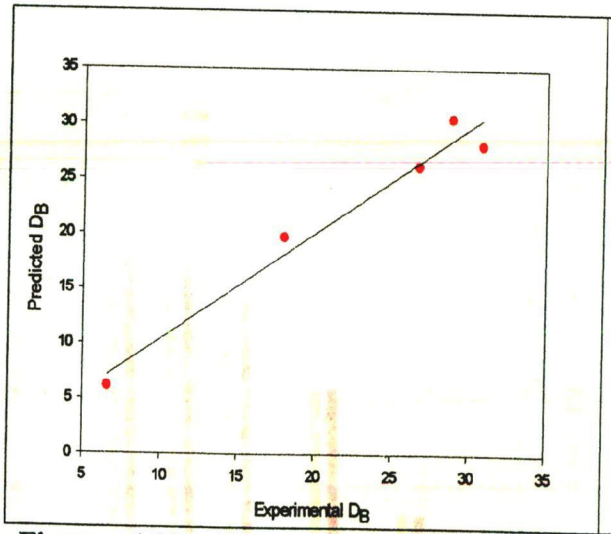


Figure 4.33: Plots of predicted values against experimental values due to variation in reaction time for the validation of deinkability factor (D_B)

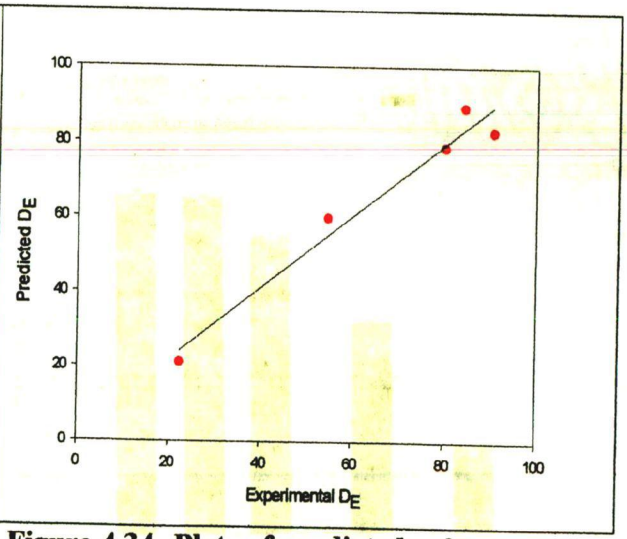


Figure 4.34: Plots of predicted values against experimental values due to variation in reaction time for the validation of deinkability factor (D_E)

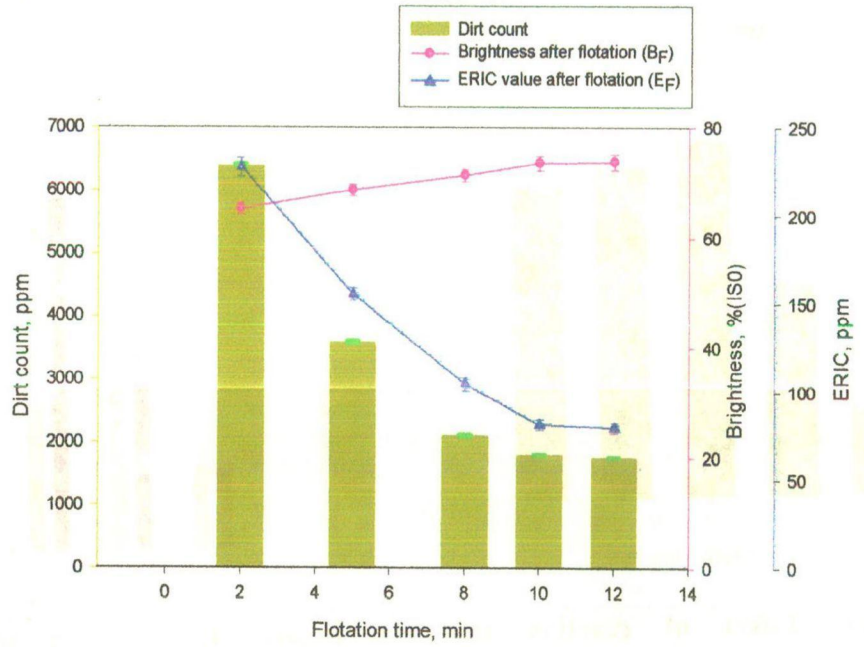


Figure 4.35: Effect of flotation time on dirt count, brightness and ERIC value during enzymatic deinking of sorted office paper

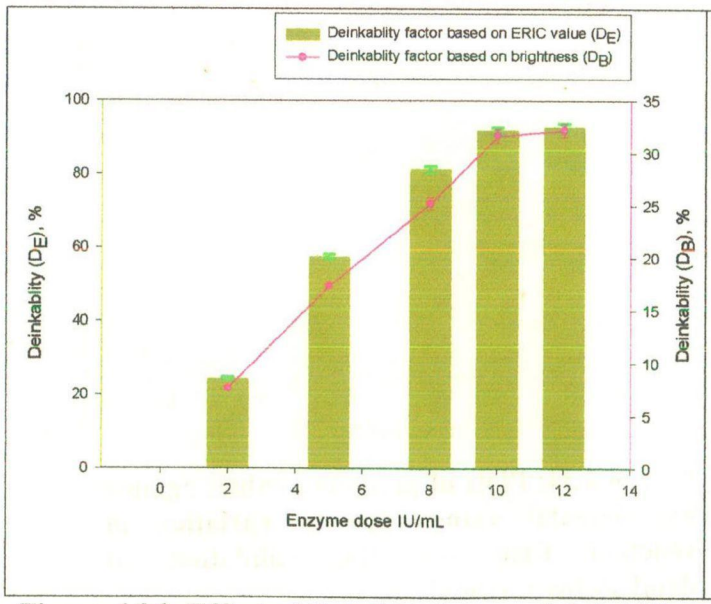


Figure 4.36: Effect of flotation time on deinkability factors (D_E and D_B) during enzymatic deinking of sorted office paper

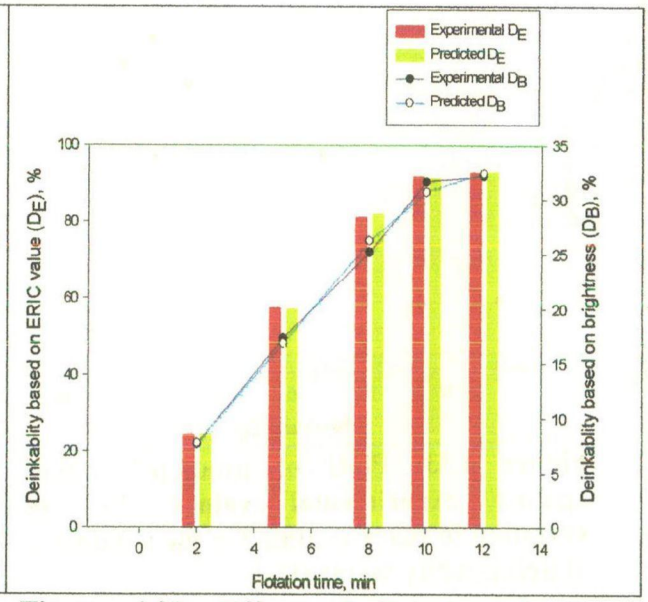


Figure 4.37: Effect of flotation time on experimental and predicted deinkability factors (D_E and D_B) during enzymatic deinking of sorted office paper

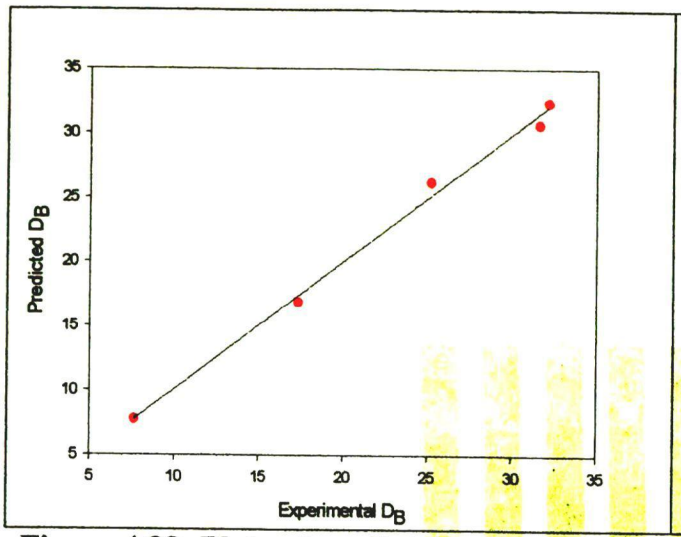


Figure 4.38: Plots of predicted values against experimental values due to variation in floatation time for the validation of deinkability factor (D_B)

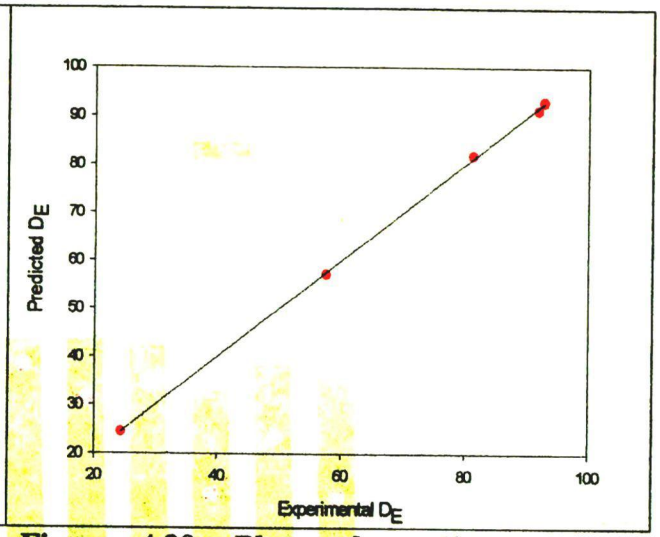


Figure 4.39: Plots of predicted values against experimental values due to variation in floatation time for the validation of deinkability factor (D_E)

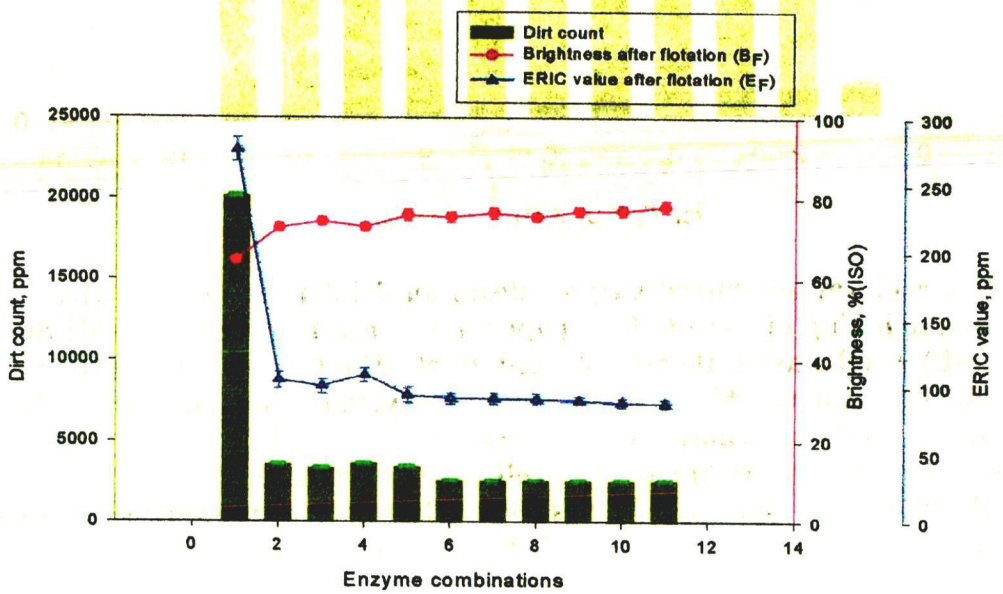


Figure 4.40: Effect of various mixed enzyme doses on dirt count, brightness and ERIC value during enzymatic deinking of sorted office paper [1= control, 2= cellulase (6IU/mL), 3= cellulase (6IU/mL) + xylanase (3IU/mL), 4= cellulose(3IU/mL) + xylanase (3IU/mL), 5= cellulose (6IU/mL) + xylanase (6IU/mL), 6= cellulose+xylanase+amylase (6+3+3 IU/mL), 7= cellulose+ xylanase+amylase, (6+3+1.5 IU/mL), 8= cellulase+ xylanase+amylase(6+1.5+1.5 IU/mL), 9= cellulase +xylanase + amylase +lipase (6+3+1.5+1.5 IU/mL), 10= cellulase +xylanase + amylase +lipase (6+3+1.5+3IU/mL) and cellulase +xylanase + amylase +lipase (6+3+1.5+6 IU/mL)]

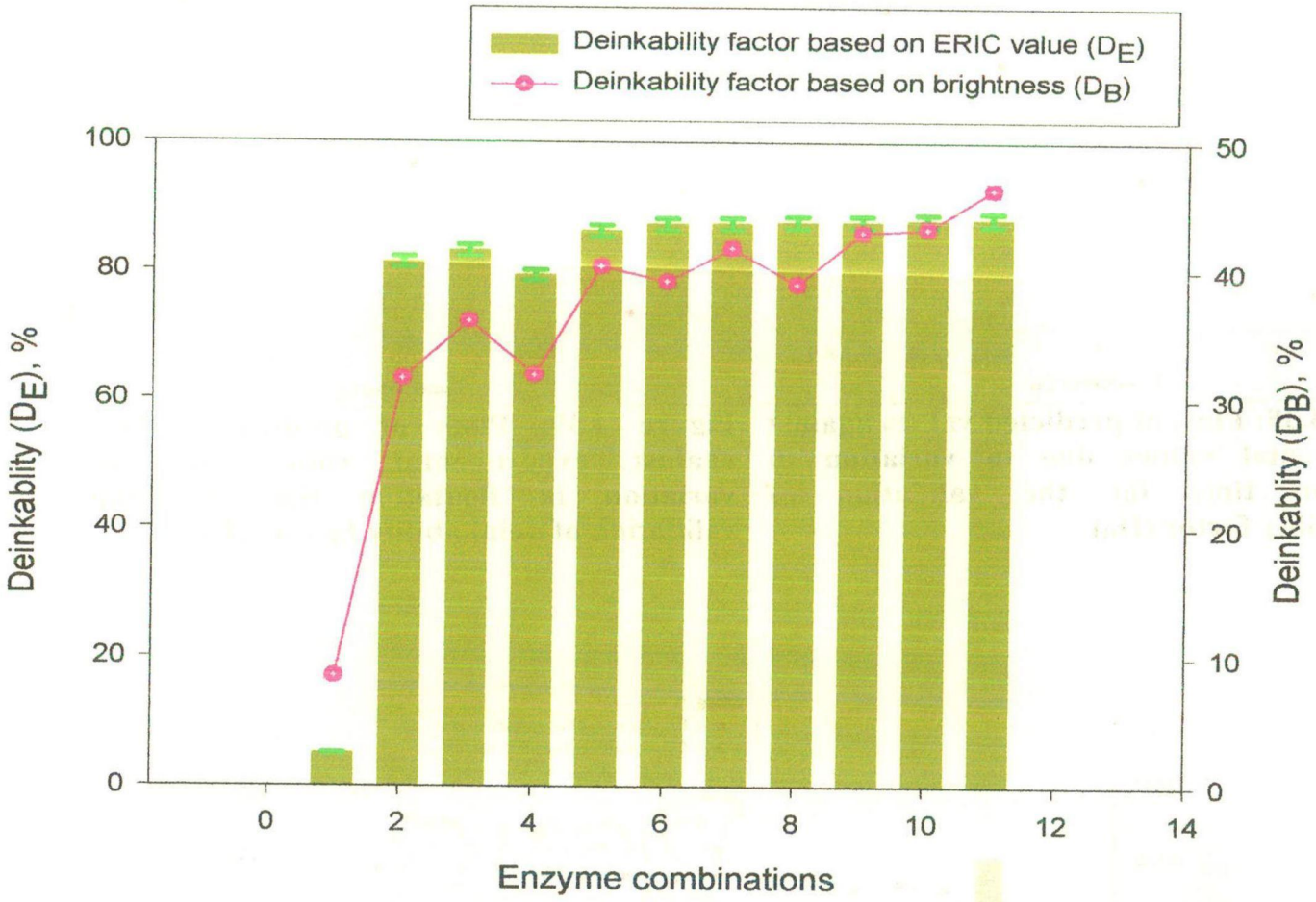


Figure 4.41: Effect of various mixed enzyme doses on deinkability factors (D_E and D_B) during enzymatic deinking of sorted office paper [1= control, 2= cellulase (6IU/mL), 3= cellulase (6IU/mL) + xylanase (3IU/mL), 4= cellulose(3IU/mL) + xylanase (3IU/mL), 5= cellulose (6IU/mL) + xylanase (6IU/mL), 6= cellulose+xylanase+amylase (6+3+3 IU/mL), 7= cellulose+ xylanase+amylase, (6+3+1.5 IU/mL), 8= cellulase+ xylanase+amylase(6+1.5+1.5 IU/mL), 9= cellulase +xylanase + amylase +lipase (6+3+1.5+1.5 IU/mL), 10= cellulase +xylanase + amylase +lipase (6+3+1.5+3IU/mL) and cellulase +xylanase + amylase +lipase (6+3+1.5+6 IU/mL)]

