IMPROVING DRAINAGE CHARACTERISTICS OF PULPS USING COMMERCIAL AND ISOLATED FUNGAL ENZYMES

Ph.D. THESIS

by

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DEPARTMENT OF PAPER TECHNOLOGY

INDIAN INSTITUTE OF TECHNOLOGY ROORKEE

ROORKEE–247 667,INDIA

MAY, 2016

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A THESIS

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

of

DOCTOR OF PHILOSOPHY

in

Paper Technology

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ROORKEE

CANDIDATE'S DECLARATION

I hereby certify that the work which is being presented in this thesis entitled **"IMPROVING DRAINAGE CHARACTERISTICS OF PULPS USING COMMERCIAL AND ISOLATED FUNGAL ENZYMES"** in partial fulfilment of the requirements for the award of the Degree of Doctor of Philosophy and submitted in the Department of Paper Technology of the Indian Institute of Technology Roorkee, Roorkee, is an authentic record of my own work carried out during a period from December, 2009 to May, 2016, under the supervision of Dr. N. K. Bhardwaj, Deputy Director, Avantha Centre for Industrial Research & Development, Yamuna Nagar and Dr. S. P. Singh, Professor, Department of Paper Technology, Saharanpur Campus, Indian Institute of Technology Roorkee, Roorkee.

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

(Piyush Kumar Verma)

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

(S. P. Singh)
Supervisor
Date:

(N. K. Bhardwaj)
Supervisor

The Ph.D. Viva-Voce Examination of **Mr. Piyush Kumar Verma**, Research Scholar, has been held on……

Signature of Supervisors Chairman, SRC External Examiner

Head of the Department/Chairman, ODC

ABSTRACT

In papermaking, the drying process at dryer section is the most energy consuming unit operation. One of the most influential problems with nonwood as well as recycled pulp is the slow drainage in different processes during papermaking. This can be the cause of slow dewatering on the wire and press sections of the paper machine leading to low production. By the improvement in pulp drainage, paper machine speed could be significantly increased along with steam savings. This research work presents a possible action of endoglucanase (EG) and cellobiohydrolase (CBH) components of cellulases on cellulosic pulps having high fines content. It covers the understanding whether the effectiveness or strength loss depends on a specific type of enzyme activity (EG or CBH). Better understanding of the enzyme−fines interaction will improve the selection of enzymes for drainage improvement of recycled and wheat straw pulps with high fines content.

The objectives of the present study are as follows.

- Evaluation of different commercial cellulase enzymes for improvement in pulp drainage and impact on pulp and paper properties
- Evaluation of enzymes and polymeric drainage aid (sequential treament) for improvement in pulp drainage and impact on pulp and paper properties
- Lab scale production of endoglucanase enzymes and their application for drainage improvement of pulps

Based on these objectives, the research work has been distributed in seven chapters.

Chapter 1 Introduction

Poor drainability i.e. less drainage of water during paper formation process is one of the major problems with recycled and wheat straw pulps. It is due to the higher relative surface area of secondary fibre fines than virgin fibre fines. The fibre fines causing lower drainage rate in recycled and wheat straw pulps are reported to consist of amorphous cellulose *(Dienes et al. 2004)*. Dewatering properties of the pulp strongly affect the energy efficiency of paper machine and thus the cost efficiency of papermaking. Conventionally, dewatering is increased in a paper machine by using drainage aids in the wet-end section and/or more intense wet pressing in the press section. But use of drainage aids can worsen formation, and high wet press levels decrease the bulk of the end-product *(Oksanen et al. 2011)*. Therefore, novel pulp modification and dewatering innovations are required with new high speed paper machines.

Chapter 2 Literature review

The fines content in recycled pulps and wheat straw ranging from 20 to 45% has already been reported in the literature *(Dienes et al. 2004, Rousu and Hytonen 2007)*. Recent studies have shown that removal of fines from recycled and wheat straw pulps either by conventional

methods such as fractionation or other methods can improve not only the drainability of the pulp suspension but also the mechanical and optical properties of the paper sheet due to the fines acting as a filler, with less contribution in bonding properties of the paper sheet. *Jackson et al. (1993)* reported that enzymes can either flocculate or hydrolyze fines and remove fibrils from the surface of large fines. The probable explanation for an increased drying rate is a more bulky and porous fibre network with enzymatic treated pulp. Reduced shrinkage forces due to hydrolysis of fines and surface hemicelluloses are a probable explanation for the increase in bulk with enzymatic treated pulps (*Oksanen et al. 2011a*). Cellulose has both crystalline and amorphous regions and it is easier to hydrolyze amorphous regions in comparison to crystalline regions *(Zuhair 2007).* In this study, the concept of monocomponent cellulase treatment of recycled-newsprint (NP), recycled- writing printing (WP) and wheat straw (WS) mill pulps, for improvement in drainage as a result of selective and controlled hydrolysis is demonstrated.

Chapter 3 Materials and methods

Bleached recycled-NP, recycled-WP and wheat straw pulps were procured from different paper mills in India. Different enzyme activities of different commercial enzymes were determined using standard assay procedures. The enzyme based products were added to pulp at varying doses ranging from 0.010 to 0.025% on dry pulp. The drainage time of the untreated and enzyme treated pulps was measured on modified °SR tester. The fines content and fibre length distributions of untreated and enzyme treated pulps were determined using Bauer McNett fibre classifier and L&W fibre tester, respectively. The pulp was also characterized for several other pulp properties such as drainage time, Canadian standard freeness, water retention value and viscosity. The thickness, tensile index, tear index, bendtsen roughness of paper was determined on L&W instruments as per different *TAPPI test methods*. The extreme effects of different enzyme treatments on pulps were evaluated using Field emission scanning electron microscope (FE-SEM). The effect of different enzymes on the crystallinity of treated pulps was also studied by X-ray diffraction. Two cellulase producing fungal strains, *Pycnoporus sanguineus (PVYA07 NFCCI 3628) and Alternaria gaisen (PVYA11 NFCCI 3629*) were isolated. Different nutritional factors (carbon sources, nitrogen sources, and different surfactants) and environmental factors (initial pHs, inoculum size, incubation days, incubation temperature of the fermentation medium) were analyzed for enzyme production by both the fungal isolates individually.

Chapter 4 Drainage improvement by commercial cellulase enzymes

The increased solubilization of amorphous cellulose mediated by endoglucanase treatments consequently improved the drainability of recycled pulp-NP, recycled pulp-WP and wheat straw pulp-WS in the range of 15.5 to 20.7%, along with better paper properties such as tensile

index and smoothness. The proportion of longer fibres (greater than 1 mm) was increased and that of shorter fibres was decreased. FE-SEM studies displayed more damage to the fibres in enzyme Fibrecare R treated fibres when compared to endoglucanase treated fibres at high enzyme dose. Treatment with the enzyme Fibercare D resulted in extensive fibre wall peeling, fibre collapse and increased fibre flexibility. X-ray diffraction studies also supported the finding that endoglucanases can improve the pulp drainage by hydrolyzing the most accessible parts of cellulose i.e. fines containing amorphous cellulose and other dissolved and colloidal substances and hemicellulose present in the fibres. The crystallinity was observed to be increased in case of endoglucanase Fibrecare D treated samples due to more action on amorphous cellulose.

Chapter 5 Drainage improvement by combined treatment of commercial cellulases and polymeric drainage aid

The interaction of various types of cellulases (Fibercare D and Fibercare R) and polymeric drainage aid for enhancing the freeness of recycled and wheat straw pulps were also explored. Polymer treatments alone were found to increase the drainage in the range of 12 to 25% at various doses but formation was adversely affected due to flocculation. It was observed that endoglucanase Fibercare D, in combination with polymeric drainage aid (P) at optimized dose levels, further enhances the freeness of recycled and wheat straw pulps by 25 to 28%. It was further concluded that by using enzyme with lower levels of polymeric drainage aid, a potentially more uniform sheet can be produced with even better pulp drainability than the enzymes alone.

Chapter 6 Lab scale production of endoglucanase enzymes and their application for drainage improvement of pulps

This chapter describes the isolation, screening and identification of two cellulase producing fungus i.e. *Pycnoporus sanguineus* PVYA07 NFCCI-3628 and *Alternaria gaisen* (PVYA11 NFCCI-3629) from 54 different fungal isolates. It also covers the production and purification of isolated endoglucanase enzymes and their evaluation of for improvement in pulp drainage. The cultures were identified based on sequencing of the genomic deoxyribonucleic acid (DNA), and deposited at National Fungal Culture Collection of India (NFCCI), Pune, India with accession numbers. Purified cellulosic substrates such as carboxymethyl cellulose (CMC) and Avicel were found to be suitable for cellulase production. Yeast extract (YE) followed by urea were found to be the suitable nitrogen source for cellulase production by *Pycnoporus sanguineus and Alternaria gaisen*. Tween 80 assisted to release the cellulase complex in the medium. The endoglucanase enzymes were purified to homogeneity from the culture filtrates of *Pycnoporus sanguineus and Alternaria gaisen* via ammonium sulfate fractionation, Sephadex G-100, and Q-Sepharose column chromatography. The molecular weights of the

enzymes from 2 different fungal strains were determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE). The effect of enzymes produced at lab scale on pulp drainage and other pulp and paper properties was also analyzed. Pulp drainage was observed to be improved by 10.1 to 19.5% using the purified enzymes from two fungal isolates. Significant improvement in tensile index and smoothness was also observed in handsheets prepared from enzyme treated pulps.

Chapter 7 Conclusions and recommendations for future work

The carried out investigations on endoglucanase treatments showed an opportunity for significant improvement in dewatering of different pulps at different enzyme doses. Significant improvement in tensile index and smoothness was also observed. The results indicated that applying specific cellulase component, i.e. endoglucanase, may be more effective for improving the drainage of pulps with high fines content, by selective hydrolysis of excess fibre fines and other dissolved colloids. The established effect of mono component endoglucanases for improving pulp drainage can be utilized for enhancing the paper productivity (increased machine speed) and decreasing the dryer steam consumption. Further research efforts in this direction should be carried out to demonstrate the commercial utility of the endoglucanase enzymes for drainage improvement at mill scale.

LIST OF PUBLICATIONS

PUBLICATIONS (4)

- **Verma PK**, Bhardwaj NK, Singh SP (2015). Selective hydrolysis of amorphous cellulosic fines for improvement in drainage of recycled pulp based on ratios of cellulase components. Journal of Industrial and Engineering Chemistry, 22, 229-239. Impact factor: 3.51
- **Verma PK**, Bhardwaj NK, Singh SP (2016). Improving the material efficiency of recycled furnish for papermaking through enzyme modifications. The Canadian Journal of Chemical Engineering, 94, 430-438. Impact factor: 1.31
- **Verma PK**, Bhardwaj NK, Singh SP (2013). Improvement in pulp dewatering through cellulases. IPPTA, 25(3), 105-108.
- **Verma PK**, Bhardwaj NK, Chakrabarti SK (2010). Enzymatic upgradation of secondary fibres, IPPTA, 22(4), 133-136.

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- ✓ **Verma PK**, Bhardwaj NK, Singh SP, "Enzymatic upgradation of cellulosic pulps for improved papermaking" National Conference on "New Avenues in Microbiology: Challenges and Prospects (NAMCAP-2015)", Maharshi Dayanand University, Rohtak, March 11, 2015

Papers communicated (2)

- **Verma PK**, Bhardwaj NK, Singh SP (2016). Upgradation of recycled pulp using endoglucanase enzyme produced by *Pycnoporus* sp.: A step towards environmental sustainability
- **Verma PK**, Bhardwaj NK, Singh SP (2016). Enzymatic upgradation of wheat straw and recycled pulps for improved papermaking: Impact of cellulosic fines and enzyme activity components

Award (1)

- First best paper award on "Improvement in pulp dewatering through cellulases" at IPPTA Zonal Seminar on "Technological Advancements in Recycling, Waste Paper Collection and Effective Reuse" on August 01-02, 2013 at Aurangabad.

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(Piyush K. Verma)

LIST OF FIGURES

LIST OF TABLES

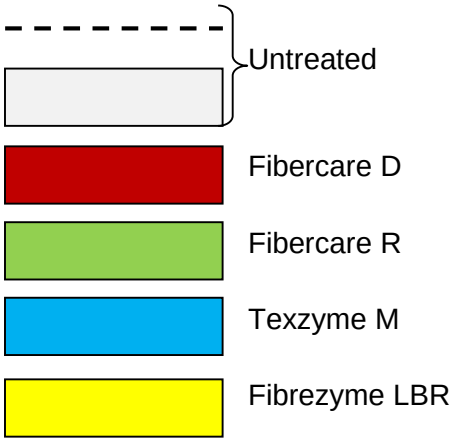
ABBREVIATIONS

ACIRD	Avantha Centre for Industrial Research and Development
ANPAM	Anionic polyacrylamide
AR	Analytical reagent
bn	Billion
BP	Bagasse pith
BSA	Bovine serum albumin
CAGR	Compound annual growth rate
CATPAM	Cationic polyacrylamide
CBD	Carbohydrate binding domain
CBH	Cellobiohydrolase
CBM	Cellulose binding modulus
CD	Catalytic domain
CMC	Carboxymethyl cellulose
CSF	Candaian standard freeness
D.W.	Distilled water
DADMAC	Poly diallyl ammonium chloride
DNA	Deoxyribonucleic acid
DNS	Dinitro salicylic acid
DP	Degree of polymerization
EC	Enzyme Commision
EG	Endoglucanase
Fc D	Fibercare D
Fc R	Fibercare R
FE-SEM	Field emission scanning electron microscope
FMCG	Fast-moving consumer goods
FPase	Filter paper
GDP	Gross Domestic Product
GSM	Grammage
IPMA	Indian Paper Manufacturers Association
IR	Infrared
ISO	International Organization for Standarization
IU	International unit
KDa	Kilodalton
L&W	Lorentzen & Wettre
mn	Million
NFCCI	National fungal culture collection of India
NP	Newsprint
NSS	Nutrient saline solution
o.d.	Oven dry
OCC	Old corrugated container
P	Polymeric drainage aid

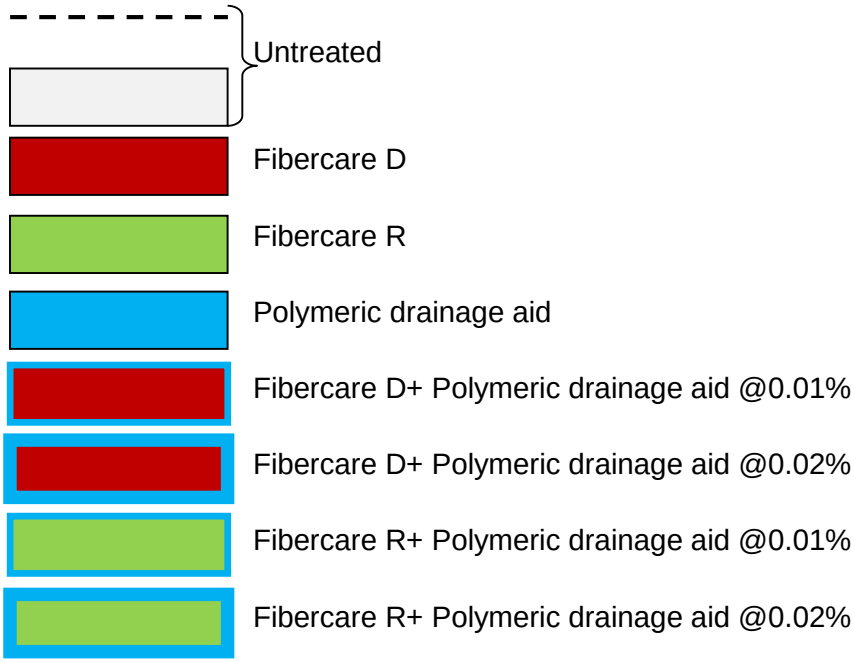
PEI	Poly ethylene imine
pNPC	Para nitrophenyl cellobioside
PP	Proteose peptone
RBA	Relative bonded area
RCF	Recycled fibre
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SSA	Specific surface area
TAPPI	Technical Association of Pup and Paper Industry
TPA	Tonnes per annum
UA	Urea
UV-Vis	Ultra violet visible
w/v	Weight/volume
WP	Writing printing
WRV	Water retention value
WS	Wheat straw
XRD	X-ray diffraction
YE	Yeast extract

COLOR DENOTIONS

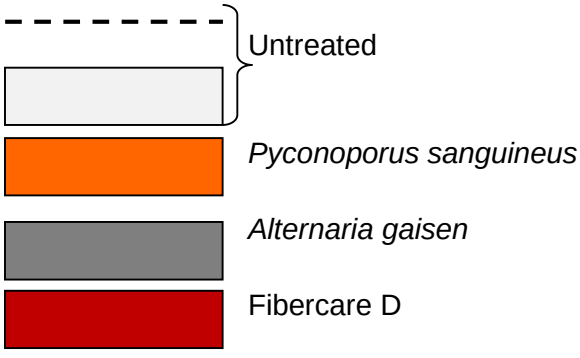
Chapter 4: Drainage improvement by commercial cellulase enzymes



Chapter 5: Drainage improvement by combined treatment of commercial cellulases and polymeric drainage aid



Chapter 6: Lab scale production of endoglucanase enzymes and their application for drainage improvement of pulps



CHAPTER 1

INTRODUCTION

1. Introduction

1.1. Paper industry: Global and Indian status

Paper as well as paperboard has emerged as an essential requirement of our life. Currently, the global paper and paperboard demand has crossed the figure of 425 mn tonnes per annum *(Moore 2015)* (Figure 1.1a). Asia and Middle East account for 43% of the industry, 172 mn tonnes and $130 bn, while rest of the world contribution is 56% (225 mn tonnes). The forecast for paper production by 2021 has been projected at 521 mn tonnes per annum by Indian Paper Manufacturers Association *(IPMA 2016)*. *Recovered paper demand and usage rate accounts for nearly 54% of the total paper and paperboard demand as reported by Moore (2015)* (Figure 1.1b).

