ENZYMATIC DEINKING OF PHOTOCOPIER WASTE PAPERS

Ph.D. THESIS

by

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A THESIS

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DOCTOR OF PHILOSOPHY

by

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Affectionately Dedicated

to

My Parents

&

My Uncle

Late Mr. Vijay Kumar

Pathak

ABSTRACT

In response to increasing environmental awareness and regulatory pressures, the paper industry expects to recover and recycle all major grades of paper. Due to inadequate availability of indigenous waste papers, Indian mills rely heavily on imported waste paper to meet the raw material demand of recycled fibres. To minimize the import of costly papers, relatively little attention has been given to develop novel methods for the recycling of indigenous waste papers such as photocopier and laser printed papers having good quality chemical pulp but hard to deink toner ink. Toner ink contains styrene and acrylate in its chemical composition and gets thermally fused with the fibre surface. Conventional chemical treatment is not effective to detach this ink from the fibre surface. Therefore, it is important to induce suitable methods, which can increase the deinking efficiency for efficient recycling of this new source of waste papers to manufacture paper products.

Consequently, the present research work aims to develop an effective, ecofriendly enzymatic deinking process for photocopier waste papers. In this regard, mainly cellulase and xylanase enzymes have been reported for the efficient ink dislodgement from the fibre surface by peeling off mechanism. The enzymatic treatment has been reported to improve as well as deteriorate the strength properties with enhanced drainage of recycled fibres. Therefore, there is an intense need to find the suitable enzyme preparations, which are able to improve the deinking efficiency (DE) and drainage, without affecting the strength properties. For this reason, this study aims to produce crude enzyme preparations, containing high activity for cellulase and xylanase enzymes from the isolated fungi and subsequently, to investigate their potential to deink photocopier waste papers in comparison to commercial enzyme and conventional chemicals. This research work has been distributed into five chapters.

- ✓ The first chapter deals with the introductory literature review of global and Indian scenario of waste papers utilization and enzyme industry. This part also covers basic information and literature review about the fungi, enzymes, fermentation and deinking.
- ✓ The second chapter describes the materials and methodology for the enzyme production and deinking experiments. Different environmental factors (Inoculum size, incubation days, incubation temperature, initial pH of the fermentation medium, particle size of the selected LCW) and nutritional factors (Carbon sources: different LCW and their combinations, nitrogen sources: different doses of soluble inorganic, soluble complex organic and insoluble plant based, surfactants) were analyzed for enzyme production by both the fungal isolates individually. Various additives like sugars, vitamins, amino acids, metal ions and chelators were evaluated. A wide range of pH and temperature values was used for the determination of their optima for the enzymatic activity. Crude enzyme

supernatant was pre-incubated at optimum pH and temperature for various times to determine the pH and thermal stability, respectively. Different parameters for deinking of photocopier waste papers were optimized using chemicals, commercial enzyme and crude enzyme of the isolated fungal strains. The deinking performance of the crude enzymes (from the isolated fungal strains) were compared with chemical and enzyme treated pulp/handsheets in terms of deinking efficiency, residual ink, yield, freeness, drainage time, optical (brightness and opacity) strength properties (tensile, burst, tear and folding endurance) dirt count and effluent load using standard tests methods. Characterization of the pulps has been done using different techniques like Scanning electron microscope (SEM), Fourier transform infrared spectroscope (FT-IR) and X-ray diffractometer (XRD).

- ✓ In the third chapter, the efforts have been made to find the optimum fermentation conditions for the enhanced production of industrially important cellulase and xylanase by the two newly isolated fungal strains *i.e. Trichoderma harzianum* PPDDN-10 NFCCI-2925 and *Coprinopsis cinerea* PPHRI-4 NFCCI-3027. This study also supports that solid-state fermentation (SSF) process is suitable for these lignocellulosic enzyme production. In the present study, *T. harzianum* and *C. cinerea* both are reported to utilize effectively cheap wheat bran (WB) as carbon source, which stimulate the production of both cellulase and xylanase. The combinations of the WB with other lignocellulosic wastes (LCWs) did not improve enzyme production. *T. harzianum* is able to produce the maximum enzyme production using ferrous ammonium sulphate (as nitrogen source) in just 4 d at 34 °C. For *C. cinerea,* mycological peptone is used as nitrogen source to produce the cellulase and xylanase enzyme in 8 d at 34 °C. The particle size of the WB has shown positive as well as negative effect on the enzyme production, but $WB_{as\ such}$ (with mix particle sizes) has shown the comparable results with the optimum particle size. Alkali pretreatment of WB has resulted in the reduced enzyme production. The addition of sugars except cellobiose acts as repressor towards enzyme production for both the fungi. The additives (vitamins, amino acids, metal ions and chelators) have influenced the enzyme production significantly. Characterization of the crude enzyme of both the fungi shows the maximum activities at optimum pH 5.5 and temperature 55–60 °C with good stability (even up to 6 h).
- ✓ Fourth chapter covers the deinking studies of photocopier waste papers. This study deals with the deinkability potential as well as pulp quality evaluation of photocopier waste paper using conventional chemicals, commercial enzyme (cellulase) and lab produced crude enzyme preparation containing cellulase and xylanase from isolated fungal strains. The chemical deinking is not able to improve DE beyond 75.9%. Different operational parameters are optimized such as point of enzyme addition, enzyme dose,

pulp consistency and reaction time to achieve the maximum possible deinking efficiency without affecting the paper strength properties. The enzymatic deinking experiments are performed in acidic range because of enzyme's optimum pH. Commercial cellulase and lab-produced enzymes have shown the maximum DE of around 93.9-95.2% under different optimum conditions. The addition of these enzymes at hydrapulping stage at medium pulp consistency is found to improve the DE. It is observed that lab produced crude enzymes are responsible for the increase in the brightness. The results have indicated that the enzymes have the potential to maintain or improve the strength properties by promoting the fibrillation, if they are used in optimized conditions of dose and time. The higher dose and longer reaction time than the optimum value found to be detrimental to the fibre surface.

Commercial cellulase and crude enzymes from the isolated fungal strains have improved ink removal efficiency by 23.6 to 25.4% and freeness by 19.6 to 21.6%, with the reduction in drainage time by 11.5 to 17.3% than chemical deinking. The strength properties like tensile and burst indices are observed to improve by 2.7 to 6.7% and 13.4 to 23.9%, respectively. The folding endurance is also 10.3 to 15.9% higher with respect to chemical treatment.The tear index is reduced by 7.6 to 21.9% in all the enzymatic treatment. The ISO brightness is improved by 3.2 to 5.2% when using crude cellulase and xylanase of the isolated fungal strains, while commercial enzyme reduces 2.1% ISO brightness than the chemical deinking.The results of deinking efficiency and freeness are comparable with commercial cellulase, but the strength properties significantly improved. The ISO brightness of crude cellulase and xylanase treated pulps are higher than the commerical cellulase treated. BOD/COD ratios of effluent from enzymatic deinking with crude enzymes (0.59 and 0.55) have been observed to be higher than that of chemical deinking (0.45) but almost equal to commercial cellulase (0.57).

Characterization of the enzymatic deinked pulps has shown better fibrillation than chemical treated (SEM analysis) thus exposing more hydroxyl groups (FT-IR analysis). The crystallinity indices are found to be higher for the enzymatic treated pulp than chemical treated pulps (XRD analysis). The lower effluent load during the enzymatic treatments makes the process ecofriendly.

- ✓ The final chapter concludes that isolated fungi *T. harzianum* PPDDN-10 NFCCI-2925 and *C. cinerea* PPHRI-4 NFCCI-3027 are capable to produce the cellulase and xylanase production to deink the photocopier waste papers. These crude enzymes and commercial cellulase have the potential to replace the conventional chemicals used to deink the photocopier waste papers without deteriorating the handsheet strength properties after the improvement in the freeness.

LIST OF PUBLICATIONS

PUBLICATIONS (3)

- ✓ **Pathak P.,** Bhardwaj N. K. and Singh A. K. (2014). Production of crude cellulase and xylanase from *Trichoderma harzianum* PPDDN10 NFCCI-2925 and its application in photocopier waste paper recycling. *Applied Biochemistry and Biotechnology*, 1-22, DOI 10.1007/s12010-014-0758-9 (Firstly online published).
- ✓ **Pathak P.,** Bhardwaj N. K. and Singh A. K. (2011). Optimization of chemical and enzymatic deinking of photocopier waste paper. *Bioresources*, *6* (1), 447-463.
- ✓ **Pathak P.,** Bhardwaj N. K. and Singh A. K. (2010). Enzymatic Deinking of Office waste Paper: An Overview. *IPPTA Journal*, *22* (2), 83-88.

CONFERENCES (2)

- ✓ **Pathak P.,** Bhardwaj N. K. and Singh A. K. (2014). Fungal cellulase and xylanase mediated toner ink removal from photocopier waste papers. *International Conference on Emerging Materials & Applications (ICEMA'14)* to be held at IIT Roorkee, Saharanpur Campus, India, 5-6 April, 2014 (Accepted for oral presentaion and Abstract).
- ✓ **Pathak P.,** Bhardwaj N. K. and Singh A. K. (2010). Effect of cellulase enzyme in deinking of photocopier waste paper, *Proceeding of Fourth International Conference on Plants and Environmental Pollution (ICPEP-4*), NBRI, Lucknow, 8 - 11 December 2010, p-161 (Oral Presentation and Abstract).
- ✓ **Pathak P.,** Bhardwaj N. K. and Singh A. K. (2009). Potentials of Microbes and their Enzymes in Pulp and Paper Industry: An Overview. *MICROCON 2009* held at Punjab University, 3-4 March, 2009 (Poster Presentation and Abstract).

Papers Communicated (3)

- **Pathak P.,** Bhardwaj N. K. and Singh A. K. (2014). "Enzymatic Deinking of Photocopier Waste Papers using Crude Cellulase and Xylanase of *Coprinopsis cinerea* PPHRI-4 NFCCI-3027" (Submitted to *APPITA Journal*: Received favorable comments and resubmitted after minor modifications).
- **Pathak P.,** Bhardwaj N. K. and Singh A. K. (2014). Enzymatic Deinking of Photocopier Waste Papers using Crude Enzyme of *Trichoderma harzianum* PPDDN10 NFCCI 2925 (Submitted to *TAPPI Journal*).
- **Pathak P.,** Bhardwaj N. K. and Singh A. K. (2014). "Production of cellulase and xylanase by *Coprinopsis cinerea* PPHRI-4 NFCCI-3027 for the deinking of photocopier waste papers" (Submitted to *Process Biochemistry* Journal).

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(Puneet Pathak)

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ABBREVIATIONS

AA	Ammonium acetate
AC	Ammonium chloride
AFS	Ammonium ferrous sulphate
AN	Ammonium nitrate
BE	Beef Extract
BOD	Biological oxygen demand
BSA	Bovine serum albumin
CC	Crude enzyme of *C. Cinerea* (in Chapter 4)
CC	Corncob (in Chapter 3)
CDA	Czapek dox agar
CE	Commercial enzyme
CEL	Cellobiose
CMCA	CMC agar
CMCase	Carboxy methyl cellulase
COCO	Coconut coir
COD	Chemical oxygen demand
CSC	Castor seed cake
Cy	Pulp consistency
D.W.	Distilled water
DE	Deinking efficiency
DNS	3, 5-dinitrosalicylic acid
DTPA	Diethylene triamine pentaacetic acid
EDTA	Ethylenediaminetetraacetic acid
ERIC	Effective residual ink concentration
FPase	Filter paper
FPU/gds	Filter paper unit per gram dry substrate
FPU/ml	Filter paper unit per milliliter
FRU	Fructose
FT-IR	Fourier transform infrared spectroscopy
GAL	Galactose
gds	Gram dry substrate
GLU	Glucose
GS	Groundnut shell
GSC	Gram seed coat
HP	Hydrapulper
IU/gds	International unit per gram dry substrate
IU/ml	International unit per milliliter
LAC	Lactose
LCWs	Lignocellulosic wastes
MC	Mustard cake
ME	Malt extract

MEA	Malt extract agar
MSC	Masoor seed coat
MTP	Meat peptone
MYP	Mycological peptone
NFCCI	National Fungal Culture Collection of India
NSS	Nutrient salt solution
O.D.	Optical density
o.d.	Oven dried
PB	Peptone bacteriological
PDA	Potato dextrose agar
PDA	Potato dextrose agar
PP	Proteose peptone
RH	Rice husk
RS	Rice straw
SBM	Soya bean meal
SCB	Sugarcane bagasse
SD	Standard deviation
SDA	Sabouraud dextrose agar
SmF	Submerged state fermentation
SmF(R)	Submerged state shaking fermentation
SmF(S)	Submerged state static fermentation
SmSF(R)	Semi-solid state shaking fermentation
SmSF(S)	Semi-solid state static fermentation
SN	Sodium nitrate
SP	Soya peptone
SSF	Solid state fermentation
SSF(R)	Solid state shaking fermentation
SSF(S)	Solid state static fermentation
TAPPI	Technical Association of the Pulp and Paper Industry
TH	Crude enzyme of *T. Harzianum*
TRP	Tryptone
UA	Urea
WB	Wheat bran
WB	Wheat bran
WBA	Wheat bran agar
WS	Wheat straw
WSD	Wood saw dust
XA	Xylan agar
XRD	X-ray diffraction
XYL	Xylose
YE	Yeast extract
YEA	Yeast extract agar

CHAPTER 1

INTRODUCTION

1.1. Global and Indian scenario

1.1.1. Paper industry

The global pulp and paper industry consists of about 5000 industrial pulp and paper mills, and an equal number of very small companies. The worldwide annual production of paper and paperboard production was 402.0 million tonnes in 2010. It is expected to increase to 490.0 million tonnes by 2020 (Bio Green Papers Limited Annual Report 2012-13). The Indian paper industry is among the top 15 global players today, with an output of more than 10.11million tonnes/annum in the year 2011 (Industry facts-IPPTA 2014). The paper industry in India is more than a century old. At present there are over 715 paper mills manufacturing a wide variety of items required by the consumers (SSEF Report, 2013). Out of these pulp and paper mills majority (551) are recycled fibre-based mills, then agro-based (139) and lastly wood-based (25) mills (SSEF Report 2013, CPPRI 2010). The Indian paper industry accounts for about 1.6% of the world's production of paper and paperboard (SSEF Report 2013). "Growth of Paper Industry in India", indicated that per capita paper consumption increased to 9.3 kg on 2011 as compared to 8.3 kg during 2008-09. Still, this figure is low (9.3 kg) as compared to 312 kg in USA and 47 kg for the World (CPPRI 2010, Industry facts-IPPTA 2014). India has emerged as the fastest growing market when it comes to consumption, posting 10.6% growth in per capita consumption of paper in 2009-10 (Goel et al. 2011).

The Indian paper industry uses wood, agricultural residues and waste papers as raw materials. In the early 70's, the share of waste paper used, as raw material was only 7%, whereas now it constitutes the major raw material base for paper industry with 47% share in total production (Figure-1.1) (SSEF Report 2013). Until now, about 551 mills in India use waste paper as primary fibre source for paper, paperboard and newsprint production. These waste papers are sourced indigenously as well as through import.

The present recovery and utilization of waste paper by paper mills in India is 3.0 million tonnes annually, which translates to a recovery of 27% of the total paper and paperboard consumed. Only 20% copier and writing/printing paper is collected. Low recovery is because of alternate use of paper in wrapping, packing, etc. Lack of collection, sorting and grading system results in contamination of waste papers and therefore it is becoming unusable. This recovery rate is very low when compared to developed countries like Germany-73%,

Sweden-69%, Japan-60%, Western Europe-56%, USA-49% and Italy-45% (Sinha and Raina 2011).

Due to inadequate availability of indigenous waste paper, Indian mills rely heavily on imported waste paper to meet the raw material demand. The import bill has increased significantly over the years. According to an estimate, the import of waste papers has increased from 5.1 million USD in 1980 to one billion USD in 2011. India imports around 4.0 million tonnes of waste paper annually, which is about 57% of its requirements (CPPRI 2010). The utilization rate of recovered fibre is only 47%. India needs a well-defined and aggressive system for collecting, sorting, grading and utilizing recyclable waste papers to minimize imports. Government intervention is necessary to encourage segregation at source and increase recycling to minimize landfill and environmental hazards.

Therefore, it is important to put in place suitable mechanisms that result in increasing the effective recycling of post consumer paper for manufacturing. The Government of India has relaxed the rules and regulations and delicensed the paper industry to encourage investment into this sector. Many joint projects in this field are allowed and some of the joint ventures have also started in India to persuade investments in paper sector. The paper industry in India is looking for the state-of-art technologies to reduce its production cost and to upgrade the technology to meet the international standards. The futuristic view is that the growth in paper consumption would be in multiples of gross domestic product (GDP) and hence an increase in consumption by one kg per capita would lead to an increase in demand by 1 million tonnes (http://ipma.co.in). It is estimated that the paper industry would be growing at the present rate of 9% of compounded rate, and would required 25 million MT by year 2020 from the existing production of around 17.2 million tonnes (Figure-1.2) (Indian-paper-industry1990-2010-Document Transcript).

1.1.2. Enzyme industry

The global market for industrial enzymes is estimated at $3.3 billion in 2010. This market is expected to reach $4.4 billion by 2015, a compound annual growth rate (CAGR) of 6% over the 5 years forecast period. Technical enzymes are valued at just over $1 billion in 2010. This sector will increase at a 6.6% compound CAGR to reach $1.5 billion in 2015. The highest sales of technical enzymes occurred in the leather market, followed by the bioethanol market (Sarrouh et al. 2012).

Indian biotechnology industry contributes to two per cent of the global biotechnology industry. The sector grew at a healthy rate of 20 per cent in 2007-2008. The industry in 2007-2008 clocked US$ 2.5 billion, registering a 30.98% growth. India is among the top 12 biotechnology markets.

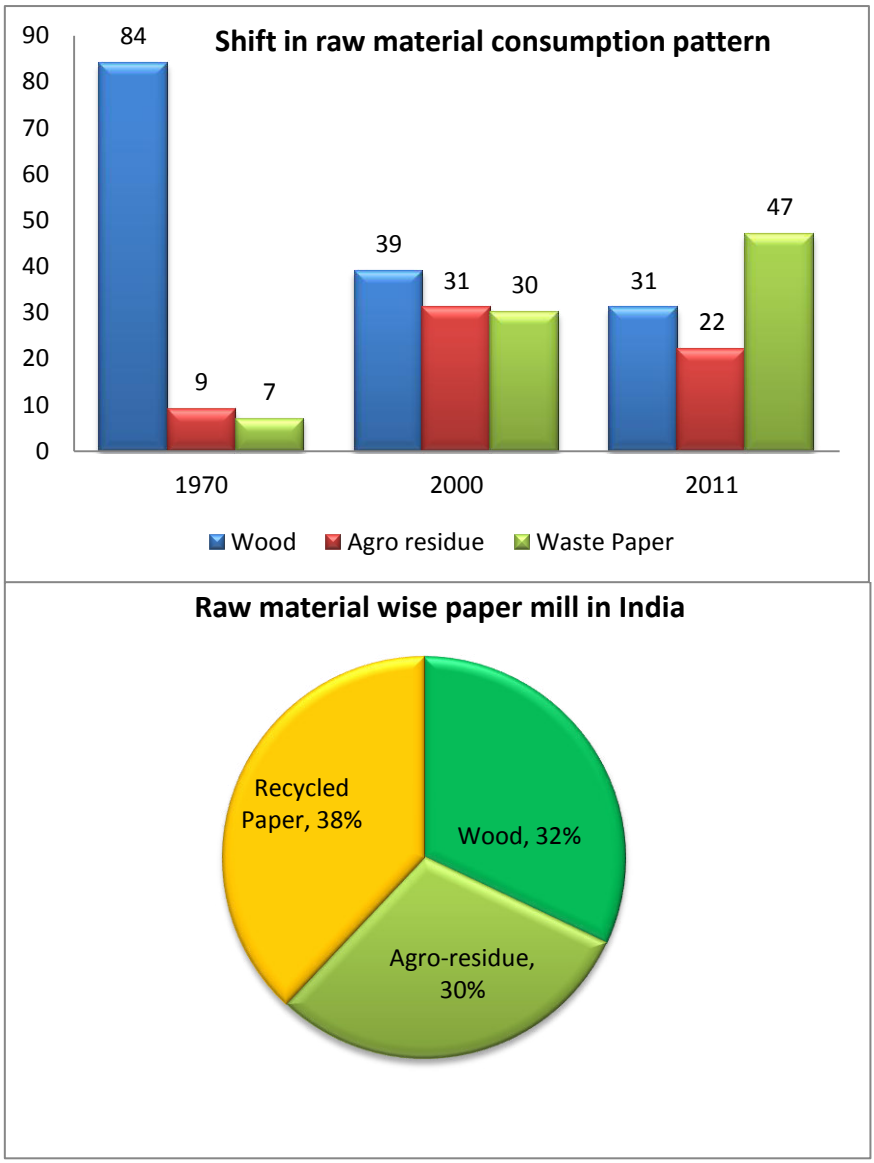


Figure-1.1 Raw material consumption and raw material based paper mills in India (Source: Paper Industry)

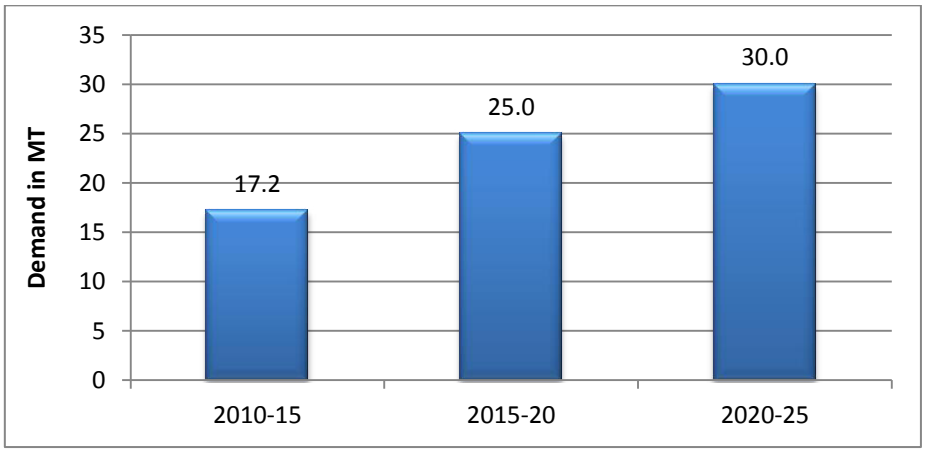


Figure-1.2 Projected demand of paper in MT

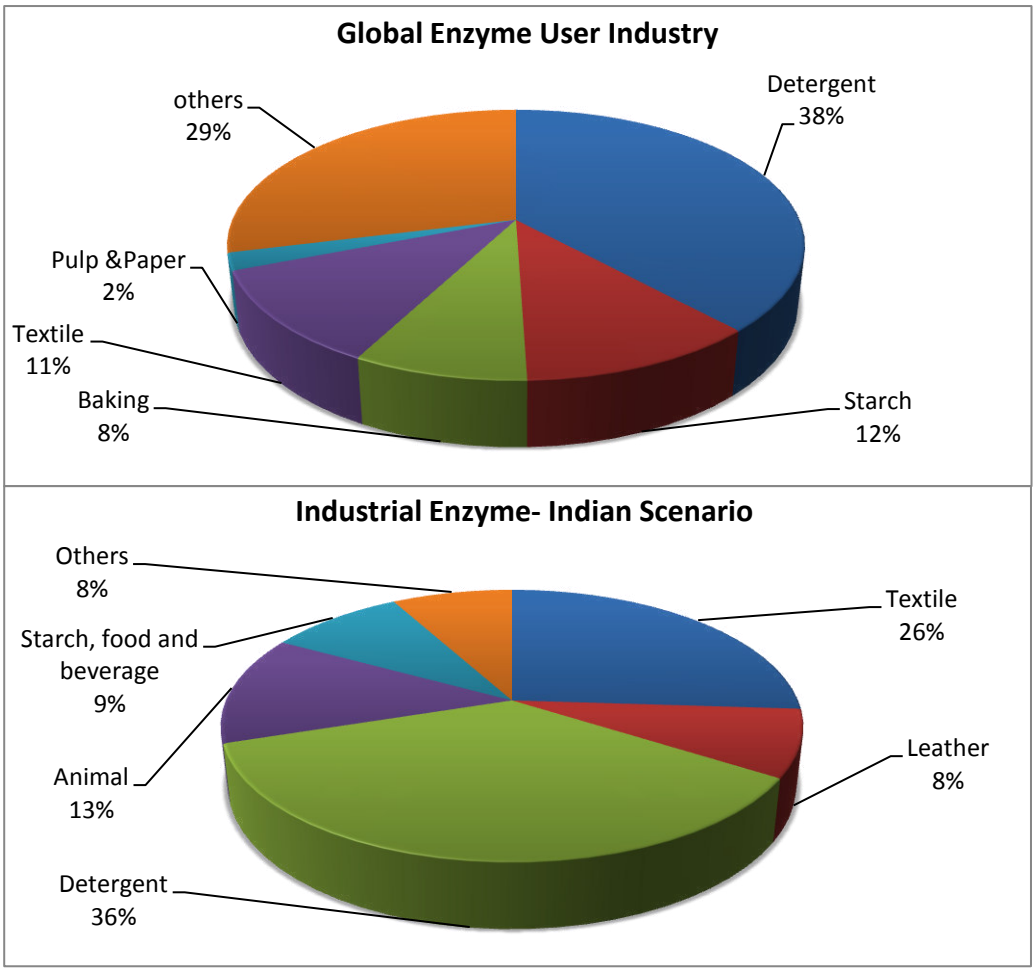


Figure-1.3 The global and Indian scenario of enzyme user industry (Source: Business Communication Company INC.)

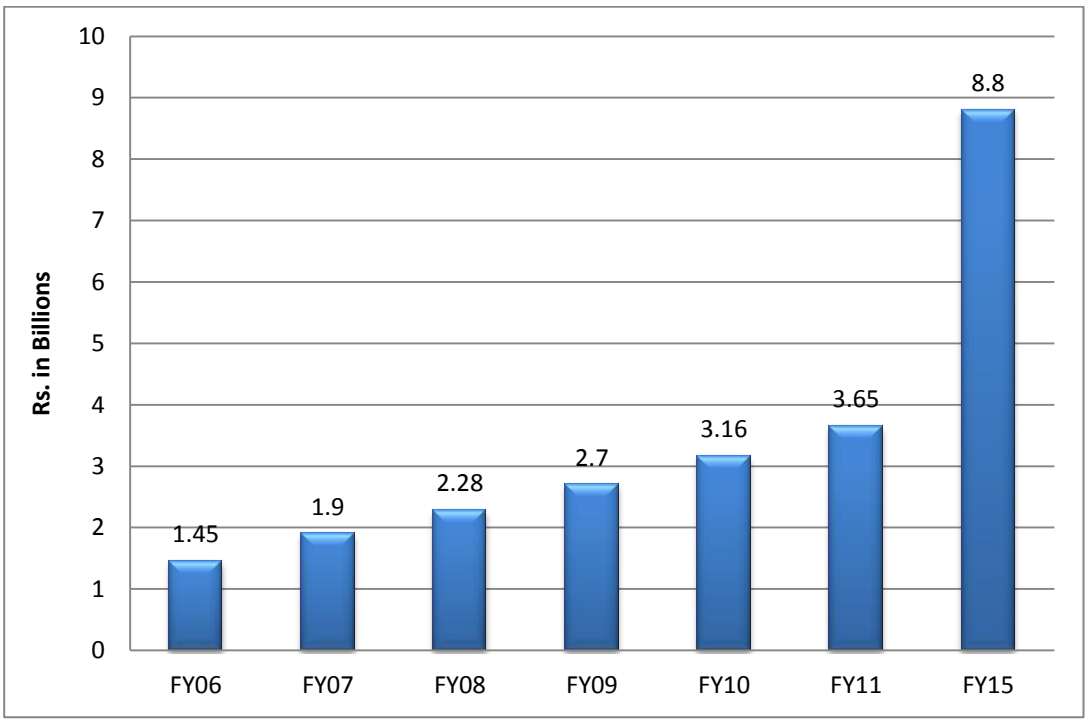


Figure-1.4 Financial growth of Indian biotechnology industry till 2015

Globally India ranks third in the Asia-Pacific, after Japan and Korea Indian market is expected to touch US$ 25 billion by 2015 (Indian-biotech-industry-presentation-010709). The global and Indian scenario of enzyme using industry figured out that the enzymes have very less consumption in the pulp and paper industry (Figure-1.3).

The bio-industrial sector in terms of venue touched almost US$ 96 million with a growth rate of 5.33 per cent in 2006-07. Despite its modest growth, the volume of the sector has been on a healthy rise and the application spectrum of industrial enzymes has been increasing. The top five companies in the bio-industrial sector contributed 87% to the sector's total market value (Indian-biotech-industry-presentation-010709).

Novozymes and Biocon, with revenues US$ 24 million and at US$ 23 million, respectively, are the largest players in the sector followed by Advanced Enzymes, Rossari Biotech and Zytex. Novozymes and Biocon account for almost 50 per cent of the sector's value. About 15 companies in India are involved in the enzymes business with some into manufacturing and others into marketing of enzymes. Traditionally, the enzyme companies have focused on chemicals, textiles, breweries and tanneries for applications of their products. However, existing companies are now looking closely into new areas of application such as food processing, agriculture, animal nutrition, dairy, aquaculture, fibre processing and marine products.

Hydrolytic enzymes constitute around 75% of the markets for industrial enzymes, with the glycosidases, including cellulases, amylases and hemicellulases, constituting the second major group after proteases (Bhat 2000). Many opportunities exist in the manufacturing of industrial enzymes for export purposes. The Government of India has also supported this sector by funding many projects related to industrial enzymes, thus helping the industry to grow and compete with global players. Some companies such as Biocon and Rossari are already involved in exporting enzymes (Indian-biotech-industry-presentation-010709).

Even though enzymes consumption in India is low compared to other countries, but their application is increasing in almost all industries in India. According to a research report on biotech market in India, Indian biotechnology industry is expected to grow at ~23% and reach USD 8.8 billion by financial year (Fy)15 (Figure-1.4) (Indian Biotechnology Market Report).

1.2. Literature review

The secretion of extracellular metabolites is the inherent practice of microorganisms, which make them unique in the nature. The diversity of microorganisms in nature is surprising. More than 400,000 of microorganisms are known in the nature, and this is just a fraction of the probable number. It is expected that there are four to five million different species of microorganism. As a result, microorganisms can be found in almost every biotope around the world. These industrially important microorganisms (e.g., enzyme production) mainly comprise of fungi, bacteria, yeast and actinomycetes.

The enzyme industries are eager to exploit this diversity by gathering different natural samples from the four corners of the Earth. Presently, the industrial enzyme companies sell enzymes for a wide variety of applications. As this research, utilizes the potential of fungi for the production of enzymes used in deinking so a brief discussion about the fungi involved in enzyme production is given below.

1.2.1. Fungi

Fungi are eukaryotic, single celled, multinucleate, or multicellular organisms including yeasts, molds and mushrooms. They are aerobic, spore forming, growth with apical anastomosis. These hetetrotrophic organisms live as saprophytes, symbionts or parasites. They require organic compounds for the nutrition. During the state of growth and metabolism, fungi secrete extracellular enzymes (e.g. cellulase, xylanase, laccase, and amylase etc.) to hydrolyze high molecular weight molecules into simpler forms in the environment. The fungi then assimilate these products. Approximately 100000 species of fungi have been identified. Fungi are valuable economically as a source of antibiotics, vitamins, enzymes and alcohols as well as for their role in fermentation processes.

1.2.1.1. *Trichoderma harzianum*

Trichoderma harzianum is cosmopolitan filamentous fungi present in the in soils, decaying wood and vegetable matter. *T. harzianum* is known to cause disease of the commercial mushroom. The fungus *Trichoderma harzianum* is a biological control organism against a wide range of soil-borne pathogens and of plant-pathogenic fungi. Mostly *Trichoderma* strains lack sexual stage, generally reffered as mitotic or clonal and produce only asexual spores. *T. Harzianum* shows the sexual stage, known as *Hypocrea lyxii* due to its close resemblance (Rubeena, et al. 2013, Kubicek and Harman 1998). Its optimum temperature for growth is at 30°C and maximum growth temperature is 36°C. This fungus is well known to produce cellulase and xylanase enzyme (Kubicek and Harman 1998). *T. harzianum* secretes a well-balanced cellulolytic, and xylanolytic lcomplex with ligninolytic, which efficiently hydrolyzes lignocellulosic materials into monomeric units. Due to its elevated cellulolytic activity, *T. harzianu*m has considerable potentials in biomass hydrolysis

applications (Seyis and Aksoz 2005, Kocher et al. 2008, Isil and Nilufer 2005, Sakthiselvan et al. 2012, Rubeena, et al. 2013, Viterbo et al. 2001).

Kingdom	Fungi	Fungi
Division	Ascomycota	Basidiomycota
Class	Sordariomycetes	Agaricomycetes
Order	Hypocreales	Agaricales
Family	*Hypocreaceae*	Psathyrellaceae
Genus	*Trichoderma*	*Coprinopsis*
Species	*harzianum*	*cinerea*

1.2.1.2. *Coprinopsis cinerea*

Coprinopsis cinerea is a saprotrophic fungus, which in nature grows on dung (Buller 1931, Uljé and Noordeloos 1999). *Coprinopsis cinerea* is a multicellular basidiomycete with a typical mushroom form that undergoes a complete sexual cycle. Unlike most mushrooms, *C. cinerea* can complete its entire life cycle (2 weeks) in the laboratory. It can also easily grow on simple artificial media based on e.g. malt extract. Generally, it is considered to be of little edible value because of the fast autolysis of the cap at maturity. This fungus also has the ability to produce cellulase, xylanase and lignin degrading enzymes (Dutt et al. 2012, Kaur et al. 2011, Navarro-González et al. 2011).

1.2.2. Enzymes

Enzymes are non-living proteins or organic chemicals that exist in all living organisms. These "bio-catalysts" are designed by nature to either carry out the synthesis of different chemical compounds essential for the proper functioning of the organism and/or to facilitate the breakdown or alteration of such chemicals under a variety of environmental and physiological conditions.

Similar to all catalysts, enzymes accelerate the rate of the reaction by lowering the activation energy for a reaction. These enzymes are neither consumed by the reactions during catalysis nor alter the equilibrium (Bairoch, 2000). The enzyme activity is influenced by temperature, chemical environment (like pH, temperature), substrate concentration and presence of inhibitors or activators. Enzymes are commonly globular proteins having three-dimensional structure, which decides the activities of the enzyme (Anfinsen, 1973). Similar to all proteins, enzymes are composed of long, linear chains of amino acids that fold to produce a three-dimensional structure. Due to their unique amino acid sequence, enzymes produce a specific structure, which has distinctive properties. Enzymes are generally very specific

towards the reactions to be catalyzed in terms of substrates that are involved in these reactions. The specificity of enzymes is mainly determined by its complementary shape, charge, hydrophilic/hydrophobic characteristics of enzymes and substrates. Enzymes can also show impressive levels of stereo-specificity, regio-selectivity and chemo-selectivity (Jaeger and Eggert 2004).

1.2.3. Cellulase

Fungal cellulase and bacterial cellulase are components of large systems or complexes that hydrolyze β-1, 4-glucosidic linkages in cellulose which produce water soluble sugars. Cellulases can be divided into three major classes (Figure-1.5). These are endocellulase (endoglucanases or endo-1,4-β-glucanase), exocellulase (exoglucanase or cellobiohydrolase) and β glucosidase. These three hydrolytic enzymes act synergistically. Endoglucanase attacks randomly along the cellulose fibre, resulting in a rapid decrease in the chain length of CM-cellulose or H_3PO_4-swollen cellulose and yielding glucose, cellobiose, cellotriose and other higher oligomers (Eriksson et al. 1990).

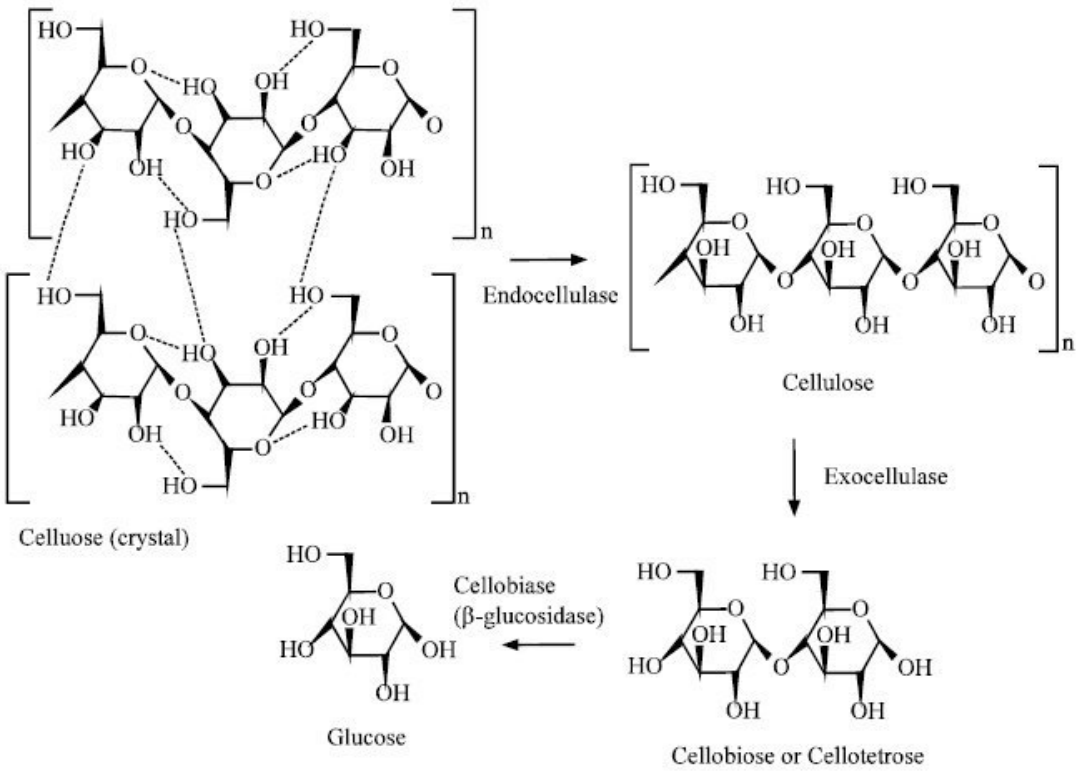


Figure-1.5 Mechanism of cellulase complex (Adapted from Karmakar et al. 2011)

Cellobiohydrolase (CBHs) acts processively on the existing chain ends and on those created by the endoglucanases, releasing cellobiose molecules. β-glucosidase cleaves the released cellobiose into two glucose molecules (Eriksson et al. 1990).

1.2.4. Hemicellulase

Due to the complex structure of hemicelluloses, several different enzymes are needed for their enzymatic degradation or modification. The two main glycosyl hydrolases depolymerising the hemicellulose backbone are endo-1,4- β-D-xylanase and endo-1,4-β-D-mannanase (Suurnakki et al. 1997). Since xylan is a complex component of the hemicelluloses, its complete hydrolysis requires the action of a complete enzyme system, which is usually composed of L-xylanase, L-xylosidase, and enzymes such as β-L-arabinofuranosidase, β-glucuronidase, acetylxylan esterase, and hydroxycinnamic acid esterases that cleave side chain residues from the xylan backbone (Figure-1.6). All these enzymes act cooperatively to convert xylan to its constituents (Sunna et al.1997). Xylanases attack randomly on the backbone of xylan to produce both substituted and non-substituted shorter chain oligomers, xylobiose and xylose (Woodward et al. 1994). Xylosidases are essential for the complete breakdown of xylan as they hydrolyse xylo-oligosaccharides to xylose. The enzymes arabinosidase, α- glucuronidase and acetylxylan esterase act in synergy with the xylanases and xylosidases by releasing the substituents on the xylan backbone to achieve a total hydrolysis of xylan to monosaccharides (Eriksson et al. 1990).

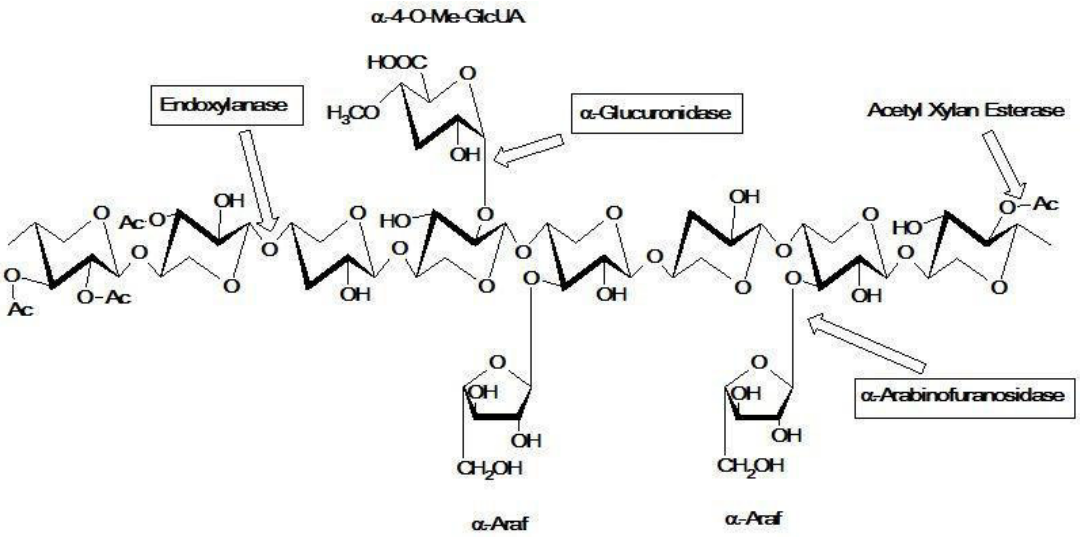


Figure-1.6 Mechanism of xylanase complex (Adapted from Sunna and Antranikian 1997)

β-Mannanases catalyse the random hydrolysis of β-D- 1,4-mannopyranosyl linkages within the main chain of mannan and various polysaccharides consisting mainly of glucomannan, galactomannan and galactoglucomannan (Suurnakki et al. 1997). Other glycosyl hydrolases that are important for the degradation of mannan include mannosidases and galactosidases. β-Mannosidase catalyses the hydrolysis of terminal non-reducing β-D-mannose residues in mannan (Suurnakki et al. 1997). α-Galactosidase hydrolyses terminal, non-reducing α-D-galactosides from galactose oligosaccharides, galactomannan and galactoglucomannan and are also capable of removing α-1,6-bound galactosyl units from polymeric galactomannan.

1.2.5. Cellulase and xylanase producers

Prokaryotes and eukaryotes produce/contain cellulase and xylanases (Dekker and Richards 1976, Sukumaran, 2005). Among higher eukaryotes, protozoa, insects, snails and germinating plant seeds demonstrate cellulase and xylanase activity (Taiz and Honigman 1976, Watanabe. and Tokuda 2010) (Table-1.1).

Among the prokaryotes, bacteria and cyanobacteria from marine environments produce xylanases (Dekker 1985). Extracellular and intracellular cellulase and xylanases have been studied extensively. Intracellular xylanases were described in rumen bacteria (Marichamy and Mattiasson 2004) and protozoa (Dekker and Richards 1976). Varieties of microorganisms including bacteria (Gessesse and Mamo 1998, Mawadza et al. 2000), yeast (Liu 1998), actinomycetes (Garg et al. 1998, Grigorevski de-Limaa, 2005) and filamentous fungi (Matsuo et al. 1998, Soni et al. 2008) have been reported to produce xylanolytic enzymes.

1.2.6. Enzyme production

For enzymes production, the two type of fermentation may be used. This includes solid state fermentation (SSF) and submerged state fermentation (SmF). It have been pointed out that SSF offers greatest possibility when fungi are used, while SmF gives better results when bacterial cultures are employed for enzyme production (Pandey 1994, 1992, Pandey et al. 2001, 2000).

1.2.7. SSF

SSF is defined as the fermentation involving solids in absence (or near absence) of free water; however, substrate must possess enough moisture to support growth and metabolism of micro-organisms (Pandey 1994, 1992, Pandey et al. 2001, 2000). SSF offers numerous opportunities in processing of agro-industrial residues. This is partly because solid state processes require low energy, produce less wastewater and are ecofriendly as they resolve the problem of solid wastes disposal (Pandey et al. 2001).

Fungal hyphae grow on the LCW to develop into a mycelial mat after sporulation, which cover the surface of LCW *i.e.* solid substrate's particles. Fungal hyphae grow in the aerial or gaseous space as well as into the liquid filled pores by penetrating the substrate. At normal moisture levels, these voids space are filled with gas (g) for the aerial hyphae and liquid (l) for mycelial mat within the substrate. Aerial hyphae take the metabolic substances through the transportation of the metabolites. The mycelium produces hydrolytic enzymes which diffuse to the solid matrix where catalyzation to degrade macromolecules into smaller monomeric units takes place. Thus, produced monomeric sugars are taken up by the fungus to serve as nutrients after consuming O_2 and release of CO_2, H_2O, heat and interesting biochemical product.

Table-1.1 Major fungi employed in cellulase and xylanase producers

Cellulase Producers		**Xylanase producers**	
Fungus	**References**	**Fungus**	**References**
Aspergillus fumigatus	Delabona et al. 2012	*Acremoniun alcalophilum*	Kazuya et al. 1997
Aspergillus niger	Pothiraj et al. 2006, Ong et al. 2004	*Aspergillus fumigatus*	Anthony et al.2003
Aspergillus niger	Delabona et al. 2012, Milala 2005	*Aspergillus kawachii*	Ito et al.1992
Aspergillus oryzae (recombinant)	Milala 2005	*Aspergillus niger*	Meagher et al.1988
Aspergillus terreus	Pothiraj et al.2006	*Aspergillus terreus*	Hrmova et al.1991
Chaetomium thermophilium	Li et al. 2003	*Coprinellus disseminatus*	Agnihotri et al. 2010
Corynascus sp.	Soni et al. 2008	*Coprinus cinerea*	Kaur et al. 2012
Emericella nidulans	Soni et al. 2008	*Gloephyllum trabeum*	Ritschkoff et al. 1994
Fomitopsis sp.	Deswal et al. 2011	*Humicola lanuginosa*	Kamra and Satyanarayana (2004
Fusarium oxysporum	Ortega 1990	*Neocallimastix patriciarum*	Lee et al. 1993
Fusarium solani	Wood and McCrae 1977	*Orpinomyces sp.*	Li et al. 1996
Humicola grisea	Takashima et al. 1996	*Penicillium herque*	Funaguma et al. 1991
Malbranchea flava (MF)	Soni et al. 2008	*Phanerochaete chrysosporium*	Cristica et al. 2012
Melanocarpus albomyces	Oinonen et al. 2004	*Pleurotus ostreatus*	Qinnghe et al.2004
Myceliophthora sp	Soni et al. 2008	*Schizophyllum commune*	Haltrich et al.1993
Neurospora crassa	Romero et al.1999,	*Sclerotium rolfsii*	Sachslehner et al.1998
Penicillium brasilianum	Jogensen et al. 2003	*Talaromyces byssochlamydoides*	Yoshioka et al. 1981
Penicillium decumbans	Yang et al. 2004, Mo 2004	*Talaromyces emersonii*	Tuohy and Coughlan 1992
Penicillium janthinellum	Adsul et al. 2004	*Termitomyces clypeatues secrets*	Ghosh et al. 1998
Penicillium occitanis	Belghith et al. 2001	*Thermoascus aurantiacus*	Oliveira et al. 2010
Penicillium occitanis.	Chaabouni et al. 1995	*Thermomyces lanuginosus*	Gomes et al.1993, Chadha et al. 1999, Sonia et al. 2005
Phanerochaete chrysosporium	Szabo et al.1996	*Trametes versicolor*	Khalil et al.2002
Rhizopus stolonifer	Pothiraj et al. 2006	*Trichoderma reesei*	Torronen et al. 1992
Torula sp.	Soni et al. 2008	*Trichoderma reesei*	Cristica et al. 2012
Trichoderma harzianum	Castro De et al. 2010, Delabona et al. 2012	*Trichoderma viride*	Cristica et al. 2012
Trichoderma longibrachiatum	Fowler et al. 1999		
Trichoderma reesei	Dashtban et al. 2011		
Trichoderma viride	Malik et al. 2010		

The transportation of oxygen and CO_2 takes place due to the developement of the gradient in biofilm (Figure-1.7). Due to heat development during metabolic activities, temperature of the system increases, this causes serious problem to the fermentation process. Heat is, therefore, removed from the substrate by conduction and evaporation, which is part of the complex balance of water in the system (Figure-1.7).

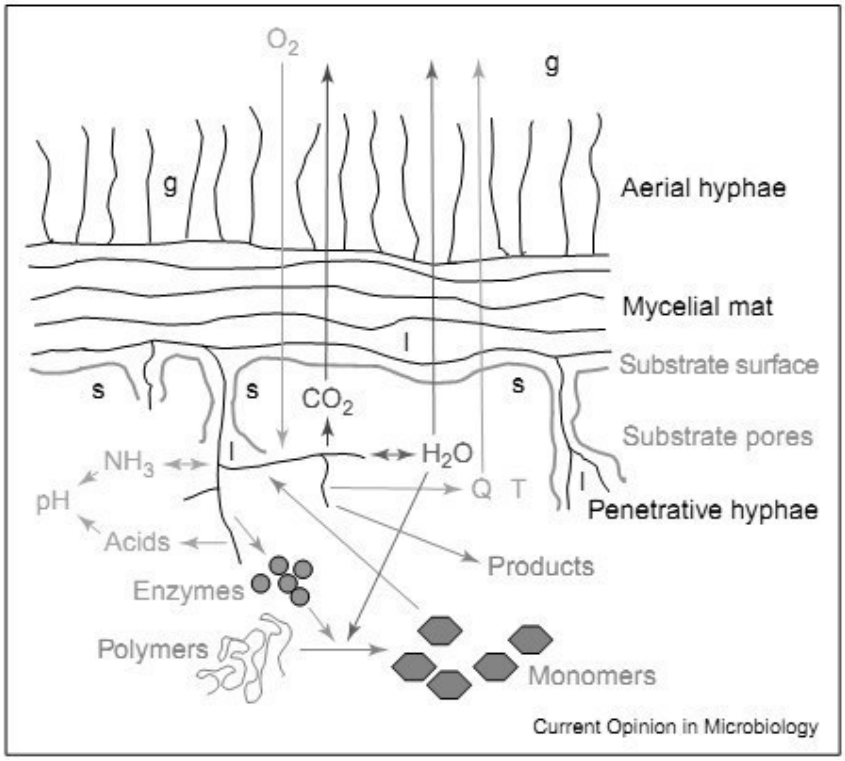


Figure-1.7 Schematic of some of the microscale processes that occur during SSF (Adapted from Holker and Lenz 2005)

At the side of evaporation, mycelium consumes water for growth and for hydrolysis reactions but it produces water through respiration. Carbon acids and the exchange of ammonia change to local pH (Figure-1.7). The biochemical products of interest are released into the solid matrix and the liquid-filled spaces during fermentation, which might absorb to the solid. These products should be extracted for further use at the end of the process. Many factors including these phenomena can strongly affect process performance of SSF (Holker and Lenz 2005).

1.2.8. Advantages of SSF over submerged fermentation (SmF)

SSF is advantageous process over SmF in terms of higher productivity, better oxygen circulation, and low-cost of media, less effort in downstream processing, reduced energy and cost requirements. It resembles the natural habitat for several microorganisms. However, there are some disadvantages also which creates difficulty on scale-up, low mixing, difficulty in controlling the process parameters (pH, heat, moisture and nutrient conditions), problems with heat build-up, and increasing recovery product costs (Couto and Sanroman 2006).

1.2.9. Different factors affecting cellulase and xylanase production in SSF

Different factors such as lignocellulose material, amount of substrate, bed height, incubation days, incubation temperature, pH, nitrogen source, initial moisture content, particle size, pretreatment of raw material, inoculum size and additional nutrients affect cellulase and xylanase production.

In SSF, solid material is non-soluble that acts both as physical support and source of nutrients. Solid material could be a naturally occurring solid substrate such as agricultural crops, agro-industrial residues (Pandey 1992). For SSF processes, different agro-industrial wastes are used as solid substrates. Selection of agro-industrial residues for utilization in SSF depends on some physical parameters such as particle size, moisture level, intra-particle spacing and nutrient composition within the substrate (Bhargav et al. 2008). It should be cheaper and easily available in mass scale in all the season. In recent years, some important agro-industrial residues such corncob, groundnut shell, coconut coir, sugarcane bagasse, rice husk, rice bran, rice straw, wheat bran, wheat straw, wood saw dust and many more have been used (Pandey 1994, 1992, Pandey et al. 2001, 2000).

Natural substrates are easily available and cheaper than synthetic substrates. However, they generally require pretreatment to make their chemical constituents more accessible and their physical structure more susceptible to mycelial penetration. Physical treatment includes chopping or grinding to reduce size and cracking to make the interior of the particle more accessible. Chemical treatment includes high temperature cooking and acid or alkali treatment (Manpreet et al. 2005).

The substrate must be in a limited size range for an optimal production of cellulase. This process can be facilitated by chipping, milling and grinding the biomass into a fine powder to increase the surface area/volume ratio of the cellulose particle. An optimal sized particle lead to better nutrient absorption, gas exchange and heat transfer thus high enzyme production. Generally low particle sizes have larger specific surface area in fine particles but low porosity property (Bahrin et al. 2011). Due to the inverse correlation of porosity and surface area factors, most researchers claimed that 400 µm substrate sized particles contribute to the optimum fungal growth and cellulase production (Tao et al. 1997, Krishna and Chandrasekaran 1996). The low porosity caused less penetration of fungus hypha into the pores of the substrate and fungal growth only can be observed on the surface of the substrate. When larger substrate particle size (> 400 µm) was applied in the fermentation, a network of aerial hypha grows into the inter-particle space low fungal growth on surface of the substrate particle and decreased the resulted enzymes (Tao et al. 1997).

The role of the water content of the substrate and the moisture content is a critical factor influencing the SSF processes because these variables has influence on growth and

biosynthesis and secretion of different metabolites. Low moisture content causes reduction in solubility of nutrients of the substrate, low degree of swelling and high water tension. On the other hand, high moisture level can cause reduction in enzyme yield due to steric hindrance of growth of the producer strain by reduction in porosity (inter-particle spaces) of the solid matrix, thus interfering oxygen transfer. For economical production of enzymes, the optimal value of moisture content depends on both the microorganism and the solid matrix used; the microorganism should be grown in optimal moisture levels for maximizing enzymes production. Lower values can induce the sporulation of the microorganism, whereas higher levels can reduce the porosity of the system, which can produce oxygen transfer limitation, and increase the risk of bacterial contamination (Pandey 1994, 1992, Pandey et al. 2001, 2000).

The increase in temperature in SSF is a consequence of the metabolic activity when the heat removal is not enough. This affects directly spores germination, growth and product formation. The temperature level reached is a function of the type of microorganism, the porosity, the particle diameter and the depth of the substrate. Temperature can rise rapidly, because there is little water to absorb the heat or in other words mean specific heat capacity of the fermenting mass is much lower than that of water. Therefore, heat generated must be dissipated immediately as most of the microorganisms used in SSF are mesophilic, having optimal temperature for growth between 20 °C and 40 °C and maximum growth below 50 °C (Manpreet et al. 2005).

The measurement and control of pH in SSF is very difficult. So, it is desirable to use microorganisms which can grow over a wide range of pH and which have broad pH optima (Manpreet et al. 2005). Nevertheless, the substrates employed in SSF usually have buffering effect due to their complex chemical composition. So the initial pH of the nutrient saline solution (NSS) are adjusted or another possibility to control the he pH is addition of a mixture of sources of nitrogen having opposite influence on the change of the pH in such a way than ones counteract the effect of the others. In this regard, ammonium salts have been used in SSF in combination with urea or nitrate salts due to the respective effects of acidification and alkalization.

1.2.10. Enzymes in paper industry

The application of enzymes in pulp and paper industry is not considered as economical and technically feasible until now due to non-availability of suitable enzyme of papermaker's choice (Bajpai 1999). Still research are ongoing regarding the application of biotechnology in different areas of the pulp and paper industry by several scientific institutions and enzyme producers companies (Demuner 2011), that led to the development of enzymes that offer significant benefits to the industry (Figure-1.8 and 1.9). Several applications have already

been commercialized; however, the majority of the potential applications are still in the pre-commercial stage.

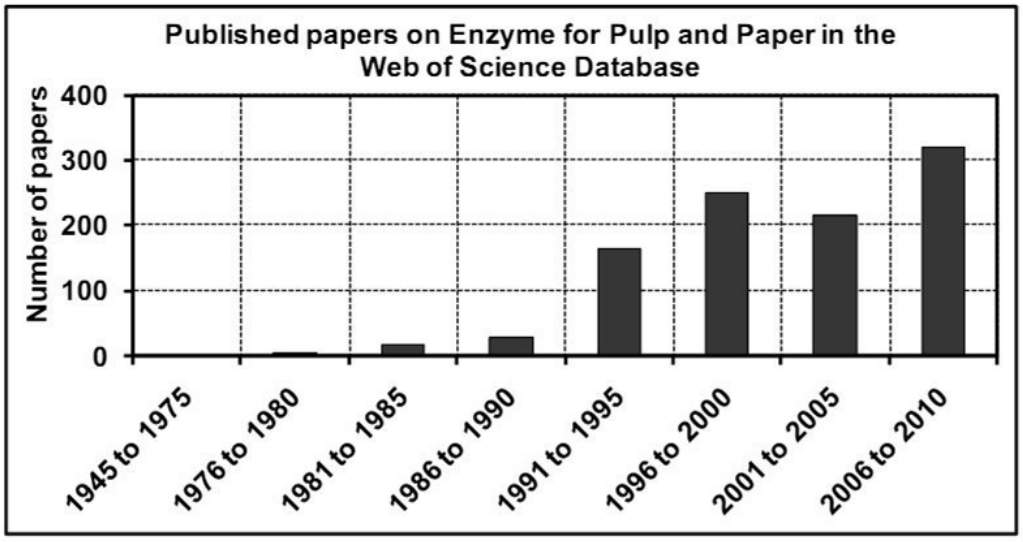


Figure-1.8 Number of published documents on the enzymes for the pulp and paper industry in the web of sciences from 1945 to July 2010 (Adapted from Demuner 2011)

As a relatively recent development, heavy increase in the innovative potential (technological intensity) on enzymes for the pulp and paper industry has continued to gain interest since 1976 when laboratory research and development studies were started (Demuner 2011).

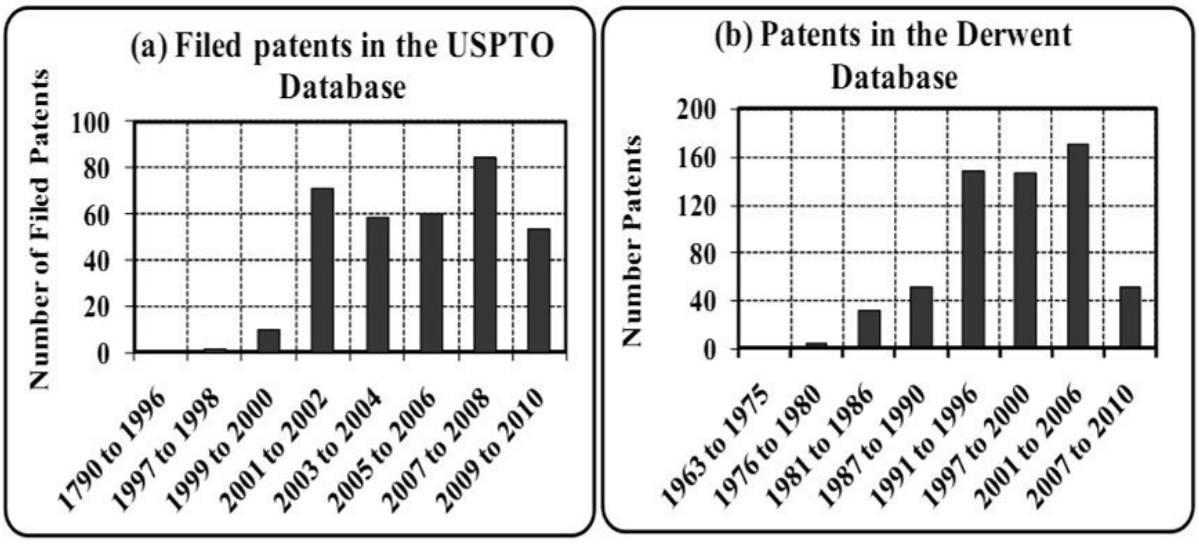


Figure-1.9 Number of patents for Pulp and Paper (a) in the USPTO from 1790 to July 2010, and (b) in the Derwant database (Adapted from Demuner 2011)

Different areas in pulp and paper industry where enzymes have been used are summarized in the Figure-1.10 and the functions and status of these enzymes are tabulated in the Table-1.3 (Bajpai 1999).

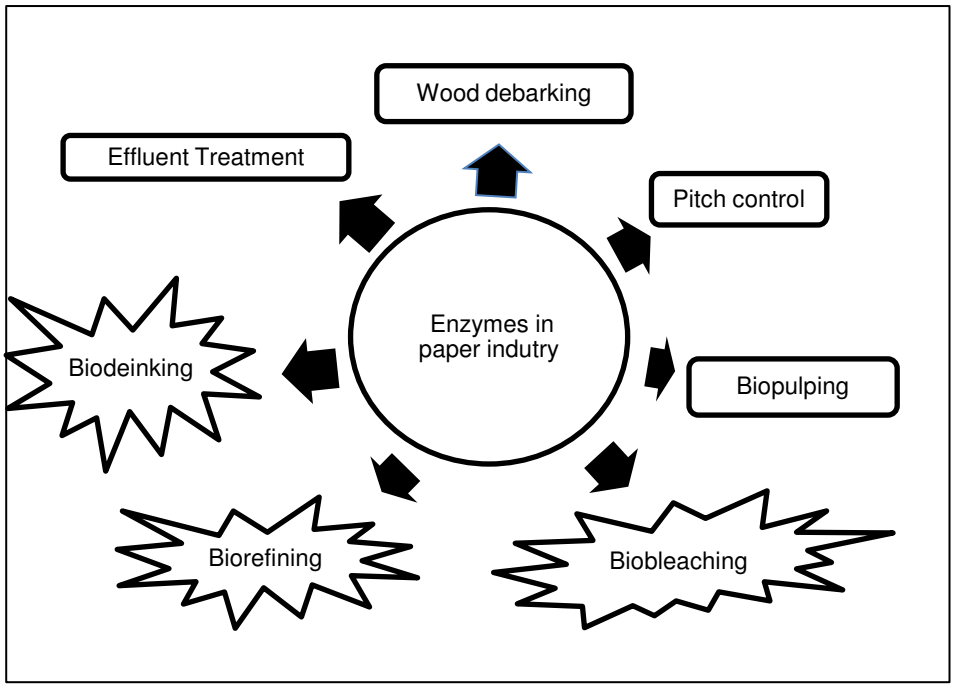


Figure-1.10 Different application of enzymes in pulp and paper industry

Figure-1.11(a) displays percentage sharing of the different types of enzymes used in the pulp and paper industry. The leading enzymes are cellulase, xylanase, laccase and lipase. The information presented in Figure-1.11(b) indicates that cellulases and lipases are

Table-1.2 Functions and status of different enzymes used in pulp and paper industry

Enzymes	Substrate	Functions	Application in paper industry	Status
Cellulase	Cellulose	Partial Hydrolysis of cellulose	Fiber modification	Commercialized
		The release of the ink from the fibre surface	De-inking	Commercialized
		Hydrolysis of the colloidal material in paper mill drainage	Drainage improvement,	
Xylanase	Xylan	Degradation of redeposited xylan and lignin carbohydrate complexes	Bleach boosting, deinking of newsprint and magazines	Commercialized
Mannanase	Gluco-mannan	Removal of glucomannan	Bleach boosting	Commercialized
Laccase	Lignins	Degradation of the lignin in the presence of mediators (transition metal complexes)	Bleaching, Polymerization of lignans in process water	Close to commercialization
Mn-peroxidase	Lignins	Degradation of the lignin in the presence of additives	Bleaching	Not commercialized
Lipase	Fat/oil	The hydrolysis of triglyceride	Pitch control, contaminant control, deinking of oil based ink	Commercialized
Amylase	Starch	Hydrolysis α-1,4 or α-1,4 and/or α-1,6 bonds of starch	Starch-coating, de-inking, drainage improvement	Commercialized
Protease	Proteins	Hydrolysis of cell wall proteins	Biofilm removal	Not commercialized
Pectinase	Pectins	Hydrolysis the cambial layer	Energy saving in debarking, decreased cationic demand	Not commercialized

primarily applied in the processing of paper, whereas xylanase and laccase are more commonly found in the bleaching and delignification processes (Demuner 2011).

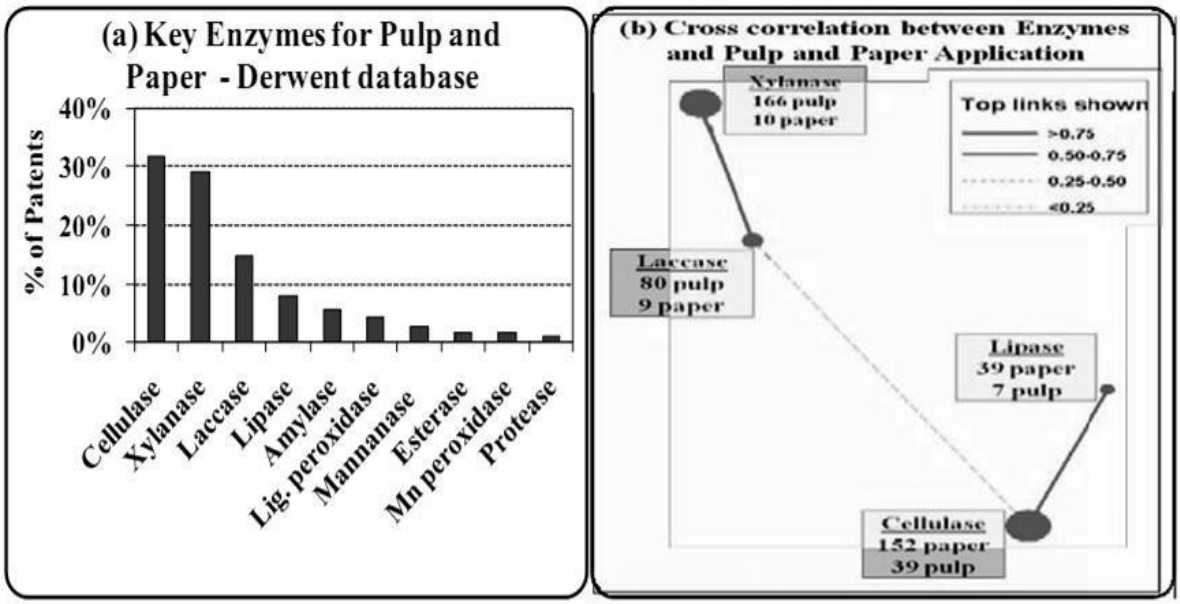


Figure-1.11 (a) Key Enzymes extracted in the patents from the derwent database (from 1963 to 2010) and (b) their major applications in pulp and paper (Adapted from Demuner2011)

1.2.11. Recycling of waste papers

The pulp and paper manufacturing industry is one of the largest wood consumers today. Along with increasing world economic growth, a substantial increase in paper consumption is expected. This means that more solid waste will be created as paper products are consumed and disposed off because of the environmental and economic concerns associated with the consumption of our forest resources. The paper industry could well experience a limited raw material resource with concurrent reduction of industry growth. Therefore, recycling of paper as a solution to this problem is attracting more and more attention since it is an effective way to preserve forest resources and save energy and landfill space (Bajpai and Bajpai 1999).

1.2.12. Advantages of recycling

There are several advantages of using recycled waste paper over virgin fibres as mentioned below (Agnihotri 2007):

1. It reduces air, water and soil pollution.
2. It is an alternate source of conventional raw materials to produce paper, thus disposal problems are reduced
3. 60% less energy is required.
4. Recycling reduces the demand for virgin fiber
5. It reduces consumption of chemicals for pulping and bleaching, thus reduction in the formation of toxic by-products takes place.

6. Recycling of waste papers reduces consumption of water (75% less water than making it from virgin fiber) thus reduces cost of treatment of effluents (90% less aqueous effluent).
7. Improved technology is available for reuse of secondary fibers to obtain better quality products.
8. It is a reliable source of pulp in times of market pulp shortage.
9. Price is usually favorable in comparison with that of corresponding grades of market pulp (Agnihotri 2007).

1.2.13. Raw material for recycling

Papermakers are focusing on recycling as an economic necessity. Due to lack of any organized sector for the waste paper collection in India, imported paper waste comprising mainly old magazine (OMG) and mixed office waste (MOW), constitutes the main ingredient used for preparing recycled paper pulp. New deinking mills established in response to this projected need are already competing for the cleanest and most homogeneous postconsumer paper source, e.g., sorted white ledger and soon will have to dip deeper into the postconsumer paper stream, e.g., unsorted MOW-the most difficult raw material for deinking, to remain competitive (Gubitz et al. 1998, Prasad et al. 1993).

1.2.14. Deinking

A process for the detaching and removing printing inks from the fibres to be recycled to improve optical characteristics of the pulp and paper using recovered printed materials. Deinking involves dislodging of the ink particles from fibre surface (ink detachment) and separating dispersed ink from fibre suspension by flotation and/or washing (ink removal) (Ferguson 1992 a and b, Zhao et al. 2004). Unsatisfactory separation gives a paper with low brightness and dirt specks. Larger ink particles >50µm are visible to the naked eye as black or colored spots in the paper. The efficiency of this method depends on the technique and printing conditions, kind of ink and kind of printing substrate (Bajpai and Bajpai 1998, Prasad et al. 1993).

1.2.15. Deinking chemicals

Different deinking chemicals are used for conventional deinking during the hydrapulping and flotation process to remove ink, firstly from the repulped fibre and then from the pulp suspension. These chemicals and their functions are summarized in the Figure-1.12 (Ferguson 1992 a and b).

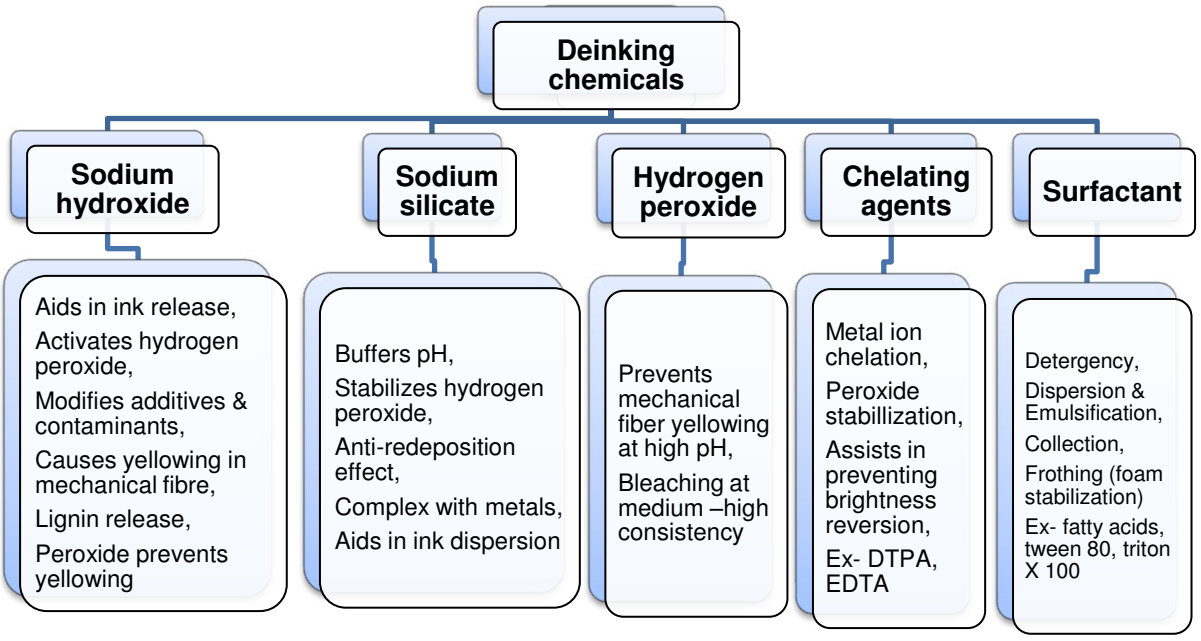


Figure-1.12 Deinking chemicals and their functions

1.2.16. Hydrapulping

Hydrapulping (Repulping) of the waste papers is carried out in the presence of sodium hydroxide, sodium silicate and hydrogen peroxide with a suitable dispersant in the hydrapulper. Hydrapulper provides the heat and mechanical agitation, which collectively helps to slush the fibres and detach the ink particles from the fibre surface (Gottsching and Pakarinen 1999).

1.2.17. Flotation deinking

Flotation is the process that separates materials based on the property of wettability. Under appropriate conditions, non polar (hydrophobic) materials are able to adhere to the air bubbles and rise to the surface. Large ink particles are desired (at least 5 microns, 10-50 microns ideally) (Snyder et al. 1993, Panek and Pfromm 1996).

It involves mainly 3 phases-

1. Collision of the ink particles with the air bubbles
2. Attachment of the ink particle- air bubbles complex
3. Separation of the ink particle-air bubble complex from the pulp

Flotation is performed generally at 1% pulp consistency (Cy), which is lower than washing. The flotation process is more chemical intensive than the washing process. It provides a high yield of fibers (Göttsching and Pakarinen 1999, Zhao et al. 2004). The combination of fatty acid and calcium alone are able to produce good deinking results due to the formation of soap which maintained the hydrophobicity of the toner particles. Even on little froth

formation using fatty acid, a stable layer consisting of soap flakes, fibers, and toner formed, is allowing the toner to be removed (Panek and Pfromm 1996).

1.2.18. Washing deinking

In washing, the particles separate primarily according to their size. Small particles such as fillers and coating pigments are easily washed out.

1. A water–ink emulsion system is formed with the particle sizes averaging below 1 micron.
2. The emulsion is washed from the pulp and the ink is removed from the wash water by flocculation. (Gottsching and Pakarinen 1999, Smook 1989)

1.2.19. Hybrid deinking processes

New deinking mills are using combined ink flotation and washing procedures. In the hybrid deinking process, bulk of the ink is removed by the flotation and residual ink by washing. Dispersants are added after the pulper but before washing. Hybrid deinking process is useful for paper wastes that contain many different types of ink formulations. Flotation removes ink by the saponification while the washing process eliminates ink by emulsification (Gottsching and Pakarinen 1999, Larson and Lafon 1997, Smook 1989).

1.2.20. Effective residual ink concentration (ERIC)

Different researchers have studied the determination of ERIC values for repulped pulp and deinked pulp obtained after ink and foam removal from flotation cell, so that residual ink remains in the sample of deinked paper can be determined in part per million (ppm) (Bansal and Agnihotri 2002, Jordan 1994, McCool 1993). This ERIC value can be calculated by measuring the reflectance in the infrared region of spectrum (950nm) and manipulating reflectance via Kubelka Munk analysis until the ERIC is computed.

Lorentzen and Wettre (071/70) Elrepho instrument uses this principle to provide these values of ERIC in sheets of paper. Reflectance measurements of paper sheets at about 950 nanometer (nm) can give a useful measure of ERIC particles in the paper sheet. The concentration of ink particles 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 over the sheet of paper. In spite of these limitations, ERIC shows a better correlation with ink removal in a deinking operation than the brightness (Bansal and Agnihotri 2004).

1.2.21. Deinking efficiency (DE)

It denotes the ratio of amount of ink removed, to the amount of ink present before deinking.

$$\mathrm{DE} = \left[\frac{\mathrm{EP} - \mathrm{EFw}}{\mathrm{EP} - \mathrm{EB}}\right] \times 100\%$$

Where,

DE= Deinking efficiency based on ERIC value (%),

EB= ERIC value in the absence of ink particles,

EFW= ERIC value after flotation and washing deinking,

EP= ERIC value of the sample before ink removal (after pulping) (Agnihotri 2007)

1.2.22. Printing processes

Paper printing can be performed either by impact or nonimpact processes. Newsprints are generally printed by impact printing in which ink does not fuse with the paper. Therefore, these inks are easy to disperse and remove during the washing/flotation process. On the other hand, non-impact printing processes are used in the photocopier, laser and inkjet printing. These inks fuse with the paper, thus making it non-dispersible, and difficult to remove by deinking processes (Jeffries et al. 1994).

1.2.23. Toner ink

The chemical composition of the toners is different from the conventional ink in that instead of oil or water based carrier, they are mainly composed of thermoplastic resin, typically a styrene/acrylate or styrene/butadiene co-polymer with 5-10 wt% dispersed carbon black as a pigment and small amount of charge carrier and dry lubricants. A large amount of iron oxide is also added in some toner to give it magnetic property (Quick and Hodgson 1986, Seldin 1985).

This toner powder is electro-statistically transferred to the paper under the heat and pressure during the printing process. As a result, toner powder is fused to itself and sticks with the paper fibres to form a flat particle on the page. During the pulping process, when sodium hydroxide was added to remove the toner from the fibres, then the fibre swells, resulting in the breakage of toner into large flat flakes of different sizes from 10 microns up to a millimeter (Borchardt et al. 1996, 1995; Shrinath et al. 1991).

1.2.24. Difficulty in removal of toners

During the toner removal, most important thing is to remove larger particles because they appear in the paper sheet as a large ink specks. These large ink particles do not float because

(i) They do not attach to the bubble after collision because either they are deflected from the bubble surface or the contact time is too short for the thin film to rupture; or

(ii) The particle initially attaches to the bubble but is not retained because the detachment forces from the gravity and the surrounding flow are greater than the attachment forces (Schmidt 1996).

Photocopier paper is the best grade of paper made up of kraft pulp and this costly paper is not easy to recycle. Less than 6% of printing and writing quality paper was recycled into any paper form. Less than 1% was returned to the previous quality. Indeed, the toners of photocopiers and laser printers are difficult and expensive to remove by conventional chemical methods (Jeffries et al. 1993, 1994). Most of the conventional deinking techniques require large amounts of chemical agents, such as sodium hydroxide, sodium carbonate, diethylenetriaminepentacetic acid, sodium silicate, hydrogen peroxide and surfactants thereby resulting in a costly wastewater treatment to meet the environmental regulations (Prasad et al. 1993, Woodward et al. 1994). Alternatively, enzyme usage has been reported to be a potentially efficient and less polluting solution to overcome this disposal problem (Prasad et al. 1993, Putz et al. 1994).

1.2.25. Enzymes used in deinking

Enzymatic treatment is a process, which gives better performance to reach desired deinked pulp properties. Many scientists perform the study of enzyme application in the deinking. Several enzymes such as cellulases, hemicellulases, pectinase, lipase, esterase, α-amylase and lignolytic enzymes have been used for deinking of various recycled fibres. However, the main enzymes used for deinking are cellulases and hemicellulases. Also many patents for the use of enzymes in deinking have been granted or filed till date (Bajpai and Bajpai 1998).

1.2.26. Mechanisms of enzymatic deinking

Different mechanisms for ink removal by enzymes have been proposed. The explanation for the performance has to be credited to the specific activity of that enzyme because enzyme cleaves specific bonds between sugar units.

Jeffries et al. (1994) proposed that deinking may be caused not by enzymes but by additives used to enhance enzyme production and stabilization. In addition, ink removal efficiency varies inversely with enzyme inhibition (Bobu and Ciolacu 2007). Kim et al. (1991) suggested that enzymes partially hydrolyze and depolymerize cellulose molecules at fibre surfaces, thereby weakening bonds between fibres and freeing them from one another. Ink particles simply are dislodged as fibres separate during pulping. Eom et al. (2007) pointed out that enzymatic treatment weakens bonds, perhaps by increasing fibrillation or removing surface layers of individual fibres. Catalytic hydrolysis may not be essential; enzymes can remove ink under non-optimal conditions. Mere cellulase binding may disrupt fibre surfaces in a manner and to an extent sufficient to release ink during pulping (Vyas and Lachke 2003). Hemicellulases facilitate deinking by breaking lignin-carbohydrate complexes and releasing lignin from fibre surfaces (Treimanis et al. 1999). Ink particles are dispersed with the lignin. Cellulase and hemicellulase treatment facilitated ink removal from newsprint, and was accompanied by release of lignin (Bobu and Ciolacu 2007). Cellulases peel fibrils from

fibre surfaces thereby freeing ink particles for dispersal in suspension (Kim et al. 1991). After enzymatic treatment of secondary fibre, pulp freeness increases due to peeling mechanism (Lee et al. 1983). Enzyme doses and reaction times, however, seem too low to cause measurable cellulose degradation (Jeffries et al. 1994). Mechanical action is also important and prerequisite to proper enzymatic activity (Zeyer et al. 1994). However, this mechanical action was also responsible for the distortion of cellulose chains at or near fibre surfaces, thereby increasing susceptibility to enzymatic attack. Assuming that fibre-fibre friction increases as the pulp consistency increases. Such an explanation appears trustworthy with earlier results that enzymatic deinking is more efficient at medium consistency than low consistency (Jeffries et al. 1994). Other research, however, disputes the importance of mechanical action (Putz et al. 1994). No improvement in brightness was observed when higher shear forces were applied at higher consistencies or for extended times. Enzymes can be denatured at higher shear forces caused by fibre-fibre friction (Bajpai and Bajpai 1998).

Enzymatic effects may be indirect, i.e., they remove microfibrils and fines, thereby improving freeness and facilitating washing or flotation (Jeffries et al. 1994). Putz et al. reported that fines content is not always reduced during enzymatic deinking (Putz et al. 1994). Ink particle hydrophobicity of the nonimpact-printed papers was increased after enzymatic treatment due to the removal of the fibrous material from ink particles, thereby facilitating separation during flotation (Jeffries et al. 1994). This promising hypothesis should be tested with a broad range of enzymes, paper grades, and inks. Zeyer et al. (1994) presented a proof that only easily accessible cellulose chains are subject to enzymatic cleavage. For the removal of a significant amount of ink, mechanical action as surface friction on the fibre is able to improve this restriction by opening the outermost layers of the fibre to fully expose the cellulose chains. Lipases and esterases degrade carrier (vegetable oil) and thus disperse pigments in ink (Morkbak et al. 1999, Morkbak and Zimmerman 1998b).

1.2.27. Factors affecting enzymatic deinking

It is necessary for the successful deinking that operating conditions must be optimized such as temperature, pH, reaction time, pulp consistency, enzyme dose and mechanical action. The activities of the cellulases and hemicellulases are mostly influenced by the pH of medium. Enzymes have the optimum value in acidic pH, alkaline or in the neutral pH. Jeffries et al. (1994) did not adjust the pH slurry (initial pH 5.5-7.5), when worked with enzymes. On the other hand, an initial slurry of the alkaline sized nonimpact paper (pH 8.5) requires adjustment of the pH levels with the help of the acid, because the enzymes works in the pH range of 5.5-7.5. Thus acidic conditions may be beneficial in terms of cost, because lesser yellowing of the paper was reported in the acidic conditions (Kim et al. 1991). Higher dose and long reaction time both can reduce the viscosity of the pulp and damage the fibres.