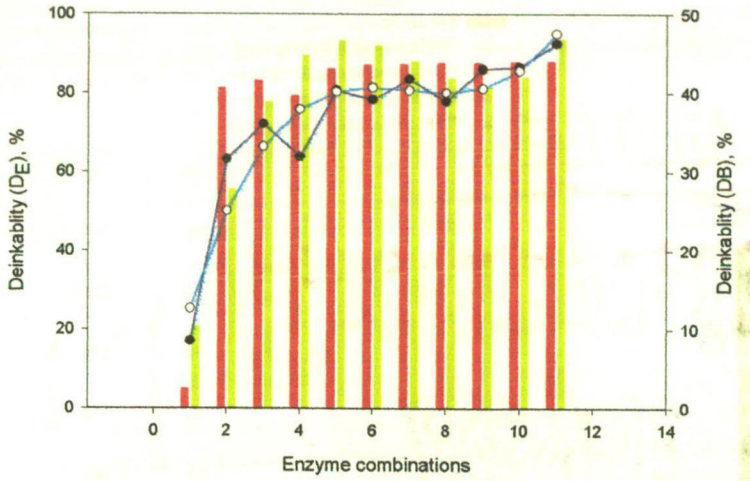


Figure 4.42: Effect of various mixed enzyme doses on experimental and predicted deinkability factors (D_E and D_B) during enzymatic deinking of sorted office paper[1= control, 2= cellulase (6IU/mL), 3= cellulase (6IU/mL) + xylanase (3IU/mL), 4= cellulose(3IU/mL) + xylanase (3IU/mL), 5= cellulose (6IU/mL) + xylanase (6IU/mL), 6= cellulose+xylanase+amylase (6+3+3 IU/mL), 7= cellulose+ xylanase+amylase, (6+3+1.5 IU/mL), 8= cellulase+ xylanase+amylase(6+1.5+1.5 IU/mL), 9= cellulase +xylanase + amylase +lipase (6+3+1.5+1.5 IU/mL), 10= cellulase +xylanase + amylase +lipase (6+3+1.5+3IU/mL) and cellulase +xylanase + amylase +lipase (6+3+1.5+6 IU/mL)]

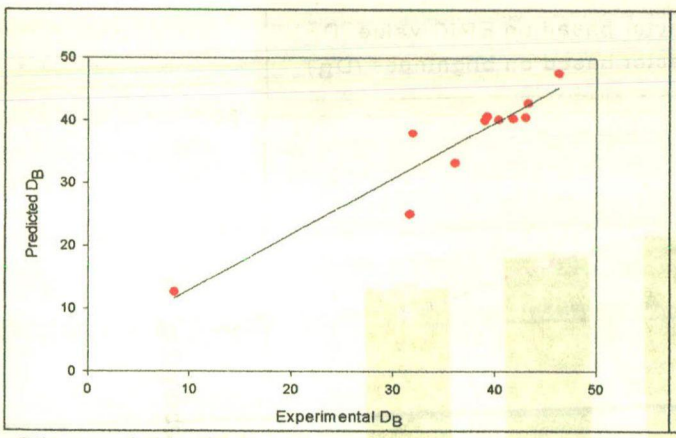


Figure 4.43: Plots of predicted values against experimental values due to variation in mixed enzyme doses for the validation of deinkability factor (D_B)

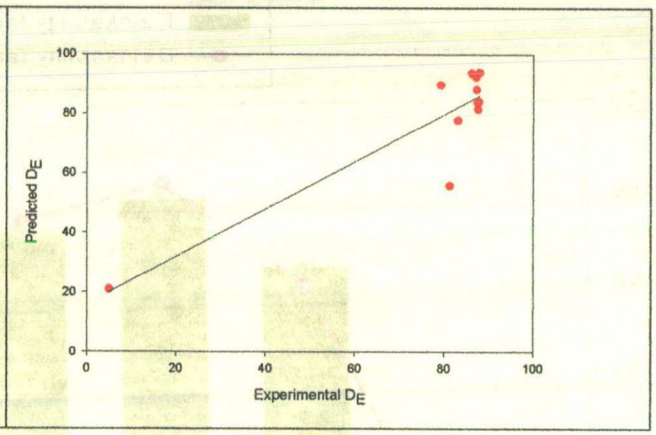


Figure 4.44: Plots of predicted values against experimental values due to variation in mixed enzyme doses for the validation of deinkability factor (D_E)

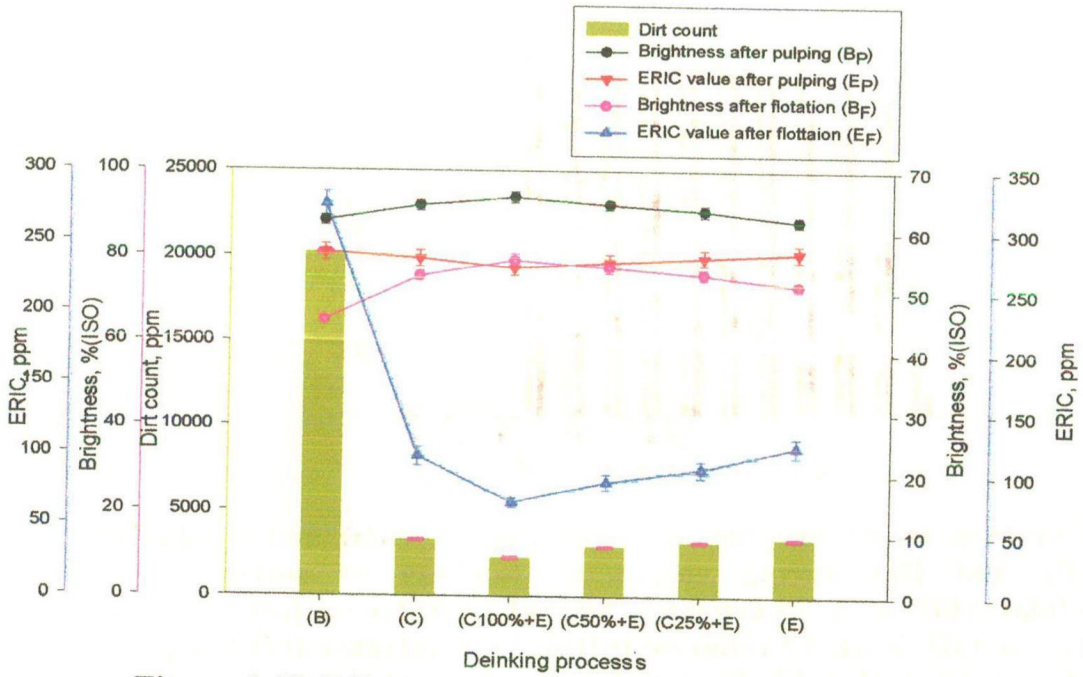


Figure 4.45: Effect of chemi-enzymatic deinking on dirt count, brightness and ERIC value (B=blank, C=chemical deinking, E=enzymatic deinking)

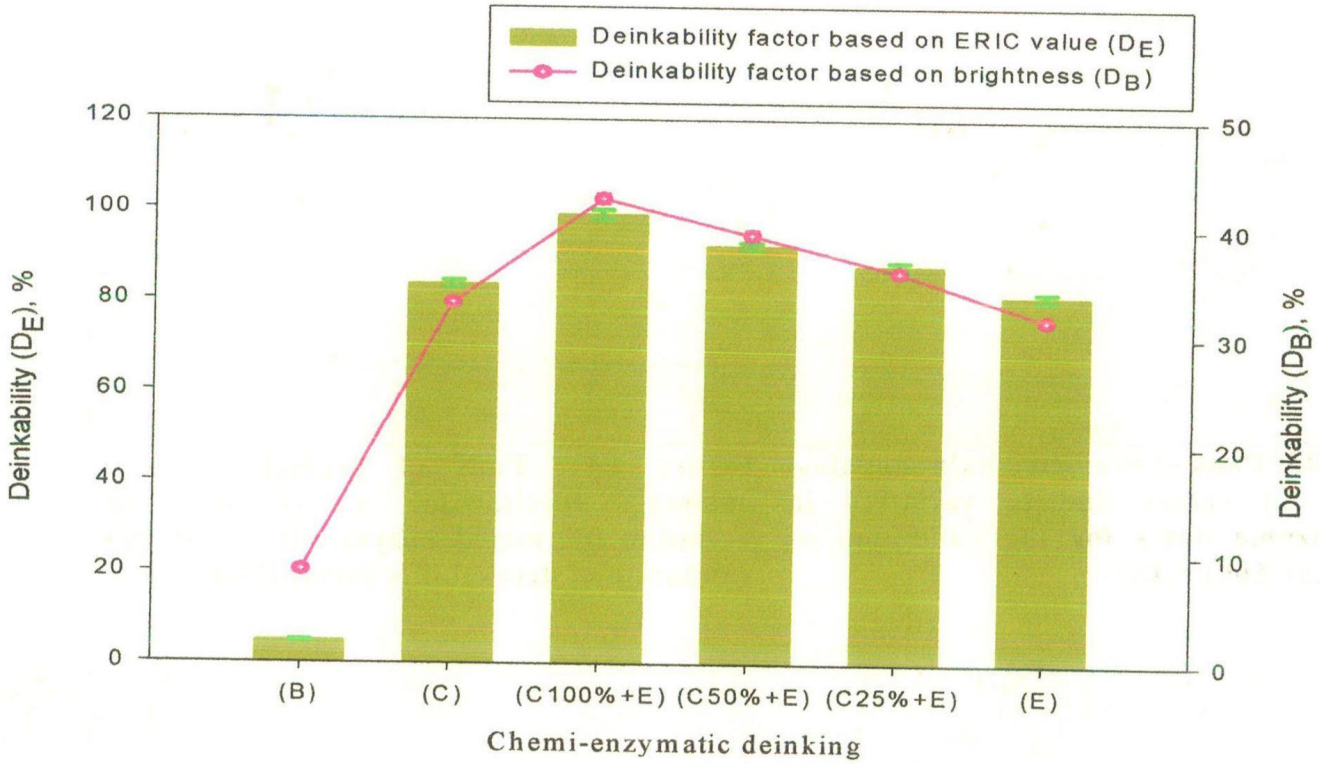


Figure 4.46: Effect of chemi-enzymatic deinking on deinkability factors (D_E and D_B)

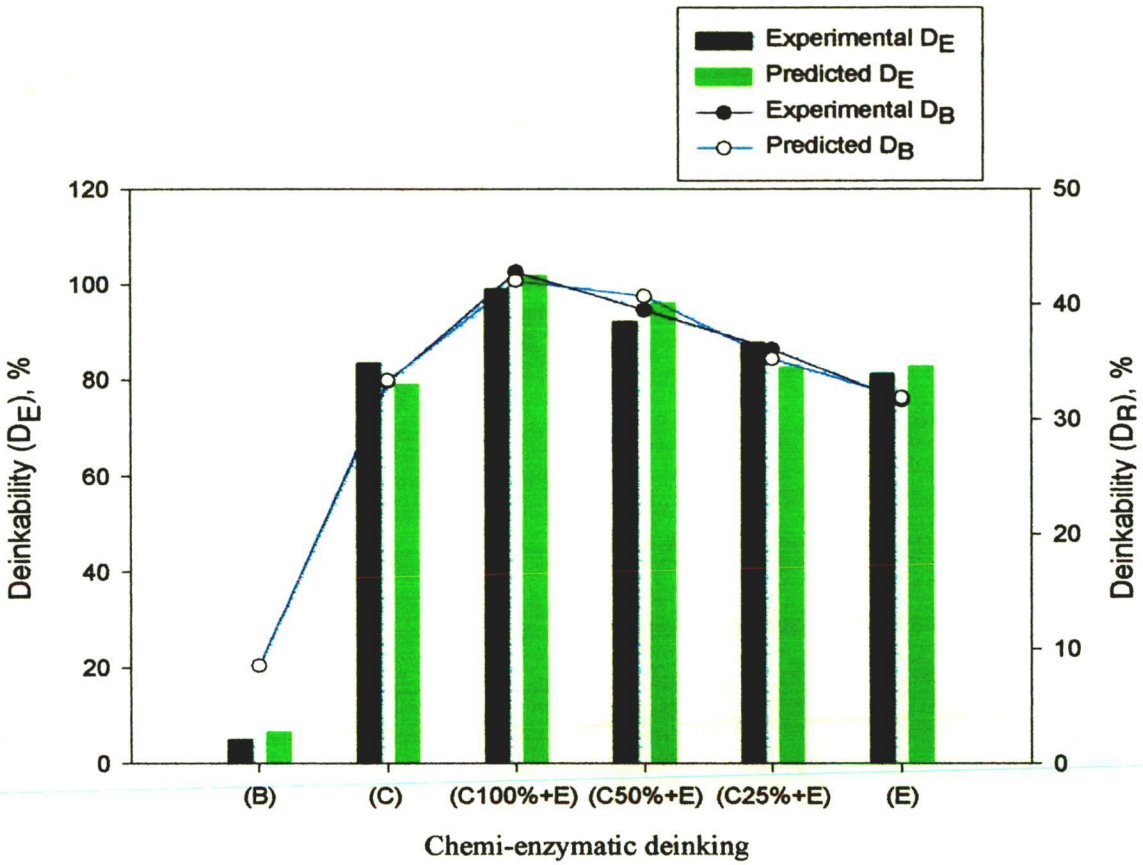


Figure 4.47: Effect of chemi-enzymatic deinking on experimental and predicted deinkablity factors (D_E and D_B)

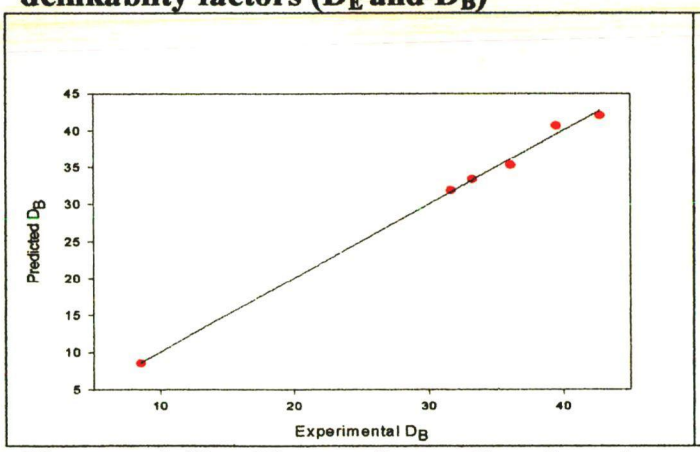


Figure 4.48: Plots of predicted values against experimental values due to effect of chemi-enzymatic deinking for the validation of deinkability factor (D_B)

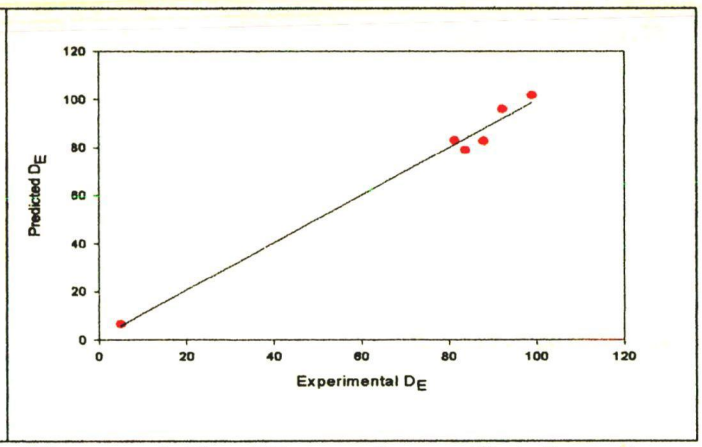
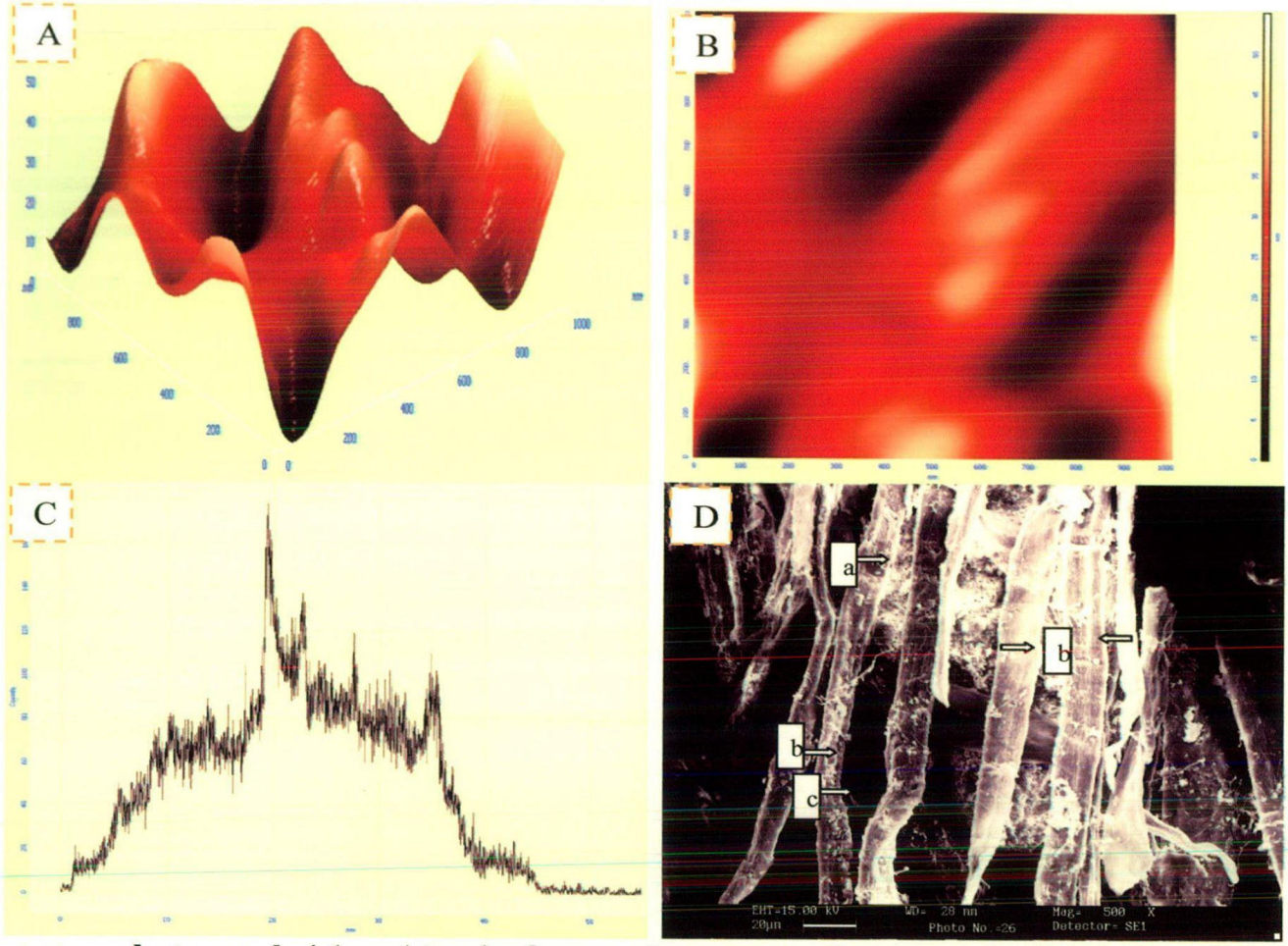
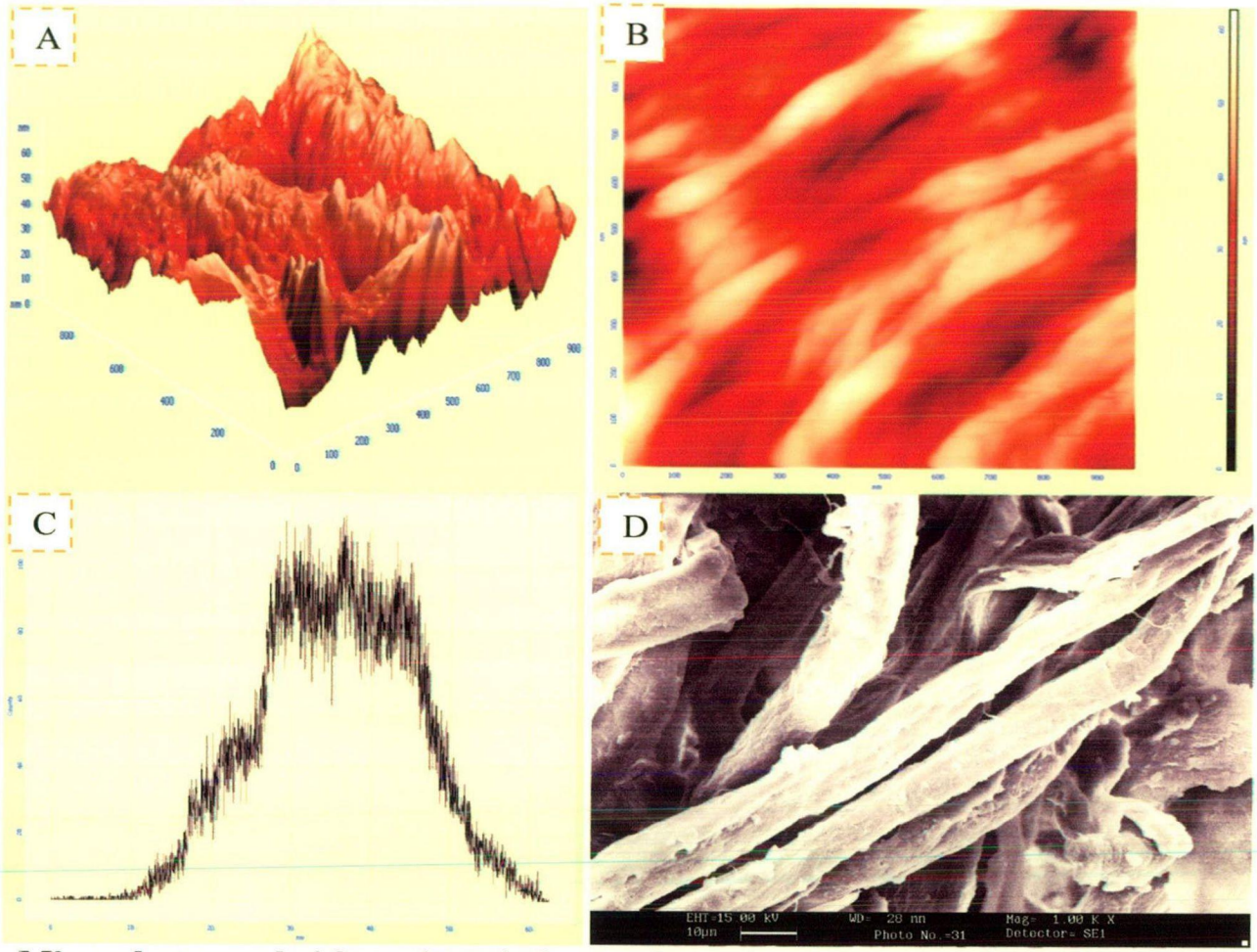