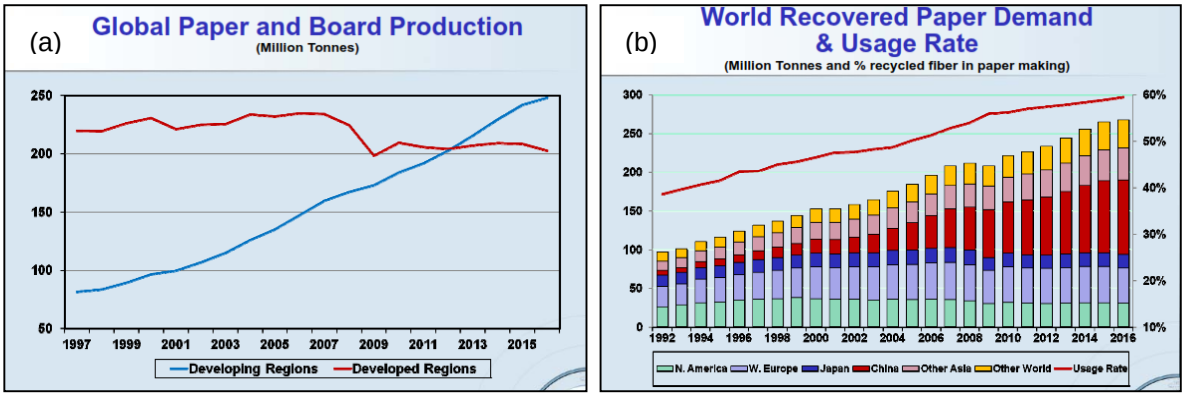


Figure 1.1 (a) Global paper and paperboard production (b) recovered paper demand (Moore 2015)

Two mega trends i.e. population growth and urbanization, will continue to transform the world and in particular, the Asia region. Both are categorized as important drivers for paper and paperboard demand growth. The focus of the pulp and paper world has been shifted to Asia due to strong growth in these emerging markets. The other demand drivers in these developing countries include education, industrial packaging, newsprint and mass marketing. This shift in production is due to the increased consumption in Asia as well as decline in paper and paperboard consumption in North America and Europe *(Wright 2015)*.

From 2010 to 2014 global paper and paperboards demand has grown by 7.6 mn tonnes. Chinese production grew from 65.5 to 77.6 mn tones *(Wright 2015)*. Rest of the world fell from 300.3 to 295.8 mn tonnes, as growth in Latin America, East Europe and other emerging Asia was offset by declines elsewhere (Figure 1.2a and b). Paper production falls in integrated

(mature markets) and grows in non-integrated regions (emerging markets). However, emerging markets have slowed considerably and the begged growth market, China, has consistently been overstated *(Wright 2015).*

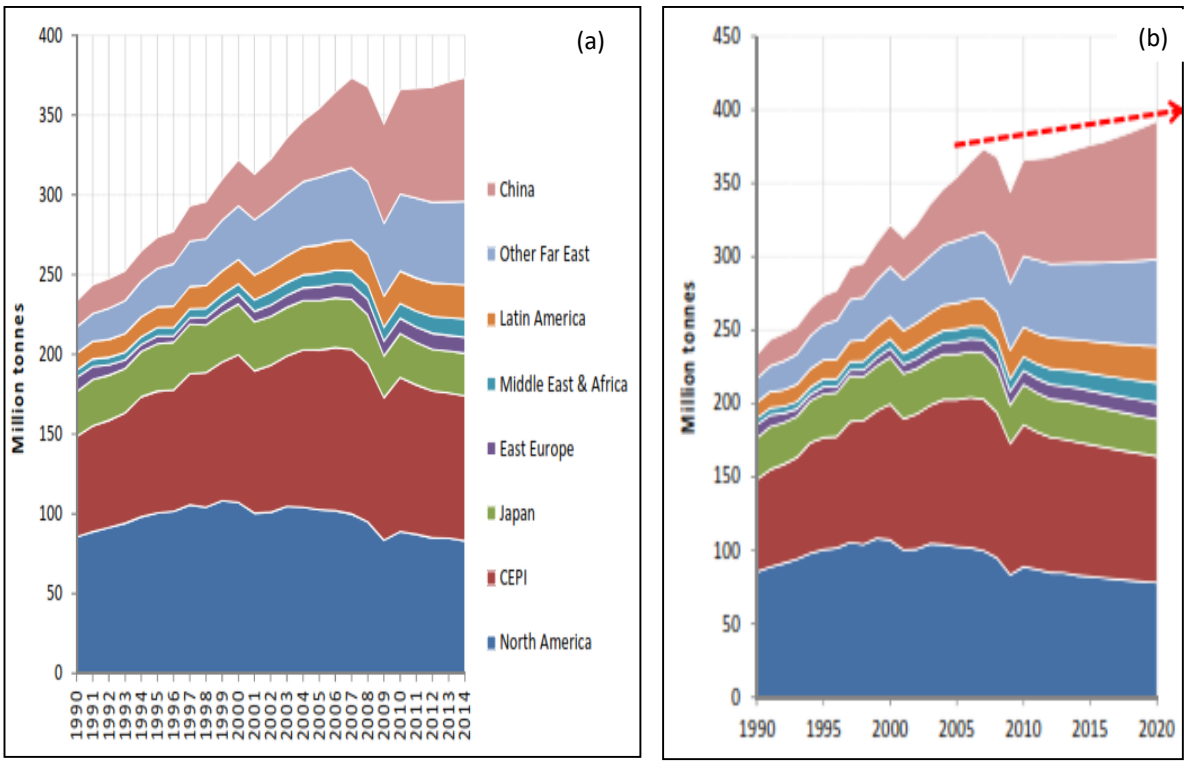


Figure1.2 Global paper and board production (a) present (b) proposed (Wright 2015)

Persistent economic troubles in Europe, slow recovery in North America, and expansion of production capacities in China are testing the global paper and forests products industry. In addition, graphic paper manufactures in already mature U.S. and Western Europe markets continue to face declining demand as consumers are gradually shifting to electronic media. Economic expansion in countries like China and India will be the key in determining the rate of global paper and paper packaging demand and growth *(Wright 2015)*.

The consumption of containerboard is expected to substantially increase in the coming years as reported by *Powlson 2014* (Figure 1.3a). The consumption of recovered paper is expected to increase in rest of Asia also (including India) although the pace of increase is expected to be less when compared to China (Figure 1.3b). Continued demand from India for recovered paper is expected, but the growth potential is expected to be satisfied locally.

India is a wood fibre deficient country; inadequate raw material availability is a major constraint for the paper industry. 90% of wood demand is met through industry driven agro/social forestry (1 mn hectares); 10% through Government sources and imports. Current demand for wood able to pulp is about 11 mn tonnes per annum (TPA) while domestic availability is 9 mn TPA (demand projected to rise to 15 mn TPA by 2024-25) as mentioned by *IPMA 2016*. Domestic

production of paper, paperboard and newsprint is reported to be 12.2 mn tonnes in 2014-15 *(IPMA 2016).* Figure 1.4a represents the estimated production of paper, paperboard and newsprint in India. The production in terms of raw material usage is divided in 3 sectors: wood/bamboo 31%, agro residue (bagasse/ wheat straw, etc.) 22% and waste paper/recycled fibre 47% (Figure 1.4b). The projected production of paper, paperboard and newsprint lies between 22.0-33.4 mn tonnes (Figure 1.4c).

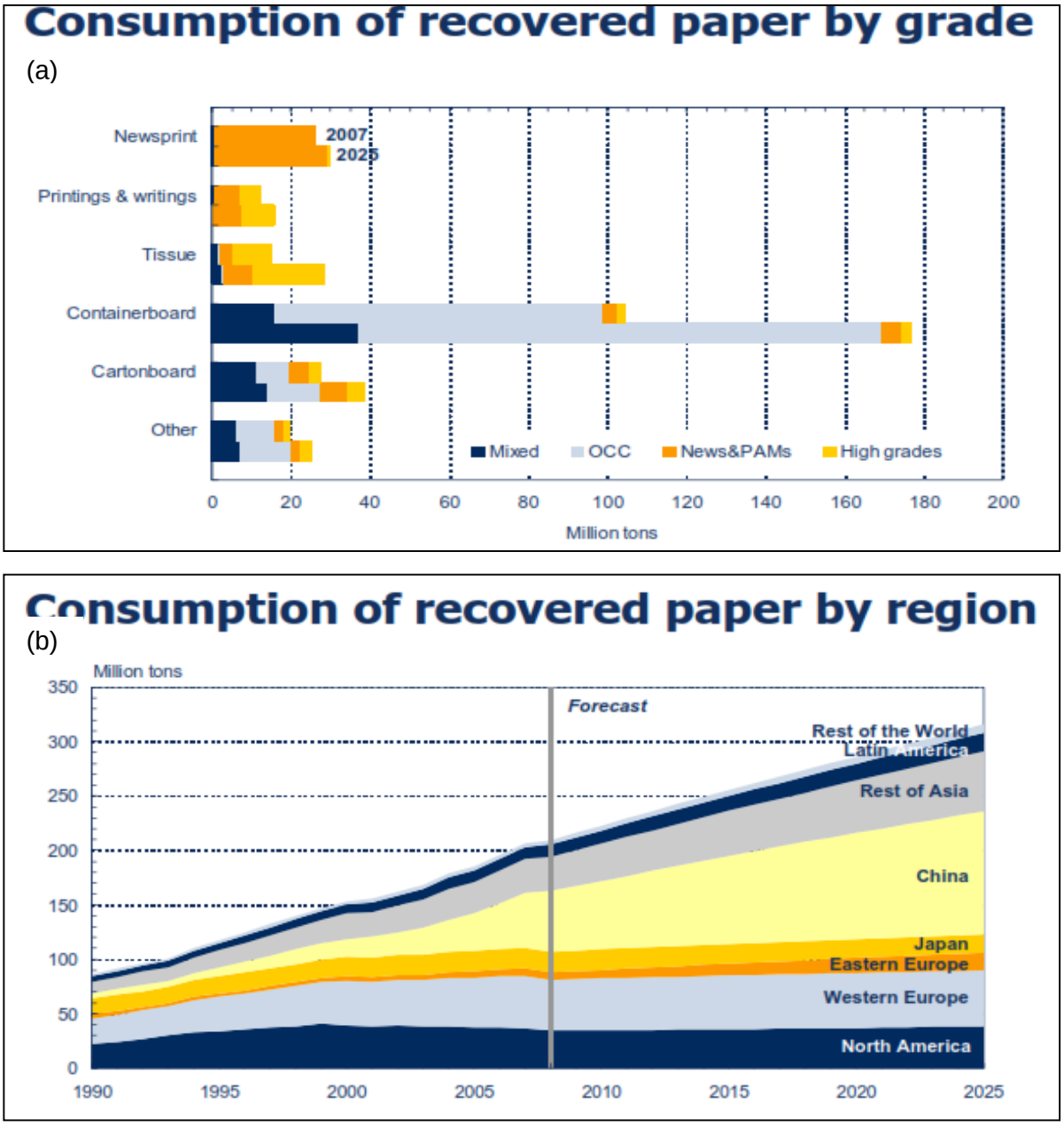


Figure 1.3 (a) Consumption of recovered paper by grade (b) consumption of recovered paper by region (Powlson 2014)

It has been reported in IPMA website that over all paper consumption has reached 13.9 mn tonnes at 10 kg per capita consumption. Paper demand has been growing around 8% per annum. Now, the Indian paper industry is growing alongwith the gross domestic product (GDP) growth *(IPMA 2016).* In India, there are approximately 750-800 paper mills having an operating capacity of 13.0 mn tones and the overall consumption is estimated to be 13.9 mn tonnes with 10.1 kg per capita consumption of paper *(IPMA 2016).* The Indian paper industry is divided into three sectors based on raw material.

Wood and Bamboo: There are 26 large integrated paper mills, using wood and bamboo which contribute 31% of the production (around 3.19 mn tonnes).

Agro-based: There are 150 mills based on agro residues like bagasse, rice straw, wheat straw etc., producing around 2.2 mn tones and contributing 22% of the total production. The agro based fibre usage has also decreased considerably during the last few years *(Kulkarni 2013).*

Recycled fibre: There are 538 mills based on waste paper which contribute almost 47% of the country's current production (around 4.72 mn tonnes) *(Kulkarni 2013).* In the recent years, recycled fibre usage has considerably increased. Although waste paper is procured locally, India does not have a well developed collection mechanism. This has resulted in low availability of waste paper. Presently the recovery rate i.e. volume of recovered paper in the country divided by paper apparent consumption is very low in the country estimated to be around 28.9% (Figure 1.4d). Hence a large quantity of waste paper is being imported and import rate has substantially increased in the last few years.

The per capita consumption in India is 9.3 kg against the world average of 56.7 kg *(IPMA 2016).* One of the main growth drivers for paper demand are enhancement of government spending on education (6% of GDP), increase in literacy rate, improvement in standard of living, booming retail sector, unprecedented growth in industries like food, pharmaceuticals and apparels, increase in packaging and advertising expenditure etc. Demand for better quality packaging of FMCG products marketed through organized retail, rising healthcare spends, over the counter medicines and increasing preference for ready to eat foods are the key drivers for paperboard demand (Figure 1.4e). In India, approximately 1.2 bn populations and changing demographic profile, which have put over 65% of the population in the working class and half of that is less than 30 years old, will further fuel the demand for paper and paperboards. It is expected that an increase in paper consumption by one kg per capita would lead to an increase in demand of 1 mn tonnes due to growth of paper consumption in multiple of GDP.