Enzymatic deinking is more efficient at medium consistency than low consistency (Jeffries et al. 1994). Enzymes have been used before pulping steps (Kim et al. 1991), during mixing (Elegir et al. 2000) and during pulping (Jeffries et al. 1994). Putz et al. (1994) reported and clearly demonstrated that enzyme addition during initial mixing of paper and reaction medium was most effective. Longer presoaking times decreased brightness, presumably due to reduced ink particle size. Longer presoaking time allowed finely dispersed ink particles to re-adhere to fibre surfaces or to penetrate into porous parts of fibers, thereby limiting effectiveness of flotation. Soaking after pulping, but before flotation, adversely affected deinking. This result was also attributed to re-adherence of ink particles to fibres (Kim et al. 1991). Most of the enzymes work within the temperature range of the 40-65°C and above this temperature the enzyme will be denatured.

1.2.28. Application of enzymes and their effectiveness

In 1991, Kim et al. showed that crude cellulases applied to pulps could facilitate the deinking process (Kim et al. 1991). A deinking efficiency of almost 73%, by the enzyme combination of cellulase and hemicellulase of *Aspergillus niger* was obtained for enzymatic deinking of laser printed waste papers on a laboratory scale (Lee et al. 1983). Selvam *et al*. (2005) has studied white rot fungi *Fomes lividus, Thelephora sp. and Trametes versicolor* and their enzymes such as lignin peroxidase (LiP), manganese dependent peroxidase (MnP), laccase and mixture of these enzymes for deinking waste papers with respect to kappa number and brightness of paper produced. Immobilized cellulase can produce superior deinking results than soluble cellulose (Zuo and Saville 2005). Previously, researchers have explained that the deinking effect of the lipase was caused by a partial degradation of the binder of the soybean oil-based inks, thereby releasing the ink particles from the paper(Morkbak et al. 1999, Morkbak and Zimmerman 1998b). For the first time, Gubitz et al. (1998) combined magnetic deinking technique with the application of enzymes. At a deinking efficiency of 94%, they measured a yield loss of only 2.8% after enzymatic–magnetic deinking, while flotation deinking was reported to reach yield losses of up to as much as 15%**.** The bacterial α-amylase produced the greatest ink particle reduction on coated colored printed magazine (Elegir et al. 2000). Viesturs et al. (1998) suggested that, for alkaline papers, the majority of inks are localized on the paper coatings and fillers consisting mainly of $CaCO_3$. Enzyme treatment improved by stock acidification and dissolution of $CaCO_3$ prior to flotation resulted in effective detachment and dispersion of toner specks ensuring a high deinking effectiveness. The enzymatic treatment is sometimes related to the ink films fragmentation (Kim et al. 1991, Treimanis et al. 1999), thus being necessary to control the enzymatic action in order to maintain the ink size in a range suitable to the separation process.

1.2.29. Role of surfactants in enzymatic deinking

Numerous studies have reported on deinking using enzymes with surfactants. Understanding the interaction and the relationships between enzymes and surfactants will be very helpful when using enzymes for the deinking. In subsequent studies, some researchers (Elegir et al. 2000, Jeffries 1993, Prasad 1993, Treimanis et al. 1999, Zeyer 1994) showed that enzymatic deinking is most effective when enzymes are used during high consistency fiberization in the presence of non-ionic surfactants. Kaya et al. (1995) showed that the surfactants could be used as both accelerators and inhibitors. The activity of the enzymes increased with nonionic and cationic surfactants. From circular dichorishm analyses and studies, it was revealed that the molecular structure of the enzymes was changed by the addition of nonionic and some anionic surfactants. As the concentration of the different anionic and some cationic surfactants increased, the activity of the enzymes decreased to different degrees. Treimanis et al. (1999) have treated the laser-printed wastepaper samples with a commercial cellulase, Celluclast 1.5L, and lipase, Resinase A2X, and reported enhanced removal of toners by promoting the toner dispersion in smaller particles. During the flotation deinking, the addition of an appropriate surfactant, such as hydrocarbon oil proved to be a necessary factor to prevent the redeposition of microink particles on the fibre surfaces and promote the separation of highly dispersed toner particles from the fibre network (Viesturs et al. 1998). Elegir et al. (2000) used a non-ionic surfactant, along with a cellulase and commercial α-amylase mixture that assisted deinking process of xerographic office wastepaper. The area coverage of the residual toner particles, measured by image analyzer, was decreased by ≤96%. Deinking of electrostatic wastepaper with commercial cellulolytic enzymes and surfactant in neutral pH was resulted into higher freeness, strength properties and lower residual ink (Eom et al. 2007). Although the use of surfactant improves deinking efficiency but it may alter the fibre's surface properties, reducing the strength of the inter-fibre bonds and decreasing the paper strength due to the adsorption of surfactant onto the fibre's surface (Elegir et al. 2000, Pala et al. 2004). Operating cost and environmental effect can be minimized if the enzymatic deinking do not use surfactants and alkaline deinking chemicals, such as the deinking of the alkaline sized nonimpact-printed paper didn't require the surfactant because calcium carbonate generates adequate froth during flotation (Prasad et al. 1993).

1.2.30. Enzymatic deinking with various mono-components of enzymes

Elegir et al. (2000) reported that among cellulases mono-component, endoglucanases were more efficient than crude cellulase for deinking of mixed office waste. Extracellular endoxylanase and endoglucanase enzymes obtained from xylan-grown *Aspergillus terreus* CCMI 498 and cellulose-grown *Trichoderma viride* CCMI 84 showed better strength and ink removal properties for mixed office waste paper deinking (Marques et al 2003). Vyas and

Lachke (2003) separated two extracellular alkali-stable 1,4-α-D-glucan-4-glucanohydrolase (EC 3.2.1.4) fractions, i.e., Endo A and Endo B from the culture filtrate of an alkali-tolerant *Fusarium* strain. The enzyme treatment resulted in the increase in brightness with the decrease in ink counts of the recycled paper. Based on the distinct properties of endoglucanases, a probable mechanism of enzymatic deinking process is presented schematically (Vyas and Lachke 2003). Furthermore, although enzymes favour ink removal, their action (in higher dose) significantly affects the paper strength properties (Magnin et al. 2002). Gubitz et al. (1998) treated laser-printed wastepaper individually and with combinations of purified endoglucanases from *Gloeophyllum sepiarium* and *Gloeophyllum trabeum* and found that pure endoglucanases were responsible for most of the success in deinking (94%) with increased freeness, slightly decreased intrinsic fibre strength and unaffected or even marginally improved hand sheet's strength.

Most cellulases comprise modular multidomain proteins containing at least three separate structural elements of different functions i.e., a catalytic domain, a cellulose binding domain (CBD), and an interdomain linker. During hydrolysis, cellulose is “captured” and brought close to the catalytic site, in the proper orientation, by the cellulose binding domain and it is then catalyzed by the catalytic domain. It is reported by Li and Xu (2002) CBD alone has a negative impact on deinking efficiency. They prepared and purified a fusion protein containing a CBD from *Cellulomonas fimi* endoglucanase for deinking of a mixed office paper (MOP). Endoglucanases lacking a CBD could result in superior deinking effects and strength properties (Geng et al. 2003).

1.2.31. Enzymatic deinking in mill trials

Knudsen et al. (1998) achieved dirt speck reduction of 35% along with a brightness increase of 2.2% for the final product (writing and printing paper was prepared from low grade office and printhouse waste at Stora Dalum Deinking Plant). Stickies were reduced by ~ >50% (indexed area from 100 to 46) and the mill throughput was increased by ~ 8 tons/day (from 215.5 to 223.7 tons/day). The conversion was at least cost-neutral. Magnin et al. (2002) using a mixture of thermoxylanase, mannanase, lipase and amylase observed lowering in specks contamination in the floated pulp to 88% lower with respect to that observed with alkaline treatment. Based on these promising results, full-scale enzymatic deinking was carried out at a French mill. Increased brightness by 4 to 6 points, lower residual ink content, maintained mechanical strength and no runnability problem on paper machine, were obtained with the enzymatic deinking as compared to the mill chemical deinking. A full scale, enzymatic deinking experiment was carried out at the Moulin Vieux Mill in France, a mill which produces deinked paper from printed coated wood-free papers (Saari et al. 2005).

With respect to the enzymes, which allow different deinking efficiencies, it was not possible

to establish a key activity (or activities) for ink removal. In fact, although the cellulolytic and/or xylanolytic activities are frequently related to effective deinking trials, it is difficult to establish the relative contribution of each one to the deinking process (Jeffries et al. 1993, Kim et al. 1991, Prasad 1993). The major contribution towards deinking process is of endoglucanases and xylanases. However, the wide range of results is probably due to the use of different type of paper samples, printing ink and enzymes obtained from different sources (Zeyer et al. 1994).

1.2.32. Environmental effects

Putz et al. (1994) found that enzymatic treatment produced almost half the chemical oxygen demand (COD) loads as compared to that produced by conventional deinking. However, some researchers have reported about 20-40% higher COD level in the process water than in the standard process (Magnin et al. 2002, Saari et al. 2005). The process water of enzyme (Novozyme 342) deinking had higher COD, but it is more easily biodegradable than that resulting from chemical deinking. Amount of generated sludge was lower in enzyme deinking and had lower inorganic content, providing potential to reduce the costs of sludge treatment (Bobu and Ciolacu 2007). Generally, it was expected that enzymatic deinking lowers the environmental load due to less or no use of chemicals. Due to the some reported cases of increased COD, it is needed that researches should be going on to reduce and find out the reason of increased COD in such cases. For the proper controlling of the biological oxygen demand (BOD) levels, it is required to control over enzyme dose and retention time more precisely to minimize cellulose hydrolysis. Reduction in pulp yields and the accompanying release of reducing sugars might be expected from hydrolytic action of cellulases and hemicellulases. The dangers are fibre loss and heightened BOD in the effluents.

1.2.33. Advantages and limitations of using enzymes in the deinking

Enzymatically deinked pulp showed superior physical properties, higher brightness and lower residual ink, no alkaline yellowing, increment in pulp freeness, improved drainage and better machine runnability than chemically deinking recycled pulp. In addition, dewatering and dispersion steps as well as subsequent reflotation and washing may not be essential. Their elimination could save capital costs and reduce electrical energy consumption. Requirement of bleach chemicals may be lower for enzymatic deinking. In general, enzymatic deinking reduces chemical use thus reducing the load on wastewater treatment.

Although there have been considerable advances in the application of biotechnology to paper recycling, enzymatic deinking processes still face problems that have limited their commercialization. Most of the commercially available enzyme products are too expensive to compete with conventional deinking chemicals. Enzymes are very sensitive to environmental

fluctuations. Enzymatic processes usually have a relatively narrow operating range with regard to pH, temperature, and storage time, and operating conditions must be precisely controlled in order to maintain enzymatic activity. Enzymatic processes are generally slower and may be difficult to retrofit into existing pulp and paper mill operations (Qian and Goodell 2005).The enzymatic process effectiveness depends more critically on the furnish characteristics than the chemical one. Considering the wide variability on the industrial wastepaper supplies, this is probably the most important disadvantage of this methodology (Pala et al. 2004). Reduction in pulp yields and the accompanying release of reducing sugars might be expected from hydrolytic action of cellulases and hemicellulases (when used in higher dose).

Keeping the above literature in mind, this research was carried out by focusing on the various points as mentioned below:

- ✓ Most of the cited studies reported the deinking of mixed office waste (MOW), consisting of photocopier papers with other type of papers, by using commercially available enzymes. Deinking agents should be chosen or prepared to facilitate deinking of those ink preparations which are hard to deink and affect the final deinking efficiency. Therefore, there is a need to study separate deinking of 100% toner containing waste papers (such as photocopier waste papers).
- ✓ The potential use of cellulase and xylanase in biotechnology has stimulated the need to discover suitable enzymes in large quantities. For the development of economically feasible process/technology, it is a prerequisite to screen suitable microorganisms as well as evaluation of nutritional (carbon and nitrogen sources) and environmental necessities (pH, temperature etc) and then, there is a need to identify various operating conditions for enzymatic treatment towards deinking to have significant positive impacts on pulp and paper properties.

In view of that, in the present study it was planned to investigate the application of crude cellulase and xylanase for deinking of photocopier waste papers and to evaluate the positive or negative impact of enzymes on the fibres with following objectives:

1. Isolation of fungi from different sources and screening of best cellulase and xylanase producers
2. To optimize different process variables for maximum cellulase and xylanase enzyme production by the isolated fungi
3. To optimize different process variables for improving the deinking efficiency of photocopier waste papers by (i) conventional chemicals (ii) commercial enzyme and (iii) crude cellulase and xylanase (produced in lab by isolated fungi) without affecting the strength and optical properties

4. To compare the deinking results of enzymatic deinking by crude cellulase and xylanase (produced in lab by isolated fungi) with the conventional chemicals and commercial enzyme
5. To develop basic mechanism of enzyme action on the fibre during deinking process

CHAPTER 2

MATERIALS AND METHODS

2.1. Materials

2.1.1. Chemicals and reagents

In the present work, the analytical grade chemicals, reagents and media were used for enzyme production and deinking experiments. These were procured from Merck (GR grade), Himedia Laboratories Pvt. Ltd. (AR Grade), Sigma Chemical Co. (St. Louis, MO, USA), and Qualigens Fine Chemicals (Fischer Scientific-AR Grade) (Annexure-1).

2.1.2. Lignocellulosic wastes (LCWs) and insoluble nitrogen sources

Different LCWs and insoluble N sources used in this study were collected from local area of Saharanpur, India.

2.1.3. Waste papers

Copier paper (sheet size 210x297mm, basis weight 75 gsm) from an Indian mill was used to produce photocopier waste paper after printing with Xerox Black Toner, 6R1046 (Part # 006R01046) used for Xerox Workcenter 238 photocopier machine. To reduce the variables in comparing repulping and flotation results, one set of ‘‘standard’’ printed sheets was prepared and used for all the experiments performed in this research work For e.g., one line of the print format used was as follows (Annexure-2):

“abcdefghijklmnopqrstuvwxyz,1234567890.?abcdefghijklmnopqrstuvwxyz,1234567890. ?!%&()<>/[]”

2.1.4. Enzymes used for deinking

For the enzymatic deinking, the commercial enzyme was supplied by the Indian supplier, while the crude enzymes were produced by isolated fungi in our laboratory. The characteristics of enzymes used for deinking were listed in the Table-2.1. The enzymes were stored at 4 °C prior to use (Annexure-3 and 4).

2.1.5. Microbiological tools, glasswares and plasticwares

The microbiological tools used for inoculation viz., cork borer, forceps, needles and loops were supplied by Hi- media, India. Cotton plugs were hand made using sterilized cotton. Glasswares such as blue cap bottles, Petri plates, beakers, conical flasks, test tubes, measuring cylinders were procured from Hi-media, Borosil and Rankem, India etc. and plasticwares like beakers, microtips, centrifuge tubes, vials etc were also obtained from Axiva, Hi-media, India (See Annexure-5).

Table-2.1 Characteristic of commercial enzymes and lab produced enzymes used for deinking

Enzyme nature	Cellulase	Crude cellulase and xylanase
Trade name	OPTIMASE™ CX 40L	N/A
Microbial source	*Trichoderma viridae*	To be isolated (two fungal strains)
Supplier	Genencor International, New York	To be produced in laboratory
pH	4.0-7.0	To be determined
Temperature, °C	40-65	
Dose, IU/g	4-18	
Activity, IU/ml	996±48	

2.2. Methods

2.2.1. Isolation and screening of fungi for the production of cellulase and xylanase enzyme

2.2.1.1. General practices

2.2.1.1.1. Sterilization of glasswares

Glasswares were washed with detergent and thoroughly rinsed with running tap water. Prior to use, these were kept in laboratory oven at 160 °C for 2 h for the dry sterilization. The glasswares along with the medium, plasticwares, cotton plugs and inoculating items were autoclaved at 15 psi for 15 min in an autoclave (wet sterilization). Inoculating items were also subjected to dry red heating in flame before inoculation.

2.2.1.1.2. Disinfection of inoculation chamber and inoculation room

Before inoculation, the inoculation chamber was first wiped with moist cotton wool with 70% ethyl alcohol, which was followed by exposure to ultra-violet (UV) radiation for 30 min. Inoculation chamber and room were disinfected by fumigation method weekly, in which about one teaspoon of potassium permanganate ($KMnO_4$) was kept on cotton wool on the bottom part of Petri-plates, and then formalin (40% formaldehyde solution) was then poured over $KMnO_4$ to wet the cotton completely. The fumes coming out in the air kill microbial spores in the surroundings.

2.2.1.2. Preparation of culture media for fungal growth and fermentation

2.2.1.2.1. Pretreatment of the LCWs

The lignocellulosic substrates were washed twice or thrice with the warm water to remove any type of impurities like dust, starch, crop residue and any other. The washed material was retained on the 100-mesh size screen and then immediately dried in sunlight. Thus, obtained dried lignocellulosic substrates were grinded in a laboratory grinder to obtain fine particles in form of fine particles (if required). These fine particles were passed through 100-mesh size

screen and the fractions so obtained on screen were stored in polyethylene bags for further use.

2.2.1.2.2. Preparation of culture media plates

The wheat bran agar (WBA) as culture medium, consisting of 4% wheat bran (WB) (w/v) and 2% agar (w/v) was used. The medium was prepared having pH 6.0±0.1 and then autoclaved at 15 psi for 15 min. After autoclaving, the medium was cooled down to about 45 °C and 185 µg/ml of chloramphenicol was added as an antibacterial agent (Pellinen et al. 1989). The medium was then poured aseptically in sterile Petri-plates and stored at 4 °C until used.

2.2.1.2.3. Fermentation media preparation

In all the experiments, the media was prepared in the 250 ml Erlenmeyer flasks for both the test fungi with 5 g of WB oven dry (o.d.) basis and 15 ml of nutrient saline solution (NSS) added in each flask unless mentioned otherwise (Table-2.2).

Table-2.2 Composition of NSS (Mandels and Reese 1957)

Ingredients	**Concentration, g/l**	**Ingredients**	**Concentration, g/l**
Yeast extract	3	$FeSO_4$	0.005
K_2HPO_4	2	$MnSO_4$	0.002
$CaCl_2$	0.3	$ZnSO_4.7H_2O$	0.0016
Tween-80	2	$CoCl_2$	0.0014
$MgSO_4$	0.3		

The pH was adjusted as required (optimum pH of both test fungi) with 1 N HCl and NaOH using pH meter. The content of these flasks were properly mixed with a glass rod and then capped with the cotton plugs. The flasks were autoclaved and kept for cooling at room temperature in the inoculation chamber under UV light exposure. Then six discs (8 mm diameter) from 6 d old culture of each test fungi were inoculated in different flasks. These flasks were incubated at optimum temperature and were harvested after optimum days of incubation.

2.2.1.2.4. Harvesting and storage of enzyme

After incubation for optimized time under SSF conditions, the flasks were taken out from incubator and 15 ml of citrate buffer (pH 4.8, 50 mM) was added to fermented matter for the extraction of enzyme. Then, the fermented matter along with the fungal mycelia was crushed with a glass rod with soft hand just to squeeze the crude extract. Thus obtained crushed fermented matter was filtered through three layered cheesecloth at room temperature. After that, this crushed fermented matter was kept for shaking for 15 minutes in shaker incubator at room temperature. This filtrate was centrifuged at 10000 x g for 15 min at 4 °C. The dark brown colored supernatant was stored at 4 °C. In all the experiments CMCase, FPase,

Xylanase activity and supernatant protein concentration were measured as described in next section.

2.2.1.3. Analytical methods for the estimation of cellulase and xylanase production

2.2.1.3.1. Endoglucanase production

The activity of endoglucanase component of the cellulase system was determined as carboxy methyl cellulase (CMCase) activity using carboxy methyl cellulose (CMC) as a substrate (Ghose 1987). The reducing sugars were measured by dinitrosalicylic acid (DNS) reagent method. 0.5 ml of enzyme preparation in appropriate dilutions as well as undiluted was thoroughly mixed in 30 ml screw capped universal sterile tubes containing 0.5 ml of 2% (w/v) CMC, as a substrate prepared in 0.05 M citrate buffer (pH 4.8). The reaction mixture was incubated in a water bath at 50 °C for 30 min. Controls were routinely included in which heat killed enzyme (20 min at 100 °C) and substrate treated similarly. 3 ml of DNS reagent was added to 1 ml of the reaction mixture. This was followed by boiling of the reaction mixture on a vigorously boiling water bath for 15 min for production of color. Afterwards, the tubes were kept in an ice bath immediately to stop the reaction. 20 ml distilled water (D.W.) was added to each tube and the mixture was thoroughly mixed and optical density (O.D.) was measured at 540nm in a UV-Vis spectrophotometer. Reducing sugars released from CMC were determined by comparing the values with the standard curve prepared by D-glucose (Annexure-6a). The enzyme activity was expressed as micromoles of D-glucose equivalents released per min at 50 °C i.e. international unit per milliliter (IU/ml). Production of endoglucanase was expressed in terms of carbon source as IU/g dry substrate (gds) as follows:

$$\text{Endoglucanase production (IU/gds)} = \frac{\text{CMCase activity (IU/ml)x Volume of enzyme extracted(ml)}}{\text{weight of LCW (g)}}$$

2.2.1.3.2. Total cellulase production

The total cellulase activity, as filter paper (FPase) activity, was determined using filter paper as a substrate and DNS as reagent (Ghose et. al. 1987). 0.5 ml of enzyme preparation in appropriate dilutions as well as undiluted was thoroughly mixed in 30 ml screw capped sterile tubes containing 1 ml of 0.05 M citrate buffer (pH 4.8). After pre-incubation, 50mg strip of whattman No. 1 filter paper (rolled) was kept in the mixture. The reaction mixture was incubated in a water bath at 50 °C for 60 min. Controls were routinely included in which heat killed enzyme (20 min at 100 °C) and substrate treated similarly. 3 ml of DNS reagent was added to 1 ml of the reaction mixture. Then the tubes were kept in on a vigorously boiling water bath for 15 min for production of color. Afterwards the tubes were kept in an ice bath immediately to stop the reaction. 20 ml D.W. was added to each tube and the mixture was thoroughly mixed and O.D. was measured at 540nm in a UV-VIS spectrophotometer.

Reducing sugars released from FP were determined by comparing the values with the standard curve prepared by D-glucose (Annexure-6b). The enzyme activity was calculated as µmoles of D-glucose equivalents released per min at 50 °C (FPU/ml). Production of total cellulase was expressed in terms of carbon source as FPU/gds as follows:

$$\text{Total cellulase production (IU/gds)} = \frac{\text{FPase activity (FPU/ml)x Volume of enzyme extracted(ml)}}{\text{weight of LCW (g)}}$$

2.2.1.3.3. Xylanase production

The xylanase activity was determined by measuring the release of reducing sugars using birchwood xylan as a substrate. The amount of reducing sugars in the reaction tubes was measured using DNS reagent method (Bailey et al. 1992). 0.2 ml of enzyme preparation (the filtrate after removing pellets) was added in a sterile tube, which contained 1.8 ml of substrate suspension (1% birchwood xylan in citrate buffer, 0.05M, pH 5.3) at 50 °C. Appropriate controls (as described above) were also included. The assay mixtures were incubated at 50 °C for 15 min. 3 ml of DNS reagent was added to it to stop the reaction. These well stirred assay mixtures were then boiled for 15 min, followed by quick cooling in cold water and the absorbance as O.D. was measured at 540 nm using a UV-Vis spectrophotometer. The amount of reducing sugars was calculated from the standard curve based on the equivalent xylose (Annexure-6c). The unit of activity was the amount of enzyme needed to release 1 µmol of xylose released per min at 50 °C.

$$\text{Total xylanase production (IU/gds)} = \frac{\text{Xylanase activity (IU/ml)x Volume of enzyme extracted(ml)}}{\text{weight of LCW (g)}}$$

2.2.1.3.4. Supernatant protein concentration

The supernatant protein concentration was determined by the Lowry method (Lowry et al. 1951). Complex forming reagent was prepared immediately before use by mixing the stock solutions A, B, C (Solution A: 2% (w/v) Na_2CO_3 in D.W, solution B: 1%$CuSO_4.5H_2O$ in D.W. Solution C: 2% (w/v) sodium potassium tartarate in D.W. in proportion 100:1:1 v/v respectively. 0.1 ml of 2N NaOH was added to 0.1 ml of sample. Then this solution was hydrolysed at 100 °C for 10 min in a boiling water bath. This hydrolysate was cooled at room temperature, and then 1 ml of the freshly mixed complex-forming reagent was added. Then the solution was kept stand still at room temperature for 10 min, afterward, 0.1 ml of Folin reagent was added using vortex mixture and kept for 30 min at room temperature. After 30 min, the absorbance was read at 750 nm using a UV-Vis spectrophotometer and the concentration of supernatant protein in each sample was estimated from a calibration curve constructed using bovine serum albumin (BSA) as a standard (Annexure-6d).

2.2.1.3.5. Biomass estimation

Glucosamine content was estimated following the protocol as described by Blix (1948). Fungal biomass was estimated by determining the N-acetyl glucosamine released by the acid hydrolysis of the chitin of fungal cell wall (Blix 1948). For this, 0.5 g (dry wt. basis) of fermented matter was mixed with concentrated sulphuric acid (2 ml) and the reaction mixture was kept for 24 h at room temperature (30 °C). This mixture was diluted with distilled water to make 1 N solution, autoclaved (at 15 psi for 1 h), neutralized with 1 N NaOH and the final volume was made to 100 ml by adding distilled water. This solution (1 ml) was mixed with 1 ml acetyl acetone reagent (freshly prepared by adding 1 ml acetyl acetone in 50 ml 0.5 N sodium carbonate). It was incubated in a boiling water bath for 20 min. After cooling, ethanol (6 ml) was added followed by the addition of 1 ml Ehrlich reagent (2.67g p-dimethyl amino benzaldehyde in 1:1 mixture of 50 ml ethanol and 50 ml concentrated hydrochloric acid). This mixture was incubated at 65 °C for 10 min. After cooling, the O.D. of the reaction mixture was read at 530 nm against the reagent blank using UV-Vis spectrophotometer. N-acetyl glucosamine was used as the standard (Annexure-6e). The results were expressed as mg glucosamine per gram dry substrate (gds).

2.2.1.4. Isolation and screening of fungi

2.2.1.4.1. Isolation of fungi

A total of 36 decaying wood, 38 humus, and 26 soil samples were collected from various sites near Saharanpur, Hardoi, Dehradun, Haridwar, Nainital, Chandigarh, Bokaro and Jaipur located in India. Out of these 100 samples, 104 strains of fungi (30 from wood, 48 from humus and 26 from soil) were isolated. The methodology for the isolation of fungi from different samples is briefed below:

(I) For the isolation of fungi from wood samples, decaying wood samples were kept in 90 mm diameter glass Petri-plates enriched with moist wheat bran. These Petri-plates were incubated at 32±2 °C in a BOD incubator and the growth was observed every day. To maintain the moisture the plates were moistened with sterile tap water carefully to avoid any free water for better growth of fungi. The mycelia from the fruiting bodies of growing fungal cultures were transferred aseptically onto potato dextrose agar (PDA) plates (enrichment method).

(II) Fungi from the soil and humus samples were isolated by serial dilution method on PDA plates.

All the plates were incubated at 32±2 °C in a BOD incubator. The purified fungi isolates were stabilized on same medium by regular sub culturing. The purified cultures of fungi were maintained at 4 °C over PDA slants. These were routinely sub cultured onto PDA slants after

every 2-3 months. The spores were preserved in 15% (v/v) sterile glycerol; and stored at -20 °C in deep freezer for further studies.

2.2.1.4.2. Screening of isolates for potent cellulase and xylanase producers

A set of highly selective procedures were used to select the fungal strains that were best among the isolates in terms of cellulase and xylanase activity.

2.2.1.4.2.1. Primary screening

2.2.1.4.2.1.1. Congo red plate assay technique (fungal inoculation method)

The 104 fungal strains isolated in the present work were screened for their abilities to produce extracellular cellulases and xylanases during growth on CMC-agar and xylan-agar medium respectively. The medium contained 1% w/v CMC or birch wood xylan (as sole carbon source) with basal salt medium and 2% agar (Addleman and Archibald 1993). Both the plates were inoculated in the centre with 6 d old pure culture of isolates grown on PDA medium and incubated at 32±2 °C until substantial growth was recorded. These Petri-plates were flooded with Congo red solution (0.5% w/v Congo red). After 15 min, this Congo red dye was discarded and the plates were washed (destained) with 1M NaCl solution. The plates were then observed with naked eye for appearance of clear zones around the fungal cultures against the red background, which has appeared only if the fungal enzymes had utilized the cellulose/ xylan (Teather and Wood 1982). The presence of clear zone indicated that the fungal enzymes had utilized the cellulose/xylan in the medium.

2.2.1.4.2.2. Secondary screening

2.2.1.4.2.2.1. Measurement of cellulase and xylanase activities after SSF

The aim of screening was to select isolates exhibiting the highest cellulase activity along with xylanase activities. The fungal isolates, exhibiting areas of clear zones in Congo red test, were further subjected to SSF conditions to determine their actual cellulase and xylanase activities. For the screening, WB was used as sole carbon source. D.W. was used in place of NSS for the SSF. The enzymes from the fermented matter were harvested on 3 to 8th day after achieving full growth on the WB (depends on growth rate of isolates) and the cellulase and xylanase activities of enzyme samples from each isolate were determined. The supernatant protein concentrations of the enzyme samples were also determined.

2.2.1.4.2.2.2. Congo red plate assay technique (enzyme pouring method)

The extracellular cellulases and xylanases enzymes produced from cellulase and xylanase positive fungi recovered in the present work were screened for their abilities to produce extracellular cellulases and xylanases, to break down the CMC-agar and xylan-agar medium. The CMC-agar and xylan-agar medium contained 1% w/v CMC and 1% w/v birch wood xylan (as sole carbon source) respectively and 2% agar (Addleman and Archibald

1993). 4 mm well was made in the centre of CMC and xylan-agar plate with the help of borer. These wells were inoculated with 20 µl crude enzymes produced by positive fungal isolates and incubated at 42 °C. After 24 h of incubation, these plates were stained with Congo red solution (0.5% w/v Congo red) for 15 min and then destained with 1M NaCl. The plates were observed with naked eye for appearance of clear zones around the fungal cultures against the red background. The diameter of the clear zone was measured by means of zone measuring scale (Hi-media) in cm. (Teather and Wood 1982).

2.2.1.5. Identification of cultures

The two selected fungal isolates were sent to National Fungal Culture Collection of India (NFCCI), Pune, India, for further identification. The cultures were identified based on sequencing of the genomic DNA, and deposited at NFCCI, Pune, India with accession numbers.

2.2.1.6. Effect of different factors on fungal growth

2.2.1.6.1. Media

Eight different agar media including yeast extract agar (YEA), czapek dox agar (CDA), sabouraud dextrose agar (SDA), malt extract agar (MEA), xylan agar (XA), potato dextrose agar (PDA), CMC agar (CMCA), WB agar (WBA) were prepared to observe its effect on fungal growth. The pH of the media was maintained at 6.0 with 1.0 N NaOH/ H_2SO_4 after autoclaving. A disc of 6 mm diameter from 6 d old culture of test fungi was aseptically inoculated in center of each Petri-plate. These were incubated at 34 °C in a BOD incubator and growth was measured every 24 h of incubation respectively in terms of colony diameter (cm). The appearance of fungi was observed with naked eye every day.

2.2.1.6.2. Temperature

The test fungi were incubated at different temperature range i.e. 26 to 50 °C (at 4 °C interval) to observe its effect on fungal growth after the inoculation on the selected media.

2.2.1.6.3. pH

The pH of the selected media for both the test fungi were maintained in the range of 3.0 to 11.0 (at the difference of 0.5) with the help of 1.0 N NaOH/ H_2SO_4 before autoclaving and inoculated plates were kept at optimized temperatures for the respective fungi.

2.2.1.7. Different factors affecting extracellular enzyme production and supernatant protein concentration

SSF was carried out to study the effect of different ecological and nutritional factors on cellulase and xylanase production (Table-2.3). This was done to determine the most

favorable conditions for achieving enhanced levels of enzyme production conditions by the test isolates. WB was used as the substrate unless mentioned otherwise.

The enzyme production was optimized using 'one factor at a time approach- (OFAT)'. In which, one independent variable was changed at a time and the other variables were kept constant. For the optimization of enzyme production process parameters, the experiments were planned according to Table-2.4 and 2.5.

In every experiment, fermentation media were prepared and inoculated with $2x10^6$ CFU/gds of *T. harzianum* PPDDN10 NFCCI 2925 (spore forming fungi) and 4 discs of *C. cinerea* PPHRI-4 NFCCI-3027 (Basidiomycetes fungi). Enzymes were harvested after incubation at desired conditions (Table-2.4 and 2.5) and then different enzyme activities CMCase, FPase, and xylanase activities and supernatant protein concentration of the same were determined as described previously (section 2.2.1.3.). A schematic representation of experimental procedure has been shown in the Figure-2.1.

2.2.1.7.1. Fermentation type

The effect of culture conditions on xylanase production by the test fungi was studied by comparing the xylanase production under SSF, semisolid state fermentation (SmSF) and SmF in static as well as in shaking conditions (Table-2.3, V1; Conditions: Table-2.4, EPA1 and Table-2.5, EPB1). The amount of NSS was kept 15 ml, 45 ml, and 60 ml for SSF, SmSF and LSF respectively.

2.2.1.7.1.1. Three step extraction

To extract the maximum enzyme from the fermented matter, crude enzyme (obtained after fermentation process) was extracted with the extraction buffer (citrate buffer pH 4.8) in different NSS**:** extraction buffer volume ratios of 1:1, 1:2, and 1:3 (15, 30 and 45 ml) in single step as well as multi consecutive extractions (three steps extraction) for SSF. Extraction buffer was not added for SmSF and SmF to avoid further dilution of enzyme. The harvesting and storage of the crude enzyme were done as described in the section 2.2.1.2.4.

2.2.1.7.2. LCWs as carbon source

Natural lignocellulosic substrates, namely, corncob, groundnut shell, coconut coir, sugarcane bagasse, rice husk, rice bran, rice straw, wheat bran, wheat straw, and wood saw dust, singly (Table-2.3, V2; Conditions: Table-2.4, EPA2 and Table-2.5, EPB2) and in different combinations (Table-2.3, V3; Conditions: Table-2.4, EPA3 and Table-2.5, EPB3) were used.

2.2.1.7.3. Inoculum size

Different inoculum size were optimized in the range of $2x10^6$ to 10^7 spores of *T. harzianum* PPDDN10 NFCCI 2925 and 2 to 10 discs/5 gds of *C. cinerea* PPHRI-4 NFCCI-3027 (Table-2.3, V4; Conditions: Table-2.4, EPA4 and Table-2.5, EPB4).

Table-2.3 Factors affecting extracellular enzyme production

Parameters	Variable code (V)	Range
Type of fermentation	V1	Solid state static [SSF(S)], semi-solid state static [SmSF(S)], submerged state static [SmF(S)], solid state shaking [SSF(R)], semi-solid state shaking [SmSF(R)], submerged state shaking [SmF(R)]
LCW	V 2	**Types:** Corncob (CC), groundnut shell (GS), coconut coir (COCO), sugarcane bagasse (SCB), rice husk (RH), rice straw (RS), wheat bran (WB), wheat straw (WS) and wood saw dust (WSD)
	V3	**Combinations:** WB with SCB, RS and WS (3:1, 1:1, 1:3) for *Trichoderma harzianum* PPDDN-10 NFCCI 2925 WB with RH, RS and WS (3:1, 1:1, 1:3) for *Coprinopsis cinerea* PPHRI-4 NFCCI-3027
Inoculum size	V4	$2x10^6$ to $10x10^6$ CFU/gds for *Trichoderma harzianum* PPDDN-10 NFCCI 2925 and 2-10 discs/5g for *Coprinopsis cinerea* PPHRI-4 NFCCI-3027
Incubation days	V5	1-16 days for *Trichoderma harzianum* PPDDN-10 (1day interval) 1-30 days for *Coprinopsis cinerea* PPHRI-4 NFCCI-3027 (1day interval for first 16 days and then 2 days interval)
Temperature	V6	26 °C to 50 °C (interval of 4 °C)
pH	V7	3.0 to 11.0 (interval of 0.5)
Nitrogen source	V8	**(a) Inorganic soluble (0.01M to 0.04M of each):** Ammonium nitrate (AN), ammonium chloride (AC), ammonium ferrous sulphate (AFS), urea (UA), sodium nitrate (SN), and ammonium acetate (AA)
	V9	**(b) Complex Organic soluble (1, 2, 3, and 4g of each):** Tryptone (TRP), meat peptone (MTP), soya peptone (SP), peptone bacteriological (PB), malt extract (ME), proteose peptone (PP), yeast extract (YE), mycological peptone (MYP), and beef Extract (BE)
	V10	**(c) Insoluble Plant based (Total 5g):** Wheat bran (WB), mustard cake (MC), gram seed coat (GSC),soya bean meal (SBM) castor seed cake (CSC) masoor seed coat (MSC), WB+ MC 1:1, WB+ MC 3:1, WB+ MC 1:3, WB+ GSC 1:1, WB+ GSC 3:1, WB+ GSC 1:3, WB+ SBM 1:1, WB+ SBM 3:1, WB+ SBM 1:3, WB+ CSC 1:1, WB+ CSC 3:1, WB+ MSC 1:1, WB+ MSC 3:1, WB+ MSC 1:3
Surfactant	V11	Tween 20, tween 40, tween 60, tween 80, triton X100
Moisture ratio	V12	1:1, 1:2, 1:3, 1:4, 1:5
Particle size	V13	**X**>14, 14<**X**>22, 22<**X**>30, 30<**X**>36, 36<**X**>44, 44<**X**>52, 52<**X**>60, 60<**X**>72, 72<**X**>100, Mix Particle *i.e* unscreened (US), mixture of each PS (1:1)
Pretreatment	V14	NaOH (0.25%, 0.50%, 0.75%, NaOH (0.25%, 0.50%, 0.75%, and 1%) followed by autoclaving (A) (15 min)
Sugars	V15	Glucose (GLU), galactose (GAL), lactose (LAC), xylose (XYL), fructose (FRU) and cellobiose (CEL) (1, 2, 3, and 4 g/l of each)
Vitamins and amino acids	V16	L-ascorbic acid, D-biotin, cyanocobalamine, calcium pantothenate, folic acid, nicotinic acid, pyridoxine, riboflavin, and thiamine Asparagine, aspartic acid, cysteine, glutamic acid, glycine, isoleucine, leucine, metheonine, tryptophan, valine
Metal ions and chelating agent	V17	Mn^{+2}, Zn^{+2}, Fe^{+3}, Al^{+3}, Hg^{+2}, Ag^{+1}, Cu^{+2}, Ba^{+2}, K^{+1}, Co^{+3}, Na^{+1}, Ni^{+2}, Pt^{+5}, Cr^{+3}, Sr^{+2}, Sn^{+2}, Pb^{+2}, EDTA, DTPA

Table-2.4 Culture conditions for the enzyme production for the *T. harzianum* PPDDN-10 NFCCI-2925

Exp. Code	Type of fermentation	LCW	Inoculum size, ($x10^6$) CFU/gds	Incubation days, d	Temperature, °C	pH	Nitrogen source	Surfactant	Moisture level	Particle size	
EPA1	**V1**	WB	2	5	34	6.0	YE	T80	1:3	US	
EPA2	SS	**V2**	2	5	34	6.0	YE	T80	1:3	US	
EPA3	SS	**V3**	2	5	34	6.0	YE	T80	1:3	US	
EPA4	SS	WB	**V4**	5	34	6.0	YE	T80	1:3	US	
EPA5	SS	WB	4	**V5**	34	6.0	YE	T80	1:3	US	
EPA6	SS	WB	4	4	**V6**	6.0	YE	T80	1:3	US	
EPA7	SS	WB	4	4	34	**V7**	YE	T80	1:3	US	
EPA8	SS	WB	4	4	34	6.0	**V8**	T80	1:3	US	
EPA9	SS	WB	4	4	34	6.0	**V9**	T80	1:3	US	
EPA10	SS	WB	4	4	34	6.0	**V10**	T80	1:3	US	
EPA11	SS	WB	4	4	34	6.0	AFS	**V11**	1:3	US	
EPA12	SS	WB	4	4	34	6.0	AFS	T80	**V12**	US	
EPA13	SS	WB	4	4	34	6.0	AFS	T80	1:3	**V13**	
EPA14	SS	WB	4	4	34	6.0	AFS	T80	1:3	US	**V14**
EPA15	SS	WB	4	4	34	6.0	AFS	T80	1:3	US	**V15**
EPA16	SS	WB	4	4	34	6.0	AFS	T80	1:3	US	**V16**
EPA17	SS	WB	4	4	34	6.0	AFS	T80	1:3	US	**V17**

Table-2.5 Culture conditions for the enzyme production for the *C. cinerea* PPHRI-4 NFCCI-3027

Exp. Code	Type of fermentation	LCW	Inoculum size, (No. of discs)/5 gds	Incubation days, d	Temperature, °C	pH	Nitrogen source	Surfactant	Moisture content	Particle size	
EPB1	**V1**	WB	4	7	34	6.0	YE	T80	1:3	US	
EPB2	SS	**V2**	4	7	34	6.0	YE	T80	1:3	US	
EPB3	SS	**V3**	4	7	34	6.0	YE	T80	1:3	US	
EPB4	SS	WB	**V4**	**7**	34	6.0	YE	T80	1:3	US	
EPB5	SS	WB	6	**V5**	34	6.0	YE	T80	1:3	US	
EPB6	SS	WB	6	8	**V6**	6.0	YE	T80	1:3	US	
EPB7	SS	WB	6	8	34	**V7**	YE	T80	1:3	US	
EPB8	SS	WB	6	8	34	6.5	**V8**	T80	1:3	US	
EPB9	SS	WB	6	8	34	6.5	**V9**	T80	1:3	US	
EPB10	SS	WB	6	8	34	6.5	**V10**	T80	1:3	US	
EPB11	SS	WB	6	8	34	6.5	MYP	**V11**	1:3	US	
EPB12	SS	WB	6	8	34	6.5	MYP	T80	**V12**	US	
EPB13	SS	WB	6	8	34	6.5	MYP	T80	1:3	**V13**	
EPB14	SS	WB	6	8	34	6.5	MYP	T80	1:3	US	**V14**
EPB15	SS	WB	6	8	34	6.5	MYP	T80	1:3	US	**V15**
EPB16	SS	WB	6	8	34	6.5	MYP	T80	1:3	US	**V16**
EPB17	SS	WB	6	8	34	6.5	MYP	T80	1:3	US	**V17**

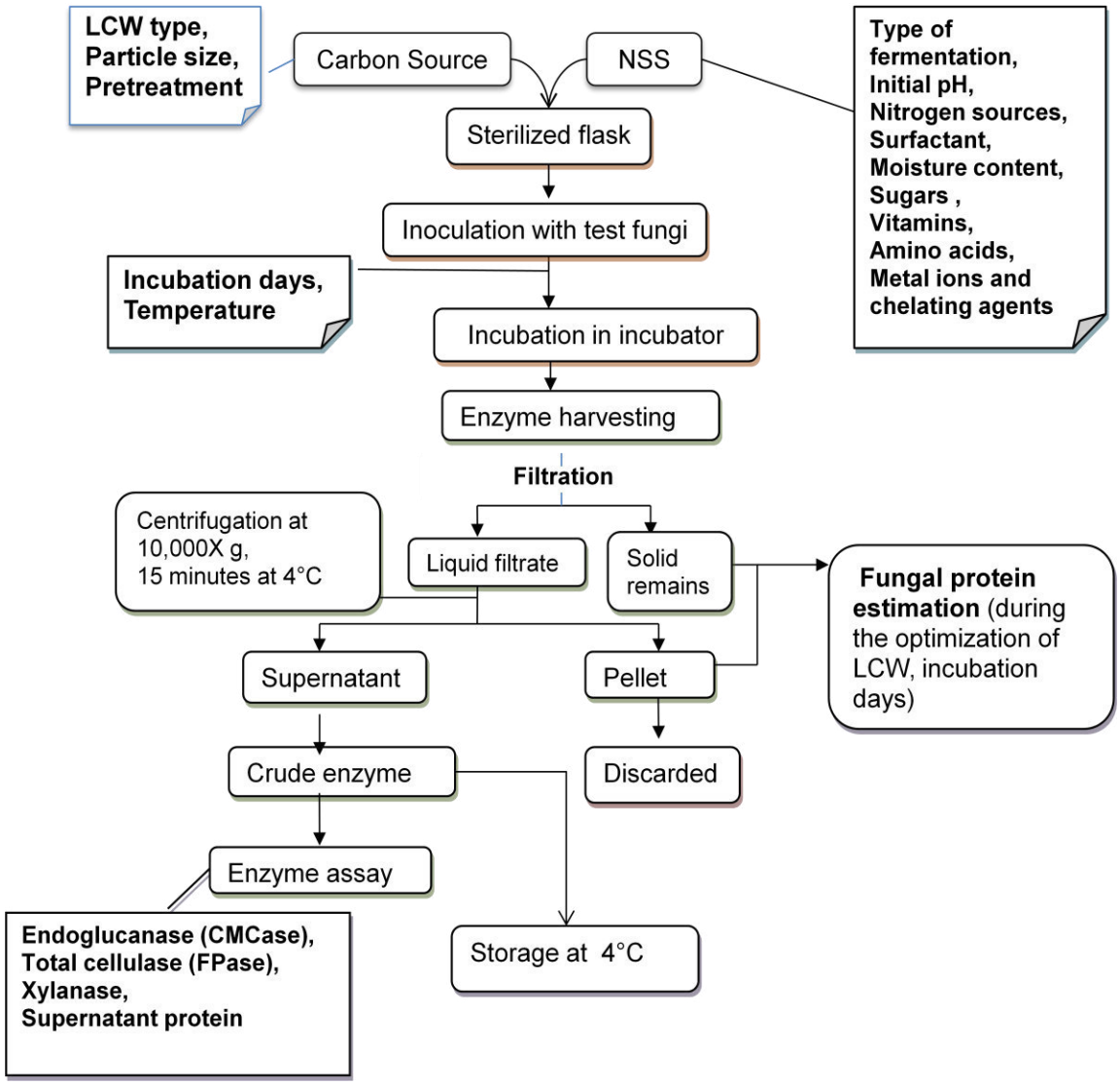


Figure-2.1 Schematic diagram showing procedure for enzyme production

2.2.1.7.4. Incubation period

The inoculated flasks were daily harvested from 1 to 16 d for *T. harzianum* PPDDN10 NFCCI 2925 and 1 to 30 d of incubation for *C. cinerea* PPHRI-4 NFCCI-3027 (Table-2.3, V5; Conditions: Table-2.4, EPA5 and Table-2.5, EPB5).

2.2.1.7.5. Incubation temperature

The effect of incubation temperature on the production of cellulase and xylanases by the test fungi was studied by incubating the inoculated flasks at different temperatures maintaining from 26 to 50 °C (Table-2.3, V6; Conditions: Table-2.4, EPA6 and Table-2.5, EPB6).

2.2.1.7.6. Initial pH

To study the effect of initial pH, the pH of the NSS, was maintained from 3.0 to 11.0 by using 1 N NaOH/H_2SO_4 separately (Table-2.3, V7; Conditions: Table-2.4, EPA7 and Table-2.5, EPB7).

2.2.1.7.7. Nitrogen Sources

2.2.1.7.7.1. Inorganic soluble nitrogen sources

The effect of complex nitrogen sources on xylanase production by the test organisms was studied by taking sodium nitrate, ammonium ferrous sulphate hexahydrate, urea, ammonium nitrate, ammonium acetate, and ammonium chloride as soluble nitrogen sources in the NSS. The concentrations were calculated to supply equivalent amounts of nitrogen (varying from 0.01M to 0.04M of each) (Table-2.3, V8; Conditions: Table-2.4, EPA8 and Table-2.5, EPB8).

2.2.1.7.7.2. Complex soluble organic nitrogen sources

The effect of complex nitrogen sources on xylanase production by the test fungi was studied by taking tryptone, meat peptone, soya peptone, peptone bacteriological, malt extract, proteose peptone, yeast extract, mycological peptone, and beef extract in the NSS with different concentrations (varying from 1 to 4 g/l of each) (Table-2.3, V9; Conditions: Table-2.4, EPA9 and Table-2.5, EPB9).

2.2.1.7.7.3. Plant based insoluble nitrogen sources

The effect of complex nitrogen sources on xylanase production by the test organisms was studied by taking mustard cake (MC), gram seed coat (GSC), soya bean meal (SBM), castor seed cake (CSC) and masoor seed coat (MSC) as a plant based insoluble nitrogen source in combination with WB. Total weight of the raw material was 5 g. The different combinations used were in the ratios of 3:1, 1:1 and 1:3 (Table-2.3, V10; Conditions: Table-2.4, EPA10 and Table-2.5, EPB10).

(**Note:** The NSS used in the insoluble plant based nitrogen source did not have any other soluble nitrogen source).

2.2.1.7.8. Surfactants

Different surfactants tween 20, tween 40, tween 60, tween 80, triton-X100, were used in NSS (concentration of 2 g/l) (Table-2.3, V11; Conditions: Table-2.4, EPA11 and Table-2.5, EPB11).

2.2.1.7.9. Moisture level

The effect of moisture level (WB:NSS) on xylanase production by the test fungi was tested by maintaining the ratio of WB amount to NSS volume from 1:1 to 1:5 (Table-2.3, V12; Conditions: Table-2.4, EPA12 and Table-2.5, EPB12).

2.2.1.7.10. Particle size of LCW

To see the effect of particle size of LCW the selected raw material was screened with different mess size 14, 22, 30, 36, 44, 52, 60, 72 and 100. In addition to this, one set of WB was kept as such i.e. unscreened (US) and one set had the mixture of each mesh size in

equal amount (MIX) (Table 1-V15). To obtain finer particle size the WB was grinded in the grinder. Thus obtained WB was used for enzyme production (Table-2.3, V13; Conditions: Table-2.4, EPA13 and Table-2.5, EPB13).

2.2.1.7.11. Pretreatment of LCW

Two sets of 50 g o.d. basis of the selected LCW was pretreated with the help of different concentrations of NaOH. During these pretreatment processes, the ratio of solid to liquid suspension was maintained as 1:10. These bottles with LCW were kept for 2 h at room temperature. One set was autoclaved at 15 Psi and 121 °C for 15 min in 1litre blue cap bottle. After the above mentioned pretreatment, the materials were washed with warm water until the chemicals were removed and the pH of the water reached to neutral. The neutralization was done with the 1 N HCl. These pretreated LCW were kept at 60 °C for 24 h (Table-2.3, V14; Conditions: Table-2.4, EPA14 and Table-2.5, EPB14).

2.2.1.7.12. Sugars

In order to investigate the effect of glucose, galactose, lactose, xylose, fructose and cellobiose on endoglucanase, total cellulase and xylanase production by the test fungi, the production medium was supplemented with different concentrations (varying from 1 to 4 g/l) of each sugar (Table-2.3, V15; Conditions: Table-2.4, EPA15 and Table-2.5, EPB15).

2.2.1.7.13. Vitamins and amino acids

Different vitamins (L-ascorbic acid, D-biotin, cyanocobalanine, calcium pantothenate, folic acid, nicotinic acid, pyridoxine, riboflavin, and thiamine) and amino acids (Asparagine, aspartic acid, cysteine, glutamic acid, isoleucine, leucine, metheonine, tryptophan, valine, and glycine) were used to study their effect on extracellular enzyme production. These vitamins and amino acids were sterilized under the UV light for 30 min and then these were added in autoclaved NSS 15 ml of the NSS with vitamins were mixed in 250 ml autoclaved Erlenmeyer flask having 5 g wheat bran (Table-2.3, V16; Conditions: Table-2.4, EPA16 and Table-2.5, EPB16).

2.1.6.14. Metal ions and chelating agents

The effect of different metal ions on enzyme production by the test fungi was studied by taking Mn^{+2}, Zn^{+2}, Fe^{+3}, Al^{+3}, Hg^{+2}, Ag^{+1}, Cu^{+2}, Ba^{+2}, K^{+1}, Co^{+3}, Na^{+1}, Ni^{+2}, Pt^{+5}, Cr^{+3}, Sr^{+2}, Sn^{+2}, and Pb^{+2} as sulphate, carbonate or chloride salts source. Ethylenediaminetetraacetic acid (EDTA) and diethylene triamine pentaacetic acid (DTPA) were taken as chelating agents. These were mixed in the NSS in concentrations of 0.2M each (Table-2.3, V17; Conditions: Table-2.4, EPA17 and Table-2.5, EPB17).

2.2.1.8. Mass production of crude enzyme from the fungal strains under optimized conditions of SSF for enzymatic deinking

Mass production of cellulase and xylanase from both the fungal strains was carried out under optimized conditions of SSF to provide the enzymes for deinking experiments. The mass production was carried out by taking 25 g WB in flasks of 1 liter capacity.

2.2.1.9. Characterization of crude enzyme produced by the fungal strains under optimized conditions of SSF

To be proven as an industrially important product, the characterization of these enzymes requires knowledge of optimum pH and temperature as well as their stability because CMCase, FPase and xylanase activities are subjected to change with varying pH as well as temperature.

2.2.1.9.1. Effect of pH on CMCase, FPase, and xylanase activity and stability

To determine the optimum pH of the enzyme, the crude enzyme obtained from both the fungal strains were incubated in different buffer range from 3-11 pH (at the difference of 0.5). Four different buffers (0.05 M) were used to maintain different pH levels. Citrate buffer was used for pH 3.0-6.0, phosphate buffer for pH 6.5-9.0, and glycine-NaOH buffer for pH 9.5-11.0. The CMCase, FPase, and xylanase activities were determined as described previously in section 2.2.1.3.

To determine the pH stability of the enzyme, the crude enzyme obtained from both the fungal strains were incubated in optimum pH buffer at temperature 40 °C after the incubation of 1 to 6 h (at 1 h interval), excluding the assay time (*i.e.* 30 min for CMCase test, 60 min for FPase test, and 15 min for xylanase test). The residual CMCase, FPase, xylanase activity was then measured by the standard assay procedures as described in section 2.2.1.3.

2.2.1.9.2. Effect of temperature on CMCase, FPase, and xylanase activity and stability

To determine the optimum temperature for the highest enzyme activity, the crude enzyme obtained from both the fungal strains were incubated at different temperature range from 20-75 °C (at the difference of 5 °C) at the optimum pH. The CMCase, FPase, and xylanase activities were determined as described previously in section 2.2.1.3.

In order to assess the temperature stability of the enzymes, the crude enzyme preparations obtained from both the fungal strains were incubated at 55, and 60 °C, under the above-mentioned conditions for determination of cellulase and xylanase activity. The residual xylanase activities were measured following the standard assay procedure as described above in section 2.2.1.3.

2.2.1.10. Statistical analysis

All the experimental analyses were replicated three times and the results are expressed as mean ± standard deviation (SD).

2.2.2. Deinking of photocopier waste papers

The optimization of the process variables was done by using OFAT approach. For the optimization of chemical and enzymatic deinking process parameters, the experiments were set as mentioned in Table-2.6 to 2.9.

2.2.2.1. Deinking experiments procedure

In all the experiments of deinking, following procedure was used (Figure-2.2).

2.2.2.1.1. Hydrapulping (Repulping)

The waste papers were torn manually into a size of approximately 1-inch squares. The hydrapulping of 250 g o.d. paper was carried out at different chemical/enzyme dose, consistency, temperature, and reaction time in HP. The process variables are listed in Table-2.6 to 2.9. The pH was maintained with 1 N H_2SO_4/NaOH during the enzymatic deinking. Before the flotation, some amount of the 20 g o.d. basis was removed to make the handsheets to analyze the total initial ink present in waste papers.

2.2.2.1.2. Flotation deinking

For flotation, the pulp was diluted to approximately 1% pulp consistency by using tap water. In all the chemical or enzymatic treatments, hydrapulping was followed by a 10 min flotation run in a 25 litre capacity laboratory flotation unit at 40±2 °C temperature to separate toner ink particles from the slushed fibers. At the end of flotation, the deinked fibers were recovered on muslin cloth from the drain valve of the flotation cell.

2.2.2.1.3. Washing deinking

After flotation, the recovered pulp was manually agitated for 5 min at 2% pulp consistency in the bucket. After this, pulp was filtered on a screen. This washing process was repeated thrice. The water obtained from the final optimized experiments was collected for the effluent studies.

2.2.2.1.4. Blank and control runs

Control runs were carried out under identical conditions without deinking chemicals for chemical deinking and replacing active enzyme by heat-denatured enzyme for enzymatic deinking. Blank samples (meant for estimating total ink) were the pulp sample treated with chemicals and enzyme but not processed by flotation and washing.

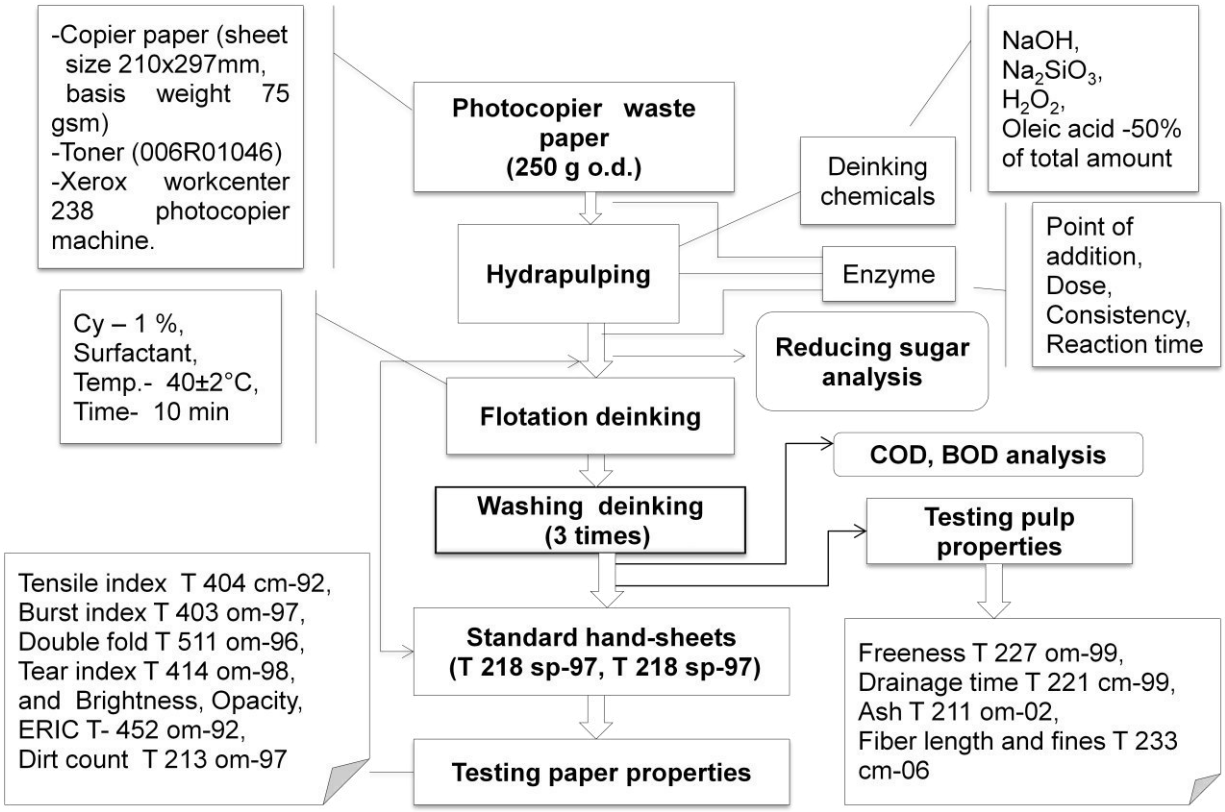


Figure-2.2 Schematic diagram showing deinking experiments procedure

2.2.2.1.5. Handsheets preparation

For preparing specimen sheets for reflectance testing of pulps Buchner funnel procedure was used following TAPPI test method, T 218 sp-97, the sheets were made at a pH of 6.5±0.5. For determining the physical properties of pulp, TAPPI test method T 205 sp-95 was used to prepare handsheets. Fifteen handsheets per run (grammage 60 g/m^2) were made on a British handsheet maker unit, and the handsheets were conditioned at 27 °C and 65% humidity.

2.2.2.2. Testing of different properties of pulp and paper

All the properties were measured by using standard TAPPI methods.

2.2.2.2.1. Pulp properties

Freeness and drainage time were determined using TAPPI standard tests T 227 om-99, T 221 cm-99. Ash in pulp was analyzed by combustion at 525 °C (T 211 om-02). Classification of weight percentage according to fiber length and fines of pulp was done by using Bauer-McNett classifier (T 233 cm-06).

2.2.2.2.2. Optical and strength properties of paper

Handsheets from chemical and enzyme treated pulp were compared for different optical and strength properties. Tensile, burst, tear indices and double fold were determined using TAPPI standard tests T 404 cm-92, T 403 om-97, T 414 om-98 and T 511 om-96,

respectively. Brightness, opacity, and ERIC were measured, using an Elerpho-070/071 device (TAPPI T 452 om-92). DE was calculated using the formula as described in the section 1.2.2.1. For dirt count TAPPI test method T 213 om-97 was used.

2.2.2.2.3. Reducing sugars analysis

For the reducing sugar analysis, after the completion of reaction time of enzyme, the pulp juice (2-3 ml) was squeezed from the pulp suspension. This solution was kept immediately in the boiling water bath to denature the enzymatic activity. After cooling, the solution wascentrifuged at 10000X g for 10 min to settle down any sort of fines or debris.1 ml of this solution was taken and 3 ml of the DNS was added in a 30 ml screw capped bottle. This was kept in the boiling water bath for 15 min followed by immediate cooling in the ice cube surroundings in order to stop the reaction. Then 20 ml of the water was added. The absorbance of the color generated was read at 540 nm with the help of UV-Visible Spectroscope against the reference blank. Reference blank contains the 1 ml of water in place of pulp solution. 1ml of water was used as a reference blank for this experiment.

2.2.2.2.4. Effluent water analysis

Effluent from the final optimized experiments were collected and analyzed for the biological oxygen demand (BOD) (by measuring dissolved oxygen before and after incubation at 20 °C for 5 d) and chemical oxygen demand (COD) (determined by closed reflux titrimetric method) (Clesceri et al. 1998).

2.2.2.3. Effect of different parameters of chemical and enzymatic deinking on the residual ink, deinking efficiency and brightness

2.2.2.3.1. Chemical deinking

For the optimization of chemical deinking, a narrow range of parameters was selected as reported by Wood et al. (1985). Accordingly, pH between 10 and 12, contact temperature between 50 °C and 75 °C, and hydrapulping time between 30 and 45 min were considered.

2.2.2.3.1.1. Sodium hydroxide (NaOH)

Chemical doses of NaOH were optimized for the concentration of 1.5, 2.0, and 2.5% (Table-2.6, Conditions: C1, C2 and C3).

2.2.2.3.1.2. Hydrapulping time

The experiments were carried out with hydrapulping time of 20, 30, and 40 min (Table-2.6, Conditions: C4, C2 and C5).

Table-2.61 Pulping process parameter variables for optimization of chemical deinking

Optimization parameters	Exp. No.	NaOH, %	Hydrapulping time, min	Temperature, °C	Na_2SiO_3, %	H_2O_2, %	Pulp consistency, %	Oleic acid, %#	DTPA, %	End pH ± 0.1 (Hydra-pulping)
NaOH	**C1**	**1.5**	30	70	2.0	1.0	6	0.8	0.8	10
	C2	**2.0**	30	70	2.0	1.0	6	0.8	0.8	10.4
	C3	**2.5**	30	70	2.0	1.0	6	0.8	0.8	10.9
Hydrapulping time	**C4**	2.0	**20**	70	2.0	1.0	6	0.8	0.8	10.4
	C2	2.0	**30**	70	2.0	1.0	6	0.8	0.8	10.5
	C5	2.0	**40**	70	2.0	1.0	6	0.8	0.8	10.5
Temperature	**C6**	2.0	30	**65**	2.0	1.0	6	0.8	0.8	10.5
	C2	2.0	30	**70**	2.0	1.0	6	0.8	0.8	10.5
	C7	2.0	30	**75**	2.0	1.0	6	0.8	0.8	10.5
Na_2SiO_3	**C8**	2.0	30	70	**1**	1.0	6	0.8	0.8	10.5
	C9	2.0	30	70	**1.5**	1.0	6	0.8	0.8	10.5
	C2	2.0	30	70	**2**	1.0	6	0.8	0.8	10.5
	C10	2.0	30	70	**2.5**	1.0	6	0.8	0.8	10.5
H_2O_2	**C11**	2.0	30	70	2.0	**0.5**	6	0.8	0.8	10.5
	C2	2.0	30	70	2.0	**1**	6	0.8	0.8	10.5
	C12	2.0	30	70	2.0	**1.5**	6	0.8	0.8	10.5
Pulp consistency	**C2**	2.0	30	70	2.0	1.0	**6**	0.8	0.8	10.5
	C13	2.0	30	70	2.0	1.0	**8**	0.8	0.8	10.5
	C14	2.0	30	70	2.0	1.0	**10**	0.8	0.8	10.5
	C15	2.0	30	70	2.0	1.0	**12**	0.8	0.8	10.5
Oleic acid	**C16**	2.0	30	70	2.0	1.0	10	**0.6**	0.8	10.5
	C14	2.0	30	70	2.0	1.0	10	**0.8**	0.8	10.5
	C17	2.0	30	70	2.0	1.0	10	**1.0**	0.8	10.5

Half (50%) of the oleic acid was added during the hydrapulping and 50% was added during the flotation stage.

2.2.2.3.1.3. Temperature

The optimization of temperature was considered for the temperatures 65, 70, and 75 °C (Table-2.6, Conditions: C6, C2 and C7).

2.2.2.3.1.4. Sodium silicate (Na_2SiO_3)

Chemical doses of the sodium silicate were optimized for the concentration of 1.0, 1.5, 2.0 and 2.5% (Table-2.6, Conditions: C8, C9, C2, and C10).

2.2.2.3.1.5. Hydrogen peroxide (H_2O_2)

Hydrogen peroxide doses were maintained as 0.5, 1.0, and 1.5% (Table-2.6, Conditions: C11, C2 and C12).

2.2.2.3.1.6. Pulp consistency

Pulp consistency was maintained from lower to medium i.e. 6, 8, 10 and 12% (Table-2.6, Conditions: C2, C13, C14 and C15).

2.2.2.3.1.7. Oleic acid

Oleic acid was added in the concentration of 0.6, 0.8, and 1.0% (Table-2.6, Conditions: C16, C14 and C17).

2.2.2.3.2. Enzymatic deinking

2.2.2.3.2.1. Point of enzyme addition

The effect of the point of inoculation of enzyme was evaluated by adding enzyme enzyme at three different stages *i.e.,* before hydrapulping (Pre-HP), during the hydrapulping (During-HP), or after hydrapulping (Post-HP). Before mixing the enzyme in all the stages, pH was maintained with H_2SO_4/ NaOH using pH strips and the temperature of pulp suspension was maintained constant at the optimized value of respective enzymes. For Pre-HP, the torn paper was kept in contact with enzyme and then before hydrapulping, the pulp was kept in boiling water for 15 min to inactivate the enzyme. In the experiment of During-HP, the enzyme was added to the torn papers in the hydrapulper and agitated for three rounds. In each round, after every 10 min of hydrapulping, agitation was interrupted for 20 min. In order to understand the process, hydrapulping time with reaction time, 'agitation' was denoted as A^+, no agitation as A^-, while the numeric value in the subscript were denoted as the time in min. The total reaction time was kept to 90 min ($\mathbf{A}^+_{10}+\mathbf{A}^-_{20}+\mathbf{A}^+_{10}+\mathbf{A}^-_{20}+\mathbf{A}^+_{10}+\mathbf{A}^-_{20}$). For the condition of Post-HP, enzymatic treatment was applied after hydrapulping. The slushed pulp was removed from the HP and then the pulp was mixed with the enzyme preparation. Poly bags filled with properly mixed enzyme and slushed pulp was kept in constant temperature water bath to allow the reaction to take place. In all the experiments, after the reaction time of enzymatic treatment, the pulp was kept in contact with boiling water for 15 min to inactivate the enzyme activity (Table-2.7, CE1-3; Table-2.8, TH 1-3; Table-2.9, CC1-3).

2.2.2.3.2.2. Enzyme dose

Experiments were carried out with different doses of different enzyme in the range of 0.2-10 IU/g of pulp (o.d. basis) depending on the enzymes preparation and types. The enzymes were added in such a way and it was properly mixed with the pulp (Table-2.7, CE2, CE4-7; Table-2.8, TH2, TH 4-7; Table-2.9, CC2, CC4-7).

Table-2.7 Pulping process parameter variables for optimization of enzymatic deinking with the commercial enzyme (CE)

Optimization parameters		**Experiment no.**	**Enzyme dose, IU/g**	**Pulp consistency, %**	**Reaction time, min**	**HP time, min**	**Temperature, °C**	**Oleic acid, %#**	**End pH ± 0.1 (Hydrapulping)**
Point of enzyme addition	**Pre-HP**	**CE1**	2	10	90	30	50	0.8	5.8
	During HP	**CE2**	2	10	90	30	50	0.8	5.8
	Post-HP	**CE3**	2	10	90	30	50	0.8	5.8
Enzyme dose		**CE2**	**2**	10	90	30	50	0.8	5.8
		CE4	**4**	10	90	30	50	0.8	5.8
		CE5	**6**	10	90	30	50	0.8	5.8
		CE6	**8**	10	90	30	50	0.8	5.8
		CE7	**10**	10	90	30	50	0.8	5.8
Pulp consistency		**CE8**	6	**6**	90	30	50	0.8	5.8
		CE9	6	**8**	90	30	50	0.8	5.8
		CE5	6	**10**	90	30	50	0.8	5.8
		CE10	6	**12**	90	30	50	0.8	5.8
		CE11	6	**14**	90	30	50	0.8	5.8
Reaction time (including 30 minutes hydrapulping time)		**CE12**	6	10	**30**	30	50	0.8	5.8
		CE13	6	10	**60**	30	50	0.8	5.8
		CE5	6	10	**90**	30	50	0.8	5.8
		CE14	6	10	**120**	30	50	0.8	5.8
		CE15	6	10	**150**	30	50	0.8	5.8
# Half (50%) of the oleic acid was added during the hydrapulping and 50% was added during the flotation stage.									

2.2.2.3.2.3. Pulp consistency

The effect of pulp consistency was examined in the range of 6 to 14% (Table-2.7, CE5, CE8-11; Table-2.8, TH5, TH 8-11; Table-2.9, CC6, CC8-11).

2.2.2.3.2.4. Reaction time

The reaction time for enzyme in the hydrapulper was optimized within the range of 30 min to 150 min (including 30 min for hydrapulping). For the optimization of 30 min reaction time, continuous agitation was carried out (A^{+}_{30}). For the rest of the time variations, the total hydrapulping time was kept equal i.e. 30 min (3 rounds of 10 min each). 60 min ($\mathbf{A}^{+}_{10}+\mathbf{A}^{-}_{10}+\mathbf{A}^{+}_{10}+\mathbf{A}^{-}_{10}+\mathbf{A}^{+}_{10}+\mathbf{A}^{-}_{10}$), 90 min ($\mathbf{A}^{+}_{10}+\mathbf{A}^{-}_{20}+\mathbf{A}^{+}_{10}+\mathbf{A}^{-}_{20}+\mathbf{A}^{+}_{10}+\mathbf{A}^{-}_{20}$), 120 min ($\mathbf{A}^{+}_{10}+\mathbf{A}^{-}_{30}+\mathbf{A}^{+}_{10}+\mathbf{A}^{-}_{30}+\mathbf{A}^{+}_{10}+\mathbf{A}^{-}_{30}$), and 150 min ($\mathbf{A}^{+}_{10}+\mathbf{A}^{-}_{40}+\mathbf{A}^{+}_{10}+\mathbf{A}^{-}_{40}+\mathbf{A}^{+}_{10}+\mathbf{A}^{-}_{40}$) (Table-2.7, CE5, CE12-15; Table-2.8, TH5, TH 12-15; Table-2.9, CC6, CC12-15).

Table-2.8 Pulping process parameter variables for optimization of enzymatic deinking with the crude enzyme of *T. harzianum* PPDDN-10 NFCCI-2925

Optimization parameters		Experiment no.	Enzyme dose, IU/g	Pulp consistency, %	Reaction time, min	HP time, min	Temperature, °C	Oleic acid, %#	End pH ± 0.1 (Hydrapulping)
Point of enzyme addition	Pre-HP	TH1	0.4	10	90	30	55	0.8	5.5
	During HP	TH2	0.4	10	90	30	55	0.8	5.5
	Post-HP	TH3	0.4	10	90	30	55	0.8	5.5
Enzyme dose		TH2	**0.2**	10	90	30	55	0.8	5.5
		TH4	**0.4**	10	90	30	55	0.8	5.5
		TH5	**0.6**	10	90	30	55	0.8	5.5
		TH6	**0.8**	10	90	30	55	0.8	5.5
		TH7	**1.0**	10	90	30	55	0.8	5.5
Pulp consistency		TH8	0.8	**6**	90	30	55	0.8	5.5
		TH9	0.8	**8**	90	30	55	0.8	5.5
		TH5	0.8	**10**	90	30	55	0.8	5.5
		TH10	0.8	**12**	90	30	55	0.8	5.5
		TH11	0.8	**14**	90	30	55	0.8	5.5
Reaction time (including 30 minutes hydrapulping time)		TH12	0.8	10	**30**	30	55	0.8	5.5
		TH13	0.8	10	**60**	30	55	0.8	5.5
		TH5	0.8	10	**90**	30	55	0.8	5.5
		TH14	0.8	10	**120**	30	55	0.8	5.5
		TH15	0.8	10	**150**	30	55	0.8	5.5

Half (50%) of the oleic acid was added during the hydrapulping and 50% was added during the flotation stage.

2.2.2.3.3. Characterization of the pulp

2.2.2.3.3.1. Scanning electron microscopy

Fiber surface was examined by using pulp handsheets through SEM (Quanta, 200 F Model, FEI, Netherland) image analysis captured at 5000X magnification. Samples were coated with gold for 50 s using sputter coater (Biotech SC 005, Switzerland) prior to scanning. Images were taken at an accelerating voltage of 15 kV.

2.2.2.3.3.2. Fourier transform infrared (FT-IR) spectroscopy

For the FT-IR spectroscopy, o.d. pulp samples were prepared by mixing and crushing with potassium bromide (KBr) for homogenization. The ratio of pulp samples and KBr was in between 1:20 to 1:50. The sample was pressed with the Nicolet economy sample press to obtain optically clear pellets. Then the pellets were analyzed using a Perkin Elmer FT-IR C91158 spectrometer system, United Kingdom. Liquid nitrogen was used as the chamber purge stream for all samples. The scanning resolution was set to 8 nm with a total of 32 scans per sample. The FTIR spectra were obtained at room temperature over a spectral

frequency range of 4000-500 cm^{-1}. The background was made against a pure KBr pellet and the data was analyzed by default inbuilt software.

2.2.2.3.3.3. X-ray diffraction (XRD)

XRD analysis of pulp samples were performed using Bruker AXS D8 Advance diffractometer (Germany) with a scanning rate of 1 °C min^{-1}. Nickel filtered CuKα radiation was used as a target having 1.54060 Å wavelength, which was operated at 40 kV and 30 mA, in the angular range (2θ) of 5° to 60°. The finely powdered samples were kept in the central cavity portion of the sample holder, which was made up of poly methyl methacrylate (PMMA). The wide-angle x-ray scattering (WAXS) patterns of the samples were obtained using 'DIFFRAC Plus XRD Commander software' and analysis was done on 'DIFFRAC Plus (Version 8.0) software'.

Table-2.9 Pulping process parameter variables for optimization of enzymatic deinking with the crude enzyme of *C. cinerea* PPHRI-4 NFCCI-3027

Optimization parameters		**Experiment no.**	**Enzyme dose, IU/g**	**Pulp consistency, %**	**Reaction time, min**	**HP time, min**	**Temperature, °C**	**Oleic acid, %#**	**End pH ± 0.1 (Hydrapulping)**
Point of enzyme addition	**Pre-HP**	**CC1**	0.2	10	90	30	55	0.8	5.5
	During HP	**CC2**	0.2	10	90	30	55	0.8	5.5
	Post-HP	**CC3**	0.2	10	90	30	55	0.8	5.5
Enzyme dose		**CC2**	**0.2**	10	90	30	55	0.8	5.5
		CC4	**0.4**	10	90	30	55	0.8	5.5
		CC5	**0.6**	10	90	30	55	0.8	5.5
		CC6	**0.8**	10	90	30	55	0.8	5.5
		CC7	**1.0**	10	90	30	55	0.8	5.5
Pulp consistency		**CC8**	0.6	**6**	90	30	55	0.8	5.5
		CC9	0.6	**8**	90	30	55	0.8	5.5
		CC6	0.6	**10**	90	30	55	0.8	5.5
		CC10	0.6	**12**	90	30	55	0.8	5.5
		CC11	0.6	**14**	90	30	55	0.8	5.5
Reaction time (including 30 minutes hydrapulping time)		**CC12**	0.6	12	**30**	30	55	0.8	5.5
		CC13	0.6	12	**60**	30	55	0.8	5.5
		CC6	0.6	12	**90**	30	55	0.8	5.5
		CC14	0.6	12	**120**	30	55	0.8	5.5
		CC15	0.6	12	**150**	30	55	0.8	5.5
# Half (50%) of the oleic acid was added during the hydrapulping and 50% was added during the flotation stage.									

CHAPTER 3

CRUDE CELLULASE AND XYLANASE ENZYME PRODUCTION BY ISOLATED FUNGAL STRAINS

3.1. Introduction

Screening of microorganisms, evaluation of their nutritional and environmental necessities as well as selection of suitable substrates for the production of enzymes are some crucial steps for the development of economically feasible process/technology (Aristidou and Penttilä 2000). The lignocellulosic biomass (wastes from forestry, agriculture and agro-industry) can provide a cheap renewable substrate for cellulase and xylanase production. The microorganisms, like fungi, bacteria and actinomycetes, may be exploited to utilize these LCWs as carbon sources to produce cellulolytic and xylanolytic enzymes (Romaní et al. 2006). *Trichoderma* and *Aspergillus* species are some of the widely reported soft rot fungi for the commercial production of these extracellular enzymes (Liu et al. 2012, Seyis and Aksoz 2005, Soni et al. 2010).

The cellulase enzyme complex is considered to be comprised of three hydrolytic enzymes for complete hydrolysis of cellulose. These are endo β-1,4-D-glucanase (endoglucanase or CMCase which cleaves glycosidic linkages of amorphous parts of cellulose randomly), exo β-1,4-glucanase (cellobiohydrolase or exoglucanase which releases cellobiose from non reducing or reducing ends from the crystalline parts of cellulose) and β-glucosidase (cellobiase-which cleaves bond between glucose dimers releasing sugar units) (Bhat 2000). Xylanolytic enzyme such as β-1,4 xylanase (1,4 β-D-xylan-xylanohydrolase or endoxylanase) had the capability of hydrolyzing the xylosidic linkages in xylan, the major component of hemicellulose (Bhat 2000, Fadel 2001).

These enzymes possess increasing demand for various biotechnological applications in different industries such as pulp and paper, textile, food, feed, beverage and biofuel production (Bhat 2000, Pandey et al. 2000, Pathak et al. 2011, 2010, Seyis and Aksoz 2005). Although, most of the industrial enzymes are produced by SmF process at commercial level, but SSF had also been considered for the better yield and the economical production of enzymes in some countries (Fadel 2001, Pandey et al. 2001, 2000). SSF is predominantly suitable for lignocellulosic enzymes production for different biotechnological applications where the crude enzyme may be used directly. Therefore, SSF produces the concentrate enzyme sample because the enzyme produced during the SSF is many folds higher than the SmF (Pandey et al. 2001, 2000). SSF has many advantages over SmF for production of enzyme by fungi such as, large volume with high concentration of product to facilitate recovery, simple process operation, better contamination control, and a less

amount of solvent for extraction of the enzymes, which considerably reduce the downstream processing cost (Pandey et al. 2000).

During the fermentation process of LCW for cellulase production, xylanase is also expected to be produced, because significant amount of xylan present in LCW stimulates the co-production of industrially important xylanase (Brijwani et al. 2010, Rahanama et al. 2013). The induction of cellulase and xylanase is likely to be under separate regulatory control in *T. harzianum* (Senior et al. 1989). Even the xylan deficient cellulosic substrates have been reported to induce the cellulase as well as xylanase production in *Trichoderma reesei* as explained by different researchers that cellulase regulator gene (ACEII) activates xylanase regulation in *T. reesei* (Aro et al. 2001, Portnoy et al. 2011). Still, majority of the available literature have reported either cellulase (Kocher et al. 2008) or xylanase production (Fadel 2001, Isil and Nilufer 2005, Saktiselvam et al. 2012, Seyis and Aksoz 2005,) by *T. harzianum* using LCW.

Therefore, the ultimate aim of this study is to optimize the various nutritional and environmental factors for maximum yield of cellulase (preferable) and co-production of xylanase enzyme by selected fungal strains through suitable fermentation process. Furthermore, optimum conditions (pH and temperature) of CMCase, FPase and xylanase activities with their stability are characterized to find out the best level of performance for enhancing the enzymatic efficiency.

3.2. Results and discussion

3.2.1. Isolation of cellulase and xylanase producing fungi

Total 104 fungal strains are isolated from decaying wood, humus soil and soil samples by using enrichment culture technique, in which the samples are kept in selective culture media under favorable conditions to isolate microorganisms directly from nature (Table-3.1).

3.2.2. Screening of isolates for potent xylanase producers

3.2.2.1. Primary screening

Out of these, 61 fungal isolates are found as cellulase and xylanase producers based on the ability to produce clear zone on CMC and xylan agar respectively (Fugure-3.1, Table-3.1).

3.2.2.2. Secondary screening

The fungal strains PPDDN-10 and PPHRI-4 are found to have the highest CMCase activity (preferable for deinking). These fungal strains are selected for the production of cellulase and xylanase, which will be used to deink the photocopier waste papers (Figure-3.1, Table-3.1).