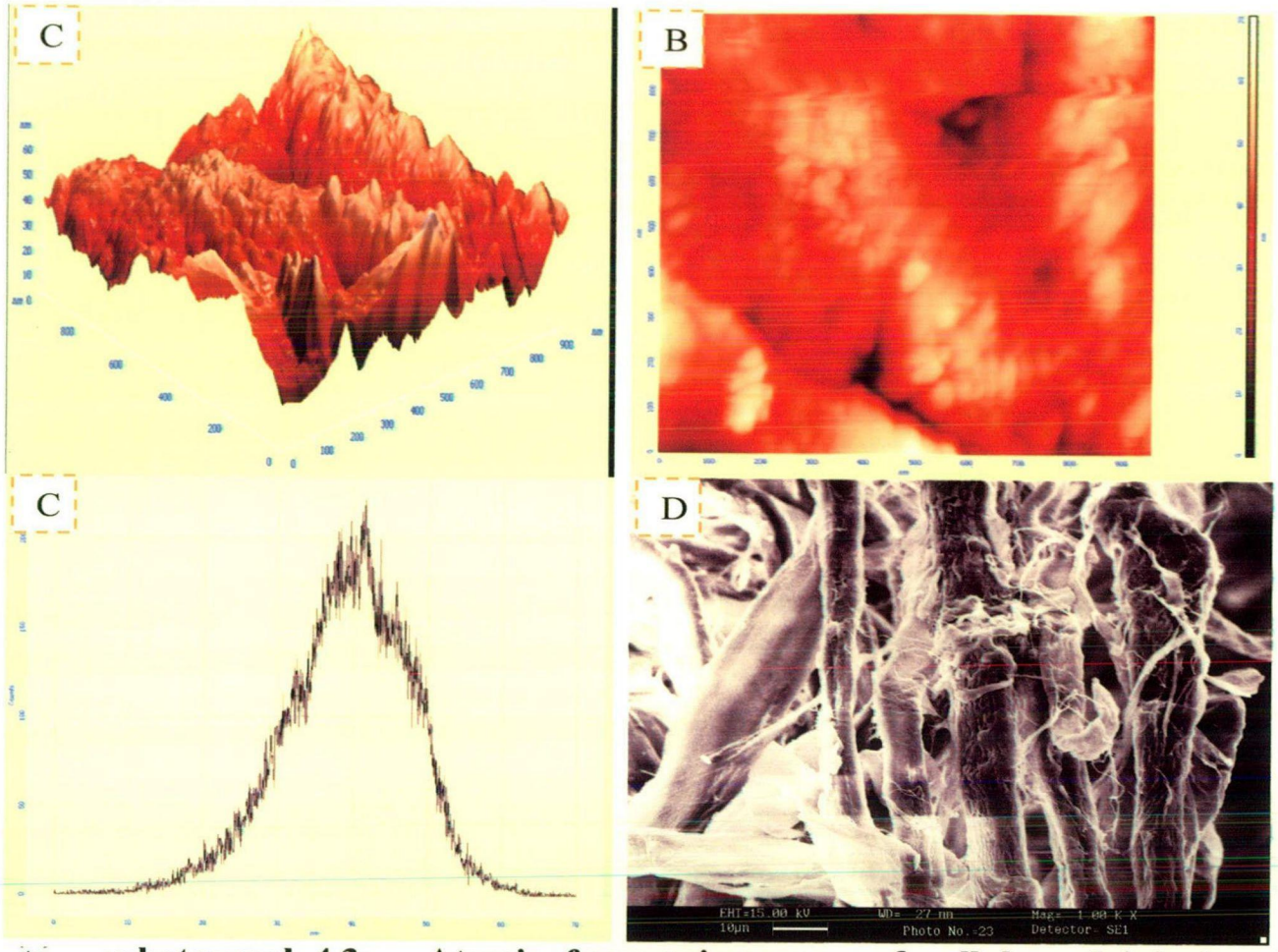
Figure 4.49: Plots of predicted values against experimental values due to effect of chemi-enzymatic deinking for the validation of deinkability factor (D_E)



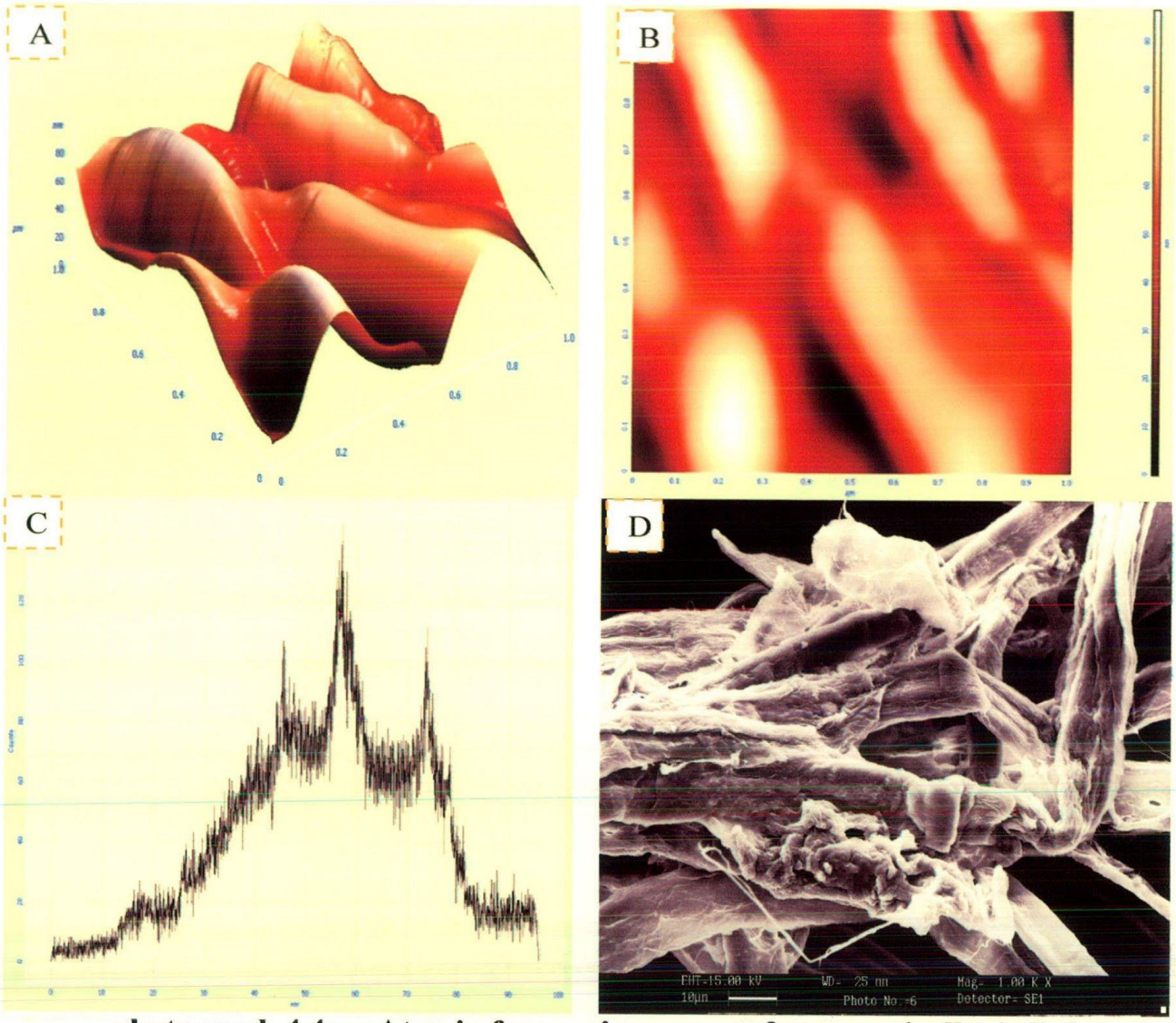
photograph 4.1: **Atomic force microscopy of enzymatic deinked pulp of SOP showing (A) 3D-structure (B) topographical structure (C) histogram (D) SEM of SOP fibers show (a) deposited non-cellulosic additives (b) swollen fissures (c) broken microfibrils at magnification of 500x**



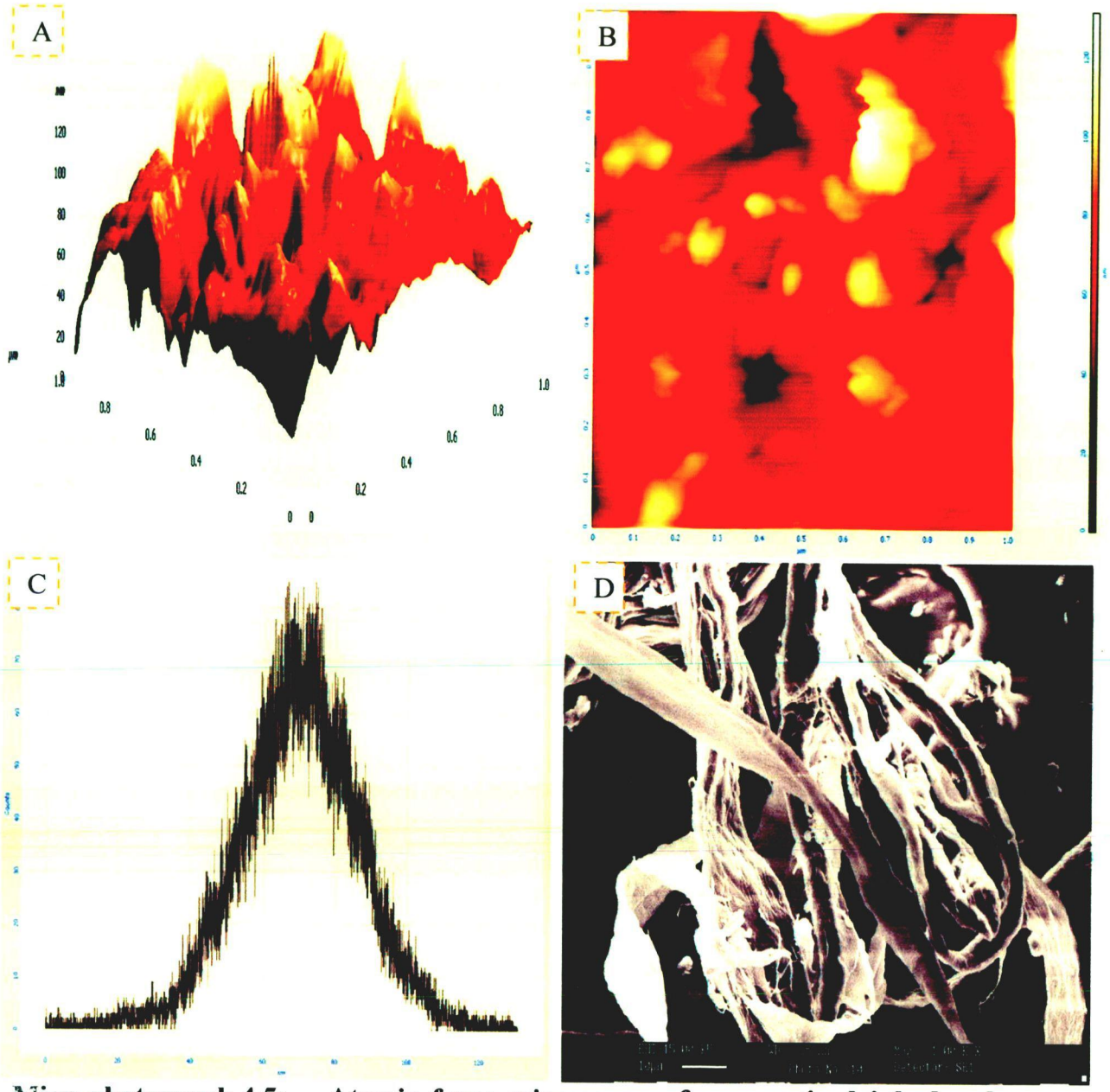
Micrograph 4.2: Atomic force microscopy of chemically deinked pulp of sorted office waste paper showing (A) 3D-structure, (B) topographical structure and (C) histogram (D)SEM of chemically deinked fibers at magnification of 1.00 KX