(a)

Pulp & Paper Industry of India

Estimated Production of Paper, Paperboard & Newsprint

Million Tonnes	2011-12	2012-13	2013-14	2014-15
Domestic Production	10.1	10.5	11.4	12.2
Exports	0.5	0.5	0.5	0.6
Imports	2.1	2.0	2.2	2.3
Domestic Market / Consumption (Production + Imports - Exports)	11.7	12.0	13.1	13.9

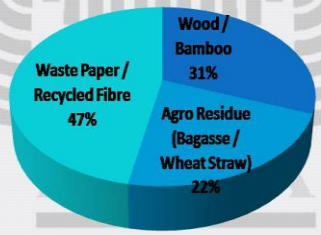
(b)

Pulp & Paper Industry of India

Industry Composition & Structure (2014-15)

Installed Capacity	13.0 million tonnes
Production of Paper, Paperboard & Newsprint	12.2 million tonnes
Domestic Consumption / Market Size	13.9 million tonnes
Domestic Production (12.2) – Exports (0.6) + Imports (2.3) = Consumption (13.9)	

Production in Terms of Raw Material Used



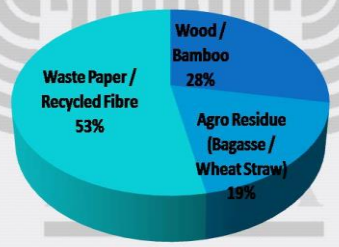
(c)

Pulp & Paper Industry of India

Projected Production & Consumption (2024-25)

Production of Paper, Paperboard & Newsprint	22.0 million tonnes (baseline scenario) 33.4 million tonnes (optimistic scenario)
Domestic Consumption	23.5 million tonnes (baseline scenario) 36.9 million tonnes (optimistic scenario)

Projected Production in Terms of Raw Material Used

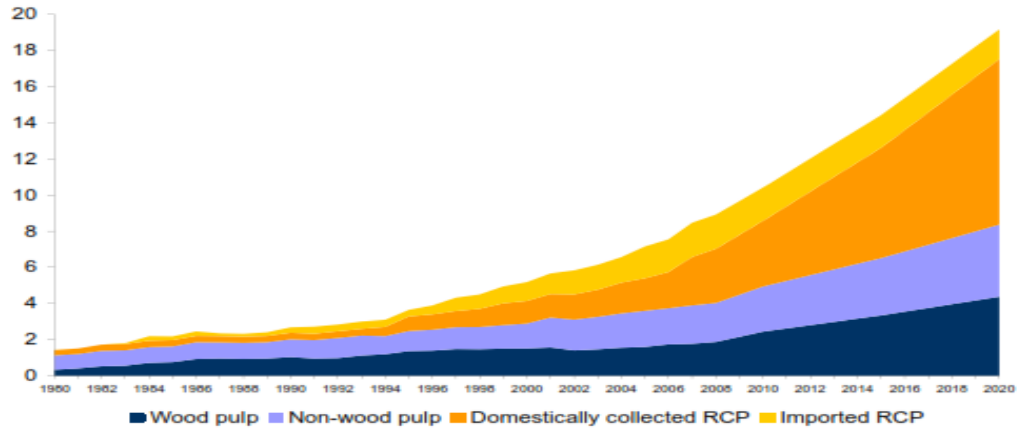


(d)

Selected Countries	Recovery Rate, %
South Korea	91.6
Germany	84.8
Japan	79.3
United States	63.6
Italy	62.8
Indonesia	53.4
Finland	48.9
Brazil	45.5
China	40.0
Russia	36.4
India	28.9

(e

India – Outlook



(f)

Pulp & Paper Industry of India

Exports & Imports of Paper, Paperboard & Newsprint (2014-15) in Thousand Tonnes

HS Code	Product Description	Exports	Imports
4801	Newsprint	10	1,336
4802	Uncoated Paper & Paperboard for Writing, Printing or Other Graphic Work	353	118
4803	Toilet or Facial Tissue Stock, Towel or Napkin Stock	15	5
4804	Uncoated Kraft Paper & Paperboard	60	174
4805	Other Uncoated Paper & Paperboard	23	71
4808	Paper & Paperboard, Corrugated, Creped, Crinkled, Embossed or Perforated	1	5
4810	Paper & Paperboard Coated on One / Both Sides	164	638
	Total	624	2,347

Figure 1.4(a) Estimated production of paper, paperboard and newsprint (b) details of installed capacity and production in terms of raw materials used (c) projected production and consumption for 2024-25 (IPMA 2016) (d) volume of recovered paper in different countries divided by paper apparent consumption (e) year wise projected production of paper, paperboard and newsprint (Powlson 2014) (f) exports and imports of paper, paperboard and newsprint in 2014-15 (IPMA 2016)

It is more important for paper to show its unique characteristics in competition with other materials: renewability, recyclability, biodegradability. In addition, the papermaking process also has to be configured in a more environmentally friendly and resource saving way. Accordingly, the paper industry has to be more globally committed for sustainability than ever. As the domestic paper production has increased over recent decades; recovered paper demand in India has also increased strongly. This trend seems likely to continue. Growth in paper demand increased by 7.4% for the fiscal year ending March 2016 and is expected to further accelerate slightly to 7.8% for up to March 2017. Import levels in October, based on Customs data were slightly increased relative to the August and September, coming in at 115,000 tonnes, and November imports seems to be further higher. It has been reported in Indian Customs data that India is getting more from Europe and the Rest of the World, but less from North America and Asia (*RISI 2016*).

The Indian paper industry will be required to meet an annual requirement of 22.0 to 33.4 mn tonnes of paper and paperboard by 2025 from the current level of over 12.2 mn tonnes, with the paper and paperboard demand, growing at an average rate of 7.8% per annum. At the same time, the share of recycled fibre (RCF) based industry is expected to increase from current level of 47% to 53% by 2025 (Figure 1.4c), thus contributing an average production of around 11.7 mn tonnes and which would eventually require an additional RCF/WP requirement of 9.3 mn tonnes as a raw material *(IPMA 2016)*.

1.2 Enzyme industry: Global and Indian Status

Enzymes are regarded as sustainable alternatives to harmful chemicals, conventionally used in many industrial processes. Enzymatic processes normally occur under moderate conditions, such as normal temperature and with minimal use of water, leading to reduced energy consumption and cost, associated with maintaining extreme environments for many chemical reactions. This decline in energy consumption can further lead to reduced greenhouse gas emissions.

Replacement of chemicals with environment-friendly enzymes can lead to cost and time savings for manufacturers and can help them to comply with various environmental norms. The demand of enzymes from various industries is likely to witness rapid and continuous growth globally as well as domestically within India due to higher emphasis on energy conservation and more stringent environmental laws (Figure 1.5a, b).

The global enzyme industry can be classified into two segments based on the end-user industries – one is industrial and other is specialty enzymes. The industrial enzymes segment includes enzymes catering to Food & beverage, textile, paper, detergents, animal feed, bio-fuel and other industries; the specialty enzymes segment consists of products catering to pharmaceuticals, diagnostics, biotechnology, research and biocatalysts.

Both industrial and specialty enzymes segments are expected to grow globally, over the next few years, at a compound annual growth rate (CAGR) of 6.9% from $5.8 bn to $11.3 bn over 2010-20. Demand for industrial enzyme segment is expected to be controlled by the animal feed and food & beverages segments. Presently, North America is considered to be the largest enzyme market worldwide (~45% of the global enzyme demand) followed by Western Europe (21.2%) and Asia Pacific (18.8%) regions. Over the past decade, demand for enzyme products in Asia Pacific and other emerging markets has considerably increased. It is forecasted that Asia Pacific by 2020, will become the second largest enzyme market in the world with an overall share of 23.2% *(Crisil 2013).*

Currently, the Indian enzymes industry is growing slowly and penetration across different end-user industries is comparatively low. Indian enzymes industry is expected to grow at a CAGR of 12% over 2010-20 *(Crisil 2013).* Different factors such as price-sensitivity and lack of awareness among the end-user industries, and the government's failure to strictly implement environmental laws have hindered the growth.

In India, most of the enzyme companies focus on producing value-added enzyme products based on simpler formulations. However, the entry of foreign manufacturers has made the environment competitive due to availability of quality products in the domestic market. On the other hand, the domestic enzyme industry is approaching the international markets for

establishing market base in foreign markets.

Pharmaceuticals, food & beverage, detergent, textile, leather and paper industries are the primary consumers of enzyme products in India. Each segment is witnessing a different stage of growth. The bio-Industrial (primarily enzyme products) market in India was worth 7.7 bn in 2013 (Figure 1.6a). The industry has registered15.1% CAGR during 2004-13. The industry is further expected to grow to $295 mn in 2020 from $96 mn in 2010 at a CAGR of 12% (Figure 1.6b) *(Crisil 2013).*

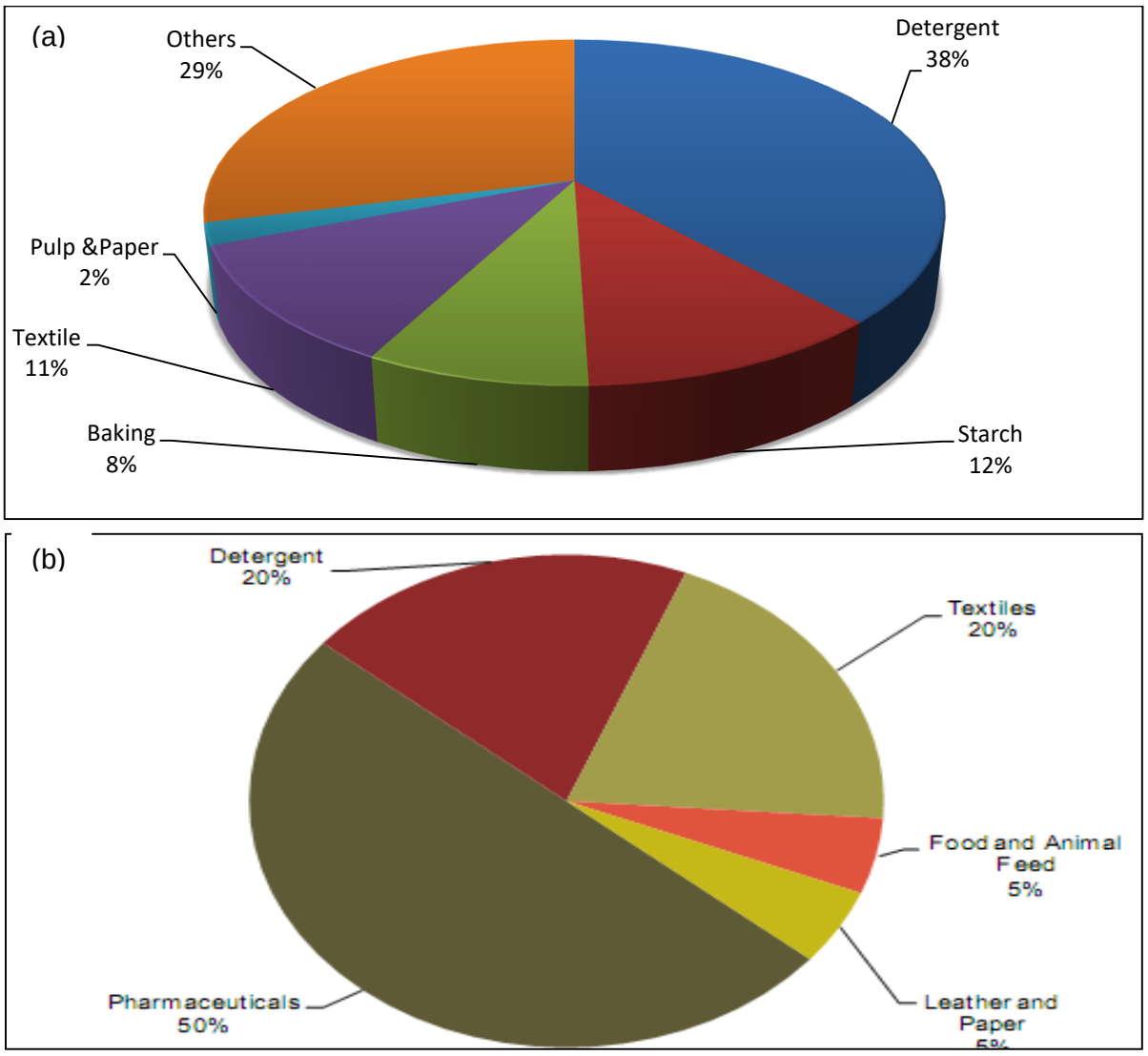


Figure 1.5(a) Global enzyme industry (b) Indian enzyme industry- demand from user segments (Crisil 2013)

Despite the modest growth in bio-industrial sector, the volume has been on a healthy rise and the application spectrum of industrial enzymes has been increasing. Novozymes and Genencor account for almost 65-70 per cent of the sector's global value (Figure 1.6c). On the other hand, in India, Novozymes is the biggest player having around 44% share followed by share of domestic companies (Figure 1.6d) *(Crisil 2013).* Traditionally, the enzyme companies have focused on chemicals, textiles, breweries and tanneries for applications of their products.

However, in last few years, companies have started exploring new areas of application such as agriculture, fibre processing, food processing, animal nutrition, dairy and marine products.The Government of India has also supported the domestic enzyme industry through various funding programs in order to help them to grow and compete with global players.

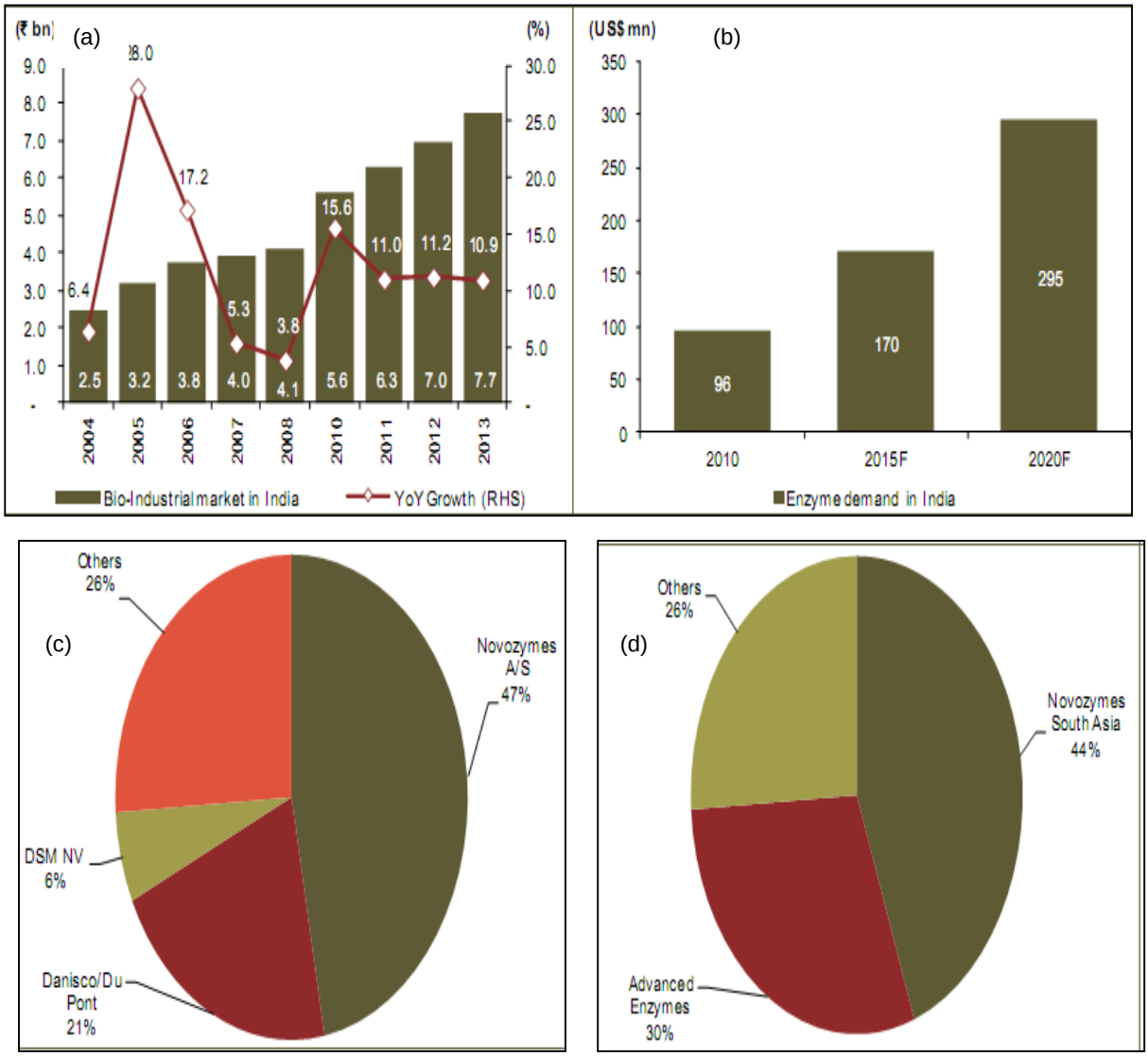


Figure 1.6(a) Expected growth of Indian enzyme industry (b) enzyme demand in India (c) global enzyme market share (d) Indian enzyme market share (Crisil 2013)

1.3 Usage of enzymes in paper industry

Due to non-availability of suitable enzymes as per indigenous raw materials and process conditions, the growth of enzymes in pulp and paper industry is slow. Still research is continued, regarding the biotechnological application in different areas of the pulp and paper industry by several scientific institutions and enzyme producers companies *(Demuner 2011)* (Figure 1.7 and 1.8). Several applications have already been commercialized; however, the many potential applications are still in pre-commercial stage.