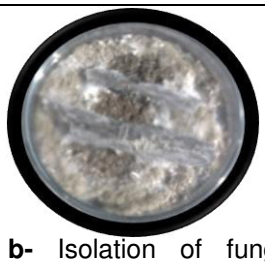
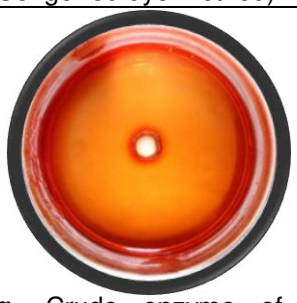
 **a-** Isolation of fungal strain PPDDN-10 from the humus soil (serial dilution method)	 **b-** Isolation of fungal strain PPHRI-4 from decaying wood sample kept in moist chamber containing wheat bran as enriched medium	 **c-** Fungal strain PPDDN-10 culture showing halo formation on CMC agar media (Primary screening- Congo red dye method)	 **d-** Fungal strain PPHRI-4 culture showing halo formation on CMC agar media(Primary screening- Congo red dye method)
 **e-** Fermentation of wheat bran with fungal strain PPDDN-10 for Congo red dye method (primary screening) and measurement of enzyme activities (secondary screening)	 **f-** Crude enzyme of fungal strain PPDDN-10 culture showing halo formation on CMC agar media (90 mm Petri-plate) (Congo red dye method)	 **g-** Crude enzyme of fungal strain PPDDN-10 culture showing halo formation on xylan agar media (52 mm Petri-plate) (Congo red dye method)	 **h-** Growth of fungal strain PPDDN-10 showing green spore formation in the centre of PDA media (identified as *Trichoderma harzianum* PPDDN-10 NFCCI 2925 at NFCCI Pune)
 **i-** Fermentation of wheat bran with fungal strain PPHRI-4 for Congo red dye method (primary screening) and measurement of enzyme activities (secondary screening)	 **j-** Crude enzyme of fungal strain PPDDN-10 culture showing halo formation on CMC agar media (90 mm Petri-plate) (primary screening- Congo red dye method)	 **k-** Crude enzyme of fungal strain PPDDN-10 culture showing halo formation on xylan agar media (52 mm Petri-plate) (primary screening- Congo red dye method)	 **l**- Growth of fungal strain PPDDN-10 showing the fruiting body on wheat bran agar (identified as *C. cinerea* PPHRI-4 NFCCI-3027 at NFCCI Pune)

Figure-3.1 Isolation, screening of fungal isolates *T. harzianum* PPDDN-10 NFCCI 2925 (a, c, e-h) and *C. cinerea* PPHRI-4 NFCCI-3027 (b, d, i-l)

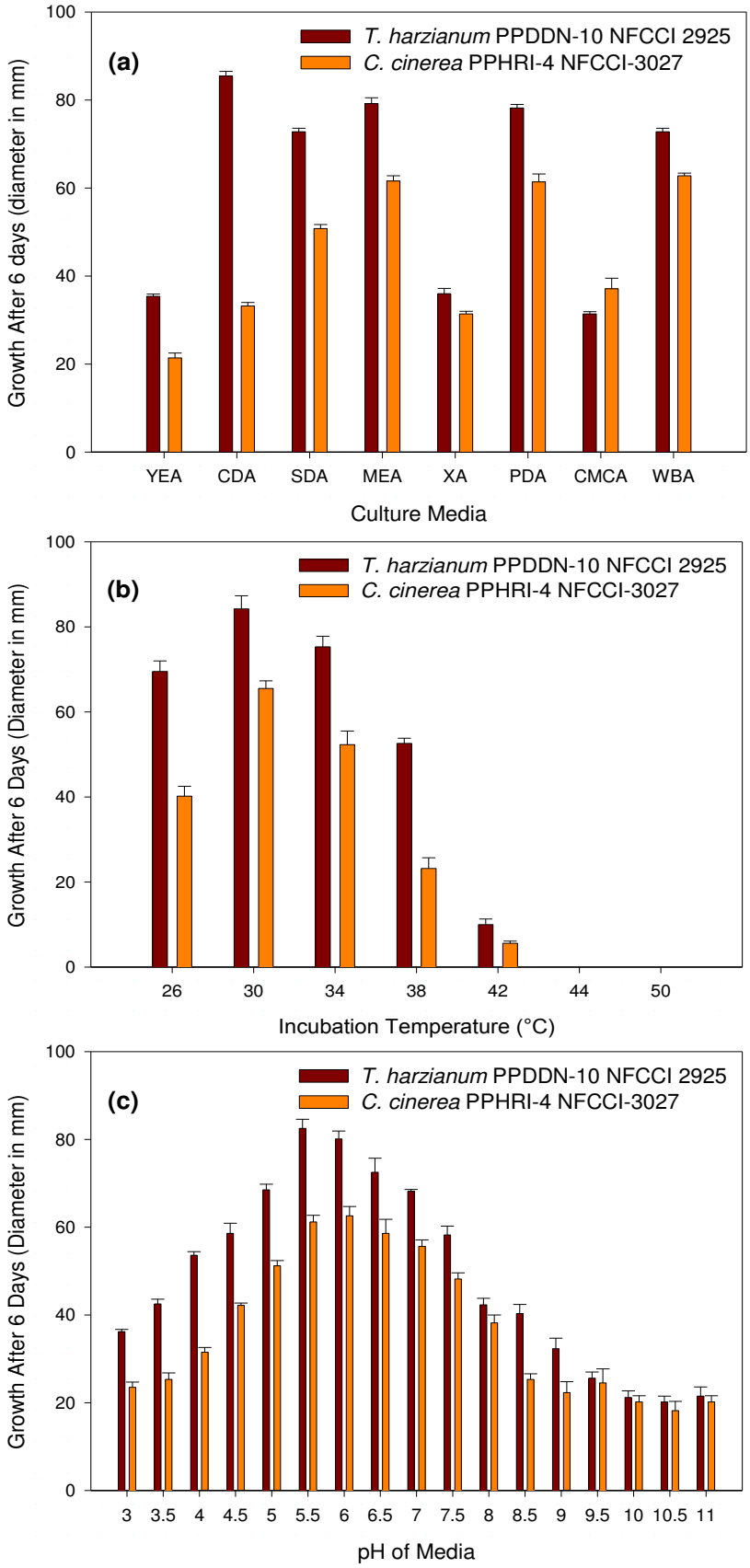


Figure-3.2 Effect of (a) culture media (b) incubation temperature (c) pH on the growth of *T. harzianum* PPDDN-10 NFCCI 2925 and *C. cinerea* PPHRI-4 NFCCI-3027

3.2.3. Identification of cultures

The fungal strains PPDDN-10 and PPHRI-4 are identified at NFCCI, Pune, India, as *Trichoderma harzianum* and *Coprinopsis cinerea,* respectively by sequencing of the genomic DNA, which showed 99% sequence similarity with genus. The same cultures are also deposited at NFCCI, with an accession number NFCCI-2925 and NFCCI-3027, respectively (Annexure-7 and Identification report).

3.2.4. Effect of different factors on fungal growth

3.2.4.1. Growth of fungal strains on different agar media

The growth and appearance of the fungal strains have been evaluated on different culture media having different composition. It is observed that the growth of the fungal strains PPDDN-10 and PPPHRI-4 are the best on CDA and WBA, respectively while poor growth is observed on YEA, CMCA and XA media. This shows that YEA, CMC and XA are less favorable for the growth of the fungal strains due to poor inducer for both the fungal strains. Thus, pure CMC and xylan have a tendency to repress cellulase and xylanase activity in these isolates. In addition, the use of CDA and other medium are not economically feasible for commercial application. The possibility of contamination by competing fungal species is also higher in case of CDA, PDA and MEA as compared to WBA because WBA acts as selective medium. Thus, WBA is chosen for cultivation of both the fungal strains (Figure-3.2 a, Table-3.2).

3.2.4.2. Growth of fungal strains at different temperature

Both the fungus has been found to grow in the temperature range of 26 to 38 °C. The best temperature for both the fungi is found to be at 30 °C. These fungi are not able to grow beyond 42 °C (Figure-3.2 b).

3.2.4.3. Growth of fungal strains at different pH

These fungi are able to grow over wide range of the pH 3.0 to 11.0, but the best growth is observed in the slightly acidic conditions i.e. 5.5 for *T. harzianum* PPDDN-10 NFCCI 2925 and 6.0 for *C. cinerea* PPHRI-4 NFCCI-3027 (Figure-3.2 c).

3.2.5. Three step extraction

T. harzianum PPDDN-10 NFCCI-2925

The extraction efficiency of enzyme is increased by 76.0 to 86.6% by three-step extraction (volume size 15 ml buffer x 3) when compared with single step extraction using 15 ml extraction buffer. It is observed that total enzyme production is enhanced by increasing the volume of extraction buffer, but enzyme activity per ml is decreased by 17.0 to 21.6% due to dilution of the crude enzyme sample. The efficiency of three-step extraction (volume size 15

ml x 3) is enhanced by 26.6 to 29.3% when compared with single step extraction using the same volume of buffer i.e. 45 ml buffer (Table-3.3).

C. cinerea PPHRI-4 NFCCI-3027

With respect to single step extraction (volume size 15 ml x 1), the three step extraction process is observed to improve the extraction efficiency from 79.1 to 88.2%. However, enzyme activities are decreased by 22.4 to 26.5% due to dilution of the enzyme. This three step extraction efficiency (volume size 15ml x 3= 45 ml) is 21.0 to 28.1% higher when compared with single step same volume size (45 ml x 1) (Table-3.4).

These results are in agreement with that of Rezende et al. (2002), who reported that the less volume of extraction buffer used consecutively could recover more enzyme than the same total volume used in a single extraction.

3.2.6. Optimization of nutritional and environmental factors for enzyme production

3.2.6.1. Fermentation type

T. harzianum PPDDN-10 NFCCI-2925

The highest enzyme yield is observed with SSF under both static and shaking conditions. Production of endoglucanase (14.80±0.69 IU/gds), total cellulase (3.96±0.28 FPU/gds), xylanase enzyme (1121.9±64.1 IU/gds), and supernatant protein (18.92±0.70 mg/gds) is slightly higher in the shaking condition than static condition for SSF. In the static SSF, the respective values are 14.12±0.54 IU/gds, 4.10±0.25 FPU/gds, 1102.4±63.5 IU/gds and 17.49±0.64 mg/gds. While for the SmSF and SmF, shaking state fermentation has a remarkable difference over static conditions (Figure-3.3a, Table-3.5).

C. cinerea PPHRI-4 NFCCI-3027

Static SSF is again selected as the best fermentation type because cellulase production (endoglucanase 14.20±0.65 IU/gds and total cellulase 3.70±0.19 FPU/gds) is maximum in this state than shaking SSF. On the other side, xylanase (2854.0±114.2 IU/gds) and soluble protein (25.78±0.95 mg/gds) are slightly higher but comparable in shaking than static SSF. It is observed that static and shaking SSF have almost same effect on the enzyme production, but SmSF and SmF in shaking conditions have shown remarkable increase in the enzyme over static condition (Figure-3.3b, Table-3.5).

Previously, different researchers have reported SSF as the best fermentation type over SmF and observed the improvement in enzyme production by 54% and 88.6% by *C. cinerea* and *C. disseminatus*, respectively (Kaur et al. 2011, Agnihotri et al. 2010). Singhania et al. (2007) have achieved 3.8 U/gds FPU using *Trichoderma reesei* RUT C30 and Wheat bran as carbon source under shaking SSF.

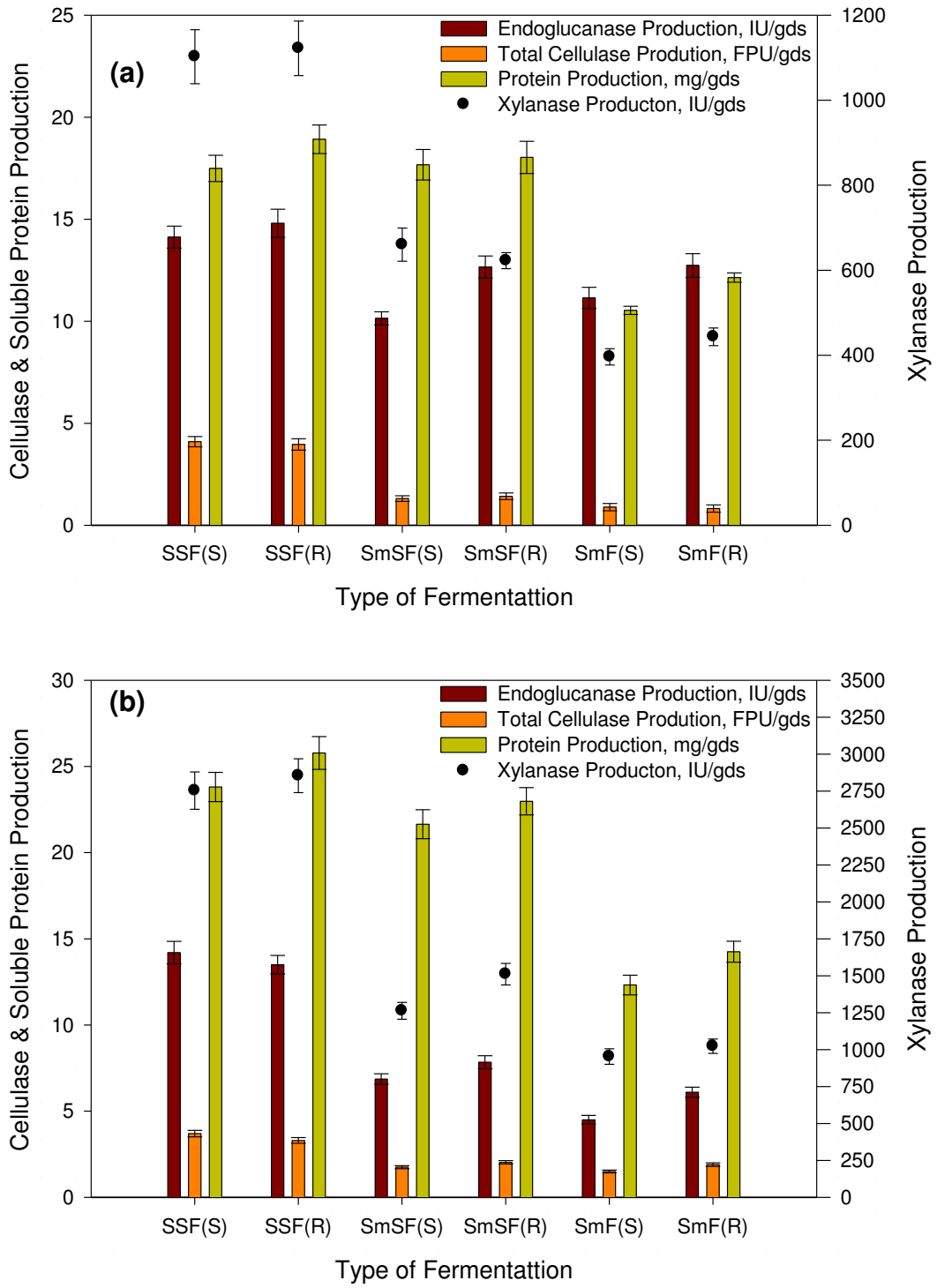


Figure-3.31 Effect of fermentation type on the cellulase and xylanase production by (a) *T. harzianum* PPDDN-10 NFCCI-2925 (b) *C. cinerea* PPHRI-4 NFCCI-3027

The greater enzyme production under SSF may be due to presence of same environment provided to the fungus i.e. closer to its natural habitat (wood and decayed organic matter) (Rühl 2009). Fungal morphology (cellular aggregation, mycelial pellet shape, size etc.) will influence the production efficiencies in SmSF and SmF, which influences the mass transfer rate (Rühl 2009, Mitchell and Lonsane 1992). In shaking SmSF and SmF, fungi are exposed to hydrodynamic forces, while in SSF growth, there are no such negative effects due to

restricted growth to the surface of the solid matrix (Silva et al. 2005). In addition, the filamentous fungi can easily penetrate into the solid substrates with their hyphae (due to less dilution of the enzymes) that results into increased accessibility of all the available nutrients within particles (Agnihotri et al. 2010, Holker 2004, Kaur et al. 2011, Pandey et al. 2000, Ramachandran et al. 2004, Singh et al. 2009). Catabolite repression problem in SmSF and SmF, is observed to be reduced or absent in SSF due to the possible existence of microscopic gradients within the mass of cell aggregates, or the changes in cell permeability to sugars (Silva et al. 2005).The SSF under static condition is selected for further experiments due to its obvious advantages.

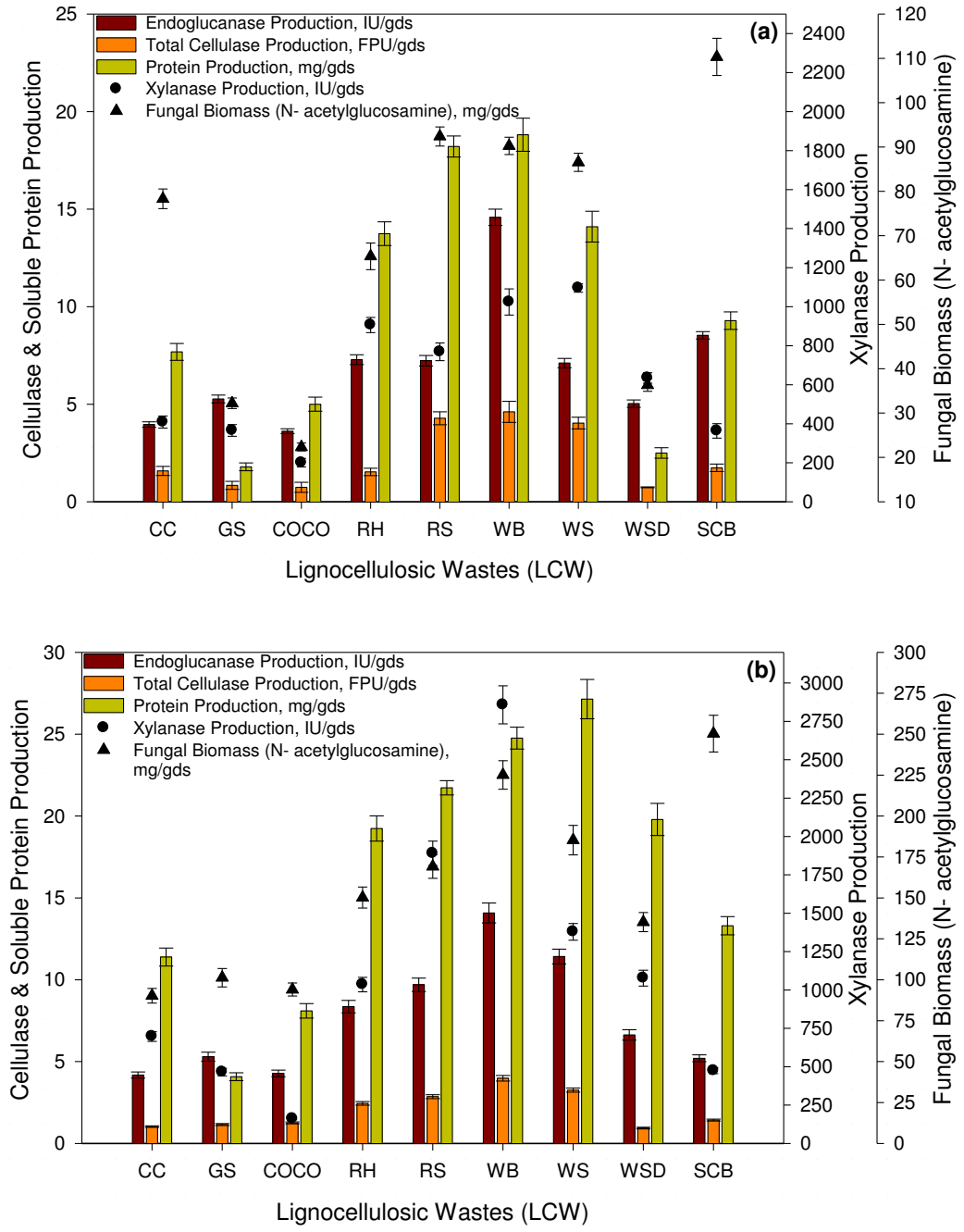


Figure-3.4 Effect of different LCWs on the cellulase and xylanase production by (a) *T. harzianum* PPDDN-10 NFCCI-2925 (b) *C. cinerea* PPHRI-4 NFCCI-3027

3.2.6.2. LCW as carbon source

T. harzianum PPDDN-10 NFCCI-2925

WB shows the highest enzyme production among various insoluble LCWs, (Figure-3.4a, Table-3.6). SCB supports the highest fungal biomass. The sequences of cellulase and xylanase production are as follows:

WB>SCB>RH=RS>WS>GS>WSD>CC>COCO (endoglucanase),

WB>RS>WS>SCB>CC>RH>GS>COCO=WSD (total cellulase),

WS>WB>RH>RS>WSD>CC>GS=SCB>COCO (xylanase production),

WB>RS>WS>RH>SCB>CC>COCO>WSD>CC (protein), and

SCB>RS>WB>WS>CC>RH>WSD>GS>COCO (Fungal biomass) (Figure-3.4a, Table-3.6).

Some researchers have used WB in combinations with other LCWs due to difference in nutrients as well as particle property to evaluate the addition effect of mixed substrate (Deschamps et al. 1985, Sherief et al. 2010). We observed that cellulase and xylanase producing efficiency of the fungus is decreased when WB is mixed in combination with RS, SCB and WS (Figure-3.5a, Table-3.7). Fungal growth is also slightly decreased in mixed substrate than individual one.

C. cinerea PPHRI-4 NFCCI-3027

Among the selected LCWs, WB is again found as the best lignocellulosic substrate as a carbon source followed by WS, RS, and RH. Although, SCB induces fungal biomass due to availability of free sugars for better growth, which is resulted in lesser enzyme production. The sequences of enzyme production are as follows:

WB>WS>RS>RH>WSD>GS>SCB>WSD>CC (endoglucanase),

WB>WS>RS>RH>SCB>COCO>GS>CC>WSD (total cellulase),

WB>RS>WS>WSD>RH>CC>SCB>GS>SCB (xylanase),

WS>WB>RS>WSD>RH>SCB>CC>COCO>GS (soluble protein) and

SCB>WB> WS>RS>RH>WSD>GS>COCO >CC (fungal biomass) (Figure-3.4b, Table-3.6).

In comparison to SSF with individual WB, the combinations of LCWs (WB+RS/WS/RH) are observed to reduce the enzyme production. This reduction is found in correlation with the amount of other LCW, i.e. WB> WB: other LCWs (3:1>1:1>1:3). On the other hand, the presence of WB is found to induce the enzyme production in other LCW (RS/WS/RH) when compared with individual RS, WS and RH (Figure-3.5b, Table-3.7).

The variation in the production may be due to compositional variation in different LCWs (i.e., amount of cellulose, hemicellulose, and lignin) and nutrient availability of the used substrate (Sherief et al. 2010). In SSF process, the solid substrate supplies the nutrients to the culture and serves as an anchorage for the filamentous fungi due to the capability of penetration into the hardest part of these solid substrates (Ramachandran et al. 2004). Esterbauer et al. (1991) did not prefer easily degradable cellulosic and lignocellulosic substrates because they are consumed rapidly causing repression of cellulase synthesis. In contrast, the growth of the organism may be retarded with a highly rigid substrate (COCO, WSD) resulting into reduced enzyme production. Therefore, equilibrium should be maintained between starvation and an excess of energy for high cellulase and xylanase production (Esterbauer et al.1991). The large variation in cellulase and xylanase yields, with various LCW is not mainly owing to the sugar composition and lignin content of these materials but due to the nature of cellulose and hemicellulose, presence of activators in the LCW, surface and pore size of the LCW particles (Senior et al. 1989). The oxygen transfer and heat dispersion should be proper within the mycelia and substrate particles (Kaur et al. 2011).

WB has been reported as good supporter due to its loosely arranged WB particles in moist conditions. WB also had nutritive value for the proper growth of fungi as it contains good amount of cellulose, hemicellulose and nitrogen source (Babu and Satyanarayana 1996, Kaur et al. 2011). The fungus grew well on the sugarcane baggase although the production of cellulase and xylanase is quite low because of catabolite repression due to possible higher sugar content. This indicates that the growth of the fungus cannot be correlated with high cellulase and xylanase production. The use of WB also eliminates the step of grinding due to its already finer particle size. Earlier, WB has been used for cellulase by *Trichoderma* and *Aspergillus* (Isil et al. 2005, Sherief et al. 2010) as well as xylanase production by *C. cinereus* and *C. disseminatus* (Agnihotri et al. 2010, Kaur et al. 2011, Singh et al. 2009,). WB, produced worldwide in big quantities as a by-product of the wheat milling industry, will be promising renewable substrates for biotechnological processes.

Some researchers have used WB in combinations with other LCW due to difference in nutrients as well as particle property to evaluate the addition effect of mixed substrate (Qinnghe et al. 2004, Sherief et al. 2010). However, WB with other LCW is not able to improve the production. Fungal growth is also slightly decreased in mixed substrate than individual one. Other researchers have also reported that WB has the property to induce enzyme production for other LCW like RS, WS, SCB, RH etc (Agnihotri et al. 2010, Kaur et al. 2011). These results are contrary with those obtained with *Aspergillus fumigatus* using mixed substrate of RS and WB (Sherief et al. 2010).

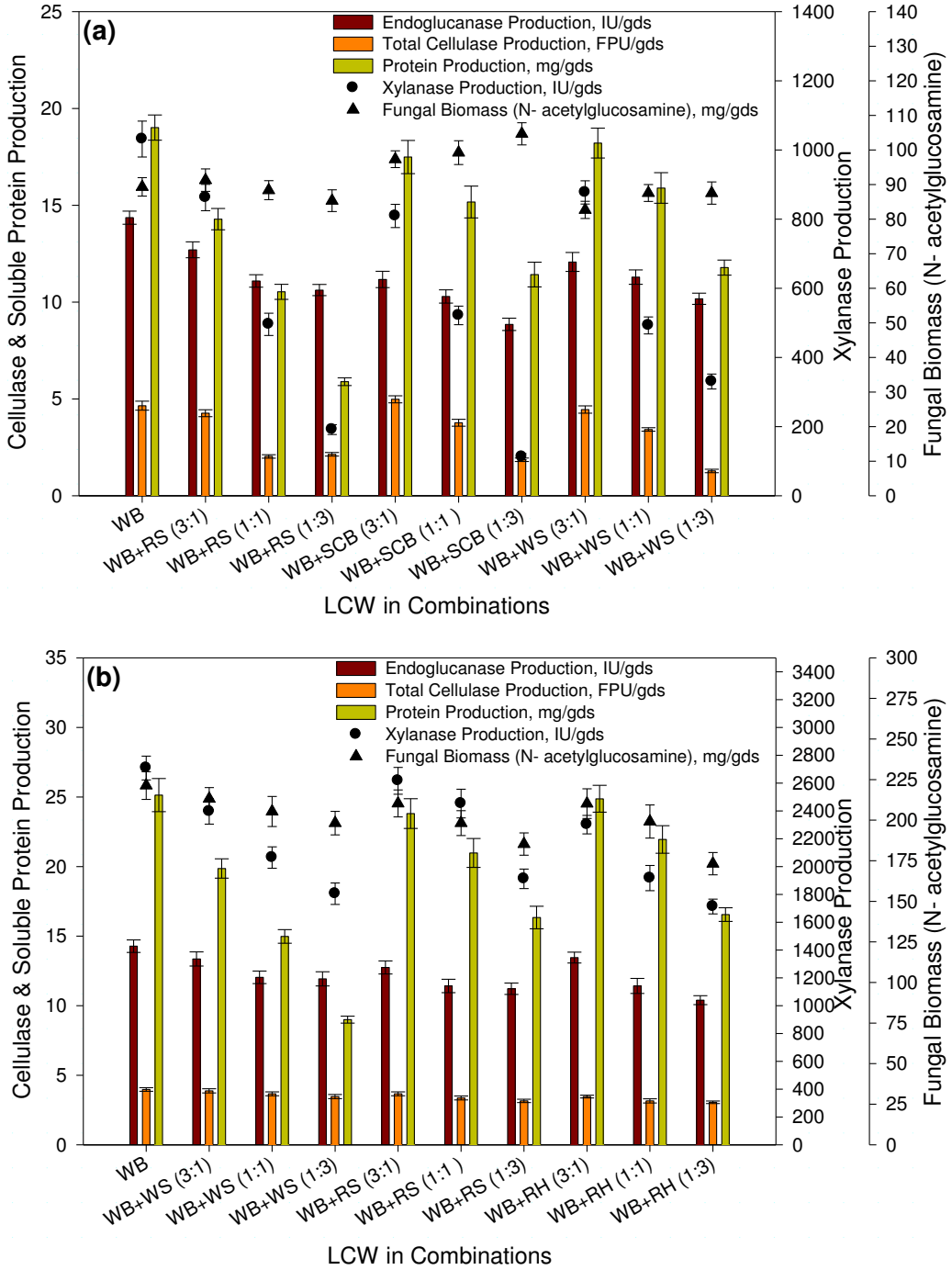


Figure-3.5 Effect of different LCWs combinations on the cellulase and xylanase production by (a) *T. harzianum* PPDDN-10 NFCCI-2925 (b) *C. cinerea* PPHRI-4 NFCCI-3027

Production of cellulolytic and xylanolytic enzymes is strongly dependent on the nature of carbon sources. The use of pure substrates as carbon source (such as CMC, cellulose and xylan) have been shown to be uneconomical, while the lignocellulosic wastes offer cost effective substrate for large-scale production of cellulase and xylanase through SSF. The induction of particular enzyme mainly depends on composition of the solid substrate but cost as well as and availability are considered to select suitable solid substrate for SSF

processes (Aristidou and Penttilä 2000, Liu et al. 2006). LCWs are about 10 times cheaper than the primary cellulosics materials (Doppelbauer et al. 1987, Esterbauer et al.1991).

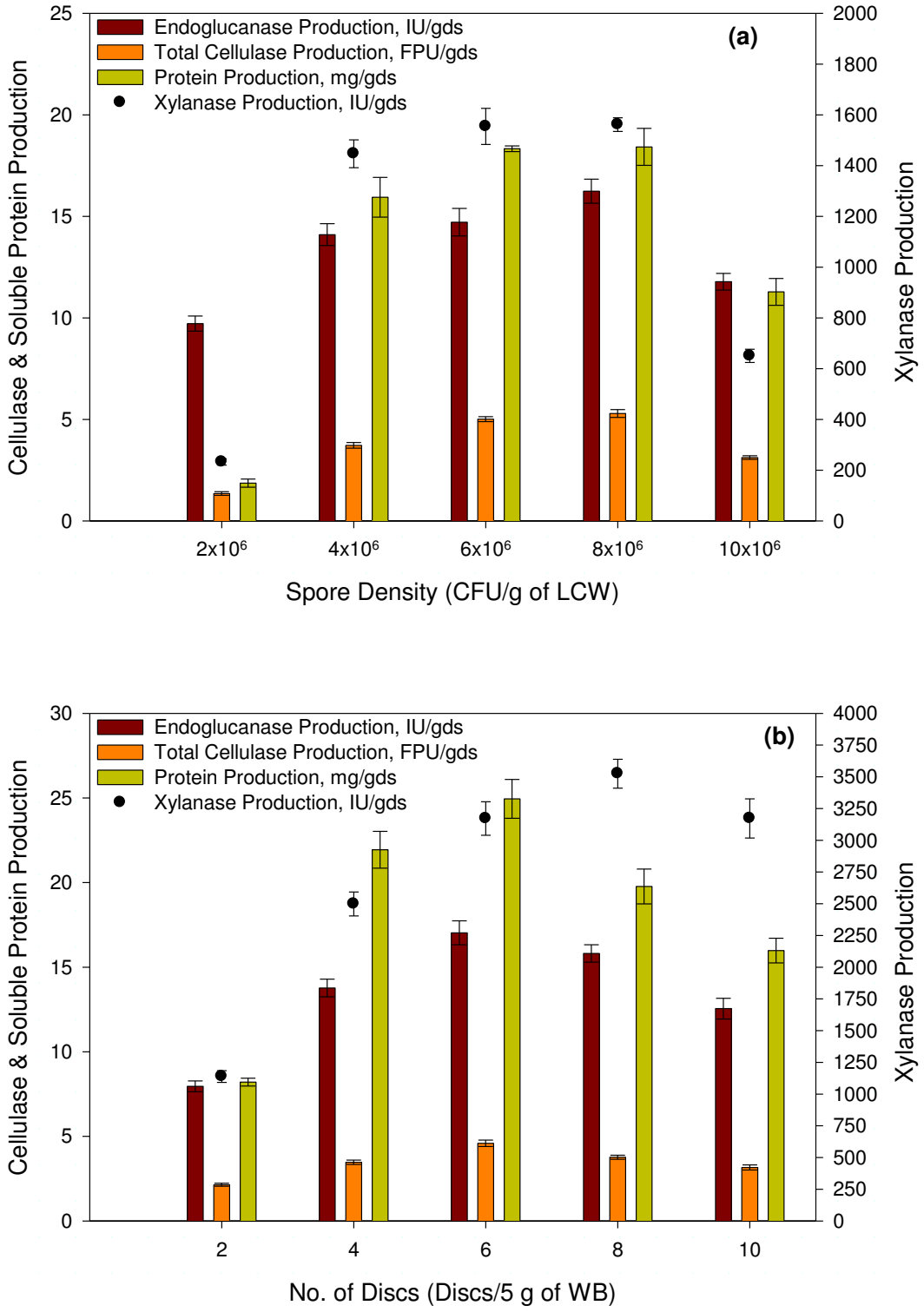


Figure-3.6 Effect of inoculum size on the cellulase and xylanase production by (a) *T. harzianum* PPDDN-10 NFCCI-2925 (b) *C. cinerea* PPHRI-4 NFCCI-3027

3.2.6.3. Inoculum size

T. harzianum PPDDN-10 NFCCI-2925

The maximum enzyme production is obtained at spore density of $8x10^6$ CFU/gds. for this spore density, the production of endoglucanase, total cellulase, xylanase and protein are 16.24±0.59 IU/gds, 5.29±0.19 FPU/gds, 1561.6±26.9 IU/gds and 18.42±0.91 mg/gds, respectively. There is no significant change in the xylanase production at the spore density of $6x10^6$ and $8x10^6$ CFU/gds (Figure-3.6a, Table-3.8).

C. cinerea PPHRI-4 NFCCI-3027

The maximum enzyme production is observed with the inoculum size of 6 discs (for 5g WB). The observed endoglucanase, total cellulase, xylanase and soluble protein production are 17.03±0.71 IU/gds, 4.59±0.19 FPU/gds, 3171.2±13.1 IU/gds and 24.95±1.15 mg/gds, respectively (Figure-3.6b, Table-3.8).

2 X 10^8 spores/flask (2 X 10^7 spores/gds) was reported as the optimum inoculum size for *Trichoderma* sp. (Sun et al. 2010).

Inoculum size is also an important factor for the enzyme production. Low inoculum level may not be sufficient to initiate the growth of microorganisms and therefore, resulting in long lag phase and slow enzyme formation. High inoculum size can reduce or deplete the lag phase and promote over growth which results in fast nutrient depletion (Ahmed et al. 2012). Optimum spore density (number of spores per gds for spore forming fungus *T. harzianum*) or filamentous inoculi (number of discs per gds for basidiomycetes fungi *C. cinerea*) are important to influence the enzyme production for SSF process. For basidiomycetes, filamentous inoculi are mostly used to avoid longer lag phase and contamination (Ahmed et al. 2012, Rühl 2009).

3.2.6.4. Incubation days

T. harzianum PPDDN-10 NFCCI-2925

The highest cellulase (endoglucanase 17.84±0.61 IU/gds and total cellulase production 6.22±0.11 FPU/gds) and xylanase (1114.9±95.3 IU/gds) production are obtained at the day 4 and 5, respectively. The maximum soluble protein production is 25.45±0.25 mg/gds at the day 4. N- acetyl glucosamine level starts to increase after the first day of fermentation to the maximum (92.68±1.65 mg/gds) at 5^{th} day, due to the growth of the fungus during the fermentation (Figure-3.7a, Table-3.9).

C. cinerea PPHRI-4 NFCCI-3027

A classic growth pattern of enzyme production is observed during this basidiomycetes fungal growth. Figure-3.7b shows 8^{th} day as the optimum time to extract enzyme. Enzyme

production is slow during the first 3 days due to slow growing nature of basidiomycetes fungus. After 3rd day, the production increases rapidly from 3rd to 9th day obtaining a maximum at 8th day, then decreases steadily thereafter. The glucosamine content is continuosly increased with the fungal growth till the 10th day. It shows that fungal growth and enzyme production are not correlated as the maximum glucosamine content is observed at 10th day, while cellulase and xylanase activities are highest at 8th day. After 13 days, the production of enzymes are slightly increased and then again decreased after 15th day (Figure-3.7b, Table-3.9).

The earlier studies with the *T. harzianum* have been reported 7 days (Rahanama et al. 2013, Rezende et al. 2002, Sakthiselvan et al. 2012, Seyis and Aksoz 2005) and 5 days for *Trichoderma* sp. (Sun et al. 2010) as the optimum time for the maximum cellulase and xylanase production. Fadel (2001) has found the maximum production in just 2 days using sorghum flour. Earlier findings with the *C. cinerea* (Kaur et al. 2011), *C. disseminatus* (Agnihotri et al. 2010, Singh et al. 2009) and *P. ostreatus* (Qinnghe et al. 2004) have reported maximum enzyme production after 7 days incubation. Deswal et al. (2011) have shown quite high incubation time of 11 days for CMCase and 15 days for FPase actities using *Fomitopsis* sp. RCK2010.

Mycelial growth indicates utilization of the substrate. The possible reason for decrease in production, after attaining the maximum, may be catabolite repression to utilize the released sugars from the LCW to avoid the attack of sugar fungi. Increasing the harvesting time may result in the accumulation of hydrolysis products such as glucose and cellobiose, which are inhibitors for cellulase-system activity and could adversely affect the rate of cellulase production (Esterbauer et al. 1991, Montenecourt and Eveleigh 1977, Rocky and Hamidi 2010, Xin and Geng 2010). After the consumption of thus accumulated sugars, the enzyme production is again increased, but not upto the optimum level due to non-availability of sufficient substrate. The decreased glucosamine content during this phase explains the possible death of fungal mycelia due to scarcity of food and action of the proteolytic enzyme present in the culture medium. Figure-3.7 (a and b) shows a trend of increasing soluble protein content correlating with the cellulase and xylanase activity, although several other types of enzymes may be secreted apart from cellulase and xylanase such as other ligninolytic enzymes (i.e., amylase, laccase, MnP and LiP etc).

3.2.6.5. Incubation temperature

The incubation temperature for the fermentation is one of the vital factors that have deep influence on products.

T. harzianum PPDDN-10 NFCCI-2925

It is observed that enzyme production increases with increase in temperature up to 34 °C showing maximum production of endoglucanase (20.06±0.86 IU/gds) and xylanase (1208.6±54.3 IU/gds) and soluble protein (26.19±1.20 mg/gds). The total cellulase production is maximum (6.01±0.18 FPU/gds) at 30 °C. Enzyme production decreases after 34 °C (Figure-3.8a, Table-3.10). This fungus is not able to grow at the temperature >42 °C.

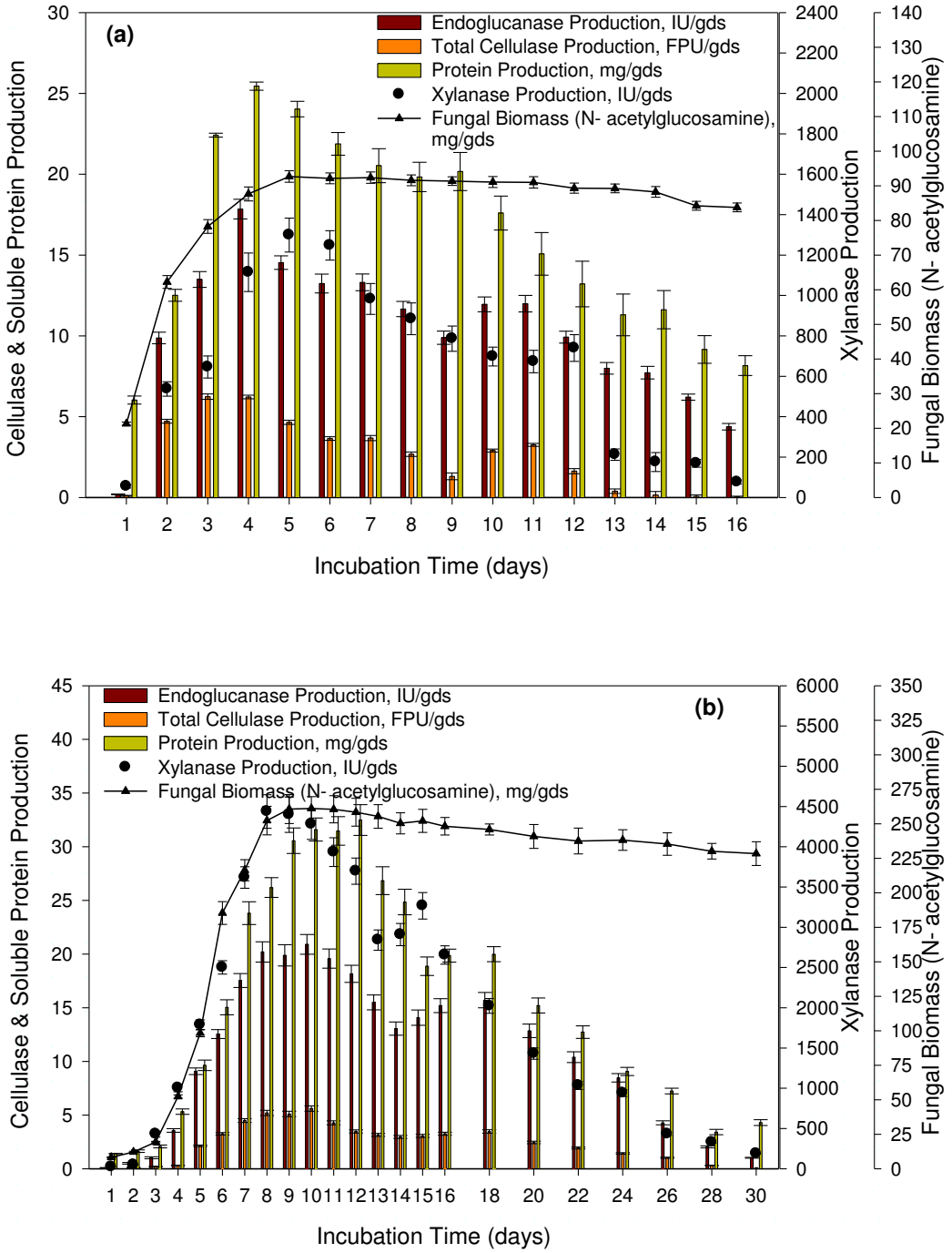


Figure-3.7 Effect of incubation time on the cellulase and xylanase production by (a) *T. harzianum* PPDDN-10 NFCCI-2925 (b) *C. cinerea* PPHRI-4 NFCCI-3027

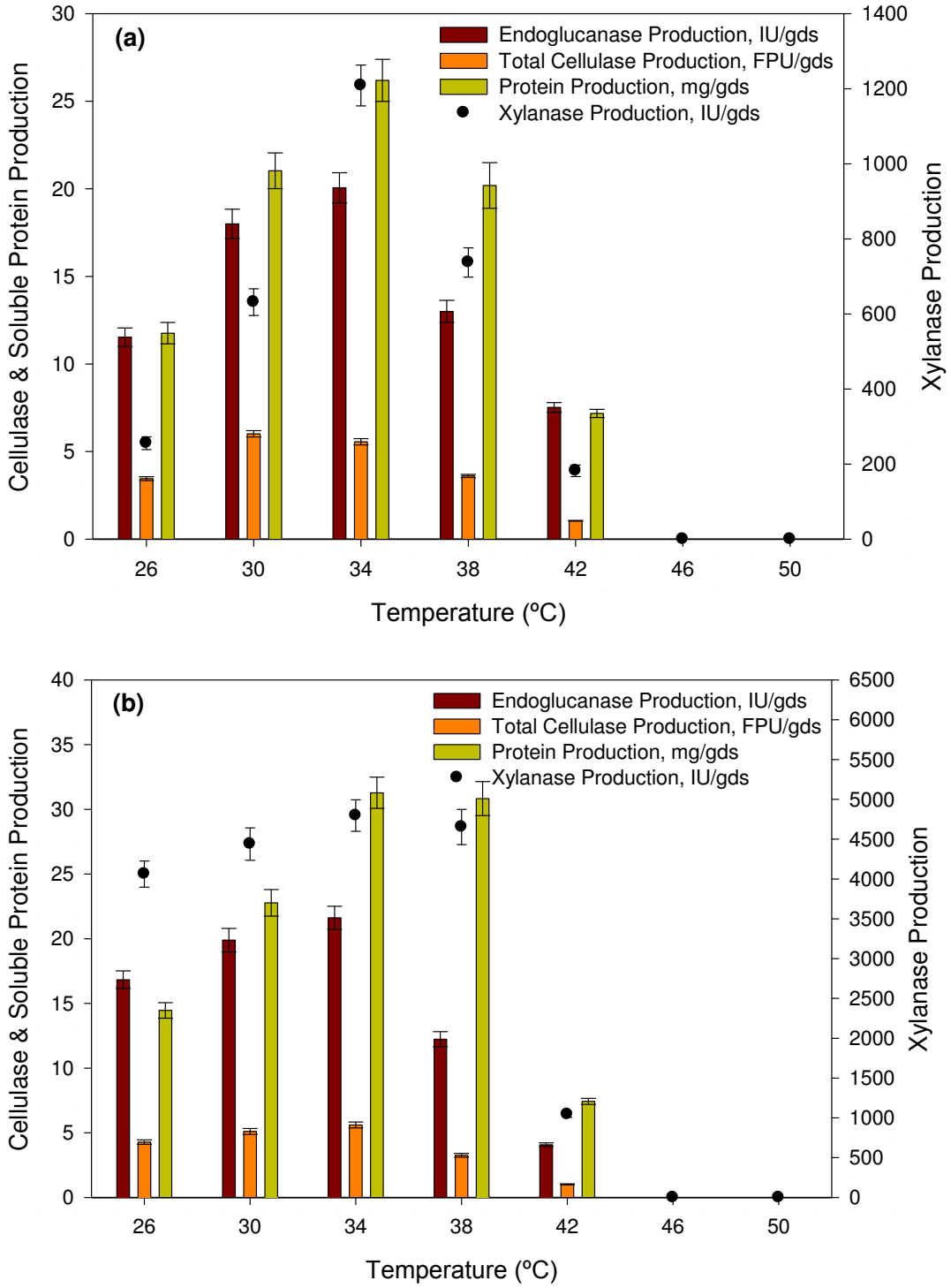


Figure-3.8 Effect of fermentation temperature on the cellulase and xylanase production by (a) *T. harzianum* PPDDN-10 NFCCI-2925 (b) *C. cinerea* PPHRI-4 NFCCI-3027

C. cinerea PPHRI-4 NFCCI-3027

The optimum temperature for the enzyme production is found to be at 34 °C, with the highest production of endoglucanase (21.62±0.89 IU/gds), total cellulase (5.61±0.23 FPU/gds), xylanase (4786.6±196.4 IU/gds), and protein content (31.28±1.21 mg/gds). The xylanase

production and protein content at 34 °C is comparable with that observed at 38 °C. Production of enzymes is rapidly decreased at high temperature (38 and 42 °C) due to reduced fungal growth (Kaur et al. 2012). This fungus is not able to grow beyond 42 °C temperature (Figure-3.8b, Table-3.10).

Mekala et al. (2008) have reported maximum cellulase production 33 °C incubation and then decreased production with high temperature. Deschamps et al. (1985) also reported the maximum FPase activity of *T. harzianum* at 30 °C. *Trichoderma* sp. has been found to produce maximum cellulase at 32 °C (Sun et al. 2010). Kaur et al. (2012) have selected 37 °C as the best temperature for the *C. cinerea* and they reported 32 to 47 °C as the best temperature range for the same fungus for the xylanase production.

Both low as well as high incubation temperature affects the enzyme production. Low temperature affects the transport of the substrates across the cells, while high temperature results into denaturation of the enzymes and proteins for different metabolic pathways (Agnihotri et al. 2010, Kaur et al. 2011, Singh et al. 2009). The optimum temperature will provide the sufficient maintenance energy by reducing the activation energy of the metabolic processes, which is essential for cell growth (Kaur et al. 2011, Pal and Khanum 2010). Under stressed conditions (*i.e.* high temperature) the microorganisms synthesize only essential proteins for theier growth and other physiological processes (Gawande and Kamat 1999)

3.2.6.6. Initial pH

T. harzianum PPDDN-10 NFCCI-2925

Maximum endoglucanase (23.52±0.94 IU/gds), total cellulase (6.85±0.25 FPU/gds), and xylanase (1191.4±73.1 IU/gds) production are observed at initial pH 6.0. The pH 6.5 was suitable for maximum xylanase production (1280.3±78.4 IU/gds) and soluble protein production (26.70±0.62 mg/gds) (Figure-3.9a, Table-3.11). Further increase or decrease in pH from this level causes denaturation of the enzyme and retards enzyme synthesis ability.

C. cinerea PPHRI-4 NFCCI-3027

Figure-3.9b reveals the effect of pH varying from 3.0 to 11.0 on enzyme production for the fungal strains. The pH range of 5.5 to 6.5 is suitable for enzyme production. The maximum enzyme production is attained at optimum pH 6.5 comprising endoglucanase (24.28±1.20 IU/gds), total cellulase (5.92±0.28 FPU/gds), xylanase (4631.7±228.9 IU/gds) and soluble protein (33.65±1.23 mg/gds). The maximum xylanase production is observed at pH 6.0. The cellulase and xylanase production are reduced below and above this pH range (Figure-3.9b, Table-3.11).

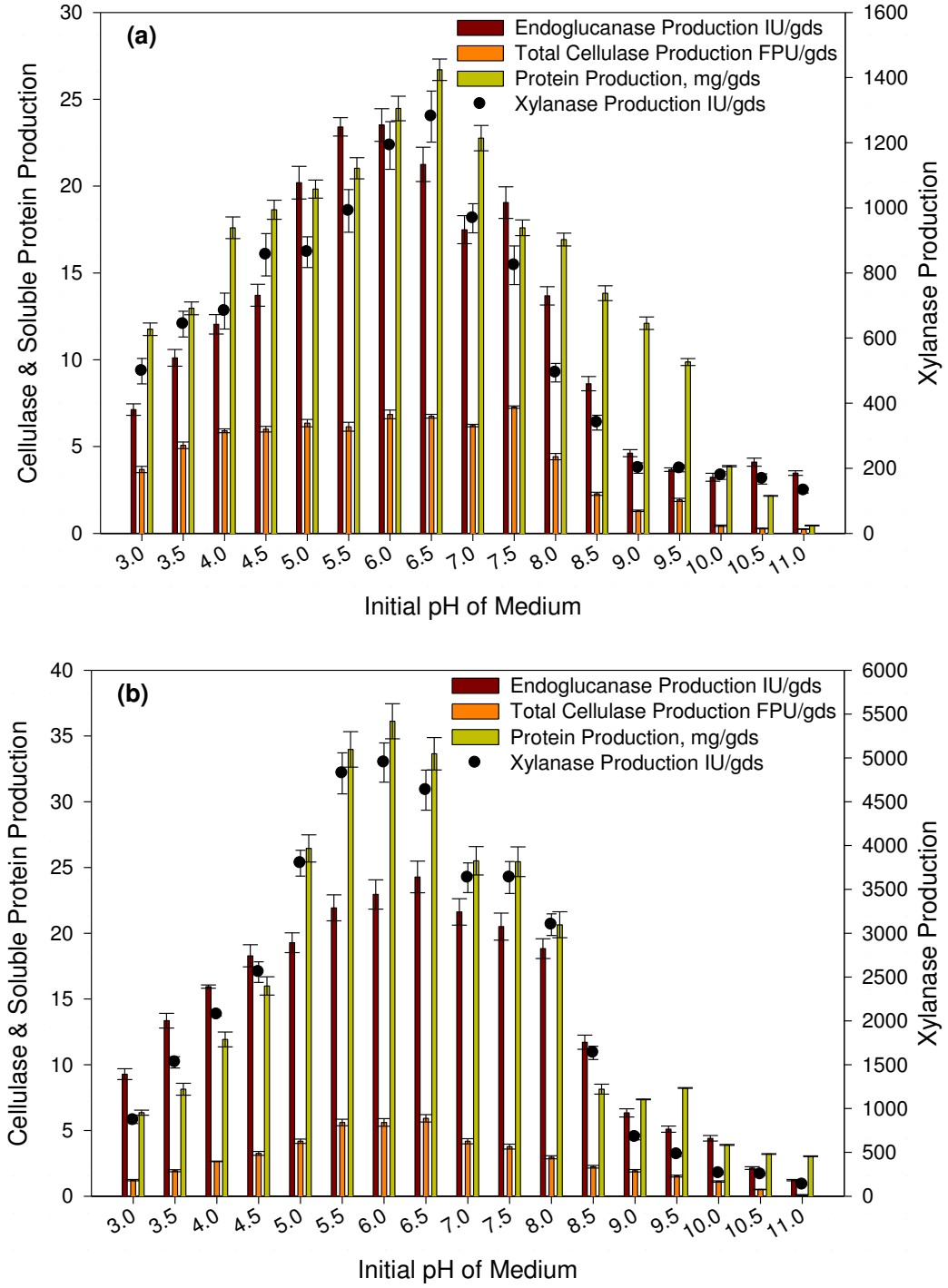


Figure-3.9 Effect of initial pH of medium on the cellulase and xylanase production by (a) *T. harzianum* PPDDN-10 NFCCI-2925 (b) *C. cinerea* PPHRI-4 NFCCI-3027

Kocher et al. (2008) found pH 5.0 to be optimum for the *Trichoderma harzianum* Rut-C 8230 using rice straw as a substrate. Rubeena et al. (2013) have reported that after the pH 4.0 cellulase production decreases rapidly for the *T. harzianum*. pH 6.4 has been reported as optimum with the *C. cinerea* (Kaur et al. 2011), while pH 5.0 for *Pleurotus ostreatus* (Qinnghe et al. 2004).

It is observed that the enzymes are produced in higher amount in the acidic range than

alkaline range. Enzymatic system and transport of enzyme across the cell wall is affected by the initial pH of the medium because of ionic nature of amino and carboxylic groups present in the amino acids chains of proteins. Any change in these groups will affect the catalytic ability of enzymes and processes (Poorna and Prema 2007, Kaur et al. 2011). The change in the pH value of the culture medium affects permeability of cells as well as stability of enzyme (Ahmed et al. 2012). Therefore, an optimum pH during fermentation will avoid the possibility of denaturation at lower and higher pH. During fermentation, any major change in pH will be controlled due to buffering action of WB (Pandey et al. 2000). Acidic pH levels (5.0–6.5) generally favor fungal cellulase and xylanase production. The reason is that the degrading wood, a natural habitat for growth of white rot fungi has an acidic environment (Furlan et al. 1997).

3.2.6.7. Nitrogen source

T. harzianum PPDDN-10 NFCCI-2925

The maximum enzyme production i.e. endoglucanase (26.22±0.81 IU/gds), total cellulase (7.42±0.07 FPU/gds), and xylanase (1942.4±84.0 IU/gds) is induced with the AFS at dose of 0.02M of available nitrogen concentration (Figure-3.10a to d, Table-3.12 to 3.15). It is comparable with the results of BE, but AFS is cheaper nitrogen source than BE. Xylanase production is found to be maximum (2137.7±68.4 IU/gds) with the MYP at dose of 2 g/l and beef extract (2119.3±67.8 IU/gds) at 3 g/l dose. The endoglucanase and total cellulase production is slightly lower than those obtained with AFS. (Figure-3.10a to c, Table-3.12 to 3.14).

The plant based nitrogen sources are able to improve the cellulase production. Among all the different plant based insoluble N sources, combination of WB and lentil seed coat in the ratio of 3:1 is observed to have maximum cellulase and low xylanase production.

WB:LSC> WB:SBM> WB:GSC> WB:CSC> WB:MC >WB (endoglucanase),

WB:SBM>WB:GSC>WB:LSC>WB:CSC>WB>WB:MC (total cellulase),

WB>WB:LSC>WB:GSC>WB:SBM>WB:MC>WB:CSC (xylanase) (Figure-3.10d, Table-3.14).

It is also observed that as the WB concentration decreased the enzyme production is also get reduced (3:1>1:1>1:3) (Figure-3.10d, Table-3.15).

C. cinerea PPHRI-4 NFCCI-3027

It is observed that all the N-sources are responsible to increase the production upto the optimum dose. Beyond the optimum dose, the production of enzyme either ceased or decreased (Figure-3.11a to d, Table-3.12 to 3.15). The optimum enzyme production i.e. endoglucanase (38.25±1.76 IU/gds), total cellulase (7.65±0.35 FPU/gds), xylanase (6841.1±275.8 IU/gds) and soluble protein (42.73±0.55 mg/gds) is found with MYP at dose

of 3 g/l. Further increase in the dose is not able to improve the production significantly (Figure-3.11a to d). It is comparable with the results of BE (3 g/l) for xylanase production but not for the cellulase production.

The plant based N-sources are observed to improve the cellulase and xylanase production as the soluble N-sources. The results of the cellulase and xylanase production are summarized as follows:

WB:SBM>WB:LSC>WB:GSC>WB:MC>WB:CSC>WB (endoglucanase)

WB:SBM>WB:CSC= WB:GSC>WB:MC>WB:CSC>WB (total cellulase)

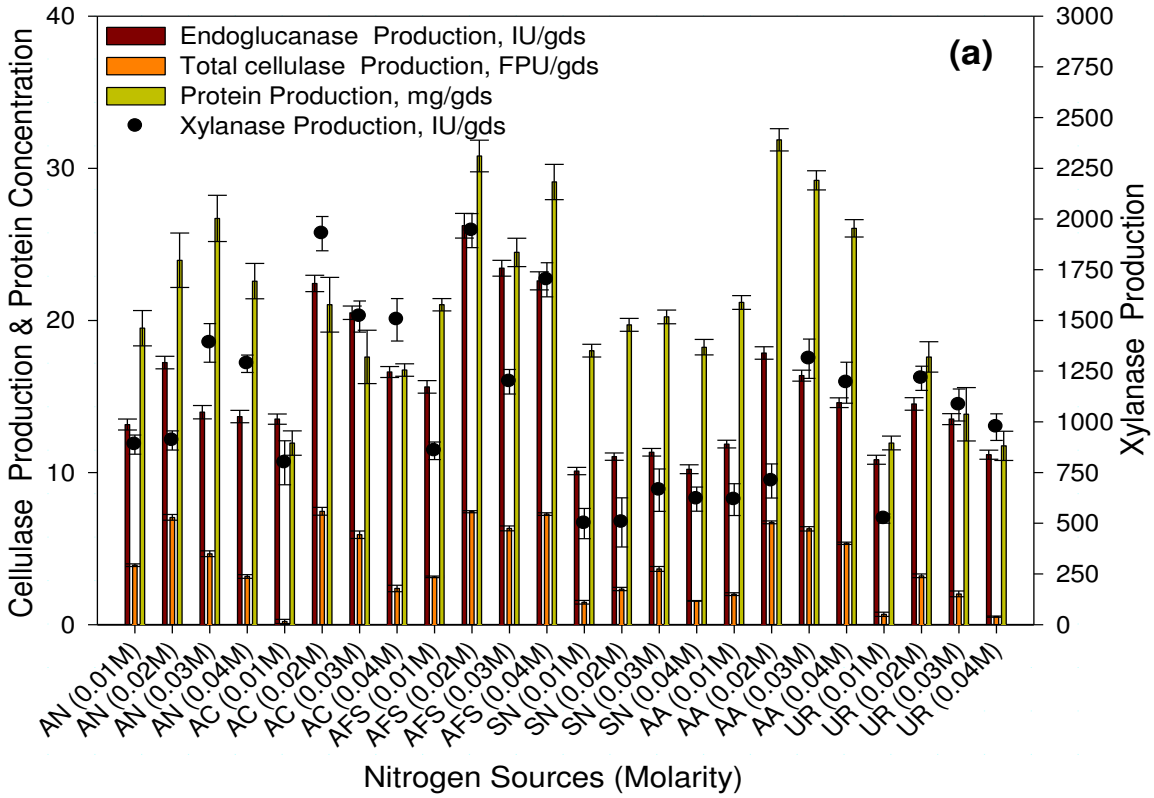
WB:LSC= WB:SBM>WB:GSC>WB:MC>WB:CSC>WB (xylanase)

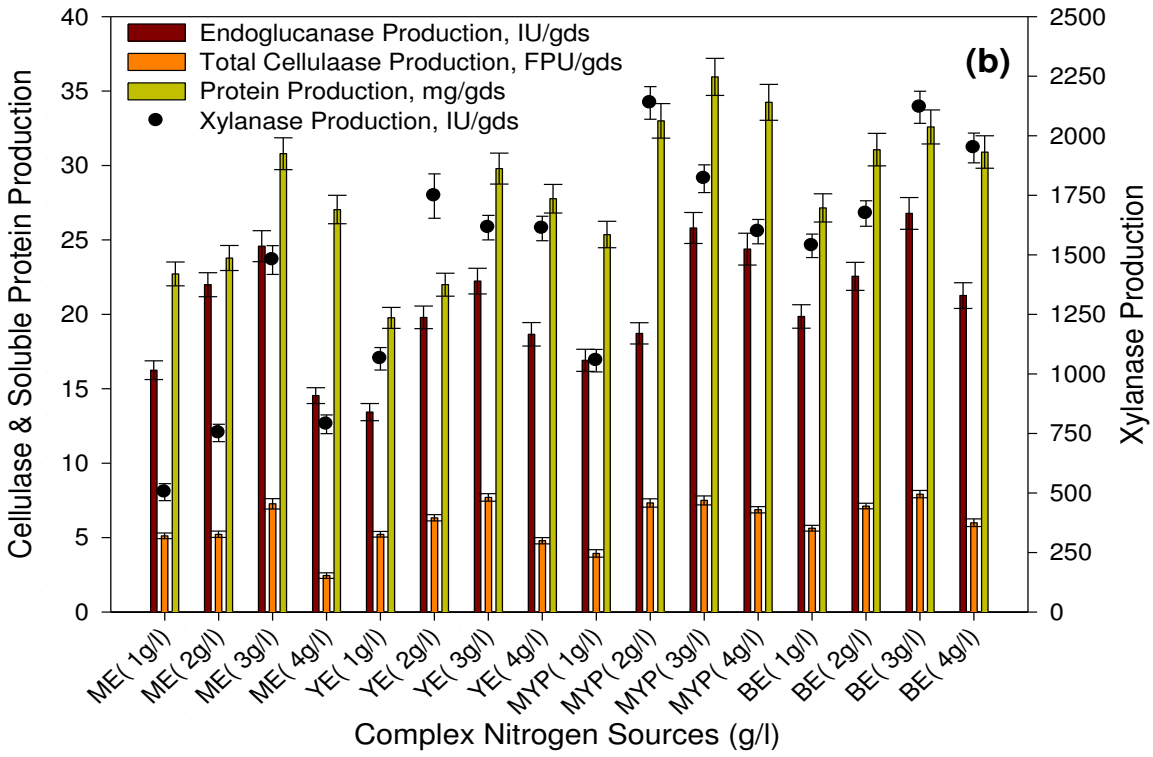
Again, the trend of enzyme production is observed to be 3:1>1:1>1:3.

Our results show that *T. harzianum* is able to use efficiently the inorganic N- source while *C. cinerea* utilizes organic N- sources. Earlier, ammonium sulphate and urea (Rahanama et al. 2013), peptone in combination with urea (Saktiselvam et al. 2012) have been identified as the best nitrogen sources for *T. harzianum*. Tong and Rajendra (1992) also found ammonium ferrous sulphate to be the best for cellulase enzyme production for *Aspergillus* sp. Other researchers have reported different organic N-sources, like beef extract for *C. cinerea* (Kaur et al. 2011), peptone for *Pleurotus ostreatus* (Qinnghe et al. 2004), and *L. edodes* (Kachlishvili et al. 2006), yeast extract for *Pleurotus dryinus* (Elisashvili et al. 2006) and soya bean meal for *C. disseminatus* (Agnihotri et al. 2010).

Cellulase and xylanase production are known to be sensitive to the nature of nitrogen source as well as to its level in the medium (Chaabouni et al. 1995, Kachlishvili et al. 2006, Maeda et al. 2010, Soni et al. 2010). The ratio of C/N is most crucial for a particular process to obtain specific production (Brijwani et al. 2010). In general, the yields of hydrolytic enzymes are increased by supplementation of medium with an additional nitrogen source (Kachlishvili et al. 2006). The production of enzymes is increased with the nitrogen content because the fungal biomass increased with the level of nitrogen concentration and excess nitrogen in the inoculum media suppresses growth (Qinnghe et al. 2004). According to Stewart and Parry (1981), cellulase production is always high when ammonium salts are used. Xylanase production is generally higher with organic nitrogen sources. However, some studies have also shown that inorganic nitrogen source resulted in improved enzyme production compared to organic nitrogen source (Kalogeris et al., 2003). The differences in production on various complex organic N sources may be due to their varying contents of amino acids, peptides, vitamins, trace elements and inducers (Lan et al. 1998). These bio-molecules can be absorbed directly by mycelia. The production of enzyme on inorganic nitrogen sources shows that these fungi have the good capability to assimilate the inorganic nitrogen to convert inorganic nitrogen to synthesize amino acids, which affects growth (Xu et al. 2005,

Qinnghe et al. 2004). These results show that fungi produce high cellulase or xylanase activities when grown on organic nitrogen sources (Bakri et al. 2003, Chaabouni et al. 1995, Lemos et al. 2001, Purkarthofer et al. 1993, Qinnghe et al. 2004, Sakthiselvan et al. 2012).





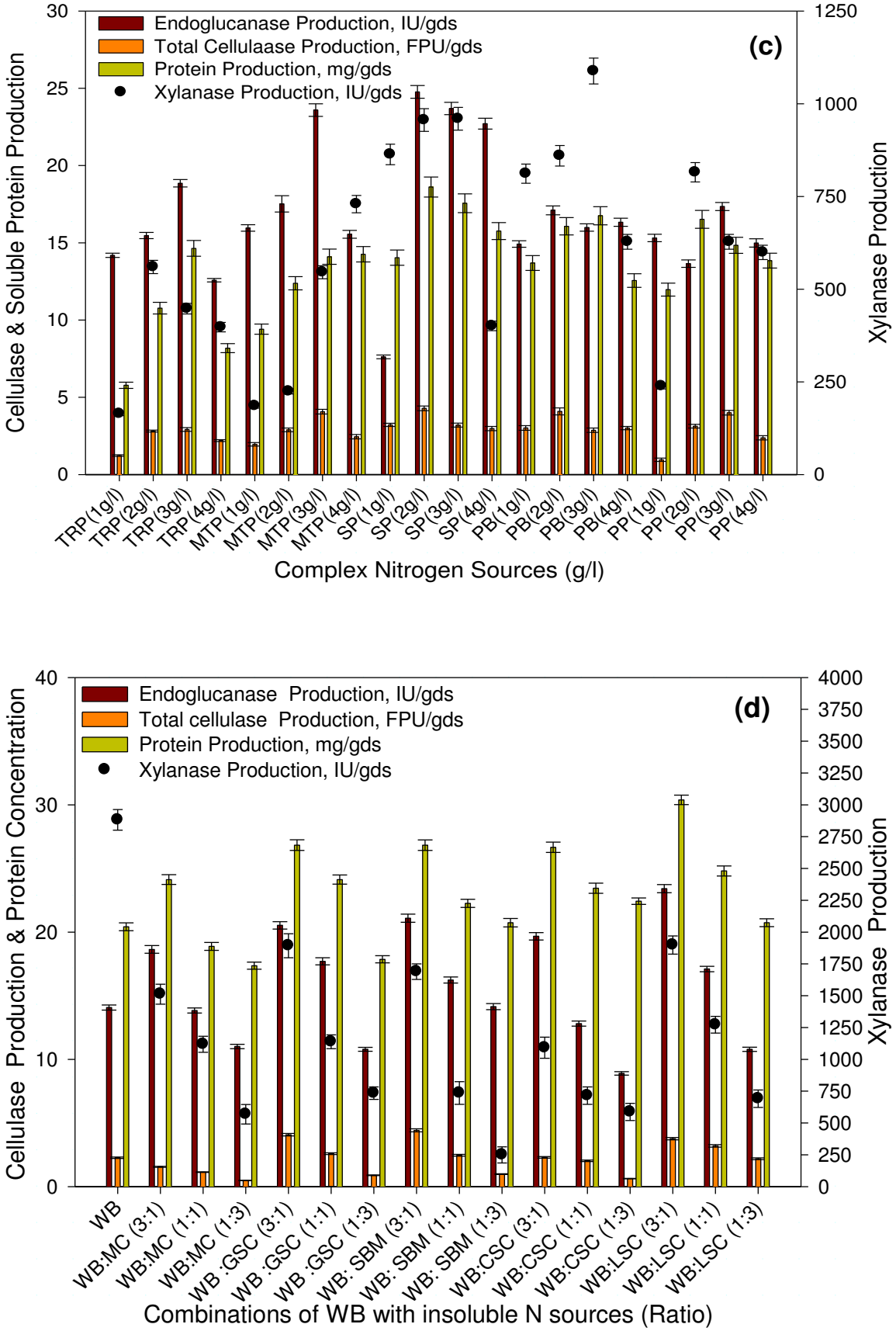
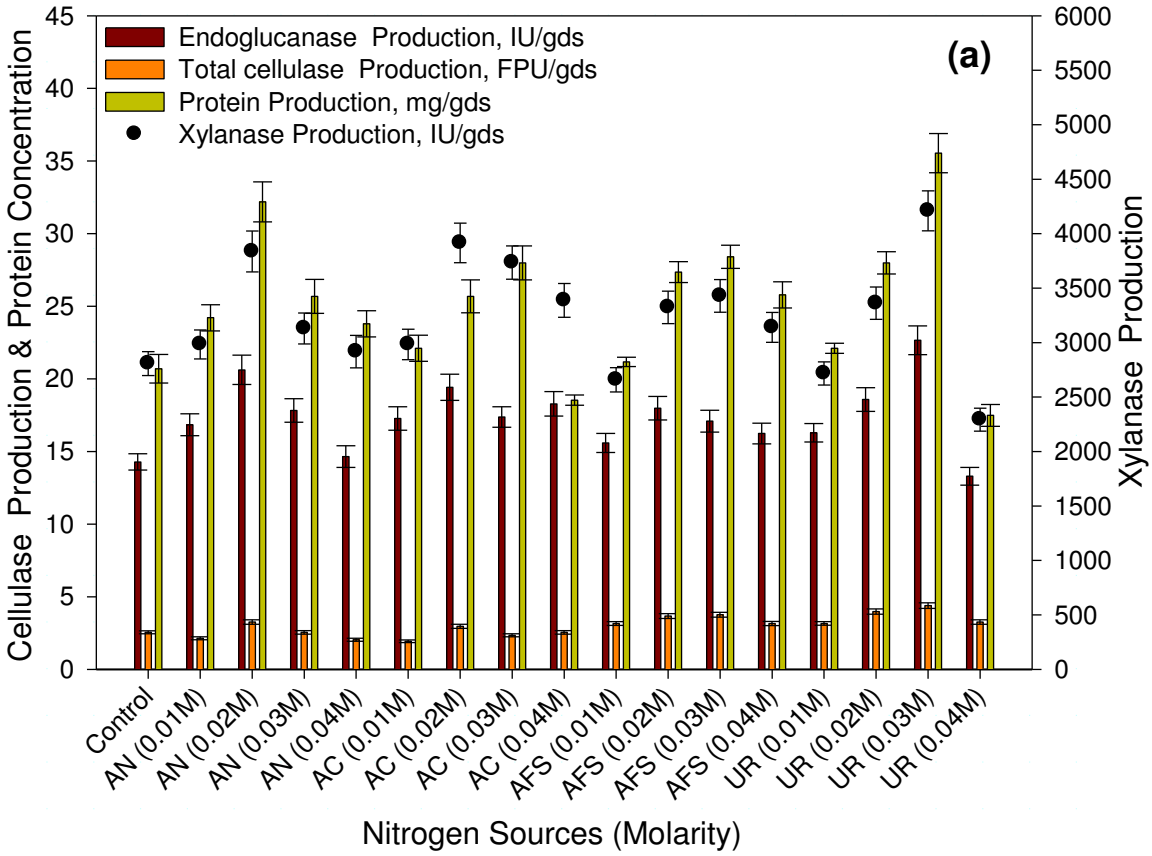
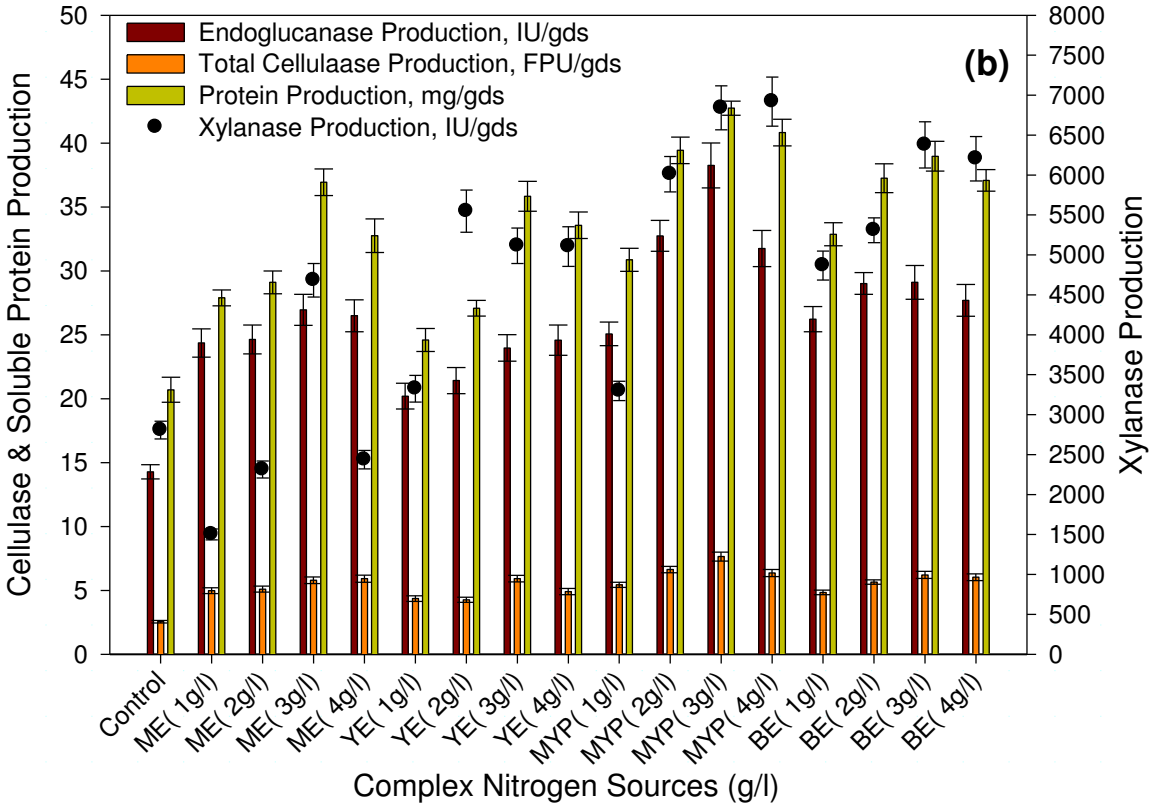
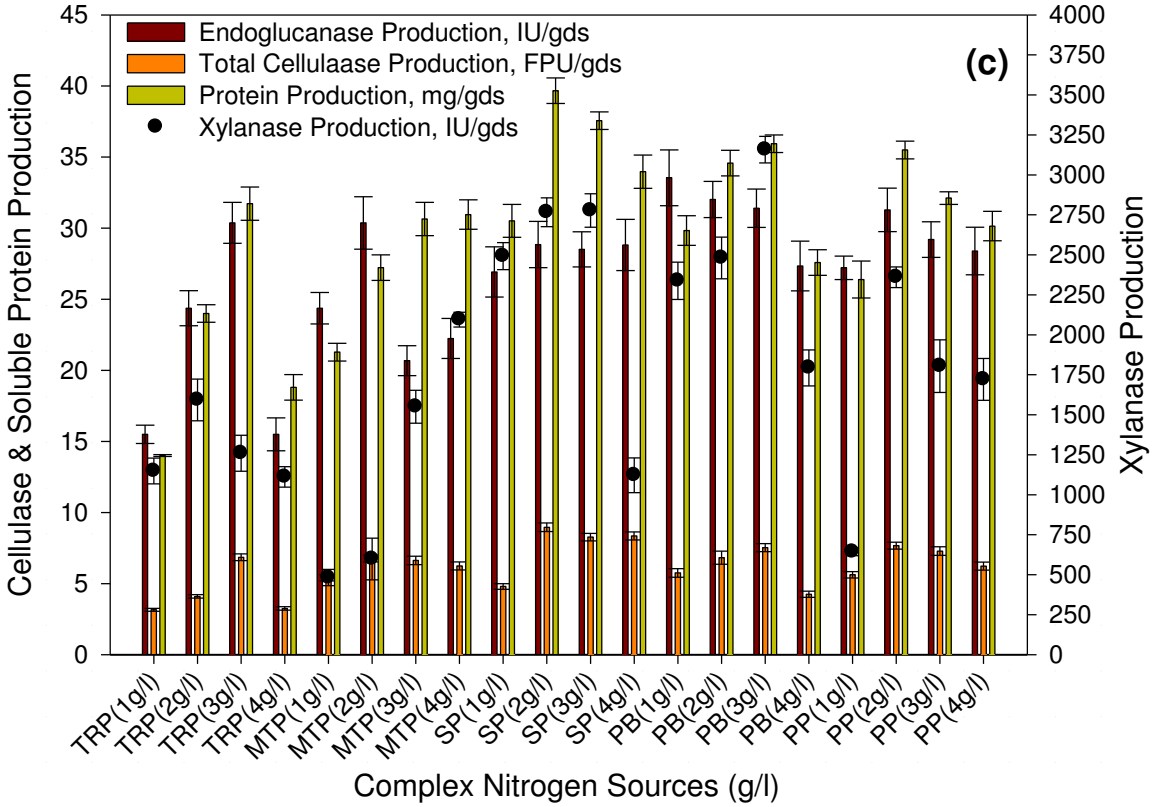


Figure-3.10 Effect of different nitrogen sources on the cellulase and xylanase production by *T. harzianum* PPDDN-10 NFCCI 2925 (a) inorganic and organic nitrogen sources, (b and c) complex organic nitrogen sources and; (d) insoluble plant based nitrogen sources


Endoglucanase Production, IU/gds
Total cellulase Production, FPU/gds
Protein Production, mg/gds
Xylanase Production, IU/gds
(a)
Cellulase Production & Protein Concentration
Xylanase Production
Control
AN (0.01M)
AN (0.02M)
AN (0.03M)
AN (0.04M)
AC (0.01M)
AC (0.02M)
AC (0.03M)
AC (0.04M)
AFS (0.01M)
AFS (0.02M)
AFS (0.03M)
AFS (0.04M)
UR (0.01M)
UR (0.02M)
UR (0.03M)
UR (0.04M)
Nitrogen Sources (Molarity)


Endoglucanase Production, IU/gds
Total Cellulaase Production, FPU/gds
Protein Production, mg/gds
Xylanase Production, IU/gds
(b)
Cellulase & Soluble Protein Production
Xylanase Production
Control
ME(1g/l)
ME(2g/l)
ME(3g/l)
ME(4g/l)
YE(1g/l)
YE(2g/l)
YE(3g/l)
YE(4g/l)
MYP(1g/l)
MYP(2g/l)
MYP(3g/l)
MYP(4g/l)
BE(1g/l)
BE(2g/l)
BE(3g/l)
BE(4g/l)
Complex Nitrogen Sources (g/l)



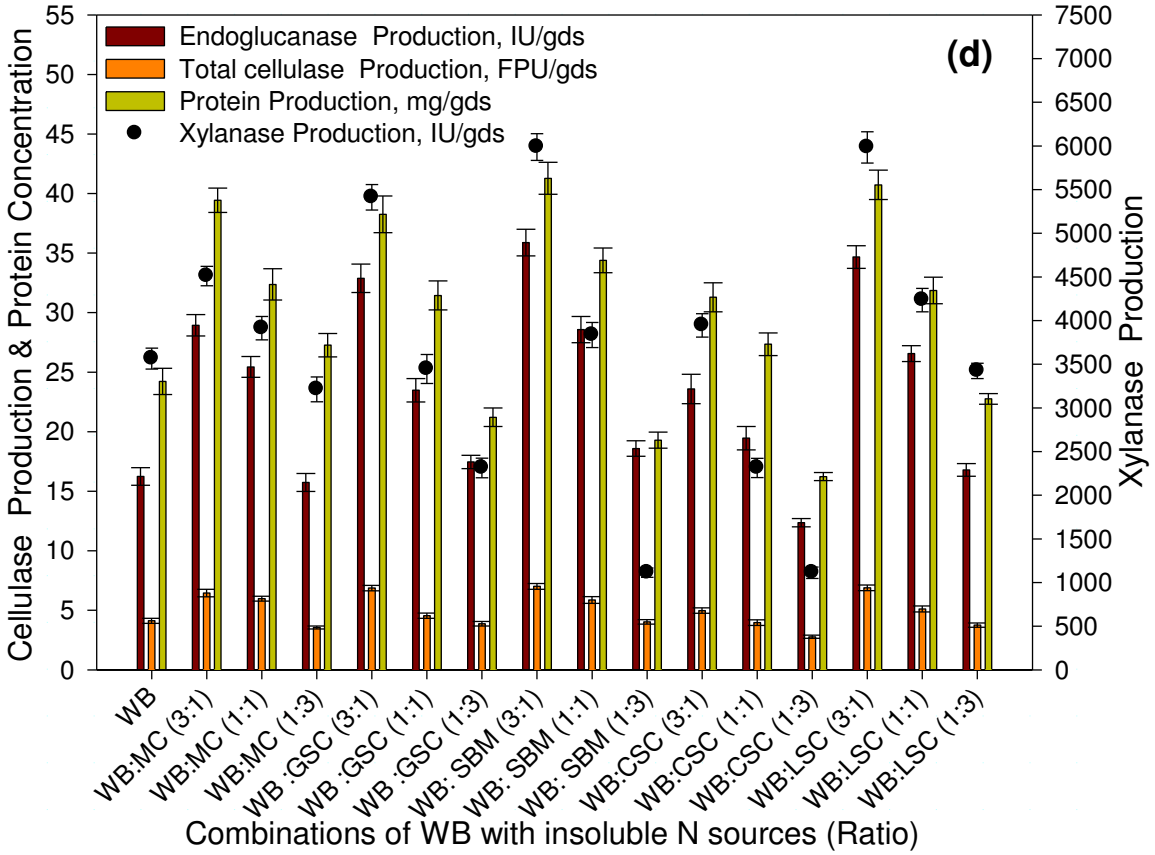


Figure-3.11 Effect of different nitrogen sources on the cellulase and xylanase production by *C. cinerea* PPHRI-4 NFCCI-3027 (a) inorganic and organic nitrogen sources, (b and c) complex organic nitrogen sources and; (d) insoluble plant based nitrogen sources

3.2.6.8. Surfactant

T. harzianum PPDDN-10 NFCCI-2925

The maximum endoglucanase is observed with the surfactant tween-80. The production of endoglucanase, total cellulase and xylanase are 27.88±0.92 IU/gds, 7.55±0.37 FPU/gds and 2069.0±159.4 IU/gds, respectively. Tween 20, tween 40, and tween 60 are observed to produce lesser enzyme production than the control one. The results of tritonX100 are comparable for the endoglucanase and xylanase but the total cellulase is lower than the tween-80 (Figure-3.12a, Table-3.16).