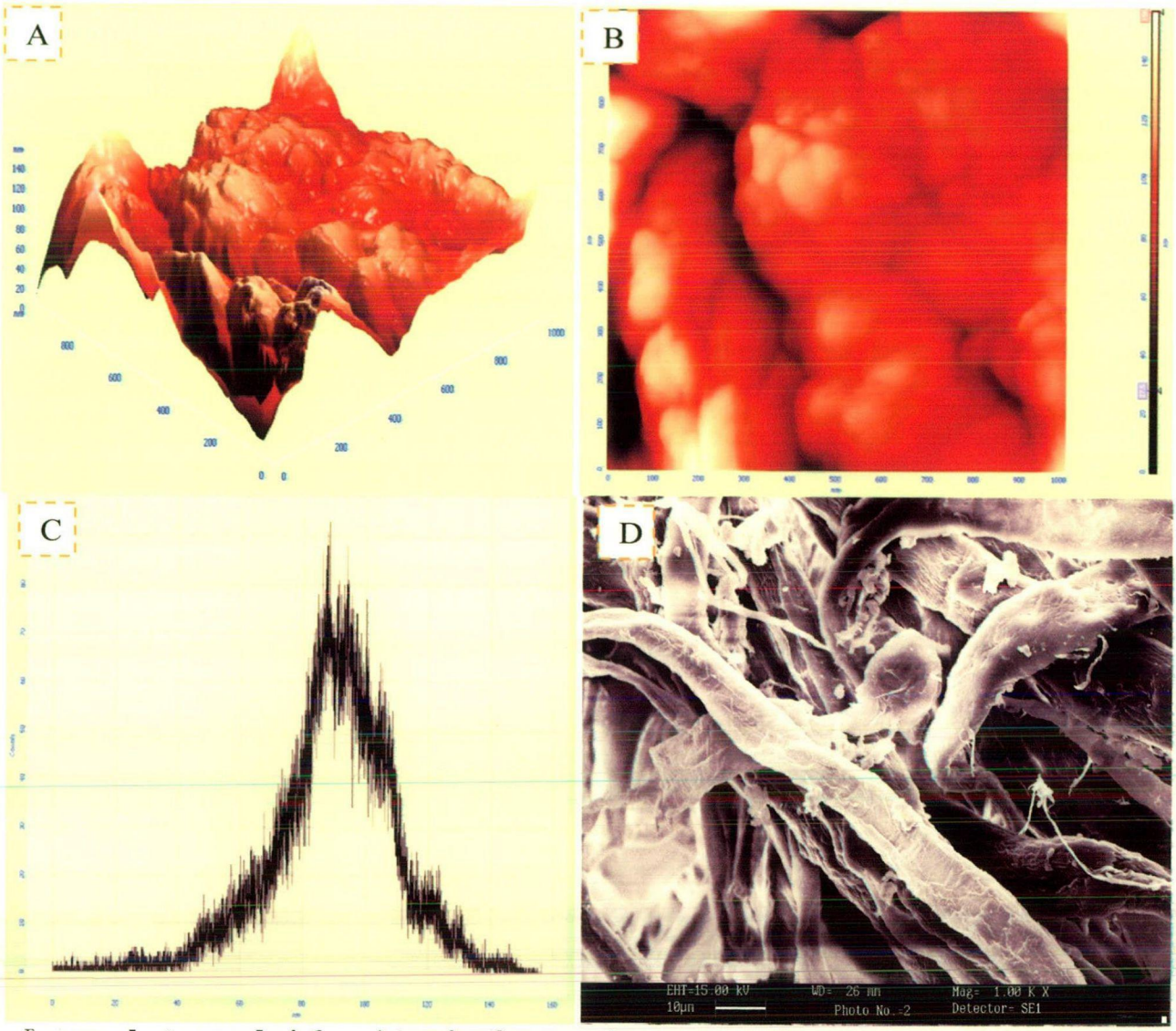
photograph 4.3: Atomic force microscopy of cellulase deinked pulp showing (A) 3D-structure, (B) topographical structure and (C) histogram (D) SEM of cellulase deinked fibers at magnification of 1.00 KX



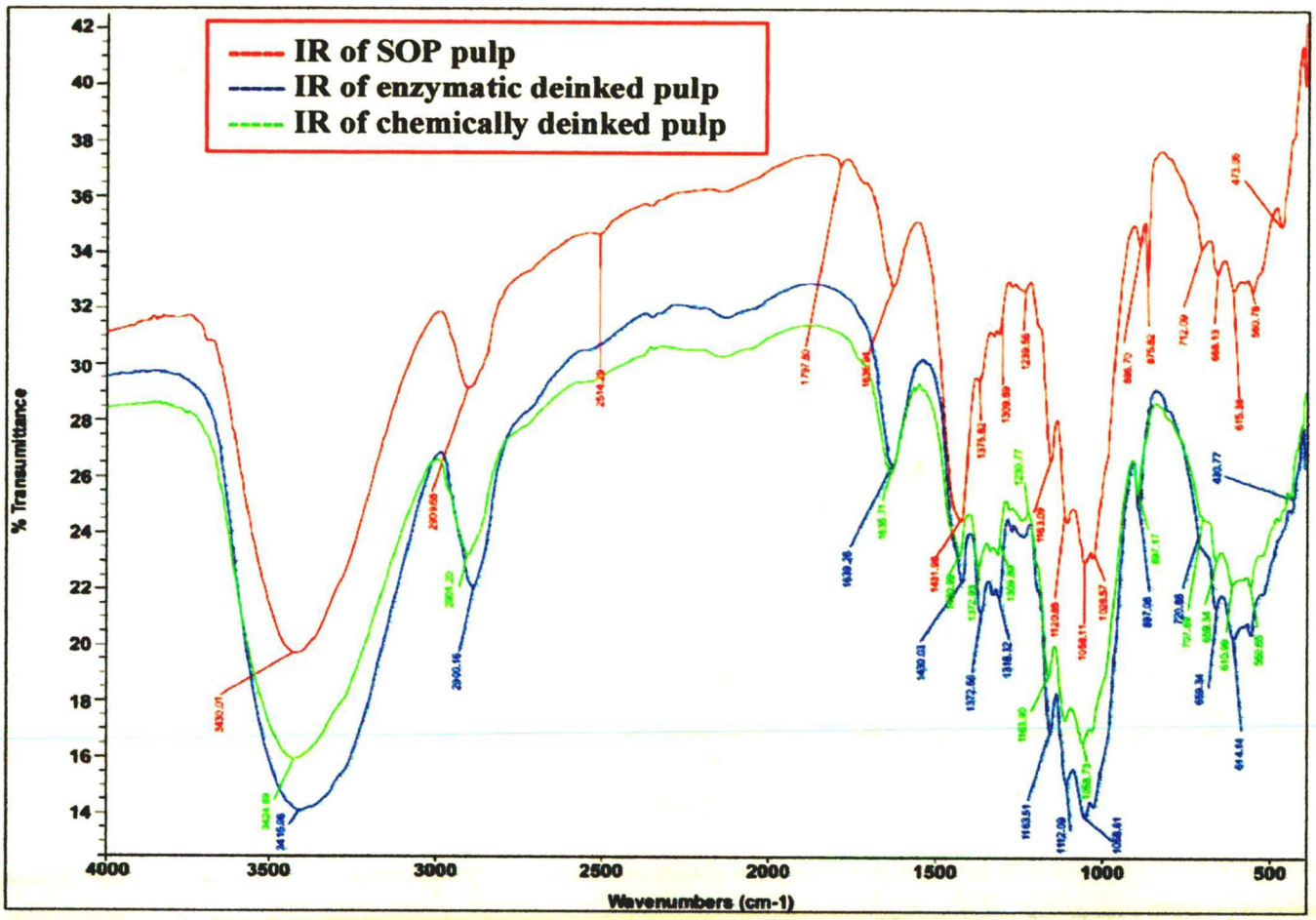
photograph 4.4: Atomic force microscopy of enzymatically (cellulase + xylanase) deinked pulp SOP showing (A) 3D-structure 3D-structure (B) topographical structure and(C) histogram (D) SEM of cellulase + xylanase deinked fibers at magnification of 1.00 KX



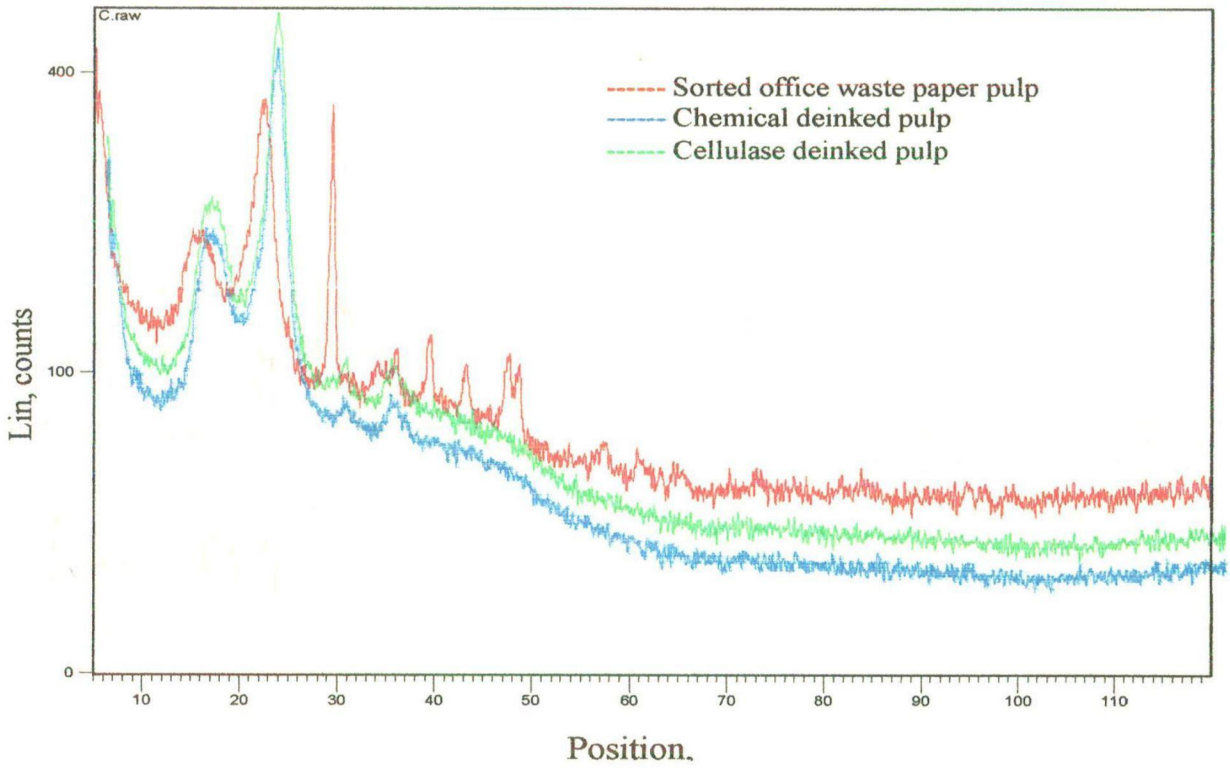
Microphotograph 4.5: **Atomic force microscopy of enzymatic deinked pulp (cellulase + xylanase + amylase) of SOP showing (A) 3D-structure (B) topographical structure and (C) histogram (D) SEM of cellulase + xylanase + amylase deinked fibers at magnification of 1.00 KX**



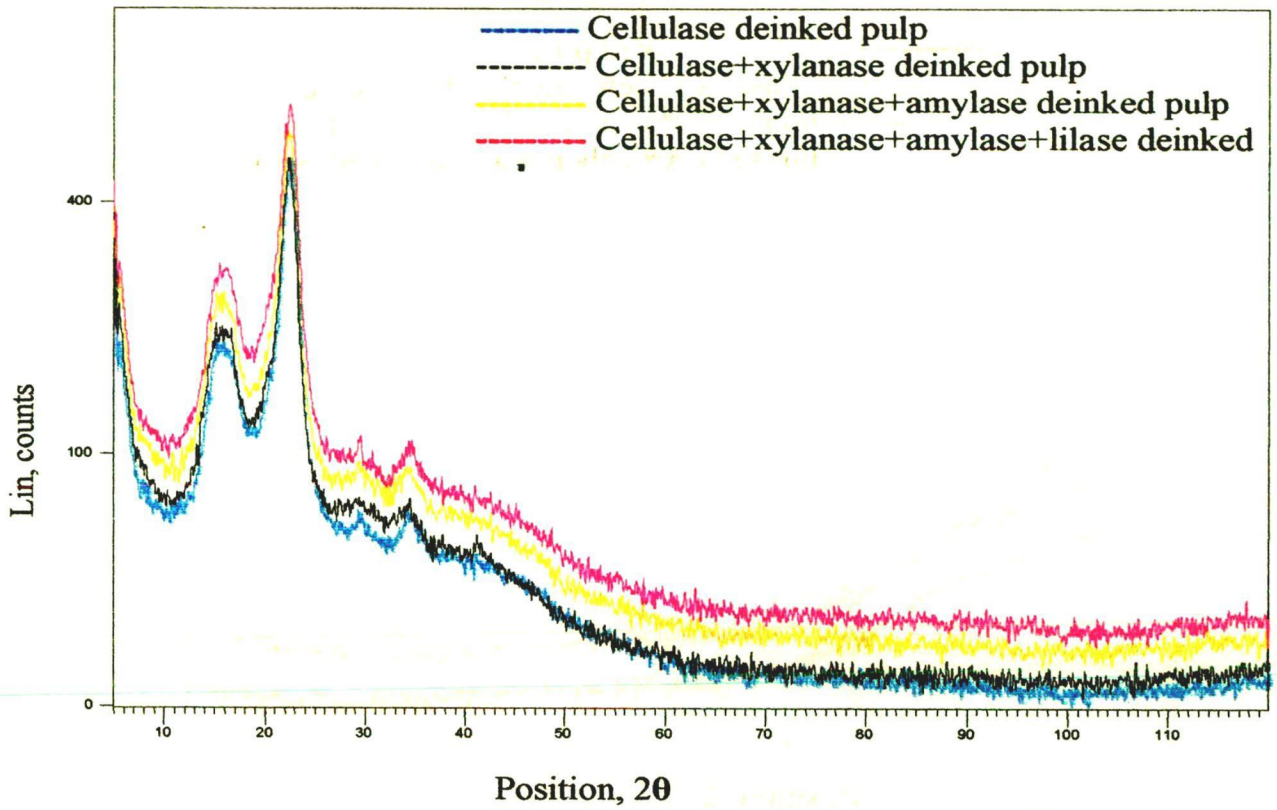
Micro photograph 4.6: **Atomic force microscopy of enzymatic deinked pulp (cellulase + xylanase + amylase + lipase) of SOP showing (A) 3D-structure, (B) topographical structure and (C) histogram (D) SEM of cellulase + xylanase + amylase + lipase deinked fibers at magnification of 1.00 KX**



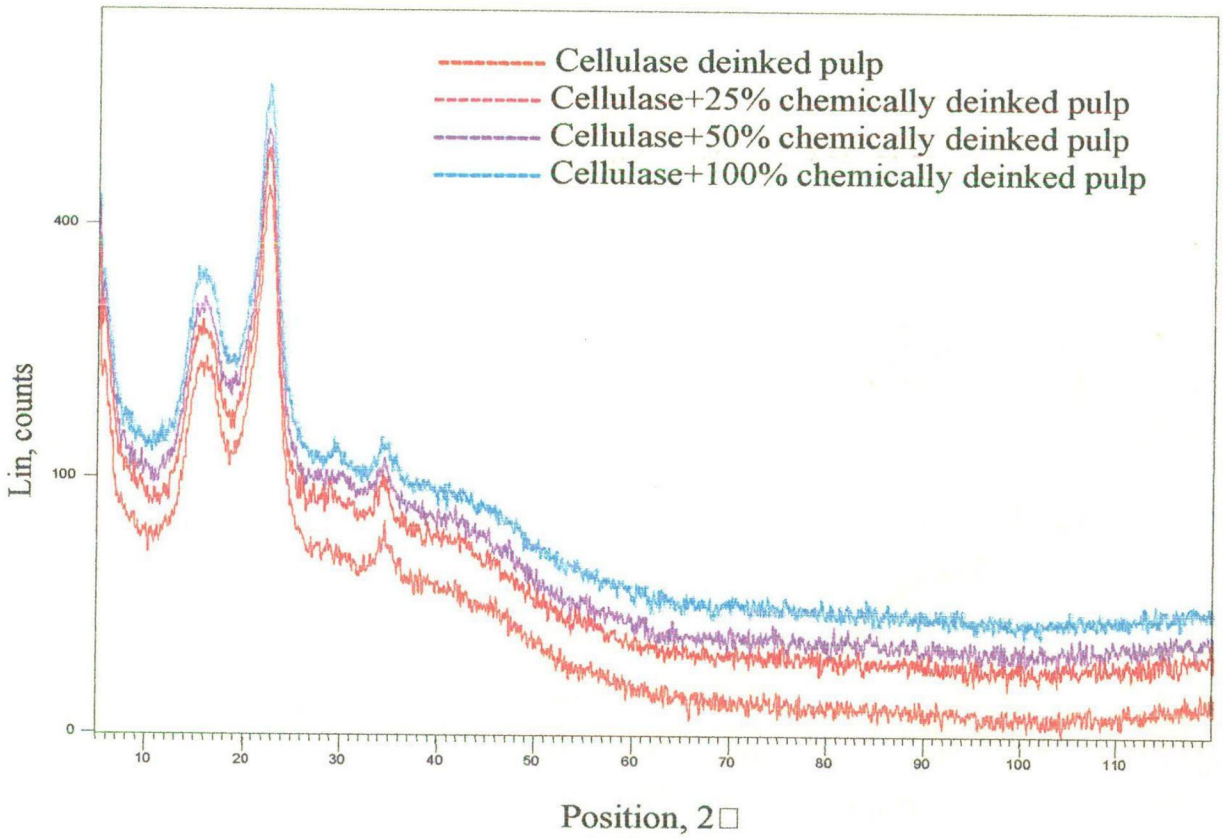
Micrograph 4.1: Infrared spectroscopy of sorted office paper (control), chemical and enzymatic deinking of SOP pulp



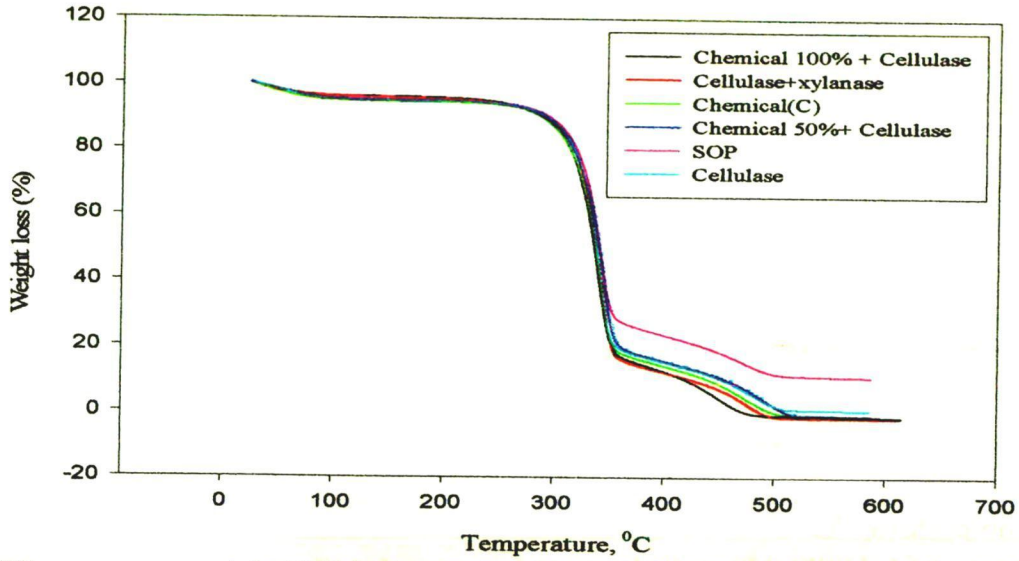
Micrograph 4.2: XRD of sorted office waste paper pulp, chemical treated and cellulase deinked pulp