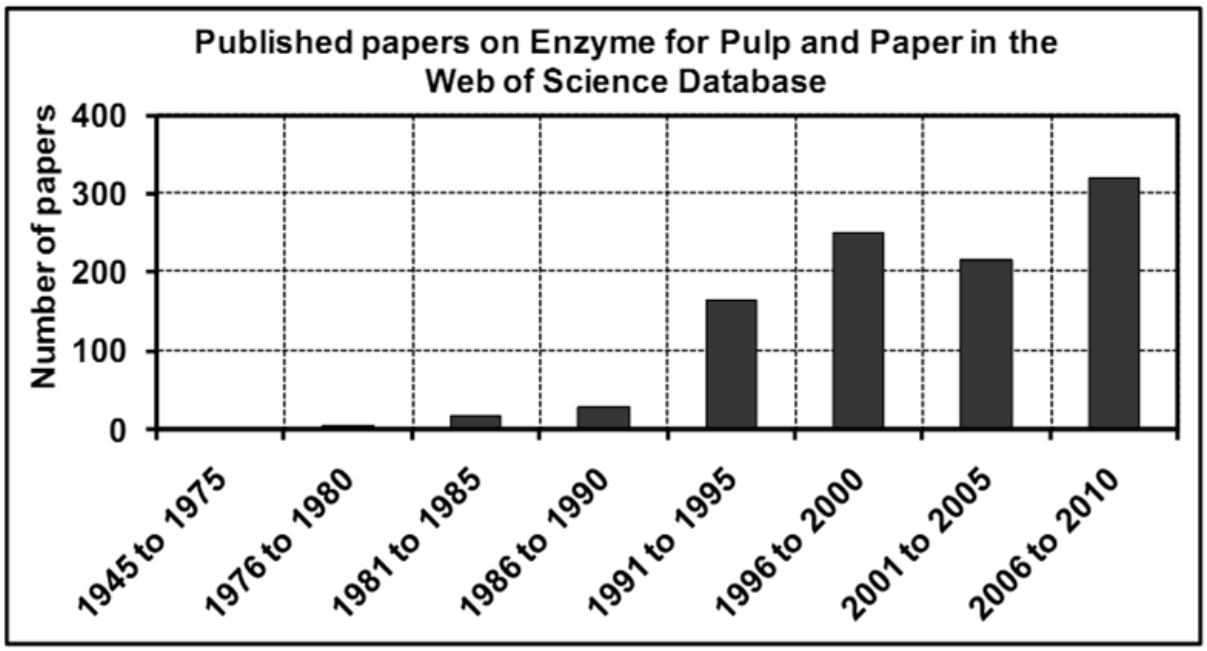


Figure 1.7 Number of published documents on the enzymes for the pulp and paper industry in the web of sciences from 1945 to July 2010 (Adapted from Demuner 2011)

Different potential areas for enzyme application in pulp and paper industry are summarized in the Figure-1.9. Figure 1.10a displays percentage sharing of the different types of enzymes used in the pulp and paper industry. The information presented in Figure 1.10b indicates that cellulases are primarily used in the paper processing, whereas xylanase and laccase are more commonly used in the bleaching and delignification processes *(Demuner 2011).*

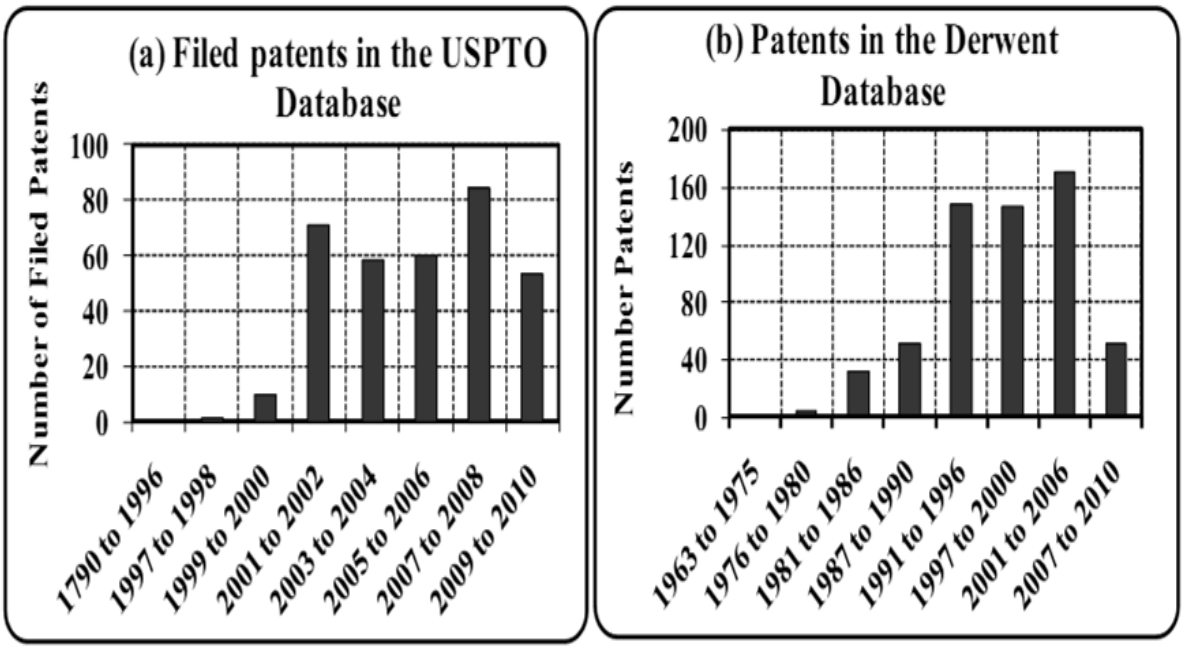


Figure 1.8 Number of patents for Pulp and Paper (a) in the USPTO from 1790 to July 2010, and (b) in the Derwant database (Adapted from Demuner 2011)

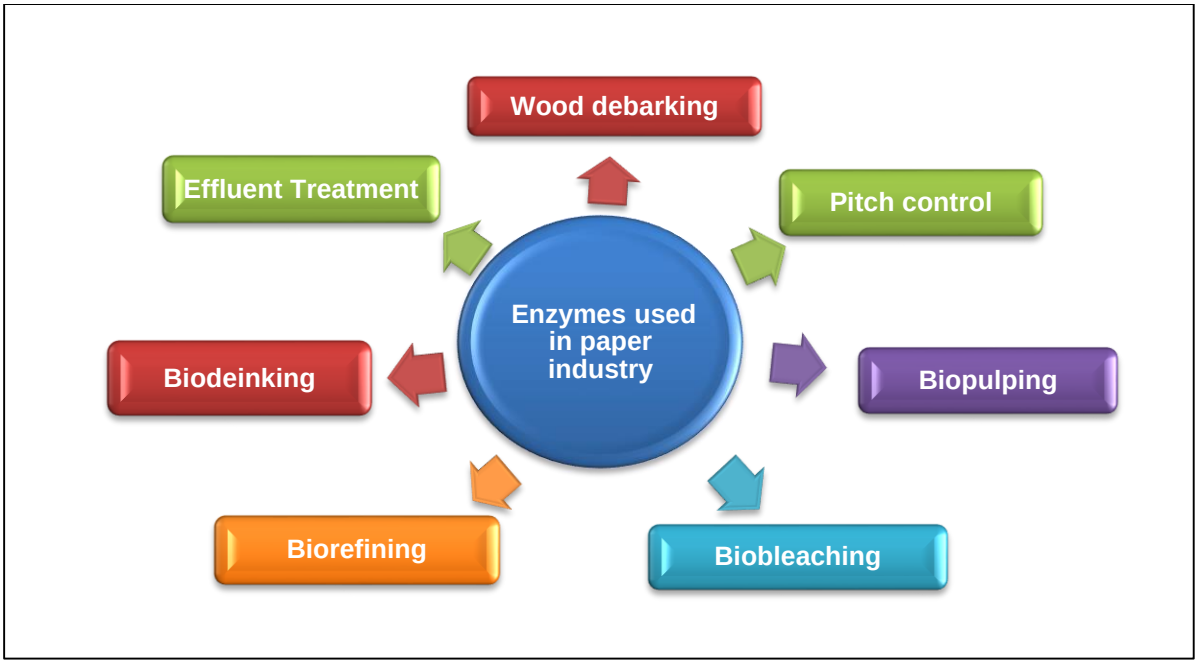


Figure 1.9 Different application of enzymes in pulp and paper industry (Adapted from Pathak 2014)

1.4 Increased usage of recycled and non wood pulps

Limited availability of raw materials is one of the biggest barriers to growth of the Indian pulp and paper industry. In India, availability of wood for paper is very low compared to the United States and Europe. The forest land in India is owned by the government and is not available for use as plantations by the pulp and paper industries. With declining forest lands, this source of much-needed fibre is declining at a rapid rate.

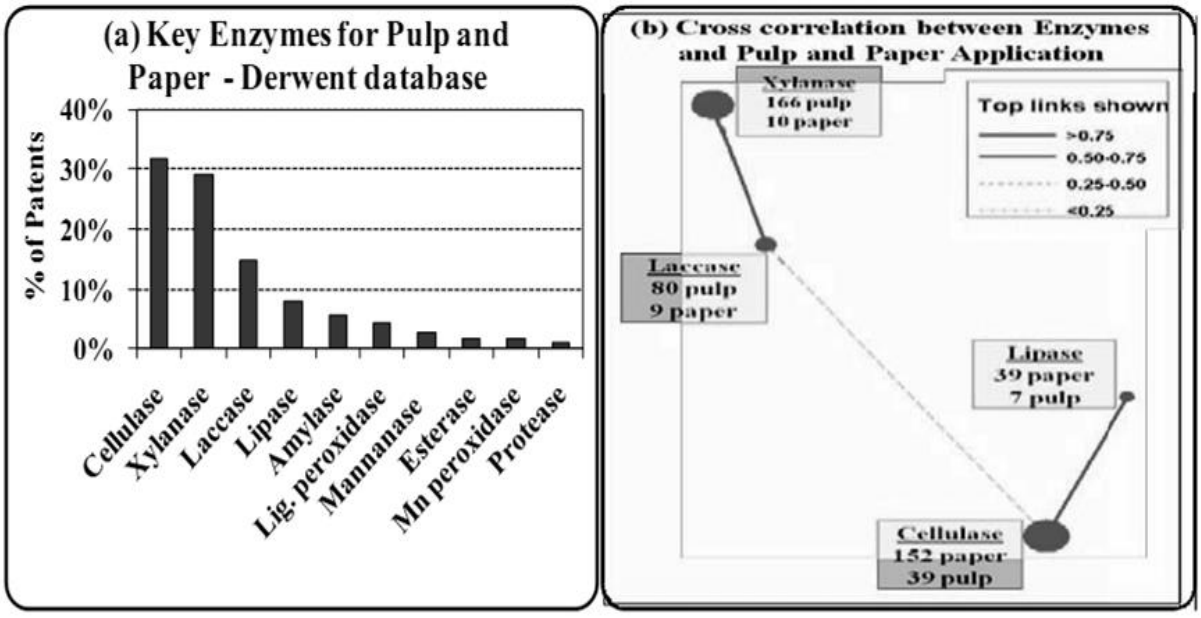


Figure1.10 (a) Key Enzymes extracted in the patents from the derwent database (from 1963 to 2010) and (b) their major applications in pulp and paper (Adapted from Demuner 2011)

Today, the pulp and paper manufacturing industry is one of the largest wood consumers. A substantial increase in paper consumption is expected along with increasing world economic growth. 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, use of recovered paper and agricultural residues to overcome the fibre shortage, to preserve forest resources and to save energy and landfill space is attracting more and more attention.

The pulp and paper industry especially in India and China uses a wide range of raw materials of both wood and non-wood origin apart from recycled pulp. Recently, there is a tendency to use more and more recycled and non-wood pulps due to depleting forest resources. Substitution of virgin pulp with recycled and non-wood pulp saves wood for making pulp resulting in the reduction of exploitation of old forests, important for their biodiversity. Every tonne of recycled fibre has been reported to save an average of 17 trees plus related pulping energy *(Bajpai 2012).* Wastepaper usage to produce new paper reduces the disposal problems. At least 30,000 liters of water, 3000 to 4000 kWh of electricity and 95% of air pollution are saved for every ton of paper used for recycling. It also saves around 2.3 m^3 of landfill volume. 28 to 70% less energy consumption and less water is involved in producing recycled paper compared to virgin paper. This is because most of the energy is used in pulping process to turn wood into paper. Recycled paper production produces fewer polluting emissions to air and water. Usually, recovered paper is not re-bleached and when it is bleached, oxygen or similar environmentally benign bleaching agents are used. High-grade papers can be recycled several times, thereby providing environmental savings each time. Recycled pulp requires less refining than virgin pulp and may also be co-refined with hardwood or combined hardwood/ softwood pulps. The deinked pulps suitable for use in printing papers usually impart special properties to the finished papers. These properties include increased opacity, less curling tendency, less fuzziness, and better formation *(Bajpai 2012)*.

The need for low-cost raw materials and the development of new process in order to boost production has been raised due to the increasing demand. Non-wood fibres i.e. agricultural residues and annual plants are considered an effective alternative cellulose source for producing pulp and paper sheets with acceptable properties. Traditional wood raw materials have been replaced with non-wood, residual and recycled materials and less polluting cooking and pulp bleaching processes have been evolved. There is a growing interest in the use of non-wood such as annual plants and agricultural residues as a raw material for pulp and paper. Less than 10% of the total pulp and paper production worldwide occurs from Non-wood raw materials *(El-Sakhawy et al. 1996)*. This 10% of the total pulp and paper production is made up of 44% straw, 18% bagasse, 14% reeds, 13% bamboo and 11% others. The production of

non-wood pulp mainly takes place in the countries facing wood shortage, such as China and India *(Oinonen and Koskivirta 1999).* China produces more than two thirds of the non-wood pulp produced worldwide *(Hammett et al., 2001).* The benefits of non-wood plants as a fibre resource include fast annual growth and presence of less amount of lignin for binding the fibres together. Additional benefit is that non-wood pulps can be produced at low temperatures with lower chemical charge. In addition, smaller mill sizes can be economically viable, giving a simplified process. Non-wood pulps are easy to refine. Moreover, non-food applications can be a source of additional income to farmers other than food crops or cattle production *(Rousu et al. 2002; Rodríguez et al. 2008; Kissinger et al. 2007).* But, increased use of recovered paper with closed water circuits can also cause biofilm development and production of volatile compounds *(Blanco 2003).*

1.5 Drainage resistance in recycled and wheat straw pulps

The wet end operation of papermaking involves the removal of water from a dilute fibre slurry stock by uniformly distributing the stock onto a moving forming wire. Aided by applied vacuum, water drains through the narrow pores of the wire while the furnish solids, comprised of fibres and other materials, are trapped atop the wire as a wet mat. As filtration proceeds and the mass of fibres and fines forming the paper mat builds up, the retention and flow resistances increase. Consequently, at a constant pressure drop, the drainage rate decreases. The factors affecting the drainage resistance are temperature of the stock, surfactants, air in the stock, wetness of the stock, fibre surface chemistry, fines and colloidal substances.

Recycled as well as wheat straw pulp contains great amount of fines having high relative surface area. By definition, fines have been identified as that fraction of furnish solids that passes through 200 mesh screen (nominally a 76 µm hole size). The effect of fines is enhanced by microfibrils and colloidal layer located on the surface.These differences limit the paper quality as well as the speed, at which paper machines can operate. Freeness reduction during beating is much faster for secondary fibres. The fines that are created after the beating of secondary fibres consist mainly of microfibrils, strongly coupled to each other when they were originally dried on the paper machine. During refining, these fines after liberation increase the specific surface area (SSA) of suspension. They start behaving as fillers, with less effect on strength, but a large effect on the drainage characterstics. As the fibres are recycled, sheet density decreases each time. The strength losses may be the result of loss in either the strength of inter-fibre bonding or in their number. As recycled fibre use and content has increased in paper and board manufacture, the need to improve the drainage rate on the paper machine while maintaining mechanical properties through refining has become more important. Similar studies with the objective to obtain improvement in drainage ofblended colored ledger, flexographic newsprint and old corrugated container (OCC) pulps while

maintaining strength properties was also done by *Eriksson et al. (1998).*

1.6 Importance of pulp drainage

Costs in paper production mainly consists of those caused by fibre 30%, chemicals 10%, fillers 10%, energy 20%, and other costs (labour, environment, etc.) 30%. Raw material costs can be reduced by using less or cheaper raw materials in paper production. But, reduction of raw material costs in papermaking is challenging due to defined paper quality standards. Deteriorated raw material quality may lead to poor printability; convertability orproduct quality.The dewatering properties of the pulp greatly affect the energy efficiency of paper machines. Sustainable objectives including energy and raw material savings are the most important objectives of every industry. In papermaking, the drying process at dryer section being the most energy consuming unit operation is definitely the largest consumer of thermal energy (~80% of all mill energy) on a paper machine as steam *(Austin et al. 2011).* Enhanced water removal in the forming and wet press section will definitely lead to lower energy consumption in the dryer section or higher speed, or alternatively shorter drying section leading to decreased investment costs. It has been estimated by *Austin et al. (2011)* that drying costs can be reduced by 4% if the dry solids content of the web after wet press can be increased by 1%. Production profitability of paper machines is strongly influenced by the runnability i.e. frequency of webbreaks, due to the low net profit of manufacturing. Therefore, new innovations that are able to enhance the runnability of the paper machine can directly improve the cost efficiency of papermaking. Due to the high-speed and width of modern paper machines, improvement in the runnability is quite challenging.