C. cinerea PPHRI-4 NFCCI-3027

For this fungus, the same tween-80 is observed to have maximum enzyme production. The endoglucanase production is 39.46±1.63, IU/gds, while total cellulase and xylanase production are 7.67±0.40 FPU/gds and 6539.8± 158.3 IU/gds, respectively. Soluble protein production is 41.55±0.90 mg/gds. The other surfactants are observed to improve the enzyme production than control but lower than tween-80 (Figure-3.12b, Table-3.16).

Our results are in accordance with Kuhad et al. (1994), who have reported that addition of Tween 80 enhanced the cellulase production by 30% using hypercellulolytic mutant of *Fusarium oxysporum*. Saktiselvam et al. (2012) have also identified tween 80 as best surfactant for *T. harzianum* MTCC 4358. Other reseachers have also added tween 80 in the production medium for *Trichoderma viride* (Mandels et al. 1971, Oberoi et al. 2010, Tangnu et al. 1981). Dwivedi (2006) has used tween 80 during the extraction of enzyme from the *C. Cinerea*. TritonX-100 was found to improve CMCase, FPase (6.711 IU/g) and b-glucosidase (65.361 IU/g) production from Fomitopsis sp. RCK2010. They also found slightly lower enzyme production with tween 80 (tween-20<tween-40<tween-60<tween-80<tritonX-100) (Deswal et al. 2011).

Most of the researchers have used tween 80 in concentrations between 0.1 and 0.2% due to obvious positive effect of the non-ionic surfactant towards the improving the permeability of the membranes for cellulases due to its interaction with the lipid components of cell membranes (Ahamed and Vermette 2008, Pardo 1996). This facilitates the contact between the fibrous portion of the substrate and mycelium (Eriksson et al. 2002, Esterbauer et al. 1991, Reese and Maguire 1969, Singh et al. 2007,) and helps to uptake of nutrient efficiently as well as promoting the release of cell-bound enzymes (Pardo 1996, Soni et al. 2010). Eukaryotic organisms release extracellular enzymes from intracellular organelles through exocytosis, so the surfactants may promote this exocytosis by interaction with cell organelles and lipid membrane components (Singh et al. 2007). According to Kruszewska et al. (1990), tween-80 has an influence on the level of glycosylation and thus on protein stability. Recently, Yang et al. (2011) have reported that tween-80 is not effective at high speed

shaking in protecting free cellulase against deactivation and decreasing cellulase proteins adsorption.

3.2.6.9. Moisture level (WB:NSS ratio)

During SSF, the moisture content plays a critical role to cause swelling of the substrate to make easy availability of nutrients.

T. harzianum PPDDN-10 NFCCI-2925

WB:NSS ratio of 1:3 is found to be optimum for the endoglucanase (27.49±0.99 IU/gds), xylanase (2165.4±158.2 IU/gds) and soluble protein production (30.81±1.34 mg/gds). Maximum total cellulase (8.03±0.41 FPU/gds) production is observed at ratio of 1:4 (Figure-3.13a, Table-3.17).

C. cinerea PPHRI-4 NFCCI-3027

For this fungus, It is again observed that the maximum enzyme production is achieved at WB:NSS ratio of 1:3 (endoglucanase 38.56±1.41 IU/gds, total cellulase 7.75±0.30 FPU/gds, xylanase 6813.6±261.1 IU/gds and soluble protein 43.42±1.04 mg/gds) (Figure-3.12b, Table-3.17).

The others researchers have maintained the moisture ratio of 1:5 for *Aspergillus terreus* strain 5 and *A. niger* strain 44 (Gawande and Kamat 1999), 1:3 for *C. cinerea* (Kaur et al. 2012) and *Coprinellus disseminatus* (Agnihotri et al. 2010).

High and low water contents adversely affect the primary metabolic activities of microbes leading to slower enzyme synthesis. The reason for this decline may be the alteration in the physical properties of the LCW *i.e.* WB (Raimbault and Alazard 1980). At low moisture level, diffusion of solutes, gases and solubility of the nutrients present in WB are reduced due to less moistened and improperly swelled WB particles. At high moisture level, low porosity results into poor oxygen transfer due to gummy texture of WB, which allows the growth of aerial hyphae. All these effects are responsible for the low enzyme production (Deschamps et al. 1985, Raimbault and Alazard 1980, Virupakshi et al. 2005, Xin and Geng 2010).

3.2.6.10. Particle size

Particle size of substrate used in fermentation affects the surface to volume ratio of particle, which decides the portion of the substrate that is initially accessible to fungi and the packing density with the surface mass.

T. harzianum PPDDN-10 NFCCI-2925

WB having 300-350 µm particle size is found to be optimal for the endoglucanase (18.22±0.45 IU/gds) and xylanase (1342.1±40.2 IU/gds) production with the maximum protein concentration (21.6±0.65 mg/gds), but the FPase production (4.43±0.12 FPU/gds) is

observed as maximum with the particle size of 350-425 µm (Figure-3.14a, Table-3.18). In the case of WB, the result of unscreened WB is found comparable with 300-350 µm particle size because there is no need to reduce or screen the particle size of the WB (which are already fine particle with different particle size), while mixture of all the particle sizes in equal amounts are resulted into lesser production of enzymes.

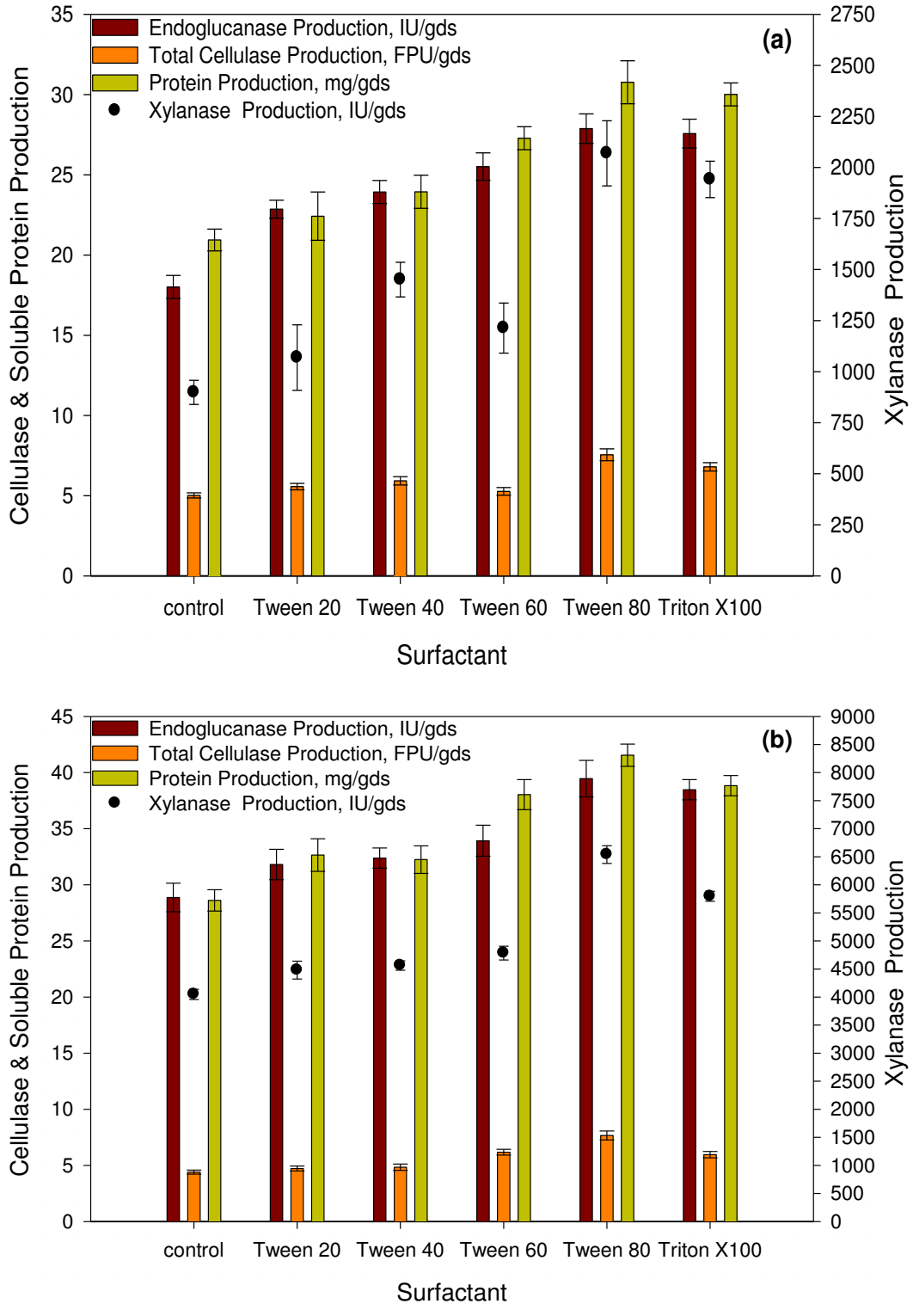


Figure-3.12 Effect of different surfactants on the cellulase and xylanase by (a) *T. harzianum* PPDDN-10 NFCCI-2925 (b) *C. cinerea* PPHRI-4 NFCCI-3027

C. cinerea PPHRI-4 NFCCI-3027

350-425 µm particle size of WB is the most suitable range for the cellulase production *i.e.,* 41.87±1.65 IU/gds endoglucanase and 9.20±0.28 FPU/gds total cellulase. It is observed that xylanase enzyme production (6155.9±231.7 IU/gds) is maximum with the slightly lower particle size *i.e.*, 300-350 µm. It is also observed that both the fungal strains are found to produce comparable results with as such WB *i.e.,* unscreened WB (Figure-3.14b, Table-3.18).

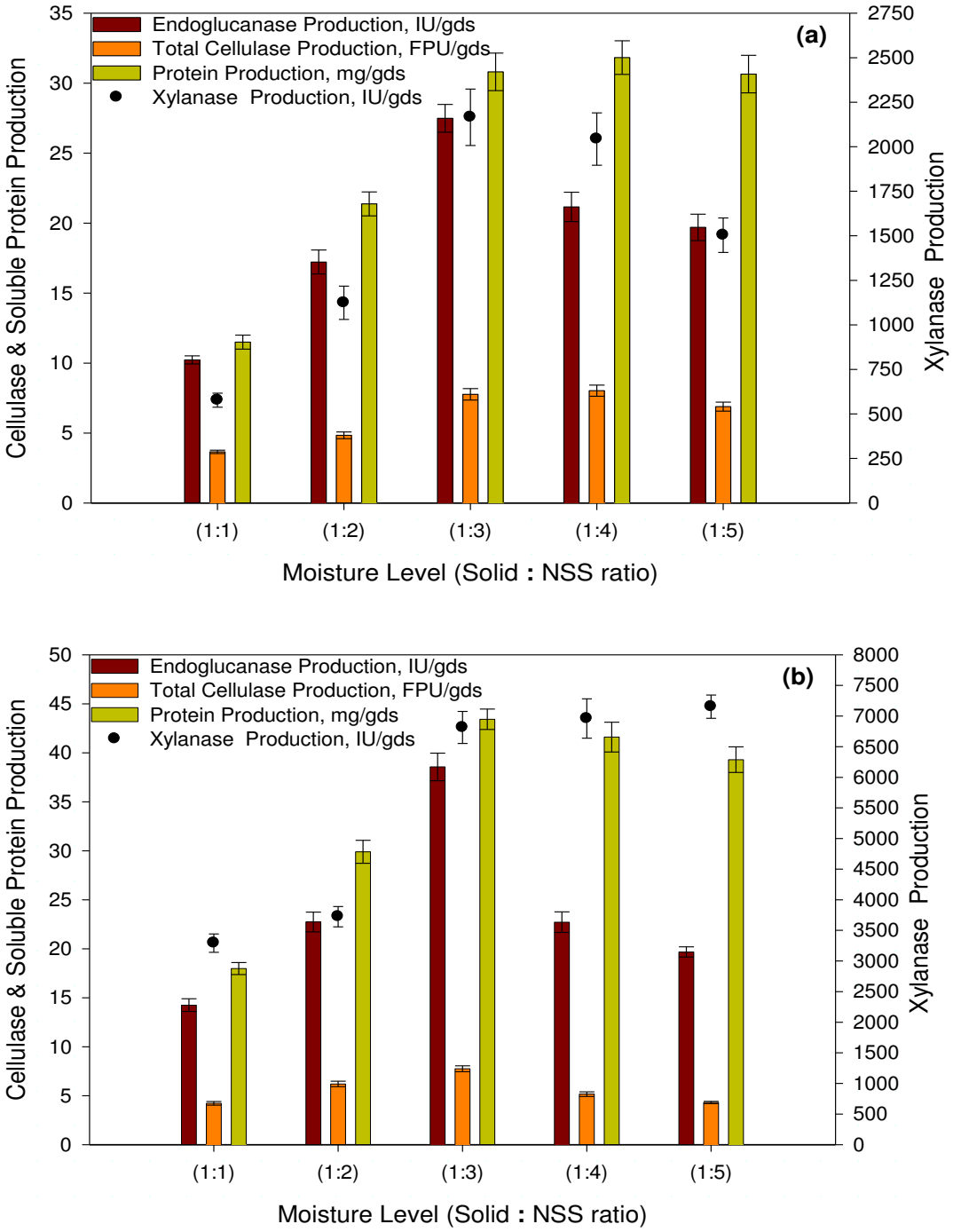
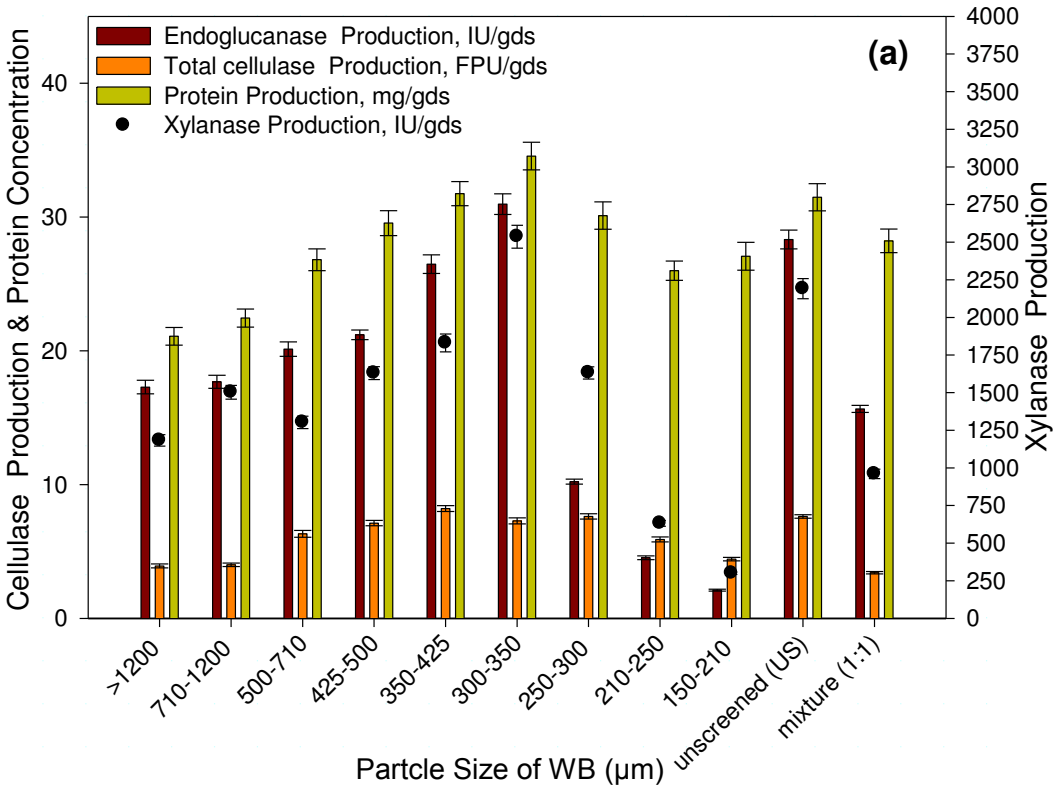


Figure-3.13 Effect of moisture level on the cellulase and xylanase production by (a) *T. harzianum* PPDDN-10 NFCCI-2925 (b) *C. cinerea* PPHRI-4 NFCCI-3027



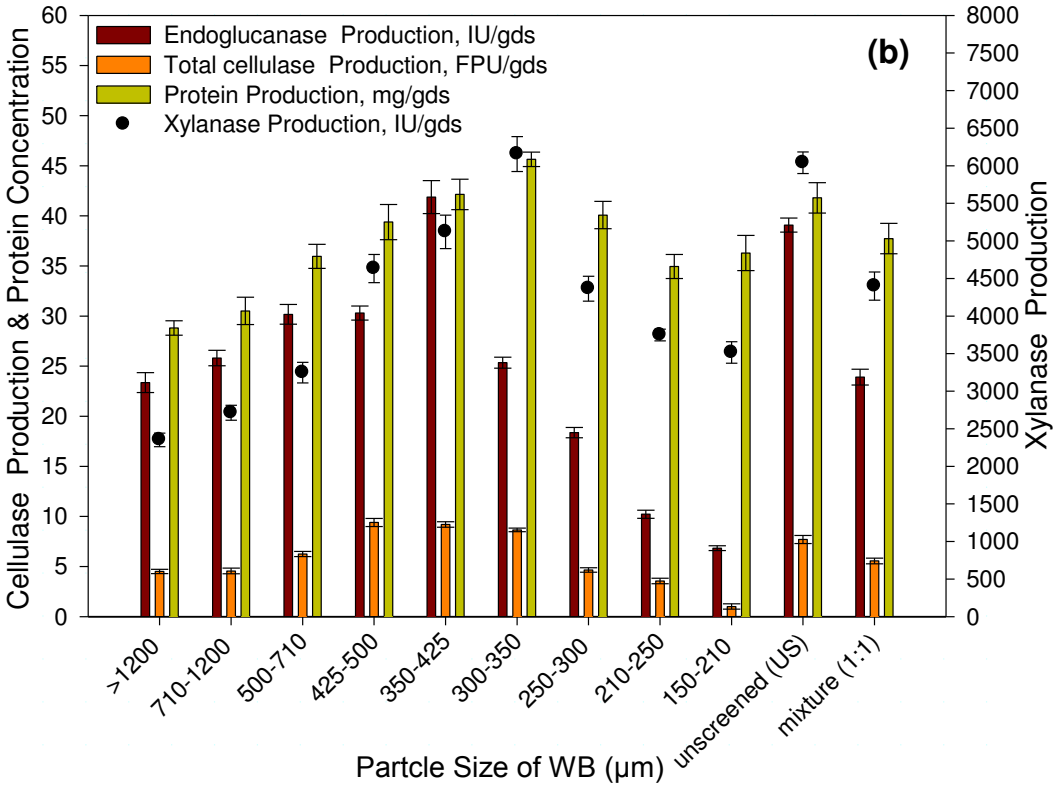


Figure-3.14 Effect of particle size of WB on the cellulase and xylanase production by (a) *T. harzianum* PPDDN-10 NFCCI-2925 (b) *C. cinerea* PPHRI-4 NFCCI-3027

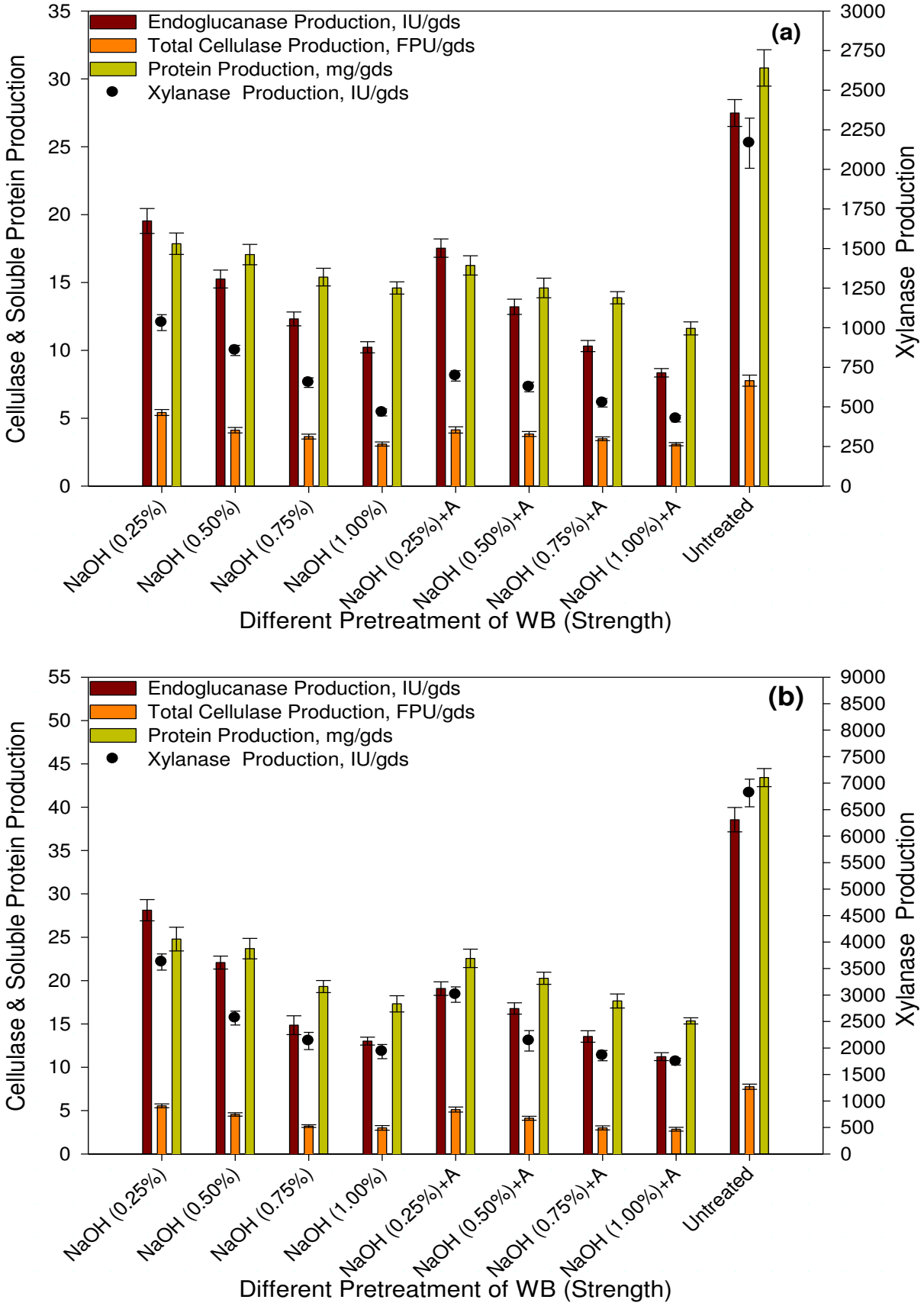


Figure-3.15 Effect of different alkali pretreatment of WB on the cellulase and xylanase production by (a) *T. harzianum* PPDDN-10 NFCCI-2925 (b) *C. cinerea* PPHRI-4 NFCCI-3027

Production on enzyme on unscreened (US) WB has shown the slightly lower enzyme production than the optimum size of WB particles. Enzyme production is decreased with mixture of different particles in equal amounts.

Bahrin et al. 2011 have found different particle sizes responsible for enzyme production from *Botryosphaeria sp. i.e.,* 0.84 to 1.0 mm for CMCase activity and 0.42 to 0.60 mm for FPase and β-glucosidase. According to Tao et al. (1997), 400 µm substrate sized particles contribute to the optimum fungal growth and cellulase production due to the inverse correlation of porosity and surface area factors. Membrillo et al. (2008) have also achieved the maximum enzyme production on different particle sizes of sugarcane baggase for *Pleurotus ostreatus* CP-50.

The exposed surface area of cellulose is more important than the actual amount of cellulose present (Fan et al. 1982). Generally, low particle sizes (400µm) facilitate the production of high cellulase and xylanase due to large specific surface area. Fine particles will have low porosity but it readily usable for the mold because of lower crystallinity and degree of polymerization. Large particle size is responsible for increased surface area and the bulk density of the raw materials (Tao et al. 1997). As such utilization of the whole WB will be economically feasible because of the complete utilization of raw material without any rejection.

3.2.6.11. Pretreatment of LCW

It is clearly shown in the Figure-3.15 that the alkali pretreatment of the WB is resulted in the decreased production of the enzymes.

T. harzianum PPDDN-10 NFCCI-2925

Alkali pretreatment with different doses (0.25 to 1.0% NaOH) has resulted into 29.0 to 62.8% endoglucanase, 30.2 to 60.1% total cellulase and 52.3 to 78.5% xylanase production reduction. After thermal degradation, the production is further reduced to 36.2 to 69.6, 46.7 to 60.2 and 67.9 to 80.4%, respectively (Figure-3.15a, Table-3.19).

C. cinerea PPHRI-4 NFCCI-3027

The endoglucanase, total cellulase and xylanase production are reduced to 27.1 to 66.2, 28.6 to 61.3 and 46.8to 71.6%, respectively. After pretreatment, the same are 50.5 to 70.9, 34.1to 63.3, and 55.8 to 74.4%, respectively. In both the fungi, the xylanase production is greatly affected by the alkali pretreatment. The thermal treatment enhanced the degradation of the LCW components (Figure-3.15b, Table-3.19).

These pretreatment processes produce the total and reducing sugars in large amounts due to dissolution of the hemicellulosic and lignin materials (data not shown) which result in the low yield of the WB (25 to 52%); and make the whole process costly.

The thermal pretreatment with alkali, results in more decrease in the enzyme production than the chemical treatment alone. The pretreatment methods give a gummy and sticky texture to the WB particles. Therefore, the porosity between WB particles is greatly influenced which in turn facilitates the surface growth on the substrate and resulted in the lower enzyme production. In the subsection 3.2.6.2. and 3.2.6.4., it is clearly shown that the cellulase and xylanase production is dependent on the growth of the filamentous fungi because the growth is dependent on the accessibility of cellulosic and hemicellulosic components present in the LCWs. According to Rahanama et al. (2013), alkali pretreatment is responsible for the dehydrated, lysed and shriveled myecelia while the untreated LCW will have better growth under SSF. All the pretreatment processes are found to have lower yield due to the solublization of the hemicellulose and lignin to generate the reducing sugars. Thus the relative cellulose percentage in the WB will be improved and the hemicellulose and lignin percentage will be decreased (Rahanama et al. 2013, Remli et al. 2014). Rahanama et al. (2013) also reported the lower enzyme production with the rice straw after alkali pretreatment. They found that the alkaline pretreatment partially disrupted the crystalline structure of the cellulose and the absolute crystallinity of the cellulase affected the cellulase production. The complex structure of the WB might act as the inducer to produce the cellulase and xylanase enzyme in greater amount in order to get the monomeric sugars (Rahanama et al. 2013). This growth might be responsible for more efforts to produce enzyme in higher amounts to degrade the cellulose and hemicellulose in comparison to easily accessible and degradable substrate achieved by pretreatment processes. This is not an ecofriendly option because of the use of chemicals.

3.2.6.12. Sugars

In order to investigate the effect of different sugars as inducer or repressor, six different sugars (glucose, galactose, lactose, xylose, fructose and cellobiose) are studied. The production of cellulase and xylanase by both the fungal strains is affected by the sugars as well as their concentration.

T. harzianum PPDDN-10 NFCCI-2925

Among these, cellobiose is found to act as inducer for the endoglucanase upto the concentration of 2 g/l. However, after this concentration, it starts working as a repressor whereas all other sugars behave as a repressor. It is also observed that all the sugars (except the cellobiose) affect the cellulase as well as xylanase production (Figure-3.16a, Table-3.20).

C. cinerea PPHRI-4 NFCCI-3027

The enzyme production is repressed by the presence of sugars. The increasing amount of sugars result in the corresponding decrease in the production of cellulase and xylanase.

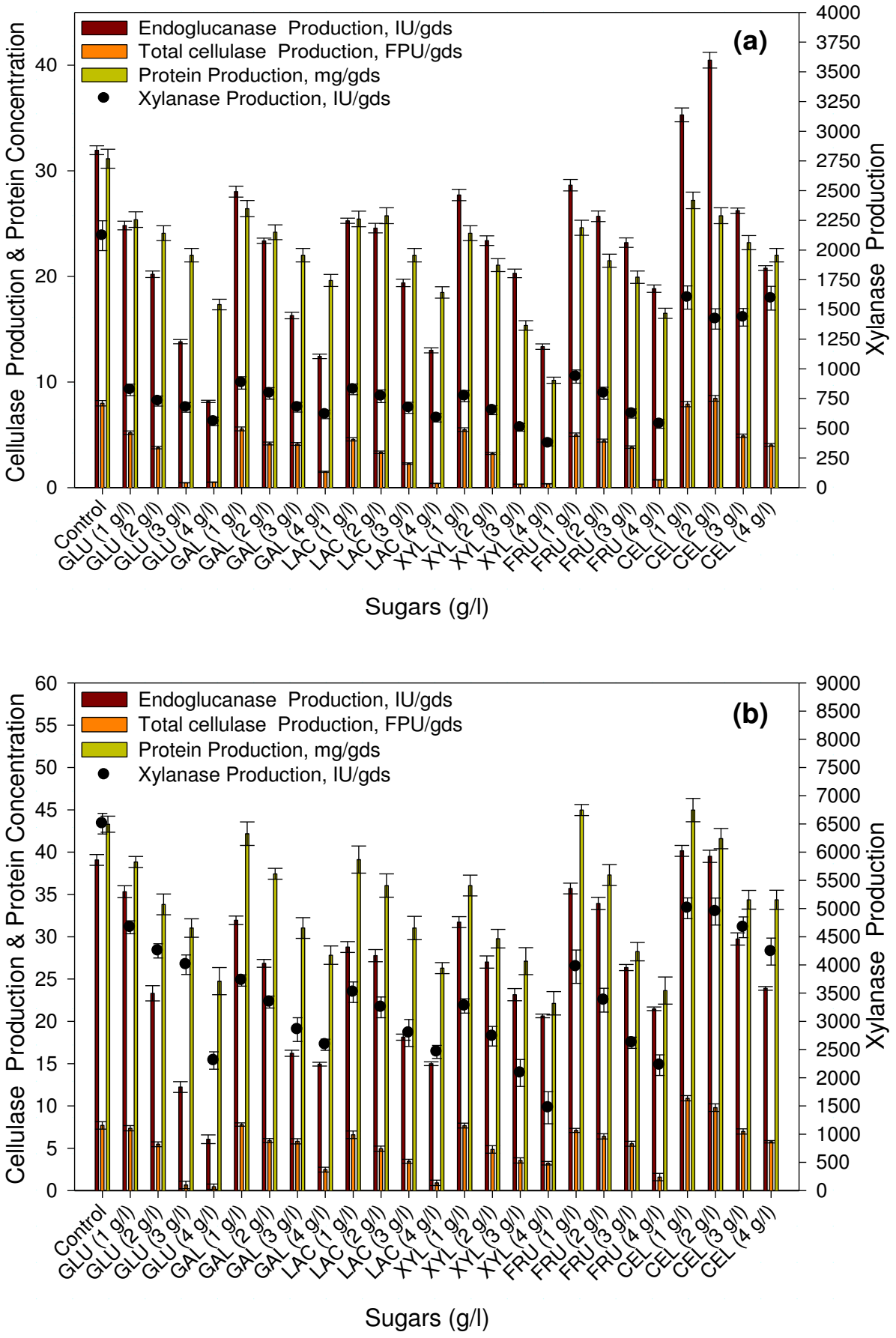


Figure-3.16 Effect of different sugars on the cellulase and xylanase production by (a) *T. harzianum* PPDDN-10 NFCCI-2925 (b) *C. cinerea* PPHRI-4 NFCCI-3027

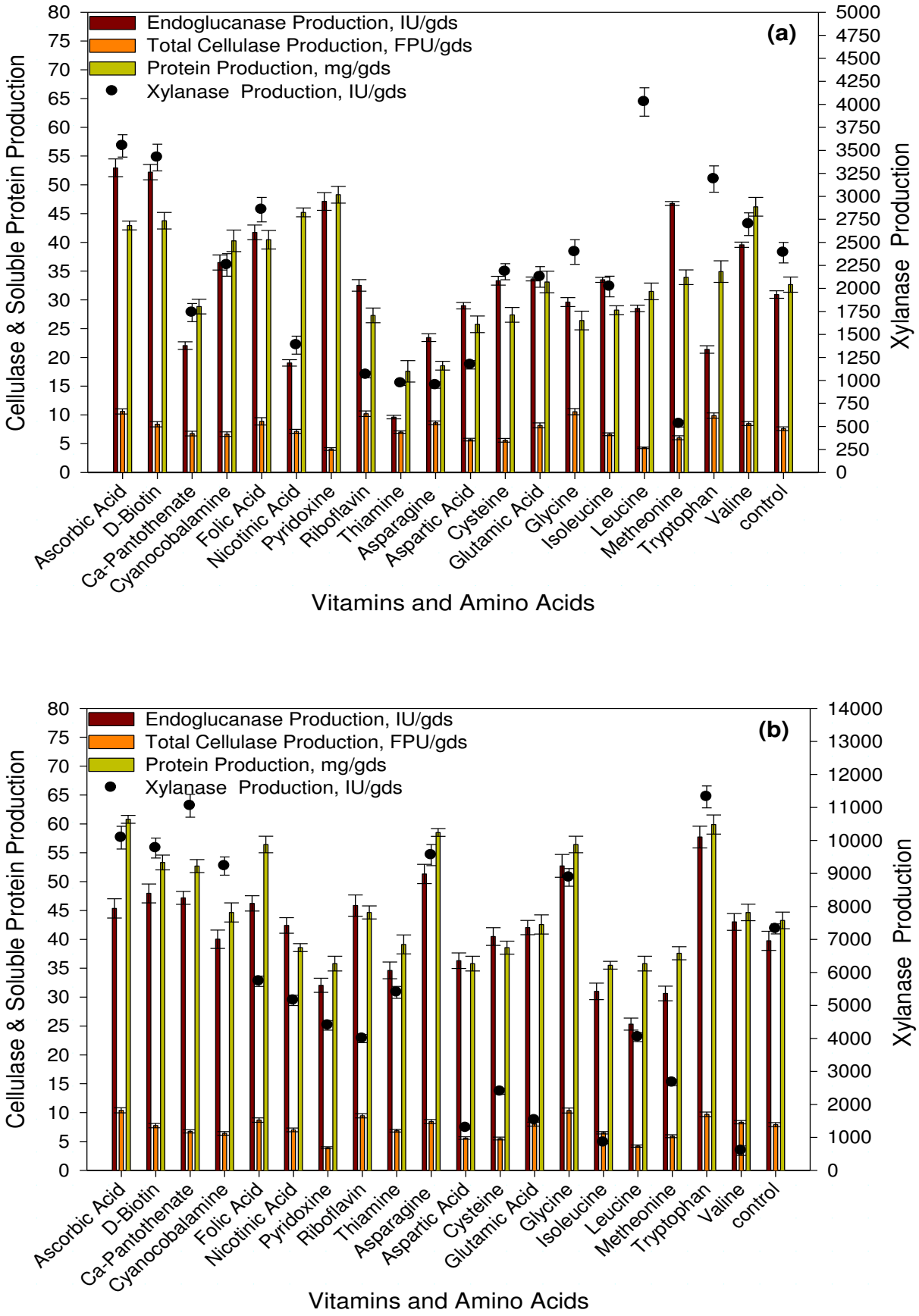


Figure-3.17 Effect of different vitamins and amino acids on the cellulase and xylanase production by (a) *T. harzianum* PPDDN-10 NFCCI-2925 (b) *C. cinerea* PPHRI-4 NFCCI-3027

The maximum production is achieved in the absence of these sugars. The extracellular production of cellulase and xylanase enzymes is found to be inducible and controlled under catabolite repression (Figure-3.16b, Table-3.20).

Cellobiose has been proposed as an inducer of cellulase activity, but it also failed to induce *Trichoderma* cellulase activity in several studies (Kawamori et al. 1986, Kubicek and Penttilä 1998, Nisizawa et al. 1971, Sternberg et al. 1979,). It is reported Cellobiose (an oxidation product ofcellulose hydrolysis) as inducer but at higher concentration it retards the cellulase production in *Trichoderma* sp.(Kubicek and Penttilä 1998, Singhania et al. 2006). On a contrary, the catabolite repression resistance from glucose is reported in a mutant strain M7 of *Thermomyces lanuginosus* (Bokhari et al. 2010). Glucose and lactose are the well-known repressors for the xylanase production in *C. cinerea* and *C. disseminatus*. The increasing doses of these sugars are responsible for the corresponding decrease in the enzyme production (Agnihotri et al. 2010, Kaur et al. 2012).

It is well known phenomenon in the fungus that the organisms stop the enzyme production when the sugars are accumulated in the fermentation medium. At that time, fungi start to utilize the accumulated sugars and then again start the production machinery (Kaur et al. 2012). The variations in the responses as inducer or repressor may be due to genetic variability of the fungi or to the different purity levels of the inducing substrates. This type of repression indicates that the transition state regulators and catabolite repression control the enzyme production machinery (Kaur et al. 2012, Sanghi et al. 2008).

3.2.6.13. Additives

The additives are added to see the inducive or suppressive effect on the enzyme production. Vitamins usually have an effect on the rate of biosynthesis of many metabolites (Deswal et al. 2011).

T. harzianum PPDDN-10 NFCCI-2925

L-Ascorbic acid, biotin and folic acid are found to enhance the production of all the three enzymes. Calcium pantothenate, nicotinic acid and thiamine suppress the production of all three enzymes. Pyridoxine enhances the endoglucanase and xylanase production and but total cellulase production is suppressed (Figure-3.17a. 3.18a, Table-3.21).

Among the amino acids, only valine shows the stimulatory effect on the all the three enzymes. Xylanase production is achieved maximum with leucine, while the FPase is observed to be the maximum with glycine. Methonine and cysteine have increased the production of the CMCase, but have negative effect on the xylanase production (Figure-3.17a. 3.18a, Table-3.21).

Among the different metal ions Mn^{+2}, Fe^{+3}, K^{+} and Na^{+} have shown the stimulatory effect on the CMCase and xylanase production. Zn^{+2} and Co^{+3} have increased the production of all three enzymes (Table 3). Chelating agent had inhibitory effect for the CMCase, FPase and xylanase production (Figure-3.19a and 3.20a, Table-3.22).

C. cinerea PPHRI-4 NFCCI-3027

The addition of ascorbic acid results into improved production of cellulase and xylanase. Pyridoxine and thiamine suppress all the three enzymes. D-biotin and Ca-pantothenate have the stimulatory effect for endoglucanase and xylanase production. Folic Acid and Riboflavin improve the cellulase production only (Figure-3.17b and 3.18b, Table-3.21).

Tryptophan and glycine have stimulatory effect, while aspartic acid, isoleucine, leucine and methionine act as suppressor for all the three enzymes (Figure-3.17b and 3.18b, Table-3.21).

Mn^{+}, Zn^{+2}, Fe^{+3} and K^{+} are found to increase both the cellulase and xylanase production. Heavy metals again repressed the cellulase as well as xylanase enzyme (Figure-3.19b and 3.20b, Table-3.22).

Deswal et al. (2011) have investigated the maximum CMCase production in the presence of L-glutamic acid; whereas, aspartic acid, asparagine and L-cystine have improved the poduction slightly lower than L-glutamic acid *Fomitopsis* sp. RCK2010. Riboflavin and biotin also exhibited a stimulatory effect on FPase production. According to them vitamins are not able to induce the CMCase production. Zinc and nickel ions have the stimulatory effect for endogluanase production, while mercury and calcium exhibited an inhibitory effect. The addition of sodium, manganese and copper has been reported to stimulate total cellulase production.

Biotin is well documented for playing important role in functioning of several physiological and metabolic enzymes including pyruvate carboxylase and several tricarboxylic acid cycle auxiliary enzymes. The stimulatory effects showed that these metal ions might be acting as cofactors for many enzymes involved in intermediatory metabolism (Jellison et al. 1997, Deswal et al. 2011). The metals ions may prevent some component necessary for induction to release the enzyme out of the cells. According to Morton and Broadbent (1955) addition of some trace metals inhibit the release of amino acids from fungal cell while addition certain trace elements to the medium are required for cellulase production, not for the fungal growth. Best cellulase production occurs in the presence of trace elements like iron, manganese, zinc and cobalt (Mandels and Reese 1957).

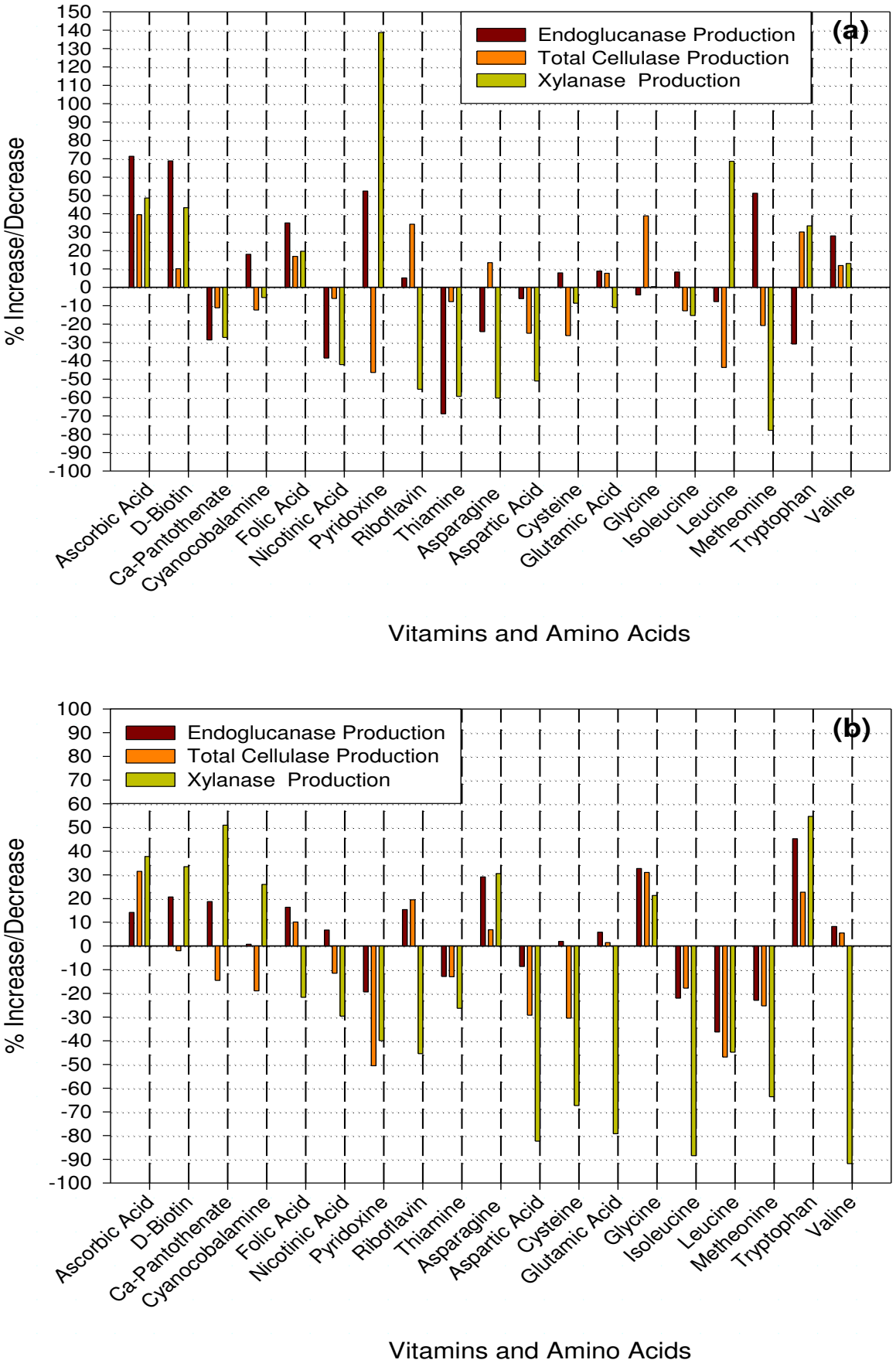


Figure-3.18 Vitamins and amino acids as stimulator and repressor for the cellulase and xylanase production by (a) *T. harzianum* PPDDN-10 NFCCI-2925 (b) *C. cinerea* PPHRI-4 NFCCI-3027

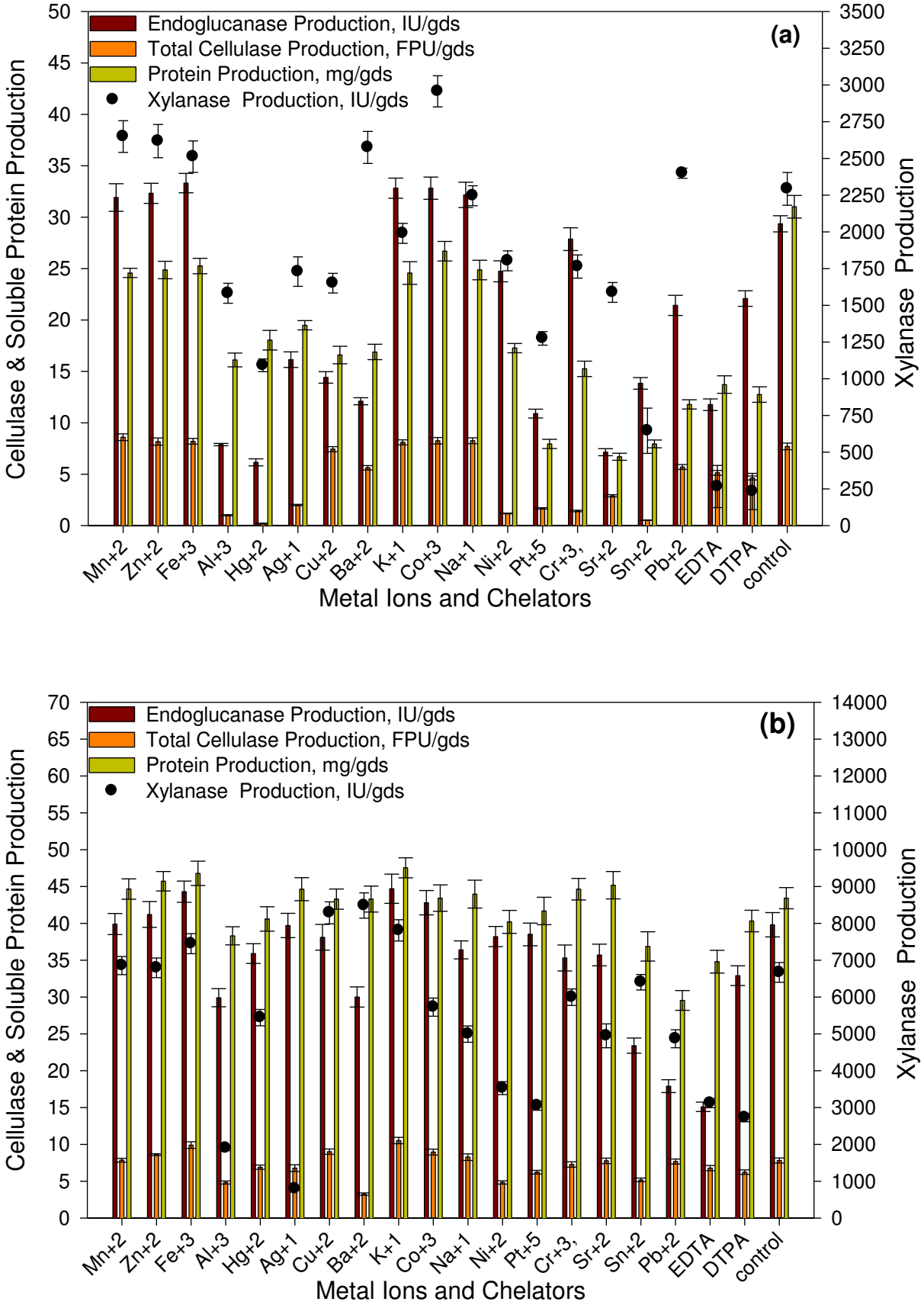


Figure-3.19 Effect of metal ions and chelators on the cellulase and xylanase production by (a) *T. harzianum* PPDDN-10 NFCCI-2925 (b) *C. cinerea* PPHRI-4 NFCCI-3027

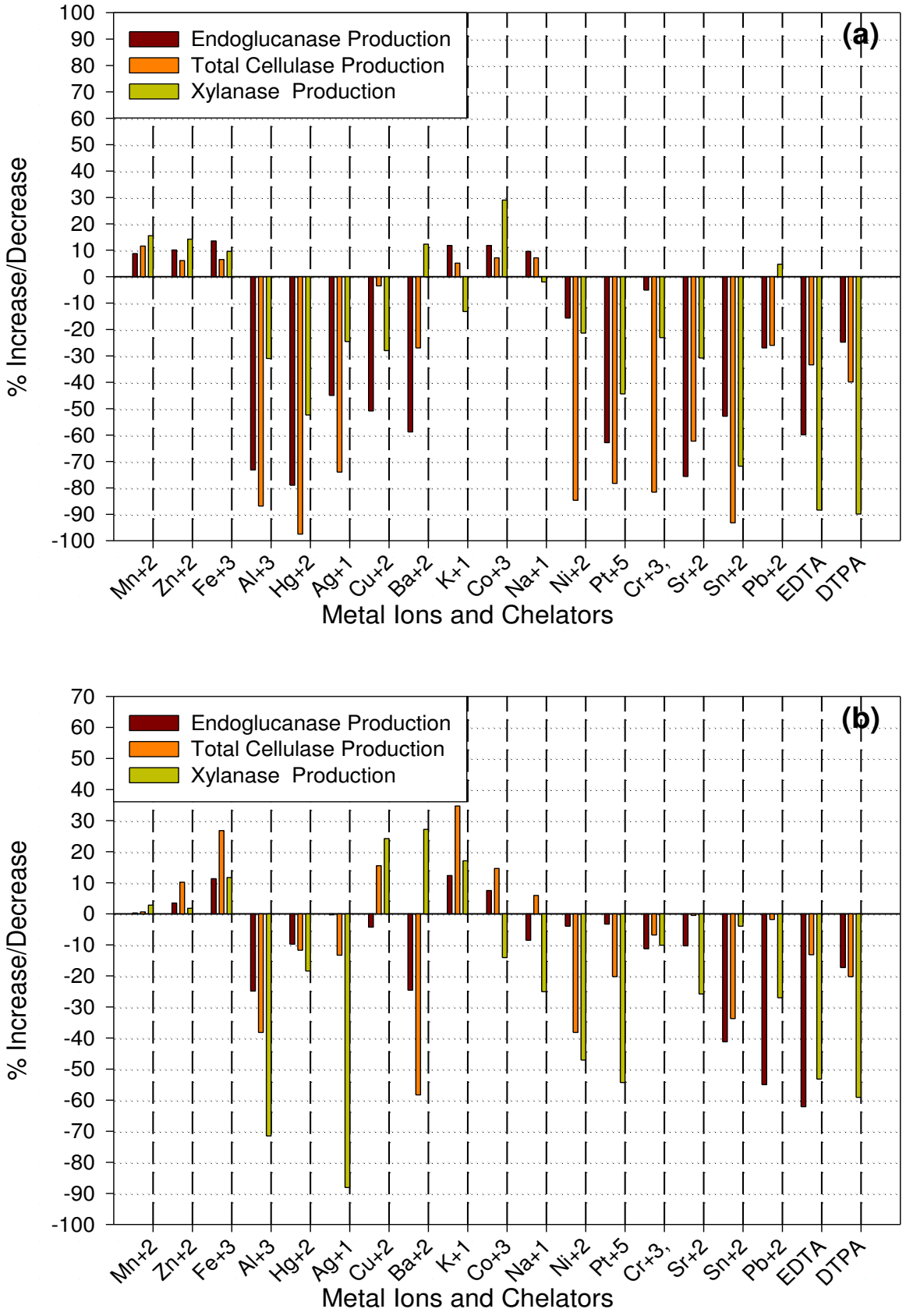


Figure-3.20 Vitamins and chelators as stimulator and repressor for the cellulase and xylanase production by (a) *T. harzianum* PPDDN-10 NFCCI-2925 (b) *C. cinerea* PPHRI-4 NFCCI-3027

3.2.7. Characterization of cellulase and xylanase enzyme for its optima and stability towards pH and temperature

To be proven as an industrially important product, the characterization of these enzymes requires knowledge of optimum pH and temperature as well as their stability because CMCase, FPase and xylanase activities are subjected to change with varying pH as well as temperature (Bhat 2000) and the optimum pH and temperature, maintained during hydrapulping, mainly depend on the enzymes to be used for deinking experiments (Lee et al. 2011b)

3.2.7.1. Optimum pH and stability

T. harzianum PPDDN-10 NFCCI-2925

pH optima profile shows that the pH of 4.5-6.0 is the best range. The optimum pH for the CMCase and xylanase activities is 5.5 while for the FPase activity it is 5.0 (Figure-3.21a). The results obtained have shown that about 50% residual activities were retained at pH 8.5. Stability of any enzymes in its optimum pH is prerequisite for any enzymatic reaction. It is observed that only 5-12% activity of CMCase, FPase and xylanase is lost at 40 °C upto 6 h i.e. more than any anticipated enzymatic reaction time. Even after 24 h, its activity is found to decrease upto 14-22% (Figure-3.21b, Table-3.23).

C. cinerea PPHRI-4 NFCCI-3027

pH optima profile of cellulase and xylanase showed that the pH of 5.0-6.0 is the best range. The maximum CMCase and FPase activities are achieved at the pH 5.5 while pH 6.0 is the best for xylanase activity (Figure-3.22a). The enzyme activities are steadily declined after pH 6.0 and it retained approximately 50% activity at pH 8.5. More than 25% of activities are observed to retain in the pH range of 3.0 to 9.0. Stability of any enzymes in its optimum pH is prerequisite for any enzymatic reaction. It is observed that only 24.6 to 28.0% activity of CMCase, FPase and xylanase is lost pH 5.5 even after 24 h i.e. more than any anticipated enzymatic reaction time (Figure-3.22b, Table-3.24).

These results are in agreement with previous studies that the activity of xylanases isolated from *Trichoderma* is the highest at pH and temperature ranges of 3.5–6.5 (Silveira et al. 1999, Aro et al. 2001). Crude cellulase and xylanase of fungal origin have optimum pH in slightly acidic range (Agnihotri et al 2010, Kaur et al. 2011, Singh et al. 2009,). Ahmed et al. (2012) have also shown the same pH range for the *T. harzianum*. Xylanase activity have been observed to be the maximum at pH 6.4 for *C. cinerea* (Kaur et al. 2011) and *C. disseminatus* (Agnihotri et al. 2010) and pH had a great effect on the activity of the enzyme due to its proteinaceous nature. At high or low pH, the charge on the amino acids present in the active sites is prone to change. Due to which the basic conformational structure of the active sites and proteins will also be changed. pKa (ionization constant) of catalytic residues

has an effect on the pH activity profile of enzymes. pKa value and pH stability have inverse relation. Amino acid residues, contributing positive charges and hydrogen bonds, lower the pKa values with shorter bonds having a more definite effect (Collins et al. 2005). As, the cellulase and xylanase enzyme have retained more than 25% activity in the alkali region. Therefore, cellulase and xylanase produced by *T. harzianum* PPDDN-10 NFCCI-2925 and *C. cinerea* PPHRI-4 NFCCI-3027 can be considered as alkali-tolerant.

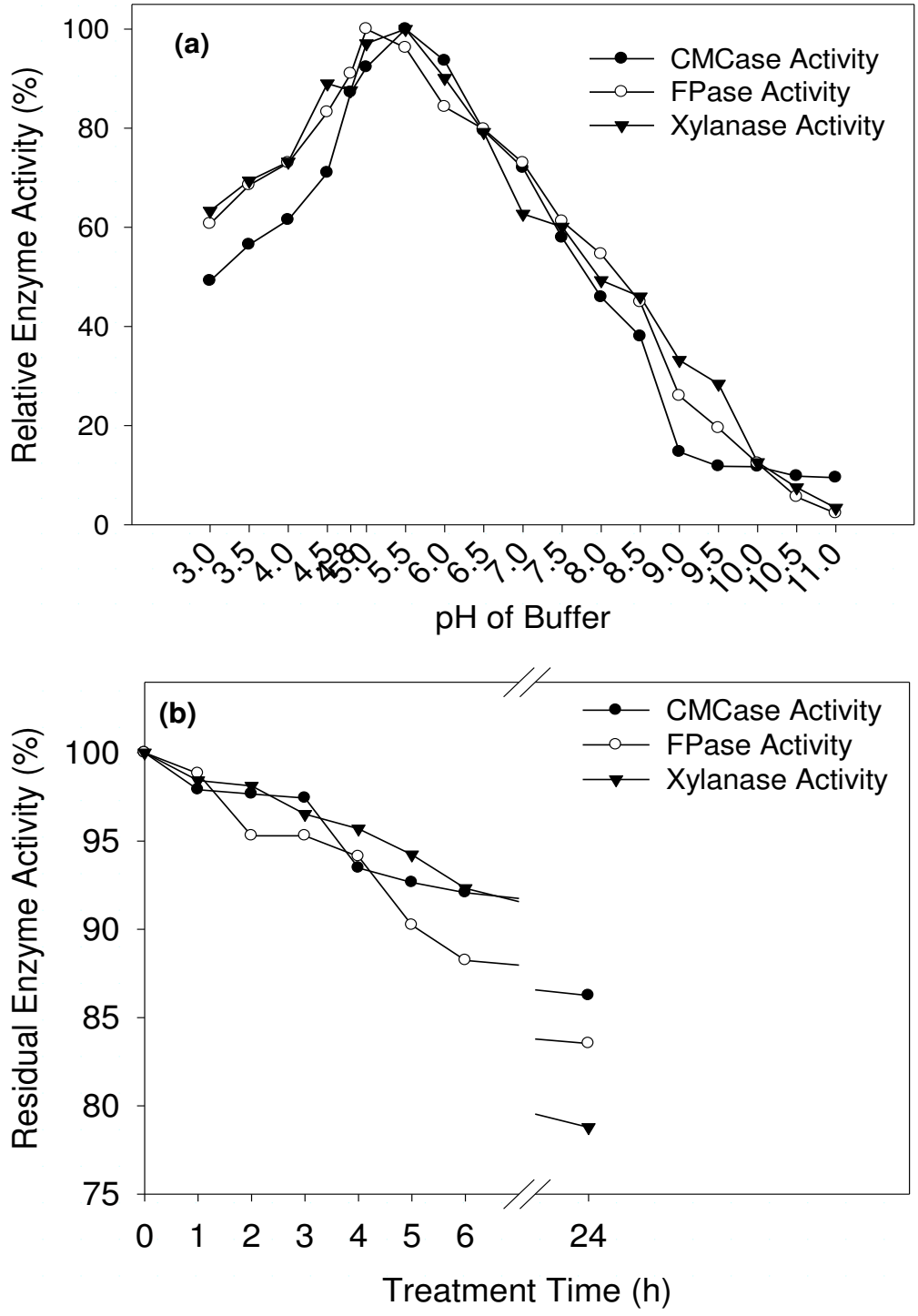


Figure-3.21 (a) Optimum pH (b) stability at the optimum pH for cellulase and xylanase of the *T. harzianum* PPDDN-10 NFCCI-2925

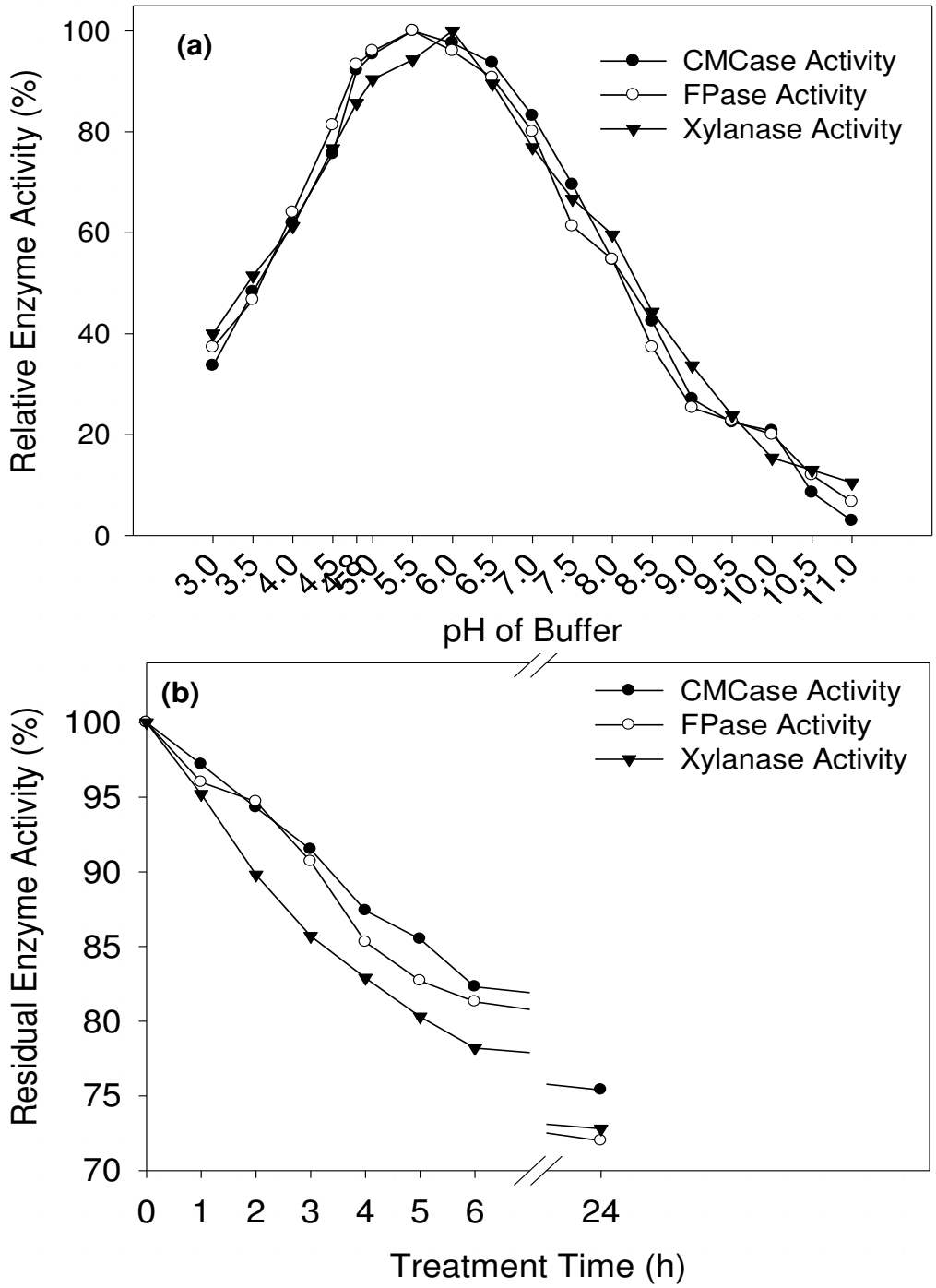


Figure-3.22 (a) Optimum pH (b) stability at the optimum pH for cellulase and xylanase of the *C. cinerea* PPHRI-4 NFCCI-3027

3.2.7.2. Optimum temperature and stability

T. harzianum PPDDN-10 NFCCI-2925

It is observed that optimum temperature is 60 °C for the CMCase and xylanase activity and 55 °C for the FPase activity. After 60 °C the enzymatic activities are decreased rapidly and at 80 °C enzyme is almost denatured i.e. having no activity (Figure-3.23a, Table-3.25). Despite the high temperature optima of enzymes, the thermo-stability of an enzyme is also a

very important property because at optimum temperature it should be stable *i.e.* it should not be denatured before the desired reaction time.

All the cellulase components are optimally active in the assay at pH 5.5 and 60 °C. CMCase, FPase and xylanase lost 35-39% of their activities when kept at 60 °C for 5 h before assaying. The enzyme gave a good temperature stability upto 6 h at 60 °C (approx. 50% retained) and 20-24% activities are lost when kept at 55 °C for 5 h (Figure-3.23b, Table-3.25). Cellulase and xylanase enzymes that are stable at high temperatures can be used for different applications in pulp and paper such as deinking of waste papers (Pathak 2010, 2011).

The maximum 4.15 ±0.42 IU/ml CMCase, 0.67±0.12 FPU/ml and 321.1±45.2 IU/ml xylanase activities are achieved at pH 5.5 and 60 °C.

C. cinerea PPHRI-4 NFCCI-3027

The performance of the enzymatic activities with temperature is as anticipated. Enzymatic activities are increased to a maximum with increasing the temperature followed by decrease. It is observed that 55 °C is optimum temperature for FPase and xylanase activities while 60 °C for the CMCase activity. After 60 °C the enzymatic activities are decreased rapidly and at 80 °C enzyme is almost denatured i.e. having no activity (<10%) (Figure-3.24a, Table-3.26). Despite the high temperature optima of enzymes, the thermo-stability of an enzyme is also a very important property because at optimum temperature it should be stable *i.e.,* it should not be denatured before the desired reaction time.

All the cellulase components are optimally active in the assay at pH 5.5 and 60 °C. CMCase, FPase and xylanase lost 35-37% of their activities when kept at 60 °C for 5 h before assaying. The enzyme gave a good temperature stability upto 6 h at 60 °C (approx. 50% retained) and 25-30% activities are lost when kept at 55 °C for 6 h (Figure-3.24b, Table-3.26).

The optimum temperatures for the fungi are in the range (45–65 °C) with the earlier reported data (Isil and Nilufer 2005, Kaur et al. 2011, Singh et al. 2009, Agnihotri et al 2010, Silveira et al. 1999, Aro et al. 2001).

As we increased the temperature from the lowest (20 °C) to optimum (60 °C), the activity of the enzymes increases due to increase in the kinetic energy of reacting molecules and ultimately the rate of the reaction. However, proteins started to denature due to higher temperature after the optimum temperature, which is resulted into loss of the activity of enzyme. The thermostable nature of the enzyme is acquired by exchange of some amino acids to modify many small structural changes. Thermo stability at high temperature is provided by hydrogen bonds, ion-pair interactions and hydrophobic interactions (Scandurra

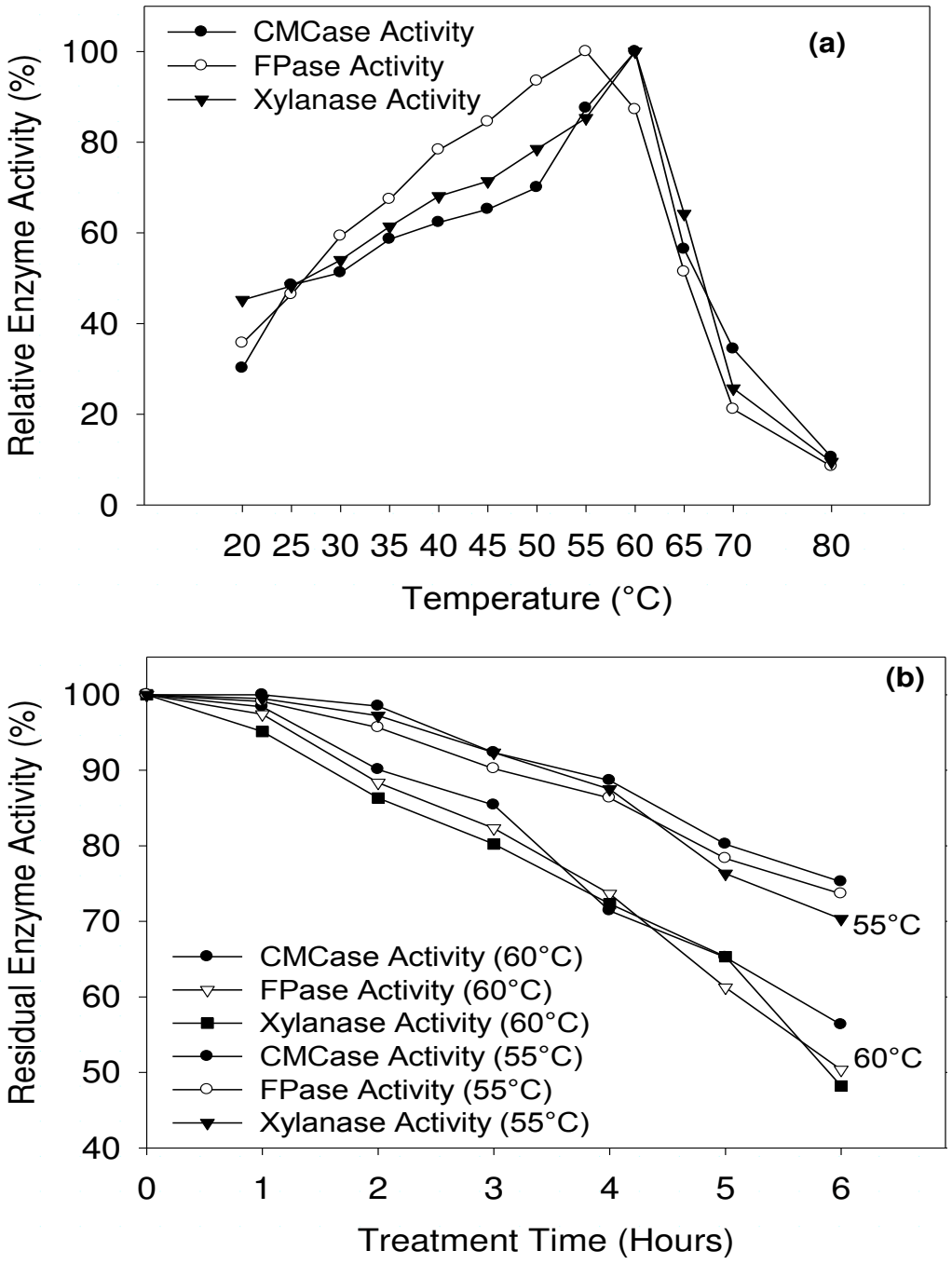


Figure-3.23 (a) Optimum temperature (b) stability at the optimum temperature for cellulase and xylanase of the *T. harzianum* PPDDN-10 NFCCI-2925

et al. 1998). Cellulase and xylanase enzymes, that are stable at high temperatures, can be used for deinking of waste papers (Pathak et al. 2011, 2010).

3.2.8. Large scale production

T. harzianum PPDDN-10 NFCCI-2925

During the large-scale production of enzyme for deinking experiments, the maximum 4.15±0.42 IU/ml (1.48±0.15IU/mg protein) CMCase, 0.67±0.12 FPU/ml (0.24±0.04 FPU/mg

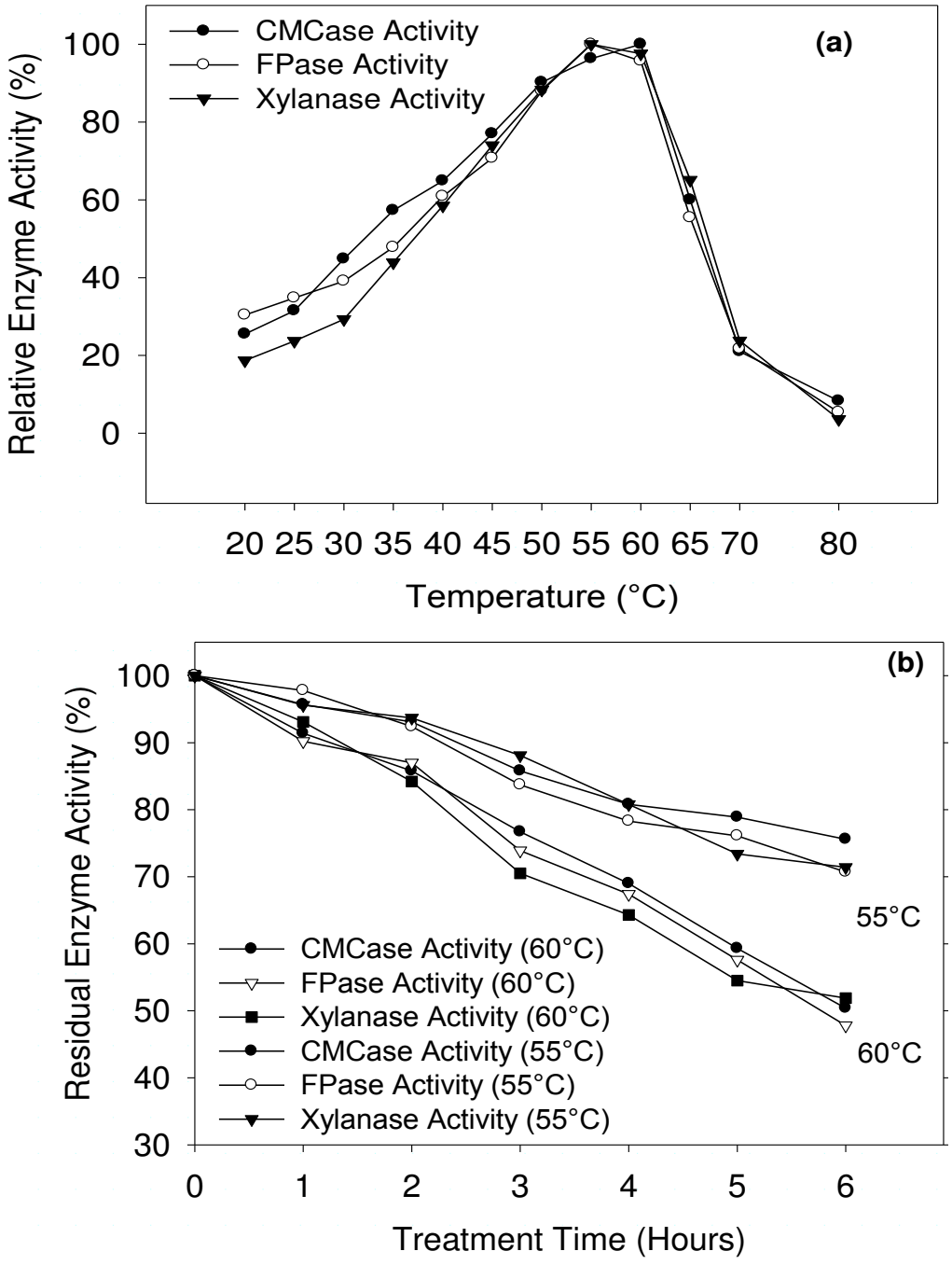


Figure-3.24 (a) Optimum temperature (b) stability at the optimum temperature for cellulase and xylanase of the *C. cinerea* PPHRI-4 NFCCI-3027

protein) FPase and 321.1±45.2 IU/ml (114.7±16.1 IU/mg protein) xylanase activities were achieved at pH 5.5 and 60°C.

C. cinerea PPHRI-4 NFCCI-3027

In the present study the CMCase, FPase and xylanase activities were found as 4.82±0.25 IU/ml, 0.88±0.09 FPU/ml and 818.4±49.5 IU/ml, respectively at the pH 5.5 and 60 °C and 4.64 IU/ml, 0.92 FPU/ml and 838.7 IU/ml at pH 5.5 and 55 ˚C.

CHAPTER 4

DEINKING OF PHOTOCOPIER WASTE PAPERS

4.1. Introduction

For paper makers, virgin fibers are the first choice over recycled fibers due to better strength. The pulping and bleaching of virgin pulps generates a huge amount colored compounds including toxic chloro-organics resulting in higher pollution load compared to waste paper processing. Due to these environmental issues, the pulp and paper industry has to introduce ecofriendly approaches (Bajpai and Bajpai 1998, Bajpai 2010, Pathak et al. 2011, 2010). Recycled fibers have become important due to limited or expensive wood supplies and because they require less chemicals and energy in comparison to virgin fiber (Bajpai 2010, Prasad 1983, Welt and Dinus 1998). About 38-40% of the total amount of produced paper is made from these recycled fibers, and this figure is continuously increasing (Bajpai 2010).

In the recycling process, deinking (dislodgement and removal of ink particles from the recycled pulp to obtain better optical and printing properties) is an important process (Bajpai and Bajpai 1998, Bajpai 2010, Pathak et al. 2011, 2010). Among different types of waste papers, non-impact ink printed papers (photocopied and laser printed) are difficult to deink due to the flat and large toners particles attached to the fiber surfaces (Gübitz et al. 1998, Pala et al. 2004, Pathak et al. 2011, 2010; Tandon et al. 2005). The ink toners used in photocopier printers are thermosetting toners consisting of non-dispersible synthetic polymers (Pathak et al. 2010, Tandon et al. 2005). Only a very small proportion of toner printed high quality fiber is being recycled back into printing and writing grades due to the technical and economic problems of deinking Conventional deinking requires large amounts of chemicals, which are not environmentally friendly (Elegir et al. 2000, Gübitz et al. 1998, Jeffries et al. 1994, Pathak et al. 2011).

It has been well recognized that biotechnology may provide some potential to deink this type of high quality waste papers by facilitating release of toners from papers. During the last few decades, the application of enzymes has attracted the attention of papermakers to develop ecofriendly process such as enzymatic or biodeinking (Bajpai 2010, Gübitz et al. 1998, Vyas and Lachke 2003). Moreover, the application of enzymes is expected to increase rapidly in near future. Among the enzymes used, cellulase and xylanase dominate over others such as amylase or lipase, because the main constituents of pulp fibers are cellulose and hemicellulose (Bajpai 2010).

For deinking, endoglucanases (component of the cellulase complex) are the most important as compared to exoglucanases or cellobiohydrolases (Soni et al. 2008). For biodeinking,

cellulases have been used singly as well as in combination with other enzymes depending on the type of inks and carriers. Xylanase for wood containing papers, lipase for papers printed with oil based inks, and amylase for starch containing/coated papers have also been used as effective biocatalysts for deinking (Bajpai and Bajpai 1998, Bajpai 2010). These enzymes deink the papers by enzymatic liberation of the ink particles from the fibre surface by making ink particles more hydrophobic, peeling mechanism, dissolution of colloidal particles, or hydrolysis of the ink carrier or coating layer. The detailed mechanism, by which enzymes can improve the deinkability of waste papers, is further discussed in the literature (Bajpai and Bajpai 1998, Pathak et al. 2011).

In most of the research papers, deinking of mixed office waste (MOW), consisting of photocopier papers with other type of papers are reported by using commercially available cellulases or xylanases (Jeffries et al. 1994, Pala et al. 2006, 2004; Prasad 1993, Wang and Menghua 2005, Viesturs 1999). On the other hand, some researchers have used cellulases (having xylanase activities) produced by the different microorganisms such as *Aspergillus, Trichoderma, Gleophyllum, Orpinomuces, Fusarium, Humicola, Coprinopsis* sp. etc (Gübitz et al. 1998, Vyas and Lachke 2003, Soni et al. 2008, Das et al. 2012, Lee et al. 2011a, Marques et al. 2003, Geng et al. 2003, Lee and Eom 1999, Morkbak and Zimmerman 1998a, Dutt et al. 2012). The crude cellulase and xylanase of *C. cinerea* have been used for the deinking of old newspapers (Lee and Eom 1999). The different types of enzyme in a mixture *i.e.* concoction of the crude xylanase of this fungus with cellulase of *Aspergillus niger* and commercial enzymes (lipase and amylase) have been used for deinking of sorted office waste paper (Dutt et al. 2012). It is noteworthy to point out here that, the deinking of photocopier waste papers alone, containing toner inks difficult to deink, using the crude enzymes of *T. harzianum* and *C. cinerea* has not been reported earlier.

In the present study, the deinking potential of the crude cellulase and xylanase enzyme produced from isolated fungus *T. harzianum* PPDDN-10 NFCCI 2925 and *C. cinerea* PPHRI-4 NFCCI-3027 has been evaluated. Different operational parameters such as point of enzyme addition, enzyme dose, pulp consistency, and reaction time are optimized using these waste papers to achieve the maximum possible deinking efficiency without affecting the paper strength properties. To check the efficiency of our newly developed enzymes, its deinking efficiency (DE), optical and strength properties are compared with conventional chemical deinking and commercial cellulase. Further, the positive or negative effects of enzyme action on paper are investigated in terms of fines content and drainage time. Another aim is to increase the pulp freeness without affecting the strength properties (Objective 3 and 4).

4.2. Results and discussion

4.2.1. Conventional chemical deinking

The chemical deinking is performed only to compare the data of enzymatic deinking with the conventional methods. Therefore, for the optimization of chemical deinking, a narrow range of parameters is selected as reported by Wood et al. (1985). It is observed to achieve 118.54±12.80 ppm residual ink with 2.0% sodium hydroxide, 2.0% sodium silicate and 1% H_2O_2 at 6% pulp Cy at 70 °C for 30 min during hydrapulping. The DE and ISO brightness are 58.4±1.7 and 78.7±0.9%. The highest DE (75.9±1.0%) and ISO brightness (80.4±0.5%) are achieved at 10% pulp Cy with 0.8% oleic acid.

4.2.1.1. Sodium hydroxide

For the 2.0% chemical dose, residual ink is lowest and DE and ISO brightness is maximum (Figure-4.1, Table-4.1 and 4.2). At higher dose (2.5%), the DE and ISO brightness both are decreased. Earlier, chemical deinking experiments are also reported with 2% NaOH for laser printed (Pala et al. 2004, 2006) and old news-papers (Agnihotri 2007, Varshney et al. 2007). Higher alkali dose may result in the accumulation of alkaline solubilized contaminants; and pulps are prone to get alkaline yellowing which reduced the ISO brightness (Morbak et al. 1999, Lee et al. 1999b). It is general assumption that chemical treatments are not able to induce the fragmentation process of polymeric toner ink (Ow et al. 1995, Azevedo et al. 1999, Wielen et al. 1999). Actually alkali is responsible for fibre swelling favouring ink detachment as large toner particles, which are not easy to remove by flotation and washing deinking (Pala et al. 2004). However, low alkali dose breaks the ink particles in the large pieces and high alkali forms the small ink particles, which may get entry inside the lumen through pits of the fibres (Ali et al. 1994). High alkali dose is observed to reduce the strength properties due to the possible weakening of the recycled fibre's structures, which has resulted into removal of microfibrills (Figure-4.1b). On increasing the alkali dose the release of reducing sugars is also increased due to its possible action on the fines, which has resulted in the slightly improvement in the freeness (Table-4.2).