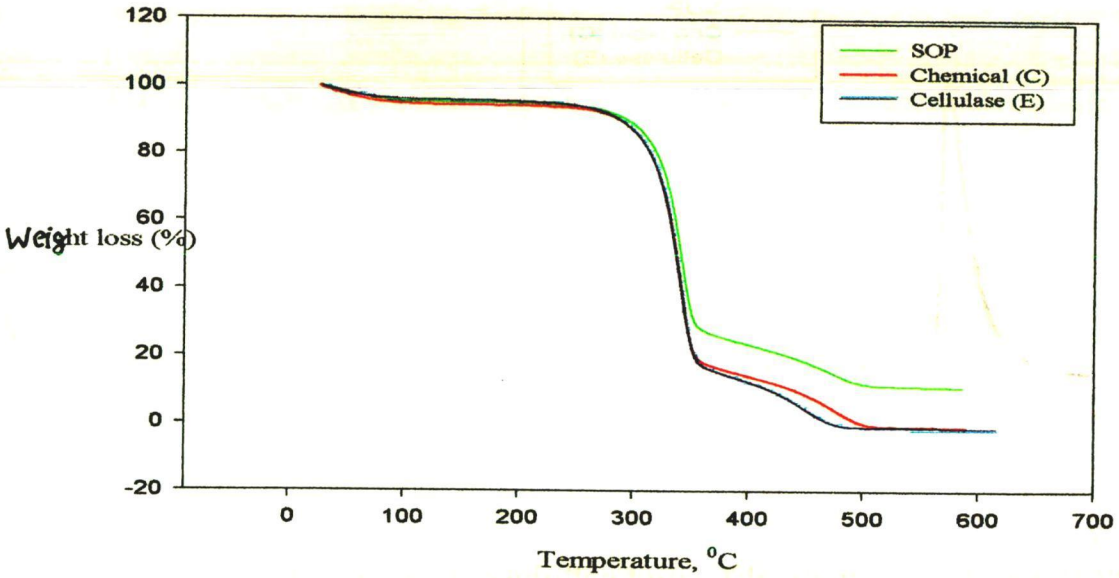
Micrograph 4.3: XRD of mixed enzyme deinked pulp of sorted office waste paper



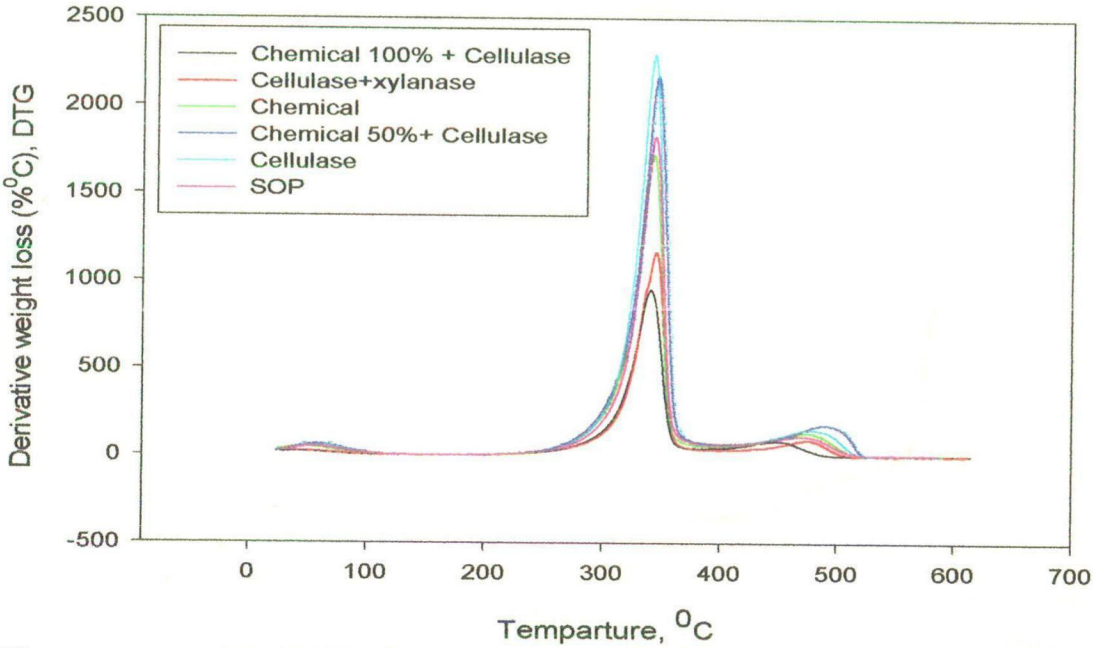
Micrograph 4.4: XRD of chemi-enzymatic deinked pulp of sorted office waste paper



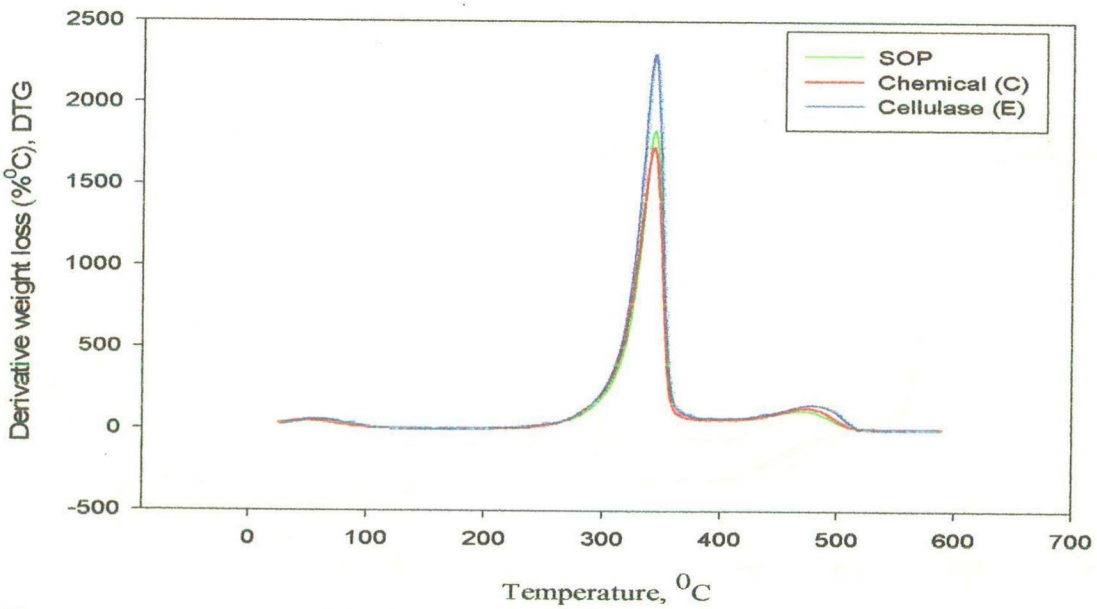
Thermogram 4.1: TGA thermograms of sorted office waste paper pulp, chemical 100% cellulase, cellulose + xylanase, chemical, chemical 50% + cellulase and cellulase deinked pulps of sorted office waste paper



Thermogram 4.2: TGA thermograms of SOP, chemical(C) and cellulase (E) deinked pulp



Thermogram 4.3: DTG thermograms of C100%+E, cellulase+xylanase, chemical, C50%+E, SOP and cellulase deinked pulp



Thermogram 4.4: DTG thermograms of SOP, chemical(C) and cellulase (E) deinked pulp

References

1. Acosta, D.A., Scamehorn, J.F. and Christian, S.D., Flotation deinking of sorted office papers using sodium octanoate and sodium dodecanoate as surfactants and calcium as the activator, *J. Pulp. Pap. Sci.*, 29(2): (2003).
2. Akin, D.E., Foulk, J.A., Dodd, R.B. and McAlister, D.D., Enzyme-retting of flax and characterization of processed fibers, *J. Biotechnol*, 89:193-203(2001).
3. Allison, R.W., Clark, T. A. and Suurnakki, A., Effect of modified kraft pulping on enzyme assisted bleaching, *Appita J.*, 49:18(1996).
4. Ali, T., McLellan, F., Adiwinata, J., May, M. and Evans, T., Functional and performance characteristics of soluble silicates in deinking, part I: alkaline deinking of newsprint/magazine, *J. Pulp. Pap. Sci.*, 20(1): J3-J8 (1994).
5. Anonymous, TAPPI Test Methods, Standard methods for pulp and paper, Technical Association of Pulp and Paper Ind., *TAPPI Press*, Technology Park, P.O.box105113, Atlanta, GA-330348-5113, USA(2007).
6. Atalla, R.H., Celluloses, In: *Comprehensive Natural Products Chemistry,* Barton, D., Nakanishi, K. and Meth-Cohn, O., (eds.), Elsevier Science Ltd., Oxford (1999) pp.529-598.
7. Awadel-Karim, S., Nazhad, M.M. and Pazsner, L., Factors affecting crystalline structure of cellulose during solvent purification treatment, *Holzforschung,* 53:1-8(1999).
8. Azevedo, M.A.D., Drelich, J. and Miller, J.D., The effect of pH on pulping and flotation of mixed office waste paper, *J. Pulp. Pap. Sci.*, 25(e): 317(1999).
9. Badar, T., Environmental impact of recycling in the paper industry, *Prog. Pap. Recycl.*, 2(3): 42(1993).
10. Bajpai, P. and Bajpai, P.K., Deinking with enzymes: a review, *Tappi J.*, 81(12):111-7(1998).
11. Bajpai, P., Application of enzymes in the pulp and paper industry, *Biotechnol. Prog.*, 15(2): 147-157 (1999).
12. Bajpai, P., Mishra, O.P., Mishra, S.P., Kumar, S. and Bajpai P.K., Enzyme assisted deinking of sorted non –impact white office waste paper, In: *9th International Conference on biotechnology in the pulp and paper industry*, Durban (South Africa) (2004) pp.121.

13. Bajpai, P., Advances in recycling and deinking, *PIRA International*, U.K., Chapter 1, (2006) pp.1-2.
14. Basta, H., Huaiyu, A. Z., Shuangfei, W., Bingyun, L., Chongxing, H. and Jaixiang, C., The use of deinking rate and cleaning rate to evaluate deinking plant performance, *Appita. J.*, 57(1): 23 (2004).
15. Bender, M., Langen, M. and Wulf, G., Tubular reactor for batches of used paper materials from a pulper, (WO 200126804) (2001).
16. Beneventi, D. and Carre, B., The mechanisms of flotation deinking and the role of fillers, *Prog. Pap. Recycl.*, 9: 77(2000).
17. Bennington, C.P.J., Sui, O.S. and smith, J.D., The effect of mechanical action on waste paper defibering and ink removal in repulping operations, *J. Pulp. Pap. Sci.*, 24(11): 341(1998).
18. Bennington, C.P.J. and Wang, M.-H., A kinetic model of ink detachment in the repulper, *J. Pulp. Pap. Sci.*, 27(10): 347-352(2001).
19. Bhat, G.R., Heitmann, J.A. and Joyce, T.W., Novel techniques for enhancing the strength of secondary fiber, *Tappi J.*, 74 (9):151-157(1991).
20. Binkley, R.W., *Modern Carbohydrate Chemistry*, Marcel Dekker, New York (1988).
21. Binnig, G., Quate, C.F. and Gerber, Ch., Atomic force microscope, *Phys. Rev. Lett.*, 56 (9): 930-933(1986).
22. Blanco, A., Negro, C., Monte, M. C., Fuente, H. and Tijero, J., Overview of two major deposit problems in recycling: slime and stickies, part II: stickies problems in recycling, *Prog. Pap. Recycl.*, 11: 26(2002).
23. Bolanca, Z., Bolanca, I. and Hladnik, A., The influence of digital printing conditions on the characteristic of the recycled fiber, proceedings on 28th EUCEPA Conference, Loureiro, A., (ed.), Lisbon, Techicelpa, (2003) pp.356-357
24. Bolaski, W., Gallatin, A. and Gallatin, J.C., Enzymatic conversion of cellulosic fibres, *United States Patent*, 3,041,246(1959).
25. Borchardt, J.K., Possible deinking mechanisms and potential analogies to laundering, *Prog. Pap. Recycl.*, 2(2):47(1993).

26. Borchardt, J.K., Ink types: The role of ink in deinking, In: *Paper Recycling Challenge-Deinking & Bleaching*, (eds.), Doschi, M.R., Dyer, J.M., Doschi & Associates Inc. ISBN 0-9657447-1-X Appleton (1997) p.13-17.
27. Borchardt, J.K., Use of flotation in removing ink from recovered paper, presented at the 87th annual meeting of The Pulp And Paper Technical Association of Canada, Preprint Book A, 71-79 (January 30, 2001)
28. Borchardt, J.K., Recent developments in paper deinking technology, May2003.
29. Call, H. P. and Strittmatter, G., Einsatz von lignolytischen enzyme in der papier- und zellstoÍndustrie, neuere ergebnisse, *Papier,* 46(10A): 32-37 (1992).
30. Call, H. and Mücke, I., History, overview and applications of mediated lignolytic systems, especially laccase-mediator-systems (Lignozyme-process), *J. Biotechnol.*, 53:163-202(1997).
31. Cao, Y., and Huimin, T., Effects of cellulase on the modification of cellulose, *Carbohydr. Res.*, 337:1291-1296(2002).
32. Carr, W.F., New trends in deinking technology, removing difficult inks from waste paper, *Tappi. J.*, 3: 127-132 (1991).
33. Carrasco, F., Pelach, M.A. and Mutje, P., Deinking of high quality offset papers: influence of consistency, agitation speed, and airflow rate in the flotation stage, *Tappi. J.*, 82(3):.125 (1999).
34. Carre, B. and Galland, G., Overview of deinking technology, *Proceedings of Ippta workshop on deinking*, Chandigarh, (2002) pp.1
35. Castanon, M. and Wilke, C.R., Effects of the surfactant Tween 80 on enzymatic hydrolysis of newspaper, *Biotechnol. Bioeng.*, 23:1365-1372(1981).
36. Catsburg, R.W., Flotation deinking research of the future, parallel process simulation, *Paper Technology J.*, 35 (1999).
37. Charm, S.E. and Wong, B.L., Shear effects on enzymes, *Enzyme Microbiol. Technol.*, 3:111-118(1981).
38. CHE-10 Spectroscopy, IR and Raman Spectra, Indira Gandhi National Open University, School of Sciences, *ISBN-81-7263-937-6* (1996)pp.36

39. Christov, L.P. and Prior, B.A., Repeated treatments with *Aureobasidium pullulans* hemicellulases and alkali enhance biobleaching of sulphite pulps, *Enzyme Microbial. Technol.*, 18:244-250(1996).
40. Deinking of Waste Paper, *TAPPI Monograph, No.*32 (1967).
41. Drelich, J., Pletka, P., Boyd, E., Raber, D., Herron, E. Luhta, H., Walqui, N., Tervo, S., Boston , J., Wieland, J., Morgan, J. and Sabo, N., Interfacial chemistry aspects of deinking flotation of mixed office paper, Preprints of the SME annual meeting, Denver, Colorado, (2001a)pp.26.
42. Drelich, J. and Miller, D., Improved flotation deinking of sorted office paper by flocculation of ink particles, *Prog. Pap. Recycl: 38* (2001b).
43. Delagoutte, T., Management and control of stickies, *Prog. Pap. Recycl.*, 15:31(2005).
44. Deng, Y., Effect of fiber surface chemistry on the fiber loss in flotation deinking, *Tappi. J.*, 83(6): 61(2000).
45. Doshi, M.R. and Dyer, J.M., Management and control of stickies, In: *Paper Recycling Challenge,* Vol. 3, Process Technology, Doshi, M.R. and Dyer, J.M.,(eds.), Doshi & Assoc., Inc., Appleton, WI(1998) pp. 195-232.
46. Elegir, G., Panizza, E. and Canetti, M., Neutral enzyme-assisted deinking of xerographic office waste with a cellulase/amylase mixture, *Tappi. J.*, 83:11-71(2000).
47. Elegir, G. and Bussini, D., Evaluation of enzymatic assisted deinking of wood containing papers versus conventional deinking, *Prog. Pap. Recycl.*, 18: 40-49(2008).
48. Eom, T.J. and Ow, S.S.K., German pat GB.3, 934,772(1990).
49. Eom, T-J. and Ow, S.K., Kami Pa Gikyoshi, 45(12): 1377-1382(1991).
50. Eriksson, K.E.L., Commercialization of enzymatic process, Personal communication, (1997).
51. Evans, J.D., Akin, D.E. and Foulk, J.A., Flax-retting by poly-galacturonase containing enzyme mixtures and effects on fiber properties, *J. Biotechnol.*, 9:223-231(2002).
52. Fabry, B., Roux, J.C. and Carré, B., Characterization of friction during pulping: An interesting tool to achieve good deinking, *J. Pulp. Pap. Sci.*, 27(8): 284(2001).
53. Fabry, B. and Carre, B., Pulping and ink detachment, 7th CTP/PTS Advanced Training Course on Deinking, Grenoble, France (2005).