During the manufacturing of paper, the ease with which water is released from cellulosic fibre material is called as pulp drainage. The pulp drainage can affect both the production rate and the energy consumption during the manufacturing process. The theoretical contributions related to dewatering phenomena based on the flow through packed beds of uniformly distributed fibres are able to explain why resistance to dewatering increases as a function of the hydrodynamic surface area of fibres. More recent studies have clearly demonstrated a critical role of fines, i.e. finely divided matter. These unattached fines tend to move freely through the fibre mat and block channels in the paper web during the dewatering process. Dewatering rate is also reported to be affected by deformability of cellulosic fibres.

The removal of water from cellulosic fibres as well as other materials in the wet web constitutes the most energy-demanding part of paper manufacturing process *(McGregor and Knight 1996)*. The cost to remove one unit of moisture in forming, pressing, and drying sections of a paper machine are reported approximately to be in the ratios 1:5:220 *(McGregor and Knight 1996).* Papermakers always try to find ways to produce more tonnes of products at a constant

input of time and energy because of the high capital costs of papermaking equipment, as well as the energy costs associated with papermaking.

The fibre fines causing lower drainage rate in recycled and wheat straw pulp are reported to decisively consist of amorphous cellulose *(Dienes et al. 2004, Oksanen et al. 2000).* Thus, the productivity of the papermaking process is considerably decreased on usage of recycled pulps as compared with virgin pulp. Dewatering properties of the pulp strongly affect the energy efficiency of paper machine and thus the cost efficiency of papermaking. Enhanced water removal in former, press and dryer sections may result either in lower energy consumption or increased paper production capacity. Alternately, due to improved drainage, a shorter drying section would lead to reduced investment costs. Although the utilization of recycled and non-wood pulps has environmental and economical advantages, the low drainability causes decreased productivity. A process that allows an increase in freeness is useful in paper mills that use recycled and non-wood pulps. A substantial gain in the drainage rate could be used in various ways like (i) to improve the speed of the paper machine (ii) to achieve greater dilution in the head box (iii) to use low grade papers more extensively as a source of recycled fibres.

The papermaking process essentially consists of forming a web of cellulosic fibres from a dilute pulp suspension and drying it to target dryness. Basically, it is a water removal process from the forming zone of the paper machine to the dryers. Obviously, substantial cost is associated with water removal, which becomes more expensive toward the pressing and drying sections. As such, the strategy should be to remove as much water as possible when the web is being formed, before the paper reaches the press and the dryer section. Papermakers always like to get any improvement in drainage because it also improves the machine productivity. Particularly, drainage is important on machines having limited drying capacity.The speed at which a paper machine can be run is limited by slow draining stock. It can also limit the extent of pulp refining which is necessary for paper strength improvement.

Several commercial cellulase enzymes are available which claim to improve the drainage of secondary fibres. But using mixtures of cellulases can be disadvantageous for certain pulp properties. Therefore, a judicious choice of the enzyme component in the new commercial enzymatic products, optimum dose levels optimum neutral pH range and lower retention time as per process requirements could make it possible to achieve desired pulp properties, while still not causing unacceptable levels of degradation to the fibres. Further knowledge on the complex “fibre-enzyme” interactions and more data on the effect of enzymatic treatments on the pulp and paper properties are necessary in order to develop a rational design of enzymatic fibre upgradation. The present research work presents a possible action of endoglucanase (EG) and cellobiohydrolase (CBH) components of cellulases (commercial as well as isolated fungal enzymes) on cellulosic pulps at process pH 7.0± 0.5 having high fines content.

In papermaking process, enzymes which can act at neutral to alkaline pH range are required for drainage improvement application, due to process pH conditions. It covers the understanding whether the effectiveness or strength loss depends on a specific type (EG or CBH) of enzyme activity. Better understanding of the enzyme−fines interaction will improve the selection of enzymes for drainage improvement of recycled and wheat straw pulps with high fines content.

The objectives of the present study are as follows.

- Evaluation of different commercial cellulase enzymes at process pH 7.0± 0.5 for improvement in pulp drainage and impact on pulp and paper properties
- Evaluation of enzymes and polymeric drainage aid (sequential treament) for improvement in pulp drainage and impact on pulp and paper properties
- Lab scale production of endoglucanase enzymes and their application for drainage improvement of pulps

CHAPTER 2

LITERATURE REVIEW

2.1 Role of fines in drainage of pulps on paper machine

Several problems are also associated with both recycled and wheat straw pulps. Continuous pulp utilization cycles diminish the bonding capacity, which leads to papers with lower strength properties. These pulp utilization cycles generate more cellulose fines and thus reduce pulp drainage rate in the paper machine *(Maximino et al. 2006)*. One of the major problems with non-wood as well as recycled pulp is the slow drainage in different processes during papermaking. This can be the cause of slow dewatering on the wire and press sections of the paper machine and thus low production. By the improvement in pulp drainage, machine speed could be increased up to 15% on dewatering limiting paper machines *(Pommier et al. 1989, 1990, Caram et al. 1996).*

The most important physical features of nonwood raw materials include low bulk density, short fibre length and high content of fines *(Oinonen and Koskivirta 1999, Paavilainen 2000).* The large amount of fines and the short fibre length affect especially the drainage properties of pulp. The fines in a wheat straw pulp, consists particularly of non-fibrous cells like parenchyma cells, epidermis cells, and vessels. These parenchyma cells are easily deformed and when present, also reduce drainage as a result of their high specific surface area *(Cheng 1994, Cheng and Paulapuro 1996 a, b).* Large specific surface area can be the reason for increased contacts between the fluid and internal surface of the fibres, leading to an increase in drainage resistance. Besides specific surface area, there may be also other shape factors that affect drainage *(Liimatainen 2009).*

The fines content in wheat straw ranging from 20 to 45% have already been reported in the literature *(Guo et al. 2009, Rousu et al. 2010, Rousu and Hytonen 2007, Rousu and Ninimaki 2007).* Recent studies have shown that removal of fines from wheat straw pulps either by conventional methods such as fractionation or other methods can improve not only the pulp drainability but also the mechanical and optical properties of the paper sheet, indicating that wheat straw fines are actually acting as a filler with low bonding properties in the paper sheet *(Guo et al. 2009, Ljusgren et al. 2006).* This differs from hard-and softwood pulps in which the fine material is known to contribute to paper strength *(Paavilainen 2000 and Ferreira et al. 2000).*

In order to better understand the mechanisms grouped under the heading “Choke Point Hypothesis”, the behavior of unattached fines and what can happen to these fines during water removal should be well considered. Several authors have proposed that unattached fibre fines,

which can move freely through the paper web during the process of dewatering, have more chances of blocking the water channels *(Hubbe and Heitmann 2007).* The choke-point hypothesis, discussed in detail by *Hubbe and Heitmann (2007),* by which fines attached and block the water channels is illustrated in Figure 2.1a. These unattached fines move freely through drainage channels and block the water flow. Figure 2.1b shows the fibre fines attached to fibre surfaces through bridging polyelectrolytes. The adverse effect of secondary fines on drainage could be more effectively overcome by treating them in such a way as to reduce their effective surface area *(Hubbe 2000).*

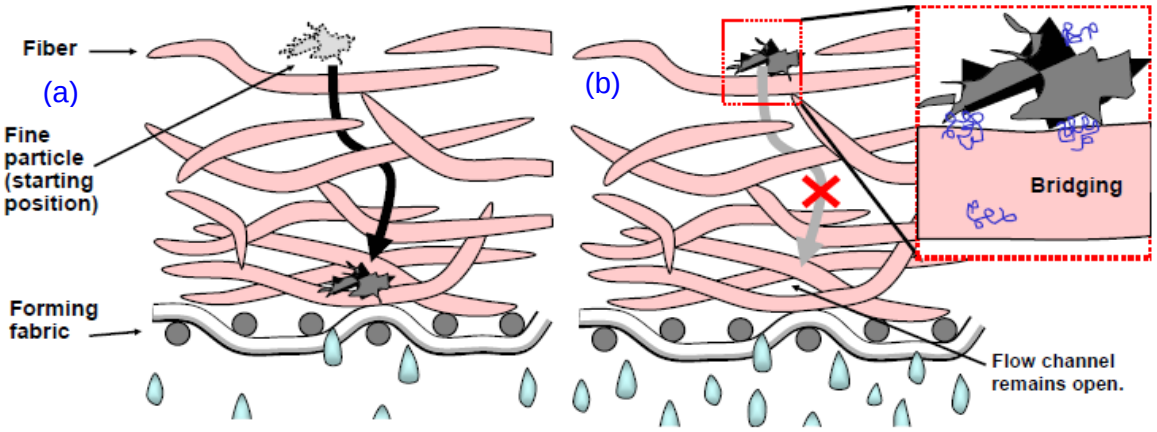


Figure 2.1 Schematic illustration of choke point mechanism (Hubbe and Heitmann 2007)

2.2 Improvement in drainage

Recycled fibres can be upgraded through refining, chemical additives, fractionation or blending with virgin pulp *(Bhat et al. 1991, Abubakr et al. 1995).* However, none of these treatments completely restore the virgin fibre properties. Through refining, the bonding ability and tensile strength are restored but drainage properties are damaged. The high molecular weight polymers sometimes used as retention/formation aids increase the flow resistance through orifices by absorbing energy from the fluid. Flocculation influences the drainage by the collection of fines and colloidal substances to the fibre surfaces and by increasing the free area for water removal. However, too strong flocculation can give voluminous flocs which are very difficult to dewater *(Hubbe et al. 2009).*

The use of enzymes for secondary fibre upgradation has also been extensively studied *(Pommier et al.1989, 1990, Putz et al. 1990, Bhardwaj et al. 1995).* Reduction in fines content, better drainability, and improvement in strength properties have also been reported for different enzyme treated pulps *(Jain et al. 2013).* In most of the studies commercial mixtures of different cellulases and hemicellulases have been used and the enzyme treatments of recycled pulps have resulted in improved drainage properties.

2.2.1 Drainage improvement using polymeric drainage aids

Drainage aids are the materials known to increase the drainage rate of water from the pulp slurry on the wire. Almost any retention aid is apt to improve the drainage rate, as fines and fillers are removed from the white water. Different drainage aids used for improvement in pulp drainage are p-DADMAC (Poly diallyl dimethyl ammonium chloride), PEI (Poly ethylene imine), CATPAM (Cationic polyacrylamide), ANPAM (Anionic polyacrylamide), micro particle system, etc *(Litchfield 1994).* Polymers can act in accordance with two mechanisms, namely patch flocculation and bridge flocculation.

Patch flocculation- It is the interaction between oppositely charged regions on the particles (Figure 2.2a). It is based upon the formation of cationic sites with a high charge density on the fibre surfaces by cationic polyelectrolytes. If there is strong interaction between the particles and polymer, the polymer can be absorbed in cationic patches on the negatively charged particle surface so that partial charge neutralization takes place *(Litchfield 1994).*

Bridge flocculation- It is the formation of polymer bridges between particles (Figure 2.2b). In this type of mechanism one part of the polymer becomes attached to one or more absorption sites, while its other part extends into the bulk solution. These extended loops and tails can be absorbed onto other particles thus forming polymer bridges *(Hubbe and Heitmann 2007, Hubbe 2009).*

Drawbacks in using drainage aids

Conventionally, dewatering is increased in a paper machine by using drainage aids in the wet-end section and/or more intense wet pressing in the press section *(Antunes et al. 2008).* Micro-particle based retention aids are reported to be used for the improvement of drainage of non-wood pulp containing furnishes *(Vishtal et al. 2011).* But use of drainage aids can worsen formation, and high wet press levels decreases the bulk of the end-product *(Oksanen et al. 2011)*. Their advantages to paper manufacture are sometimes limited as they do not assure the simultaneous increase in paper resistance and pulp drainage ability *(Marton et al., 1993).* The formation is adversely affected by the use of drainage aids, especially at high levels of addition. The presence of fibre flocs results in less uniform sheet and a flocculated sheet does not generally respond well to vacuum dewatering *(Räisänen et al.1995).*

Strong interrelationships between retention, drainage, flocculation and reduced strength have been observed *(Horn and Linhart 1996).* Therefore, novel pulp modification and dewatering innovations are required with new high speed paper machines. Selective application of cellulase enzyme components could improve the drainability in an environmentally friendly way *(Verma et al. 2010, Singh et al. 2011).*

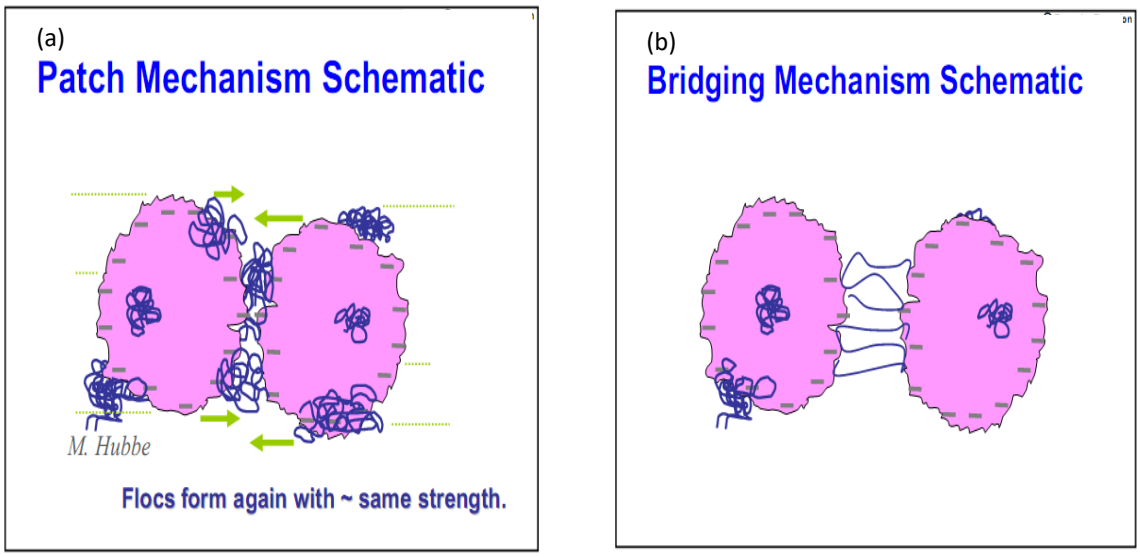


Figure 2.2 (a) Patch mechanism (b) bridging mechanism (Hubbe and Heitmann 2007)

2.2.2 Drainage improvement using enzymes

The concept of cellulose degradation by cellulolytic enzymes was first proposed by *Reese et al. (1950).* Since then, the enzymatic degradation of cellulose has been studied extensively. The initial studies were mainly aimed to better understand the fundamental mechanism of degradation. Later, the objective of investigations focused on the actual transformation of cellulose into soluble and fermentable sugars and thus a source of renewable energy and chemicals. Mild and selective modification of cellulosic fibres was another area of cellulose application that was investigated, and to some extent implemented in textile as well as in the paper manufacturing industry. At the turn of the century, the interest in the enzymatic hydrolysis of cellulose increased dramatically. This renewed attention was a result of increased awareness of the critically declining resources of fossil fuels and an urgent need for finding alternative and sustainable sources of energy. The appeal of cellulose assisted hydrolysis of cellulose was further enhanced by recent advances in the mechanistic understanding of cellulase action as well as by progress in the production of the enzymes. The cost of cellulase, or cost of the actual hydrolysis, has been identified as one of the main determinants in the overall economics of the process; therefore, more efficient cellulase production at lower cost is essential for commercial feasibility of the process. In general, a better understanding of the cellulose hydrolysis is crucial for designing efficient and cost competitive bioconversion of biomass, justifying the ongoing research in the area.

Cellulases: classes and mode of substrate action

The term cellulase complex normally refers to the complex of enzymes involved in cellulose hydrolysis; a complete cellulase complex is required in the complete hydrolysis of crystalline cellulose.