4.2.1.2. Hydrapulping time

The maximum DE and ISO pulp brightness with lowest residual ink content have been achieved for 30 min hydrapulping time (Figure-4.2, Table-4.1 and 4.2). Mechanical forces for less hydrapulping time, are not able to defibre the waste-papers completely. Generally, a minimum of 20 to 30 min hydrapulping time is required for complete separation of the recycled waste papers (Ali et al. 1994, Jobbins et al. 1997). For ONP relatively lesser hydrapulping time (15 min) was given by Varshney et al. (2007). Mechanical forces applied for higher time will be responsible for the lower ink particle size and fines content that will reduce the freeness of the pulp as observed in the Table-4.2. The tensile and tear indices

are almost constant, but the burst index is significantly improved at higher reaction time due to the possible generation of the fines (Figure-4.2b, Table-4.1 and 4.2). Fines are generated during both the fibre separation and fibre development processes (Kangas et al. 2004). The reducing sugars are also observed to increase along with the hydrapulping time.

4.2.1.3. Temperature

The maximum DE, ISO brightness and minimum residual ink are obtained for 70 °C temperature during hydrapulping (Figure-4.3, Table-4.1 and 4.2). Temperature beyond 70 °C is considered to be unsuitable as thermal reversion and accelerated alkali darkening can occur (Magnin et al. 2002). The temperature variation is not found to affect the reducing sugars liberation (Table4.2). The strength properties have the mixed effect as tensile strength is high at 70 °C, but the tear index is reduced. The reason may be the good inter-fibre bonding (Pathak et al. 2011).

4.2.1.4. Sodium silicate

2.0% sodium silicate is selected as the best dose due to the maximum DE, ISO brightness and lowest residual ink (Figure-4.4a, Table-4.1 and 4.2). Previously, 2.0 and 2.5% concentration is also used for laser printed papers and ONP, respectively (Pala et al. 2004, 2006, Varshney et al. 2007). Sodium silicate is able to slightly improve the DE with increasing dose, but at higher dose, DE is decreased. Different researchers have used sodium silicate to absorb the metal ions. Sodium silicate acts as a buffer, peroxide stabilization, dispersing agent, emulsification and wetting agent. The half of the role of silicate is to stabilize the hydrogen peroxide and other half is as detergent and buffer (Ali et al. 1994). Low dose of silicate generates ink particles of small size and higher dose has the large ink particles (Ali et al. 1994). The strength properties are found to be almost same, but at 2.5% dose, the reduction in these properties is observed. The freeness values are slightly improved at high doses, although reducing sugar analysis shows initial increase and then drastic reduction (Figure-4.4b, Table-4.1 and 4.2). The reason for this type of pattern is unclear.

4.2.1.5. Hydrogen peroxide

The maximum DE and lowest residual ink are achieved for the dose of 1.0% (Figure-4.5a, Table-4.1 and 4.2). As expected, the maximum ISO brightness is observed at high dose of H_2O_2 *i.e.*1.5%. H_2O_2 is not observed to affect the reducing sugars and freeness significantly although decrease in the burst and tear indices is observed at the optimum dose (Figure-4.5b, Table-4.1 and 4.2). Varshney et al. (2007) have also conducted their deinking experiments with1.0% H_2O_2 for ONP.

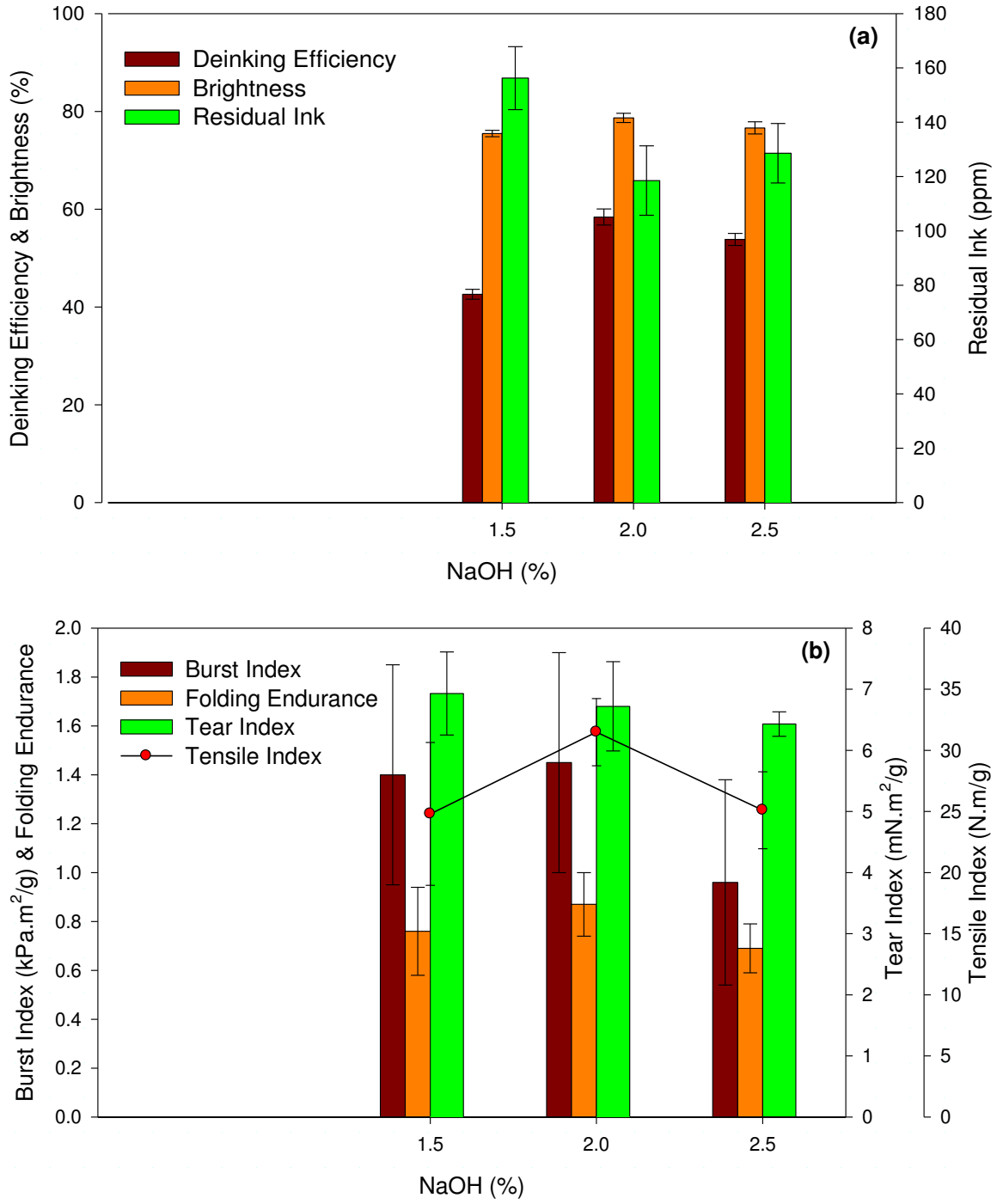


Figure-4.1 Deinking results with chemicals showing the effect of dose of sodium hydroxide on **(a)** DE, ISO brightness and residual ink **(b)** strength properties of handsheets

4.2.1.6. Pulp consistency (Cy)

On increasing the pulp Cy from low to medium *i.e.,* from 6 to 10%, DE and ISO brightness are found to improve, then decrease at higher Cy (12%). The highest DE (75.9±1.0%) and ISO brightness (80.4±0.5%) are achieved at 10% pulp Cy (Figure-4.6, Table-4.1 and 4.2). Ali et al. also reported that 10% Cy is favorable for the complete defibreization (Ali et al. 1994). Higher Cy will result in the limited volume of liquid phase that is insufficient to suspend the

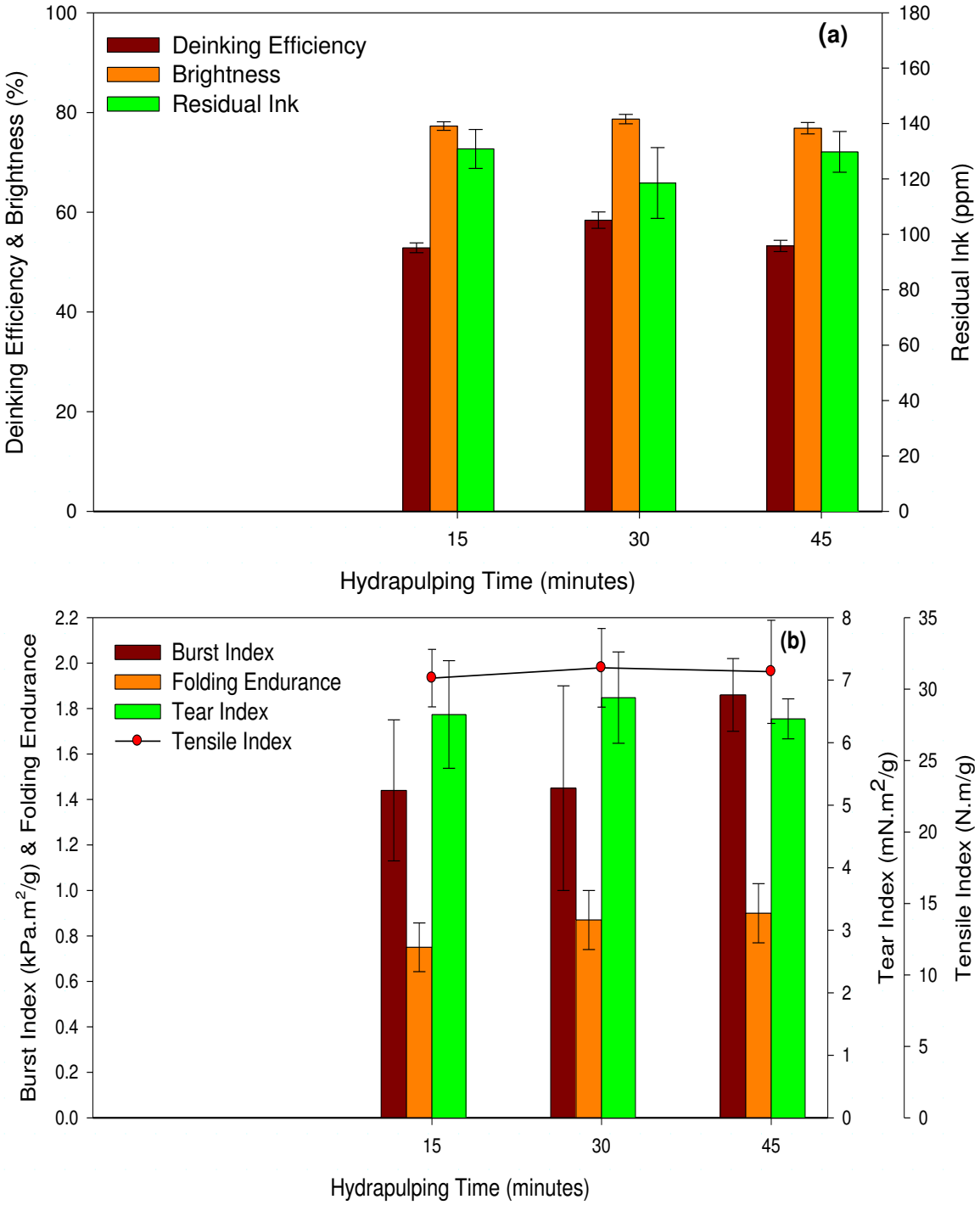


Figure-4.1 Deinking results with chemicals showing the effect of hydrapulping time on **(a)** DE, ISO brightness and residual ink **(b)** strength properties of handsheets

ink. Therefore, ink is smeared and prone to redeposition back on the fibre surface (Ali et al. 1994).

The relatively high Cy of 10% is chosen because it facilitates fibre/fibre attrition, favoring ink detachment; additionally, it would be advantageous for industrial usage. The strength properties are observed to be reduced with increase in the pulp Cy. The reason may be the removal of microfibrills due to friction forces applied at increased Cy. The freeness is

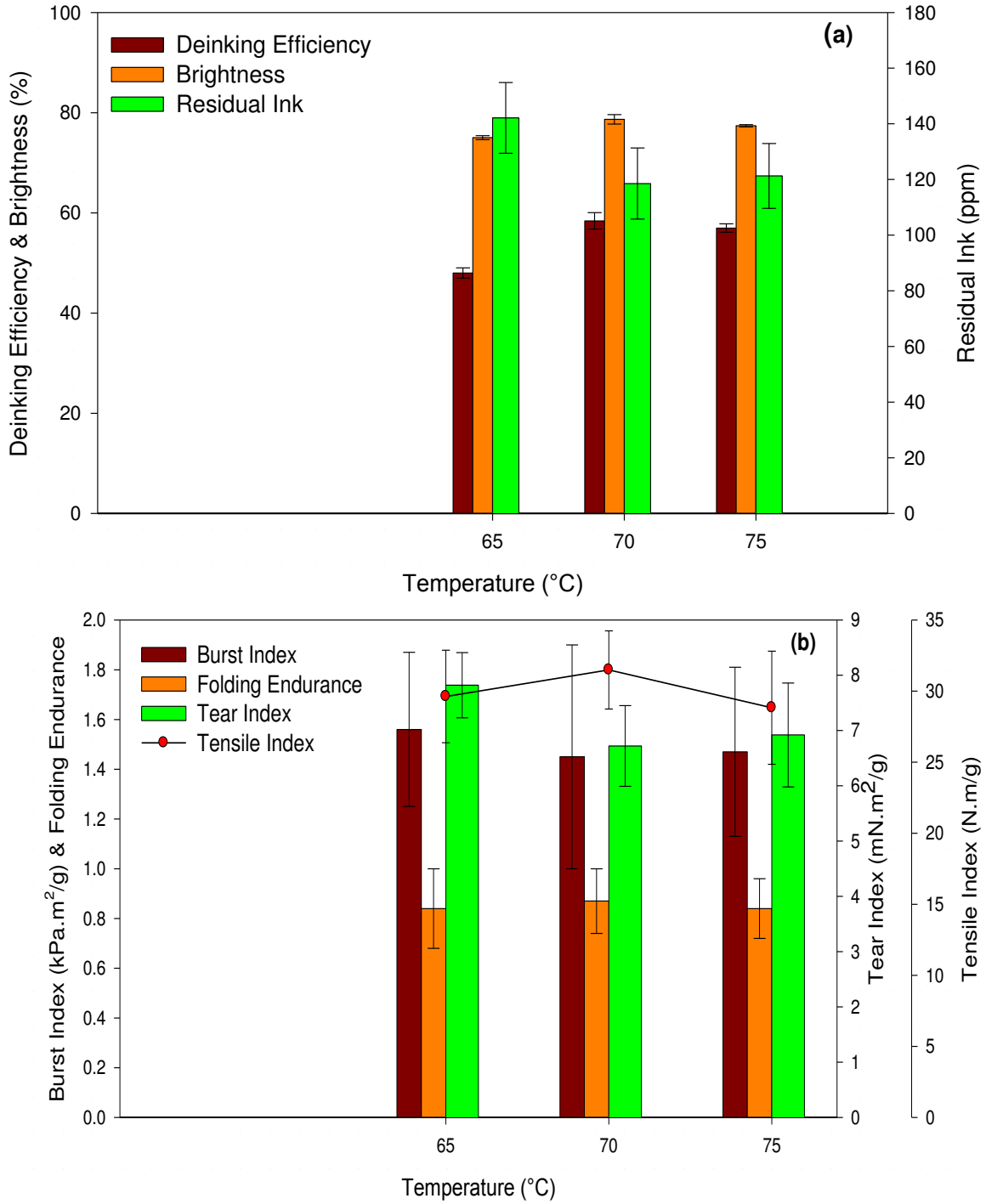


Figure-4.3 Deinking results with chemicals showing the effect of temperature on **(a)** DE, ISO brightness and residual ink **(b)** strength properties of handsheets

observed to be slightly improved with the increase in the Cy, but the reducing sugars are significantly improved as observed in the Table-4.2. Higher Cy are responsible for the reduction in the tensile and burst indices along with folding endurance significantly but the tear index is slightly improved. The reason may be removal of microfibrills due to the higher shear forces (Table-4.2). Pala et al. (2004, 2006) have repulped the MOW and photocopier papers at 11% Cy.

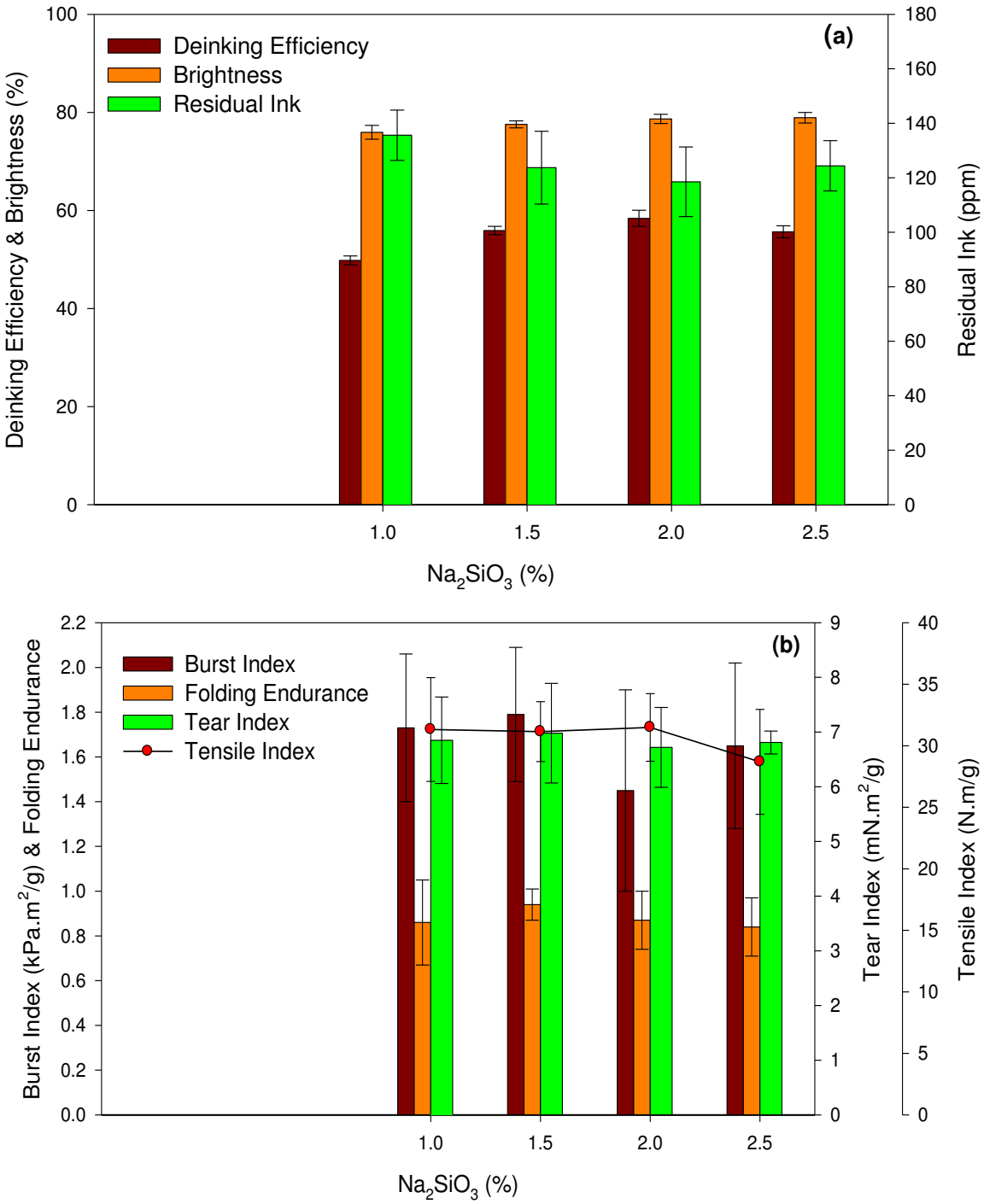


Figure-4.4 Deinking results with chemicals showing the effect of dose of sodium silicate on **(a)** DE, ISO brightness and residual ink **(b)** strength properties of handsheets

4.2.1.7. Oleic acid

0.8% oleic acid is selected as optimized dose having the maximum DE (75.9%) with yield of 77.9% (Figure-4.7, Table-4.1 and 4.2). Varshney et al. (2007) have identified 1.2 % oleic acid as the optimum dose for highest ink removal from the ONP. Higher oleic acid dose resulted in higher pulp removal with the froth, so the net yield decreased. The main role of surfactant systems is to release ink particles from the fibres surface and stabilize detached ink particles, aggregate dispersed ink particles and/or modify the surface properties of

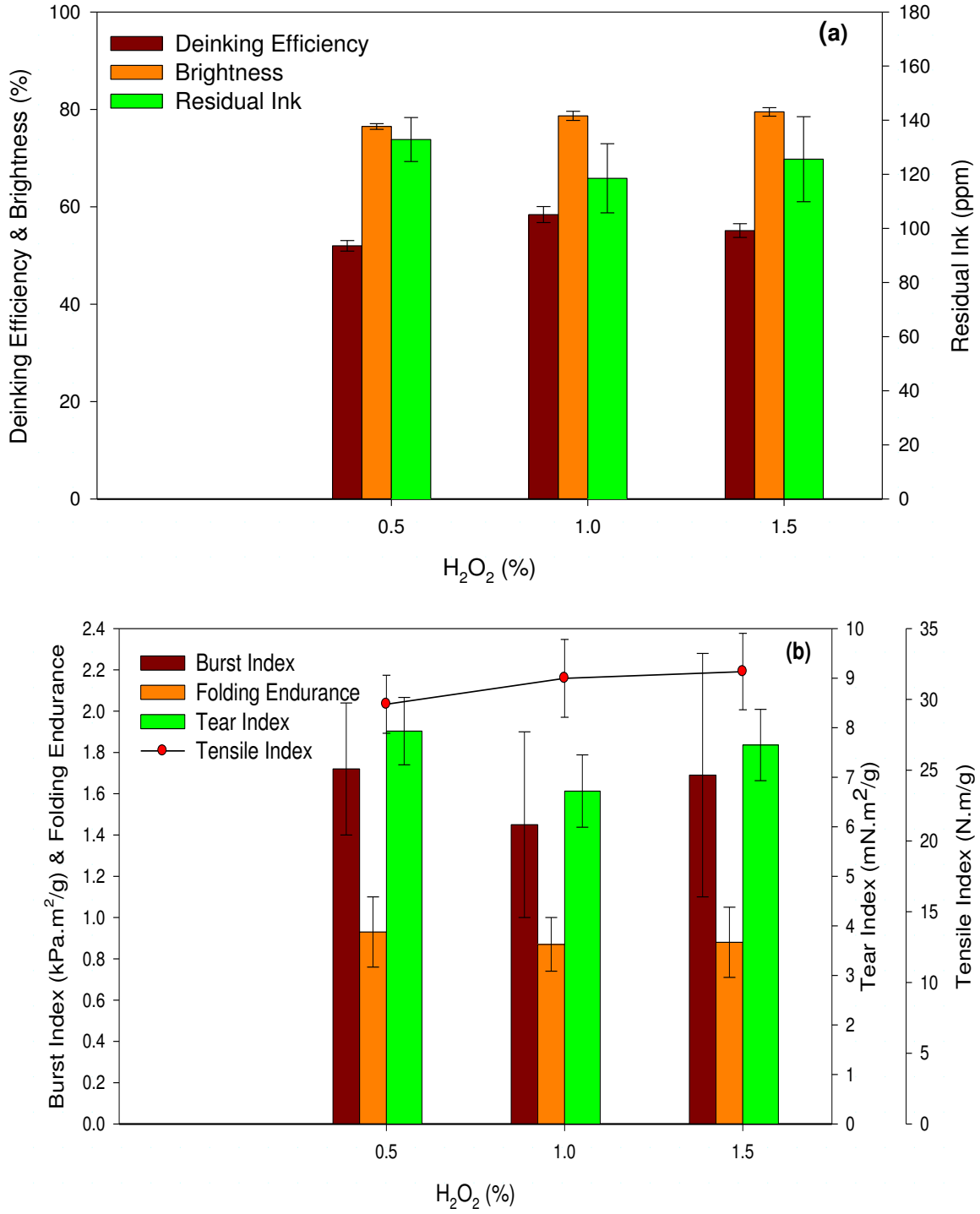


Figure-4.5 Deinking results with chemicals showing the effect of dose of hydrogen peroxide on **(a)** DE, ISO brightness and residual ink **(b)** strength properties of handsheets

released ink particles, these entire phenomenon are responsible for improvement in the overall ink removal of a flotation process (Viesturs et al. 1999, Lee et al. 2011b, Zhao et al. 2004). Oleic acid is not observed to affect the release of the reducing sugars (Table-4.2).

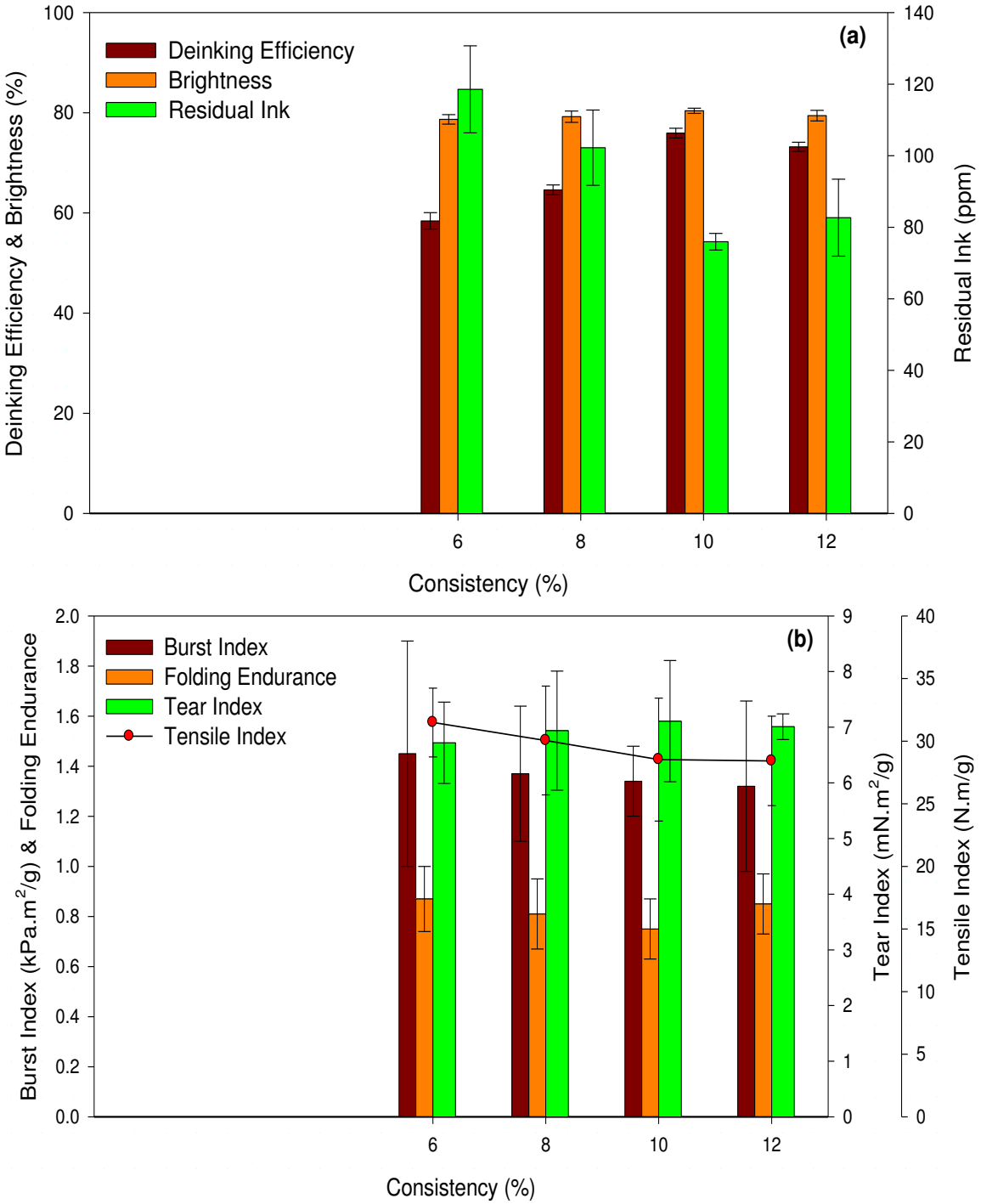


Figure-4.6 Deinking results with chemicals showing the effect of pulp consistency on **(a)** DE, ISO brightness and residual ink **(b)** strength properties of handsheets

4.4.2. Enzymatic deinking

4.2.2.1. Point of enzyme addition

The first and the most decisive part of enzymatic deinking is the point of addition of enzyme. This is because there is no meaning to add the enzyme where its efficiency is reduced due to either non-availability of substrate or other post-reaction effect. It is observed that the highest deinking performance is achieved when enzyme is added 'during the hydrapulping

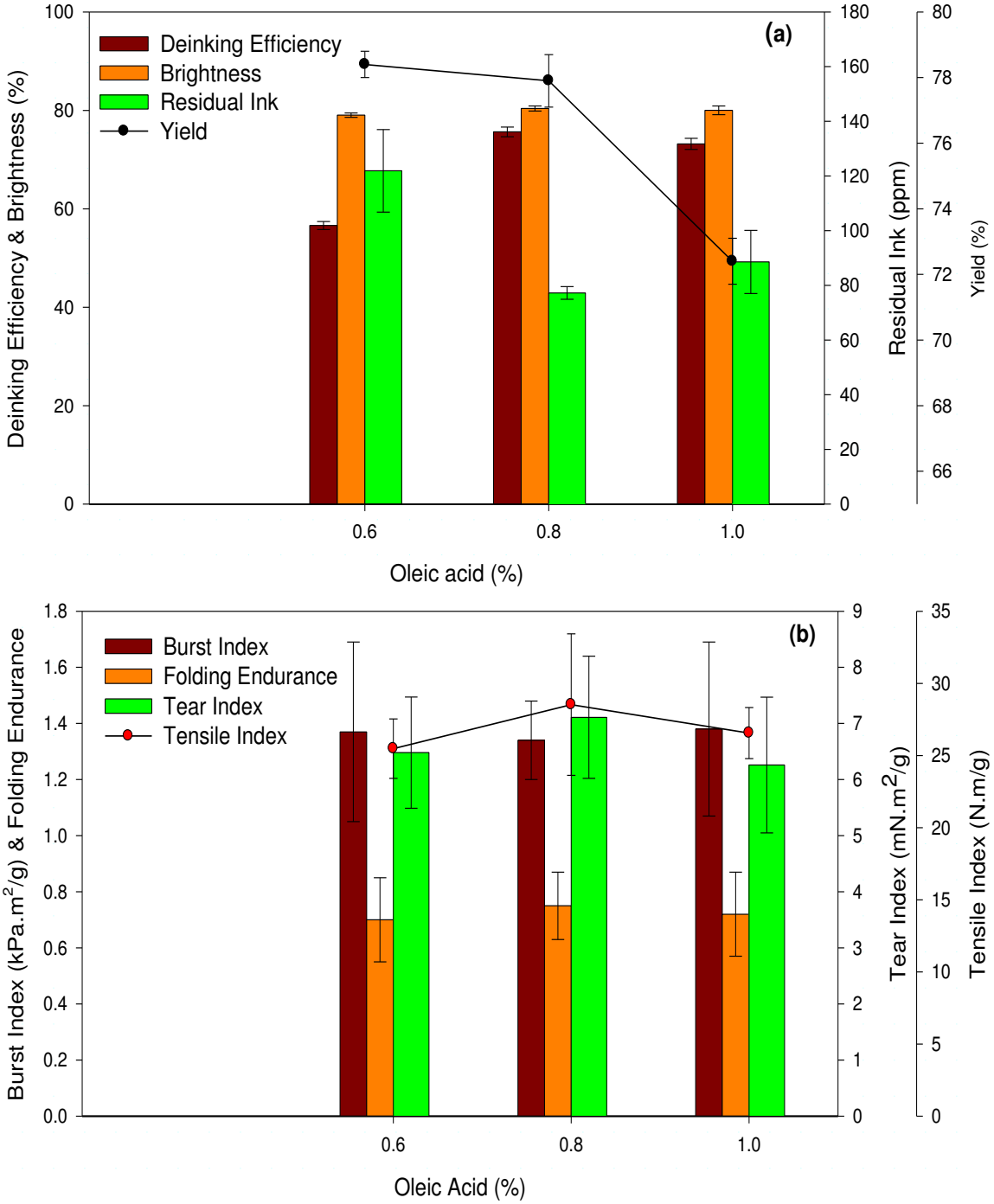


Figure-4.7 Deinking results with chemicals showing the effect of dose of oleic acid on **(a)** DE, ISO brightness, residual ink and yield **(b)** strength properties of handsheets

(during-HP)' in all the enzymatic treatments. The results obtained with different enzymatic treatments are as follows:

Commercial cellulase

The maximum DE (69.7±1.2%) and ISO brightness (74.8±0.5%) with lowest residual ink count (102.67±4.56% ppm) is obtained when enzyme is added in the hydrapulping stage (Figure-4.8, Table-4.3 and 4.4).

Crude enzyme of *T. harzianum* PPDDN-10 NFCCI-2925

The DE (66.4±1.0%) and ISO brightness (77.8±0.7%), having the lowest residual ink of 123.25±8.33 ppm, are observed to be higher than the 'pre-HP' and 'post-HP' enzyme addition (Figure-4.9, Table-4.3 and 4.4).

Crude enzyme of *C. cinerea* PPHRI-4 NFCCI-3027

The maximum DE (63.4±2.0%) with lowest residual ink count (135.02±3.02 ppm) is obtained when enzyme is added in the hydrapulping stage (Figure-4.10). Post hydrapulping treatment also showed almost same DE. The strength properties of 'during and post-HP' enzyme addition are also slightly better than 'pre-HP', which may be due to better enzymatic action on fibre that results in better fibrillation. Lower freeness is observed with 'pre-HP' enzyme addition while 'post-HP' and 'during-HP' addition have slightly higher but same freeness value (Table 3, Table-4.3 and 4.4).

In all the experiments of enzymatic deinking, it is observed that the maximum freeness is achieved during-HP addition of the enzyme. About 2.4 to 2.8 and 2.3 to 3.2% higher amount of the reducing sugars is observed with 'during-HP' and 'post-HP' enzyme addition, respectively over Pre-HP. The reason may be the continuous contact of enzyme with the fibre surface. Pre-HP addition is not able to improve the freeness (Table-4.4).

The significance of point of mixing for the enzyme action has previously been reported (Zeyer et al. 1994). Although different researchers have performed the deinking experiments by adding enzymes in 'Post-HP' stage and achieve the maximum DE of 40.5 to 53.0% (Soni et al. 2008), 70.2% (Lee and Eom 1999), and 80% by (Dutt et al. 2012). Gubitz et al. (1998) were able to achieve around 95% deinking efficiency using enzymatic deinking followed by magnetic separation of the ink particles.

In the pre-hydrapulper stage, the enzyme is not able to reach all the fibres present in the torn papers because all the fibres are still bonded together and cellulose chains are not easily accessible for the enzymatic action (Zeyer et al. 1994). To alleviate this restriction, mechanical action is required to open the outermost layers of the fibre to expose cellulose chains completely. Thus produced surface friction on fibre will cause the loosening of ink particles. The release of ink particles are further facilitated due to cellulase action and only then the removal of significant amount of ink become possible to achieve considerable deinking with enzymes (Zeyer et al. 1994). During hydrapulping, all the fibres tend to separate so it becomes easier for the enzyme to reach each fibre surface. According to Zeyer et al. (1994), the enzyme activity must be applied at the same time as the surface friction *i.e.,* during hydrapulping. Enzymatic treatment after hydrapulping, has almost the same results but the detached ink particles may have the possibility of redeposition on the fibre surface or entry inside the pits of the fibres (Pathak et al. 2011).

It is to be mentioned here that, in all the successive experiments, these enzymes are added in the hydrapulping stage with interrupted agitation.

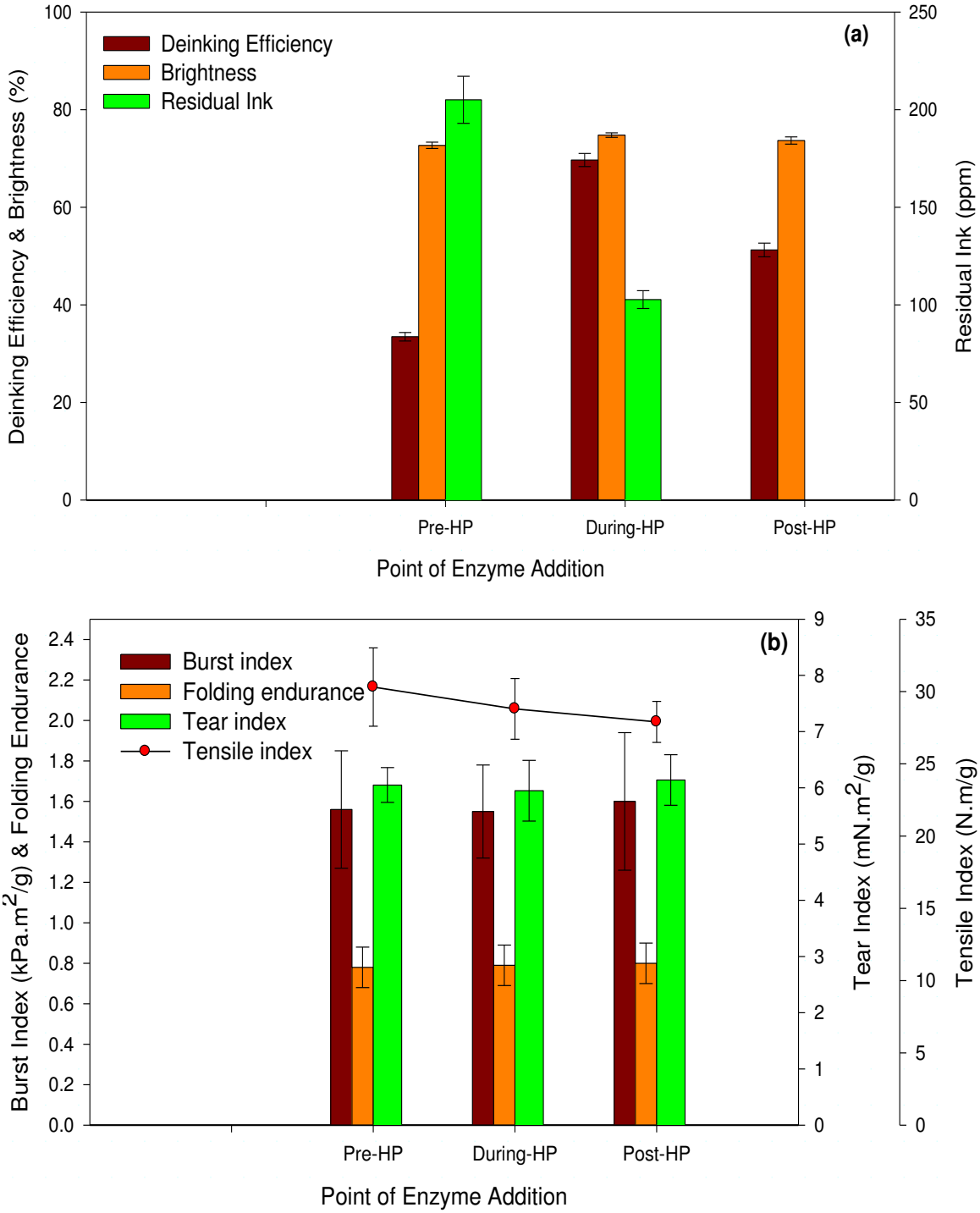


Figure-4.8 Deinking results with the 'commercial cellulase' showing the effect of point of addition of enzyme on **(a)** DE, ISO brightness and residual ink **(b)** strength properties of handsheets

4.2.2.2. Enzyme dose

The selection of the optimum enzyme dose is an important aspect in any enzymatic process since excessive enzymes may be detrimental to the fibres and thus affect the strength of the paper and its quality (Lee et al. 2007). Optimum dose may vary with variety of enzyme type,

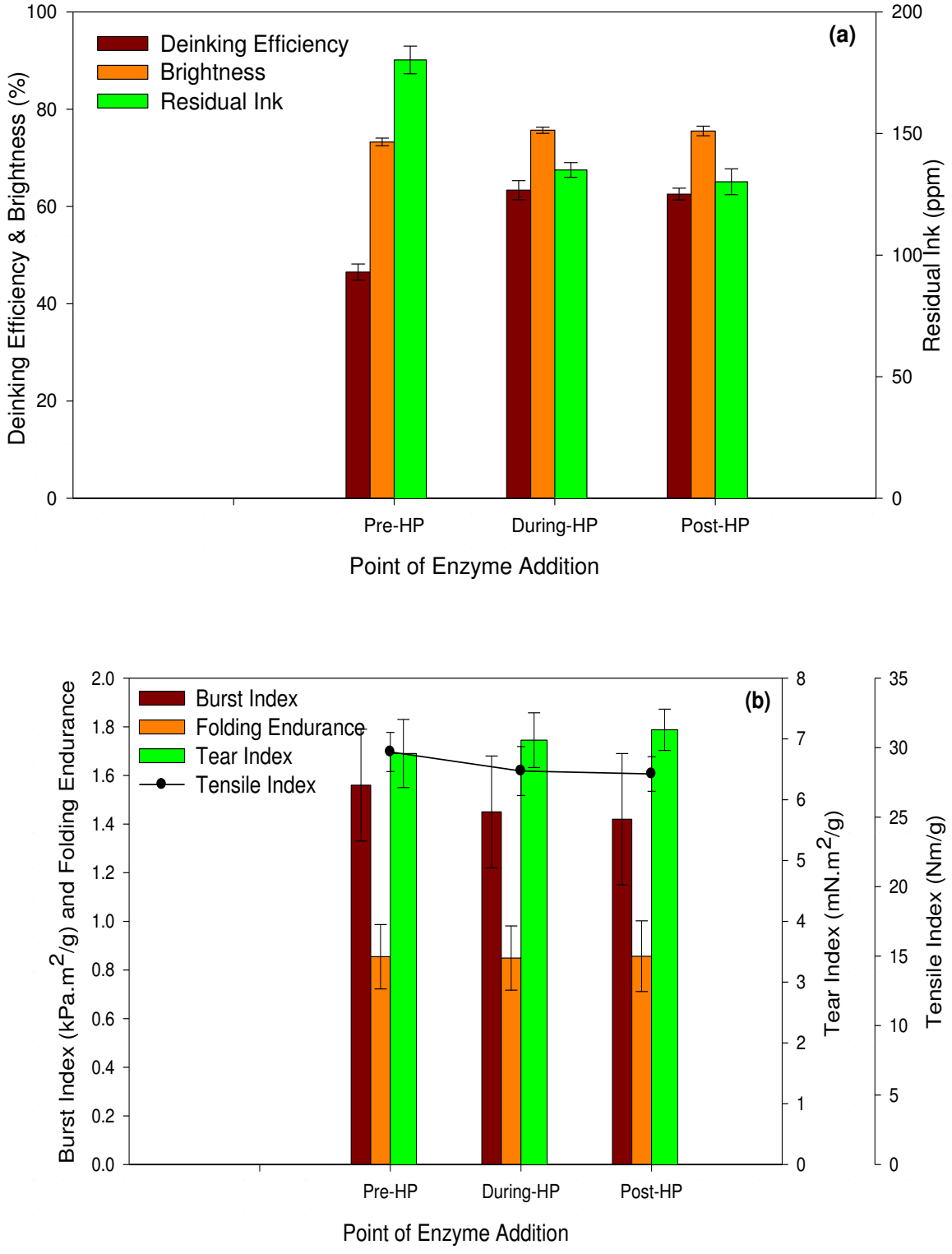


Figure-4.9 Deinking results with the 'crude enzyme of *T. harzianum*' showing the effect of point of addition of enzyme on **(a)** DE, ISO brightness and residual ink **(b)** strength properties of handsheets

enzyme source, paper and ink. The enzyme doses are selected after performing experiments in the different range of doses (i.e. 0.01 to 0.1, 0.1-1.0, 1.0-10.0 IU/g). The doses are decided on the basis of endoglucanase activities. The results shown here are of the selected range for that enzyme.

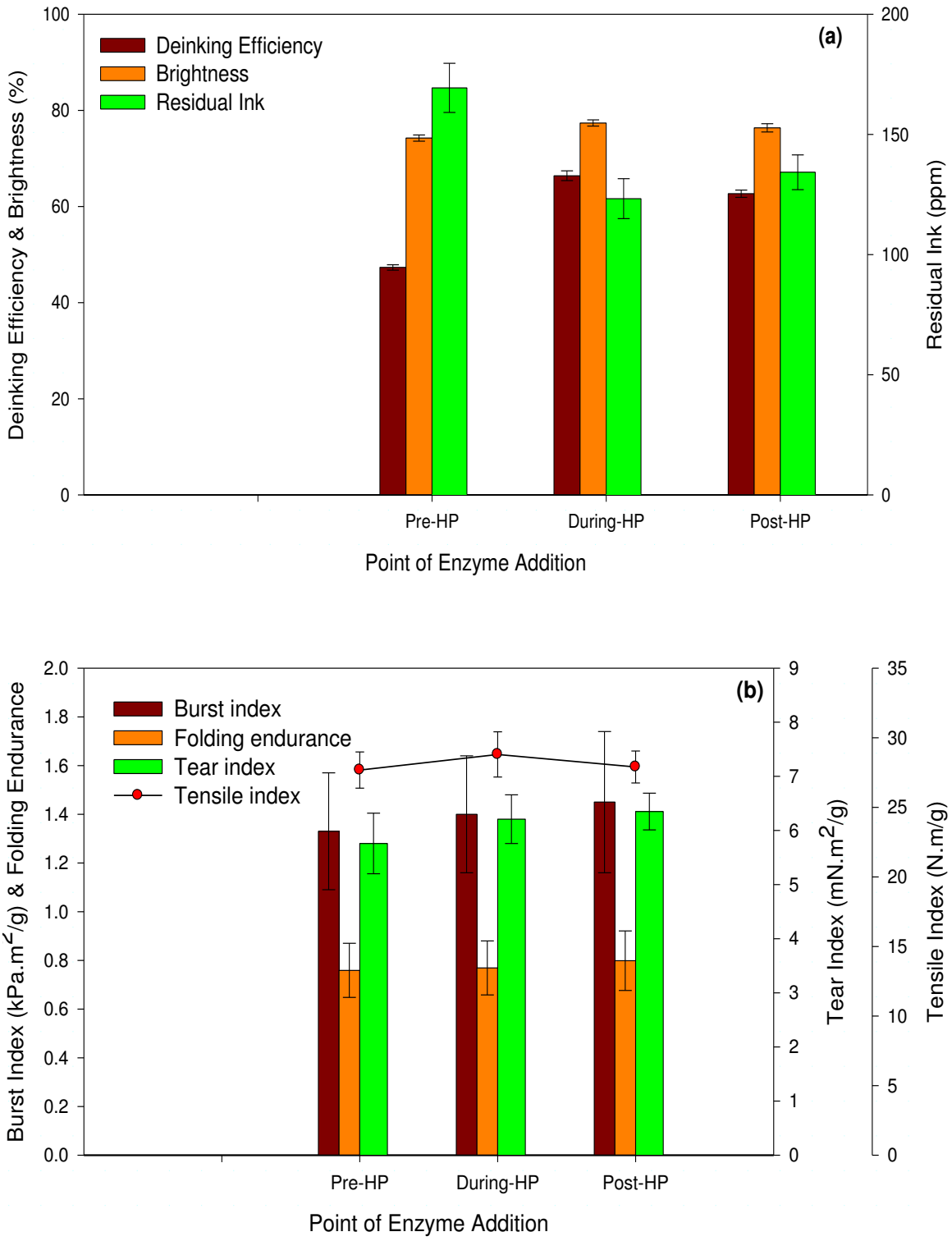
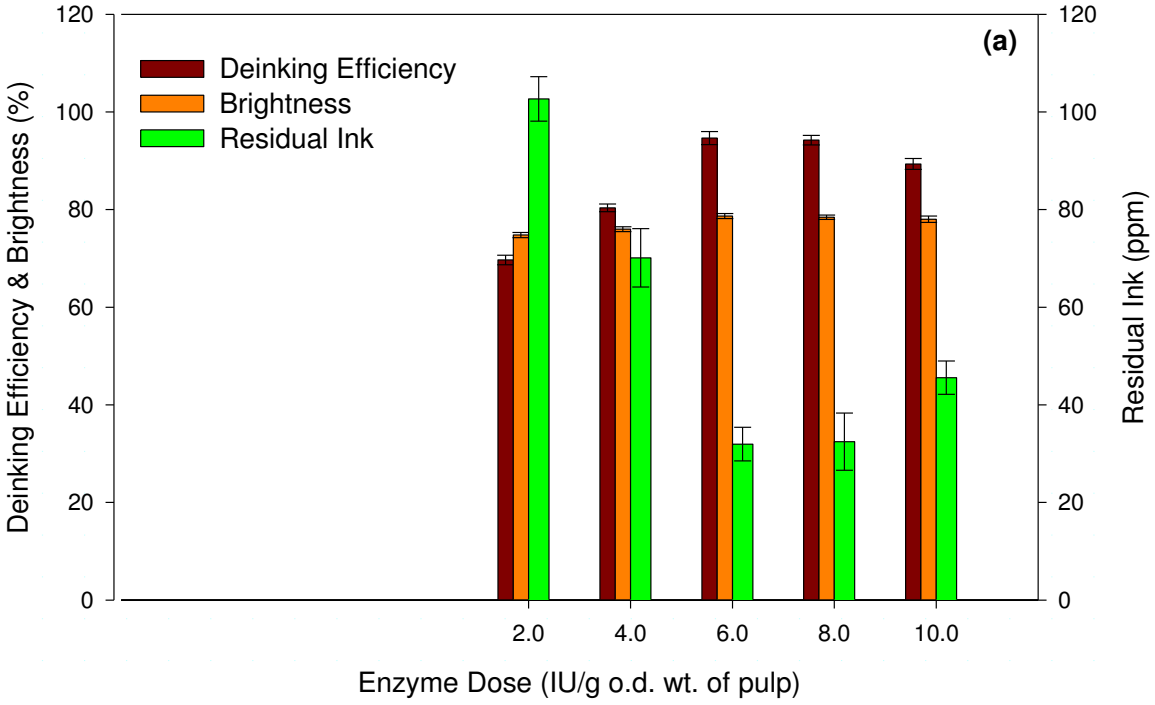


Figure-4.10 Deinking results with the 'crude enzyme of *C. cinerea*' showing the effect of point of addition of enzyme on **(a)** DE, ISO brightness and residual ink **(b)** strength properties of handsheets

Commercial cellulase

Figure-4.11 shows that 6 IU/g o.d. wt. pulp enzyme dose results to achieve maximum DE and ISO brightness (94.6±1.3% and 78.7±0.5%, respectively) and lowest residual ink (31.95±3.45 ppm), but further increase in enzyme dose results in reduced DE (Figure-4.11, Table-4.5 and 4.6). The strength properties are observed to decrease from the low (2.0 IU/g)

to high (10.0 IU/g) enzyme dose. Freeness value is improved from the 600 to 650 ml during this optimization and 2.5 times higher release of the reducing sugars than the lowest enzyme dose.



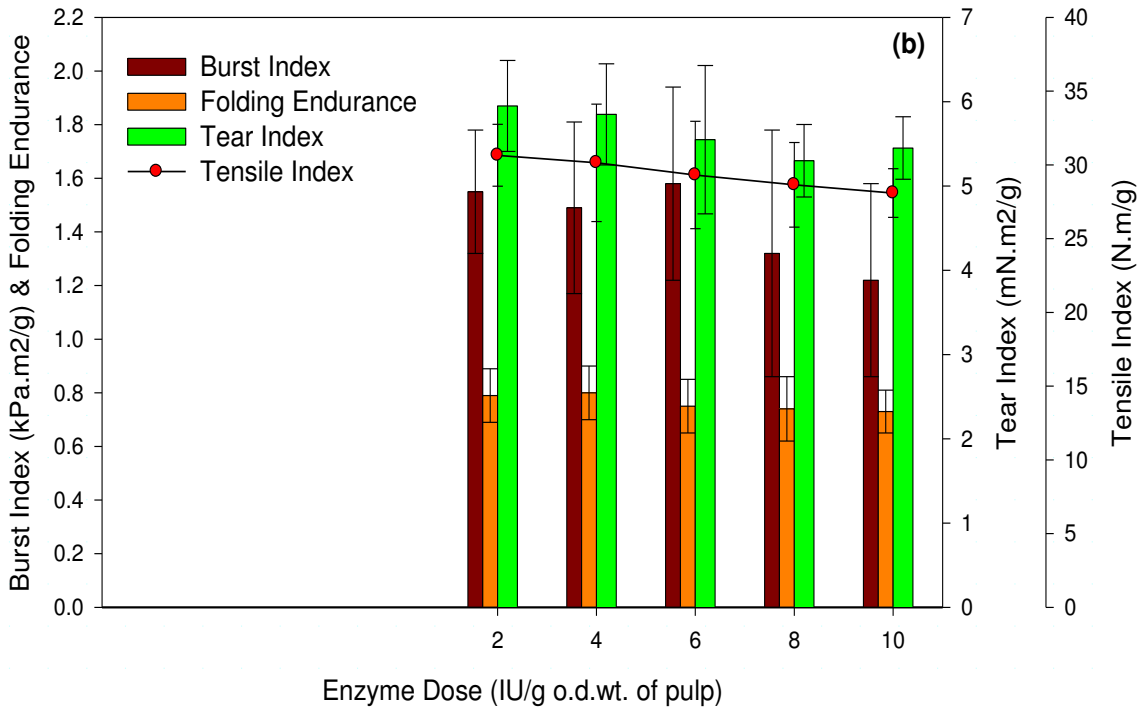


Figure-4.11 Deinking results with the 'commercial cellulase' showing the effect of enzyme dose on **(a)** DE, ISO brightness and residual ink **(b)** strength properties of handsheets

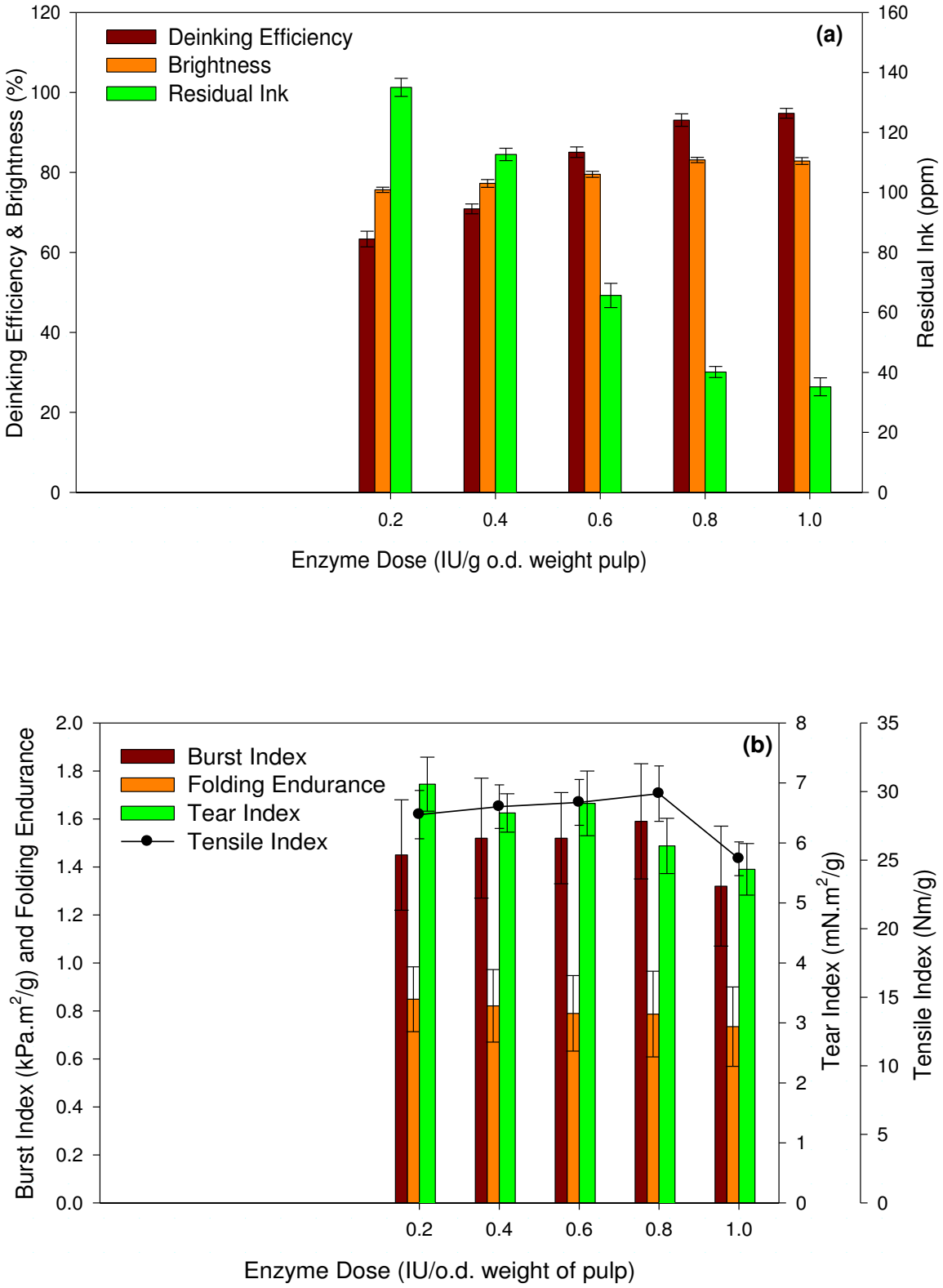


Figure-4.12 Deinking results with the 'crude enzyme of *T. harzianum*' showing the effect of enzyme dose on **(a)** DE, ISO brightness and residual ink **(b)** strength properties of handsheets

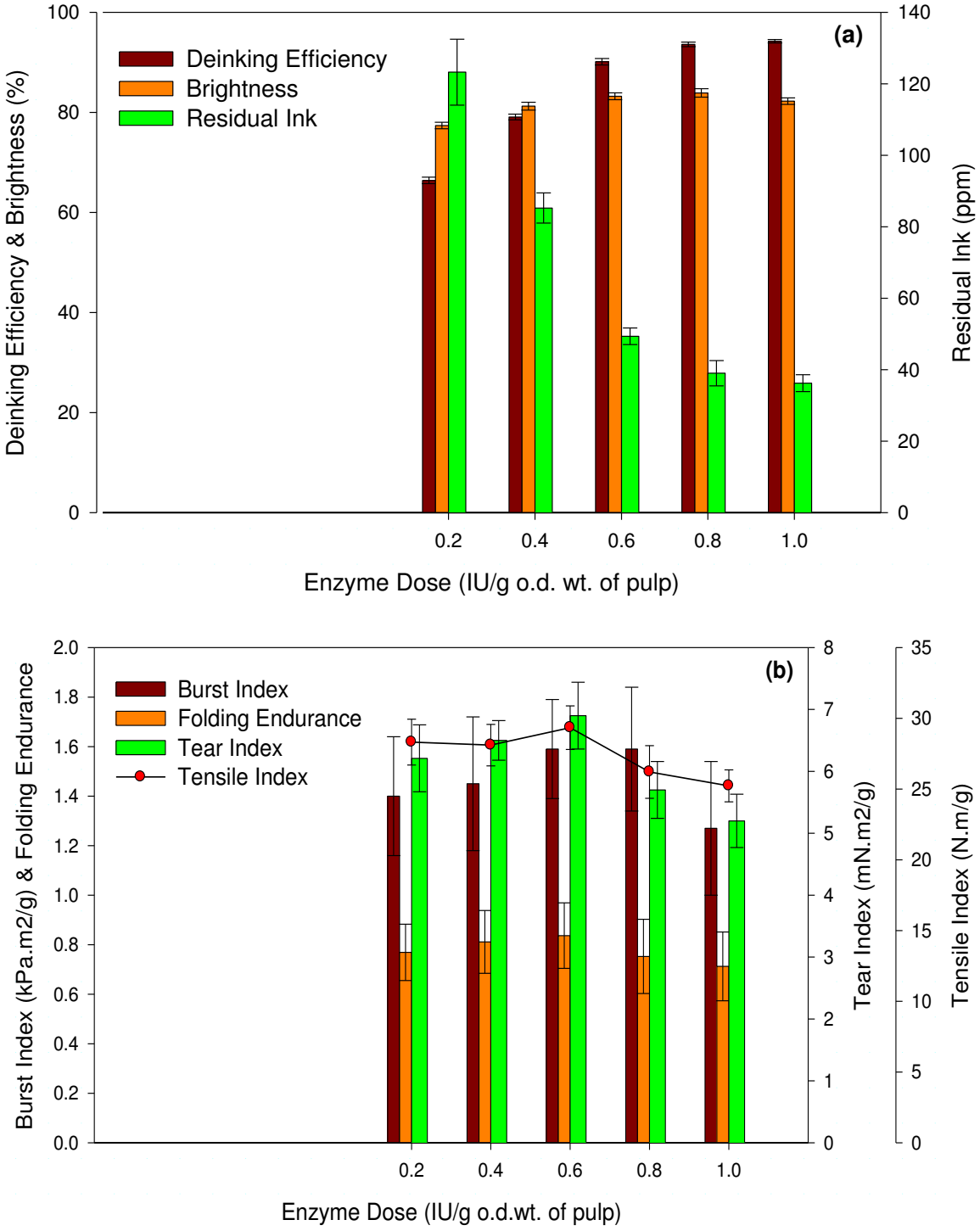


Figure-4.13 Deinking results with the 'crude enzyme of *C. cinerea*' showing the effect of enzyme dose on **(a)** DE, ISO brightness and residual ink **(b)** strength properties of handsheets

Crude enzyme of *T. harzianum* PPDDN-10 NFCCI-2925

The experiments show the increase in the DE with the enzyme dose at 1.0 IU/g, however, both ISO brightness and strength properties are observed to decrease after 0.8 IU/g. At enzyme dose of 0.8 IU/g, DE of 93.1±1.6% and 83.1±0.7% ISO brightness with 40.l2±1.85 ppm residual ink are observed (Figure-4.12a, Table-4.5). The reducing sugars analysis shows that high enzyme doses are responsible for the dissolution of more fines resulting into

higher reducing sugars and freeness of the pulp with lower drainage time (Table-4.6). Freeness is found to improve from 520 to 620 ml during this optimization process and 2 time more release of the reducing sugars from the lowest to the highest enzyme dose. The maximum amount of reducing sugars is observed at 1 IU/g enzyme dose. As a result, lower strength properties are reported at high enzyme loading (1.0 IU/g) responsible for the removal of microfibrills from the surface (Figure-4.12b, 4.24d, Table-4.6).

Crude enzyme of *C. cinerea* PPHRI-4 NFCCI-3027

The highest DE and ISO brightness are obtained at the enzyme dose of 0.8 IU/g, but at this dose, the strength properties are also deteriorated. Therefore, 0.6 IU/g is chosen as optimum dose. At this dose, DE, ISO brightness and residual ink are 90.1±0.7%, 83.2±0.7% and 49.35±2.32 ppm, respectively (Figure-4.13a, Table-4.5). It is observed that there is a narrow balance of enzyme dose to enhance or deteriorate the strength properties of papers and quality, as the detected benefit tends to diminish with increasing enzyme dose i.e. after 0.6 IU/g (Figure-4.13b). Freeness is observed to increase from 530 to 640 ml for 0.2 to 1.0 IU/g enzyme dose. Reducing sugars analysis has also revealed the gradual increase in release of sugars from 0.2 to 1.0 IU/g and at the enzyme dose of 1.0 IU/g; it is approximately 1.8X increase than that of 0.2 IU/g (Table-4.6).

Pala et al. (2004) have used different commercial enzymes in the doses of 0.03 to 1.03 FPU/g and 1 to 654 IU/g o.d. pulp (based on xylanase activity). Lee et al. (1999) reported DE of 70.2% using 0.1% enzyme dose (i.e. 0.04 IU/g pulp) for newsprint papers using the cellulase and xylanase of *C. cinerea*. Kumar et al. (2013) have given the cellulase treatment to the laser printed papers with the dose of 1.2 U/mg of paper (*i.e.*1200 U/g) ,while for MOW 1.2 U/g have also been (Lee et al. 2011b).

High enzyme loading may cause the reduction in the ISO brightness of the paper due to accumulation of pigments or enzyme particles on the surfaces of the fibres (Jeffries et al. 1994). Reduction in strength properties at higher enzyme dose is also reported in literature, it may be due to deterioration of the fibres (Lee at al. 2007). Freeness is observed to increase with the enzyme dose due to higher dissolution of fines and micbrofibrills. Das et al. have also observed the same effect on reducing sugars with enzyme (Das et al. 2012). Welt and Dinus (1994) have reported that fines are dissolved at low enzyme dose while the intact fibre remains without deterioration. High enzyme dose is destructive for the fibres surface, which will make fibre prone to break, removal of microfibrils and more dissolution of the fines.

4.2.2.3. Pulp consistency (Cy)

The effect of pulp Cy is examined in the range of 6 to 14%.

Commercial cellulase

The results indicate that highest DE (94.6±1.3%) and ISO brightness (78.7±0.5%) are achieved when the Cy is kept at 10% (Figure-4.14, Table-4.7). Further increase in Cy resulted in decreased DE and ISO brightness with the deteriorated strength properties. The freeness is improved from 560 (6% Cy) to 600 ml (12% Cy), but at 14% Cy it is reduced to 580 ml. The reducing sugars released at 14% Cy are approximately 1.3% higher than that of 6% Cy (Table-4.8).

Crude enzyme of *T. harzianum* PPDDN-10 NFCCI-2925

Maximum DE (93.0±1.7%) and ISO brightness (83.1±0.7%) are obtained at 10% Cy during hydrapulping. High Cy (12 and 14%) results in decreased DE and ISO brightness. The strength properties are improved when Cy is increased from 6% to 10% but after that no significant improvement is observed at high Cy of 12 and 14% (Figure-4.15a, Table-4.7). Reducing sugars analysis also shows that at higher Cy, efficiency of the enzyme is slightly decreased as shown by slightly low amount of the released sugars (Table-4.8). At higher Cy, the strength properties are observed to be almost constant (Figure-4.15b).

Crude enzyme of *C. cinerea* PPHRI-4 NFCCI-3027

The maximum DE (93.0±0.5%) and ISO brightness (83.7±0.7%) with minimum residual ink (39.53±2.58 ppm) are achieved at 12% Cy (Figure-4.16a, Table-4.7). The strength properties are slightly improved at 10% Cy and the results of strength properties are comparable with the 12% Cy.

It is observed that at higher Cy (14%), less reducing sugars (24.3% lower than 12% Cy) are released due to the possible reduced enzymatic action, which is resulted in decreased strength properties (Figure-4.16b) and freeness (Table-4.8). The freeness value is observed to improve with increasing Cy from 6 to 10%, but after that the freeness is reduced to 570 ml at 14% Cy (Table-4.8).

Different reseachers have given the enzymatic treatment at various Cy such as 4% (Lee and Eom 1999, Lee et al. 2011b), 11% (Pala et al. 2004) and 12% (Dutt et al. 2012).

Reduced DE and ISO brightness achieved at high Cy could be related to the substrate inhibition or enzyme inadequacy for higher substrate. It would be advantageous for industrial usage, to operate hydrapulper at medium Cy (10 to 12%). In this range, the enzymes are in better contact with the fibre surface than lower or higher Cy. Fibre-fibre friction also increases with pulp Cy and there should be an optimum fibre surface friction to avoid denaturation of the enzyme due to shear (Zeyer et al. 1994). Such an explanation seems consistent with earlier finding that enzymatic deinking is more effective at medium Cy (Jeffries et al. 1994) as opposed to low Cy (Pelach et al. 2003, Lee at al. 2007).

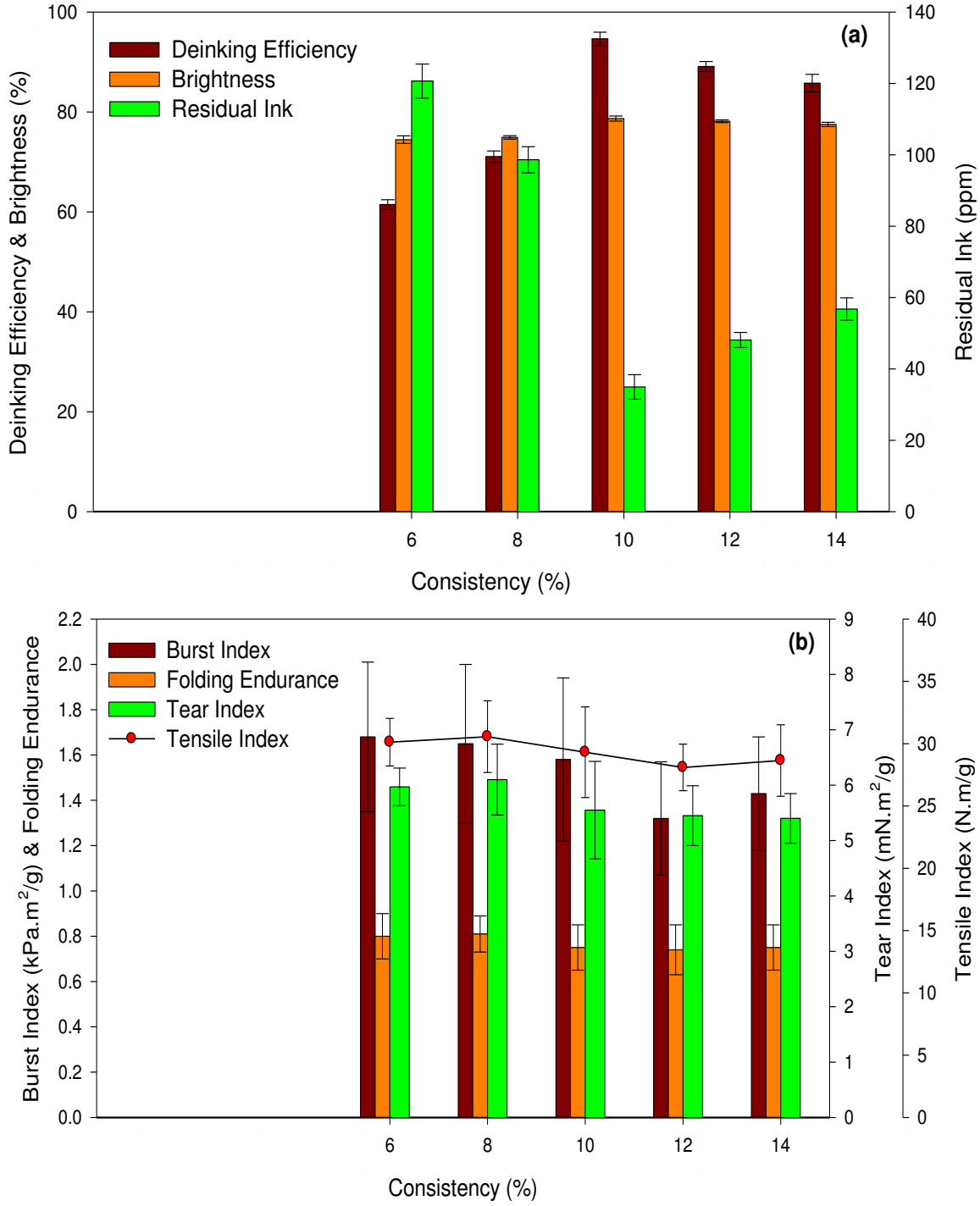


Figure-4.14 Deinking results with the 'commercial cellulase' showing the effect of pulp consistency on **(a)** DE, ISO brightness and residual ink **(b)** strength properties of handsheets

4.2.2. Reaction time

Commercial cellulase

The maximum DE (94.6±1.3%) and ISO brightness (78.7±0.5%) are achieved for 90 min reaction time. The lowest residual ink count is 31.95±3.45 ppm. Further increase in reaction time resulted in decreased DE and ISO brightness (Figure-4.17, Table-4.9 and 4.10). The strength properties are also decreased for long reaction time (beyond 90 min) (Figure-4.17,

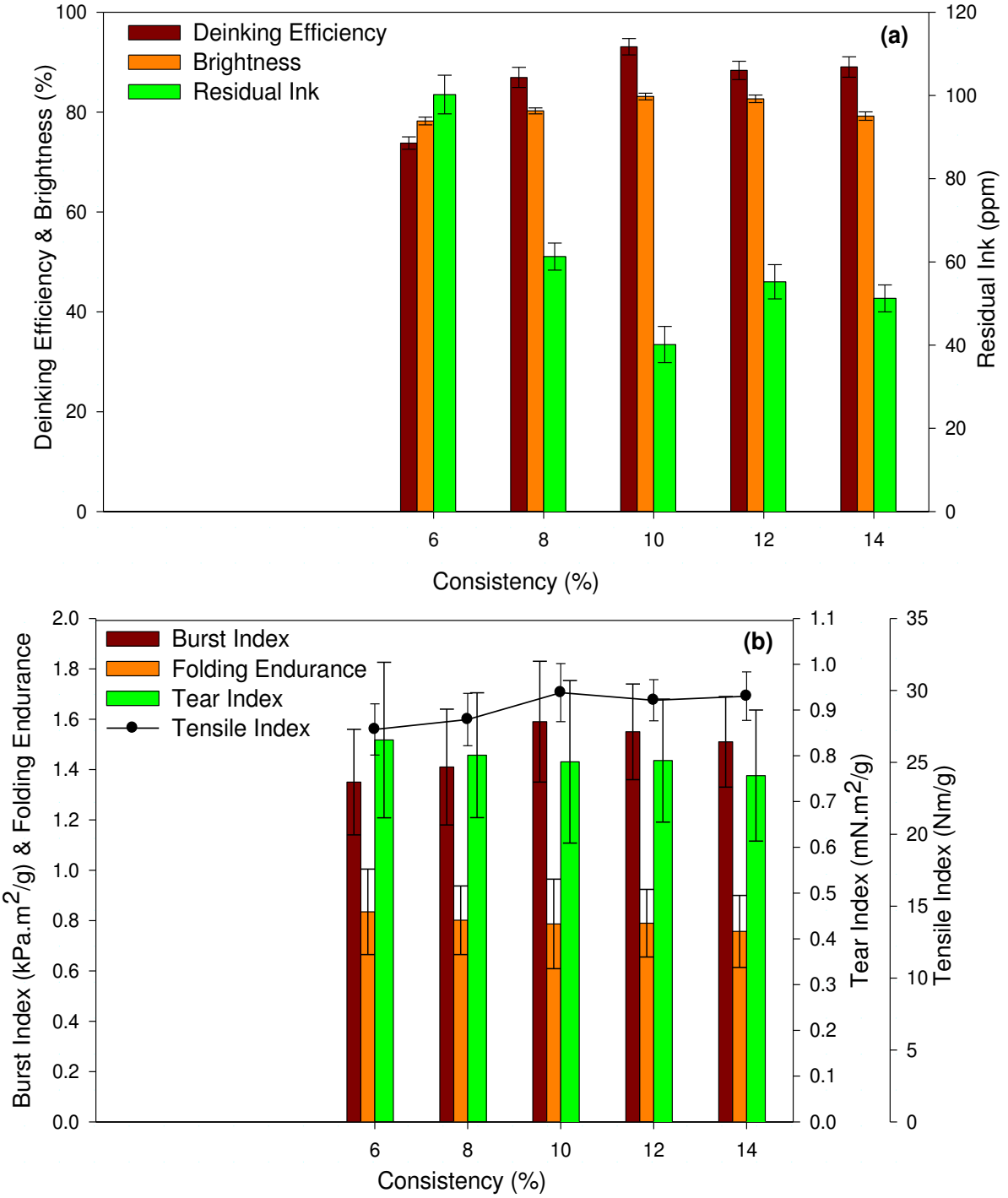


Figure-4.15 Deinking results with the 'crude enzyme of *T. harzianum*' showing the effect of pulp consistency on **(a)** DE, ISO brightness and residual ink **(b)** strength properties of handsheets

Table-4.9 and 4.10). Reducing sugars released after 150 min are about 2.4 and 1.2 times higher than the 30 min and 90 min reaction time, respectively (Table-4.10). The freeness is observed to improve from 480 to 620 ml for 90 min but after that it is reduced to 580 ml. The reason may low stability of the enzyme at these hydrapulping conditions.

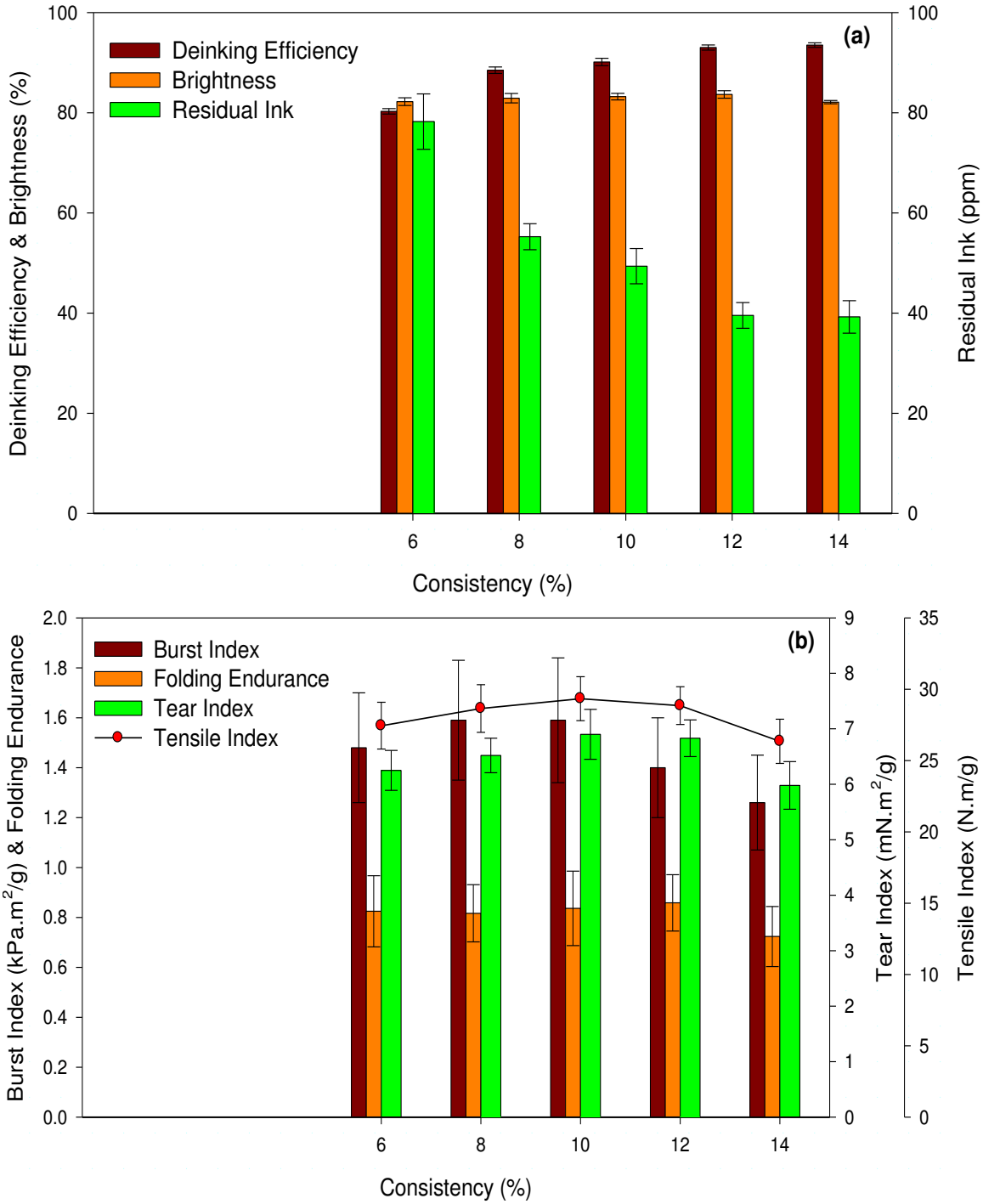


Figure-4.16 Deinking results with the 'crude enzyme of *C. cinerea*' showing the effect of pulp consistency on **(a)** DE, ISO brightness and residual ink **(b)** strength properties of handsheets

Crude enzyme of *T. harzianum* PPDDN-10 NFCCI-2925

The maximum DE (93.9±1.5%) and ISO brightness (83.0±0.4%) are achieved for 60 min reaction time. The lowest residual ink count is 37.52±3.23 ppm (Figure-4.18a). Further increase or decrease in reaction time resulted in slightly decreased DE and ISO brightness. Like the high enzyme dose, optimum enzyme dose for long reaction time also has negative effect on the fibre as observed in the form of high amount of reducing sugars and deteriorated strength (Figure-4.18b, Table-4.9 and 4.10). The amount of reducing sugars for

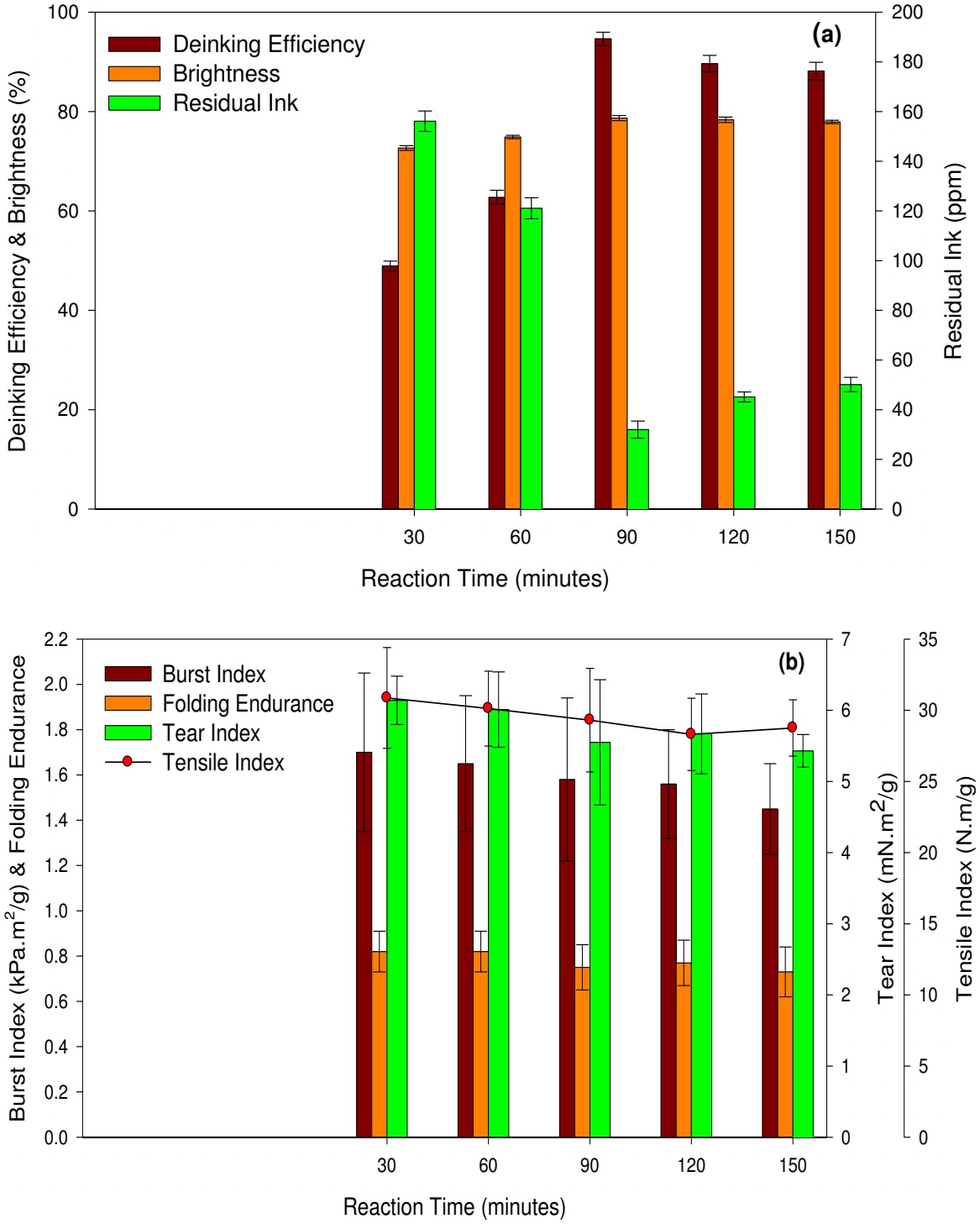


Figure-4.17 Deinking results with the 'commercial cellulase' showing the effect of reaction time on **(a)** DE, ISO brightness and residual ink **(b)** strength properties of handsheets

150min reaction time is 1.4 times higher than that of the 150 min (Table-4.10) while the freeness is changed from 550 to 630 ml for the same.