54. Faix, O., Fourier transform infrared spectroscopy, In: *Methods in Lignin Chemistry*, T.E. Timell (eds.), *Springer-Verlag*, Germany (1992).
55. Ferguson, L.D., Flotation deinking technology, *In Deinking Short Course Notes, TAPPI*, TAPPI PRESS, Atlanta (1995) (Chap.10).
56. Franks, N.E. and Munk, N., In: *TAPPI Pulping Conference Proceeding*, Alkaline cellulase and the enzymatic deinking of mixed office waste, *TAPPI PRESS*, Atlanta,(1995) pp.343.
57. Franks, N. E., Holm, H.C. and Munk, N., Enzymatic deinking. *World Pulp Pap. Technol.*, 93-96(1995).
58. Froix, M.F. and Nelson, R., The Interaction of water with cellulose from nuclear magnetic resonance relaxation times, *Macromolecules*, 8:726-730(1975).
59. Fujioka, S., Naito, K., Okuyama, T., Sano, K. and Takayama, S., Decolorizable ink and printer, US Patent No 5922115(1999).
60. Fujioka, S., Machida, S., Naito, K., Okuyama, T., Sano, K. and Takayama, S., Decolorizable ink and printer, US Patent No 6017386(2000).
61. Fukuda, S., Hayashi, S., Ochiai, H., Iiizumi, T. and Nakamura, K., Improvers for deinking of wastepaper, Japanese Patent 2,229,290(1990).
62. Gabriel, B.L., In: *Biological scanning electron microscopy*, Von Nostrand Reinhold Company, New York (1982) pp.186.
63. Galland, G., Saint Amand, F. J., Vernac, Y. and Bernard, E., Deinking of varnished printed papers: characterization and removal of specks, 4th PTS Deinking Symposium, Munich, April (1990) pp.3.
64. Galland, G., Vernac, Y., Carre, B. and Rousset, X., In: *Effect of pulping conditions on ink redeposition and ink removal when recycling water based ink printed papers, TAPPI Pulping Conference,* Seattle, WA (2001).
65. Goodenough, P.W., Clark, D.C. and Durrant, A.J., Gilbert, H.J., Hazlewood, G.P. and Waksman, G., Structural analysis by circular dichroism of some enzymes involved in plant cell wall degradation, *FEBS letters*, 282(2):355-8(1991).
66. Goto, S. and Miyanishi, T., Effects of structures and properties of surfactants on office waste deinking, *Res. Forum. Recycl. Proc.*, 171(2004).

67. Goto, S. and Miyanishi, T., Effects of non-ionic deinking agents and fatty acids on foaming properties in flotation deinking of old magazine, *Jpn. Tappi J.*, 58(2): 88(2004).
68. Göttsching, L. and Putz, H.-J., Deinking chemistry, Wochenblatt für papierfabrikation, 127(10): 650-654(1999).
69. Göttsching, L. and Pakarinen, H., *Recycled fiber and deinking, papermaking science and technology,* Paulapuro, H., (ed.), *Fapet Oy,* 7: Helsinki, Finland (1999).
70. Gu, Q.P., Yong, Q., Ye, H.L. and YOU, J.X., Study on deinking of the used newsprint with blended cellulase and xylanase, *Chemistry and Industry of Forest Products.*, 23: p.19-22(2003).
71. Gübitz, G.M., Mansfield, S.D., Böhm, D. and Saddler J.N., Effect of endoglucanases and hemicellulases in magnetic and flotation deinking of xerographic and laser-printed papers, *J. Biotechnol.*, 65:209-15(1998).
72. Gübitz, G.M., Mansfield, S.D. and Saddler, J.N., Effectiveness of two endoglucanases from *Gloeophyllum* species in deinking mixed office waste paper, In: 47th *International Conference on Biotechnology in the Pulp and Paper Industry*, C: (1998)135-8.
73. Gümüskaya, E., Usta, M., and Kirci, H., The effects of various pulping conditions on crystalline structure of cellulose in cotton linters, *Polymer Degradation and Stability.*, 81:559–564(2003).
74. Haarhoff, J., Moes, C.J., Cerff, C., Wyk van, W.J., Gerischer, G. and Janse, B.J.H., Characterization and bio-bleaching effect of hemicellulases produced by thermophilic fungi, *Biotechnol. Lett.*, 21:415-420(1999).
75. Heise, O. U., Unwin, J. P., Klungness, J. H., Fineran, W. G., Sykes, M., Abubark, S., Industrial scale up of enzyme-enhanced deinking of nonimpact printed toners, *Tappi. J.*, 79: 207-212(1996).
76. Heitmann, J., Joyce, T. and Prasad, D., In: 5th *International Conference on Biotechnology in the Pulp and Paper Industry*, Enzymatic deinking of newsprint waste, Kyoto, (1992) pp.175.
77. Helle, S.S., Duff, S.J.B., and Cooper, D.G., *Biotech. Bioeng.*, 42: 611(1993).
78. Henrissat, B., Cellulases and their interaction with cellulose, *Cellulose,* 1:169-196(1994).
79. Hindeleh, A.M. and Johnson, D.J., Crystallinity and crystallite size measurements in polyamide and polyester fibers, *Polymer*, 19:27-32(1978).

80. Hinrichs, D.D., The effect of kraft pulping variables on the delignification, *Tappi. J.*, 50(4): 173(1967).

81. Holik, H., Unit operations and equipments in recycled fiber processing, In: *Papermaking Science and Technology,* Göttsching, L. and Pakarinen, H. (eds.), Book 7. Recycled Fiber and Deinking, *Fapet Oy,* (2000) pp.91-134.

82. Hua, Y-fen., Zhang, M., Fu, C-xin., Chen, Z-hui. and Chan, G.Y.S., Structural characterization of a 2-O-acetylglucomannan from *Dendrobium officinale* stem, *Carbohydr. Res.*, 339: 2219-2224(2004).

83. Hubbe, M.A., Venditti, R.A and Rojas, O.J., What happens to cellulosic fibers during papermaking and recycling? A review, *BioResources*, 2(4): 739-788(2007).

84. Iannazzi, F., *Proceedings of waste paper 4th Conference*, Supply, Demand, and Future Prices For OWP, Miller Freeman Inc., Sect.18 (1993).

85. Ince, P. and Mckeever, D., *TAPPI International Environmental Conference Proceedings*, TAPPI Press, Atlanta, (1995) pp.315

86. Ishikawa, A., Sugiyama, J., Okano, T., Fine structure and tensile properties of ramie fibers in the crystalline form of cellulose I, II and III, *Wood Research,* 81:16-18(1994).

87. Jackson, L.S., Heitmann, J.A. and Joyce, T.W., Enzymatic modifications of secondary fiber, *Tappi. J.*, 76(3): 147-154(1993).

88. Jeffries, T.W., Klungness, J.H., Sykes, M.S. and Rutledge, C.K.R., Preliminary results of enzyme-enhanced versus conventional deinking of xerographic printed paper, *In: Tappi, 1993 Recycling Symposium Notes,* Tappi Press, Atlanta, USA, (1993) pp.183-188.

89. Jeffries, T.W., Klungness, J.H., Sykes, M.S. and Rutledge, C.K.R., Comparison of enzyme-enhanced with conventional deinking of xerographic and laser printed paper, *Tappi. J.*, 77(4): 173-179(1994).

90. Jeffries, T.W., Sykes, M.S., Rutledge, C.K.R., Klungness, J.H. and Abubakr, S., Enhanced removal of toners from office waste papers by microbial cellulases, In: *The 6th International Conference on Biotechnology and Pulp Paper Industry,* (1995) pp. 141-144.

91. Jeffries, T.W., Sykes, M.S., Rutledge-Cropsey, K., Klungness, J.H. and Abubakr, S., In: *Biotechnology in the Pulp and Paper Industry*: Recent Advances in Applied and

Fundamental Research, Srebotnik, E., Messner, K., (eds.), Facultas-Universitäts Verlag, Vienna, Austria (1996).pp.141-144.

92. Jensen, R.G., Galluzzo, D.R. and Bush, V.J., Selectivity is an important characteristic of lipases (acyl-glycerol hydrolases), *Biocatalysis,* 3:307-316(1990).
93. Jobbins, J.M. and Franks, N.E., Enzymatic deinking of mixed office waste: process condition optimization. *Tappi. J.*, 80 (9): 73-78(1997).
94. Johansson, B. and Strom, G., Surface chemistry of flotation deinking: effect of various chemical conditions on ink agglomerate character and floatability, *Nordic Pulp Paper Research J.,* 13(1):37(1998).
95. Johansson, B. and Strom G., Floatability of model offset ink, *Appita. J.,* 52(1): 37(1999).
96. Johansson, B., Pulping chemistry- effects and adaptation to regional differences in waste paper furnish, Proceedings from the 10th PTS-CTP Deinking Symposium, Munich, Paper no. 13, 23-25 April 2002.
97. Joshi, G. K., Kumar, S., Tripathi, B.N. and Sharma, V., Production of alkaline lipase by *Corynebacterium paurometabolum* MTCC 6841 isolated from lake Naukuchiatal, Uttaranchal State, India, *Current Microbiology*, 52: 354–358(2006).
98. Julien, S. A. F. and Perrin B., The effect of particle size on ink and speck removal efficiency of the deinking steps part l, Paper presented at the 1st Research Forum on Recycling, Toronto, Ontario 1991.
99. Julien, S. A. F. and Perrin B., The effect of particles size on ink and speck removal efficiency of and speck removal efficiency of the deinking step part1: flotation, *Pulp and Paper Canada Journal*, 94(10):25(1993).
100. Julien, S. A. F. and Perrin B., Ink removal by flotation and washing: hydrodynamic and technological aspect, The 3rd advanced training course on deinking technology Course Note, Centre Technique, *du Papier*, (1997).
101. Kanhekar, S.K., Agnihotri, P.K., Sharma, C. and Bansal M.C., Effects of surfactants in flotation deinking of old newsprint, *Ippta. J.,* 17(3):63(2005).
102. Kaya, F., Heitmann Jr. J.A. and Joyce, T. W., Cellulase binding to cellulose fibers in high shear fields, *Journal of biotechnology,* 36:1-10(1994).
103. Kaya, F., Heitmann Jr. J. A. and Joyce, T. W., Influence of surfactants on enzymatic hydrolysis of xylan and cellulose, *Tappi. J.*, 78(10):150(1995).

104. Khalil, H.P.A., Ismail, H., Rozman, H.D. and Ahmad, M.N., The effect of acetylation on interfacial shear strength between plant fiber and various matrices, *Eur. Polym. J.*, 37(5):1037-1045(2001

105. Kibblewhite, R. P. and Clark T. A., Proc. 50th Appita Ann. Gen. Conf., 65(1996).

106. Kim, M.H., Lee, S.B., Ryu, D.D.Y. and Reese, E.T., Surface deactivation of cellulase and its prevention, *Enzyme Microbial. Technol.*, 4: 99-103(1982).

107. Kim, T-J., Ow, S.K. and Eom, T-J., TAPPI Pulping Conference Proceedings, Enzymatic deinking of wastepaper, TAPPI *Press* Atlanta, (1991) pp.1023-1030.

108. Kirk, T. K. and Thomas, W. J., *In: Roles for microbial enzymes in pulp and paper processing,* Enzyme for pulp and paper processing, Published by, *American Chemical Society*, Madison W153705(1996).

109. Klungness, J.H. and Sykes, M.S., Environmentally sound alternatives for upgrading mixed office waste, In: *Proceedings of the 1995 International Environmental Conference, TAPPI Press Atlanta,* (1995) pp.445-448.

110. Klungness, J.H. and Sykes, M.S., Bleachability of recycled fibers deinked with enzyme preparations, *Recycling symposium,* (1996) pp.63-67.

111. Kookurek, M.J., Joint text book committee of paper technology, pulp & paper manufacture, secondary fiber and non wood pulping, TAPPI Press, USA & Canada, 3 (1993) pp.214.

112. Laivins, G. and Scallan, A., The mechanism of hornification of wood pulps, Products of papermaking, *Trans. Fundam. Res. Symp.* Oxf, 2:1235–1260(1993).

113. Larsson, A., Stenius, P. and Odberg, L., Surface chemistry flotation deinking, part 2, the importance of ink particle size, *Svensk Papperstidning,* 87 (18): R1158 (1984).

114. Lassus, A., Deinking chemistry, Recycled fiber and deinking, Göttsching, L. and Pakarinen, H., (eds.), *TAPPI Press,* (2000) pp.24.

115. Learner, T.J.S, The analysis of synthetic resins found in twentieth century paint media, In: *Resins*, Ancient and Modern SSCR conference, SSCR, Edinburgh (1995).

116. Lee. S.B, Kim, K.H., Ryu, J.D. and Taguchi, H., Structural properties of cellulose and cellulase reaction mechanism, *Biotechnol. Bioeng.*, 25: 33-52(1983).

117. Lee, I., Evans, B. R. and Woodward, J., The mechanism of cellulase action on cotton fibers: evidence from atomic force microscopy, *Ultramicroscopy,* 82:213-221(2000).

118. Lee, C. K., Darah, I., Ibrahim, C.O., Enzymatic deinking of laser printed office waste papers: some governing parameters on deinking efficiency, *Biores. Technol.*, 98: 1684-1689(2007).

119. Liphard, M., Schreck, B. and Hornfeck, K., Surface- chemical aspects of filler flotation in waste paper recycling, *Pulp paper Canada J.*, 94(8):27(1993).

120. Liu, R-H., Dai, S.A., Chang, F-J., Cheng, W-T and Shih, Y-F., Investigation on solubility of polymeric binder of xerographic toner and de-inking by emulsion process, *Journal of the Taiwan Institute of Chemical Engineers,* 40: 84-90(2009).

121. Long, F. A. and Richman, D. J., *Am. Chem. Soc.,* 82: 513–519(1960).

122. Lopez Daniel, Colom Jose F., Vidal, T., Pastor. J. and Torres, A. L., Flotation deinking of xerographic- printed-paper: a study of the dispersant, collector and cellulases on handsheet visual appearance, *Appita. J.*, 56(6) 449(2003).

123. Luthfi, H., Enzymatic deinking of pulp from laser print paper, IPB-Bogor Agricultural University-2011, Bogor (Indonesia), URI http://repository.ipb.ac.id/handle/123456789/9531 (2007).

124. Macdonald, J.G. and Nohr, R.S., Method for mutating a colorant by irradiation, US Patent No 5721287(1998).

125. Magnin, L., Delpech, P., and Lantto, R., Potential of enzymatic deinking, In: *Biotechnology in the pulp and paper industry* (Viikari, L. and Lantto, R., (eds.), published by Elsevier Science B.V. (2002) pp.328.

126. Marchildon, L., Lapointe, M. and Chabot, B., The influence of, particulate size in flotation deinking of newsprint, *Pulp and Paper Canada Journal*, 4: 90(1989).

127. Marques, S., Pala, H., Alves, L., Amaral Collaco, M.T., Gama, F.M. and Gírio, F.M., Characterization and application of glycanases secreted by *Aspergillus terreus* CCMI 498 and *Trichoderma viride* CCMI 84 for enzymatic deinking of mixed office wastepaper, *J. Biotechnol.*, 100(3): 209-219(2003).

128. Martin, Y., Williams, C.C. and Wickramasinghe, H.K., Atomic force microscope force mapping and profiling on a sub 100Å scale, *J. Appl. Phys.*, 61(10):4723-4729(1987).

129. Marton, R., Brown, A., Granzow, S., Koeppicus, R. and Tomlinson, S., Recycling and fibre structure, *Prog. Paper. Recycl.*, 3(2): 58-70(1993).

130. Mathur, I., Chelant optimization in deinking formulation, *Pulp and Paper Canada Journal*, 94(10):55(1993).

131. McCool, M.A. and Silveri, L., Removal of specks and non-dispersed ink from a deinking furnish, *Tappi. J.*, 70(11): 75(1987).

132. McCool, M. A., Flotation deinking in secondary fiber recycling, Spangenberg, R.J., (ed.), *TAPPI Press*, Atlanta, (1993) pp.141.

133. McGinnis, G.D. and Shafizadeh, F., In: *Pulp and Paper Chemistry and Chemical Technology*, Casey, J.P., (ed.), vol. 1, 3rd edn., Chap.1, *Wiley-Interscience,* New York (1980).

134. Mika, Körkkö. and Ossi, Laitinen., A report on deinking primer pulping fibre and particle engineering laboratory, University of Oulu (2008).

135. Miller, G.L., Use of dinitrosalicylic acid reagent for determination of reducing sugar, *Anal. Chem.*, 31:426-428(1959).

136. Moore, B., Recovered paper market global commodities, In: *Paper international,* 6-14(2007).

137. Morkbak, A.L. and Zimmermann, W., Deinking of mixed office paper, old newspaper and vegetable oil-based ink printed paper using cellulases, xylanases and lipases, *Prog. Pap. Recycl.*, 7:14-21(1998).

138. Morkbak, A.L. and Zimmermann, W., Applications of enzymes in paper deinking processes, *ACS Symp Ser.*, 687:133–41(1998).

139. Morkbak, A.L. and Zimmermann, W., Deinking of recovered mixed office paper by treatment with a single endoglucanase I from *Humicola insolens* and a neutral surfactant, *Prog. Pap. Recycl.*, 73:33-7(1998).

140. Morkbak, A.L., Degn, P. and Zimmermann, W., Deinking of soybean oil based ink-printed paper with lipases and a neutral surfactant, *J. Biotechnol.*, 67:229-36(1999).

141. Morkbak, A.L., Degn, P. and Zimmermann, W., Deinking of soybean oil based ink-printed paper with lipases and a neutral surfactant, J. Biotechnol., 67: 229-36 (1999).

142. Muller-Hagedorn, M. and Bockhorn, H., Pyrolytic behaviour of different biomasses (angiosperms) (maize plants, straws, and wood) in low temperature pyrolysis, *J. Analyt. App. Pyrolysis,* 79:136-146(2007).

143. N.N., Plastics- Thermogravimetry (TG) of Polymers- General Principles, ISO 11358(1997).
144. N.N., Thermal analysis (TA), Thermogravimetry (TG) - Principles, DIN 51006(2000).
145. N.N., Standard test method for Compositional Analysis by Thermogravimetry, ASTME 1131(2003).
146. Nakamura, K., Hatakeyama, T. and Hatakeyama, H., *Textile Res. J.*, 53:607-613(1981).
147. Nakano, J., Recent research trends in pulping chemistry, *J. Korea. Tappi,* 25: 85-91(1993).
148. Nielsen, J.E. and Borchert, T.V., Protein engineering of bacterial α -amylases, *Biochim. Biophys. Acta.*, 1543:253-74(2000).
149. Nigam, P. and Singh, D., Enzymes and microbial systems involved in starch processing, *Enzyme Microbiol. Technol.*, 17:770–8(1995).
150. Oates, C.G., Towards an understanding of starch granule structure and hydrolysis, *Trend Food Sci. Technol.*, 8:375-382(1997).
151. Oh, J-T., Park, K-N., Park, J-W. and Kim, J-H., Effect of HLB value of surfactant on enzymatic deinking process, *J. Ind. Eng. Chemistry,* 3(1):7-12(1997).
152. Oksanen, Tu., Pere, J., Paavilainen, L., Buchert, J. and Viikari, L., Treatment of recycled kraft pulps with *Trichoderma reesei*, hemicellulases and cellulases, *J. Biotechnol.*,78: 39-48(2000).
153. Olson, C.R. and Marybeth, L.K., Increasing the use of secondary fiber: an overview of deinking chemistry and stickies control, *Appita. J.*, 45(2): 125(1992).
154. Olson, C.R., Richmann, S.K., Sutman, F.J. and Letscher, M.B., Deinking of laser printed stock using chemical densification and forward cleaning, *Tappi. J.*, 76:136-143(1993).
155. Oltus, E., Mato, J., Bauer, S. and Farkas, V., Enzymatic hydrolysis of waste paper, *Cellulose Chem. Technol.*, 21:663-72(1987).
156. Ooshima, H., Sakata, M. and Harano, Y., Enhancement of enzymatic hydrolysis of cellulose by surfactant, *Biotech. Bioeng.*, 28: 1727-1734(1986).
157. Ow, S-K., Park, J-M. and Han, S-H., Effects of enzyme on ink size and distribution during enzymatic deinking process of old newsprint, In: *The 6th International Conference on Biotechnology and Pulp Paper Industry*, (1995) pp.163-168.