1) 1, 4-β-D-glucan-4-glucanohydrolases (endoglucanases EC 3.2.1.4), randomly hydrolyze β-(1, 4)-glucosidic linkages, although the degree of randomness may vary amongst the several different endoglucanases which are normally produced by a single organism *(Erikkson 1990).*

2) 1, 4-β-D-glucancellobiohydrolases (Exo-β-(1,4)-glucanases, EC 3.2.1.91) cleave cellobiose units from the non-reducing ends of cellulose molecules. The hydrolysis of exoglucanases is restricted to the ends of cellulose chains as their structure hinders their access to the substrate *(Meinke et al. 1995).*

3) 1, 4-β-D-Glucanglucohydrolases (Exo-β-(1, 4)-glucosidases, EC 3.2.1.21) cleave glucose units successively from the non-reducing end of the glucan. They differ from β-glucosidase because of their preference for substrates of longer chain length and by inversion of their products *(Erikkson 1990).*

It is generally accepted that cellulose degradation by cellulases is initiated in the amorphous regions by endoglucanase, randomly cleaving internal glucosidic bonds along the cellulose chain length, decreasing the degree of polymerization (DP) of substrate. Then the newly created chain ends become available sites for action of cellobiohydrolase. Cellobiohydrolases are known to be processive enzymes initiating their action from ends of the cellulose chains, producing primarily cellobiose that is then released to solution. They attack the crystalline parts of the substrate and decrease the DP of the substrate, although slowly. Cellobiose is known to inhibit both exoglucanases and endoglucanases and makes this action rate limiting step in cellulose degradation *(Lee 1997).* The β-D-glucosidase (cellobiase) converts cellooligosaccharides and cellobiose to glucose. Therefore, the presence of β-glucosidase, which converts cellobiose to glucose, propels the reaction in the forward direction.

The activity of cellulase systems is greater than the collective sum of individual activities, a phenomenon known as synergism. Synergism is a function of multiple forms of cellulases and the kind of cellulose i.e. amorphous or crystalline. There are different types of synergism: exo-endo, exo-exo, exo-gluco, etc. Out of all these synergistic actions, synergism between exoglucanase and endoglucanase is the most important *(Lynd et al. 2002, Kumar et al. 2008).* As mentioned earlier, cellulose has both crystalline and amorphous regions and it is easier to hydrolyze amorphous regions in comparison to crystalline regions *(Coughlan 1992).*

Mechanism of cellulase action

Endoglucanases randomly attack in the middle of the more disordered amorphous regions of cellulose and produce oligosaccharides of varying lengths and create new chain ends for exoglucanases. Crystalline regions are resistant to attack by endoglucanases and the bonds cleaved are re-formed owing to stability of crystalline glucan chains. Exoglucanases attack the crystalline areas and chop off cellobiose units from newly created ends formed by

endoglucanase, thereby preventing the reformation of bonds. A schematic of cellulase action is shown in Figure 2.3.

The hydrolysis of exoglucanases is restricted to the ends of cellulose chains as their access to substrate is hindered by their structure. β-glucosidase hydrolyzes cellobiose units to form glucose units (Figure 2.3). Cellobiose is known to inhibit both exoglucanases and endoglucanases and makes this action rate limiting step in cellulose degradation. Therefore, the presence of β-glucosidase, which converts cellobiose to glucose, propels the reaction in the forward direction.

Commercially available cellulases often display optimum activity over a pH range from 4 to 6. Recently, enzymes that are active and stable in neutral to alkaline conditions have gained significant commercial importance due to their ecofriendly applications in detergent, textile and paper industries. Thermostable cellulases having high alkali tolerance are known to reduce the substrate viscosity in bulk processes, leading to higher reaction velocities, better substrate conversion and lower energy consumption. Alkalophilic microorganisms (fungi) growing in alkaline habitats in nature should be screened for selection of promising cultures that produce extracellular cellulase enzymes, both cellobiohydrolase as well as endoglucanase, of desired properties *(Vyas and Lachke 2003).*

Effect of structural features on enzymatic hydrolysis

Although the composition of cellulase system influences enzymatic hydrolysis, the dominant factor is the dependency on structural features of cellulose such as crystallinity, lignin content, particle size, pore volume and accessible surface area which are specific to source and nature of substrate *(Fan et al. 1980, Dusterhoft et al. 1993, Lee 1997).* The initial rate of enzymatic hydrolysis is relatively rapid and decreases over time. The biphasic behavior of enzymatic hydrolysis has been investigated by researchers for a long time; however there is still no definite answer and the mechanism is not clearly understood. It is a complex process which is a function of many variables and is further complicated by changing reaction dynamics. The fine structure variability of cellulose makes it difficult to obtain a discrete population of particles with identical structural features. Because of a great variability in the shape and size of the particles within any cellulose sample, measurements of structural features are only the average values of those features. Thus, experiments are limited to comparing measurements of hydrolysis among cellulose particles of average structural features and it is hard to study the effect of a particular structural feature on hydrolysis rates *(Lynd et al. 2002).*

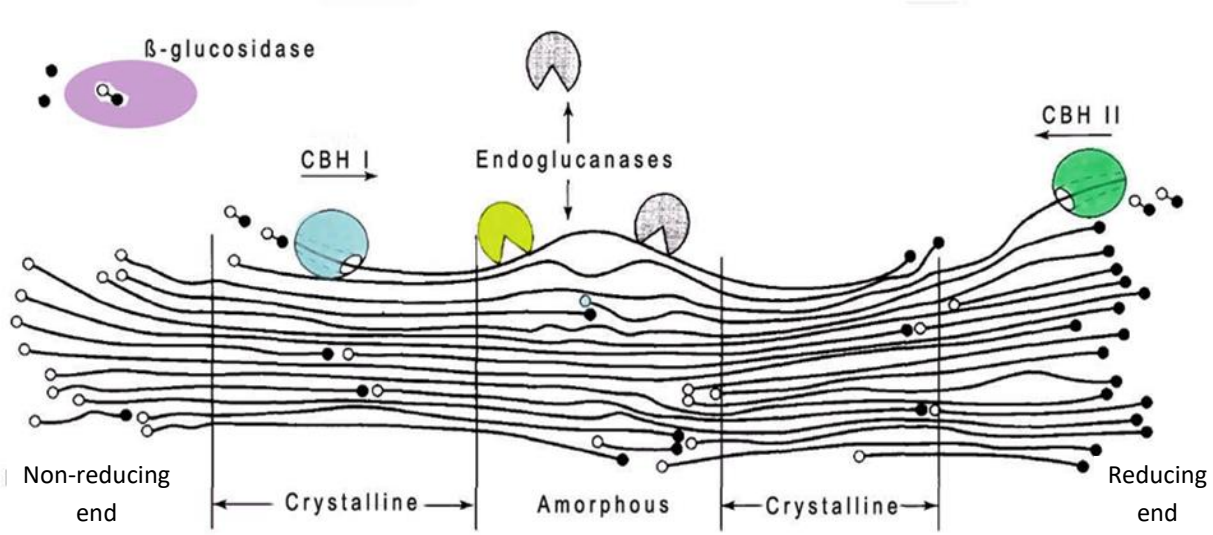


Figure 2.3 Mechanism of enzymatic hydrolysis of cellulose (Teeri 1997)

Because of interrelationships among various structural features, one cannot identify a particular structural feature as the reason for slowing down of hydrolysis rates. For example, mechanical treatment of fibres to reduce crystallinity also increases the accessible surface area of cellulose. Therefore the increase in enzymatic hydrolysis can be due to either a decrease in crystallinity or an increase in surface area or a combination of both these factors. Similarly, structural discontinuities that contribute to an increase in pore volume also lower the average crystallinity. It is impossible to alter one fine structural feature without altering others *(Lynd et al. 2002).*

The most common methods for the characterization of crystalline cellulose structure are based on X-ray *(Kolpak and Blackwell 1976, Krassig 1993)* or *infrared (IR) absorption (Fink et al. 1985, Oh et al. 2005).* As mentioned earlier, crystalline regions are more difficult to hydrolyze than amorphous regions. It has been reported by several researchers that the rate of hydrolysis of amorphous cellulose is five to thirty times higher than that of crystalline cellulose *(Ghana et al.1993, Lynd et al. 2002; Ortega et al. 2007, Zuhair 2007).* It has been reported that a lower starting crystallinity index produces higher saccharification rates *(Fan et al. 1980).* Studies have also been conducted by ball milling of crystalline cellulose, which decreases both particle size and crystallinity which confirm an enhancement in enzymatic hydrolysis rate with a reduction in crystallinity *(Chang and Holtzapple 2007, Yoshida 2008).* However, it is not clear if this increase in hydrolysis rate is a concerted effect of decrease in particle size and reduction in crystallinity. Cellulose is made up of both crystalline and amorphous fractions and if enzymes preferentially attack amorphous regions then one expects crystallinity to increase over the course of hydrolysis *(Fan et al. 1980).* There are conflicting results in support of this postulation. A few groups of investigators have reported an increase in crystallinity as hydrolysis proceeds *(Saddler 1986, Park et al. 2007).* Several other studies did not find any appreciable increase

in crystallinity during the course of enzymatic hydrolysis *(Lynd et al. 2002).* However, *Sinitsyn et al. (1989)* found that crystallinity initially increases, then decreases and finally levels off.

Papers that have reported a direct correlation between hydrolysis rates and crystallinity have mostly used pure cellulosic substrates. The equivocal results and uncertainty of methodologies used to measure crystallinity make it difficult to conclude at this time if crystallinity index is a major determinant of the rate of enzymatic hydrolysis *(Lynd et al. 2002; Mansfield et al. 1999; Yoshida et al. 2008).*

Fungal cellulases

Cellulases are produced by wide variety of bacteria, fungi, actinomycetes, aerobes and anaerobes, mesophiles and thermophiles. However, fungi are the most studied organisms and are mostly used in industrial processes because of their higher enzyme yields and capacities to produce complete cellulase complex. Cellulolytic filamentous fungi having the ability to penetrate cellulosic substrates through their hyphal extensions, thus reach all portions of the cellulosic particles. This ability to reach out for the substrate with or without the presence of cellulose binding domain (CBDs) shows the efficiency of the cellulose hydrolysis. The enzymes in this cellulase system do not form stable high- molecular weight complexes and therefore are termed “Non-complex” systems *(Lynd et al. 2002).*

The enzymes produced by aerobic fungus, *Trichoderma reesei* are broadly studied for enzymatic hydrolysis of cellulose. These enzymes constitute non-complexed cellulase systems i.e. systems based on synergistic discrete action of individual components rather than that of a stable complex *(Meinke et al. 1995).* The general structure of most of the cellulases can be broken down into two structural parts: the catalytic domain (CD) and the carbohydrate binding domain (CBD), both of which are connected via a flexible linker peptide. CBD promotes the adsorption of the cellulase to the crystalline region of the cellulosic substrate and facilitates the hydrolysis by bringing its catalytic domain in close proximity with cellulose chains *(Abuja et al.1988).* The adsorption of cellulases onto cellulosic substrates is affected by the degree of polymerization, crystallinity, pH and temperature *(Lynd et al. 2002, Zhang and Lynd, 2004).*

Trichoderma reesei is reported to produce at least two exoglucanases, five endoglucanases and two beta-glucosidases *(Vinzant et al. 2001).* The two exoglucanases are explained by the two ends, i.e. reducing and non-reducing ends of crystalline cellulose and are also supported by synergy between exo-exoglucanases. However, the role of five endoglucanases is not clearly understood and is attributed to different nature of binding sites available on cellulose chains. Two β-glucosidases are explained by enzymes needed for breaking down cellobioses and short oligosaccharides to glucose. Although enzyme composition is an important factor which influences hydrolysis but accessibility of substrates has been found to be the overriding factor which affects hydrolysis *(Ortega et al. 2001).*

Cellobiohydrolase I (CBH I) is the most abundant enzyme produced by *T. reesei*, making up 60% of the total cellulolytic protein and thus accounting for majority of its cellulolytic activity *(Teeri 1997).* The structure of isolated CBH I and its mechanism of action have been thoroughly investigated. The CBH I and other *T. reesei* cellulases consist of two distinct domains– a cellulose binding modulus (CBM) and an active catalytic core. These two domains are linked together by a flexible linker peptide *(Tomme et al. 1988).* The CBM does not possess hydrolytic activity and has no effect on the hydrolysis of soluble substrates. Its role is mainly the adsorption of the active catalytic core onto an insoluble substrate and it is essential for enzymatic activity on crystalline cellulose. The structure, studied by multidimensional NMR, was shown to be wedge shaped with one of the two sides being very flat *(Kraulis et al. 1989)*. The flat side (face) is composed of three aromatic residues (tyrosines) that are approximately 1.04 nm apart from each other, which coincides with the size of glucose unit and suggests that the flat size would bind to cellulose through these residues (Henrissat 1994). The catalytic core is responsible for the actual hydrolysis of the substrate, and its structure has been shown to be in the shape of a tunnel, approximately 50 A long *(Divne et al. 1994).*

Trametes versicolor is reported to be one of the most competent wood-degrading white-rot fungi. It *is* a colorful bracket fungus, also known as Turkey Tail. It belongs to division Basidiomycota, class Agaricomycetes, Polyporales order and family Polyporaceae. Due to its ability to degrade lignocelluloses *(Collin and Dobson 1997, Tanaka et al. 1999),* different enzymes like cellobiose dehydrogenase, laccase, lignin peroxidases and manganese peroxidases are reported to be secreted *(Dodson 1987, Dumonceaux 1998, Johansson et al. 2002, Paice 1993)*.The said fungus is commonly found on standing dead or fallen hardwood trees.

Alternaria species has long been reported for their cellulolytic potential *(Logan and Siehr 1966).* It has also been proved that some of the species of *Alternaria* induce plant invasion by elaborating the cellulolytic enzymes and genes encoding endoglucanases from this organism have been characterized *(Eshel et al. 2002).* The hydrolytic potential of an indigenous strain MS28 of *Alternaria* has been reported by *Sohail et al. 2009.* Some *Alternaria* strains had previously been mutagenized for improved *productivity (Macris 1984).* Cellulase enzyme production and optimization study were reported from different *Alternaria* sps including *Alternaria sesame, Alternaria helianthi, Alternaria triticina (Bhaskaran and Kandaswamy 1978, Jha and Gupta 1988, Jahangeer et al. 2005, Dawar and Jain 2010)*, A. alternate *(Anand et al. 2008, Hubballi et al. 2011, Gautam et al. 2012, Saleem et al. 2013), Alternaria* brassicae *(Jain and Dhawan 2008).* Major fungi reported to produce cellulase and Xylanase enzymes are given in Table 2.2.

2.3 Enzymatic up-gradation of pulps having high fine content

Enzymatic technologies can provide a natural and ecofriendly solutions for various problems encountered in the papermaking process especially related to bleaching, deinking, drainability, refining etc. *(Sharma et al. 2014, Thakur et al. 2012, Pathak et al. 2014, Gupta et al. 2011,Torres et al. 2012).* Application of cellulase free xylano-pectinolytic enzymes from bacterial isolate was reported to work well in biobleaching of kraft pulps *(Kaur et al. 2010)*. *Cadena et al. (2010)* used Cel9B as a highly efficient biocatalyst in pulp refining to reduce the refining energy. Published results vary in response to the various enzymatic activities present in the cellulase preparations. Other carbohydrate-modifying enzymes are often present in commercial preparations as side activities *(Garcia et al. 2013)*. Knowledge of endoglucanase and cellobiohydrolase action on cellulosic fibres is needed for selective and controlled fibre modification. The action of cellulase on the fibre components can result in improved inter-fibre bonding and decreased fibre-water interactions. The enzyme specificity plays a very important role in drainage improvement. Endoglucanases are more active on amorphous cellulose and randomly attack the inner part of the cellulosic chain, whereas exoglucanases can hydrolyze both crystalline and amorphous cellulose by removing cellobiose from the terminal part of cellulose chains *(Henrissat and Davies 1995)*. It has also been suggested that shorter fibres with a larger specific surface area are more susceptible to the enzymatic attack than longer ones *(Pala et al. 2001).*

The adverse effect of secondary fines on drainage could be more effectively overcome by treating them in such a way as to reduce their effective surface area. It was suggested by *Jackson et al. (1993)* that enzymes can either flocculate or hydrolyze fines and remove fibrils from the surface of large fines. At a low enzyme dose, fines and small fibre particles tend to aggregate with each other or with the larger fibres. Low enzyme dosages produced fines content reductions, which were related to a possible effect of flocculation produced by the enzyme, similar to the one of polymeric drainage aids. These aggregations decrease the amount of small particles in pulp and consequently improve pulp drainage. The highest enzyme dosages led to increased fines contents, which were attributed to the fiber disintegration produced by cellulases *(Jackson et al. 1993).*

Enzyme usage can be an elegant way to demonstrate the effect of specific surface area on dewatering resistance of cellulosic material. Optimization of cellulose treatments can be done to systematically clean up or “polish” the surfaces of fibres and fibre fines, thereby removing fibrillar material, projecting outwards from such surfaces. In such a way, cellulose treatment after refining can provide a significant increase in drainage rates *(Gong and Bi 2005).*

Table 2.1 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*	*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	*Jorgensen 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 viride*	*Malik et al. 2010*
Trichoderma reesei	*Dashtban et al. 2011*		

When cellulolytic enzymes are used for partial hydrolysis of cellulose chains, and thus to form a better wheat straw and recycled fibre structure, a balance is required between two opposite directions. On one hand, by the hydrolysis of fines, improved dewatering rate is obtained. On the other hand, enough fines have to be left in the pulp in order to obtain optimal interfiber bonding, required for good strength properties of the end product. Besides, action of enzyme should not result in excessive hydrolysis, as it may lead to loss of weight and thus production.