Crude enzyme of *C. cinerea* PPHRI-4 NFCCI-3027

The maximum DE (95.2±1.0%), ISO brightness (84.6±0.5%) and the lowest residual ink (33.23±2.36 ppm) are achieved for 60 min reaction time (Figure-4.19a, Table-4.9). The 30 min reaction time is not enough for enzymatic action, which results into the decreased

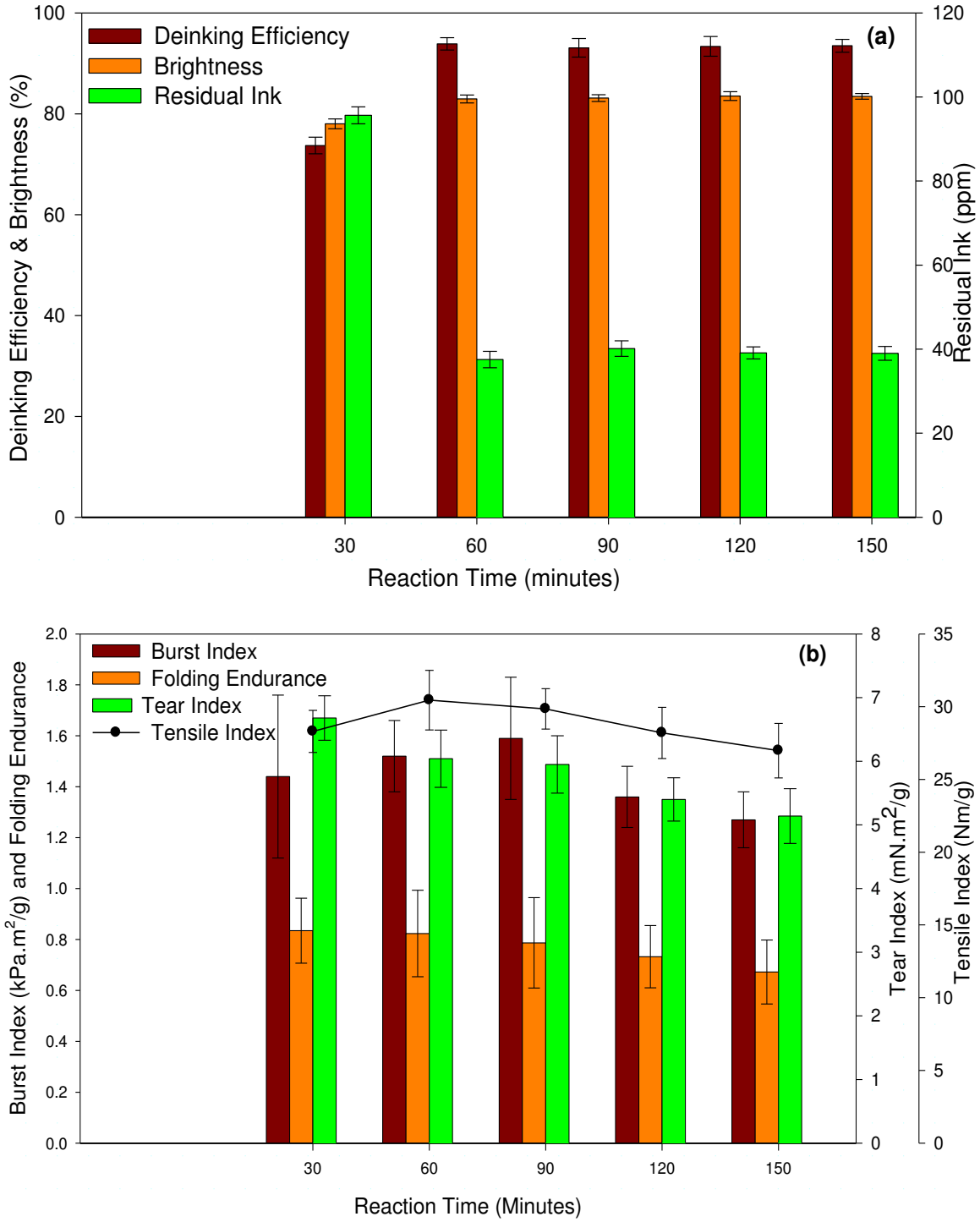


Figure-4.18 Deinking results with the 'crude enzyme of *T. harzianum*' showing the effect of reaction time on **(a)** DE, ISO brightness and residual ink **(b)** strength properties of handsheets

efficiency of the process. DE and ISO brightness are slightly decreased with an increase in reaction time from 60 to 150 min. At longer reaction time (90 to 150 min), optimum enzyme dose of 0.6 IU/g shows negative effect on the fibre i.e. deteriorated strength properties (Figure-4.19b, Table-4.9). The release of reducing sugars is increased upto 2.15 times when the reaction time is increased from 30 to 150 min (Table-4.10).

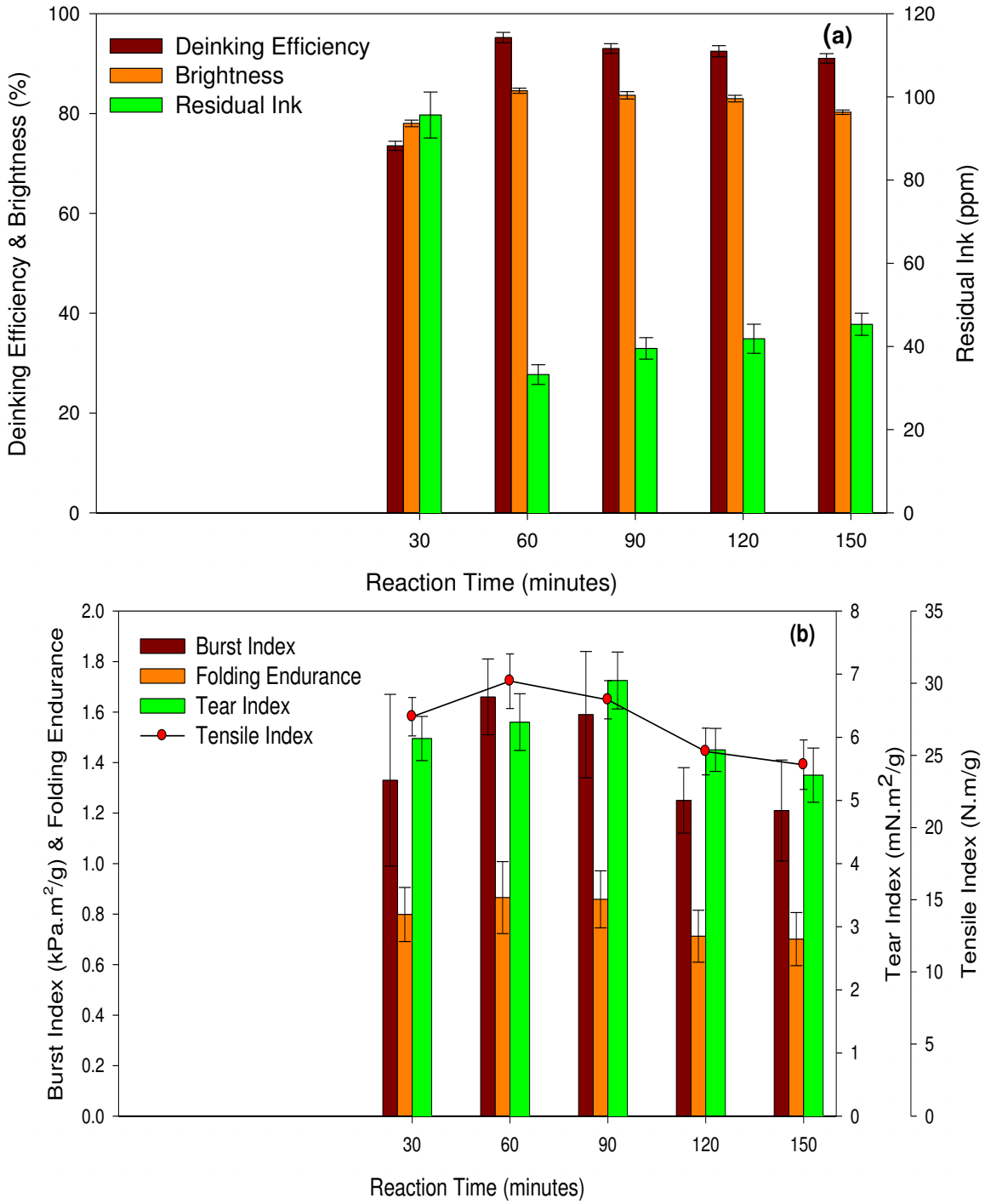


Figure-4.19 Deinking results with the 'crude enzyme of *C. cinerea*' showing the effect of reaction time on **(a)** DE, ISO brightness and residual ink **(b)** strength properties of handsheets

Earlier deinking studies with the crude enzyme of *C. cinerea* showed that 30 min reaction time was effective to remove ink from old newspapers with DE 70.2% (Lee and Eom 1999), whereas 60 min was effective for sorted office waste papers with DE 80% (Dutt et al. 2012). According to Dienes et al. (2004), Reaction time of 1.0 to 1.5 h, 35 to 50˚C temperature and a pH of 4.5 seem to be more efficient for industrial conditions. Maity et al. (2012) and Thomas et al. (2014) have reported quite high reaction time of 47.2 h and overnight tratment, respectively to deink laser printed papers with bacterial xylanse enzymes to show best DE.

The reaction time of 60 min, obtained in this study, to deink toner printed papers is reasonable. Lee et al. (2011b) have given 45 min hydrolysis time for enzymes after 60 minutes hydrapulping of MOW.

In presence of cellulase, long pulping time is responsible for redeposition of ink particles on the fibre surface and break down of detached ink particles into smaller size, which may get entry into pits or lumen due to mechanical forces (Bajpai 1998, Pelach et al. 2003, Vyas and Lachke 2003, Welt and Dinus 1998, Zeyer at al. 1994,). The optimum reaction time and enzyme dose have an inverse relation for any enzymatic reaction. Like the higher enzyme dose, optimum enzyme dose for the long reaction time will also have negative effect on the fibre as observed in the form of higher reducing sugars and deteriorated strength. Lesser reaction time may also result into the decreased efficiency of the process due to insufficient time for reaction. With the increase in the reaction time, reducing sugars and drainage of pulp are also increased. The released reducing sugars are primarily generated by dissolution of fines, which is resulted in increased freeness due to enzymatic action for long time (Table-4.10). It is clearly shown that there should be balance between the enzyme dose and reaction time. Pala et al. (2001) also advised to use low enzyme doses and short reaction periods, which results in lower sugar solubilisation.

4.2.3. Comparison of results of deinking using crude enzyme with the chemical and commercial cellulase deinking

The deinking results obtained with crude enzymes of both the fungal strains are compared with the conventional chemicals and commercial enzyme to analyze the enzymatic action (objective 5) (Figure-4.20 and 4.21). The optimized conditions for the chemical and enzymatic deinking are tabulated in the Table-4.11. Enzymatic deinking can result into improved, exacerbated or with no effect on the strength properties. The reason may be owing to different microorganisms as sources of enzyme production, types of waste papers used in the deinking processes, enzyme dose and incubation time during the treatments. To avoid any ambiguity in the results during comparison, the handsheets with the formation index of 160±5 are selected.

4.2.3.1. DE

Crude enzyme of *T. harzianum* PPDDN-10 NFCCI-2925

DE of lab produced crude enzyme has shown significant improvement (23.6%) than conventional chemical deinking but slightly lower (0.8%) than commercial cellulase (Figure-4.20, Table-4.12 and 4.13).

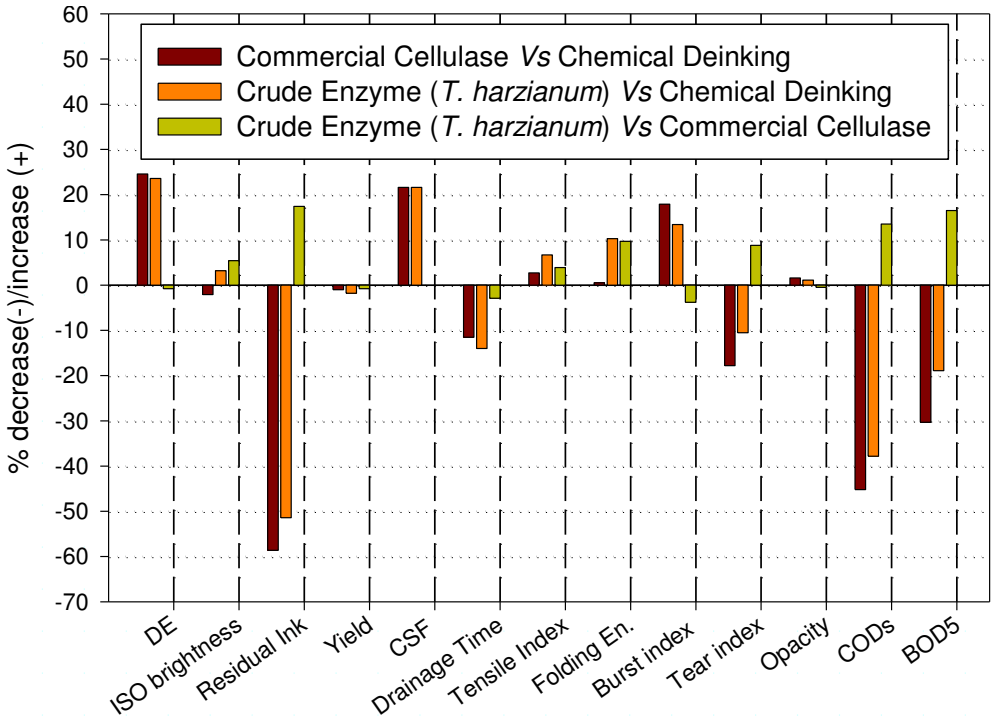


Figure-4.20 Comparison of the results of DE, pulp and handsheet properties obtained with chemical and commercial cellulase to evaluate the positive and negative effect of crude enzyme of *T. harzianum* PPDDN-10 NFCCI-2925

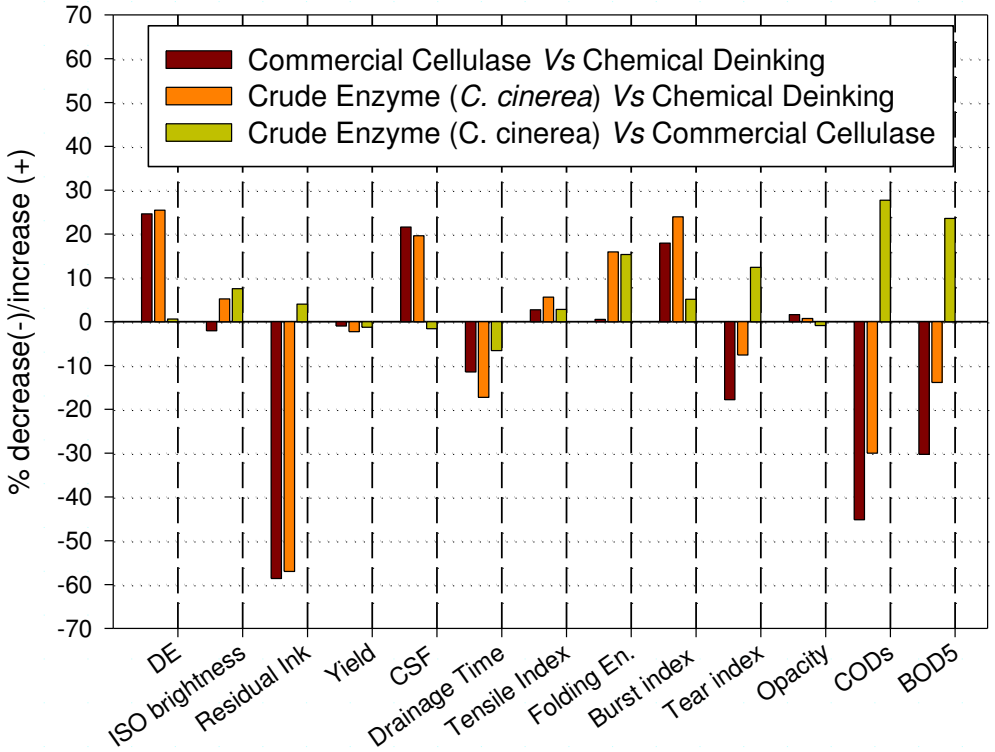


Figure-4.21 Comparison of the results of DE, pulp and handsheet properties obtained with chemical and commercial cellulase to evaluate the positive and negative effect of crude enzyme of *C. cinerea* PPHRI-4 NFCCI-3027

Crude enzyme of *C. cinerea* PPHRI-4 NFCCI-3027

It is observed that deinking with crude enzyme is resulted into significant 25.4% higher DE when compared with chemical deinking. DE is almost same (+0.6%) as observed with commercial cellulase (Figure-4.21, Table-4.12 and 4.13).

Earlier reported data also showed 94-96% DE for photocopier and laser printed waste papers with the cellulase and xylanase preparations (Gubitz et al.1998, Jeffries et al. 1994). Pala et al. (2004) reported 22% DE (with respect to control) for the photocopier waste papers using commercial cellulase and xylanase which was lower than chemical deinking (DE 37%).

These improvements may be owing to peeling mechanism of cellulase and xylanase on fibre surface, which has resulted into detachment of ink particle from the fibre surface (Das et al. 2012). On the other hand, these enzymes may also be responsible for freeing the entrapped toner particles within the loosely microfibrils which facilitates separation during the flotation process due to increased hydrophobicity of the toner particles (Bajpai and Bajpai 1998, Das et al. 2012, Jeffries et al. 1994, Maity et al. 2012, Pathak et al. 2010, 2011, Thomas et al. 2014). Earlier reported data also showed 94-96% DE for photocopier and laser printed waste papers with the cellulase and xylanase preparations (Gubitz et al.1998, Jeffries et al 1994). Pala et al. (2004) reported 22% DE (with respect to control) for the photocopier waste papers using commercial cellulase and xylanase, which was lower than chemical deinking (DE 37%). Recently, bacterial xylanases have also been used to deink the toner printed papers with higher DE than the control at alkaline pH (Maity et al. 2012, Thomas et al. 2014). Shifting of pH from alkaline to acidic can assist the ink removal during flotation process due to the dissolution of the removed calcium carbonate coatings (Jeffries et al. 1994) and reduction of size of toner particles due to low pH (Lee et al. 2007).

In this work, the enzymatic deinking shifted the deinking to acidic range due to its optimum pH (5.5-5.8). It is observed with the results of the control experiments which show the control experiments of the enzymatic deinking have higher deinking efficiency than the chemical control (Table-4.13). The reason may be the dissolution of the removed calcium carbonate coatings in acidic conditions, which facilitates the ink removal during flotation process (Jeffries et al. 1994). This synergistic effect of acidification of pulp slurry and enzymatic hydrolysis will reduce the ink particle size (Lee et al. 2007, 2011b, Viesturs et al. 1999) which subsequently helps the removal of the toner from the surfaces of the paper fibres during the flotation process. Although a numbers of researchers have preffered alkaline deinking (Prasad 1993, Vyas and Lachke 2003). This work suggests acidic deinking for the photocopier waste papers because the optimum pH of enzymes, used for deinking in this study, lies in this range which is supported by Lee et al. (2011b).

4.2.3.2. ISO brightness

Commercial cellulase

Enzymatically deinked pulp samples are found to obtain maximum ISO brightness of 78.67±0.65% which is 2.1% lower than chemically deinked pulp (80.38±0.52%) (Figure-4.20, Table-4.12 and 4.13).

Crude enzyme of *T. harzianum* PPDDN-10 NFCCI-2925

Enzymatically deinked samples are found to have 82.95% ISO brightness value, which is 5.2% higher than the commercial cellulase and 3.2% higher chemically treated pulp (Figure-4.20, Table-4.12 and 4.13).

Crude enzyme of *C. cinerea* PPHRI-4 NFCCI-3027

It is observed that the pulp after crude enzyme deinking has 84.56% ISO brightness value which is higher (+5.2%) than the chemical and commercial cellulase (+7.0%) based deinking, respectively (Figure-4.21, Table-4.12 and 4.13).

It is observed that all the control of enzymatic and chemical experiments have almost same brightness although they differ significantly in DE. The reason may be the deposition of the denatured enzyme particles on the fibre surface which counterbalance the expected improvement of the brightness due to higher DE (Table-4.13).

Different researchers have found miscellaneous results with optical properties of enzymatic deinked pulp handsheets. Some researchers have reported improved (Gubitz et al.1998, Heise et al. 1996, Jeffries et al. 1994, Mayeli and Talaeipour 2010, Vyas and Lachke 2003, Wang and Menghua 2005) brightness due to xylanase along with cellulase. Reduced ISO brightness is also observed due to accumulation of pigments present in the crude enzyme on the surface of the fibres at high enzyme loading (Das et al. 2012, Pathak et al. 2011, Singh et al. 2012, Soni et al. 2010). According to Bajpai (2010), improvement in the freeness of the cellulase, treated recycled pulp generally results in reduced ISO brightness and strength properties. It is found that crude enzymes are responsible for the increase in the ISO brightness while with the commercial cellulase decreased ISO brightness is reported. Several researchers also observed this enhanced ISO brightness, when they use cellulase with xylanase (Gubitz et al.1998, Heise et al. 1996, Jeffries et al. 1994, Vyas and Lachke 2003, Wang and Qin 2005). Lee et al. (2011b) have achieved the 83.6% ISO brightness with the enzymatic deinking of MOW. Jeffries et al. (1994) have reported 80.2% ISO brightness with commercial xylanase while the cellulase preparations achieved 76.0-78.0%. They also reported better DE with cellulase than the xylanase preparations. Xylanase has been proven to free the xylan and lignin bond to facilitate the extraction of residual lignin (Manimaran et al. 2009).

Improvement in ISO brightness due to xylanase was also observed using only commercial xylanase enzyme during deinking experiments in our laboratory (full data not shown due to unsatisfactory results with the deinking). The maximum DE of 70.4±0.8% is obtained which is lower than the chemical deinked pulp but the ISO brightness value is 79.9±0.5%. It is compared with the almost same DE of commercial cellulase (DE=69.68±0.62%) with the xylanase treated (DE=70.4±0.8%) (data not shown). Cellulase treated pulp has 74.8±0.5% ISO brightness at the approximately same DE, which is lower than solely xylanase treated pulp (79.9±0.5%). This data gave the reason of brightness improvement due to xylanase activity. In our experiments, the crude enzymes overcome the reduced ISO brightness problem, which is obtained with commercial cellulase based deinking. Therefore, it may be concluded that increase or decrease in ISO brightness depends on the enzyme source, enzyme type and composition. In this study, no correlation between the DE and ISO brightness is observed which is similar to Jeffries et al. (1994) because the highest degree of deinking is not resulted in the highest ISO brightness in our study.

4.2.3.3. Freeness

Commercial cellulase

Freeness is improved by 21.6%, and consequently drainage time is reduced by 11.5% in the case of enzymatic deinking as compared to chemical deinking (Figure-4.20, Table-4.12 and 4.13).

Crude enzyme of *T. harzianum* PPDDN-10 NFCCI-2925

This crude enzyme is found to be responsible for increased freeness (+21.6%) with reduced drainage time (-14.0%) than chemical deinking. The freeness (620 ml) values are same for both the crude and commercial cellulase treated pulp, but the drainage time is slightly lower (-2.9%) than the commercial cellulase (Figure-4.20, Table-4.12 and 4.13).

Crude enzyme of *C. cinerea* PPHRI-4 NFCCI-3027

The freeness enzyme treated pulp is improved by 19.6% with 17.3% less drainage time when compared with chemical treatment. However, it has slightly lower freeness (-1.6%) as obtained with the commercial cellulase based deinking (Figure-4.21, Table-4.12 and 4.13).

The endoglucanase (CMCase) activity of cellulase is the prerequisite for the improvement in the drainage of the recycled pulp (Bajpai and Bajpai 1998, Bajpai 2010, Elegir et al. 2000). Previously different researchers (Elegir et al. 2000, Gubitz et al.1998, Jeffries et al. 1994, Lee et al. 2007, Pathak et al. 2011, Park and Park 2001, Lee et al. 2013) have proposed that such an increase in freeness is due to selective removal of fine fibres by controlled peeling action. Endoglucanase preferentially acts on the amorphous region and low molecular components of the cellulose (Bajpai 2010). During the enzymatic deinking, enzymes detach

the ink particles with some part of microfibrils as shown in the Figure-4.24a. As a result, the removal of sufficient hydrophilic material also improves dewatering of the pulp as these particles hold maximum water because of their high specific surface area (Lee and Eom 1999, Pathak et al. 2011). Other reasons for increased freeness may be the removal of small colloidal particles (additives) due to enzymatic action (Putz et al. 1990). The increase in the freeness can increase the production of the recycled paper mill by improving the formation rate on the high speed machine and pulp dilution in the head box, thus finally the production of better quality paper (Bajpai 2010).

4.2.3.4. Strength properties

The strength properties are compared for deinked pulps obtained as such after the washing stage.

Commercial cellulase

Tensile and burst indices of enzymatically treated pulp handsheets are found to be 2.7% and 17.9% higher, while tear index is 17.8% lower than chemically deinked pulp handsheets (Figure-4.20, Table-4.12 and 4.13).

Crude enzyme of *T. harzianum* PPDDN-10 NFCCI-2925

Tensile index, burst index and folding endurance of enzymatically treated pulp handsheets are observed to be higher (+6.7%, +13.4% and +10.2%, respectively) in comparison to chemically deinked pulp handsheets and the same are +3.7%, -3.9% and +8.8% than the commercial cellulase treated pulp handsheets. The tear index is 10.5% lower than that of the chemical treated pulp while it is 8.1% higher than the commercial cellulase treated pulp (Figure-4.20, Table-4.12 and 4.13).

Crude enzyme of *C. cinerea* PPHRI-4 NFCCI-3027

The strength properties are found to be significantly improved in comparison to chemical deinked pulp. The tensile index, burst index and folding endurance are improved by 5.6, 23.9, and 15.9%, respectively. In comparison to commercial cellulase, the improvement is 2.8, 5.1 and 15.3%, respectively, which shows the better efficacy of this newly developed enzyme. In contrast, tear index is again found to be decreased by 7.6% than chemical deinking (Pathak et al. 2011). This reduction is slightly lower than the drop observed with commercial cellulase (Figure-4.21, Table-4.12 and 4.13).

In general, higher freeness is obtained at the expense of the strength properties (Putz et al. 1990). According to Pala et al. (2001), the xylanase activity is not considered to interfere with the strength propertie, unless cellulase activity is also present. Xylan removal from the fibers surface may reduce the total charge. The presence of non-identified secondary activities (processitivity, adsorption, etc) may have a significant synergistic effect on the overall

performance over the pulp. The combined action of the two activities of cellulase and xylanase may be helpful to provide a better upgradation of the recycled fibres because the overall effect is dependent of the applied dose and reaction time (Pala et al. 2001). However, if strength properties are improving, then it may be due to action of cellulase and xylanase enzymes on the surface of the fibres during enzymatic deinking. Cellulases are principle enzyme for dissolving the fines and facilitate surface microfibrillation of fibres which is contributed towards higher inter fibre bonding due to exposure of more hydroxyl groups (Bajpai 2010). According to Pala et al. (2002), enzymes facilitate to increase the fibres surface hydrophilicity, probably by increasing the exposure of number of hydrophilic groups, which contribute for a strong bond between the water molecules and the enzyme treated surfaces. This effect enhances fibres stability in aqueous suspension, which may lead to better paper sheet formation (better formation index). The high amount of hydroxyl groups is confirmed by the FT-IR analysis (will be explained in the subsection 4.2.3.7.) Increased strength can also be signed to internal as well as surface fibrillation of the fibre by enzymatic action as shown in SEM photographs (Figure-4.22 to 4.25). This development enhances surface area of fibre and hence improves inter-fibre bonding in spite of observed increased freeness.

The Bauer-McNett classification also shows that enzyme deinked pulps have higher proportion of longer fibres with less fine content when compared with chemical deinked pulp which indicates that enzyme are able to maintain fibre structure under optimum conditions (Table-4.12 and 4.13).The enzymatic action is considered to depend on the length of the fibre. Generally, the shorter fibers are more susceptible to the enzymatic attack than the longer, as they present a wider specific surface area to the enzymes (Pala et al. 2001).

Many researchers have reported similar increased strength properties (Heise et al. 1996, Mayeli and Talaeipour 2010, Pathak et al. 2011, Soni et al. 2010) as well as decreased (Gübitz et al. 1998) and both (Pala et al. 2006, 2004). Gubitz and coworkers have found the decrease in the tensile index with endoglucanase and hemicellulase, when compared with control (Gubitz et al. 1998). Pala et al. (2004) have reported that some enzymes are responsible for the improvement in the tensile and burst index while some showed the reduction. The cause of the reduced tear index may be attributed to improved inter fibre bonding, which resulted in breakage of more fibres rather than breaking of fibre-fibre bonds during tearing process (Pathak et al. 2011). Reduced tear indices have also observed by researchers (Lee et al. 2007, Pathak et al. 2011, Vyas and Lachke 2003,), while some of them have reported the increased tear index (Morkbak et al. 1999, Morkbak and Zimmerman 1998b).

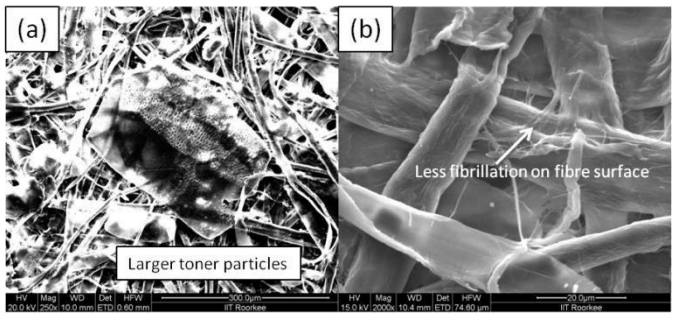


Figure-4.22 SEM photographs of chemical deinked pulp showing:
(a) toner particles attached with fibre (500X),
(b) degree of fibrillation on surface of fibre obtained (2000X)

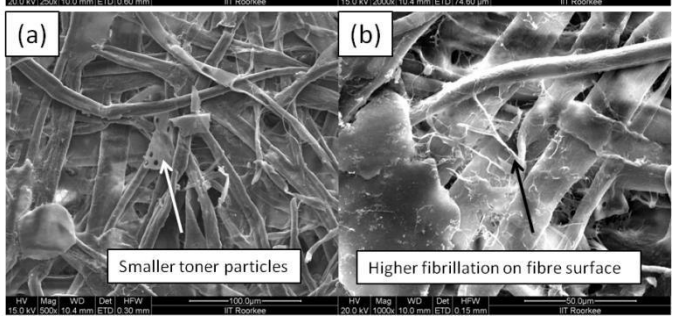


Figure-4.23 SEM photographs of commercial cellulase deinked pulp showing:
(a) toner particles attached with fibre (500X),
(b) degree of fibrillation on surface of fibre obtained (2000X)

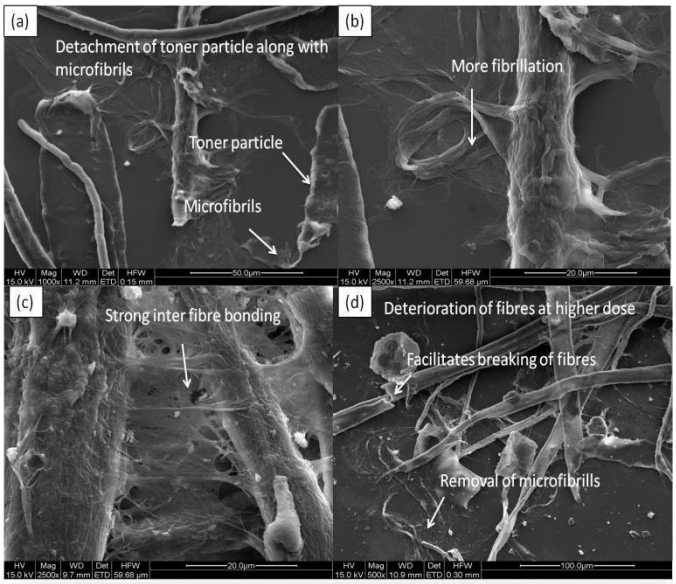


Figure-4.242 SEM photographs of deinked pulp (with crude enzyme of *T. harzianum* PPDDN-10 NFCCI-2925) showing:
(a) detachments of toner particles along-with some part of microfibrills (1000X)
(b) fibrillation on the fibre surface (2500X)
(c) strong inter-fibre bonding which results in the higher strength properties (2500X)
(d) deterioration of the fibre surface at higher dose resulting in removal of micro-fibrils

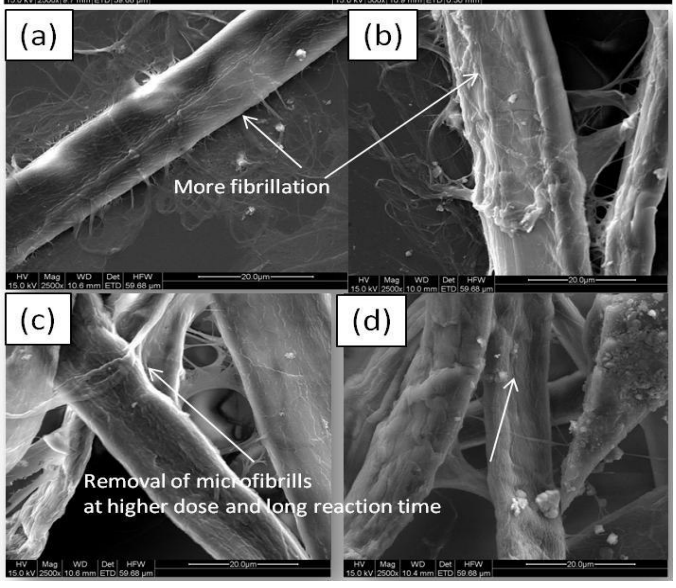


Figure-4.25 SEM photographs of deinked pulp (with crude enzyme of *C. cinerea* PPHRI-4 NFCCI-3027) showing:
(a) and **(b)** fibrillation on the fibre surface (2500X)
(c) deterioration of the fibre surface at higher dose resulting in removal of micro-fibrils (2500X)
(d) deterioration of the fibre surface for long reaction time resulting in removal of microfibrills (2500X)

4.2.3.5. Ash

Crude enzyme of *T. harzianum* PPDDN-10 NFCCI-2925

The ash content in crude enzyme deinked pulp (0.7%) is lower than the chemically (2.4%) and commercial cellulase deinked pulp (0.9%). This reduction in ash content is reflected in the 1.8% lower yield than chemical deinking.

Crude enzyme of *C. cinerea* PPHRI-4 NFCCI-3027

The ash content in enzymatically deinked pulp (0.8%) is lower than the chemically deinked pulp (2.4%) and almost equal to commercial cellulase deinked pulp (0.9%). This reduction is reflected in the 2.3% lower yield than chemical deinking.

The ash content in the enzyme deinked pulp is reduced due to dissolution of filler by acid used to lower pH. According to Viesture et al. even a small acidification during cellulase treatment of the stock to adjust the pH 5.0, resulted in up to 95% loss of ash from the initial ash content (Viesturs et al. 1999). In the flotation process, the removal of non-fibrous material (such as inorganic) would facilitate the interfibre bonds resulting into improved strength properties (Viesture et al. 1999, Mayeli and Talaiepour 2010).

4.2.3.6. Effluent load

Commercial cellulase

The effluent load produced during the deinking with commercial cellulase has been found to have lower pollution load than that of conventional chemical deinking. COD and BOD_5 are 45.2 and 30.3% lower with respect to effluent of chemical deinking. The BOD:COD ratio of effluent of the enzymatic deinking (0.57) is higher than that of chemical deinking (0.47) (Figure-4.20, Table-4.12 and 4.13).

Crude enzyme of *T. harzianum* PPDDN-10 NFCCI-2925

The effluent generated during the enzymatic deinking has 37.8% lesser COD and 18.9% lesser BOD_5 value than effluent of chemical deinking and 45.2 and 30.3%, respectively than the effluent of commercial enzyme deinking (Figure-4.20, Table-4.12 and 4.13)

Crude enzyme of *C. cinerea* PPHRI-4 NFCCI-3027

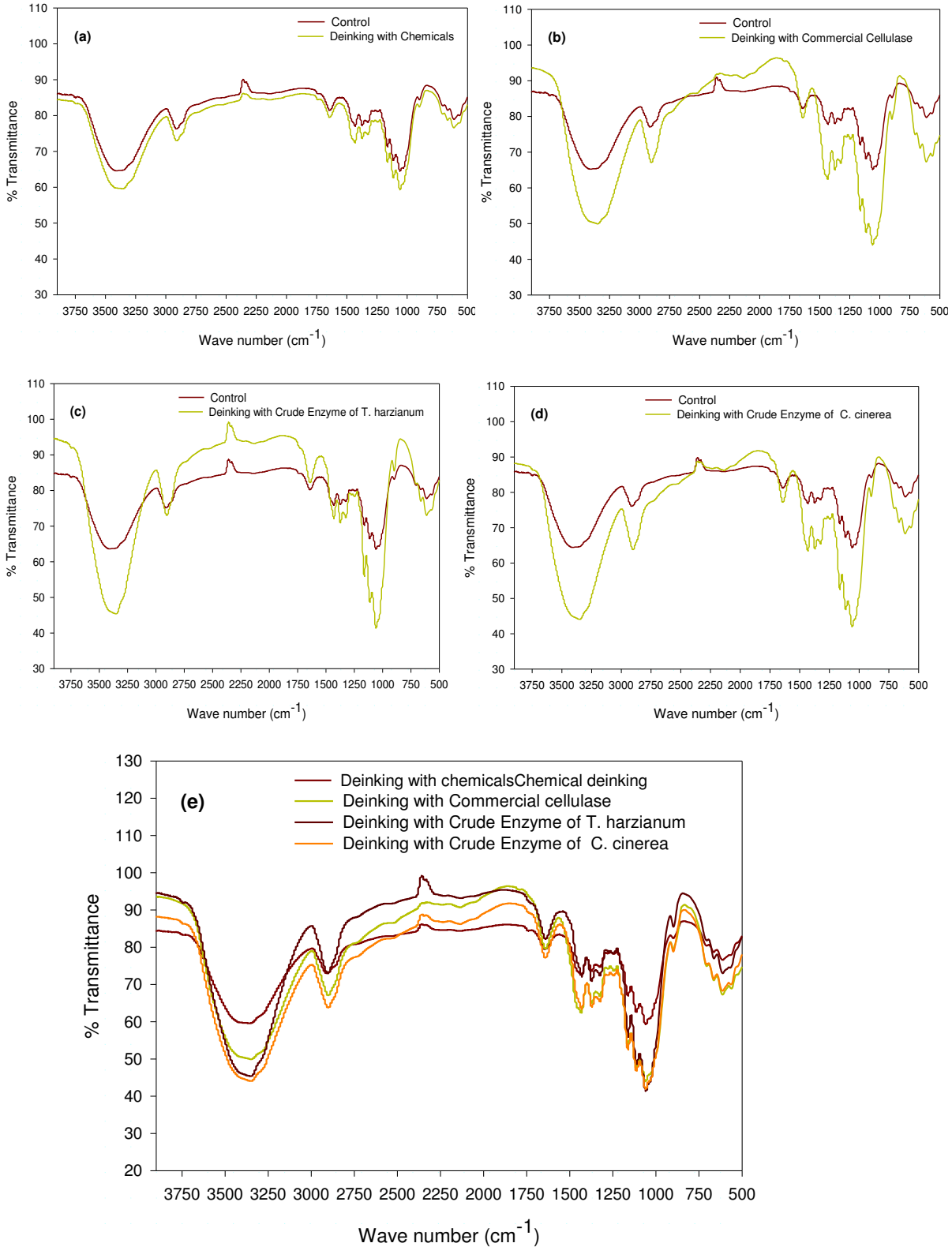
The effluent after enzymatic deinking is observed to have 30.0% less COD and 13.9% less BOD_5 value than that of chemical deinking. These values are 45.2 and 30.3%, respectively for the commercial enzyme (Figure-4.21, Table-4.12 and 4.13).

It is expected that enzymatic deinking lowers the environmental load due to less or no use of chemicals. The BOD/COD ratio is high for the enzymatic deinking, which shows that the effluents have more biodegradable components (enzymes, fines and fine cellulosic fibres). Putz et al. (1990) have resulted that enzymatic treatment produces almost half the COD load

as compared to that produced by conventional deinking. However, some researchers have reported about 20-40% higher COD level in the process water than in the standard process (Dutt et al. 2012, Magnin et al. 2002, Saari 2005). Heise et al. (1996) also reported that enzymatic treatment produces lower COD and BOD loads than conventional deinking due to less or no use of chemicals. In one of the runs, Heise et al. (1996) reported that although the process water of enzymatic deinking has higher COD, but it is more easily biodegradable than control. Bobu and Ciolacu (2007) also reported the same with enzyme Novozyme 342 based deinking. Amount of generated sludge is lower in enzyme deinking and has lower inorganic content, providing potential to reduce the costs of sludge treatment.

4.2.3.7. FT-IR analysis

A broad band at around 3401–3352 cm−1 depicts stretching vibrations of hydroxyl group (–OH) hydrogen group for hydrogen bonding (H-bonding) (Meng et al. 2013, Virk et al. 2013). The maximum absorbance of these peaks is observed to shift from higher wave number to lower wave number after chemical and enzymatic deinking as presented in the Figure-4.26. It is reported in previous studies that the shifting of these band is influenced by the transformation related to the change of H bonding (intra as well as intermolecular H-bonds) (Meng et al. 2013). The bands have the tendency to shift towards lower wave number as the intensity of intermolecular H bonding increases (Figure-4.26). The increase in the relative intensities of band (3401–3352 cm^{-1}) after enzymatic treatments is attributed to the increase in open cellulosic content of the pulp (Virk et al. 2013). Thus, the results of this FTIR analysis shows that the enzymatic deinking process has resulted into more fibrillation than chemical and control, which exposes more hydroxyl groups on the microfibrills. These groups facilitate the H- bonding and as a result, the strength properties are observed to improve (as described in subsection 4.2.3.4.). These results are contradictory with the results of Meng et al. (2013) who have found the higher crystallinity index with the alkaline deinking than the enzymatic or neutral deinking. It was observed that the peak intentities at 2921–2917 cm^{-1} and 617 cm^{-1} of enzymatic treated pulp are lower than the chemical treated pulp. Virk et al. (2013) have explained this decrease as the degradation of aliphatic side chains, these bands corresponds to CH asymmetrical stretching vibration in CH_3, CH_2, CH, in cellulase and xylanase treated pulps. The bands that appeared at 1737 cm^{-1} (C = O stretching vibration in β-C = O, COOH, ester) and 1162 cm^{-1} (degradation of syringyl groups) indicate that the presence of residual lignin (although very less proportion in photocopier waste papers). After crude enzyme treatments, the pulps are observed to more exposure of these types of functional groups due to the action of xylanase and cellulase along with possible ligninolytic enzymes, which degrade the residual lignin (Virk et al. 2013). These combined effects may be responsible for higher brightness in the crude enzyme treated pulps.



	Control	Chemical	Commercial cellulase	Crude enzyme of *T. harzianum*	Crude enzyme of *C. cinerea*
Wave number (cm^{-1})	3410-3420	3370-3400	3350	3350-3360	3340-3360
% transmittance	64.6-62.5	59.7	49.9	45.4	44.1

Figure-4.236 FT-IR analysis of the chemical and enzymatic treated pulps a) chemical (b) commercial cellulase (c) crude enzyme of *T. harzianum* (d) crude enzyme of *C. cinerea*, and (e) comparative study

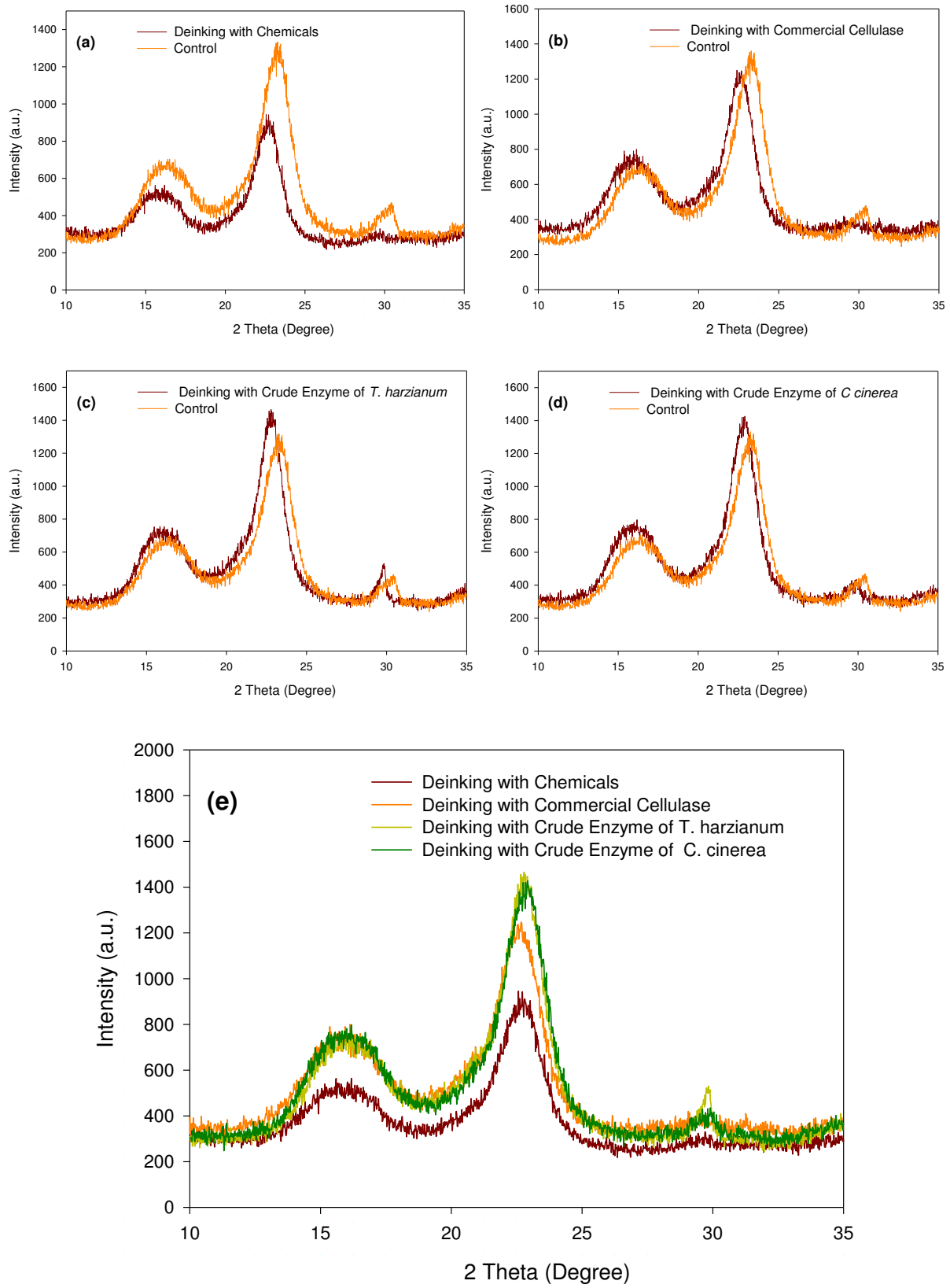


Figure-4.27 XRD analysis of the deinked pulp obtained after treatment with (a) chemical (b) commercial cellulase (c) crude enzyme of *T. harzianum* (d) crude enzyme of *C. cinerea*, and (e) comparative study

4.2.3.8. XRD analysis

Crystalline content of deinked pulp (holocellulose) is calculated on the basis of amorphous subtraction method. In the chemical deinking process, photocopier waste papers are deinked using 2% NaOH with other chemicals. These chemicals are non-selective they acts on both the crystalline as well as amorphous regions. Alkali charge of 2% is only able to partial removal of hemicellulose and cellulose. According to Pan et al. (2008), the chemicals used in conventional deinking destroy the hydrogen bonds and crystalline structure of the cellulosic component of the fibre, which results in the lower cellulose crystallinity (CI=36.99%), and higher deformation of fibres because of swelling phenomenon.

The commercial cellulase treatment is responsible for the partial damage of crystalline regions due to surface fibrillation (as observed in the SEM photographs) and pore formation at the cross section (CI=40.68%). In comparison to crystalline portion, the amorphous regions of the pulp fibres are more prone to attack by the cellulase to hydrolyze micro-fibres (Yang et al. 2011, Vyas and Lachke 2003). On the other side, the crude enzyme of *T. harzianum* and *C. Cinere*a contains both cellulase and xylanase. The endoglucanase attacks on selectively attacks on the amorphous region of the cellulose chains. The xylanase attacks on the hemicellulosic components of the photocopier pulp and thereby some of the hemicellulosic components are removed during washing process of ink removal. Thus, the percentage of crystallinity of the pulp, deinked with the crude enzyme of *T. harzianum* (CI=42.22%) and *C. Cinere*a (CI=43.66%) are observed to be higher than that of chemical deinked pulp (CI=36.99%) because the residue enriched in cellulosic components. Therefore, the percentage of crystallinity of chemical deinked pulp is observed to be less than the enzyme treated pulp (Figure-4.27, Table-4.15). Both the chemical and enzymatic treatments have the lower crystallinity index than the respective controls. Virk et al. (2013) have also observed the higher crystalline with the enzymatic treated pulp of ONP.

4.2.3.9. Dirt count

Dirt is defined as any foreign matter embedded in the sheet which, when examined with reflected light, has a contrasting colour of greater than 10% of full scale relative to the local background around an individual dirt speck. A higher ppm (mm^2/m^2) indicates greater dirt content. (Figure-4.28 to 4.31) showed that the enzyme mixtures have a significant effect on pulp cleanliness. In comparison to chemical deinking, enzymatic treatment generally produces smaller, finer ink particles. But in our study, the chemical treated pulps generated the fine particles of the dirt specks as shown in the table and fig. After flotation and washing steps, the surprisingly enzyme treated pulps have lower dirt speck area as well as dirt counts than the chemical treated pulp. The enzymatic deinked pulps have reached nearer to the dirt count limit than the chemical deinked pulp. According to Faul (2004), deinked paper should

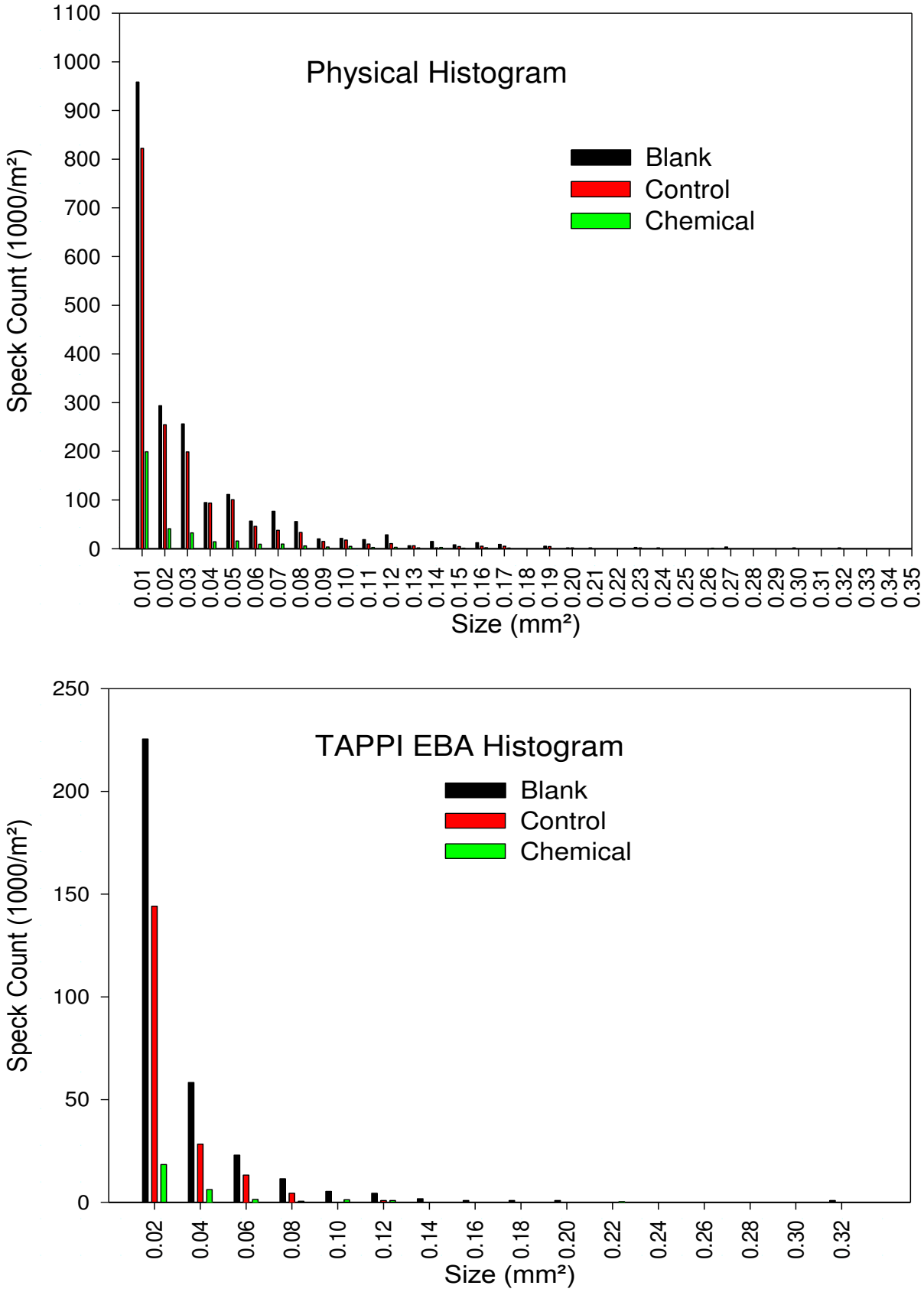


Figure-4.28 Physical and TAPPI EBA histograms of chemical deinked pulp

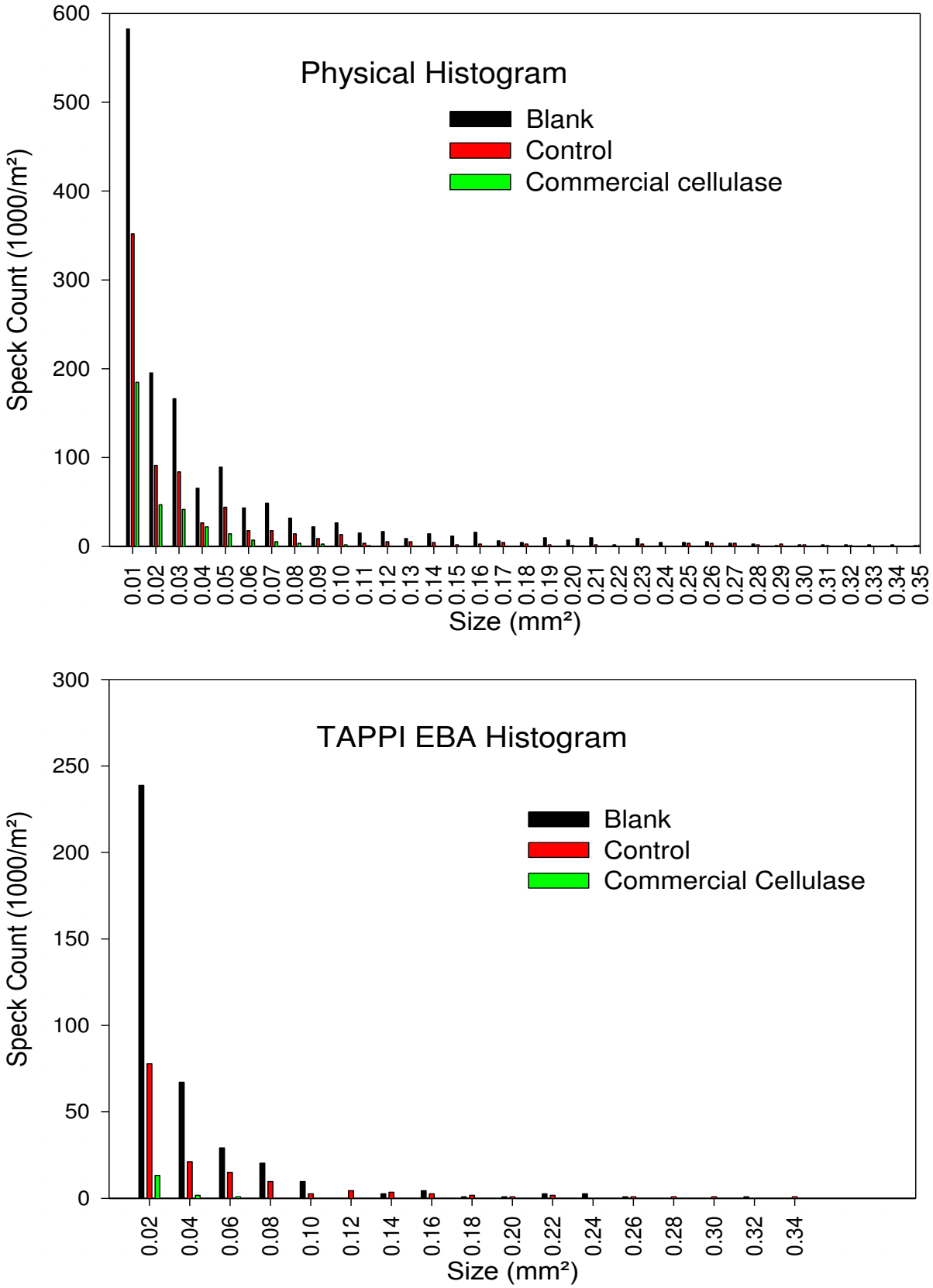


Figure-4.29 Physical and TAPPI EBA histograms of enzymatic deinked pulp with the commercial cellulase

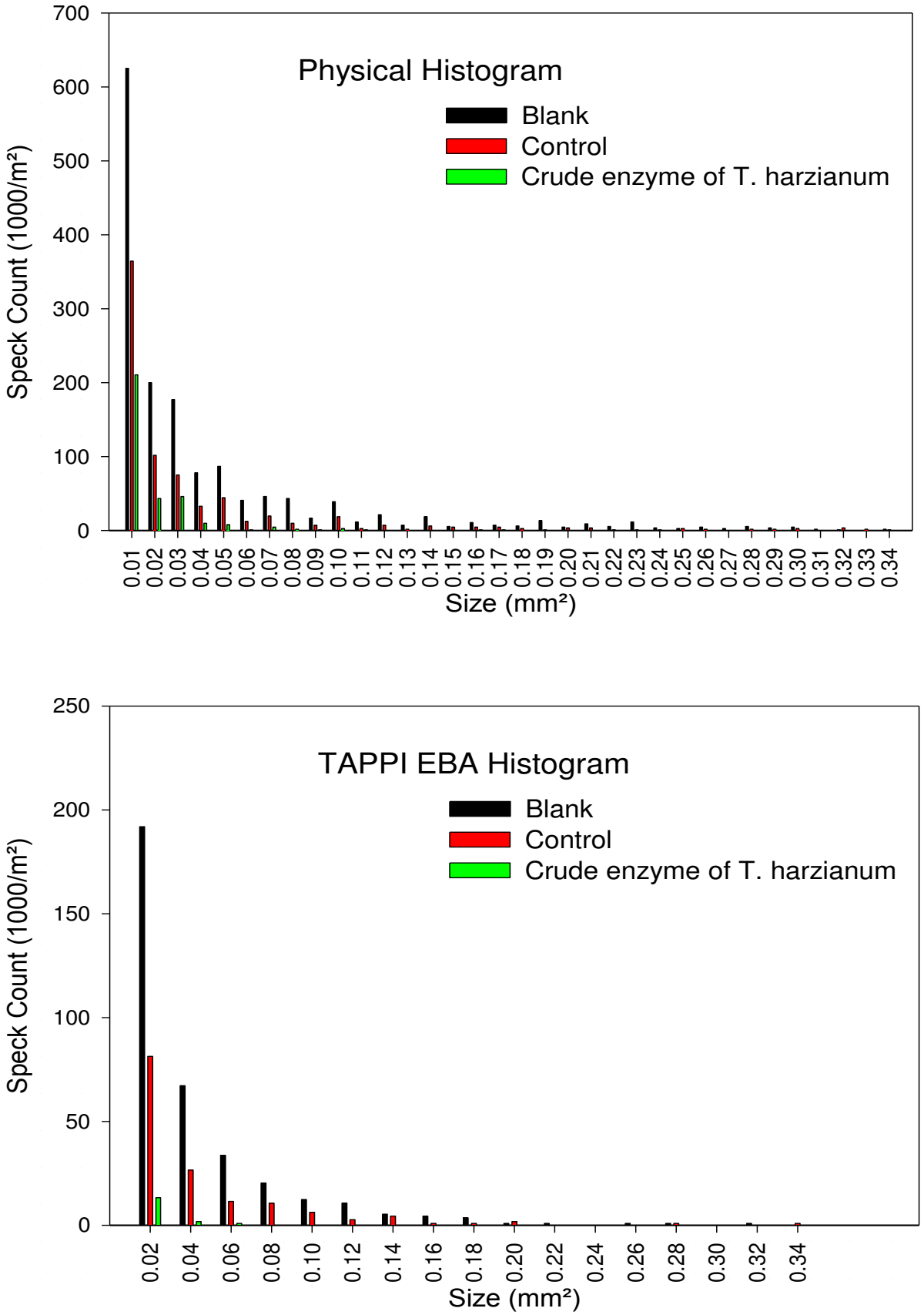


Figure-4.30 Physical and TAPPI EBA histograms of enzymatic deinked pulp with the crude enzyme of *T. harzianum*

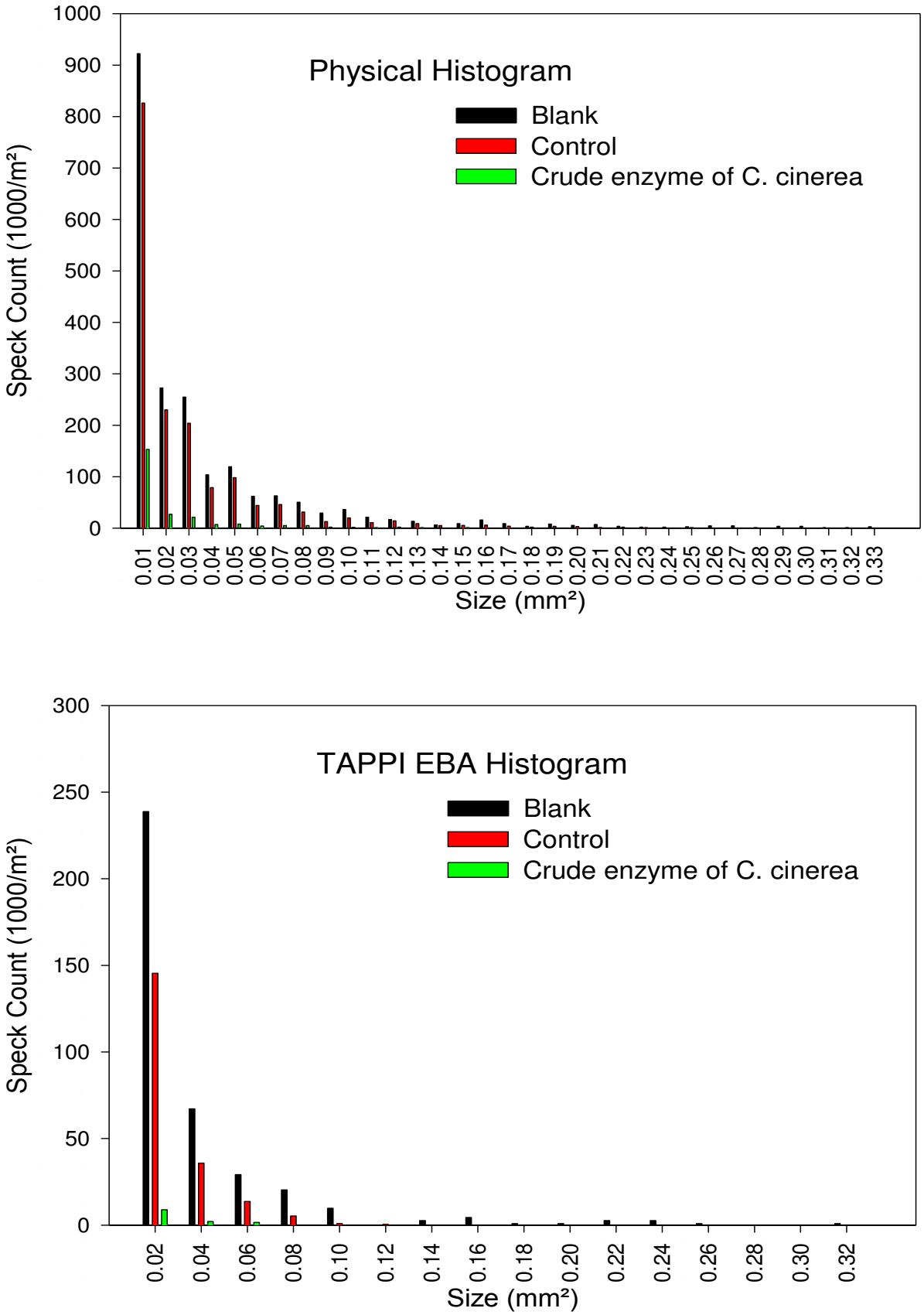


Figure-4.31 Physical and TAPPI EBA histograms of enzymatic deinked pulp with the crude enzyme of *C. cinerea*

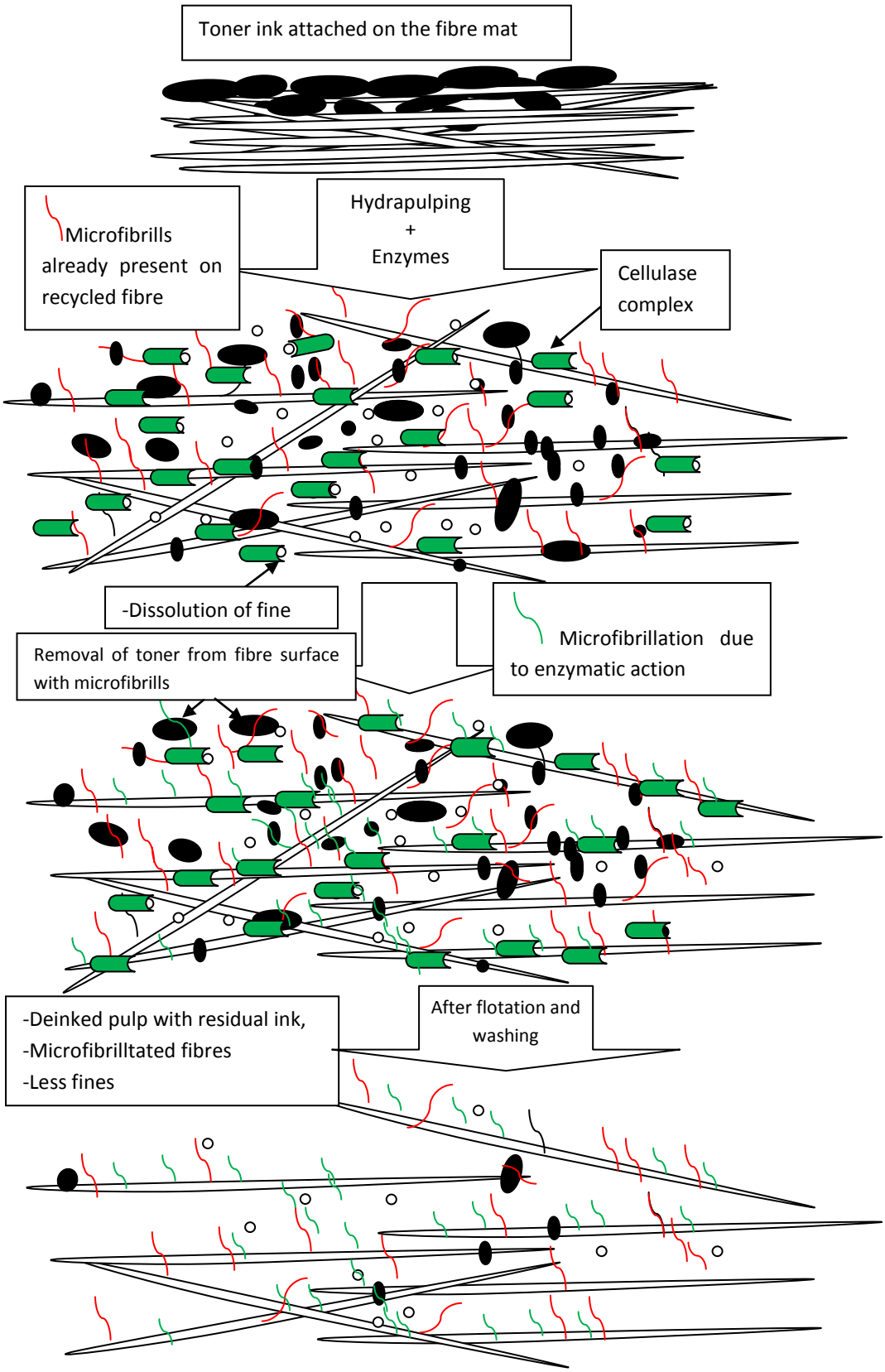


Figure-4.32 Diagram showing basic mechanism of the enzymatic action to deink toner from fibre surface

have a dirt count of < 500 mm^2/m^2. The commercial cellulase and crude enzyme of *T. harzianum* have the lower dirt count (639 and 523 mm^2/m^2, respectively) than the chemical deinked pulp (1380 mm^2/m^2). The pulp deinked with the crude enzyme of *C. cinerea* have shown the dirt count within limit *i.e.* 466 mm^2/m^2. Other researchers have also found the reduction in dirt count after enzymatic treatment (Dutt et al 2012, Tauske 2005).

4.2.4. Basic mechanism of enzymatic action on the fibre

Based on the deinking results and characterization of the pulp, a basic mechanism has been developed (objective 5) (Figure-4.32). During the enzymatic deinking, the cellulase and xylanase enzyme both act on the fibre surface and they primarily attack on the amorphous region exposed by the microfibrills and on the fibre surface. During photocopying process, the toner ink particles are entrapped in microfibrils, present at the surface of the fibre as well as on the fibre surface. This can be observed from scanning electron microscopic images (Figure-4.22 to 4.25). During the hydrapulping, the fibres are separated due to mechanical action. This mechanical action released some microfibrills. Thus, some of ink attached with the microbrills and filler particles is removed during the flotation and washing processes, respectively (results of control experiments). Still most of the ink is firmly attached with the microfibrills and on the fibre surface. The endoglucanase is well known component of cellulase system to split the cellulose fibres at several amorphous sites to generate non- reducing ends of the chain. This action supports loosening of fibres to release the toner particles entrapped within the loosely bound microfibrills. The microfibrills attached with the toner particles enhanced its hydrophillicity, so the endoglucanases released short fibres from surface of hairy toners to enhance the hydrophobicity of ink particles, which facilitates its better separation during the flotation process (Figure-4.32). On the other side, the cellulase and xylanase also act on the fibre surface, which improved the fibrillation on the fibre surface to expose more hydroxyl groups for hydrogen bonding as shown in the SEM and FT-IR analysis. This activity will maintain the strength properties of the fibres. The enzymes will act on the fibre surface for the initial treatment time. The recycled fibres already have primary fines as well as recycled fines generated during hydrapulping. The endoglucanase and β glucosidase activities will dissolve these fines, which will enhance the freeness of the pulp. Longer reaction time and higher enzyme dose cause severe damage to fibre as it degrade the microfibrils and make the fibre surface prone to breakage (cross breakage) which ultimately decreases the average fibre length due to the synergistic behavior of the endoglucanases and cellobiohydrolases.

CHAPTER 5

CONCLUSIONS AND RECOMMENDATIONS

The present chapter concludes this thesis by summarizing the results and the achievements of this research and recommends future work in this area.

5.1. Conclusions

The broad research objectives (mentioned in Chapter 1), successively attained by the research work presented within this dissertation, can be briefly listed as:

5.1.1. Crude cellulase and xylanase enzyme pruduction by the isolated fungal strains

This research work has optimized the conditions for the enhanced production of cellulase and xylanase by newly isolated fungal strains *T. harzianum* PPDDN-10 NFCCI-2925 and *C. cinerea* PPHRI-4 NFCCI-3027 (objective 1 and 2).

1. Two fungal strains *T. harzianum* PPDDN-10 NFCCI-2925 and *C. cinerea* PPHRI-4 NFCCI-3027 have been selected among the 104 isolated strains as the best cellulase (preferrable) and xylanase producers.
2. For the SSF, three-step extraction has been found suitable to extract maximum enzyme from the fermented matter resulting into the dilution of the enzymes.
3. Static SSF is strongly recommended for lignocellulosic enzymes higher production for both the fungal strains than SmF and SmSF.
4. WB is the best carbon source among the evaluated ones for both the fungal strains. The use of WB eliminates the use of costly carbon sources cellulose, CMC and xylan.
5. WB in combination with other carbon sources is not effective to improve the enzyme production but it induces the enzyme production in other carbon sources.
6. *T. harzianum* PPDDN-10 NFCCI-2925 has produced the maximum enzyme at spore density of 8×10^6 CFU/gds. Inoculum size of 6 discs/ 5 g of WB has been selected as the optimum for the *C. cinerea* PPHRI-4 NFCCI-3027.
7. The maximum cellulase and xylanase production has been obtained at the day 4 the *T. harzianum* PPDDN-10 NFCCI 2925 and day 8 for the *C. cinerea* PPHRI-4 NFCCI-3027.
8. Both the fungi have ability to produce maximum enzyme at the incubation temperature of 34 °C during fermentation and these are not able to grow above 42 °C.
9. The initial pH range of 6.0 to 6.5 is preferable for the fermentation of WB by these fungi.
10. The nature and amounts of N-sources have been observed to affect the enzyme production significantly. For the *T. harzianum* PPDDN-10 NFCCI-2925 maximum enzyme production has been found with AFS at a dose of 0.02M of available nitrogen concentration. MYP is the best nitrogen source for the *C. cinerea* PPHRI-4 NFCCI-3027

at the concentration of 3 g/l. High xylanase production can be achieved with complex organic N source. SBM may be used as cheaper N source for the *C. cinerea* PPHRI-4 NFCCI-3027 but it results in lower xylanase production.

11. Tween 80 is found to be the best surfactant to increase the cellulase and xylanase production.

12. Wheat bran to nutrient salt solution ratio (WB:NSS) of 1:3 is found to be optimum for the SSF for both the fungi.

13. Alkali pretreatment of WB is not effective to improve cellulase and production. The pretreatment processes made the WB particles gummy and sticky.

14. The 300-350 µm and 350-425 µm particle size of the WB are the optimum for *T. harzianum* PPDDN-10 NFCCI-2925 and *C. cinerea* PPHRI-4 NFCCI-3027, respectively but the whole WB (*i.e.* unscreened) can be used to produce comparable amount of the enzyme.

15. Most of the vitamins and amino acids have significant stimulatory effect on enzyme production but these may not be feasible economically.

16. Mn^{+2}, Fe^{+3}, K^{+} and Na^{+} have shown the stimulatory effect on the cellulase and xylanase production, while heavy metals were found to reduce the enzyme production in both the fungi.

17. The crude enzyme of *T. harzianum* PPDDN-10 NFCCI-2925 and *C. cinerea* PPHRI-4 NFCCI-3027 clearly have good activitities (upto 50% residual activity) in a wide range of pH 3.5-8.5 (optimum 5.5-6.0), temperature range of 30-65 °C (optimum at 55-60 °C).

18. After optimization (excluding additives), the production of the endoglucanase, total cellulase and xylanase enzyme is enhanced by 3.2, 1.9 and 3.2 folds, respectively for *T. harzianum* PPDDN-10 NFCCI 2925. While the same enhanced by 3.5, 2.4 and 3.0 folds for *C. cinerea* PPHRI-4 NFCCI 3027.

5.1.2. Deinking of photocopier waste papers

The crude enzymes, studied in present case, are found to have potential towards the efficient deinking of photocopier waste papers (objective 3 to 5) as concluded below:

1. Chemical deinking is not effective to remove the toner ink from the photocopier waste papers efficiently. Under optimum conditions, only 75.9±1.0% deinking efficiency was achieved using 2% NaOH, 2% Na_2SiO_3, 1% H_2O_2, 0.8% oleic acid, 0.8% DTPA for 30 min hydrapulping at 70 °C followed by 10 min flotation at 40±2 °C.

2. The optimum pH and temperature of these crude enzymes is suitable for deinking photocopier waste papers. The optimum pH of enzyme activities in acidic range has helped to eliminate the alkaline deinking. The stability of crude enzymes has been found

good (upto 50% residual activity) at the optimized pH and temperature even after 6 h *i.e.* more than reaction time for deinking process.

3. The enzymes should be added preferably during the hydrapulping stage. Post hydrapulping addition had comparable results for the deinking with the crude enzymes of *C. cinerea* PPHRI-4 NFCCI 3027. Pre-hydrapulping addition of enzymes is not suitable for effective ink removal.
4. The enzymes used for the deinking should be used at optimized dose. Different optimum doses have been applied according to enzyme type, composition and type of ink and waste papers. All the three enzymes used in this study had different compositions and had different doses i.e. 6.0, 0.8 and 0.6 IU/ g o.d. weight of pulp for commercial cellulase and crude enzyme of *T. harzianum* PPDDN-10 NFCCI 2925 and *C. cinerea* PPHRI-4 NFCCI 3027, respectively.
5. Optimum doses of enzyme primarily act on the fines and microfibrills but they also facilitate fibrillation on the surface. But at high doses, the enzymes have the capability to deteriorate the strength properties due to more dissolution of fine and removal of microfibrills. Enzyme dose higher than the optimum dose have shown detrimental effect to the fibres, and resulted in fibre breakage, removal of most of the microfibrils and more dissolution of fines.
6. 10-12% pulp consistency is the best for enzymatic treatment and it will be advantageous for industrial applications.
7. Reaction time of 60 (for crude enzymes of *T. harzianum* PPDDN-10 NFCCI 2925 and *C. cinerea* PPHRI-4 NFCCI 3027) to 90 minutes (for commercial cellulase) is the optimum to obtain maximum DE. Beyond these optimized reaction times, even the optimized doses are responsible for the reduced strength properties.
8. The crude enzyme preparations and commercial cellulase have shown to remove 93.9 to 95.2% toner from photocopier waste papers, which is 23.6 to 25.4% higher than the conventional chemical deinking. This efficiency can be slightly improved but at the cost of strength properties.
9. Under optimized conditions, crude enzymes had shown the better strength properties than chemicals due to selective action. Crude enzymes have the capability to facilitate fibrillation on the fibre surface and to preserve fibre integrity. The tear index is reduced in all the enzymatic treatments due to better inter fibre hydrogen bonding.
10. Enzymatic treatment with the optimum enzyme dose for optimum reaction time is responsible for surface as well as possible internal fibrillation of the fiber, which is resulted into increased strength of the handsheets (SEM analysis).
11. Crude enzymes having cellulase with xylanase have positive effect towards the brightness improvement, which is not achieved during commercial cellulase, based deinking.

12. Enzymatic treatment is resulted into higher fibrillation than chemical treatment, which exposes more hydroxyl groups on the microfibrills. These groups facilitate the H-bonding to provide the strength to the handsheets (FTIR analysis).
13. Crude cellulase and xylanase preparations are responsible for higher crystalline index than the chemical deinking because cellulase and xylanase act on the amorphous region of the fibres, which improves the relative proportion of crystalline regions (XRD analysis).
14. Enzymatic treatments improve the freeness with the reduction in the drainage time due to the dissolution of fines (Bauer Mc-Nett classification). The improved freeness may be helpful to make paper on higher speed machines.
15. Dirt count analysis shows that enzymatic deinked pulp has better cleanliness over chemical treated pulp.
16. Enzymatic approach has the potential to reduce the pollution load in comparison to conventional chemical deinking.

Therefore, conventional chemicals can be replaced with the crude cellulase and xylanase enzyme of *T. harzianum* PPDDN-10 NFCCI 2925 and *C. cinerea* PPHRI-4 NFCCI 3027 to deink the photocopier waste papers. These crude enzyme preparations will be better option for the commercial cellulase due to higher brightness.

5.2. Recommendations for future work

Nature of this work is multidisciplinary type and involves different areas like fermentation technology, enzyme technology, and paper technology (waste paper recycling). It is impossible to work in all the possible aspects of these areas in a single research study either due to the shortage of time or due to lack of the required facilities. The research that has been undertaken for this thesis has highlighted a number of topics on which further research would be beneficial. These areas have been identified as recommendations for further work, which have been listed as follows:

1. In the present study, photocopier waste paper was used for the deinking experiments, which is one of the sources of mixed office waste papers. The other sources are the laser printed papers and coated papers. It is recommended to evaluate the performance of these enzymes with the combination of both types of papers as well as with the other conventional sources like newspapers and old magazines.
2. A study on the immobilization of enzymes for the deinking with the enzymes is also recommended to reuse the enzymes.
3. Stickies are the major problems associated with the recycling of waste papers, thus, a study on the stickies control is also recommended to explore the effectiveness of the enzymes over conventional methods.