158. Paik, K.H. and Park, J.Y., Enzyme deinking of newsprint waste (I) - effect of cellulase and xylanase on brightness, yield and physical properties of deinked pulps, *J. Korean Tappi.*, 25(3): 42-52(1993).

159. Pala, H., Mota, M., Gama, F.M. Enzymatic versus chemical deinking of non-impact ink printed paper, *J. Biotechnol,* 108(1):79-89(2004).

160. Pala, H., Mota, M. and Gama, F.M., Factors influencing MOW deinking: Laboratory scale studies, *Enzyme Microbial. Technol.*, 38: 81-87(2006).

161. Pandey, A. and Nigam, P., Advances in microbial amylases. *Biotechnol. Appl. Biochem.*, 31:135-52(2000).

162. Pandit, N., Garg, M. and Singh S.P., Response of offset news inks to deinking, *Ippta J.*, 13(3):67(2001).

163. Park, J.W., Takahata, Y., Kajiuchi, T. and Akehata, T., Effects of non-ionic surfactant on enzymatic hydrolysis of used newspaper, *Biotechnol. Bioeng.*, 39:117–20(1992).

164. Park, J. and Park, K., Improvement of the physical properties of reprocessed paper by using biological treatment with modified cellulase, *Biores. Technol.*, 79: 91-94(2001).

165. Park, S-B., Lee, J-M., and Eom, T-J., The control of sticky contaminants with enzymes in the recycling of waste paper, *J. Ind. Eng. Chem.*, 10(1): 72-77(2004).

166. Pélach, M.A., Procés de destintatge del paper per flotació, Avaluació de léficácia dèliminació de tinta, Tesis Doctoral, Universitat de Girona, 1998.

167. Pélach, M.A., Puig, J., Vilaseca, F. and Mutjé, P., Influence of chemicals on deinkability of wood-free fully coated paper, *J. Pulp Paper Sci.*, 27 (10): 353-358 (2001).

168. Pèlach, M.A., Pastor, F.J., Puig, J., Vilaseca, F. and Mutje, P., Enzymatic deinking of old newspapers with cellulase. *Process. Biochem,* 38:1063-1067(2003a).

169. Pèlach, M. A., Puig, J., Roux, J-C., Vilaseca, F. and Mutje, P., The use of cationic surfactants to enhance the deinking of coated fine papers, *Appita. J.*, 56(5): 351(2003b).

170. Pere, J., Siika-aho, M., Buchert, J. and Viikari, L., Effects of purified *Trichoderma reesei* cellulases on the fiber properties of Kraft pulp, *Tappi. J.*, 78 (6): (1995).

171. Phoung, D.T. and Nazhad, M.M., Effect of swelling or surface agents on strength of old corrugated containers (OCC), *The 60th Appita annual conference and exhibition, Melbourne, Australia*, (2006).

172. Ping, G.Q., Xue, Y.J., Quiang,Y. and Yuan, Y.S., Enzymatic deinking with lipase, cellulase/ xylanase, *China Pulp and Paper,* 23: 7-9(2004)

173. Pirttinen, E. and Stenius, P., The effects of chemical conditions on newsprint ink detachment and fragmentation, *Tappi, J.,* (2000).

174. Pletka, J., Gosiewska, A., Chee, K.Y., Mcguire, J.P., Drelich, J. and Groleau, L., Interfacial effect of poly alkylene oxide/ fatty acid surfactant blend in flotation deinking of mixed office waste paper, *Prog. Pap. Recycl.,* 9:40-48(2000).

175. Pommier, J.C., Fuentes, J.L. and Goma, G., Using enzymes to improve the process and the product quality in the recycled paper industry, *Tappi. J.,* 72(6): 187-191(1989).

176. Pommier, J.C., Goma, G., Fuentes, J.L., Rousset, C. and Jokinen, O., Using of enzymes to improve the process and the product quality in the recycled paper industry, part 2: industrial applications, *Tappi. J.,* 73:197–202(1990).

177. Prasad, D.Y., Heitmann, J.A. and Joyce, T.W., Enzyme deinking of black and white letterpress printed newsprint waste, *Prog. Pap. Recycl.,* 1(3): 21-30(1992).

178. Prasad, D.Y., Heitmann, J.A. and Joyce, T.W. Enzymatic deinking of coloured offset newsprint, *Nordic Pulp and Paper Research Journal,* 2: 284-286(1993).

179. Prasad, D.Y., Enzymatic deinking of laser and xerographic office wastes, *Appita. J.,* 46: 289-292(1993).

180. Pundir, M., Mahto, S.S., Naithani, A. and Mathur, G., Deinking of waste paper- a case study, *Proceedings of IPPTA Workshop on Deinking,* Chandigarh (2002) pp.193.

181. Putz, H. J., Renner, K. and Göttsching, L., Pulping conference proceedings, TAPPI Press, Atlanta, *TAPPI* (1994) pp.877.

182. Quick, T.H. and Hodgson, K.T., Xerography deinking-a fundamental approach, *Tappi. J.,* 69(3): 102-106(1986).

183. Rheng-Huan, Liu, Shenghong, A. Dai, Feng-Jyi Chang, Wen-Tung Cheng and Yeng-Fong Shih, Investigation on solubility of polymeric binder of xerographic toner and de-inking by emulsion process, *Journal of the Taiwan Institute of Chemical Engineers.,* 40: 84-90(2009).

184. Rosen, M. J., Surfactants and interfacial phenomena, 3rd ed., Wiley, New York, USA (2004) pp.253.

185. Rushing, W., Joyce, T.W. and Heitmann, J.A., *Proceedings of 7th International Symposium on Wood and Pulping Chemistry*, China TAPPI, Beijing (1993) pp.233-238.

186. Rutledge-Cropsey, K., Jeffries, T., Klungness, J. H. and Sykes, M., Preliminary results of effect of sizings on enzyme-enhanced deinking, *TAPPI Recycling Symposium proceedings, TAPPI Press*, Atlanta (1994) pp.103-105.

187. Sanciolo, P., Warnock, H., Harding, I., Forbes, L. and Lonergan, G., Microscopy study: liberation of ink from fibers during enzymatic deinking of mixed office papers, *Prog. Pap. Recycl.*, 9: 22–30(2000).

188. Sarja, T., Measurement, nature and removal of stickies in deinked pulp, University of Oulu, Oulu (Finland) 2007, C 275 ISBN 978-951-42-8463-2, ISSN 0355-3213

189. Scot, W. E. and Gerber, P., Using ultrasound to deink xerographic waste, *Tappi J.*, 77(3): 125-130(1995).

190. Scrap Specification Circular, Institute of scrap recycling industries, Inc. Washington, DC 20005-3104(2005).

191. Sengupta, D.K. and Rao, R.B., Deinking of Indian newspaper by agglomerate floatation, *IE(I) Journal- CH*, 85: 36(2004)

192. Serres, A., Method for de-inking paper pulp from recycled paper, Assigned to Lamort Fils, US 6197153) March 6(2001).

193. Shrinath, A., Szewczak, J.T. and Bowen, L.J., A review of ink-removal techniques in current deinking technology, *Tappi. J.*, 85-93(1991).

194. Singh S., Dutt, D., Tyagi, C. H. and Upadhyaya, J. S., Bio-conventional bleaching of wheat straw soda-AQ pulp with crude xylanases from SH-1 NTCC-1163 and SH-2 NTCC-1164 strains of *Coprinellus disseminatus* to mitigate AOX generation, *New Biotechnology.*, 28(1): 47-57(2011).

195. Skaya, E.G., Usta, M. and Kirci, H. S., The effects of various pulping conditions on crystalline structure of cellulose in cotton linters, *Polymer Degradation and Stability*, 81:559–564(2003).

196. Somasundaran, P., Zhang, L., Krishna, K.S. and Slepetys, R., Floatation deinking- a review of the principles and techniques, *Prog. Pap. Recycl.*, 8(3): 22-36(1999).

197. Soni, R., Najir, A., Chadha, B.S. and Saini, H.S., Novel source of fungal cellulase for efficient deinking of composite paper wastes, *BioResources*, 3: 234-246(2008).

198. Sreenath, H.K., Yang, V.W., Burdsall, H. and Jeffries, T.W. Toner removal by alkaline active cellulases from desert Basidiomycetes, In: *Enzymes for pulp and paper processing, American Chemical Society,* 207-19(1996).

199. Sridhar, P. Sundaram, T.S. and Bhargrava, S.C., Flotation deinking process, Proceedings of IPPTA workshop on deinking, Chandigarh (2002) pp.169.

200. Stork, G., Pereira, H., Wood, T., Düsterhöft, E. M., Toft, A. and Puls, J., Upgrading recycled pulps using enzymatic treatment, *Tappi. J.*, 78(2): 79-88(1995).

201. Suurnäkki, A., Tenkanen, M., Buchert, J. and Viikari, L., Hemicellulases in the bleaching of chemical pulps, *Adv. Biochem. eng. Biotechnol.*, 57:261-287(1997).

202. Suurnäkki, A., Tenkanen, M., Siikaaho, M., NikuPaavola, M.L.,Viikari, L. and Buchert, J., *Trichoderma reesei* cellulases and their core domains in the hydrolysis and modification of chemical pulp, *Cellulose,* 7(2):189-209(2000).

203. Sykes, M., Klungness, J., Jeffries, T., Cropsey, K. and Abubakr, S., Enzymatic deinking of sorted office waste : Recommendation for scale-up, Recycling Symposium Proceedings, TAPPI Press, Atlanta, TAPPI (1995) pp.61.

204. Sykes, M., Klungness, J., Tan, F. and Abubakr, S., Environmentally sound alternatives for upgrading mixed office waste, *Tappi International Environmental Conference Proceedings,* (1995).

205. Sykes, S. M. and Klungness, J. H., Enzymatic removal of stickies contaminants, *Tappi Pulping Conference,* (1997)687 -691

206. Taleb, M.C and Maximino, M.G., Effect of enzymatic treatment on cellulosic fibers from recycled papers, analysis using a response curves experimental design, *Appita. J.,* 60: 296-300(2007).

207. Tandon, R., Thakur., V.T., Mathur, R.M. and Kulkarni, A.G., Effect of multi-component enzyme in deinking of mixed office waste, Proceedings of 59th APPITA annual conference and exhibition, International symposium on wood fiber and pulping chemistry held on 16-19th May, 2005, Auckaland, New Zealand, (2005) pp.383-389.

208. Tandon, R., Thakur, V.V., Chisty Mohamed, H., Mathur, R.M. and Kulkarni, A.G., Enzymatic deinking an alternative technology for quality upgradation of mix office waste, *Ippta J.*, 17(3): 51-55(2005b).

209. Training manual on COD analysis, E. Merck (I) Ltd., Mumbai 400022(352).

210. Trip, V.W., In: *Cellulose and cellulose derivatives*, measurement of crystallinity, Bikales, X. and Segal, L., (eds.), New York: *Wiley Interscience*, (1971) pp.305-23.

211. Turvey, R.W., Why do fibers float? Proceeding 1st Research Forum on Recycling, CPPA, Toronto, (1991) pp.123.

212. Verma, D., Katti, K. and Katti, D., Photoacoustic FTIR spectroscopic study of undisturbed nacre from red abalone, *Spectrochimica acta Part A Molecular and bimolecular spectroscopy*, *64*(4), 1051-1057. Retrieved from http://linkinghub.elsevier.com/retrieve/pii/S1386142505005111

213. Viesturs, U. and Leite, M., Pulp and wastepaper bleaching using xylanases and other enzymatic complexes, In: *Latvian State Institute of Wood Chemistry Yearbook*, (1999) pp. 49-50.

214. Viikari, L., Suurnäkki, A. and Büchert, J., In: *Enzymes for pulp and paper processing*, Enzyme-aided bleaching of kraft pulp: fundamental mechanisms and practical applications, *ACS symposium Series*, 655(2): (1996) pp15-24.

215. Vyas, S. and Lachke, A., Biodeinking of mixed office waste papers by alkaline active cellulases from alkalotolerant *Fusarium Sp, Enzyme Microbial. Technol.*, 2: 236-245(2003).

216. Vyas, S., Pradhan, S. D., Pavaskar, N. R. and Lachke, A., Differential thermal and thermo-gravimetric analyses of bound water content in cellulosic substrates and its significance during cellulose hydrolysis by alkaline active fungal cellulases, *Applied Biochem. Biotechnol.*, 118:177-188(2004).

217. Walmsley, M., Yu, C.J. and Silveri, L., Effect of ink specks on brightness of recycled paper, Proceedings of the TAPPI Recycling Symposium, TAPPI Press, Atlanta, (1993) pp.417-441.

218. Welt, T. and Dinus, R.J., Enzymatic deinking- a review, *IPST Technical Paper Series* Number 542 (1994)

219. Welt, T. and Dinus, R.J., Enzymatic deinking - a review, *Prog. Pap. Recycl.*, 4(2): 36-47(1995).

220. Widjanarko, S.B., Nugroho, A. and Estiasih, T., Functional interaction components of protein isolates and glucomannan in food bars by FTIR and SEM studies, *African Journal of Food Science*, 5(1): 12-21(2011).

221. Widsten, P. and Kandelbauer, A., Laccase applications in the forest products industry: a review, *Enzyme Microbial. Technol.*, 42(4):293-307(2008).

222. Wielen, L.C.V., Panek, J.C. and Pfromm, P.H., Fracture of toner due to paper swelling. *Tappi. J.*, 82(5):115-21(1999).

223. Wong, K.K.Y., Tan, L.U.L. and Saddler, J.N., Multiplicity of β-1, 4-xylanases in microorganisms: functions and applications, *Microbiol. Rev.*, 52:305-317(1988).

224. Woodward, J., Stephan, L.M., Koran, L.J., Wong, K.K.Y. and Saddler, J.N., Enzymatic separation of high quality un-inked pulp fibers from recycled newspaper, *Biotechnology*, 12(9): 905-908(1994).

225. WTW instruction manual for photometer MPM 2010 and thermo-reactor CR 2010, Wissenschaftlich-Technische Wekstatten, D-82362, Weilheim, Gmbh.(378)

226. Xia, Z., Beaudry, A.R. and Bourbonnais, R., Effect of cellulase on the surfactant assisted acid deinking of ONP and OMG, *Prog. Pap. Recycl.*, 5:46-57(1996).

227. Xu, Q., Fu, Y., Qin, M. and Qiu, H., Surface properties of old news print laccase-violuric acid system deinked pulp, *Appita. J.*, 60: 372-377(2007).

228. Xu, Q., Fu, Y., Gao, Y. and Qin, M., Performance and efficiency of old newspaper deinking by combining cellulase/hemicellulase with laccase-violuric acid system, *Waste Management*, 29:1486-1490(2009).

229. Yamada, N., Okano, T., Sakai, H., Karikusa, F., Sawasaki, Y. and Sakurai, Y., *Makromol. Chem. Rapid. Commun.*, 11: 571(1990).

230. Yang, H., Yan, R., Chen, H., Lee, D.H. and Zheng, C., Characteristics of hemicelluloses, cellulose and lignin pyrolysis, *Fuel*, 86(12-13):1781-1788 (2007).

231. Zeyer, C., Joyce, T.W., Rucker, J.W. and Heitmann, J.A., Enzymatic deinking of cellulose fabrics: a model study for enzymatic paper deinking, *Prog. Pap. Recycl.*, 3(1):36-44 (1993).

232. Zeyer, C., Joyce, T.W., Heitmann, J.A. and Rucker, J.W., Factors influencing enzyme deinking of recycled fiber, *Tappi. J.*, 77: 169-177(1994).

233. Zhang, X., Renaud, S. and Paice, M., Cellulase deinking of fresh and aged recycled newsprint/magazines (ONP/OMG), *Enzyme Microbial. Technol.*, 43:103-108(2008).

234. Zhao, Y., Deng, Y. and Zhu, J.Y., Roles of surfactants in flotation deinking, *Prog. Pap. Recycl.*, 14(1): 41 (2004).

235. Zhao, X., Heide E. van der., Zhang, T. and Liu, D., Delignification of sugarcane bagasse with alkali and per-acetic acid and characterization of pulp, *BioResources,* 5(3):1565-1580(2010)

236. Zhenying, S., Shijin, D., Xuejun, C., Yan, G., Junfeng, L., Hongyan, W. and Zhang, Sean X., Combined de-inking technology applied on laser printed paper, *Chemical Engineering and Processing.,* 48: 587–591(2009).

237. Zhong, Q., Innis, D., Kjoller, K. and Elings, V.B., Fractured polymer silica fiber surface studied by tapping mode atomic force microscopy, *Surf. Sci.,* 290(12): L688-L692 (1993).

238. Zobel, H.F., Molecules to granules: A comprehensive starch review, *Starch/Stärke.*, 40: 44-50(1988).

239. Zollner, H.K., Schroeder, L.R., Enzymatic deinking of nonimpact printed white office paper with α-amylase, *Tappi J.,* 81(3):166-70(1998).