Pulp drainability can be improved through application of cellulases and hemicellulases, in an environmentally friendly way. Several explanations along with various experimental observations, for the possible effects of enzymes have been suggested. It has also been reported that although enzymatic treatment of fibres causes the decrease of the amount of amorphous and gel-like polysaccharide layer on the surface, it did not affect the fines content *(Kantelinen and Jokinen 1997).* On the other hand, enzymes may behave like the retention aids and polymers, facilitating the flocculation of the small fibre particles *(Mansfield et al. 1998, Mansfield and Wong 1999).*

Bhardwaj et al. (1997) examined several chemical additives as well as enzymes such as modified acrylamide and modified starches, and a preparation of cellulase and hemicellulases enzymes to improve the drainage and strength of secondary fibres, containing corrugated Kraft cuttings and corrugated boxes. Using enzymes, improvement in drainage by 40% was observed when compared to control, without any appreciable change in pulp strength properties.

Enzymatic action was described as a “peeling effect” by *Pommier et al. (1989).* These authors suggested that selective enzymes are able to defibrillate the fibres by removing the molecules having high water affinity, but contributing less to the overall hydrogen bonding potential of the fibres. This reduction in pulp – water interactions allows better pulp drainage, without affecting the final mechanical properties of paper. An intensive enzymatic reaction leads to both intrinsic fibre strength and fibre length reduction, and excessive fines production. As a result, the paper strength is dramatically affected.

It was suggested by *Jackson et al. (1993)* that enzymes can either flocculate or hydrolyze fines and are capable to remove fibrils from the surface of large fines. When a low enzyme dose is used, enzyme aided flocculation occurs in which fines and small fibre particles aggregate with each other or with the larger fibres, decreasing the amount of small particles in pulp and thus improve pulp drainage. On the other hand, at higher enzyme concentration, flocculation becomes less significant, and hydrolysis of fines begins to predominate.

The endoglucanases (EG I and EG II) of *Trichoderma reesei* have been reported to significantly improve the pulp drainage even at low dose levels *(Oksanen et al. 2000).* Combined treatment of hemicellulose and endoglucanase enzymes are reported to further increase the pulp drainage. However, as a result of endoglucanase treatments at high dose,

slight loss in strength was also observed. It was reported that endoglucanases enhance dewatering by hydrolyzing the amorphous hydrophilic cellulose which is the main constituent of the fines formed during refining *(Oksanen et al. 2000)*. Significant increase in drainage rate along with improvement in smoothness and tensile index using endoglucanase enzymes was also reported by *Oksanen et al. (2009)*. The probable explanation for an increased drying rate is a more bulky and porous fibre network with enzymatic treated pulp. Reduced shrinkage forces due to hydrolysis of fines and surface hemicelluloses are a probable explanation for the increase in bulk with enzymatic treated pulps *(Oksanen et al. 2011)*. Xyloglucan treatment has also been reported to improve the runnability, end product quality by improving smoothness and dry strength properties, and decreasing air permeability *(Oksanen et al. 2011)*.

The effectiveness of a cellulase enzyme, having predominantly endoglucanase (EG) activity, for improving the freeness and drainability of different types of recycled pulps was evaluated by *Shaikh and Luo (2009)* in laboratory as well as in paper mills. On treating the refined pulps with endoglucanase, Canadian standard freeness (CSF) was observed to be improved by 13.1%, 19.3% and 40.5% in recycled (NP); old corrugated container (OCC) mixed waste (MW) pulps, respectively.

It was shown by *Pere et al. (1995)* that endo and exo glucanases influence the paper properties differently. Endoglucanase can lower the pulp viscosity and thus dramatically reduce the pulp strength. Endoglucanases are more active on amorphous cellulose and randomly attack the inner part of cellulosic chain, whereas exoglucanases hydrolyze both crystalline and amorphous cellulose by removing cellobiose from terminal part of cellulose chains *(Henrissat et al. 1995)*.

Early attempts to utilize enzymes in the pulp and paper industry started in mid 1980s. At that time, the need of the industry to adopt environmentally benign technologies in pulp production and processing stimulated the interest in the use of enzymes. Later, a better understanding of the enzyme mechanism, environment friendly nature and the promise of more efficient production of commercial enzymes at lower cost were the main drivers of the extensive research and efforts in the final decade of last century. At that time, several authors reviewed the status of enzyme applications in the pulp and paper industry *(Viikari et al. 1998, Bajpai 1999, Mansfield and Wong 1999)*. The potential applications of cellulases in paper industry were identified for reducing the refining energy demands of mechanical pulps, modifications of chemical fibre properties, enhanced de-inking of recycled fibres, and process water treatment. Many of these enzymatic applications showed potential, but only a few applications found their way to a successful commercial implementation.

Traditionally, cellulases have been considered detrimental to pulp and paper properties, particularly yield and strength properties. However, selective and controlled hydrolysis of fibre

carbohydrates can have positive effects. The extent of the hydrolytic action can be controlled either by adjusting the dose, treatment time, or by eliminating the synergism of the cellulase components. As reported earlier, cellulase components act in a synergistic manner and complete hydrolysis of cellulose requires the presence of both endo- and exocellulases *(Henrissat 1994, Medve et al. 1994).* The use of monocomponent cellulases for selective and more targeted action can have tremendous potential in many pulp and paper applications. *Pere et al. (1995)* initially evaluated the concept of using individual components of cellulase. The individual cellulose components exhibited significant differences in their mode of action and the resultant effects on pulp properties.

Since, the presence of fines and highly fibrillated fibres are related with low pulp freeness, several theories have been proposed to explain the increase in freeness, after enzymatic treatments. It has been reported that enzymatic attack may involve a peeling mechanism, which removes fibrils and fibre bundles that have a high affinity for water, and leaves the fibres less hydrophilic and easier to drain. On the other hand, it has also been suggested that enzymes act preferably on fines, having a tendency to block up interstices in the fibre network. The increase in drainage has also been recognized to the cleaving of amorphous cellulose on the surface of fines *(Stork et al. 1995*). One of the explanations suggested that due to the high specific surface area of the fines, the attack of cellulases was specific towards this fraction i.e. fines of the pulp. It has been pointed out in literature that the fibre surface is exposed through the enzymatic hydrolysis of subsequent layers or fibrils *(Jackson et al 1993).*

Multicomponent cellulases vs Monocomponent cellulases

In secondary fibres the fines and fibrils, which cause low rate of drainage, decisively consist of amorphous cellulose. Since, the amorphous cellulose is known to be more accessible than crystalline cellulose, it is not necessary to use the whole cellulose complex for the hydrolysis. Several cellulase enzymes, which claim to improve the drainage of pulp, are available in the market. But, using mixtures of cellulases can be disadvantageous for certain pulp properties. By the application of purified enzymes, specific regions of cellulose fibres can be attacked, the desired part of the pulp could be modified *(Dienes et al. 2004).* Therefore, applying specific cellulase component may be effective enough for selective and controlled modification of fibres. Several researchers have postulated that endoglucanases could be the best candidate for drainage improvement in pulp and paper industry *(Pala et al. 2001; Dienes et al. 2004).*

Interestingly, the increased solubilisation of amorphous cellulose mediated by endoglucanase treatments (EGI and EGII) are reported to improve water retention values, and consequently improved drainability *(Dienes et al. 2004).* Furthermore, combinations of endoglucanases and hemicellulases acted synergistically to improve the drainage beyond that improved by single enzymes alone. In contrast, cellobiohydrolase treatments (CBH I) failed to improve the fibre

drainage, and only in combination with hemicellulases, there were slight positive effects observed *(Oksanen et al 2009).*

The combined enzyme and polymer approach have also been reported not only to enhance the freeness of the recycled pulp, but also to increase the strength of paper *(Sarkar et al. 1995)*. A likely explanation may be that the enzymes attack colloidal cellulosic material i.e. fines, and as their quantity decreases, interstitial water flows more easily. Another possibility is that the enzyme acts on the fibre surface and remove small components that have a high affinity for water *(Sarkar and Cosper 1992).* Enzyme treatment in combination with a high molecular weight cationic polymer was studied by *Sarkar et al. (1995)*. An enzyme and polymer treated furnish showed a significant increase in filtration rate. This increase was not achieved by using polymer alone.

Considering all these findings, it can be postulated that the improvement of drainage of recycled and wheat straw pulps, having high fines content can be achieved by the selective and controlled hydrolysis of amorphous cellulosic fines. By applying purified enzymes on specific regions of the cellulose fibres, the desired part of the pulp could be modified in a controlled manner, for drainage improvement. Further knowledge on the interactions between fibres and enzymes and more data on the effect of enzymatic treatments on the pulp and paper properties is required for further understanding of fibre upgradation through enzymes. The following research investigates the impact of different commercial and isolated cellulase enzymes on the structure, freeness, drainage, viscosity and paper strength for recycled pulp-Newsprint-NP, recycled pulp-writing printing-WP and wheat straw-WS pulp.

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

3.1.1 Chemicals and reagents

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

3.1.2 Cellulosic substrates as carbon source

Bagasse pith and rice bran used in this study as cellulosic substrates, were collected from Yamuna Nagar and Saharanpur, India.

3.1.3 Cellulosic pulps

(a*) Recycled pulp-NP*

Deinked recycled pulp constituting 75% old newsprint, 5-8% old record and 17-20% coated book stock recovered papers was procured from a paper mill in India. The recycled pulp was meant for newsprint recycled paper production and will be referred as recycled pulp-NP in subsequent discussions.

(b) Recycled pulp-WP

Wood-free deinked recycled pulp constituting sorted office pack, coated book stock and old record recovered papers was procured from a recovered paper based paper mill in Northern India. The recycled pulp was meant for production of writing printing grade recycled paper and will be referred as recycled pulp-WP in subsequent discussions.

(c) Wheat straw pulp-WS

Never dried bleached wheat straw pulp, meant for writing printing grade paper production was procured from a paper mill in Northern India.

3.1.4 Commercial cellulase enzymes

The four different commercial enzyme products used for this study were procured from different enzyme manufacturing companies, as given below.

Commercial enzyme products	**Manufacturer**
Fibercare R	Novozymes (Denmark)
Fibercare D	
Texbio M	Tex Biosciences (India)
Fibrezyme LBR	Dyadic (U.S.A.)

While the four enzymes contain both major classes of cellulases, they differ significantly in their relative activity of endoglucanases, exoglucanases (cellobiohydrolases) and activity shown relative to degradation of filter paper (denoted as Filter paper (FPase) activity). The enzyme preparations were characterized by their activity against standard substrates purchased from Sigma Aldrich, USA. The activities are presented in international units (IU) per mL of enzyme preparation. Fibercare D and Fibercare R from Novozymes, Denmark were engineered blend of mainly endoglucanases with side activities of cellobiohydrolase and mixed blend of both endoglucanase and cellobiohydrolase enzymes components in sufficient quantity, respectively. Fibrezyme LBR from Dyadic, U.S.A was a natural blend of enzymes having more cellobiohydrolase activity.

3.1.5 Polymeric drainage aid: A cationic, medium to high molecular weight commercial (BASF) polymeric drainage aid, Percol 47was used in the study.

3.2 Methods

3.2.1 Characterization of commercial and isolated fungal enzymes

CMCase assay

The endoglucanase activity of enzyme preparations was determined using sodium carboxymethyl cellulose (CMC) as a substrate *(Ghose 1987)*. 0.5 mL of suitably diluted enzyme solution (commercial or isolated fungal) was incubated together with 0.5 mL of 2% CMC solution in sodium phosphate buffer (0.05M, pH 7.0) for 30 min. at 45°C. Controls were routinely included in which heat killed enzyme (20 min at 100°C) and substrate treated similarly. After 30 min. of incubation, 3 mL of dinitro salicylic acid (DNS) reagent was added to terminate the reaction. This was followed by boiling of the reaction mixture on a vigorously boiling water bath for 5 min. for color production. After boiling, the tubes were cooled to room temperature. 20 mL distilled water (D.W.) was added to each tube and the mixture was thoroughly mixed and optical density was measured at 540 nm in an ultra violet -visible (UV-Vis) spectrophotometer. Reducing sugars released from CMC were determined by comparing the values with the standard curve prepared by D-glucose *(Annexure-2)*. The enzyme activity was expressed as micromoles of D-glucose equivalents released per min. at 50°C i.e. international unit per mL (IU/mL).

Cellobiohydrolase, CBH 1 assay

The exocellulase activity (cellobiohydrolase, CBH 1, referred as CBH assay and CBH activity in subsequent discusiions) was determined with p-nitrophenyl-β-D-cellobioside (pNPC) (Sigma Aldrich, USA) as a substrate *(Gusakov et al. 2005)*. Aliquot of 10mM substrate stock solution (0.05 mL) was mixed with 0.85 mL of 0.1M acetate buffer, pH 5.0, and preheated at 40°C for 5 min. The enzymatic reaction was started by the addition of 0.1 mL of suitably diluted enzyme and preheated at 40°C for 5 min. After exactly 10 min. of incubation of the

mixture at 40°C, the reaction was stopped by the addition of 0.5 mL of 1M sodium carbonate solution. Then the absorbance at 400 nm was measured on a spectrophotometer against a substrate blank that was prepared and incubated in the same way as the sample with enzyme, except the acetate buffer (0.1 mL) was added to the blank instead of enzyme. The quantity of p-nitrophenol released was determined using its extinction coefficient (18,300 $M^{-1}cm^{-1}$), and then the enzyme activity was calculated.

Filter paper assay

The FPase activity was determined by incubating 0.5 mL of suitably diluted enzyme with 50 mg of Whatman No. 1 filter paper of 1 cm x 6 cm size *(Ghose 1987).* After 60 min. of incubation at 45°C, 3.0 mL DNS was added and mixed. The tubes were boiled for exactly 5.0 min. in a vigorously boiling water bath. All samples, enzyme blanks, glucose standards and the spectro zero were boiled together. After boiling, the tubes were transferred immediately to a cold water bath. 20 mL deionised or distilled water was added and mixed by completely inverting the tube several times so that the solution separated from the bottom of the tube at each inversion. The absorbance was measured at 540nm in a UV-VIS spectrophotometer. Reducing sugars released from filter paper were determined by comparing the values with the standard curve prepared by D-glucose *(Annexure-2)*. The enzyme activity was calculated as µmoles of D-glucose equivalents released per min. at 50°C (FPU/mL).

Xylanase assay

Xylanase activity was determined according to Bailey method *(Bailey et al. 1992)*. The activity was determined by measuring the release of reducing sugars using birchwood xylan as a substrate. 0.2 mL of suitably diluted enzyme (commercial or isolated fungal) was added in a sterile 50 mL tube, which contained 1.8 mL of substrate suspension (1% birchwood xylan in 0.05M phosphate buffer, pH 7.0) at 45°C. The assay solutions were incubated for 5 min. at 45°C and after completion of 5 min., 3 mL of DNS (Dinitro salicylic acid) reagent was added to terminate the reaction. The solution was boiled for 5 min. in constant water bath, followed by quick cooling in cold water and the absorbance was measured at 540 nm using a UV-Vis spectrophotometer. One international unit (IU) of enzyme was defined as the amount of enzyme releasing 1 µmole of xylose sugars per min. per mL of enzyme.

Protein concentration

The 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, Solution B: 1% $CuSO_4.5H_2O$, Solution C: 2% (w/v) sodium potassium tartarate) 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 hydrolyzed at 100°C for 10 min. in a boiling water bath. This hydrolyzate 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-2).*

3.2.2 Enzyme treatments

All enzyme treatments were done at 4% consistency, 45°C and pH~7 as per the industrial conditions available in the plants. Pulp suspension was warmed up to desired temperature and the pH was adjusted by addition of aluminum sulfate, which is used as retention aid and flocculating agent in pulp and paper industry. The enzyme based products were added at varying doses ranging from 0.010 to 0.025% (w/w). Reaction mixtures were continuously stirred using an IKA laboratory stirrer at 500 rpm and incubated with the pulp slurry for 30 min. prior to filtering the slurry through a Buchner funnel using a Whatman no.1 filter paper. The filtrate was collected and amount of reducing sugar (as glucose equivalents) released from pulp was determined spectrophotometrically at 540 nm according to the DNS method *(Miller 1959)*. Then the enzyme reaction was stopped by keeping the pulp at 90°C temperature for 10 min. The pulp was then washed thoroughly. The control sample was treated in the same manner as the enzyme treated samples with the exception of enzyme addition.