4. Multivariate analysis can also be done for the optimization of the different parameters for enzyme production and deinking experiments to see the combined effect.
5. It is recommended to study the large-scale production of these enzymes in the fermentor and purification of the crude enzyme to explore these enzymes for other application like bio-refining (cellulase), bio-bleaching (xylanase) etc.

TABLES

Table-3.1 Primary and secondary screening of cellulase and xylanase producers isolated from different sources

	S. No.	Code No.	Location	CMC (IU/ml)	Xylan (IU/ml)	S. No.	Code No.	Location	CMC (IU/ml)	Xylan (IU/ml)
Wood sample	1	PPSRE1	Saharanpur	+ (0.25)	+(65.2)	16	PPNTL3	Nainital	+(1.19)	+(211.2)
	2	PPSRE2	Saharanpur	-	+(52.9)	17	PPCDG1	Chandigarh	+(0.95)	+(95.3)
	3	PPSRE3	Saharanpur	+(1.01)	+(98.2)	18	PPCDG2	Chandigarh	-	-
	4	PPSRE4	Saharanpur	-	-	19	PPCDG3	Chandigarh	+(0.65)	+(102.3)
	5	PPDDN1	Dehradun	+(1.15)	+(185.5)	20	PPHRI1	Hardoi	+(0.89)	+(305.2)
	6	PPDDN2	Dehradun	+(1.35)	-	21	PPHRI2	Hardoi	-	-
	7	PPDDN3	Dehradun	-	-	22	PPHRI3	Hardoi	+(1.35)	+(195.2)
	8	PPDDN4	Dehradun	+(1.23)	+(255.3)	23	**PPHRI4**	**Hardoi**	**+(1.82)**	**+(298.2)**
	9	PPDDN5	Dehradun	+(0.26)	+(112.3)	24	PPBKSC1	Bokaro	-	+(22.0)
	10	PPHW1	Haridwar	-	-	25	PPBKSC2	Bokaro	+(0.42)	+(151.2)
	11	PPHW2	Haridwar	+(0.22)	+(233.2)	26	PPBKSC3	Bokaro	-	-
	12	PPHW3	Haridwar	-	-	27	PPJP1	Jaipur	+(0.38)	+(11.2)
	13	PPHW4	Haridwar	+(1.36)	+(252.3)	28	PPJP2	Jaipur	-	-
	14	PPNTL1	Nainital	+(0.31)	+(123.2)	29	PPJP3	Jaipur	+(0.88)	+(156.3)
	15	PPNTL2	Nainital	-	-	30	PPJP4	Jaipur	-	+(23.5)
Humus sample	31	PPSRE5	Saharanpur	+(1.52)	+(112.3)	55	PPNTL4	Nainital	-	-
	32	PPSRE6	Saharanpur	-	-	56	PPNTL5	Nainital	+(0.39)	+(112.2)
	33	PPSRE7	Saharanpur	-	-	57	PPNTL6	Nainital	-	-
	34	PPSRE8	Saharanpur	+(1.23)	+(23.2)	58	PPCDG4	Chandigarh	+(0.98)	+(112.3)
	35	PPSRE9	Saharanpur	+(1.54)	+(11.2)	59	PPCDG5	Chandigarh	+(1.53)	+(22.3)
	36	PPSRE10	Saharanpur	-	-	60	PPCDG6	Chandigarh	-	-
	37	PPSRE11	Saharanpur	+(0.25)	-	61	PPCDG7	Chandigarh	-	-
	38	PPSRE12	Saharanpur	-	-	62	PPCDG8	Chandigarh	-	-
	39	PPSRE13	Saharanpur	+(0.95)	+(112.3)	63	PPHRI5	Hardoi	+(1.18)	+(32.3)
	40	PPDDN6	Dehradun	-	-	64	PPHRI6	Hardoi	-	-
	41	PPDDN7	Dehradun	+(1.22)	+(285.2)	65	PPHRI7	Hardoi	+(1.28)	+(232.3)
	42	PPDDN8	Dehradun	+(1.23)	+(21.3)	66	PPHRI8	Hardoi	+(1.12)	+(11.3)
	43	PPDDN9	Dehradun			67	PPHRI9	Hardoi	+(0.22)	-
	44	**PPDDN10**	**Dehradun**	**+(1.62)**	**+(112.3)**	68	PPHRI10	Hardoi	-	-
	45	PPDDN11	Dehradun	-	-	69	PPHRI11	Hardoi	+(0.52)	+(125.3)
	46	PPDDN12	Dehradun	+(1.45)	-	70	PPBKSC4	Bokaro	-	-
	47	PPDDN13	Dehradun	-	-	71	PPBKSC5	Bokaro	+(0.65)	+(108.2)
	48	PPDDN14	Dehradun	+(0.38)	+(122.3)	72	PPBKSC6	Bokaro	-	-
	49	PPDDN15	Dehradun	-	-	73	PPBKSC7	Bokaro	+(0.74)	+(115.3)
	50	PPHW5	Haridwar	+(0.68)	+(213.2)	74	PPJP5	Jaipur	-	-
	51	PPHW6	Haridwar	+(0.98)	-	75	PPJP6	Jaipur	+(0.52)	+(123.2)
	52	PPHW7	Haridwar	+(0.38)	+(102.3)	76	PPJP7	Jaipur	-	-
	53	PPHW8	Haridwar	+(0.26)	+(85.5)	77	PPJP8	Jaipur	+(1.42)	+(156.2)
	54	PPHW9	Haridwar	-	-	78	PPJP9	Jaipur	-	-
Soil sample	79	PPSRE14	Saharanpur	+(1.23)	+(23.2)	92	PPNTL8	Nainital	+(1.32)	+(112.3)
	80	PPSRE15	Saharanpur	-	-	93	PPNTL9	Nainital	-	-
	81	PPSRE16	Saharanpur	+(0.95)	+(45.8)	94	PPCDG9	Chandigarh	+(0.23)	+(102.3)
	82	PPDDN16	Dehradun	-	-	95	PPCDG10	Chandigarh	-	-
	83	PPDDN17	Dehradun	+(0.25)	+(118.6)	96	PPCDG11	Chandigarh	+(0.32)	+(65.2)
	84	PPDDN18	Dehradun	+(0.35)	-	97	PPHRI12	Hardoi	-	-
	85	PPDDN19	Dehradun	+(0.98)	+(158.3)	98	PPHRI13	Hardoi	+(0.85)	+(112.1)
	86	PPDDN20	Dehradun	-	-	99	PPHRI14	Hardoi	-	-
	87	PPHW10	Haridwar	+(0.84)	+(12.3)	100	PPHRI15	Hardoi	+(0.42)	+(52.3)
	88	PPHW11	Haridwar	-	-	101	PPBKSC8	Bokaro	+(0.65)	+(38.9)
	89	PPHW12	Haridwar	-	-	102	PPBKSC9	Bokaro	+(0.85)	+(65.5)
	90	PPHW13	Haridwar	+(1.19)	+(22.3)	103	PPJP10	Jaipur	+(0.45)	+(101.2)
	91	PPNTL7	Nainital	-	-	104	PPJP11	Jaipur	-	-

+ sign showed 'producer strains' and – sign showed 'non-producer strains'
The values in brackets show the CMCase and xylanase activities

Table-3.2 Effect of different culture media on the growth of the *T. harzianum* PPDDN-10 NFCCI-2925 and *C. cinerea* PPHRI-4 NFCCI-3027

Name of the media	***T. harzianum* PPDDN-10 NFCCI-2925**		***C. cinerea* PPHRI-4 NFCCI-3027**	
	Growth characteristic after full growth	**Growth in mm as on the 6th day**	**Growth characteristic after full growth**	**Growth in mm as on the 6th day**
Yeast extract agar (YEA)	Moderate mycelial growth, spore in the centre	35.4 (0.5)	Growth in the centre but poor mycelial growth towards periphery	21.4 (1.1)
Czapek dox agar (CDA)	Full mycelial growth, spore formation in centre	85.4 (1.1)	Growth in the centre but poor mycelial growth towards periphery	33.2 (0.8)
Sabouraud dextrose agar (SDA)	Moderate growth, wavy and light brownish color after 7 days	72.8 (0.8)	Circular, poor mycelial growth	50.8 (0.9)
Malt extract agar (MEA)	Less mycelia , circular, green spore in the centre	79.2 (1.3)	Circular cottony growth	61.8 (1.2)
Xylan agar (XA)	Wavy, Less mycelial growth towards periphery	36.0 (1.2)	Sparse and irregular	31.4 (0.6)
Potato dextrose agar (PDA)	Dark green color in the centre, light color towards periphery	78.2 (0.8)	Circular, granular and diffused mycelial growth	61.4 (1.8)
CMC agar (CMCA)	Less mycelial growth	31.4 (0.5)	Poor mycelial growth	37.1 (2.4)
Wheat bran agar (WBA)	Light green spore formation in the centre	72.8 (0.8)	Circular, cottony mycelial growth	62.8 (0.6)
pH=6.0, Temperaure= 34°C, 90 mm Petriplate				

Digits in parenthesis indicate the standard deviation at 95% confidence limit.

Table-3.3 Effect of volume of extraction buffer on the enzyme extraction efficiency and enzyme titer of *T. harzianum* PPDDN-10 NFCCI 2925

	Volume of buffer used, ml x No. of extraction steps		Enzyme production			Enzyme activities		
			CMCase, IU/gds	FPase, FPU/gds	Xylanase, IU/gds	CMCase, IU/ml	FPase, FPU/ml	Xylanase, IU/ml
Single step extraction	**15 x 1**		8.34 (0.35)	2.34 (0.11)	610.3 (21.2)	1.67 (0.05)	0.47 (0.04)	122.0 (5.5)
	30 x 1		10.32 (0.41)	2.95 (0.13)	780.7 (28.3)	1.29 (0.04)	0.37 (0.03)	97.5 (6.2)
	45 x 1		11.35 (0.45)	3.25 (0.09)	899.2 (35.3)	1.03 (0.05)	0.30 (0.03)	81.7 (4.2)
Three step extraction	**15 x 3**	**15 ml (Ist)**	8.44 (0.21)	2.34 (0.11)	610.3 (21.3)	1.67 (0.04)	0.47 (0.04)	122.1 (5.4)
		15 ml (IInd)	3.98 (0.19)	1.12 (0.06)	369.4 (19.5)	1.33 (0.04)	0.37 (0.03)	123.1 (4.6)
		15 ml (IIIrd)	1.86 (0.06)	0.66 (0.05)	150.7 (9.2)	0.62 (0.03)	0.22 (0.03)	50.0 (3.0)
		Total	14.68 (0.49)	4.20 (0.25)	1138.7 (55.2)	1.31 (0.09)	0.39 (0.02)	100.7 (2.4)
% increase/ decrease	**(15 x3) *Vs* (45x1)**		+29.3	+29.2	+26.6	+27.2	+30.0	+23.2
	(15 x3) *Vs* (15x 1)		+76.0	+79.5	+86.6	-21.6	-17.0	-17.5

Table-3.4 Effect of volume of extraction buffer on the enzyme extraction efficiency and enzyme titer of *C. cinerea* PPHRI-4 NFCCI-3027

	Volume of buffer used, ml x No. of extraction steps		Enzyme production			Enzyme activities		
			CMCase, IU/gds)	FPase, FPU/gds	Xylanase, IU/gds	CMCase, IU/ml	FPase, FPU/ml	Xylanase, IU/ml
Single step extraction	**15 x 1**		7.52 (0.31)	1.96 (0.10)	1320.8 (56.8)	1.88 (0.07)	0.49 (0.04)	330.2 (15.6)
	30 x 1		11.06 (0.36)	2.73 (0.11)	1723.5 (76.4)	1.58 (0.06)	0.39 (0.05)	250.5 (12.2)
	45 x 1		11.30 (0.42)	2.90 (0.15)	1927.0 (83.8)	1.13 (0.04)	0.29 (0.04)	192.7 (10.2)
Three step extraction	**15 x 3**	**15 ml (Ist)**	7.52 (0.29)	1.96 (0.10)	1320.4 (21.3)	1.88 (0.08)	0.49 (0.04)	330.1 (13.5)
		15 ml (IInd)	4.47 (0.22)	0.96 (0.05)	812.4 (19.5)	1.49 (0.07)	0.32 (0.03)	270.8 (10.4)
		15 ml (IIIrd)	2.10 (0.09)	0.57 (0.04)	316.2 (9.2)	0.70 (0.04)	0.19 (0.04)	105.4 (5.0)
		Total	14.15 (0.49)	3.51 (0.16)	2468.0 (55.2)	1.42 (0.08)	0.36 (0.03)	256.0 (13.5)
% Increase/ decrease	**(15 x3) *Vs* (45x1)**		+25.2	+21.0	+28.1	+25.7	+24.1	+32.8
	(15 x3) *Vs* (15x 1)		+88.2	+79.1	+86.9	-24.5	-26.5	-22.4

Digits in parenthesis indicate the standard deviation at 95% confidence limit.

Table-3.5 Effect of fermentation type on the cellulase and xylanase production by *T. harzianum* PPDDN-10 NFCCI-2925 and *C. cinerea* PPHRI-4 NFCCI-3027

State of Fermentation	***T. harzianum* PPDDN-10 NFCCI-2925**				***C. cinerea* PPHRI-4 NFCCI-3027**			
	Endoglucanase, IU/gds	**Total cellulase, FPU/gds**	**Xylanase, IU/gds**	**Soluble protein, mg/gds**	**Endoglucanase, IU/gds**	**Total cellulase, FPU/gds**	**Xylanase, IU/gds**	**Soluble protein, mg/gds**
Solid static SSF(S)	14.12 (0.54)	4.10 (0.25)	1102.3 (63.5)	17.49 (0.64)	14.20 (0.65)	3.70 (0.19)	2753.0 (126.0)	23.81 (0.85)
Solid shaking SSF(R)	14.80 (0.69)	3.96 (0.28)	1121.9 (64.1)	18.92 (0.70)	13.50 (0.54)	3.30 (0.17)	2854.00 (114.2)	25.78 (0.95)
Semisolid static SmSF(S)	10.14 (0.32)	1.31 (0.14)	660.2 (39.1)	17.67 (0.75)	6.86 (0.31)	1.75 (0.09)	1262.10 (57.0)	21.65 (0.84)
Semisolid shaking SmSF(R)	12.66 (0.54)	1.42 (0.16)	622.4 (18.8)	18.03 (0.79)	7.84 (0.38)	2.03 (0.10)	1510.60 (73.2)	22.98 (0.79)
Submerged static SmF(S)	11.14 (0.52)	0.89 (0.18)	396.1 (19.3)	10.53 (0.20)	4.50 (0.25)	1.50 (0.08)	953.00 (52.9)	12.32 (0.57)
Submerged shaking SmF(R)	12.73 (0.58)	0.82 (0.18)	443.3 (20.8)	12.14 (0.23)	6.10 (0.29)	1.90 (0.10)	1023.00 (49.6)	14.25 (0.61)

Table-3.6 Effect of different LCWs on the cellulase and xylanase production by *T. harzianum* PPDDN-10 NFCCI-2925 and *C. cinerea* PPHRI-4 NFCCI-3027

LCW	***T. harzianum* PPDDN-10 NFCCI-2925**					***C. cinerea* PPHRI-4 NFCCI-3027**				
	Endoglucanase, IU/gds	**Total cellulase, FPU/gds**	**Xylanase, IU/gds**	**Soluble protein, mg/gds**	**N- acetyl glucosamine, mg/gds**	**Endoglucanase, IU/gds**	**Total cellulase, FPU/gds**	**Xylanase, IU/gds**	**Soluble protein, mg/gds**	**N- acetyl glucosamine, mg/gds**
Corncob (CC)	3.96 (0.14)	1.58 (0.23)	408.5 (30.9)	7.68 (0.43)	78.32 (2.21)	4.18 (0.19)	1.03 (0.05)	696.7 (31.7)	11.39 (0.55)	90.2 (4.5)
Groundnut shell (GS)	5.27 (0.20)	0.84 (0.21)	365.5 (29.9)	1.79 (0.20)	32.21 (1.20)	5.30 (0.28)	1.15 (0.06)	465.1 (24.6)	4.08 (0.24)	101.2 (5.7)
Coconut coir (COCO)	3.62 (0.12)	0.74 (0.26)	199.9 (21.8)	5.00 (0.36)	22.35 (0.90)	4.28 (0.20)	1.26 (0.06)	161.2 (7.5)	8.10 (0.44)	94.0 (3.9)
Rice husk (RH)	7.28 (0.25)	1.53 (0.19)	905.8 (39.2)	13.74 (0.61)	65.36 (3.00)	8.36 (0.38)	2.45 (0.11)	1035.3 (47.0)	19.24 (0.77)	150.2 (6.42)
Rice straw (RS)	7.23 (0.27)	4.28 (0.33)	768.3 (45.3)	18.21 (0.54)	92.38 (2.15)	9.69 (0.41)	2.86 (0.12)	1890.3 (80.0)	21.73 (0.44)	169.2 (7.25)
Wheat bran (WB)	14.58 (0.42)	4.61 (0.54)	1023.8 (67.1)	18.81 (0.85)	90.24 (1.95)	14.08 (0.31)	3.98 (0.17)	2858.0 (123.9)	24.76 (0.67)	225.1 (8.69)
Wheat straw (WS)	7.11 (0.24)	4.03 (0.30)	1095.3 (21.4)	14.10 (0.79)	86.54 (2.05)	11.42 (0.45)	3.26 (0.13)	1379.0 (54.3)	27.14 (1.20)	185.3 (9.0)
Wood saw dust (WSD)	5.03 (0.19)	0.74 (0.02)	634.5 (26.9)	2.50 (0.27)	36.32 (1.42)	6.63 (0.32)	0.94 (0.05)	1077.1 (52.0)	19.79 (0.98)	135.2 (5.8)
Sugarcane bagasse (SCB)	8.53 (0.19)	1.74 (0.19)	363.3 (37.9)	9.28 (0.45)	110.32 (4.20)	5.20 (0.21)	1.43 (0.06)	474.3 (19.2)	13.30 (0.56)	250.3 (11.2)

Digits in parenthesis indicate the standard deviation at 95% confidence limit.

Table-3.7 Effect of combination of different LCWs on the cellulase and xylanase production by *T. harzianum* PPDDN-10 NFCCI-2925 and *C. cinerea* PPHRI-4 NFCCI-3027

***T. harzianum* PPDDN-10 NFCCI-2925**						***C. cinerea* PPHRI-4 NFCCI-3027**					
LCW (Ratio)	**Endoglucanase, IU/gds**	**Total cellulase, FPU/gds**	**Xylanase, IU/gds**	**Soluble protein, mg/gds**	**N- acetyl glucosamine, mg/gds**	**LCW (Ratio)**	**Endoglucanase, IU/gds**	**Total cellulase, FPU/gds**	**Xylanase, IU/gds**	**Soluble protein, mg/gds**	**N- acetyl glucosamine, mg/gds**
WB	14.36 (0.34)	4.65 (0.23)	1031.5 (52.0)	19.01 (0.65)	89.32 (2.65)	**WB**	14.28 (0.45)	3.98 (0.13)	2708.1 (85.3)	25.14 (11.9)	221.3 (8.5)
WB+RS (3:1)	12.70 (0.41)	4.26 (0.18)	862.2 (37.9)	14.28 (0.55)	91.25 (3.23)	**WB+WS (3:1)**	13.36 (0.51)	3.88 (0.15)	2396.0 (91.5)	19.86 (0.70)	213.2 (6.9)
WB+RS (1:1)	11.09 (0.32)	2.03 (0.09)	495.7 (32.1)	10.53 (0.39)	88.35 (2.74)	**WB+WS (1:1)**	12.04 (0.45)	3.67 (0.14)	2064.5 (77.2)	14.98 (0.49)	205.3 (9.3)
WB+RS (1:3)	10.62 (0.29)	2.14 (0.09)	191.2 (14.2)	5.89 (0.20)	85.32 (3.14)	**WB+WS (1:3)**	11.93 (0.51)	3.47 (0.15)	1804.4 (77.1)	9.00 (0.25)	198.2 (7.2)
WB+SCB (3:1)	11.17 (0.42)	4.98 (0.18)	809.1 (33.6)	17.49 (0.86)	97.32 (2.41)	**WB+RS (3:1)**	12.75 (0.47)	3.67 (0.14)	2616.3 (96.4)	23.81 (1.07)	210.3 (8.4)
WB+SCB (1:1)	10.29 (0.34)	3.77 (0.18)	521.5 (26.9)	15.17 (0.82)	99.23 (3.44)	**WB+RS (1:1)**	11.42 (0.48)	3.37 (0.14)	2452.1 (103.0)	20.98 (1.04)	198.2 (7.7)
WB+SCB (1:3)	8.85 (0.32)	1.86 (0.09)	112.8 (9.1)	11.42 (0.64)	104.65 (3.21)	**WB+RS (1:3)**	11.22 (0.41)	3.16 (0.12)	1911.5 (69.9)	16.34 (0.81)	185.3 (6.9)
WB+WS (3:1)	12.07 (0.49)	4.45 (0.19)	877.0 (33.6)	18.21 (0.77)	82.65 (2.49)	**WB+RH (3:1)**	13.46 (0.39)	3.47 (0.10)	2301.1 (66.7)	24.8 7 (0.96)	210.2 (9.1)
WB+WS (1:1)	11.29 (0.37)	3.42 (0.09)	492.5 (24.7)	15.89 (0.79)	87.55 (2.45)	**WB+RH (1:1)**	11.42 (0.54)	3.16 (0.15)	1916.6 (90.6)	21.95 (0.99)	199.2 (10.2)
WB+WS (1:3)	10.17 (0.29)	1.28 (0.09)	330.1 (21.6)	11.78 (0.39)	87.52 (3.23)	**WB+RH (1:3)**	10.40 (0.32)	3.06 (0.09)	1712.6 (52.7)	16.55 (0.49)	173.20 (6.9)

Table-3.8 Effect of inoculum size on the cellulase and xylanase production by *T. harzianum* PPDDN-10 NFCCI-2925 and *C. cinerea* PPHRI-4 NFCCI-3027

***T. harzianum* PPDDN-10 NFCCI-2925**					***C. cinerea* PPHRI-4 NFCCI-3027**				
Inoculum size (CFU/gds)	**Endoglucanas, IU/gds**	**Total cellulase, FPU/gds**	**Xylanase, IU/gds**	**Soluble protein, mg/gds**	**Inoculum size (Discs/5g WB)**	**Endoglucanase, IU/gds**	**Total cellulase, FPU/gds**	**Xylanase, IU/gds**	**Soluble protein, mg/gds**
$2x10^6$	9.72 (0..371)	1.35 (0.09)	232.34 (12.7)	1.86 (0.20)	**2**	7.96 (0.32)	2.14 (0.09)	1138.3 (45.8)	8.21 (0.23)
$4x10^6$	14.10 (3.72)	3.72 (0.14)	1446.60 (54.6)	15.94 (0.98)	**4**	13.77 (0.52)	3.47 (0.13)	2498.0 (94.3)	21.94 (1.09)
$6x10^6$	14.71 (0.68)	5.01 (0.12)	1554.18 (71.2)	18.33 (0.14)	**6**	17.03 (0.71)	4.59 (0.19)	3171.2 (132.2)	24.95 (1.15)
$8x10^6$	16.24 (0.59)	5.29 (0.19)	1561.61 (26.9)	18.42 (0.91)	**8**	15.81 (0.51)	3.77 (0.12)	3525.1 (113.7)	19.77 (1.03)
$1x10^7$	11.78 (0.41)	3.12 (0.09)	650.28 (26.1)	11.28 ()0.66	**10**	12.55 (0.61)	3.16 (0.15)	3172.2 (154.2)	15.98 (0.73)

Digits in parenthesis indicate the standard deviation at 95% confidence limit.

Table-3.9 Effect of incubation days on the cellulase and xylanase production by *T. harzianum* PPDDN-10 NFCCI-2925 and *C. cinerea* PPHRI-4 NFCCI-3027

***T. harzianum* PPDDN-10 NFCCI-2925**						***C. cinerea* PPHRI-4 NFCCI-3027**					
Days	**Endoglucanase, IU/gds**	**Total cellulase, FPU/gds**	**Xylanase, IU/gds**	**Soluble protein, mg/gds**	**N- acetyl glucosamine, mg/gds**	**Days**	**Endoglucanase, IU/gds**	**Total cellulase, FPU/gds**	**Xylanase, IU/gds**	**Soluble protein, mg/gds**	**N- acetyl glucosamine, mg/gds**
1	0.20 (0.02)	0.11 (0.02)	55.1 (8.4)	6.03 (0.25)	21.32 (0.45)	**1**	0.10 (0.01)	0.00 (0.00)	23.5 (2.30)	1.41 (0.01)	8.23 (0.32)
2	9.87 (0.36)	4.72 (0.12)	537.1 (35.7)	12.51 (0.37)	62.23 (1.85)	**2**	0.51 (0.08)	0.10 (0.02)	49.0 (7.7)	1.64 (0.12)	12.20 (0.45)
3	13.49 (0.49)	6.24 (0.17)	645.9 (54.7)	22.42 (0.12)	78.24 (1.98)	**3**	1.02 (0.09)	0.20 (0.02)	431.5 (38.1)	2.08 (0.13)	19.35 (0.48)
4	17.84 (0.61)	6.22 (0.11)	1114.9 (9534)	25.45 (0.25)	87.65 (2.01)	**4**	3.57 (0.17)	0.31 (0.01)	1003.7 ()	5.32 (0.25)	52.32 (1.25)
5	14.53 (0.42)	4.65 (0.13)	1298.8 (84.0)	24.03 (0.48)	92.68 (1.65)	**5**	9.08 (0.31)	2.14 (0.07)	1787.0 (61.0)	9.64 (0.49)	98.32 (2.65)
6	13.24 (0.58)	3.65 (0.11)	1247.7 (72.7)	21.88 (0.70)	92.14 (1.57)	**6**	12.55 (0.42)	3.26 (0.11)	2501.0 (83.7)	15.04 (0.70)	185.32 (8.23)
7	13.31 (0.52)	3.68 (0.16)	982.6 (76.2)	20.53 (1.05)	92.35 (1.65)	**7**	17.54 (0.65)	4.49 (0.17)	3617.9 (134.0)	23.81 (1.06)	216.35 (7.65)
8	11.66 (0.47)	2.68 (0.14)	884.4 (78.6)	19.83 (0.91)	91.58 (1.56)	**8**	20.20 (0.95)	5.20 (0.24)	4438.0 (208)	26.20 (0.92)	252.32 (10.32)
9	9.89 (0.41)	1.31 (0.21)	786.1 (62.4)	20.17 (1.18)	91.35 (1.24)	**9**	19.89 (0.99)	5.10 (.25)	4398.2 (218.9)	30.56 (30.56)	260.65 (10.52)
10	11.95 (0.46)	2.91 (0.09)	697.7 (46.7)	17.60 (1.05)	91.08 (1.58)	**10**	20.91 (0.92)	5.61 (0.25)	4278.9 (188.3)	31.60 (1.07)	261.01 (8.65)
11	11.99 (0.51)	3.26 (0.09)	673.2 (55.5)	15.07 (1.32)	90.98 (1.64)	**11**	19.58 (0.89)	4.28 (0.19)	3933.1 (178.7)	31.47 (1.33)	260.50 (9.84)
12	9.92 (0.37)	1.63 (0.16)	739.6 (66.1)	13.21 (1.41)	89.32 (1.46)	**12**	18.16 (0.80)	3.47 (0.15)	3697.5 (162.9)	32.49 (1.43)	258.35 (10.12)
13	7.99 (0.36)	0.39 (0.14)	212.4 (28.2)	11.30 (1.29)	89.24 (1.25)	**13**	15.50 (0.69)	3.16 (0.14)	2840.7 (126.4)	26.84 (1.30)	255.32 (8.52)
14	7.72 (0.39)	0.16 (0.21)	175.6 (47.1)	11.62 (1.18)	88.24 (1.54)	**14**	13.06 (0.62)	2.96 (0.14)	2909.0 (138.1)	24.85 (1.19)	250.32 (7.65)
15	6.21 (0.19)	0.07 (0.09)	169.0 (19.0)	9.16 (0.86)	84.22 (1.27)	**15**	14.08 ()0.71	3.06 (0.15)	3266.0 (164.7)	18.87 (0.86)	252.10 (8.33)
16	4.38 (0.20)	0.04 (0.04)	76.6 (9.9)	8.16 (0.61)	83.76 (1.26)	**16**	15.20 (0.65)	3.26 (0.14)	2655.1 (113.6)	19.86 (0.60)	248.20 (6.32)
						18	15.71 (0.71)	3.47 (0.16)	2022.7 (91.4)	19.99 (0.70)	245.84 (4.01)
						20	12.85 (0.63)	2.45 (0.12)	1430.0 (70.1)	15.21 (0.70)	240.85 (8.65)
						22	10.40 (0.50)	1.94 (0.09)	1035.3 (49.8)	12.73 (0.58)	237.50 (9.33)
						24	8.47 (0.39)	1.43 (0.07)	940.4 (43.3)	9.07 (0.38)	238.20 (7.45)
						26	4.28 (0.19)	1.02 (0.05)	431.5 (19.1)	7.24 (0.26)	235.32 (8.13)
						28	2.04 (0.09)	0.31 (0.01)	328.4 (14.5)	3.42 (0.25)	230.10 (5.68)
						30	1.02 (0.04)	0.10 (0.01)	187.7 (7.4)	4.30 (0.27)	228.35 (8.64)

Digits in parenthesis indicate the standard deviation at 95% confidence limit.

Table-3.10 Effect of incubation temperature on the cellulase and xylanase production by *T. harzianum* PPDDN-10 NFCCI-2925 and *C. cinerea* PPHRI-4 NFCCI-3027

Incubation temperature (°C)	***T. harzianum* PPDDN-10 NFCCI-2925**				***C. cinerea* PPHRI-4 NFCCI-3027**			
	Endoglucanase, IU/gds	**Total cellulase, FPU/gds**	**Xylanase, IU/gds**	**Soluble protein, mg/gds**	**Endoglucanase, IU/gds**	**Total cellulase, FPU/gds**	**Xylanase, IU/gds**	**Soluble protein, mg/gds**
26	11.53 (0.53)	3.45 (0.11)	255.6 (16.9)	11.76 (0.61)	16.83 (0.68)	4.28 (0.17)	4061.6 (164.1)	14.46 (0.60)
30	18.00 (0.84)	6.01 (0.18)	631.4 (35.7)	21.03 (1.02)	19.89 (0.91)	5.10 (0.23)	4439.0 (203.1)	22.77 (1.03)
34	20.06 (0.86)	5.56 (0.18)	1208.6 (54.3)	26.19 (1.20)	21.62 (0.89)	5.61 (0.23)	4796.6 (197.4)	31.28 (1.21)
38	13.00 (0.63)	3.61 (0.09)	737.2 (39.1)	20.19 (1.30)	12.24 (0.58)	3.26 (0.15)	4653.2 (220.5)	30.83 (1.31)
42	7.52 (0.28)	1.05 (0.02)	182.16 (15.4)	7.18 (0.23)	4.08 (0.14)	1.02 (0.04)	1043.5 (35.8)	7.43 (0.23)
46	No growth	No growth	No growth	No growth	No growth	No growth	No growth	No growth
50	No growth	No growth	No growth	No growth	No growth	No growth	No growth	No growth

Digits in parenthesis indicate the standard deviation at 95% confidence limit.

Table-3.11 Effect of initial pH on the cellulase and xylanase production by *T. harzianum* PPDDN-10 NFCCI-2925 and *C. cinerea* PPHRI-4 NFCCI-3027

Initial pH	***T. harzianum* PPDDN-10 NFCCI-2925**				***C. cinerea* PPHRI-4 NFCCI-3027**			
	Endoglucanase, IU/gds	**Total cellulase, FPU/gds**	**Xylanase, IU/gds**	**Soluble protein, mg/gds**	**Endoglucanase, IU/gds**	**Total cellulase, FPU/gds**	**Xylanase, IU/gds**	**Soluble protein, mg/gds**
3.0	7.13 (0.33)	3.68 (0.18)	498.3 (39.1)	11.76 (0.36)	9.28 (0.41)	1.22 (0.05)	867.2 (38.3)	6.35 (0.19)
3.5	10.11 (0.48)	5.07 (0.19)	642.7 (39.6)	12.96 (0.37)	13.35 (0.56)	1.94 (0.08)	1527.4 (64.1)	8.14 (0.45)
4.0	12.04 (0.56)	5.91 (0.11)	682.9 (55.1)	17.60 (0.62)	15.94 (0.12)	2.65 (0.02)	2073.2 (15.6)	11.92 (0.56)
4.5	13.71 (0.63)	6.00 (0.16)	855.9 (65.1)	18.64 (0.55)	18.28 (0.84)	3.26 (0.15)	2556.4 (117.9)	15.99 (0.69)
5.0	20.20 (0.94)	6.35 (0.21)	863.8 (47.0)	19.83 (0.52)	19.28 (0.75)	4.18 (0.16)	3799.0 (147.8)	26.45 (1.03)
5.5	23.41 (0.53)	6.14 (0.26)	990.5 (64.5)	21.03 (0.61)	21.93 (0.99)	5.61 (0.25)	4823.7 (233.3)	33.98 (1.35)
6.0	23.52 (0.94)	6.85 (0.25)	1191.4 (73.1)	24.47 (0.71)	22.95 (1.11)	5.61 (0.29)	4946.7 (223.3)	36.12 (1.33)
6.5	21.25 (0.99)	6.75 (0.11)	1280.3 (78.4)	26.70 (0.62)	24.28 (1.20)	5.92 (0.28)	4631.7 (229.0)	33.65 (1.23)
7.0	17.49 (0.81)	6.21 (0.07)	967.9 44.6()	22.76 (0.73)	21.62 (1.01)	4.18 (0.20)	3632.9 (169.7)	25.51 (1.08)
7.5	19.05 (0.91)	7.27 (0.07)	823.5 (59.5)	17.60 (0.45)	20.50 (1.03)	3.77 (0.19)	3635.0 (182.6)	25.44 (1.13)
8.0	13.68 (0.53)	4.42 (0.18)	493.4 (28.5)	16.92 (0.37)	18.83 (0.75)	2.96 (0.12)	3097.9 (123.4)	20.64 (0.99)
8.5	8.62 (0.41)	2.28 (0.09)	340.1 (23.0)	13.83 (0.43)	11.71 (0.54)	2.24 (0.10)	1635.2 (75.4)	8.14 (0.38)
9.0	4.62 (0.20)	1.30 (0.04)	200.6 (16.1)	12.10 (0.36)	6.34 (0.31)	1.94 (0.09)	675.2 (33.0)	7.36 (0.03)
9.5	3.67 (0.10)	1.93 (0.09)	199.6 (10.5)	9.87 (0.20)	5.10 (0.24)	1.53 (0.07)	476.8 (22.4)	8.23 (0.03)
10.0	3.23 (0.23)	0.44 (0.04)	178.1 (11.8)	3.87 (0.04)	4.40 (0.21)	1.12 (0.05)	261.0 (12.5)	3.90 (0.03)
10.5	4.10 (0.23)	0.28 (0.02)	167.2 (15.5)	2.16 (0.02)	2.14 (0.10)	0.51 (0.02)	248.1 (11.6)	3.21 (0.03)
11.0	3.47 (0.13)	0.25 (0.02)	131.8 (8.0)	0.45 (0.02)	1.22 (0.05)	0.10 (0.02)	131.1 (5.4)	3.04 (0.03)

Digits in parenthesis indicate the standard deviation at 95% confidence limit.

Table-3.12 Effect of different inorganic nitrogen sources on the cellulase and xylanase production by *T. harzianum* PPDDN-10 NFCCI-2925 and *C. cinerea* PPHRI-4 NFCCI-3027

Nitrogen source	Amounts of nitrogen (M)	*T. harzianum* PPDDN-10 NFCCI-2925				*C. cinerea* PPHRI-4 NFCCI-3027			
		Endoglucanase, IU/gds	Total cellulase, FPU/gds	Xylanase, IU/gds	Soluble protein, mg/gds	Endoglucanase, IU/gds	Total cellulase, FPU/gds	Xylanase, IU/gds	Soluble protein, mg/gds
Control	-	14.28 (0.56)	2.55 (0.10)	2807.0 (110.1)	20.7 (0.98)				
Ammonium nitrate (AN)	**0.01**	13.15 (0.36)	3.91 (0.09)	887.3 (47.0)	19.49 (1.16)	16.84 (0.75)	2.14 (0.10)	2983.5 (132.9)	24.21 (0.90)
	0.02	17.23 (0.41)	7.05 (0.19)	907.9 (45.2)	23.95 (1.79)	20.62 (1.01)	3.26 (0.16)	3836.1 (187.9)	32.19 (1.38)
	0.03	13.97 (0.44)	4.66 (0.19)	1389.3 (95.2)	26.70 (1.52)	17.83 (0.81)	2.55 (0.12)	3129.8 (142.2)	25.68 (1.17)
	0.04	13.68 (0.41)	3.17 (0.12)	1286.2 (43.2)	22.58 (1.16)	14.65 (0.75)	2.04 (0.10)	2917.1 (149.4)	23.79 (0.90)
Ammonium chloride (AC)	**0.01**	13.51 (0.34)	0.21 (0.14)	797.9 (108.2)	11.94 (0.80)	17.28 (0.81)	1.94 (0.09)	2983.5 (139.8)	22.11 (0.90)
	0.02	22.43 (0.54)	7.45 (0.26)	1927.7 (84.0)	21.03 (1.80)	19.42 (0.90)	2.96 (0.14)	3914.6 (181.4)	25.68 (1.13)
	0.03	20.50 (0.44)	5.91 (0.25)	1519.0 (76.6)	17.60 (1.75)	17.38 (0.71)	2.35 (0.10)	3735.1 (152.6)	27.99 (1.12)
	0.04	16.61 (0.36)	2.38 (0.21)	1503.3 (104.5)	16.74 (0.41)	18.28 (0.84)	2.55 (0.12)	3387.4 (155.7)	18.54 (0.35)
Ammonium ferrous sulphate (AFS)	**0.01**	15.63 (0.41)	3.14 (0.05)	856.9 (43.2)	21.03 (0.41)	15.59 (0.65)	3.16 (0.13)	2658.2 (110.8)	21.17 (0.32)
	0.02	26.22 (0.81)	7.42 (0.07)	1942.4 (84.0)	30.81 (1.04)	17.98 (0.81)	3.67 (0.17)	3323.4 (149.7)	27.36 (0.72)
	0.03	23.43 (0.52)	6.33 (0.16)	1197.8 (60.4)	24.47 (0.93)	17.09 (0.75)	3.76 (0.17)	3428.3 (150.5)	28.41 (0.80)
	0.04	22.60 (0.59)	7.27 (0.09)	1699.8 (84.0)	29.10 (1.16)	16.24 (0.71)	3.16 (0.14)	3139.6 (137.8)	25.79 (0.90)
Sodium nitrate (SN)	**0.01**	11.87 (0.25)	2.01 (0.09)	616.2 (78.6)	21.18 (0.45)	15.42 (0.51)	2.52 (0.15)	2917.4 (93.1)	26.53 (0.90)
	0.02	17.85 (0.41)	6.73 (0.09)	708.5 (84.0)	31.**7** (0.73)	19.12 (0.79)	3.66 (0.18)	3340.4 (99.5)	30.82 (1.13)
	0.03	16.37 (0.36)	6.30 (0.14)	1310.7 (96.9)	29.21 (0.63)	18.59 (0.55)	4.91 (0.25)	3641.4 (114.7)	33.59 (1.17)
	0.04	11.87 (0.25)	2.01 (0.09)	616.2 (78.6)	21.18 (0.45)	13.60 (0.55)	2.73 (0.16)	2575.9 (119.5)	22.25 (0.35)
Ammonium acetate (AA)	**0.01**	10.09 (0.24)	1.47 (0.12)	498.3 (74.5)	18.01 (0.42)	15.74 (0.55)	3.80 (0.15)	2575.9 (88.3)	22.25 (0.76)
	0.02	11.04 (0.24)	2.34 (0.11)	504.2 (121.2)	19.71 (0.42)	16.71 (0.57)	4.70 (0.21)	2762.0 (143.5)	25.40 (0.32)
	0.03	11.33 (0.25)	3.66 (0.16)	663.3 (104.6)	20.23 (0.45)	16.55 (0.70)	5.95 (0.24)	3229.6 (123.9)	29.18 (0.77)
	0.04	10.22 (0.29)	1.56 (0.02)	619.1 (59.7)	18.24 (0.51)	15.22 (0.55)	2.91 (0.09)	2989.2 (70.7)	22.34 (0.62)
Urea (UA)	**0.01**	10.84 (0.30)	0.68 (0.14)	522.8 (22.7)	11.94 (0.45)	16.29 (0.64)	3.16 (0.12)	2716.1 (106.7)	22.11 (0.35)
	0.02	14.51 (0.41)	3.21 (0.12)	1214.5 (59.9)	17.60 (1.00)	18.58 (0.82)	3.98 (0.18)	3361.0 (148.4)	27.99 (0.77)
	0.03	13.51 (0.36)	2.02 (0.19)	1082.8 (78.6)	13.83 (1.75)	22.66 (0.99)	4.39 (0.19)	4209.4 (183.9)	35.55 (1.35)
	0.04	11.17 (0.30)	0.53 (0.04)	973.8 (65.6)	11.76 (0.96)	13.30 (0.61)	3.26 (0.15)	2292.3 (105.1)	17.49 (0.75)

Digits in parenthesis indicate the standard deviation at 95% confidence limit.

Table-3.13 Effect of different complex organic nitrogen sources on the cellulase and xylanase production by *T. harzianum* PPDDN-10 NFCCI-2925 and *C. cinerea* PPHRI-4 NFCCI-3027

Nitrogen source	Amounts of nitrogen source (g/l)	*T. harzianum* PPDDN-10 NFCCI-2925				*C. cinerea* PPHRI-4 NFCCI-3027			
		Endoglucanase, IU/gds	Total cellulase, FPU/gds	Xylanase, IU/gds	Soluble protein, mg/gds	Endoglucanase, IU/gds	Total cellulase, FPU/gds	Xylanase, IU/gds	Soluble protein, mg/gds
Malt extract (ME)	**1.0**	16.25 (0.63)	5.12 (0.19)	503.1 (35.7)	22.71 (0.80)	24.36 (1.11)	4.98 (0.23)	1501.5 (68.4)	27.89 (0.62)
	2.0	21.99 (0.81)	5.22 (0.21)	751.78 (36.6)	23.78 (0.84)	24.64 (1.13)	5.10 (0.23)	2312.8 (106.1)	29.10 (0.90)
	3.0	24.58 (1.04)	7.27 (0.35)	1477.9 (60.7)	30.79 (1.07)	26.95 (1.21)	5.79 (0.26)	4682.0 (210.2)	36.94 (1.04)
	4.0	14.54 (0.53)	2.45 (0.19)	788.2 (39.6)	27.04 (0.95)	26.49 (1.25)	5.92 (0.28)	2436.4 (115.0)	32.75 (1.32)
Yeast extract (YE)	**1.0**	13.43 (0.58)	5.22 (0.19)	1063.4 (47.0)	19.76 (0.77)	20.20 (1.01)	4.36 (0.22)	3324.4 (166.3)	24.59 (0.90)
	2.0	19.79 (0.76)	6.33 (0.21)	1746.5 (93.5)	21.99 (0.77)	21.42 (1.02)	4.27 (0.20)	5546.4 (264.1)	27.08 (0.62)
	3.0	22.23 (0.86)	7.70 (0.25)	1613.8 (51.6)	29.79 (1.04)	23.97 (1.04)	5.92 (0.26)	5114.9 (221.9)	35.84 (1.17)
	4.0	18.66 (0.79)	4.79 (0.21)	1610.5 (51.5)	27.77 (0.96)	24.58 (1.19)	4.91 (0.24)	5104.2 (247.1)	33.57 (1.04)
Mycological peptone (MYP)	**1.0**	16.91 (0.74)	3.94 (0.25)	1055.1 (47.1)	25.36 (0.89)	25.07 (0.92)	5.44 (0.20)	3297.4 (121.0)	30.87 (0.90)
	2.0	18.72 (0.71)	7.33 (0.28)	2137.7 (68.4)	33.00 (1.16)	32.74 (1.21)	6.63 (0.25)	6010.9 (222.1)	39.43 (1.04)
	3.0	25.80 (1.04)	7.50 (0.30)	1819.4 (58.2)	35.95 (1.25)	38.25 (1.76)	7.65 (0.35)	6841.1 (275.8)	42.73 (0.55)
	4.0	24.38 (1.07)	6.87 (0.21)	1597.2 (51.1)	34.24 (1.20)	31.75 (1.41)	6.36 (0.28)	6919.7 (307.3)	40.82 (1.04)
Beef extract (BE)	**1.0**	19.86 (0.79)	5.63 (0.19)	1537.5 (49.2)	27.15 (0.95)	26.22 (0.98)	4.84 (0.18)	4866.9 (181.9)	32.86 (0.90)
	2.0	22.55 (0.94)	7.12 (0.19)	1673.5 (53.6)	31.06 (1.09)	29.01 (0.85)	5.65 (0.17)	5309.1 (155.6)	37.25 (1.13)
	3.0	26.78 (1.07)	7.92 (0.25)	2119.3 (67.8)	32.59 (1.14)	29.10 (1.32)	6.21 (0.28)	6377.0 (289.3)	38.97 (1.16)
	4.0	21.26 (0.86)	6.00 (0.26)	1948.7 (62.4)	30.90 (1.09)	27.69 (1.24)	6.03 (0.27)	6204.4 (277.8)	37.08 (0.84)

Digits in parenthesis indicate the standard deviation at 95% confidence limit.

Table-3.14 Effect of different complex organic nitrogen sources on the cellulase and xylanase production by *T. harzianum* PPDDN-10 NFCCI-2925 and *C. cinerea* PPHRI-4 NFCCI-3027

Nitrogen source	Amounts of nitrogen source (g/l)	*T. harzianum* PPDDN-10 NFCCI-2925				*C. cinerea* PPHRI-4 NFCCI-3027			
		Endoglucanase, IU/gds	Total cellulase, FPU/gds	Xylanase, IU/gds	Soluble protein, mg/gds	Endoglucanase, IU/gds	Total cellulase, FPU/gds	Xylanase, IU/gds	Soluble protein, mg/gds
Tryptone (TRP)	**1.0**	14.19 (0.14)	1.23 (0.05)	163.98 (5.25)	5.78 (0.20)	15.50 (0.64)	3.16 (0.10)	1148.4 (80.2)	14.01 (0.06)
	2.0	15.47 (0.20)	2.82 (0.06)	559.85 (17.92)	10.77 (0.38)	24.37 (1.23)	4.10 (0.12)	1592.6 (130.4)	24.00 (0.62)
	3.0	18.85 (0.24)	2.92 (0.12)	447.43 (14.32)	14.64 (0.51)	30.37 (1.44)	6.85 (0.24)	1259.5 (112.6)	31.73 (1.17)
	4.0	12.58 (0.11)	2.20 (0.06)	397.46 (12.72)	8.18 (0.29)	15.50 (1.16)	3.25 (0.12)	1111.4 (64.2)	18.81 (0.90)
Meat peptone (MTP)	**1.0**	15.96 (0.21)	1.96 (0.11)	185.10 (5.92)	9.41 (0.33)	24.37 (1.10)	5.41 (0.22)	482.2 (50.6)	21.28 (0.62)
	2.0	17.52 (0.53)	2.89 (0.12)	224.36 (7.18)	12.38 (0.43)	30.37 (1.85)	6.77 (0.24)	598.5 (130.6)	27.22 (0.90)
	3.0	23.59 (0.41)	4.07 (0.15)	545.58 (17.46)	14.10 (0.49)	20.68 (1.05)	6.63 (0.30)	1550.3 (102.5)	30.65 (1.17)
	4.0	15.55 (0.25)	2.45 (0.14)	729.38 (23.34)	14.26 (0.50)	22.24 (1.41)	6.24 (0.28)	2094.9 (46.5)	30.96 (1.04)
Soya peptone (SP)	**1.0**	7.62 (0.12)	3.22 (0.10)	863.22 (27.62)	14.04 (0.49)	26.92 (1.77)	4.80 (0.20)	2491.5 (84.6)	30.52 (1.16)
	2.0	24.77 (0.41)	4.29 (0.15)	956.02 (30.59)	18.61 (0.65)	28.85 (1.63)	8.96 (0.30)	2766.4 (90.5)	39.67 (0.90)
	3.0	23.69 (0.40)	3.20 (0.13)	959.59 (30.71)	17.56 (0.61)	28.51 (1.24)	8.27 (0.26)	2777.0 (104.3)	37.56 (0.62)
	4.0	22.71 (0.35)	2.98 (0.14)	401.03 (12.83)	15.76 (0.55)	28.82 (1.80)	8.35 (0.28)	1122.0 (108.6)	33.97 (1.17)
Peptone bacteriological (PB)	**1.0**	14.92 (0.21)	3.02 (0.15)	811.47 (25.97)	13.69 (0.48)	33.55 (1.96)	5.75 (0.30)	2338.1 (116.5)	29.84 (1.04)
	2.0	17.10 (0.29)	4.09 (0.23)	859.65 (27.51)	16.07 (0.56)	32.02 (1.27)	6.82 (0.46)	2480.9 (130.5)	34.58 (0.90)
	3.0	15.99 (0.24)	2.87 (0.14)	1088.07 (34.82)	16.75 (0.59)	31.41 (1.35)	7.53 (0.28)	3157.7 (82.5)	35.94 (0.62)
	4.0	16.33 (0.26)	3.01 (0.11)	627.66 (20.09)	12.56 (0.44)	27.34 (1.75)	4.25 (0.22)	1793.5 (112.5)	27.58 (0.90)
Proteose peptone (PP)	**1.0**	15.31 (0.24)	0.96 (0.11)	238.64 (7.64)	11.97 (0.42)	27.21 (0.82)	5.62 (0.22)	643.4 (24.5)	26.39 (1.30)
	2.0	13.65 (0.24)	3.13 (0.12)	815.04 (26.08)	16.52 (0.58)	31.29 (1.53)	7.67 (0.24)	2360.4 (64.5)	35.50 (0.62)
	3.0	17.35 (0.26)	4.00 (0.15)	627.66 (20.09)	14.84 (0.52)	29.20 (1.25)	7.29 (0.30)	1804.3 (164.6)	32.12 (0.44)
	4.0	14.99 (0.27)	2.38 (0.14)	599.11 (19.17)	13.85 (0.48)	28.40 (1.67)	6.23 (0.28)	1720.9 (130.6)	30.15 (1.04)

Digits in parenthesis indicate the standard deviation at 95% confidence limit.

Table-3.15 Effect of different insoluble plant based nitrogen sources on the cellulase and xylanase production by *T. harzianum* PPDDN-10 NFCCI-2925 and *C. cinerea* PPHRI-4 NFCCI-3027

WB+ plant based N source	Ratio (total 5 g)	*T. harzianum* PPDDN-10 NFCCI-2925				*C. cinerea* PPHRI-4 NFCCI-3027			
		Endoglucanase, IU/gds	Total cellulase, FPU/gds	Xylanase, IU/gds	Soluble protein, mg/gds	Endoglucanase, IU/gds	Total cellulase, FPU/gds	Xylanase, IU/gds	Soluble protein, mg/gds
Wheat bran (WB)		14.07 (0.20)	2.27 (0.06)	2882.0 (81.0)	20.42 (0.31)	16.24 (0.74)	4.12 (0.21)	3565.1 (120.3)	24.23 (1.10)
Wheat bran (WB) : Mustard cake (MC)	**3:1**	18.64 (0.30)	1.56 (0.04)	1512.5 (78.2)	24.12 (0.38)	28.94 (0.89)	6.45 (0.32)	4509.2 (111.2)	39.44 (1.02)
	1:1	13.84 (0.20)	1.14 (0.02)	1118.1 (63.2)	18.88 (0.31)	25.44 (0.87)	5.98 (0.21)	3912.3 (134.3)	32.37 (1.31)
	1:3	11.01 (0.17)	0.48 (0.02)	569.1 (76.7)	17.35 (0.28)	15.74 (0.76)	3.56 (0.13)	3212.3 (143.2)	27.27 (0.98)
Wheat bran (WB) : Gram seed coat (GSC)	**3:1**	20.53 (0.29)	4.08 (0.10)	1892.8 (94.3)	26.83 (0.42)	32.88 (1.20)	6.87 (0.23)	5412.2 (145.6)	38.25 (1.54)
	1:1	17.70 (0.28)	2.59 (0.07)	1137.9 (54.1)	24.12 (0.36)	23.48 (0.98)	4.55 (0.22)	3445.4 (165.5)	31.44 (1.22)
	1:3	10.79 (0.15)	0.89 (0.03)	734.2 (49.4)	17.86 (0.28)	17.46 (0.56)	3.87 (0.19)	2312.3 (112.4)	21.21 (0.78)
Wheat bran (WB) : Soya bean meal (SBM)	**3:1**	21.09 (0.32)	4.42 (0.12)	1689.3 (61.3)	26.83 (0.41)	35.88 (1.12)	7.01 (0.24)	5987.3 (153.3)	41.28 (1.34)
	1:1	16.24 (0.24)	2.46 (0.07)	735.4 (89.3)	22.26 (0.32)	28.58 (1.10)	5.87 (0.28)	3834.7 (143.4)	34.39 (1.04)
	1:3	14.14 (0.24)	0.98 (0.03)	249.2 (63.3)	20.74 (0.33)	18.59 (0.65)	4.03 (0.19)	1113.3 (54.4)	19.29 (0.67)
Wheat bran (WB) : Castor seed cake (CSC)	**3:1**	19.67 (0.29)	2.30 (0.06)	1091.4 (83.3)	26.66 (0.41)	23.59 (1.23)	4.98 (0.23)	3945.3 (134.3)	31.29 (1.22)
	1:1	12.81 (0.20)	2.03 (0.06)	714.5 (68.5)	23.45 (0.40)	19.46 (0.98)	3.97 (0.23)	2312.2 (110.3)	27.35 (0.95)
	1:3	8.90 (0.14)	0.63 (0.02)	586.5 (67.6)	22.43 (0.25)	12.36 (0.34)	2.78 (0.13)	1112.3 (65.4)	16.23 (0.34)
Wheat bran (WB) : Lentil seed coat (LSC)	**3:1**	23.41 (0.32)	3.77 (0.09)	1898.7 (71.3)	30.39 (0.37)	34.67 (0.95)	6.89 (0.24)	5983.3 (178.5)	40.73 (1.23)
	1:1	17.10 (0.21)	3.20 (0.10)	1271.7 (65.7)	24.80 (0.39)	26.56 (0.67)	5.12 (0.25)	4234.4 (134.2)	31.86 (1.11)
	1:3	10.79 (0.17)	2.19 (0.07)	691.2 (68.7)	20.74 (0.31)	16.79 (0.53)	3.76 (0.18)	3423.3 (87.5)	22.76 (0.45)

Digits in parenthesis indicate the standard deviation at 95% confidence limit.

Table-3.16 Effect of different surfactant on the cellulase and xylanase production by *T. harzianum* PPDDN-10 NFCCI-2925 and *C. cinerea* PPHRI-4 NFCCI-3027

Surfactant	***T. harzianum* PPDDN-10 NFCCI-2925**				***C. cinerea* PPHRI-4 NFCCI-3027**			
	Endoglucanase, IU/gds	**Total cellulase, FPU/gds**	**Xylanase, IU/gds**	**Soluble protein, mg/gds**	**Endoglucanase, IU/gds**	**Total cellulase, FPU/gds**	**Xylanase, IU/gds**	**Soluble protein, mg/gds**
Control	18.01 (0.72)	5.02 (0.16)	898.6 (58.8)	20.94 (0.68)	28.87 (1.27)	4.40 (0.18)	4048.4 (91.4)	28.61 (0.95)
Tween 20	22.86 (0.56)	5.57 (0.20)	1068.8 (160.4)	22.42 (1.50)	31.81 (1.36)	4.73 (0.22)	4480.8 (159.3)	32.65 (1.45)
Tween 40	23.92 (0.72)	5.93 (0.26)	1451.1 (84.9)	23.94 (1.03)	32.38 (0.90)	4.84 (0.28)	4562.2 (84.3)	32.25 (1.23)
Tween 60	25.51 (0.85)	5.27 (0.24)	1213.5 (122.3)	27.28 (0.71)	33.93 (1.38)	6.18 (0.26)	4783.3 (121.5)	38.04 (1.34)
Tween 80	27.88 (0.92)	7.55 (0.37)	2069.0 (159.4)	30.77 (1.34)	39.46 (1.63)	7.67 (0.40)	6539.8 (158.3)	41.55 (0.99)
TritonX100	27.57 (0.90)	6.80 (0.26)	1941.3 (89.2)	30.01 (0.71)	38.48 (0.90)	5.96 (0.28)	5795.3 (88.6)	38.84 (0.90)

Table-3.17 Effect of moisture ratio on the cellulase and xylanase production by *T. harzianum* PPDDN-10 NFCCI-2925 and *C. cinerea* PPHRI-4 NFCCI-3027

Moisture ratio	***T. harzianum* PPDDN-10 NFCCI-2925**				***C. cinerea* PPHRI-4 NFCCI-3027**			
	Endoglucanase, IU/gds	**Total cellulase, FPU/gds**	**Xylanase, IU/gds**	**Soluble protein, mg/gds**	**Endoglucanase, IU/gds**	**Total cellulase, FPU/gds**	**Xylanase, IU/gds**	**Soluble protein, mg/gds**
1:1	10.23 (0.29)	3.66 (0.12)	577.2 (39.6)	11.50 (0.50)	14.24 (0.65)	4.22 (0.19)	3292.0 (150.3)	17.98 (0.62)
1:2	17.22 (0.85)	4.84 (0.24)	1124.0 (93.3)	21.37 (0.86)	22.73 (1.01)	6.20 (0.28)	3723.5 (165.4)	29.89 (1.17)
1:3	27.49 (0.99)	7.77 (0.41)	2165.4 (158.2)	30.81 (1.34)	38.56 (1.41)	7.75 (0.30)	6813.6 (261.1)	43.42 (1.04)
1:4	21.16 (1.05)	8.03 (0.40)	2043.3 (147.3)	31.83 (1.20)	22.71 (1.05)	5.16 (0.24)	6959.5 (321.7)	41.60 (1.52)
1:5	19.69 (0.95)	6.89 (0.32)	1503.6 (96.9)	30.65 (1.34)	19.67 (0.52)	4.31 (0.11)	7153.3 (189.1)	39.30 (1.31)

Digits in parenthesis indicate the standard deviation at 95% confidence limit.

Table-3.18 Effect of particle size (PS) of WB the cellulase and xylanase production by *T. harzianum* PPDDN-10 NFCCI-2925 and *C. cinerea* PPHRI-4 NFCCI-3027

Particle size of WB	***T. harzianum* PPDDN-10 NFCCI-2925**				***C. cinerea* PPHRI-4 NFCCI-3027**			
	Endoglucanase, IU/gds	**Total cellulase, FPU/gds**	**Xylanase, IU/gds**	**Soluble protein, mg/gds**	**Endoglucanase, IU/gds**	**Total cellulase, FPU/gds**	**Xylanase, IU/gds**	**Soluble protein, mg/gds**
X>14	17.29 (0.51)	3.92 (0.15)	1182.6 (38.4)	21.09 (0.66)	23.36 (0.99)	4.51 (0.22)	2352.1 (90.5)	28.81 (0.72)
14<**X**>22	17.69 (0.49)	4.01 (0.13)	1502.2 (45.9)	22.45 (0.67)	25.81 (0.77)	4.56 (0.28)	2712.7 (99.0)	30.51 (1.36)
22<**X**>30	20.13 (0.54)	6.32 (0.26)	1302.3 (40.7)	26.81 (0.82)	30.17 (0.99)	6.25 (0.26)	3247.8 (137.1)	35.96 (1.20)
30<**X**>36	21.19 (0.36)	7.12 (0.20)	1629.4 (43.0)	29.55 (0.93)	30.30 (0.70)	9.40 (0.40)	4632.0 (187.5)	39.38 (1.76)
36<**X**>44	26.48 (0.70)	8.21 (0.22)	1830.0 ()59.2	31.75 (0.90)	41.87 (1.65)	9.20 (0.28)	5120.6 (222.9)	42.14 (1.52)
44<**X**>52	30.97 (0.77)	7.29 (0.22)	2536.5 (76.0)	34.56 (1.04)	25.34 (0.55)	8.65 (0.18)	6155.9 (231.7)	45.65 (0.72)
52<**X**>60	10.22 (0.19)	7.62 (0.20)	1631.1 (40.3)	30.11 (1.02)	18.37 (0.51)	4.66 (0.22)	4364.5 (165.7)	40.08 (1.36)
60>**X**<72	4.53 (0.14)	5.90 (0.19)	631.8 (19.5)	25.99 (0.72)	10.22 (0.40)	3.56 (0.28)	3748.0 (77.7)	34.94 (1.20)
72<**X**>100	2.11 (0.07)	4.43 (0.13)	300.0 (7.7)	27.07 (1.04)	6.84 (0.24)	1.01 (0.26)	3515.3 (143.5)	36.29 (1.76)
Unscreened (US)	28.32 (0.70)	7.62 (0.13)	2190.7 (66.8)	31.48 (1.02)	39.08 (0.70)	7.70 (0.40)	6039.6 (143.7)	41.80 (1.52)
Mixture of each PS (1:1)	15.66 (0.26)	3.42 (0.09)	959.6 (31.3)	28.22 (0.88)	23.90 (0.79)	5.56 (0.28)	4399.4 (187.7)	37.73 (1.52)

Digits in parenthesis indicate the standard deviation at 95% confidence limit.

Table-3.19 Effect of alkali pretreatment of WB on the cellulase and xylanase production by *T. harzianum* PPDDN-10 NFCCI-2925 and *C. cinerea* PPHRI-4 NFCCI-3027

Pretreatment Methods	***T. harzianum* PPDDN-10 NFCCI-2925**				***C. cinerea* PPHRI-4 NFCCI-3027**			
	Endoglucanase, IU/gds	**Total cellulase, FPU/gds**	**Xylanase, IU/gds**	**Soluble protein, mg/gds**	**Endoglucanase, IU/gds**	**Total cellulase, FPU/gds**	**Xylanase, IU/gds**	**Soluble protein, mg/gds**
NaOH (0.25%)	19.53 (0.91)	5.42 (0.22)	1032.1 (50.2)	17.86 (0.79)	28.12 (1.22)	5.55 (0.22)	3622.7 (153.5)	24.79 (1.37)
NaOH (0.50%)	15.26 (0.66)	4.12 (0.20)	856.3 (32.5)	17.06 (0.75)	22.08 (0.75)	4.56 (0.20)	2565.3 (130.8)	23.69 (1.18)
NaOH (0.75%)	12.32 (0.51)	3.65 (0.18)	653.2 (30.2)	15.40 (0.65)	14.86 (1.08)	3.21 (0.16)	2132.2 (162.3)	19.32 (0.70)
NaOH (1.00%)	10.23 (0.41)	3.10 (0.15)	465.2 (22.3)	14.60 (0.45)	13.02 (0.46)	3.01 (0.26)	1932.5 (132.9)	17.32 (0.93)
NaOH (0.25%) + Autoclave (A)	17.53 (0.67)	4.14 (0.23)	695.8 (32.5)	16.26 (0.71)	19.08 (0.78)	5.12 (0.29)	3008.5 (144.6)	22.56 (1.06)
NaOH (0.50%)+A	13.21 (0.56)	3.84 (0.18)	625.4 (30.5)	14.60 (0.72)	16.78 (0.65)	4.12 (0.23)	2135.6 (191.7)	20.27 (0.70)
NaOH (0.75%)+A	10.32 (0.41)	3.49 (0.14)	525.6 (26.5)	13.88 (0.45)	13.56 (0.66)	3.01 (0.24)	1856.2 (95.6)	17.65 (0.81)
NaOH (1.00%)+A	8.35 (0.31)	3.09 (0.12)	425.3 (20.3)	11.62 (0.48)	11.23 (0.46)	2.85 (0.22)	1742.2 (65.2)	15.35 (0.35)
Untreated	27.49 (0.99)	7.77 (0.41)	2165.4 (158.1)	30.81 (1.34)	38.56 (1.41)	7.75 (0.30)	6813.6 (261.0)	43.42 (1.04)

Digits in parenthesis indicate the standard deviation at 95% confidence limit.

Table-3.20 Effect of different sugars on the cellulase and xylanase production

Sugar	Concentration (g/l)	*T. harzianum* PPDDN-10 NFCCI-2925				*C. cinerea* PPHRI-4 NFCCI-3027			
		Endoglucanase, IU/gds	Total cellulase, FPU/gds	Xylanase, IU/gds	Soluble protein, mg/gds	Endoglucanase, IU/gds	Total cellulase, FPU/gds	Xylanase, IU/gds	Soluble protein, mg/gds
Control		31.95 (0.42)	7.99 (0.26)	2120.2 (125.6)	31.2 (0.90)	39.08 (0.64)	7.70 (0.44)	6504.9 (182.9)	43.30 (0.95)
Glucose	**1.0**	24.82 (0.41)	5.20 (0.17)	822.9 (48.9)	25.38 (0.74)	35.32 (0.70)	7.36 (0.31)	4666.9 (115.1)	38.84 (0.65)
	2.0	20.21 (0.31)	3.79 (0.12)	728.7 (42.7)	24.09 (0.70)	23.31 (0.90)	5.47 (0.29)	4248.2 (126.0)	33.82 (1.23)
	3.0	13.82 (0.20)	0.46 (0.01)	675.4 (40.4)	22.01 (0.64)	12.23 (0.64)	0.66 (0.44)	4003.9 (174.5)	31.03 (1.09)
	4.0	8.17 (0.11)	0.52 (0.02)	556.8 (34.7)	17.34 (0.50)	6.06 (0.51)	0.46 (0.31)	2305.6 (153.4)	24.75 (1.60)
Galactose	**1.0**	28.03 (0.52)	5.56 (0.18)	881.3 (52.4)	26.42 (0.77)	31.94 (0.50)	7.80 (0.20)	3724.7 (101.3)	42.18 (1.38)
	2.0	23.37 (0.25)	4.18 (0.13)	793.2 (49.0)	24.19 (0.70)	26.85 (0.46)	5.91 (0.24)	3340.8 (105.3)	37.44 (0.65)
	3.0	16.30 (0.31)	4.14 (0.13)	676.3 (40.4)	22.01 (0.64)	16.23 (0.36)	5.82 (0.31)	2852.3 (210.9)	31.03 (1.23)
	4.0	12.43 (0.21)	1.50 (0.05)	616.1 (35.6)	19.62 (0.57)	14.94 (0.22)	2.47 (0.29)	2584.7 (98.9)	27.82 (1.09)
Lactose	**1.0**	25.27 (0.24)	4.57 (0.15)	827.2 (44.9)	25.44 (0.74)	28.78 (0.64)	6.59 (0.44)	3515.3 (182.6)	39.12 (1.60)
	2.0	24.58 (0.45)	3.35 (0.11)	768.8 (53.0)	25.75 (0.75)	27.76 (0.72)	4.95 (0.31)	3247.8 (182.9)	36.05 (1.38)
	3.0	19.39 (0.35)	2.28 (0.07)	671.9 (46.4)	22.01 (0.64)	18.13 (0.36)	3.46 (0.24)	2794.1 (238.9)	31.03 (1.38)
	4.0	13.00 (0.24)	0.39 (0.01)	587.3 (34.6)	18.48 (0.54)	14.99 (0.22)	0.91 (0.31)	2456.8 (115.1)	26.29 (0.65)
Xylose	**1.0**	27.72 (0.54)	5.49 (0.18)	770.5 (47.6)	24.09 (0.70)	31.73 (0.64)	7.68 (0.29)	3271.0 (126.0)	36.05 (1.23)
	2.0	23.39 (0.46)	3.24 (0.10)	651.9 (38.0)	21.07 (0.61)	27.01 (0.72)	4.86 (0.44)	2736.0 (174.5)	29.77 (1.09)
	3.0	20.29 (0.40)	0.32 (0.01)	507.1 (31.7)	15.36 (0.45)	23.15 (0.72)	3.56 (0.31)	2084.5 (238.6)	27.11 (1.60)
	4.0	13.35 (0.26)	0.37 (0.01)	373.6 (23.0)	10.17 (0.29)	20.64 (0.22)	3.23 (0.20)	1468.0 (283.7)	22.13 (1.38)
Fructose	**1.0**	28.64 (0.54)	5.01 (0.16)	934.6 (55.4)	24.61 (0.71)	35.70 (0.64)	7.10 (0.24)	3969.0 (294.9)	44.97 (0.65)
	2.0	25.69 (0.50)	4.46 (0.14)	794.1 (50.9)	21.49 (0.62)	33.93 (0.72)	6.40 (0.31)	3375.7 (210.9)	37.30 (1.23)
	3.0	23.20 (0.45)	3.84 (0.12)	624.0 (38.6)	19.93 (0.58)	26.36 (0.36)	5.53 (0.29)	2619.6 (98.9)	28.24 (1.09)
	4.0	18.84 (0.35)	0.74 (0.02)	536.7 (37.7)	16.51 (0.48)	21.48 (0.22)	1.59 (0.44)	2224.1 (182.6)	23.64 (1.60)
Cellobiose	**1.0**	35.30 (0.65)	7.91 (0.25)	1600.2 (97.7)	27.20 (0.79)	40.15 (0.64)	10.91 (0.31)	5004.3 (182.9)	44.97 (1.38)
	2.0	40.48 (0.75)	8.46 (0.27)	1419.6 (85.7)	25.75 (0.75)	39.51 (0.72)	9.80 (0.44)	4946.1 (238.9)	41.60 (1.20)
	3.0	26.23 (0.25)	4.91 (0.16)	1433.6 (74.0)	23.20 (0.67)	29.74 (0.72)	6.98 (0.31)	4666.9 (182.9)	34.37 (1.11)
	4.0	20.79 (0.22)	4.05 (0.13)	1594.1 (99.9)	22.01 (0.64)	23.90 (0.22)	5.77 (0.13)	4236.5 (238.9)	34.37 (1.13)

Digits in parenthesis indicate the standard deviation at 95% confidence limit.

Table-3.21 Effect of different vitamins and amino acids on the cellulase and xylanase production

Vitamins and amino acids	*T. harzianum* PPDDN-10 NFCCI-2925				*C. cinerea* PPHRI-4 NFCCI-3027			
	Endoglucanase, IU/gds	Total cellulase, FPU/gds	Xylanase, IU/gds	Soluble protein, mg/gds	Endoglucanase, IU/gds	Total cellulase, FPU/gds	Xylanase, IU/gds	Soluble protein, mg/gds
Control	30.92 (0.65)	7.60 (0.32)	3548.1 (121.4)	42.92 (0.77)	39.75 (1.65)	7.91 (0.36)	7319.1 (151.3)	43.28 (1.43)
L-Ascorbic acid	52.96 (1.54)	10.60 (0.45)	3421.6 (145.4)	43.76 (1.45)	45.37 (1.67)	10.40 (0.45)	10081.9 (342.3)	60.79 (0.68)
D-Biotin	52.20 (1.34)	8.37 (0.46)	1736.0 (98.5)	28.84 (1.28)	47.96 (1.63)	7.75 (0.37)	9767.8 (301.2)	53.31 (1.28)
Calcium pantothenate	22.05 (0.67)	6.75 (0.41)	2254.1 (122.3)	40.27 (1.87)	47.19 (1.11)	6.76 (0.28)	11047.3 (345.4)	52.69 (1.13)
Cyanocobalamine	36.50 (1.32)	6.66 (0.40)	2854.9 (132.3)	40.46 (1.62)	40.03 (1.59)	6.41 (0.31)	9221.0 (276.3)	44.66 (1.65)
Folic acid	41.73 (1.27)	8.88 (0.65)	1382.9 (98.3)	45.21 (0.77)	46.23 (1.33)	8.71 (0.38)	5731.3 (156.6)	56.43 (1.43)
Nicotinic acid	19.03 (0.56)	7.14 (0.34)	5698.9 (176.4)	48.28 (1.45)	42.41 (1.34)	7.00 (0.32)	5149.7 (156.0)	38.57 (0.68)
Pyridoxine	47.11 (1.54)	4.07 (0.23)	1061.6 (34.4)	27.30 (1.28)	32.04 (1.22)	3.91 (0.17)	4393.6 (145.5)	35.80 (1.28)
Riboflavin	32.51 (1.01)	10.21 (0.48)	968.8 (23.5)	17.58 (1.87)	45.86 (1.84)	9.45 (0.37)	3992.3 (123.4)	44.66 (1.13)
Thiamine	9.62 (0.31)	7.01 (0.22)	948.6 (34.3)	18.56 (0.77)	34.62 (1.45)	6.88 (0.23)	5394.0 (178.4)	39.12 (1.65)
Asparagine	23.43 (0.67)	8.62 (0.34)	1168.9 (45.5)	25.75 (1.45)	51.33 (1.67)	8.45 (0.34)	9554.9 (321.1)	58.51 (0.68)
Aspartic acid	28.99 (0.56)	5.70 (0.23)	2180.9 (87.5)	27.39 (1.28)	36.31 (1.34)	5.60 (0.22)	1291.2 (45.5)	35.80 (1.28)
Cysteine	33.33 (0.76)	5.60 (0.32)	2124.0 (112.3)	33.11 (1.87)	40.50 (1.53)	5.50 (0.24)	2391.6 (87.7)	38.57 (1.13)
Glutamic acid	33.64 (0.34)	8.18 (0.43)	2395.6 (134.2)	26.41 (1.62)	42.05 (1.23)	8.02 (0.34)	1521.5 (57.8)	42.58 (1.65)
Glycine	29.61 (0.78)	10.56 (0.54)	2019.6 (112.2)	28.21 (0.77)	52.73 (1.98)	10.36 (0.43)	8877.9 (267.6)	56.43 (1.43)
Isoleucine	33.49 (0.45)	6.63 (0.25)	4025.0 (154.3)	31.47 (1.45)	31.00 (1.43)	6.50 (0.21)	844.5 (34.9)	35.52 (0.68)
Leucine	28.52 (0.56)	4.28 (0.13)	527.4 (34.5)	33.93 (1.28)	25.32 (1.04)	4.20 (0.19)	4036.5 (134.9)	35.80 (1.28)
Metheonine	46.75 (0.34)	6.02 (0.34)	3187.8 (143.4)	34.91 (1.87)	30.63 (1.26)	5.91 (0.26)	2661.5 (89.8)	37.60 (1.13)
Tryptophan	21.38 (0.65)	9.89 (0.45)	2697.2 (123.4)	46.19 (1.62)	57.72 (1.89)	9.70 (0.39)	11318.4 (333.5)	59.89 (1.65)
Valine	39.58 (0.45)	8.50 (0.34)	2387.5 (111.3)	32.65 (1.33)	43.02 (1.45)	8.34 (0.29)	597.9 (145.8)	44.66 (1.43)

Digits in parenthesis indicate the standard deviation at 95% confidence limit.

Table-3.22 Effect of different metal ions and chelating agents on the cellulase and xylanase production

Metal ions and chelating agents	*T. harzianum* PPDDN-10 NFCCI-2925				*C. cinerea* PPHRI-4 NFCCI-3027			
	Endoglucanase, IU/gds	Total cellulase, FPU/gds	Xylanase, IU/gds	Soluble protein, mg/gds	Endoglucanase, IU/gds	Total cellulase, FPU/gds	Xylanase, IU/gds	Soluble protein, mg/gds
Control	30.92 (0.65)	7.60 (0.32)	3548.1 (121.4)	42.92 (0.77)	39.75 (1.65)	7.91 (0.36)	7319.1 (151.3)	43.28 (1.43)
Mn^{+2}	31.91 (1.34)	8.59 (0.33)	2648.2 (107.9)	24.57 (0.45)	39.90 (1.43)	7.86 (0.26)	6853.5 (245.7)	44.65 (1.38)
Zn^{+2}	32.32 (0.98)	8.17 (0.35)	2617.5 (112.7)	24.86 (0.85)	41.20 (1.76)	8.61 (0.12)	6790.6 (265.7)	45.71 (1.32)
Fe^{+3}	33.31 (0.94)	8.20 (0.28)	2511.4 (106.8)	25.25 (0.75)	44.31 (1.44)	9.90 (0.45)	7447.1 (272.5)	46.78 (1.65)
Al^{+3}	7.88 (0.12)	1.01 (0.06)	1581.5 (68.0)	16.11 (0.67)	29.92 (1.23)	4.83 (0.21)	1898.4 (67.8)	38.30 (1.23)
Hg^{+2}	6.15 (0.34)	0.19 (0.04)	1091.0 (43.9)	18.03 (0.95)	35.91 (1.34)	6.90 (0.29)	5440.5 (221.8)	40.59 (1.67)
Ag^{+1}	16.14 (0.76)	2.00 (0.07)	1729.0 (100.8)	19.48 (0.45)	39.72 (1.65)	6.77 (0.46)	793.3 (42.6)	44.63 (1.56)
Cu^{+2}	14.41 (0.56)	7.43 (0.25)	1650.3 (66.9)	16.59 (0.85)	38.13 (1.76)	9.02 (0.36)	8284.6 (298.6)	43.29 (1.37)
Ba^{+2}	12.09 (0.34)	5.62 (0.21)	2574.6 (108.6)	16.88 (0.75)	30.02 (1.37)	3.26 (0.16)	8482.4 (344.8)	43.29 (1.76)
K^{+1}	32.82 (0.98)	8.09 (0.24)	1989.8 (67.8)	24.57 (1.10)	44.73 (1.98)	10.52 (0.43)	7807.7 (289.9)	47.54 (1.36)
Co^{+3}	32.82 (1.08)	8.25 (0.32)	2956.6 (105.8)	26.69 (0.95)	42.83 (1.65)	8.95 (0.40)	5725.5 (245.8)	43.42 (1.78)
Na^{+1}	32.16 (1.23)	8.25 (0.29)	2245.7 (67.8)	24.86 (0.95)	36.45 (1.22)	8.27 (0.45)	4992.7 (221.7)	43.96 (1.89)
Ni^{+2}	24.73 (1.02)	1.18 (0.03)	1802.7 (67.9)	17.26 (0.45)	38.20 (1.37)	4.83 (0.22)	3527.0 (176.8)	40.19 (1.56)
Pt^{+5}	10.89 (0.43)	1.67 (0.06)	1274.3 (45.9)	7.94 (0.45)	38.53 (1.54)	6.23 (0.25)	3050.0 (123.7)	41.67 (1.87)
Cr^{+3}	27.86 (1.11)	1.42 (0.07)	1763.6 (78.9)	15.25 (0.75)	35.31 (1.76)	7.28 (0.36)	5993.0 (223.8)	44.63 (1.45)
Sr^{+2}	7.14 (0.34)	2.90 (0.11)	1587.2 (67.7)	6.69 (0.34)	35.70 (1.47)	7.77 (0.37)	4947.3 (323.8)	45.17 (1.87)
Sn^{+2}	13.83 (0.56)	0.52 (0.01)	645.7 (154.3)	7.94 (0.38)	23.44 (1.03)	5.18 (0.25)	6400.2 (210.7)	36.86 (1.99)
Pb^{+2}	21.42 (0.98)	5.70 (0.23)	2399.5 (34.5)	11.78 (0.45)	17.91 (0.87)	7.67 (0.34)	4864.7 (243.8)	29.54 (1.34)
EDTA	11.76 (0.56)	5.13 (0.23)	265.9 (143.4)	13.71 (0.85)	15.11 (0.64)	6.78 (0.35)	3119.8 (123.9)	34.80 (1.54)
DTPA	22.08 (0.76)	4.63 (0.19)	232.2 (123.4)	12.75 (0.75)	32.90 (1.34)	6.23 (0.30)	2724.3 (111.8)	40.32 (1.46)

Digits in parenthesis indicate the standard deviation at 95% confidence limit.

Table-3.23 Effect of pH of the medium on the CMCase, FPase and xylanase activities of the crude enzyme of *T. harzianum* PPDDN-10 NFCCI-2925 and their stability at optimum pH

pH of buffer	Relative activity,%			Treatment hours	Residual activity,%		
	CMCase	FPase	xylanase		CMCase	FPase	xylanase
3.0	49.2	60.7	63.3	**0**	100.0	100.0	100.0
3.5	56.5	68.5	69.4	**1**	97.9	98.8	98.4
4.0	61.5	73.0	73.2	**2**	97.7	95.3	98.1
4.5	71.0	83.2	89.0	**3**	97.4	95.3	96.5
5.0	87.2	91.0	87.6	**4**	93.5	94.1	95.7
5.5	92.3	100.0	97.1	**5**	92.7	90.2	94.2
6.0	100.0	96.2	100.0	**6**	92.1	88.2	92.3
6.5	93.6	84.3	90.1	**24**	86.2	83.5	78.8
7.0	79.5	79.8	79.2				
7.5	72.0	73.0	62.7				
8.0	57.9	61.2	60.1				
8.5	45.9	54.6	49.3				
9.0	38.0	44.9	46.0				
9.5	14.7	26.0	33.2				
10.0	11.8	19.5	28.4				
10.5	11.7	12.4	12.6				
11.0	9.8	5.6	7.5				

Table-3.24 Effect of pH of the medium on the CMCase, FPase and xylanase activities of the crude enzyme of *C. cinerea* PPHRI-4 NFCCI-3027 and their stability at optimum pH

pH of buffer	Relative activity,%			Treatment hours	Residual activity,%		
	CMCase	FPase	xylanase		CMCase	FPase	xylanase
3.0	33.7	37.3	40.0	**0**	100.0	100.0	100.0
3.5	48.3	46.7	51.5	**1**	97.2	96.0	95.2
4.0	62.0	64.0	61.3	**2**	94.3	94.7	89.8
4.5	75.6	81.3	76.7	**3**	91.5	90.7	85.7
5.0	92.2	93.3	85.7	**4**	87.4	85.3	82.9
5.5	95.4	96.0	90.4	**5**	85.5	82.7	80.3
6.0	100.0	100.0	94.3	**6**	82.3	81.3	78.2
6.5	97.6	96.0	100.0	**24**	75.4	72.0	72.8
7.0	93.7	90.7	89.5				
7.5	83.2	80.0	76.9				
8.0	69.5	61.3	66.7				
8.5	54.6	54.7	59.6				
9.0	42.4	37.3	44.3				
9.5	27.1	25.3	33.7				
10.0	22.4	22.7	23.8				
10.5	20.7	20.0	15.4				
11.0	8.5	12.0	13.0				

Digits in parenthesis indicate the standard deviation at 95% confidence limit.