CHAPTER 5

CONCLUSIONS AND SUGGESTIONS

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5.1 CONCLUSIONS

From the present study, with regard to their unexploited potential in the pulp and paper industry and applying environmentally benign deinking technologies following the use of crude cellulase from A. *flavus* AT-2 A. *niger* AT-3 and crude xylanase from white rot fungus *Coprinus cinereus* AT-1 , following are the major conclusions drawn:

1. In order to find out a potential strain for bio-conversion, 12 microbial strains (fungal) were isolated from the decomposing cellulosic waste materials. Among these strains, two fungal strain AT-2 and AT-3 were finally selected based on its ability of maximum cellulase production. These strains were identified as A*spergillus flavus* and A*spergillus niger* from Indian Agricultural Research Institute, New Delhi and designated as A*spergillus flavus* AT-2 and *Aspergillus niger* AT-3 for further study.
2. Based on morphological and microscopic investigation, it was found that the two strains differed with respect to their colony colour, conidia type uniseriate in *A. flavus AT-2* while biseriate in *A. niger* AT-3 while both had a white coloured mycelium, thin walled in *A. flavus AT-2* and thick walled in *A. niger* AT-3 and conidiogenesis was copious found in both the test isolates which is a characteristic feature of all the members of ascomycotina group.
3. Among the five different commercially available ligninocellulosic substrates, since CMC was less crystalline, more amorphous in nature therefore, it was more susceptible to enzymatic attack on its surface as compared to other substrates.
4. The XRD analysis indicates that rice straw among five different naturally available ligninocellulosic substrates shows a significant increase in crystallinity due to sufficient removal of lignin and other hemicellulosic contents thus offering an increase in the amorphosity in cellulose regions and was highly susceptible to enzyme attack which resulted into its bioconversion to reducing sugars.

5. SEM studies indicates that untreated rice straw had compact and rigid structure compared to the treated rice straw, which was loose and fibrous. XRD studies and further SEM analysis valorized rice straw (pretreated) as the carbon substrate to be used for further enzyme (cellulases) optimization studies.
6. The CMC_{ase} activities of *A. flavus* AT-2 and *A. niger* AT-3 under SSF conditions were 42.69 and 40.08 % respectively higher than that of SmF conditions. In the same way, FP_{ase} respectively of *A. flavus* AT-2 and *A. niger* AT-3 increased by 44.00 and 45.86 % respectively in SSF compared to SmF. Therefore, SSF was selected for further investigations.
7. A fungal inoculum dose of 5%, incubation period 5 days, temperature and pH 30 ^{0}C and 4.8 for *A. flavus* AT-2 and 35 ^{0}C and 5.3 for *A. niger* AT-3 respectively, solid substrate: moisture content 1:3, rice straw (treated) as the carbon source, $(NH_4)_2 SO_4$ + yeast extract as the nitrogen source, KH_2PO_4 as the phosphorous source, succinic acid as the source of organic acid, Tween-80 as the surfactant and cellobiose as the source of soluble sugar might be taken as optimum parameters for cellulase production by both the fungal strains
8. The optimum CMC_{ase} (17.24 IU/mL), FP_{ase} (1.92 IU/mL), β-glucosidase (0.69 IU/mL) and xylanase activities (5.73 IU/mL) and fungal protein concentration (2.90 mg/L) for *A. flavus* AT-2 and CMC_{ase} (24.32 IU/mL), FP_{ase} (2.47 IU/mL), β-glucosidase (0.82 IU/mL) and xylanase activities (4.80 IU/mL) and protein concentration (3.07 mg/L) for *A. niger* AT-3 were observed under optimum SSF conditions.
9. *A. flavus* AT-2 and *A. niger* AT-3 were active in the pH range of 4.0 to 7.5 and maximum cellulase activity was obtained at pH 5.0 for *A. flavus* AT-2 (17.31IU/mL) and at pH 5.5 for *A. niger* AT-3 (24.50 IU/mL). Cellulase activities of *A. flavus* AT-2 and *A. niger* AT-3 started decreasing above buffer pH 5.0 and 5.5 respectively. It was observed that at neutral pH (7.0), *A. flavus* AT-2 retained about 54% of its optimum cellulase activity while *A. niger* AT-3 retained only 65% of its optimum cellulase activity. At pH 4.0, *A. flavus* AT-2 retained 84% and *A. niger* AT-3 retained 90% of its activity compared to optimum. The cellulase produced by *A. niger* AT-3 maintained its high stability (more than 60% of its optimum cellulase activity) over a

pH range of 4.0 to 7.0. Therefore, cellulase produced by strain *A. niger* AT-3 was less acidic compared to *A. flavus* AT-2.

10. The optimum temperature for the crude cellulase activities of both the fungal strains (*A. flavus* AT-2, IU/mL and *A. niger* AT-3, IU/mL) were 50 ^{0}C and beyond that cellulase activities were found to decrease. At temperature 60 ^{0}C, cellulase produced from *A. flavus* AT-2 retained about 51% of its cellulase activity while that of *A. niger* AT-3 exhibited about 64% of its cellulase activity compared to that at optimum temperature i.e. 50 ^{0}C. When assayed at higher temperatures (70 and 80 ^{0}C), both of the strains lost a large amount of their cellulase activities; *A. flavus* AT-2 could maintain 19 and 4% while, *A. niger* AT-3 retained only 29 and 7% of its cellulase activities respectively, as compared to optimum cellulase activity at 50 ^{0}C. *A. niger* AT-3 was found to be slightly more thermo-tolerant as it showed more tolerance to higher temperature as compared to *A. flavus* AT-2.
11. SDS PAGE analysis and Zymogram indicates that of molecular weight of cellulase produced by *A. niger* AT-3 was 29 kDa.
12. Among twelve fungal strains isolated from different lignocellulosic sources, nine isolates have been selected as xylanase producers on the basis of clear zone-formation on xylan-agar (XA) plates. These strains have distinct morphological features, color appearance and growth patterns. Finally, one strains i.e. AT-1 has been selected, resulting into maximum xylanase along with minimum cellulase production and were identified initially wild mushroom from IARI, New Delhi and finally identification was done up to species level on the basis of ITS sequence analysis from IMTECH, Chandigarh (India), and acknowledged as to be the strain of *Coprinus cinereus*.
13. The basidiospores produced by C. *cinereus* AT-1 were club-shaped that is a characteristic feature of all the members of basidiomycotina group. In natural habitat a cap is present in wild mushroom, initially white in colour but soon begins to turn grayish brown, with a brownish center. It was easily recognized as a wild mushroom by its cap shape and grayish black gills.
14. The xylanase activity of strain *C. cinereus* AT-1 under SSF conditions was 54.55 % higher than that of SmF conditions. Therefore, SSF was selected for further investigations.

15. The optimum physico-chemical parameters for crude xylanase production by *C. cinereus* AT-1 were: incubation period 7 days, incubation temperature 37 ^{0}C, pH 6.4, solid substrate to moisture ratio 1:3, carbon source wheat bran and nitrogen source yeast extract. The xylanase, cellulase and laccase activities of *C. cinereus* AT-1 at optimum conditions were 698.75 IU/mL, 1.01 IU/mL and 25.6 IU/mL respectively while protein biomass was 5.7 mg/mL.
16. The crude xylanase produced by *C. cinereus* AT-1 was active in the pH range of 6.0 to 9.0 with the maximum xylanase activity at pH 6.4 (699.60 IU/mL). At pH 6.0, 67% of the maximal activity was maintained. At neutral pH (7.0), xylanase retained about 64% of its optimum activity while at a pH of 7.5, 56% of the optimum activity was retained. Even at pH 8.0, the enzyme could retain 30% of its optimum activity which showed its alkali-tolerant nature.
17. The optimum temperature for the crude xylanase activity of the fungal strain was 55 ^{0}C (702.00 IU/mL). At a temperature of 65 ^{0}C, xylanase from *C. cinereus* AT-1 retained about 74% of its optimum xylanase activity. When assayed at higher temperatures i.e. 75 and 85 ^{0}C, the xylanase lost most of its activity by retaining just 17 and 11% of its optimum activity respectively.
18. Two bands showing xylanolytic activity were detected as the hydrolysis zones in the zymogram. The molecular weights of these proteins were found to be about 50 kDa and 88kDa respectively for fungal strain *C. cinereus* AT-1.
19. It was observed that out of three distinct addition points of enzyme and surfactant, excellent results were obtained in case when enzyme and surfactant were added after pulping.
20. Enzymatic deinking of SOP indicates that maximum deinking efficiency in terms of both visible dirt counts and effective residual ink contents was achieved when enzymes were used along with surfactant and surfactant addition was also found to improve pulp brightness and dirt removal.
21. Maximum brightness and minimum ERIC values were obtained at a surfactant dose of 0.2%, pulping time of 20 min, temperature of 60 ^{0}C and consistency of 12% during pulping of SOP. Deinkability factors based ERIC values and brightness i.e. D_E and

D_B showed minimum regression errors up to 3rd order of polynomial regression analysis and passed the normality test and constant variance test.

22. An enzyme dose of 6IU/mL at a reaction time of 60 min was found optimum for maximum gain in brightness and decrease in dirt counts and ERIC values during enzymatic treatment. Similarly, D_E and D_B showed minimum regression errors up to 3rd order of polynomial regression analysis and passed the normality test and constant variance test.
23. A flotation time of 10 min during deinking of SOP was found optimum for improving pulp brightness and reducing dirt counts and ERIC values.
24. A concoction of cellulase, xylanase, amylase and lipase at a dosing of 6, 3, 1.5 and 6 IU/mL respectively increased the pulp brightness, D_B and D_E by 13.3, 37.79 and 83.00% respectively compared to control and 2.58, 7.10 and 0.7% respectively compared to the concoction of cellulase, xylanase and amylase in the ratio of 6, 3 and 1.5 IU/mL. The introduction of cellulase (alone) treatment improved all the mechanical strength properties with freeness level and pulp viscosity except double fold numbers compared to control. This mixture (cellulase, xylanase, amylase and lipase) showed an improvement in tear index where all other properties showed a declining trend and on contrary to that total solids, COD and BOD increased compared to control as well as the concoction of cellulase, xylanase and amylase (6, 3 and 1.5 IU/mL).
25. In chemi-enzymatic deinking trials, the dosages of chemicals were reduced to from 100% to 75, 50 and 0% while keeping the dosage of cellulase constant. The pulp brightness, D_B, and D_E increased with increasing chemical dosing and conversely, dirt count and ERIC value decreased accordingly during chemi-enzymatic deinking compared to enzymatic deinking. Similarly, all the strength properties mitigated as a result of increasing chemical dosing and total solids, COD and BOD of combined effluent increased in chemi-enzymatic deinking compared to enzymatic deinking.
26. Mechanical strength properties like burst index, tensile index and double fold number decreased as a result of repeated recycling whereas tear index increased up to 3rd recycling with further recycling (4th) produced an insignificant increase in tear index. Bulk and opacity increased up to 3rd recycling and then remained almost constant. In

case of enzymatic deinked pulp, strength properties follow the same pattern but they were inferior compared to chemical deinking.

27. Cellulase alone improved the stickies removal by 4% as compared to control. Further, xylanase and amylase introduced in the mixture did not bring a significant improvement (0.06 and 0.23% respectively) in stickies removal. Lipase addition was also significant in improving the stickies removal.
28. Ink particles size in terms of number of specks and number of specks/cm^3 in chemical and enzymatic deinking processes reduced in the following order: chemical>cellulase>cellulase+xylanase>cellulase+xylanase+amylanase>cellulase+xylanase + amylase + lipase.
29. AFM and SEM studies indicate various morphological changes in fiber surface brought about during conventional, enzymatic and chemi-enzymatic deinking trials. The surface roughness increased in the following descending order of enzymatic concoction: control< cellulase< cellulase+xylanase< cellulase xylanase+amylase< cellulase xylanase+amylase. The introduction of lipase to the mixture of cellulase, xylanase and amylase caused surface roughness increased by 159% compared to control.
30. Based on FTIR studies of SOP pulp it was concluded that the appearance of band at 875 cm^{-1}. The disappearance of this band at 875.82 cm^{-1} showed the dissolution of xylan by crude enzymes during enzymatic deinking and by peeling reactions during chemical deinking. The four sharp absorptions at 2524 cm^{-1}, 1797 cm^{-1}, 875 cm^{-1} and 712 cm^{-1} and the strong but broad absorption between 1400 cm^{-1} and 1500 cm^{-1} were characteristic of chalk ($CaCO_3$), used as a filler in the paper. The peak cantered at 1770-1800 cm^{-1} corresponded to vinyl ester group, which came under strong intensity region. The band at a wavelength of 1797.80 cm^{-1} was observed in FTIR spectrum of SOP (control) which disappeared in chemical and enzymatic deinked pulp.
31. XRD analysis of deinked pulp showed that the crystalline behaviour increased with enzyme concoction and found to be maximum in case of deinking process having cellulase + xylanase + amylase + lipase. There was an insignificant increase in crystallinity of pulps with increasing chemical dosing i.e. from 41.3 to 42% in chemi-enzymatic deinking process.

32. TGA analysis indicated the weight loss in SOP at a temperature between 27-100 ^{0}C was 5.40% and weight losses decreased with increasing crystallinity of deinked pulps in the following order: chemical deinked pulp>cellulase deinked pulp> cellulase+chemical (50%)>cellulase+xylanase deinked pulp>cellulase+chemical (100%) deinked pulp.
33. In case of enzymatic and chemi-enzymatic deinking the weight losses decreased in the following order: cellulase>cellulase+xylanase>cellulase+chemical (50%)>cellulase+ chemical (100%). At 500 ^{0}C, SOP, chemically and cellulase deinked pulps showed a residual mass of 11.86, 2.10 and 0.11% respectively
34. The fist DTG peak temperatures observed for SOP, cellulase, chemical, cellulase+xylanase, cellulase+chemical (100%) and cellulase+chemical (50%) deinked pulps were 344, 343, 342, 344, 341 and 345 ^{0}C and their degradation rate per min was 1.83, 2.29, 1.73, 1.16, 0.95 and 0.17 mg/min respectively. Whereas, second DTG peaks were observed at 476, 482, 475, 477, 450 and 496 ^{0}C with degradation rate per min as 0.11. 0.15, 0.13, 0.09, 0.08 and 0.17 mg/min respectively for the above mentioned pulps.

Realistic cost estimate and improvement in process economics are the key factors for the commercial success of any technology. The enzyme production was designed in a way so as to keep the process as cost effective as possible, like cheap lignocellulosic substrates were used for enzyme production under SSF and crude cellulase and xylanase were used in enzymatic deinking process. Still it must be clearly understood that no enzyme based process for deinking can be inexpensive as using different chemicals during conventional deinking. Waste paper recycling by the use of enzymes must be viewed in terms of their accrued direct benefits in terms of saving tress, biodiversity and indirect benefits like prevention of environmental derangement and reduced health hazards to mankind. At the same time, cellulase and xylanase treatment can easily be applied to any traditional or major process changes.

5.2 Future perspectives

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

1. Microbes exploration and isolation in natural habitats, such as geothermal sites, alkaline soda lakes for enzymes that can function at high temperatures and in industrial environments.
2. Test strains (A. *flavus* AT-2, A. *niger* AT-3 and C. *cinereus*) may be genetically modified for making their enzyme preparation more thermo and alkali stable to be used in various pulp and paper making process. Different approaches such as cloning and over expression of cellulase genes, mutagenesis, however these techniques have limitations such as complexity, use of hazardous chemical agents and scale up difficulties respectively.
3. The following strategies can be applied for reducing the cellulase production cost by: strain improvement methods, isolating novel and overproducing strains, using mixed cultures or co cultures of organisms, using fed batch system and applying recent trends in solid state fermentation. This will make the fermentation process more cost effective and commercially viable
4. Further work on purification and characterization of cellulase and xylanase obtained from the test strains by various chromatography techniques is suggested for a better understanding of their complex enzyme system and function of individual component of enzyme during enzymatic deinking mechanism.
5. Further research in this area demands studies on the kinetic parameters of enzymes systems; scale up of immobilization protocols and their applications in various industrial processes.
6. By genetic engineering and recombinant DNA technology it is also possible to transfer cellulase genes(from *Aspergillus*) in to other host like *Saccharomyces cerevisiae* to produce "Super strains" , capable of hydrolyzing cellulose and xylan along with fermentation of glucose and xylose into ethanol

7. Since relatively high yield losses are associated with washing, reducing yield loss would appear to be a valuable area of research.
8. More work is needed with different type of ink and the disposal of the foam generated from environmental consideration.
9. Removal of stickies and other contaminants which affect th equality of paper must be considered in deinking process.
10. Realistic cost estimate for enzymatic deinking studies should be made for better evaluation of the process economics.

RESEARCH PUBLICATIONS

1. Dutt, D., Upadhyaya, J.S., Tyagi, C.H. and Kumar, A. and Siddhartha, Alkoxygen and alkoxygen-AQ delignification of *Ipomea carnea* and *Cannabis sativa,* ***Indian Journal of Chemical Technology*****, 48** (6): 523-528 (2009)

2. Dutt, D., Tyagi, C. H., Agnihotri, S., Kumar, A. and Siddhartha, Bio-soda pulping of lignocellulosic residues of palma rosa grass: An attempt towards energy conversion, ***Indian Journal of Chemical Technology*****, 17** (1): 60-70 (2010).

3. Agnihotri, S., Dutt, D., Tyagi, C.H., Kumar, A. and Upadhyaya, J.S., Production and biochemical characterization of a novel cellulase-poor alkali-thermo-tolerant xylanase from *Coprinellus disseminatus* SW-1 NTCC 1165, ***World J Microbiol Biotechnol*****, 26,** 1349-1359(2010).

4. Dharm Dutt, C. H. Tyagi Alok Kumar and Amit Kumar, Envirinmentally friendly and cost-effective methods for the manufacturing absorbent grade paper, Communicated to Bioresource Technology.

5. Mohan Lal, Dharm Dutt, C. H. Tyagi and Alok Kumar, FEQP bleaching of *Anthocephalus cadamba* kraft-AQ pulp and its effect on pulp properties and bleach effluent characteristics, Communicated to Bioresource Technology.

6. Shalini Singh, Dharm Dutt, C. H. Tyagi, Alok Kumar, Mitigation of adsorbable organic halides in combined effluents of wheat straw soda-AQ pulp bleached with cellulase-poor crude xylanases from novel strains of *Coprinellus disseminatus* in elemental chlorine free bleaching sequences, Communicated to Bioprocess and Biosystems Engineering