Table-3.25 Effect of incubation temperature on the CMCase, FPase and xylanase activities of the crude enzyme of *T. harzianum* PPDDN-10 NFCCI-2925 and their stability at optimum temperature

Incubation temperature, °C	Relative activity,%			Treatment hours	Residual activity,%					
					At 55 °C			At 60 °C		
	CMCase	FPase	Xylanase		CMCase	FPase	Xylanase	CMCase	FPase	Xylanase
20	30.2	35.7	45.2	**0**	100.0	100.0	100.0	100.0	100.0	100.0
25	48.5	46.4	48.3	**1**	98.4	97.4	95.1	100.0	99.1	99.5
30	51.2	59.3	54.0	**2**	90.1	88.4	86.3	98.5	95.7	97.2
35	58.6	67.4	61.4	**3**	85.4	82.3	80.2	92.4	90.2	92.4
40	62.3	78.3	68.1	**4**	71.4	73.7	72.3	88.7	86.3	87.5
45	65.2	84.5	71.4	**5**	65.2	61.2	65.3	80.2	78.3	76.3
50	70.0	93.5	78.5	**6**	56.3	50.4	48.2	75.2	73.7	70.3
55	87.5	100.0	85.3							
60	100.0	87.2	100.0							
65	56.4	51.4	64.2							
70	34.4	21.1	25.7							
80	10.5	8.5	9.5							

Table-3.26 Effect of incubation temperature on the CMCase, FPase and xylanase activities of the crude enzyme of *C. cinerea* PPHRI-4 NFCCI-3027 and their stability at optimum temperature

Incubation temperature, °C	Relative activity,%			Treatment hours	Residual activity,%					
					At 55 °C			At 60 °C		
	CMCase	FPase	Xylanase		CMCase	FPase	Xylanase	CMCase	FPase	Xylanase
20	25.5	30.4	18.7	**0**	100.0	100.0	100.0	100.0	100.0	100.0
25	31.5	34.8	23.7	**1**	91.4	90.2	93.1	95.7	97.8	95.6
30	44.8	39.1	29.3	**2**	85.8	87.0	84.2	93.1	92.4	93.7
35	57.3	47.8	43.9	**3**	76.7	73.9	70.5	85.8	83.7	88.1
40	64.9	60.9	58.5	**4**	69.0	67.4	64.3	80.8	78.3	80.8
45	77.0	70.7	74.0	**5**	59.3	57.6	54.5	78.9	76.1	73.4
50	90.2	88.0	88.3	**6**	50.4	47.8	51.9	75.6	70.7	71.4
55	96.3	100.0	100.0							
60	100.0	95.7	97.6							
65	60.0	55.4	65.1							
70	21.0	21.7	23.8							
80	8.3	5.4	3.6							

Digits in parenthesis indicate the standard deviation at 95% confidence limit.

Table-4.1 Effect of chemical deinking on paper properties

Parameters		Exp. no.	Deinking efficiency, %	Residual ink, ppm	ISO Brightness, %	Opacity, %	Tensile index, N.m/g	Folding endurance	Burst index, $kPa.m^2/g$	Tear index, $mN.m^2/g$	initial ink, ppm (EP)[#]
NaOH, %	**1.5**	**C1**	42.6 (1.0)	156.25 (11.61)	75.5 (0.7)	90.5 (0.6)	24.80 (3.85)	0.76 (0.18)	1.15 (0.45)	6.93 (0.68)	256.45 (5.65)
	2.0	**C2**	58.4 (1.7)	118.54 (12.80)	78.7 (0.9)	90.1 (0.8)	31.49 (2.75)	0.87 (0.13)	1.45 (0.45)	6.72 (0.73)	254.78 (3.78)
	2.5	**C3**	53.8 (1.2)	128.61 (10.96)	76.6 (1.2)	91.5 (0.7)	25.1 (3.15)	0.69 (0.10)	0.96 (0.42)	6.43 (0.20)	249.54 (3.87)
Hydrapulping time, min	**15**	**C4**	52.9 (1.0)	130.84 (7.02)	77.3 (0.9)	90.6 (0.8)	30.77 (3.01)	0.75 (0.19)	1.44 (0.31)	6.45 (0.86)	256.45 (3.98)
	30	**C2**	58.4 (1.7)	118.54 (12.80)	78.7 (0.9)	90.1 (0.8)	31.49 (2.75)	0.87 (0.13)	1.45 (0.45)	6.72 (0.73)	254.78 (3.78)
	45	**C5**	53.2 (1.1)	129.79 (7.37)	76.9 (1.1)	91.3 (0.9)	31.21 (3.61)	0.90 (0.13)	1.86 (0.16)	6.38 (0.32)	251.67 (5.87)
Temperature, °C	**65**	**C6**	48.0 (1.0)	142.14 (12.7)	75.0 (0.4)	88.9 (0.9)	29.62 (3.25)	0.84 (0.16)	1.56 (0.31)	7.82 (0.59)	257.34 (6.34)
	70	**C2**	58.4 (1.7)	118.54 (12.80)	78.7 (0.9)	90.1 (0.8)	31.49 (2.75)	0.87 (0.13)	1.45 (0.45)	6.72 (0.73)	254.78 (3.78)
	75	**C7**	57.0 (0.9)	121.27 (11.65)	77.4 (0.2)	91.8 (2.1)	28.83 (3.98)	0.84 (0.12)	1.47 (0.34)	6.92 (0.94)	250.23 (3.87)
Na_2SiO_3,%	**1.0**	**C8**	49.8 (0.9)	135.62 (9.24)	75.9 (1.4)	92.3 (0.5)	31.32 (4.22)	0.86 (0.19)	1.73 (0.33)	6.85 (0.79)	249.76 (4.98)
	1.5	**C9**	55.9 (0.9)	123.72 (13.37)	77.6 (0.7)	91.6 (0.6)	31.15 (2.43)	0.94 (0.07)	1.79 (0.30)	6.98 (0.91)	250.45 (6.98)
	2.0	**C2**	58.4 (1.7)	118.54 (12.80	78.7 (0.9)	90.1 (0.9)	31.49 (2.75)	0.87 (0.13)	1.45 (0.45)	6.72 (0.73)	254.78 (3.78)
	2.5	**C10**	55.6 (1.2)	124.39 (9.23)	78.9 (1.1)	91.0 (0.7)	28.69 (4.26	0.84 (0.13)	1.65 (0.37)	6.81 (0.21)	257.31 (4.87)
H_2O_2, %	**0.5**	**C11**	52.0 (1.1)	132.87 (8.11)	76.5 (0.6)	90.9 (1.1)	29.66 (2.05)	0.93 (0.17)	1.72 (0.32)	7.93 (0.68)	249.76 (5.26)
	1.0	**C2**	58.4 (1.7)	118.54 (12.80)	78.7 (0.9)	90.1 (0.8)	31.49 (2.75)	0.87 (0.13)	1.45 (0.45)	6.72 (0.73)	254.78 (3.78)
	1.5	**C12**	55.1 (1.4)	125.59 (15.73)	79.5 (0.9)	90.7 (1.0)	31.96 (2.70)	0.88 (0.17)	1.69 (0.59)	7.65 (0.72)	257.56 (4.98)
Pulp consistency, %	**6**	**C2**	58.4 (1.7)	118.54 (12.18)	78.7 (0.9)	90.1 (0.8)	31.49 (2.75)	0.87 (0.13)	1.45 (0.45)	6.72 (0.73)	254.78 (3.78)
	8	**C13**	64.6 (1.0)	102.22 (10.52)	79.2 (1.1)	91.1 (1.2)	30.05 (4.34)	0.81 (0.14)	1.37 (0.27)	6.94 (1.07)	249.78 (6.89)
	10	**C14**	75.9 (1.0)	77.22 (2.32)	80.4 (0.5)	88.7 (0.6)	28.53 (4.91	0.75 (0.12)	1.34 (0.14)	7.11 (1.09)	253.65 (6.56)
	12	**C15**	73.2 (0.9)	82.65 (10.76)	79.4 (1.1)	89.3 (0.9)	28.42 (3.57)	0.85 (0.12)	1.32 (0.34)	7.01 (0.23)	248.45 (7.45)
Oleic acid, %	**0.6**	**C16**	56.6 (0.8)	121.89 (15.11)	79.0 (0.4)	91.2 (0.5)	25.48 (2.06	0.70 (0.15)	1.37 (0.32)	6.48 (0.99))	253.19 (8.67)
	0.8	**C14**	75.9 (1.0)	77.22 (2.32)	80.4 (0.5)	88.7 (0.6)	28.53 (4.91	0.75 (0.12)	1.34 (0.14)	7.11 (1.09)	253.65 (6.56)
	1.0	**C17**	73.2 (1.1)	88.57 (11.54)	80.0 (0.9)	90.1 (0.8)	26.56 (1.77	0.72 (0.15)	1.38 (0.31)	6.26 (1.21)	249.67 (7.89)

[#]EB = 21.3 ppm

Digits in parenthesis indicate the standard deviation at 95% confidence limit.

Table-4.2 Effect of chemical deinking on the yield, freeness, drainage rate and reducing sugars

Parameters		Exp. no.	Yield , %	CSF, ml	Drainage rate, sec	Reducing sugars, mg/ml
NaOH, %	**1.5**	**C1**	76.7 (0.7)	460	8.17 (0.63)	0.134 (0.013)
	2.0	**C2**	77.1 (0.5)	500	6.63 (0.31)	0.235 (0.018)
	2.5	**C3**	77.7 (0.8)	510	6.57 (0.35)	0.246 (0.023)
Time, min	**15**	**C4**	77.3 (0.9)	480	7.25 (0.49)	0.185 (0.014)
	30	**C2**	77.1 (0.5)	500	6.63 (0.31)	0.235 (0.018)
	45	**C5**	77.2 (0.5)	460	7.99 (0.32)	0.268 (0.021)
Temperature, °C	**65**	**C6**	77.6 (0.6)	480	7.23 (0.30)	0.252 (0.012)
	70	**C2**	77.1 (0.5)	500	6.63 (0.31)	0.245 (0.018)
	75	**C7**	76.0 (0.4)	470	7.87 (0.15)	0.254 (0.021)
Na_2SiO_3, %	**1.0**	**C8**	79.3 (0.5)	480	7.74 (0.47)	0.321 (0.029)
	1.5	**C9**	77.9 (0.4)	480	7.81 (0.37)	0.398 (0.024)
	2.0	**C2**	77.1 (0.5)	500	6.63 (0.31)	0.235 (0.018)
	2.5	**C10**	78.9 (0.5)	500	6.75 (0.17)	0.278 (0.025)
H_2O_2, %	**0.5**	**C11**	77.4 (0.4)	490	6.98 (0.30)	0.212 (0.021)
	1.0	**C2**	77.1 (0.5)	500	6.63 (0.31)	0.235 (0.018)
	1.5	**C12**	77.5 (0.7)	500	6.82 (0.67)	0.222 (0.018)
Pulp consistency, %	**6**	**C2**	76.1 (0.7)	500	6.63 (0.31)	0.235 (0.018)
	8	**C13**	76.7 (0.5)	500	6.84 (0.37)	0.285 (0.023)
	10	**C14**	77.9 (0.8)	510	6.72 (0.67)	0.312 (0.028)
	12	**C15**	77.1 (0.7)	520	6.56 (0.45)	0.325 (0.032)
Oleic acid, %	**0.6**	**C16**	78.4 (0.4)	490	6.59 (0.37)	0.336 (0.012)
	0.8	**C14**	77.9 (0.8)	510	6.72 (0.67)	0.312 (0.028)
	1.0	**C17**	72.4 (0.7)	480	7.10 (0.57)	0.342 (0.010)

Digits in parenthesis indicate the standard deviation at 95% confidence limit.

Table-4.3 Effect of point of addition of enzymes on paper properties

Source of enzyme		Point of addition of enzyme	Exp. no.	Deinking efficiency, %	Residual ink, ppm	ISO Brightness, %	Opacity, %	Tensile index, N.m/g	Folding endurance	Burst index, kPa.m^2/g	Tear index, mN.m^2/g	Initial ink, ppm (EP)[#]
Commercial cellulase (CE)		Pre-HP	CE1	33.5 (0.9)	205.05 (12.08)	72.7 (0.6)	90.4 (1.0)	30.31 (2.71)	0.78 (0.10)	1.56 (0.29)	6.05 (0.31)	300.04 (3.56)
		During-HP	CE2	69.7 (1.3)	102.67 (4.56)	74.8 (0.5)	90.0 (0.7)	30.65 (2.1)	0.79 (0.10)	1.55 (0.23)	5.95 (0.54)	301.24 (4.67)
		Post-HP	CE3	51.2 (1.4)	152.86 (8.97)	73.7 (0.8)	89.7 (0.5)	31.04 (1.42)	0.80 (0.10)	1.60 (0.34)	6.14 (0.45)	295.95 (6.35)
Crude enzyme	*T. harzianum* (TH)	Pre-HP	TH1	46.5 (1.7)	180.23 (5.68)	73.3 (0.8)	90.1 (0.1)	29.69 (1.41)	0.85 (0.13)	1.56 (0.23)	6.76 (0.56)	320.21 (5.68)
		During-HP	TH2	63.4 (2.0)	135.02 (3.02)	75.7 (0.6)	90.3 (0.2)	28.32 (1.76)	0.82 (0.13)	1.45 (0.23)	6.98 (0.45)	335.20 (8.45)
		Post-HP	TH3	62.5 (1.2)	130.12 (5.32)	75.5 (1.0)	90.0 (0.2)	28.10 (1.25)	0.86 (0.15)	1.42 (0.27)	7.15 (0.34)	315.32 (10.1)
	C. cinerea (CC)	Pre-HP	CC1	47.3 (0.6)	169.35 (10.23)	74.2 (0.6)	90.2 (0.2)	27.68 (1.30)	0.76 (0.11)	1.33 (0.24)	5.76 (0.56)	305.32 (12.3)
		During-HP	CC2	66.4 (1.0)	123.25 (8.33)	77.4 (0.7)	90.0 (0.2)	28.80 (1.62)	0.77 (0.14)	1.40 (0.22)	6.21 (0.45)	331.25 (10.2)
		Post-HP	CC3	62.7 (0.8)	134.23 (7.22)	76.4 (0.9)	89.7 (0.3)	27.90 (1.15)	0.80 (0.12)	1.45 (0.29)	6.35 (0.34)	329.32 (12.6)

[#] EB value for commercial cellulase= 16.3 ppm, Crude enzyme of *T. harzianum*=19.21 ppm, Crude enzyme of *C. cin*erea=17.98

Table-4.4 Effect of point of addition of enzymes on the yield, freeness, drainage rate and reducing sugars

Source of enzyme		Point of addition of enzyme	Exp. no.	Yield , %	CSF, ml	Drainage rate, sec	Reducing sugars, mg/ml
Commercial cellulase (CE)		Pre-HP	CE1	79.2 (1.2)	510	6.65 (0.29)	0.145 (0.029)
		During-HP	CE2	77.9 (1.4)	560	6.21 (0.21)	0.412 (0.043)
		Post-HP	CE3	78.3 (1.1))	540	6.34 (0.34)	0.465 (0.026)
Crude enzyme	*T. harzianum* (TH)	Pre-HP	TH1	79.3 (1.0)	480	6.10 (0.21)	0.123 (0.025)
		During-HP	TH2	78.3 (1.3)	520	5.95 (0.11)	0.321 (0.034)
		Post-HP	TH3	78.6 (1.1)	520	5.98 (0.14)	0.365 (0.022)
	C. cinerea (CC)	Pre-HP	CC1	79.8 (1.1)	490	6.70 (0.25)	0.165 (0.026)
		During-HP	CC2	78.2 (1.3)	530	6.24 (0.13)	0.399 (0.025)
		Post-HP	CC3	78.9 (1.6)	530	6.12 (0.17)	0.384 (0.013)

Digits in parenthesis indicate the standard deviation at 95% confidence limit.

Table-4.5 Effect of enzyme dose on paper properties

Source of enzyme		Enzyme dose, IU/g o.d. wt. pulp*	Exp. no.	Deinking efficiency, %	Residual ink, ppm	ISO Brightness, %	Opacity, %	Tensile index, N.m/g	Folding endurance	Burst index, $kPa.m^2/g$	Tear index, $mN.m^2/g$	Initial ink, ppm (EP)#
Commercial cellulase (CE)		2.0	CE2	69.7 (1.0)	102.67 (4.56)	74.8 (0.5)	90.0 (0.7)	30.65 (2.10)	0.79 (0.10)	1.55 (0.23)	5.95 (0.54)	301.24 (4.67)
		4.0	CE4	80.4 (0.8)	70.11 (5.98)	76.0 (0.49)	90.6 (0.5)	30.14 (3.98)	0.80 (0.10)	1.49 (0.32)	5.85 (0.60)	290.11 (6.61)
		6.0	CE5	94.6 (1.3)	31.95 (3.45)	78.7 (0.5)	90.1 (1.0)	29.31 (3.64)	0.75 (0.10)	1.58 (0.36)	5.55 (0.88)	308.46 (4.78)
		8.0	CE6	94.2 (1.0)	32.45 (5.87)	78.4 (0.5)	89.3 (0.4)	28.65 (2.87)	0.74 (0.12)	1.32 (0.46)	5.30 (0.43)	295.67 (4.87)
		10.0	CE7	89.3 (1.1)	45.56 (3.42)	78.0 (0.7)	89.1 (0.7)	28.09 (1.65)	0.73 (0.08)	1.22 (0.36)	5.45 (0.37)	290.87 (3.98)
Crude enzyme	*T. harzianum* (TH)	0.2	TH2	63.4 (2.0)	135.02 (3.02)	75.7 (0.7)	90.3 (0.2)	28.32 (1.76)	0.85 (0.14)	1.45 (0.23)	6.98 (0.45)	335.2 (4.98)
		0.4	TH4	70.9 (1.2)	112.65 (2.05)	77.3 (1.0)	89.7 (0.2)	28.90 (1.59)	0.82 (0.15)	1.52 (0.25)	6.50 (0.32)	340.21 (5.32)
		0.6	TH5	85.1 (1.0)	65.65 (4.05)	79.5 (0.8)	89.3 (0.3)	29.21 (1.67)	0.79 0.16()	1.52 (0.19)	6.66 (0.54)	330.12 (2.65)
		0.8	TH6	93.1 (1.6)	40.12 (1.85)	83.1 (0.7)	89.7 (0.4)	29.85 (2.02)	0.79 (0.18)	1.59 (0.24)	5.95 (0.46)	321.25 (5.49)
		1.0	TH7	94.8 (1.2)	35.20 (2.98)	82.9 (0.9)	88.2 (0.3)	25.10 (1.23)	0.73 (0.17)	1.32 (0.25)	5.56 (0.43)	326.32 (4.65)
	C. cinerea (CC)	0.2	CC2	66.4 (0.7)	123.25 (9.23)	77.4 (0.7)	90.0 (0.2)	28.32 (1.62)	0.77 (0.11)	1.40 (0.24)	6.21 (0.54)	331.25 (2.95)
		0.4	CC4	79.1 (0.6)	85.23 (4.23)	81.2 (0.8)	90.0 (0.3)	28.10 (1.46)	0.81 (0.13)	1.45 (0.27)	6.50 (0.32)	339.23 (5.12)
		0.6	CC5	90.1 (0.7)	49.35 (2.32)	83.2 (0.7)	89.6 (0.3)	29.34 (1.54)	0.84 (0.13)	1.59 (0.20)	6.90 (0.54)	335.39 (2.39)
		0.8	CC6	93.6 (0.5)	39.02 (3.54)	83.9 (0.8)	90.0 (0.15)	26.21 (1.86)	0.75 (0.15)	1.59 (0.25)	5.70 (0.46)	346.32 (7.23)
		1.0	CC7	94.2 (0.4)	36.23 (2.35)	82.3 (0.7)	88.1 (0.3)	25.23 (1.13)	0.71 (0.14)	1.27 (0.27)	5.20 (0.43)	333.21 (4.32)

EB value for commercial cellulase= 16.3 ppm, Crude enzyme of *T. harzianum*=19.21 ppm, Crude enzyme of *C. cin*erea=17.98

*Enzyme doses were decided based on CMCase activities

Digits in parenthesis indicate the standard deviation at 95% confidence limit.

Table-4.6 Effect of enzyme dose on the yield, freeness, drainage rate and reducing sugars

Source of enzyme		Enzyme dose, IU/g o.d. wt. pulp	Exp. no.	Yield, %	CSF, ml	Drainage rate, sec	Reducing sugars, mg/ml
Commercial cellulase (CE)		**2.0**	**CE2**	77.9 (1.4)	600	6.21 (0.21)	0.214 (0.025)
		4.0	**CE4**	78.01 (0.8)	620	6.20 (0.30)	0.325 (0.031)
		6.0	**CE5**	77.1 (1.1)	660	5.95 (0.17)	0.498 (0.043)
		8.0	**CE6**	76.4 (1.4)	650	6.01 (0.29)	0.515 (0.045)
		10.0	**CE7**	75.9 (1.2)	650	5.80 (0.21)	0.542 (0.034)
Crude enzyme	*T. harzianum* (TH)	**0.2**	**TH2**	78.3 (1.3)	520	5.95 (0.11)	0.321 (0.034)
		0.4	**TH4**	77.0 (1.5)	540	5.90 (0.19)	0.398 (0.029)
		0.6	**TH5**	77.1 (1.1)	590	5.84 (0.17)	0.423 (0.038)
		0.8	**TH6**	76.4 (1.4)	610	5.75 (0.16)	0.454 (0.045)
		1.0	**TH7**	75.9 (1.2)	620	5.46 (0.13)	0.643 (0.075)
	C. cinerea (CC)	**0.2**	**CC2**	78.2 (1.3)	530	6.24 (0.13)	0.399 (0.025)
		0.4	**CC4**	78.3 (1.0)	570	6.14 (0.23)	0.456 (0.032)
		0.6	**CC5**	78.0 (1.5)	630	5.75 (0.20)	0.498 (0.041)
		0.8	**CC6**	77.9 (1.0)	630	5.71 (0.19)	0.605 (0.054)
		1.0	**CC7**	76.8 (1.5)	640	5.62 (0.16)	0.708 (0.084)

Digits in parenthesis indicate the standard deviation at 95% confidence limit.

Table-4.7 Effect of pulp consistency on paper properties

Source of enzyme		Pulp consistency, %	Exp. no.	Deinking efficiency, %	Residual ink, ppm	Brightness, %	Opacity, %	Tensile index, N.m/g	Folding endurance	Burst index, kPa.m^2/g	Tear index, mN.m^2/g	Initial ink, ppm (EP)
Commercial cellulase (CE)		6	CE8	61.5 (1.0)	120.65 (4.78)	74.5 (0.8)	90.8 (0.8)	30.12 (1.91)	0.80 (0.10)	1.68 (0.33)	5.97 (0.34)	287.09 (5.37)
		8	CE9	71.1 (1.1)	98.01 (3.65)	74.9 (0.3)	90.2 (0.8)	30.56 (2.87)	0.81 (0.08)	1.65 (0.35)	6.10 (0.64)	298.65 (5.45)
		10	CE5	94.6 (1.3)	31.95 (3.45)	78.7 (0.5)	90.1 (1.0)	29.31 (3.64)	0.75 (0.10)	1.58 (0.36)	5.55 (0.88)	308.46 (4.78)
		12	CE10	89.1 (1.0)	48.09 (2.12)	78.1 (0.3)	89.9 (0.5)	28.09 (1.87)	0.74 (0.11)	1.32 (0.25)	5.45 (0.54)	307.98 (3.67)
		14	CE11	85.8 (1.8)	56.8 (3.12)	77.5 (0.4)	89.3 (0.4)	28.65 (2.87)	0.75 (0.10)	1.43 (0.25)	5.40 (0.45)	300.98 (4.34)
Crude enzyme	*T. harzianum* (TH)	6	TH8	73.8 (1.2)	100.21 (4.65)	78.2 (0.8)	90.2 (0.2)	27.29 (1.78)	0.83 (0.17)	1.35 (0.21)	6.25 (0.36)	328.32 (5.68)
		8	TH9	86.9 (2.0)	61.27 (3.25)	80.2 (0.6)	90.1 (0.3)	27.98 (1.82)	0.80 (0.14)	1.41 (0.23)	6.15 (0.31)	341.32 (6.25)
		10	TH6	93.1 (1.7)	40.12 (4.35)	83.1 (0.7)	89.7 (0.4)	29.85 (2.02)	0.79 (0.18)	1.59 (0.24)	5.95 (0.45)	321.25 (4.25)
		12	TH10	88.4 (1.9)	55.21 (4.12)	82.7 (0.8)	88.8 (0.3)	29.32 (1.43)	0.79 (0.13)	1.55 (0.19)	5.80 (0.33)	328.21 (3.85)
		14	TH11	89.0 (2.0)	51.23 (3.25)	79.2 (0.9)	89.2 (0.2)	29.60 (1.68)	0.76 (0.14)	1.51 (0.18)	5.85 (0.43)	311.02 (7.58)
	C. cinerea (CC)	6	CC8	80.8 (0.6)	78.23 (5.52)	82.2 (0.8)	90.7 (0.3)	27.44 (1.64)	0.82 (0.14)	1.48 (0.22)	6.25 (0.36)	323.32 (5.65)
		8	CC9	88.5 (0.6)	55.23 (2.60)	82.9 (1.0)	90.4 (0.2)	28.65 (1.67)	0.82 (0.11)	1.59 (0.24)	6.52 (0.31)	341.32 (3.65)
		10	CC4	90.1 (0.7)	49.35 (3.51)	83.2 (0.7)	89.6 (0.3)	29.34 (1.54)	0.84 (0.15)	1.59 (0.25)	6.90 (0.45)	335.39 (5.95)
		12	CC10	93.0 (0.5)	39.53 (2.58)	83.7 (0.7)	89.7 (0.1)	28.85 (1.32)	0.86 (0.11)	1.40 (0.20)	6.83 (0.33)	326.32 (4.68)
		14	CC11	93.5 (0.5)	39.23 (3.25)	82.1 (0.3)	89.1 (0.4)	26.35 (1.55)	0.72 (0.12)	1.26 (0.19)	5.98 (0.43)	345.02 (4.95)

[#] EB value for commercial cellulase= 16.3 ppm, Crude enzyme of *T. harzianum*=19.21 ppm, Crude enzyme of *C. cin*erea=17.98

Digits in parenthesis indicate the standard deviation at 95% confidence limit.

Table-4.8 Effect of pulp consistency on the yield, freeness, drainage rate and reducing sugars

Source of enzyme		Pulp consistency, %	Exp. no.	Yield ,%	CSF±10, ml	Drainage rate, sec	Reducing sugars, mg/ml
Commercial cellulase (CE)		**6**	**CE8**	79.4 (0.9)	560	6.31 (0.20)	0.365 (0.035)
		8	**CE9**	78.9 (0.6)	570	6.30 (0.36)	0.405 (0.042)
		10	**CE5**	77.1 (1.1)	620	5.95 (0.17)	0.498 (0.043)
		12	**CE10**	77.6 (1.0)	600	6.14 (0.34)	0.475 (0.065)
		14	**CE11**	76.8 (1.5)	580	6.20 (0.18)	0.491 (0.054)
Crude enzyme	*T. harzianum* (TH)	**6**	**TH8**	79.1 (0.6)	590	5.80 (0.26)	0.400 (0.045)
		8	**TH9**	77.8 (1.1)	590	5.82 (0.21)	0.410 (0.041)
		10	**TH6**	76.4 (1.4)	610	5.75 (0.16)	0.454 (0.045)
		12	**TH10**	77.1 (1.2)	610	5.67 (0.22)	0.440 (0.035)
		14	**TH11**	77.5 (0.8)	590	5.64 (0.15)	0.425 (0.028)
	C. cinerea (CC)	**6**	**CC8**	78.9 (1.2)	570	6.35 (0.31)	0.395 (0.041)
		8	**CC9**	77.8 (0.7)	580	6.05 (0.25)	0.465 (0.049
		10	**CC4**	78.0 (1.5)	630	5.75 (0.20)	0.498 (0.041)
		12	**CC10**	77.5 (1.1)	610	5.86 (0.26)	0.548 (0.031)
		14	**CC11**	76.8 (0.8)	570	5.69 (0.18)	0.415 (0.039)

Digits in parenthesis indicate the standard deviation at 95% confidence limit.

Table-4.9 Effect of reaction time on paper properties

Source of enzyme		Reaction time, min	Exp. no.	Deinking efficiency, %	Residual ink, ppm	ISO Brightness, %	Opacity , %	Tensile index, N.m/g	Folding endurance	Burst index, kPa.m^2/g	Tear index, mN.m^2/g	Initial ink, ppm (EP)[#]
Commercial cellulase (CE)		30	CE12	48.9 (1.0)	156.09 (4.10)	72.7 (0.5)	90.1 (0.5)	30.87 (3.54)	0.82 (0.09)	1.70 (0.35)	6.14 (0.34)	289.98 (2.76)
		60	CE13	62.7 (1.4)	121.07 (4.23)	74.9 (0.4)	89.9 (0.3)	30.12 (2.63)	0.82 (0.09)	1.65 (0.30)	6.01 (0.53)	297.45 (3.87)
		90	CE5	94.6 (1.3)	31.95 (3.45)	78.7 (0.5)	90.1 (1.0)	29.31 (3.64)	0.75 (0.10)	1.58 (0.36)	5.55 (0.88)	308.46 (4.78)
		120	CE14	89.8 (1.7)	45.11 (1.98)	78.3 (0.5)	88.7 (0.6)	28.31 (2.54)	0.77 (0.10)	1.56 (0.24)	5.67 (0.56)	298.45 (5.43)
		150	CE15	88.2 (1.8)	50.11 (2.87)	77.9 (0.3)	89.0 (0.8)	28.76 (1.98)	0.73 (0.11)	1.45 (0.26)	5.43 (0.23)	301.65 (3.56)
Crude enzyme	*T. harzianum* (TH)	30	TH12	73.7 (1.7)	95.65 (2.03)	78.0 (1.0)	89.3 (0.9)	28.30 (1.45)	1.44 (0.32)	6.68 (0.35)	0.83 (0.13)	310.00 (3.65)
		60	TH13	93.9 (1.2)	37.52 (1.95)	83.0 (0.8)	89.7 (0.4)	30.45 (2.05)	1.52 (0.14)	6.04 (0.45)	0.82 (0.17)	318.32 (7.56)
		90	TH6	93.1 (1.9)	40.12 (1.85)	83.1 (0.7)	89.7 (0.4)	29.85 (1.39)	1.59 (0.24)	5.95 (0.45)	0.79 (0.18)	321.25 (4.68)
		120	TH14	93.4 (2.0)	39.10 (1.45)	83.5 (0.9)	90.2 (1.0)	28.20 (1.76)	1.36 (0.12)	5.40 (0.34)	0.73 (0.12)	319.32 (8.25)
		150	TH15	93.5 (1.3)	39.00 (1.65)	83.5 (0.6)	90.0 (0.6)	26.98 (1.87)	1.27 (0.11)	5.14 (0.43)	0.67 (0.13)	323.23 (4.02)
	C. cinerea (CC)	30	CC12	73.5 (0.9)	95.65 (5.52)	78.0 (0.7)	90.6 (0.5)	27.68 (1.33)	0.80 (0.11)	1.33 (0.34)	5.98 (0.35)	311.32 (6.25)
		60	CC13	95.2 (1.0)	33.23 (2.36)	84.6 (0.5)	89.3 (0.7)	30.14 (1.89)	0.87 (0.14)	1.66 (0.15)	6.24 (0.45)	336.6 (6.00)
		90	CC10	93.0 (1.0)	39.53 (2.58)	83.6 (0.7)	89.7 (0.6)	28.85 (1.32)	0.86 (0.11)	1.59 (0.25)	6.90 (0.45)	326.32 (4.12)
		120	CC14	92.5 (1.1)	41.85 (3.50)	83.0 (0.7)	88.7 (0.5)	25.27 (1.62)	0.71 (0.10)	1.25 (0.13)	5.80 (0.34)	334.95 (3.29)
		150	CC15	91.0 (1.0)	45.32 (2.65)	80.3 (0.5)	88.4 (0.4)	24.35 (1.72)	0.70 (0.11)	1.21 (0.20)	5.40 (0.43)	323.23 (5.28)

[#] EB value for commercial cellulase= 16.3 ppm, Crude enzyme of *T. harzianum*=19.21 ppm, Crude enzyme of *C. cin*erea=17.98

Digits in parenthesis indicate the standard deviation at 95% confidence limit.

Table-4.10 Effect of reaction time on the yield, freeness, drainage rate and reducing sugars

Source of enzyme		Reaction time, min	Exp. no.	Yield , %	CSF±10, ml	Drainage rate, sec	Reducing sugars, mg/ml
Commercial cellulase (CE)		**30**	**CE12**	79.0 (0.5)	480	6.98 (0.43)	0.235 (0.021)
		60	**CE13**	77.5 (0.9)	540	6.50 (0.34)	0.389 (0.041)
		90	**CE5**	77.1 (1.1)	620	5.95 (0.17)	0.498 (0.043)
		120	**CE14**	75.6 (1.5)	600	6.28 (0.25)	0.532 (0.032)
		150	**CE15**	75.2 (1.9)	580	6.38 (0.28)	0.568 (0.045)
Crude enzyme	*T. harzianum* (TH)	**30**	**TH12**	78.6 (0.6)	550	5.98 (0.11)	0.387 (0.011)
		60	**TH13**	76.5 (0.7)	620	5.78 (0.13)	0.470 (0.021)
		90	**TH6**	76.4 (1.4)	610	5.75 (0.16)	0.454 (0.045)
		120	**TH14**	76.1 (0.5)	630	5.57 (0.21)	0.601 (0.085)
		150	**TH15**	75.8 (0.9)	630	5.6 (0.12)	0.676 (0.032)
	C. cinerea (CC)	**30**	**CC12**	77.5 (0.9)	550	6.32 (0.13)	0.360 (0.015)
		60	**CC13**	76.1 (0.5)	610	5.60 (0.16)	0.482 (0.035)
		90	**CC10**	76.5 (1.1)	610	5.86 (0.26)	0.548 (0.031)
		120	**CC14**	75.2 (0.9)	620	5.50 (0.25)	0.698 (0.098)
		150	**CC15**	74.5 (1.1)	630	5.62 (0.14)	0.777 (0.065)

Digits in parenthesis indicate the standard deviation at 95% confidence limit.

Table-4.11 Optimized parameters of the chemical and enzymatic deinking

Parameters optimized	Chemical Deinking	Enzymatic deinking		
		Commercial enzyme	Crude enzyme of *T. harzianum*	Crude enzyme of *C. cinerea*
Pulp consistency,%	10	10	10	12
Temperature, °C	70	50	55	55
Hydrapulping time, min	30	30	30	30
NaOH,%	2	0.25	0.25	0.25
Na_2SiO_3,%	2	NA	NA	NA
DTPA,%	0.08	NA	NA	NA
Oleic acid,%	0.8	0.8	NA	NA
H_2O_2, %	1	NA	NA	NA
Enzyme dose, IU/g o.d. pulp	NA	6	0.8	0.6
End pH±0.1 (hydrapulping)	10.5	5.8	5.5	5.5
Hydrolysis time, min	NA	90	60	60
Flotation time, min	10	10	10	10
Flotation temperature±2, °C	40	40	40	40

Table-4.12 Comparison of enzymatic and chemical deinking in terms of deinking performance and pulp and handsheets properties

Properties	Chemical deinking	Enzymatic deinking		
		Commercial enzyme	Crude enzyme of *T. harzianum*	Crude enzyme of *C. cinerea*
Deinking efficiency,%	75.9 (1.0)	94.6 (1.3)	93.9 (1.1)	95.2 (1.0)
ISO brightness,%	80.4 (0.5)	78.7 (0.7)	83.0 (0.8)	84.6 (0.5)
Residual ink, ppm	77.22 (2.32)	31.95 (1.56)	37.52 (2.31)	33.23 (2.36)
Yield,%	77.9 (0.8)	77.1 (0.7)	76.5 (0.7)	76.1 (0.5)
CSF , ml	510±10	620±10	620±10	610±10
Drainage time, sec	6.72 (0.67)	5.95 (0.17)	5.78 (0.13)	5.56 (0.13)
Tensile index, N.m/g	28.53 (4.91)	29.31 (3.64)	30.45 (2.05)	30.14 (1.89)
Folding endurance	0.746 (0.123)	0.750 (0.103)	0.823 (0.169)	0.865 (0.142)
Burst index, kPa.m^2/g	1.34 (0.14)	1.58 (0.36)	1.52 (0.14)	1.66 (0.15)
Tear index, mN.m^2/g	6.75 (0.85)	5.55 (0.88)	6.04 (0.45)	6.24 (0.45)
Opacity,%	88.66 (0.62)	90.11 (1.01)	89.65 (0.37)	89.32 (1.06)
Formation index	155.6 (5.6)	161.6 (4.7)	162.09 (9.6)	160.10 (2.9)
Contrast intensity,%	7.71 (0.16)	7.40 (0.11)	7.29 (0.26)	7.44 (0.10)
Specific perimeter, 1/mm	3.20 (0.05)	3.20 (0.04)	3.16 (0.09)	3.18 (0.02)
Bauer McNett fiber classification (wt% basis)				
+40 mesh	16.2	18.2	19.8	19.2
+100 mesh	53.5	57.5	58.9	56.2
+150 mesh	6.6	6.5	7.5	6.5
+200 mesh	6.6	4	1.2	5.5
Fines	17.1	13.8	12.6	12.6
Effluent characteristics				
Soluble COD, ppm	270 (9)	148 (4)	168 (7)	189 (5)
BOD_5, ppm	122 (5)	85 (7)	99 (5)	105 (4)
BOD/COD ratio	0.45	0.57	0.59	0.55

Table-4.13 Comparison of control experiments of enzymatic and chemical deinking in terms of deinking performance and pulp and handsheets properties

Properties	Control (Chemical deinking)	Control (Enzymatic deinking)		
		Commercial enzyme	Crude enzyme of *T. harzianum*	Crude enzyme of *C. cinerea*
Deinking efficiency,%	22.9 (2.6)	38.4 (1.0)	42.4 (1.3)	43.7 (0.9)
ISO brightness,%	72.3 (1.2)	73.0 (0.9)	72.9 (0.7)	73.1 (0.4)
Residual ink, ppm	200.56 (6.32)	196.32 (4.68)	192.78 (2.32)	185.24 (8.32)
Yield,%	80.1 (0.6)	80.2 (0.3)	79.5 (0.5)	79.1 (0.5)
CSF, ml	480±10	470±10	490±10	470±10
Drainage time, sec	7.23 (0.32)	7.49 (0.21)	7.29 (0.31)	7.38± 0.21)
Tensile index, N.m/g	27.96 (3.85)	27.65 (3.65)	26.65 (2.65)	26.95 (2.45)
Folding endurance	0.746 (0.123)	0.761 (0.102)	0.791 (0.102)	0.701 (0.142)
Burst index, kPa.m^2/g	1.34 (0.14)	1.35 (0.24)	1.35 (0.31)	1.41 (0.21)
Tear index, mN.m^2/g	6.75 (0.85)	6.85 (1.01)	7.01 (0.91)	7.11 (0.88)
Opacity,%	92.32 (0.32)	90.89 (0.86)	90.65 (0.52)	90.25 (0.62)
Bauer McNett fiber classification (wt% basis)				
+40 mesh	20.2	19.6	20.5	20.5
+100 mesh	60.3	58.9	57.6	57.6
+150 mesh	5.5	4.2	3.3	4.3
+200 mesh	1.4	4.3	4.5	3.2
Fines	12.6	13.0	14.1	14.4
Effluent characteristics				
Soluble COD, ppm	148 (8)	152 (9)	149 (5)	155 (6)
BOD_5, ppm	72 (5)	79 (6)	74 (3)	78 (4)
BOD/COD ratio	0.48	0.51	0.50	0.50

Table-4.14 Crystallinity index (CI) of the deinked pulp obtained after treatment with chemicals, commercial cellulase, crude enzyme of *T. harzianum*, and crude enzyme of *C. cinerea* (XRD analysis)

	CI,% (Treated pulp)	**CI,% (Control pulp)**
Chemical	37.0	46.8
Commercial cellulase	40.7	47.8
Crude enzyme of *T. harzianum*	42.2	46.2
Crude enzyme of *C. cinerea*	43.7	46.7

Table-4.15 Comparison of enzymatic and chemical deinking in terms of number of dirt specks, average area and dirt area

Deinking approach		**Number of specks/m²**		**Average area (mm²)**		**ppm (mm²/m²)**	
		Physical	TAPPI	Physical	TAPPI	Physical	TAPPI
Chemical	**$Blank_C$**	2081344	334218	0.042	0.045	87364	15072
	$Control_C$	1676393	190981	0.033	0.035	55848	6675
	Chemical	360212	29355	0.041	0.047	14771	1380
Commercial cellulase	**$Blank_{CE}$**	1453581	351901	0.056	0.052	81624	18308
	$Control_{CE}$	742706	145004	0.054	0.061	40036	8889
	Commercial cellulase	330681	22989	0.026	0.028	8683	639
Crude enzymes	**$Blank_{TH}$**	1529620	352785	0.054	0.052	82591	18501
	$Control_{TH}$	756852	148541	0.050	0.055	37826	8208
	T. harzianum	330681	15915	0.024	0.033	7857	523
	$Blank_{CC}$	2076039	381079	0.046	0.030	95878	18142
	$Control_{CC}$	1659593	201592	0.034	0.036	56718	7259
	C. cinerea	244209	12732	0.031	0.037	7566	466

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Annexure

Annexure-1 List of the chemicals

Name of the chemicals	Name of the company	Name of the chemicals	Name of the company
3, 5, dinitro salicyclic acid	Merck	Mycological peptone	Hi media
Acetyl acetone	Qualigens	N-acetyl glucosamine	Hi media
Agar- agar	Hi media	Nicotinic acid	Merck
Ammonium acetate	Merck	Oleic acid	Merck
Ammonium chloride	Hi media	P- dimethyl amino benzaldehyde	Hi media
Ammonium ferrous sulphate hexahydrate	Qualigens	Parafilm	Hi media
Ammonium nitrate	Merck	Peptone	Hi media
Ammonium sulphate	Merck	Peptone bacteriological	Hi media
Asparagine	Qualigens	Phenol crystals	Qualigens
Aspartic acid	Merck	Potassium chloride	Hi media
Beef extract	Hi media	Potassium dihydrogen phosphate	Hi media
Bovine serum albumin	Hi media	Potassium hydrogen phosphate	Hi media
Birch wood xylan	Sigma-Aldrich	Potassium permanganate	Qualigens
Calcium chloride, dihydrate	Merck	Potassium sodium tartarate tetrahydrate	Merck
Calcium pantothenate	Qualigens	Potato dextrose agar	Hi media
Carboxy methyl cellulose	Sigma-Aldrich	Proteose peptone	Hi media
Cellobiose	Hi media	Pyridoxine	Merck
Citric acid	Hi media	Riboflavin	Merck
Cobalt chloride	Qualigens	Sodium carbonate	Qualigens
Congo red dye	Hi media	Sodium chloride	Hi media
Copper sulphate pentahydrate	Hi media	Sodium hydrogen phosphate	Hi media
Cyanocobalamine	Merck	Sodium hydroxide	Qualigens
Cysteine	Qualigens	Sodium hydroxide pellets	Merck
D-biotin	Merck	Sodium nitrate	Hi media
Dextrose	Hi media	Sodium phosphate	Hi media
DTPA	Qualigens	Sodium silicate	Qualigens
EDTA	Qualigens	Sodium sulphite anhydrous	Qualigens
Ethanol	Qualigens	Soya peptone	Hi media
Folic acid	Qualigens	Sucrose	Qualigens
Folin reagent	Qualigens	Sulphuric acid Conc.	Qualigens
Formaldehyde	Qualigens	Thiamine hydrochloride	Merck
Fructose	Merck	Triton X100	Hi media
Galactose	Hi media	Tryptone	Hi media
Hydrochloric acid Conc.	Qualigens	Tryptophan	Merck
N acetyl Glucosamine	Sigma- Aldrich	Tween 20	Hi media
Glucose	Hi media	Tween 40	Hi media
Glutamic acid	Merck	Tween 60	Hi media
Glutarldehyde	Qualigens	Tween-80	Merck
Glycerol	Merck	Urea	Merck
Glycine	Hi media	Valine	Qualigens
Hydrogen peroxide	Qualigens	Whatman no. 1 filter paper	Camlab. Ltd.
Iron sulphate heptahydrate	Merck	Xylan	Sigma-Aldrich
Isoleucine	Qualigens	Xylose	Sigma-Aldrich
Lactose	Hi media	Yeast extract	Hi media
L-ascorbic acid,	Merck	Zinc sulphate	Qualigens
Leucine	Qualigens		
Magnesium sulphate, heptahydrate	Merck		
Malt extract	Hi media		
Manganese sulphate	Merck		
Meat peptone	Hi media		
Metheonine	Qualigens		

Annexure-2 Print format to print photocopier papers used as waste papers for deinking

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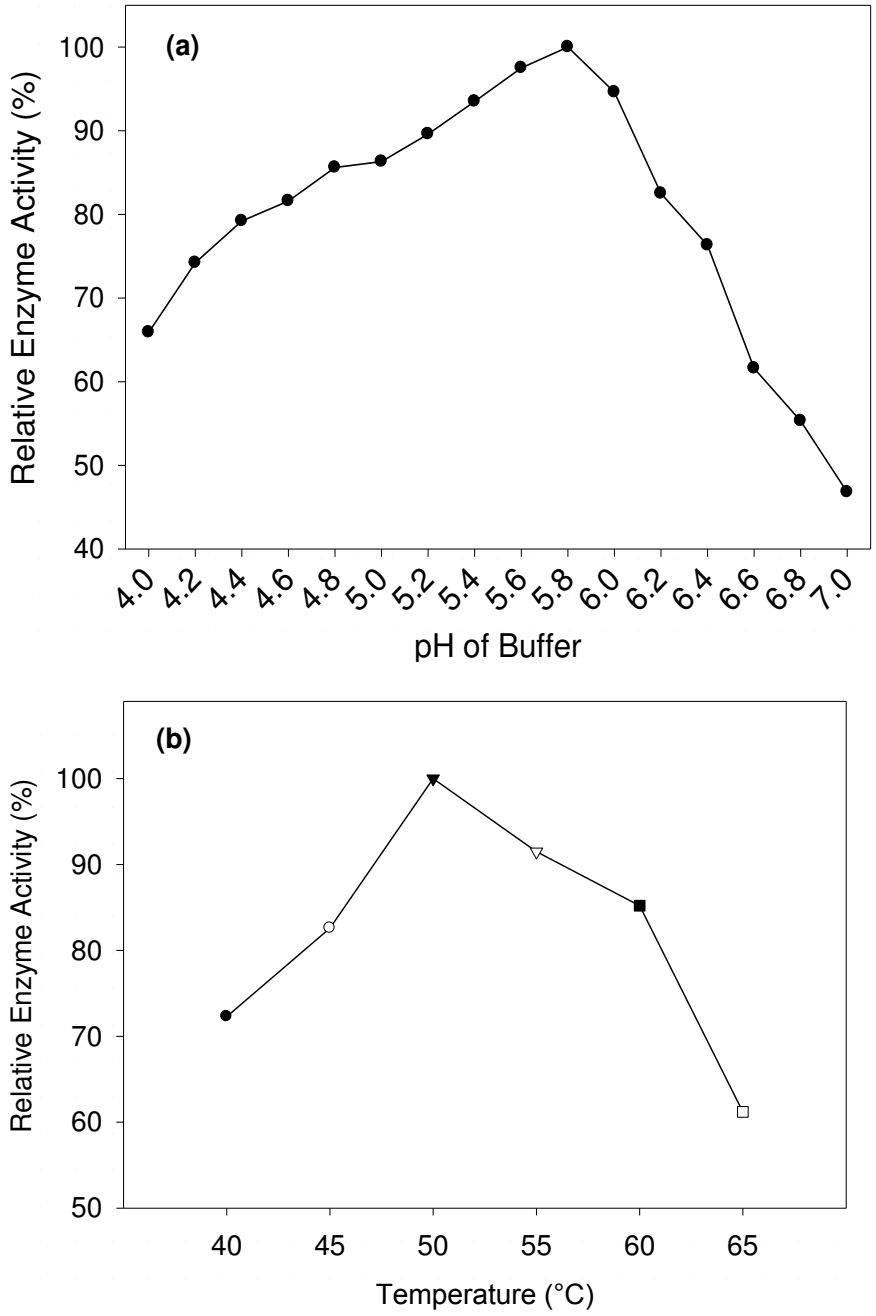
Annexure-3 Properties of initial unprinted paper

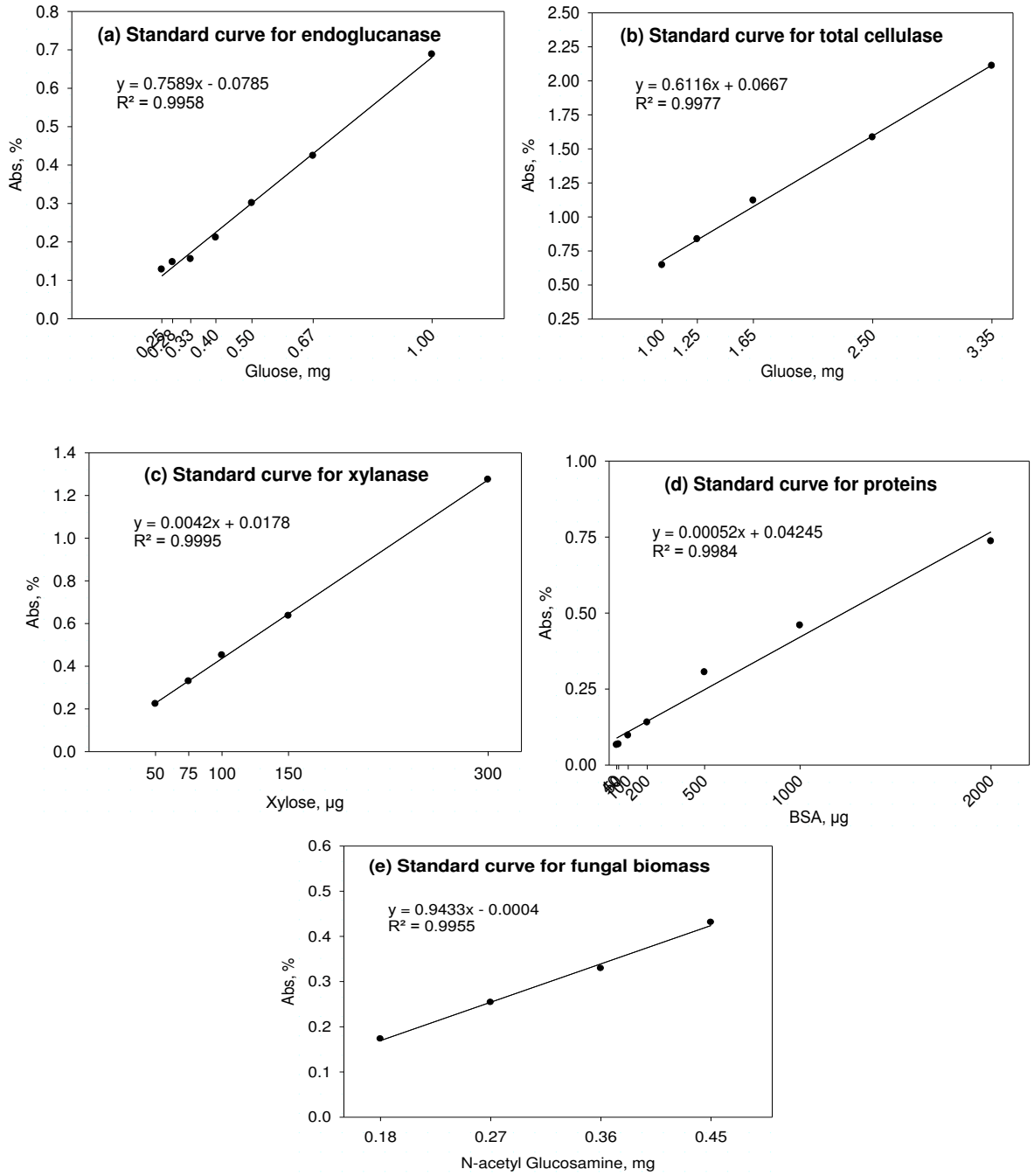
Grammage, g/m^2	75
Ash,%	12.2 (0.3
Brightness	85.63 (0.35
Tensile index (N.m/g)	45.71 (2.45
Folding endurance	1.1461 (0.1527
Burst index (kPa.m^2/g)	1.44 (0.27
Tear index (mN.m^2/g)	5.95 (0.53



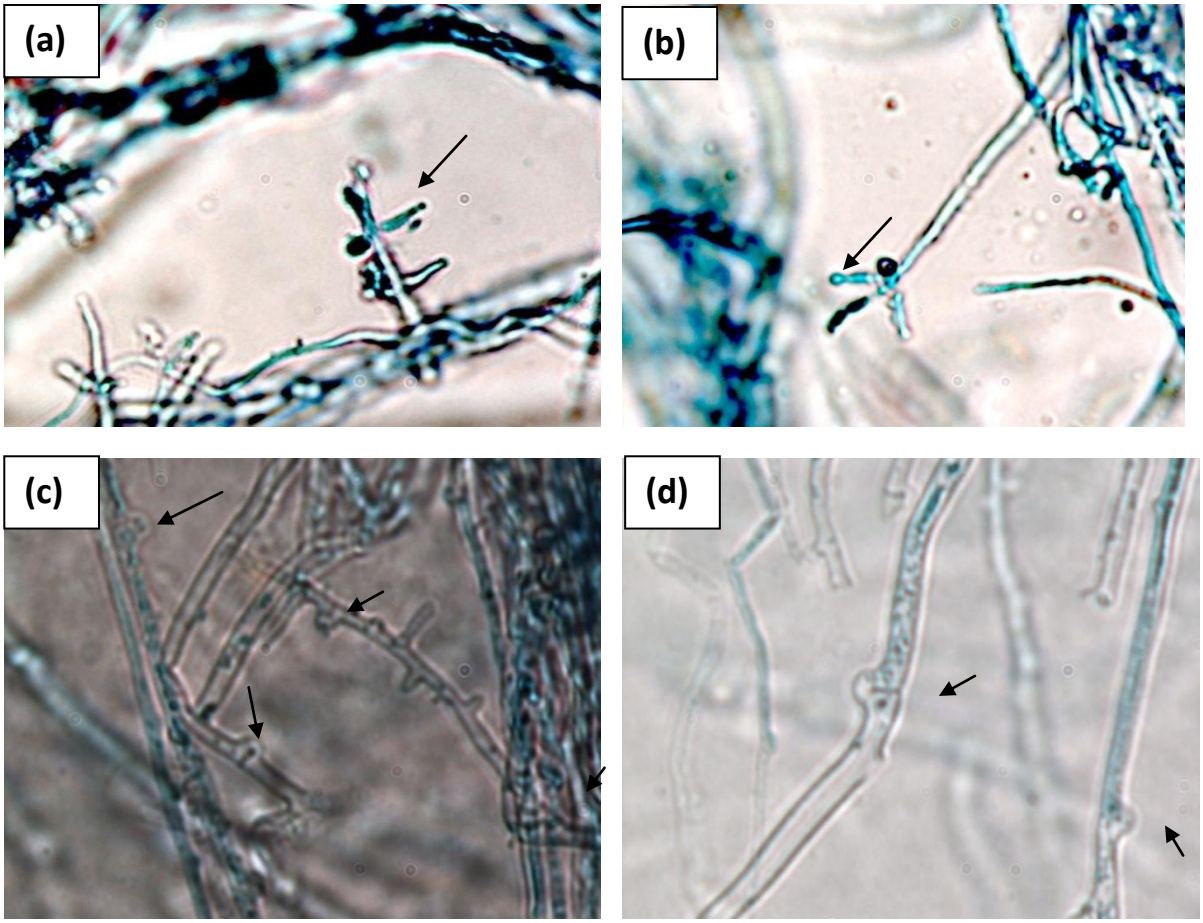
Annexure-4 (a) pH and (b) temperature profile of the commercial cellulase used for enzymatic deinking

Annexure-5 List of the instruments

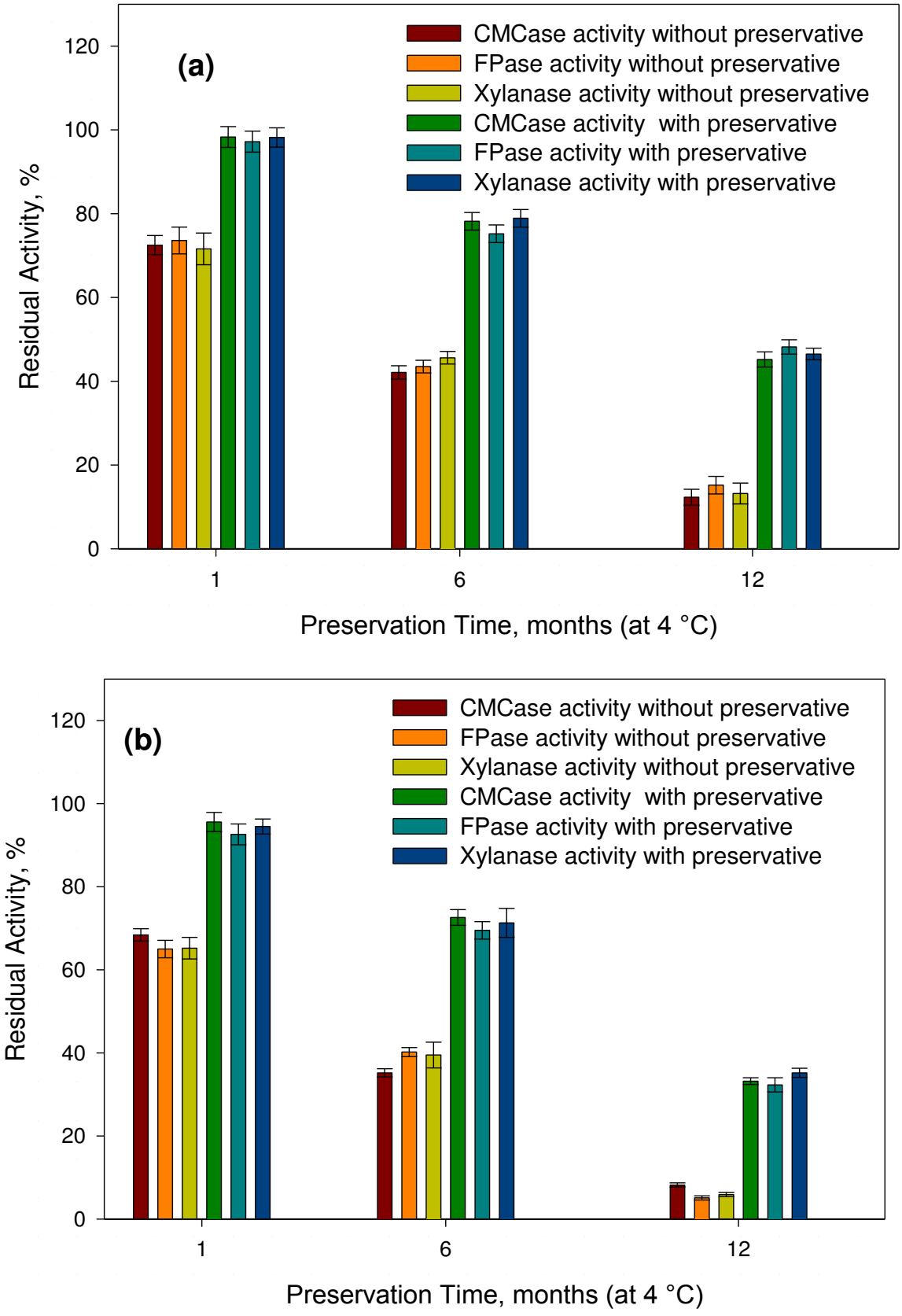
Instrument name/Test	**Make**
Autoclave	HC, Jas- Anz
BOD incubator	Widsons Scientific work, New Delhi, India
Brightness, opacity and ERIC tester	Elrepho Lorentzen & Wetters 070/071,
British handsheet former	Universal Engineering Corporation, Saharanpur, India
Burst tester (Mullen)	Aktiebolaget, Maskinaffer Lorentzen & Wetters, Stockholm Sweden
Centrifuge, R23	Remi Instruments, New Delhi
COD digester	Spectralab COD Digestion Appratus,Model No. 2015D
CSF tester	Lorentzen & Wetters, Stockholm Sweden
Disintegrator	Universal Engineering Corporation, Saharanpur, India
Folding endurance (Kohler- Mullin)	Aktiebolaget, Maskinaffer Lorentzen & Wetters, Stockholm Sweden
Fourier transform infrared spectroscope	PerkinElmer FT-IR C91158 spectrometer system, United Kingdom.
High consistency laboratory pulper	FORMAX, 450H, Adirondack Machine Corporation, Queensbury, New York
Image analyser	Zeiss Axio Scope A.1, Carl Zeiss Microimaging, Gmbh, Germany with Axiocam MRc 5 camera.
Laboratory flotation cell	Delta25, Voith Paper Fiber Systems GmbH & Co. Germany
Laminar air flow	HC, Jas- Anz
Mc Baur Net fibre classifier	Universal Engineering Corporation, Saharanpur, India
Oven	Vaxelstrom, Sweden & Perfit, Ambala
pH meter	Toshniwal Instruments manufacturing Pvt. Ltd. Ajmer, India
Pressing machine	Weverk Lorentzen & Wetters,
Refrigerator	Whirlpool
Scanning electron microsope, Quanta, 200 F Model,	FEI, Netherland,
Tear tester (Elmendorf)	Aktiebolaget, Maskinaffer Lorentzen & Wetters, Stockholm Sweden
Tensile tester (Electro hydraulic)	Aktiebolaget, Maskinaffer Lorentzen & Wetters, Stockholm Sweden
The PAPRICAN Micro-scanner version 1.2 (Rel. RI214, RI515)	OpTest® Equipment Inc., Canada
UV-Vis spectrophotometer	Systronics UV-Vis Spectrophotometer 118,
Vortex, CM 101 cyclomixer	Remi Equipments Pvt, Ltd.
Water bath	Narang Scientific Works Pvt. Ltd.
Weighing balance	Santorius (max 210g & 5kg)
X-ray diffractometer,	Bruker AXS D8 Advance Diffractrometer (Germany)



Annexure-6 Standard curves used for the determination of (a) endoglucanase; (b) total cellulase; (c) xylanase; (d) proteins and (e) fungal biomass



Annexure-7 Photographs of the isolated fungal strains after staining with lactophenol cotton blue (a, b) arrows indicating mycelia bearing conidiophores and conidiospores, of *T. harzianum* PPDDN-10 NFCCI 2925 (c) arrows indicating clamp connections at the septa of a basidiomycota hypha of *C. cinerea* PPHRI-4 NFCCI-3027



Annexure-8 Residual activities of crude enzyme stored at 4 °C with and without preservative (Sodium azide-0.02% w/v) (a) crude enzyme of *T. harzianum* PPDDN-10 NFCCI 2925 *(b)* crude enzyme of *C. cinerea* PPHRI-4 NFCCI-3027

MOLECULAR IDENTIFICATION REPORT

Name : Mr. Punit Pathak
INSTITUTE/ORGANIZATION : Department of Paper Technology, IIT Roorkee, Saharanpur, 247001, Uttar Pradesh
Data Sheet Number : 141/1579/**16/04**/2012/P Pathak/Uttar Pradesh/-/---
JOB TITLE : To identify the culture PPDDN-10
PROCEDURE:

- Genomic DNA was isolated in pure form, from the culture provided by the sender.
- The nearly ~550 bp rDNA fragments were successfully amplified using universal primers.
- The sequencing PCR was set up with ABI-BigDye® Terminatorv3.1 Cycle Sequencing Kit.
- The raw sequence obtained from ABI 3100 automated DNA sequencer was manually edited for inconsistency.
- The sequence data was aligned with publicly available sequences & analyzed to reach identity.

RESULT for Sample:

Strain 1579 showed 99% sequence similarity with genus *Hypocrea* Fr. (1825) species ***Hypocrea lixii*** Pat. (1891). The genus currently has 171 species as mentioned in the *Dictionary of Fungi*, 10th edn. edited by Paul Kirk et al. in 2008.

STATUS OF YOUR SENT FUNGUS No. **1579** (QUERY)

Hypocrea lixii

SEQUENCE ANALYSIS (**536 bases**) with NCBI sequence Accession JX436467.1

```
NCBI Accession JX436467.1/ strain PBCA / Hypocrea lixii
Length=624
Score = 966 bits (523), Expect = 0.0
Identities = 527/529 (99%), Gaps = 1/529 (0%)
Strand=Plus/Plus

Query  8    CGAGTTTACAACTCCCAAACCCAATGTGAACGTTACCAAACTGTTGCCTCGGCGGGATCT  67
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Sbjct  58   CGAGTTTACAACTCCCAAACCCAATGTGAACGTTACCAAACTGTTGCCTCGGCGGGATCT  117

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Query  188  GCGTTTCGAAAATGAATCAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAA  247
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Query  248  GAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTT  307
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Sbjct  298  GAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTT  357

Query  308  TGAACGCACATTGCGCCCGCCAGTATTCTGGCGGGCATGCCTGTCCGAGCGTCATTTCAA  367
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  358  TGAACGCACATTGCGCCCGCCAGTATTCTGGCGGGCATGCCTGTCCGAGCGTCATTTCAA  417

Query  368  CCCTCGAACCCCTCCGGGGGGGTCGGCGTTGGGGATCGGCCCTGCCTTGGCGGTGGCCGTC  427
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Query  428  TCCGAAATACAGTGGCGGTCTCGCCGCAGCCTCTCCTGCGCANTAGTTTGCACACTCGCA  487
            |||||||||||||||||||||||||||||||||||||||||| ||||||||||||||||||
Sbjct  478  TCCGAAATACAGTGGCGGTCTCGCCGCAGCCTCTCCTGCGCAGTAGTTTGCACACTCGCA  537
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Sbjct  538  TCGGGAGCGCGGCGCGTCCACAGCCGTTAAACACCCAACTTCTG-AAAT  585
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Sequences producing significant alignments:

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
JX436467.1	Hypocrea lixii strain PBCA 18S ribosomal RNA gene, partial sequence;	966	966	98%	0.0	99%
JQ040359.1	Hypocrea lixii strain SHSJ5003 18S ribosomal RNA gene, partial seque	966	966	98%	0.0	99%
JN943373.1	Hypocrea nigricans strain NBRC 31290 internal transcribed spacer 1,	966	966	98%	0.0	99%
JN618345.1	Hypocrea lixii strain BHU150 internal transcribed spacer 1, partial sec	966	966	98%	0.0	99%
JN618344.1	Hypocrea lixii strain BHU105 internal transcribed spacer 1, partial sec	966	966	98%	0.0	99%
JN618343.1	Hypocrea lixii strain BHU51 18S ribosomal RNA gene, partial sequenc	966	966	98%	0.0	99%
JN618342.1	Hypocrea lixii strain BHU199 18S ribosomal RNA gene, partial sequen	966	966	98%	0.0	99%
JN618339.1	Hypocrea lixii strain BHU166 18S ribosomal RNA gene, partial sequen	966	966	98%	0.0	99%
JN604838.1	Hypocrea lixii strain BHU162 18S ribosomal RNA gene, partial sequen	966	966	98%	0.0	99%
JN604836.1	Hypocrea lixii strain BHU110 18S ribosomal RNA gene, partial sequen	966	966	98%	0.0	99%
JN604835.1	Hypocrea lixii strain BHU18 18S ribosomal RNA gene, partial sequenc	966	966	98%	0.0	99%
JN604834.1	Hypocrea lixii strain BHU226 18S ribosomal RNA gene, partial sequen	966	966	98%	0.0	99%
JN604833.1	Hypocrea lixii strain BHU221 18S ribosomal RNA gene, partial sequen	966	966	98%	0.0	99%
JF923805.1	Hypocrea lixii isolate NRCfBA-44 18S ribosomal RNA gene, partial seq	966	966	98%	0.0	99%
JF923802.1	Hypocrea lixii isolate NRCfBA-37 18S ribosomal RNA gene, partial seq	966	966	98%	0.0	99%
JF923801.1	Hypocrea lixii isolate NRCfBA-31 18S ribosomal RNA gene, partial seq	966	966	98%	0.0	99%

Additional References:

1. Zhang et al. Mycol. 98, 1076, 2006, phylogeny.
2. Jaklitsch et al., Stud. Mycol. 56, 135, 2006.
3. Gams, Stud. Mycol. 56, 177pp, 2006.

CONDITIONS AND REMARKS:

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Kind Attn:

Mr. Puneet Pathak
C/o Dr. Satish Kumar, Prof. & Head,
Dept. of Paper Technology
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(S.K. Singh)

महाराष्ट्र विज्ञान वर्धिनी
आघारकर अनुसंधान संस्था
Maharashtra Association for the Cultivation of Science
AGHARKAR RESEARCH INSTITUTE
(An Autonomous Grant-in-Aid Institute under the Department of Science and Technology, Govt. of India)

National Fungal Culture Collection of India (NFCCI)-A National Facility

Sender -Mr. Puneet Pathak, C/o Dr. Satish Kumar, Professor and Head, Department of Paper Technology, IIT Rorkee, Saharanpur Campus, Saharanpur-247001 (UP)

Details of Fungus identified

Sr. No.	Culture	Source	NFCCI Accession	Identification Remarks
1.	PPDDN-10	Wood	2925	*Hypocrea lixii* Pat.

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for

Dr. S.K. Singh
Scientist & Coordinator
DST-National Facility (NFCCI & FIS)
Mycology and Plant Pathology Group
E-mail: nfcci.ari@gmail.com, singhsksingh@gmail.com
Phone: 020-25653680

ARI/NFCCI/FIS/ 2013/Add Reg/ Sl No. 1579/14.01.2013/PNS/VMW/SVS

MOLECULAR IDENTIFICATION REPORT

Name : Mr. Puneet Pathak
Institute/Organization : Dept. of Paper Technology, IIT Roorkee
Saharanpur Campus, Saharanpur UP-247001
Data Sheet Number : 140/1579/**16/04**/2012/P Pathak/Uttar Pradesh/-/---
Job Title : To identify the culture PPHRI-4
Procedure:

- Genomic DNA was isolated in pure form, from the culture provided by the sender.
- The nearly ~650 bp rDNA fragments were successfully amplified using universal primers.
- The sequencing PCR was set up with ABI-BigDye® Terminatorv3.1 Cycle Sequencing Kit.
- The raw sequence obtained from ABI 3100 automated DNA sequencer was manually edited for inconsistency.
- The sequence data was aligned with publicly available sequences & analyzed to reach identity.

RESULT for Sample:

Strain 1579 PPHRI-4 showed 97% sequence similarity with genus *Coprinopsis* P. Karst (1881). The genus currently has 200 species as mentioned in the *Dictionary of Fungi*, 10th edn. edited by Paul Kirk et al. in 2008.

STATUS OF YOUR SENT FUNGUS No. **1579-PPHRI-4** (QUERY)

***Coprinopsis* sp. close to *C. Cinerea* [NFCCI Accession No. 3027]**

SEQUENCE ANALYSIS (654 bases) with NCBI sequence Accession AB499044.1

NCBI Accession AB499044.1/ strain TW 06-150/ *Coprinopsis* sp
Length=688
Score = 1098 bits(594), Expect = 0.0
Identities = 636/656 (97%), Gaps = 3/656 (0%)
Strand=Plus/Minus

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Query  60   AGTAACCAACCCAATCCACGGCGTACATAATTATCACACCAATAGATTAGGGGCACAACC  119
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Query  180  CTGCACACAACAAAGTTGGTGAGGTTGAGAATTTAATGACACTCAAACAGGCATGCTCCT  239
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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Query  240  CGGAATACCAAGGAGCGCAAGGTGCGTTCAAAGATTCGATGATTCACTGAATTCTGCAAT  299
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Sbjct  424  CGGAATACCAAGGAGCGCAAGGTGCGTTCAAAGATTCGATGATTCACTGAATTCTGCAAT  365

Query  300  TCACATTACTTATCGCATTTCGCTGCGTTCTTCATCGATGCGAGAGCCAAGAGATCCGTT  359
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  364  TCACATTACTTATCGCATTTCGCTGCGTTCTTCATCGATGCGAGAGCCAAGAGATCCGTT  305

Query  360  GCTGAAAGTTGTATAGGTTTATAGGCACAAGGCCTTGAGATGACATTCCCTAACATTCAG  419
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Query  420  TTTGGGGTGTGTTAGAAATCATAGACCTGGGAATTCATGGCAAGGCGGCCTTCTTTCTAA  479
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Query  480  AGCCACAGCTAATCACGCATCCGGCTTGCGACCAGAGGTATTCAGGCCACCAAGTGTGTG  539
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Query  540  CACAGGTGGAGAAATAAAGGTGACGGGCGTGCACATGCTCCCGGGGGAGCCAGCGACCAC  599
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Sbjct  125  CACAGGTGGAGAAATAAAGGTGACGGGCGTGCACATGCTCCCGGGGGAGCCAGCGACAAC  66
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Query  600  CACGTCAGATTTATTCAATAATGATCCTTCCGCAGGTTCACCTACGGAA-CCTTGT  654
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Description	Query cover	Max ident	Accession
Coprinopsis sp. TW 06-150	99%	97%	AB499044.1
Uncultured fungus clone L042884-122-064-C05	99%	97%	GQ851873.1
Coprinopsis cinerea strain ATCC MYA-4618	98%	97%	GU327634.1
Uncultured compost fungus clone NK012_054	99%	97%	FM177671.1
Coprinopsis cinerea SFSU DEH2065	99%	97%	AY461825.1
Coprinopsis cinerea	98%	97%	HQ433353.1
Coprinopsis cinerea strain HN08	98%	97%	JQ796875.1
Coprinopsis cinerea strain CNRMA/F 07-32	98%	97%	GQ131575.1
Coprinopsis cinerea voucher:NBRC100011	99%	96%	AB097563.1
Coprinopsis cinerea voucher:NBRC30628	99%	96%	AB097562.1
Coprinopsis aff. brunneofibrillosa Nicholson426	94%	97%	HQ847018.1
Coprinus cinereus KACC49356	95%	97%	AF345819.1
Coprinopsis calospora strain CBS612.91	96%	96%	GQ249275.1

Additional References:

1. Redhead et al., Taxon, 50, 203, 2001, nomenclature.
2. Keirle et al. Fungal Diversity, 15, 33, 2004, Hawaii.
3. Gams, Taxon, 54, 520, 2005, Nomenclature.

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Kind Attn:

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Appl Biochem Biotechnol
DOI 10.1007/s12010-014-0758-9

Production of Crude Cellulase and Xylanase From *Trichoderma harzianum* PPDDN10 NFCCI-2925 and Its Application in Photocopier Waste Paper Recycling

Puneet Pathak · Nishi Kant Bhardwaj · Ajay Kumar Singh

Received: 17 September 2013 / Accepted: 27 January 2014

Abstract This paper implies production of cellulase and xylanase enzyme using a potent strain of *Trichoderma harzianum* for the efficient deinking of photocopier waste papers. Different nutritional and environmental factors were optimized for higher production of cellulase along with xylanase. After fermentation, maximum enzyme extraction was achieved from fermented matter using a three-step extraction process with increased efficiency by 26.6–29.3 % over single-step extraction. Static solid state was found as the best fermentation type using wheat bran (WB) as carbon source and ammonium ferrous sulfate (0.02 M) as nitrogen source. Subsequently, inoculum size (8×10^6 CFU/gds), incubation days (4 days), temperature (34 °C), initial pH (6.0), and moisture ratio (1:3) significantly affected the enzyme production. Cellulase and xylanase activities were found to be maximum at pH 5.5 and temperature 55–60 °C with good stability (even up to 6 h). Furthermore, this crude enzyme was evaluated for the deinking of photocopier waste papers without affecting the strength properties with improved drainage as an additional advantage. The crude enzyme-deinked pulp showed 23.6 % higher deinking efficiency and 3.2 % higher brightness than chemically deinked pulp. Strength properties like tensile, burst indices, and folding endurance were also observed to improve by 6.7, 13.4, and 10.3 %, respectively, for enzyme-deinked pulp. However, the tear index was decreased by 10.5 %. The freeness of the pulp was also increased by 21.6 % with reduced drainage time by 13.9 %.

Keywords Cellulase · Xylanase · *Trichoderma harzianum* · Deinking · Drainage · Photocopier waste paper

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Published online: 27 February 2014

OPTIMIZATION OF CHEMICAL AND ENZYMATIC DEINKING OF PHOTOCOPIER WASTE PAPER

Puneet Pathak, Nishi K. Bhardwaj,* and Ajay K. Singh

The utilization of post-consumer papers in the production of new paper products is increasing all over the world in recent years. Recycling of photocopier paper is a major problem due to difficulty in removal of non-impact ink. Enzymes offer potential advantages in ecofriendly deinking of recovered paper. In this study the deinking of photocopier paper was examined using chemicals and a commercial cellulase enzyme. Parameters of deinking experiments were optimized for hydrapulping. The ink was removed by flotation and washing processes. Then these parameters were compared in terms of ink removal ability of the process, as well as optical and strength properties of the deinked paper. The application of enzymatic deinking improved ink removal efficiency by 24.6% and freeness by 21.6% with a reduction in drainage time of 11.5% in comparison to those obtained with chemical deinking. The physical properties, namely burst index and tensile index, were observed to improve by 15.3% and 2.7%, respectively and brightness and tear index decreased by 2.1% and 21.9%, respectively. Results of deinking efficiency of photocopier paper showed that the enzyme used in the present work performed better than the conventional chemicals used for deinking.

Keywords: Photocopier paper; Deinking; Cellulase; Deinking efficiency; ISO brightness; Residual ink.

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INTRODUCTION

The pulp and paper manufacturing industry is one of the largest consumers of wood today. Along with increasing world economic growth, a substantial increase in paper consumption is expected. As a result, due to more harvesting of trees, the paper industry could well experience a limited raw material resource, with concurrent reduction of industry growth. Therefore, "recycling of paper", as a solution to this problem, is attracting more and more attention. It offers an effective way to preserve forest resources and save energy and landfill space. Furthermore, papermakers are focusing on recycling as an economic necessity.

One of the important processes in recycling of paper is the deinking i.e. the removal of the printing ink from the used paper to obtain brighter pulp. The process of deinking involves ink particles' dislodgement from the fiber surface and the separation of the dispersed ink from fiber suspensions by washing or flotation (Bajpai and Bajpai 1998; Prasad *et al.*1993). The efficiency of this method depends on the technique, printing conditions, kind of ink, and kind of printing substrate. The photocopier printers use thermosetting toners, consisting of non-dispersible synthetic polymers, as ink for printing

Enzymatic Deinking Of Office Waste Paper:An Overview

Pathak Puneet, Bhardwaj Nishi K., Singh Ajay K.

ABSTRACT

The utilization of the post consumer paper product in the production of new paper products is increasing all over the world in recent years. The use of enzymes in ecofriendly deinking of the recovered paper is one of the potential enzymatic application in the pulp and paper industry. Carbohydrate hydrolyzing enzymes (cellulases and xylanases) and lipases can deink office waste papers by enzymatic liberation of the ink particles from the fibre surface or hydrolysis of the ink carrier or coating layer. Enzymes have optimal deinking activity when presoaked before pulping at mostly acidic pH and medium consistency pulping. Enzymatic deinked pulp shows improved drainage, better physical properties, increased brightness, lower residual ink, reduced chemical consumption, lower COD as compared to chemically recycled pulps. Enzymes are supposed to retard redeposition of ink particles onto the fibres. Use of cellulases and hemicellulases, in excessive amount may cause the depolymerization resulting in fibre loss and high BOD in effluents. Accordingly, although the applications of the enzymes have been reported recently at mill scale but more research is needed to optimize process parameters and to tailor the enzymes through genetic engineering techniques according to the interest of the papermakers.

Keywords:- cellulases, xylanases, deinking efficiency, mixed office waste, residual ink

Introduction

The pulp and paper manufacturing industry is one of the largest wood consumers today. Along with increasing world economic growth, a substantial increase in paper consumption is expected. This means that more trees will be harvested and more solid waste will be created as paper products are consumed and disposed off because of the environmental and economic concerns associated with the consumption of our forest resources. The paper industry could well experience a limited raw material resource with concurrent reduction of industry growth. Therefore, "recycling of paper" as a solution to this problem is attracting more and more attention since it is an effective way to preserve forest resources and save energy and landfill space. One of the important processes in recycling of paper is the removal of the printing ink, also called deinking, from the used paper to obtain brighter pulp. Deinking involves the ink particles dislodgement from the fibre surface and the separation of the dispersed ink from fibre suspensions by washing or flotation (1,2). The efficiency of this method depends on the technique and printing conditions, kind of ink and kind of printing substrate. Papermakers are focusing on recycling as an economic necessity. Due to lack of any organized sector for the waste paper collection in India, imported paper waste comprising mainly Old Magazine (OMG) and Mixed Office Waste (MOW), constitutes the main ingredient used for preparing recycled paper pulp. New deinking mills established in response to this projected need are already competing for the cleanest and most homogeneous postconsumer paper source, e.g., sorted white ledger and soon will have to dip deeper into the postconsumer paper stream, e.g., unsorted (MOW -The most difficult raw material for deinking), to remain competitive (2,3). Indeed, photocopiers and laser printers physically bind the ink (thermosetting toners consisting of non-dispersible synthetic polymers) to the fibres as a result of high heat, making it difficult and expensive to remove by conventional chemical methods (4,5). Most of the conventional deinking techniques require large amounts of chemical agents, such as sodium hydroxide, sodium carbonate, diethylenetriaminepentacetic acid, sodium silicate, hydrogen peroxide and surfactants resulting in a costly wastewater treatment to meet the environmental regulations (2,6). Alternatively, enzyme usage has been reported to be a potentially efficient and less polluting solution to overcome this disposal problem (2,7).

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Saharanpur-247001, U.P, India

ENZYMES USED IN DEINKING:-

Enzymatic treatment is a recent process which gives better performance to reach desired deinked pulp properties. The study of enzyme application in the deinking is performed by many scientists. Several enzymes such as cellulases, hemicellulases, Pectinase, lipase, esterase, -amylase and lignolytic enzymes have been used for deinking of various recycled fibres. But the main enzymes used for deinking are cellulases and hemicellulases. Also many patents for the use of enzymes in deinking have been granted or applied for (1).

Cellulases:

Fungal cellulases and bacterial cellulases are components of large systems or complexes that hydrolyze -1, 4-glucosidic linkages in cellulose which produce water soluble sugars. Cellulases can be divided into three major classes. These are endoglucanases or endo-1,4--glucanase, cellobiohydrolase and glucosidase. Endoglucanases, attack randomly along the cellulose fibre, resulting in a rapid decrease in the chain length of CM-cellulose or H_3PO_4-swollen cellulose and yielding glucose, cellobiose, cellotriose and other higher oligomers (9). These three hydrolytic enzymes act synergistically. Endoglucanases (EG) hydrolyze internal bonds, producing oligomers with new chain ends. EGs act preferentially in the amorphous regions of the microfibrills. Cellobiohydrolases (CBHs) act processively on the existing chain ends and on those created by the endoglucanases, releasing cellobiose molecules. glucosidase cleaves the released cellobiose to two glucose molecules (9).