3.2.3 Characterization of untreated and enzyme treated pulps

Canadian standard freeness (CSF)

Canadian Standard Freeness is designed to give a measure of the rate at which a dilute suspension of pulp drains through a fibre mat, which during the test, forms on a perforated screen plate (TAPPI Test Method T 227 om-99). CSF number is the volume expressed in mL of the filtrate collected from the side orifice of the Canadian Standard Freeness tester.

Drainability

This method describes a procedure to determine numerically a measure of slowness of stock, particularly beaten pulps. The drainage time of the pulp slurry was measured on modified °SR tester using the method used by previous researchers *(Litchfield 1994, Bhardwaj et al. 1997, 1999, Hubbe 2003, Verma et al. 2013).* The equivalent amount of 2 g oven dry (o.d.) pulp from the pulp slurry was taken and made-up to 1 L using cold filtered water to maintain the final temperature of pulp stock at around 20°C. The back (vertical) orifice of the tester was closed using a rubber stopper and the pulp slurry was taken in the jar of the tester. The time required to drain the water from pulp slurry for collection of 800 mL filtrate from the front orifice was measured and reported as the drainage time in seconds.

Water retention value

The measurement of the water retention value (WRV) of pulps was done using the centrifugal method, which is simple and popular. The measurement of the WRV used a slightly modified version of the standard method proposed by TAPPI Useful Method 256. It was measured according to the following procedure: one gram of pulp of known water content was disintegrated, put into a 200 cm^3 Erlenmeyer flask and suspended in 100 cm^3 of distilled water. The suspension was shaken for 1h at 20°C, and then transferred to a G3 sintered-glass funnel to remove excess water applying a gentle vacuum (TAPPI Useful Method 256) as low as 100 to 200 mm Hg. Within 2 to 3 min., a pulp pad of uniform grammage was formed. The sintered-glass funnel was then transferred to a centrifuge tube and centrifuged at 2000 g for 15 min. Then, WRV was calculated according to formula given below *(Rom et al. 2007, Xiao et al. 2001)*,

$$WRV = \frac{(m2 - m1)100}{\mathrm{m1}}$$

where m1 equals the mass of dry sample (o.d.), and m2 equals the mass of moist sample. Four WRV tests were conducted in each experimental series to calculate the average value.

Viscosity

The solution viscosity of a pulp gives an indication of the average degree of polymerization of the cellulose. Such a test therefore gives a relative indication of the degradation (decrease in cellulose molecular weight) resulting from the pulping and/or bleaching process. This method (TAPPI Test Method T 230 om-99) describes a procedure for determining the viscosity of 0.5% cellulose solutions, using 0.5M cupriethylenediamine as a solvent and a capillary viscometer.

Fibre classification

The fibre length distributions were determined for the untreated and enzyme treated pulps using Lorentzen & Wettre (L&W) Fibre tester.

Bauer McNett fibre classification of the untreated and enzyme treated recycled and wheat straw pulps were carried out as per TAPPI test method T 233 cm-95. Fines fraction passed through 200 mesh screen was collected on 300 mesh screen and used as the fines component in the experiments.

X-Ray diffraction

The enzyme treatments of cellulose pulp were performed at 4% consistency at 45°C for 30 min. with dose of 0.025%. Both untreated and treated pulps were thoroughly washed and filtered through Buchner funnel with machine wire mesh of 76 µm. The filtrate was recycled 2 to 3 times unless a clear filtrate was obtained. The pulps were shredded and air dried for 8-12 h at ambient temperature. The dried pulp samples were powdered using a pulverizer. X-ray diffraction was performed with a Bruker AXS D8 Advance diffractrometer (Germany) with a

scanning rate of 1°/min. The radiation was Ni-filtered CuKα of wavelength 0.1542 nm. The X-ray unit operated at 40 kV and 30 mA. Angular scanning was conducted from 5° to 50° at 1°/min. 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'. The crystallinity of cellulose in pulp samples was calculated from diffraction intensity data *(Segal et al.1959)*.

$$CrI = \frac{(I\ 002 - I\ amorph)100}{I\ 002}$$

where CrI is the degree of crystallinity, *I 002* is the maximum intensity of the (002) lattice diffraction (at 2θ = 22.6°), and *I amorph* is the peak intensity of amorphous phase diffraction (at 2θ = 18°).

Scanning electron microscopy (SEM)

In order to study the extreme effects/action of cellobiohydrolase and endoglucanase components, the enzyme treatments were done at a higher dose of 0.25%, 4% substrate consistency at 45°C for 2 h. A small portion of the dilute pulp stock slurry (0.3% consistency) was placed onto the surface of a carbon adhesive tab that was mounted on a 25 mm aluminum stub. The liquid slurry was allowed to air dry at room temperature to immobilize the fibers. The prepared specimens were then sputter gold coated. Both untreated and the enzyme treated samples were imaged for comparison. All imaging was performed using a Quanta, 200 F Model, FEI, scanning electron microscope equipped with a solid state secondary electron detector and a tungsten filament. Numerous micrographs were obtained for each treatment at various magnifications to document any physical changes to the fibers. The electrographs were made using 15 KV accelerating voltage.

3.2.4 Preparation of paper sheets and effect on paper properties

The enzyme treated pulps were first filtered to remove excess water and avoid extended contact time of the pulp fibres with the enzyme. The pulp was diluted to a consistency of 0.3% and laboratory handsheets of 70 g/m^2 were prepared according to TAPPI test method T 205 sp-02. The handsheets were conditioned at 27°C and 65% relative humidity for 24 h.

Apparent density

Thickness is defined as the perpendicular distance between the two principal surfaces of the paper or paperboard under prescribed conditions, as measured between hard metal plates. The method (TAPPI Test Method T 411 om-97) involves measuring the thickness of a single sheet of paper, paperboard, or combined board by the use of an automatically operated micrometer when a specified static load is applied for a minimum specified time. Bulk is

calculated as average thickness in microns divided by grammage, GSM. Apparent density was then calculated from the ratio of grammage and thickness.

Tensile strength

Tensile strength is the maximum tensile force developed in a test specimen before rupture on a tensile test carried to rupture under prescribed conditions (TAPPI Test Method T 494 om-01). Tensile strength is the force per unit width (usually 15 mm) of test specimen. Tensile strength is an indicative of the strength derived from factors such as fibre strength, fibre length and bonding. Tensile index is calculated as the tensile strength in N/m divided by grammage, GSM. The tensile index of paper was determined on L&W Tensile strength tester.

Tearing strength

The tear index was determined on L&W Tearing tester (model: SE 009) as per TAPPI test method T 414 om-98. This method measures the force perpendicular to the plane of the paper required to tear multiple plies through a specified distance after the tear has been started.

Bursting strength

The burst strength is widely used as a measure of resistance to rupture in many kinds of paper. The burst index was determined on L&W Bursting strength tester (model: SE 181) as per TAPPI test method T 403 om-97. This method is applicable to measure the maximum bursting strength of paper having a bursting strength of 50 kPa up to 1200 kPa and in the form of flat sheets of up to 0.6 mm thick.

Bendsten smoothness

Smoothness is a measure of the airflow between the specimen (backed by flat glass on the bottom side) and two pressurized, concentric annular heads that are impressed into the sample from the topside. Smoothness has an important influence on printing quality and others such as gloss and coefficient of friction. The Bendtsen roughness of the sheets was determined on L&W Bendtsen tester as per ISO 8791-2 test method. The test is expressed in millilitres per min. (mL/min.).

Air permeance

The air permeance of the handsheets was determined on L&W Air permeance tester (model: SE 166) as per TAPPI test method T 460 om-02. The time (in seconds) required to pass the 100 mL of air through the paper sheet was measured by the instrument and reported as s/100 ml. The higher value means the lower air permeability and porosity, and vice-versa.

Formation index

Formation value, most often used to quantify formation quality, is a ratio that is made up of both the contrast and size distribution components of the sheet formation. A higher formation index implies a more uniform sheet. Formation index was analyzed through Paprican Mirco-Scanner (LAD07). The program determines a topographical map of the optical densities of paper. From this map both the floc sizes and the contrast of the optical densities are used to measure the formation quality.

All the experiments were carried out in triplicate and the bars shown in figures represent the standard deviation on either side of the mean.

3.2.5 Lab scale production of endoglucanase enzyme

3.2.5.1 General practices

Sterilization of glasswares

Before usage, all the labware were washed with detergent (laboline) and rinsed thoroughly under running tap water. Washed glasswares were sterilized by keeping in a laboratory oven for 2 h at 105°C for the dry sterilization prior to usage. The glasswares along with the medium, plasticwares, cotton plugs and inoculating items were further sterilized in an autoclave at 121°C (15 psi) for 15 min. (wet sterilization). The sterilized inoculating items were also subjected to dry red heating in flame before inoculation.

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.

Pretreatment of the lignocellulosic substrates

Lignocellulosic substrates (bagasse pith, rice bran) were washed properly with warm water in order to remove impurities like dust, starch, crop residue etc. The washed materials were retained on 100-mesh screen and then dried in sunlight. Dried lignocellulosic substrates were grinded in a laboratory grinder to obtain fine particles. These fine particles were passed through 100-mesh screen and the fractions so obtained on screen were stored in polyethylene bags for further use.

Preparation of culture media plates

Rice bran agar as culture medium, consisting of 5% rice bran (RB) (w/v) and 2% agar (w/v) was used. The medium was prepared having pH 7.0±0.1 and then autoclaved at 15 psi for 15 min. After autoclaving, the medium was cooled down to about 40-45°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.

Fermentation media preparation

The two fungal strains i.e. *Pycnoporus sanguineus* and *Alternaria gaisen* were maintained by periodic transfer on potato dextrose agar slants at 30°C. The two fungus were grown in M-1 medium *(Reese and Mandels 1963)* supplemented with rice bran at a concentration of 5 g/L. The basal M-1 medium contained different ingredients mentioned in Table-3.1.

Table 3.1 Media Composition (Reese and Mandels 1963)

Ingredients	Concentration, g/L	Ingredients	Concentration, g/L
K_2HPO_4	2.0	$FeSO_4$	0.005
$(NH_4)_2\ SO_4$	2.0	$MnSO_4$	0.006
$CaCl_2$	0.3	$ZnSO_4.7H_2O$	0.003
$MgSO_4$	0.3	$CoCl_2$	0.002
Tween-80	1.0		

The pH of the medium was adjusted to pH 7.0 with 1N HCl / NaOH using pH meter. The enzyme production was carried out in 250 mL conical flasks (Erlenmeyer) containing 100 mL M-1 medium. The contents of these flasks were mixed with a glass rod and then capped properly with cotton plugs. The flasks were autoclaved and kept for cooling at room temperature in the inoculation chamber under UV light exposure. After cooling, five discs of 8 mm diameter from 6 day old culture of each test fungi were inoculated in different flasks. These flasks were incubated at optimum temperature on a rotary shaker at 200 rpm. The samples were withdrawn at regular intervals. After optimum days of incubation, the mycelium was removed by centrifugation at 6000 rpm at 4°C and a clear crude broth was obtained. The said broth was used for measurement of enzyme activities. The mean of at least duplicate experiments was shown in the results.

3.2.5.2 Isolation and screening of fungi for the production of cellulase enzyme

Isolation of fungi

A total of 62 decaying wood and 34 humus samples were collected from various sites near Saharanpur, Meerut and Yamuna Nagar located in India, using enrichment technique. Out of these 96 samples, 54 different strains (appearance wise) of fungi (35 from wood and 19 from humus) were isolated. Serially diluted samples prepared from different sources were spread

on surface of potato dextrose agar and incubated for 7 days at 30°C. Colonies were picked and sub-cultured to obtain a pure culture. The cellulolytic nature of 54 fungal strains obtained as above was confirmed by cultivation on CMC agar culture plate and by subsequent staining using Congo red staining method *(Teather and Wood 1982)*. The strains that showed a clearing zone around the colony were isolated as potential alkaline cellulase producing fungi. The experiments were done in triplicates.

Screening of isolates for potent cellulase producers

Selective procedures were used for selection of best fungal strains in terms of cellulase activity among the isolates.

Primary screening: Congo red plate assay technique (fungal inoculation method)

The 54 fungal strains isolated in the present work were screened for their abilities to produce extracellular cellulases during growth on CMC-agar medium. The medium contained 1% w/v CMC (as sole carbon source) with basal salt medium and 2% agar *(Addleman and Archibald 1993)*. The plates were inoculated in the centre with 6 day 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). 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 as well as on colony counter 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 *(Teather and Wood 1982)*. The presence of clear zone indicated that the fungal enzymes had utilized the cellulose in the medium.

Secondary screening: Measurement of CMCase and Xylanase activities after submerged fermentation

The aim of screening was to select isolates exhibiting the highest CMCase. The fungal isolates, exhibiting areas of clear zones in Congo red test, were further subjected to submerged fermentation conditions to determine their actual cellulase and xylanase activities. For the screening, malt extract agar was used as sole carbon source. The enzymes from the fermented matter were harvested on 3 to 8th day after achieving full growth (depending on growth rate of isolates) and the cellulase activities of enzyme samples from each isolate were determined.

3.2.5.3 Identification of cultures

The two selected fungal isolates selected by primary (Congo red) and secondary screening (CMCase activities) were sent to National Fungal Culture Collection of India (NFCCI), Pune, India, for further identification. The cultures were identified as *Trametes sanguinea (*PVYA07 NFCCI-3628*, current name Pycnoporus sanguineus)* and *Alternaria gaisen (*PVYA11 NFCCI-

3629*)* based on sequencing of the genomic deoxyribonucleic acid (DNA), and were deposited at NFCCI, Pune, India with accession numbers.

3.2.5.4 Effect of culture conditions on enzyme production

Carbon source (Cellulosic substrate)

The fungus was grown in M-1 basal medium supplemented with different cellulosic substrate at 30°C. Different cellulosic substrates used in the study include bagasse pith, rice bran, carboxymethyl cellulose and Avicel (Sigma Chemical Co., St. Louis, MO, USA)at a dose of 0.5% (Table 3.2, V1; Conditions: Table 3.3, A1 and Table 3.4, B1). The enzyme production and change in pH was monitored for 10 days.

Initial pH

To study the effect of initial pH, the pH of the nutrient saline solution (NSS), was maintained from 5.0 to 9.0 by using 1 N NaOH/H_2SO_4 separately (Table 3.2, V2; Conditions: Table 3.3, A2 and Table 3.4, B2).

Nitrogen source

The effect of inorganic and organic nitrogen source was studied similarly by replacing ammonium sulphate from the medium by different inorganic and organic nitrogen sources i.e. urea (inorganic), proteose peptone (organic) and yeast extract (organic) (Table 3.2, V3; Conditions: Table 3.3, A3 and Table 3.4, B3).

Surfactants

Different surfactants Tween 20, Tween 80, Triton-X100, were used in NSS (concentration of 2 g/L) (Table 3.2, V4; Conditions: Table 3.3, A4 and Table 3.4, B4).

Inoculum size

Different inoculum sizes were optimized in the range of 2 to 10 discs/5 gds of *Pycnoporus sanguineus PYA07 NFCCI-3628* and *Alternaria gaisen PVYA11 NFCCI-3629* (Table 3.2, V5; Conditions: Table 3.3, A5 and Table 3.4, B5).

Incubation days

The inoculated flasks were daily harvested from 1 to 12 d for *Pycnoporus sanguineus* PVYA07 NFCCI-3628 and *Alternaria gaisen* PVYA11 NFCCI-3629 (Table 3.2, V6; Conditions: Table 3.3, A6 and Table 3.4, B6).

Table 3.2 Factors affecting extracellular enzyme production

Parameters	Variable code (V)	Range
Carbon source	V1	Bagasse pith (BP), Rice bran (RB), Carboxymethyl cellulose (CMC), Avicel at 0.5% each
pH	V2	5.0 to 9.0
Nitrogen source	V3	Urea (UA), Proteose peptone (PP), Yeast extract (YE) at 2.0 g/L each
Surfactant	V4	Tween 20, Tween 80, Triton X100
Inoculum size	V5	2 to 10 discs/5g RB
Incubation days	V6	1 to12 days (1 day interval)
Temperature	V7	26 °C to 42 °C (interval of 4°C)

Table 3.3 Culture conditions for the enzyme production for the Pycnoporus sanguineus PVYA07 NFCCI- 3628

Exp. Code	Carbon source	pH	Nitrogen source	Surfactant	Inoculum size, ($x10^6$) CFU/gds	Incubation days, days	Temperature, °C
A1	**V1**	6.0	AS	T80	2	6	30
A2	RB	**V2**	AS	T80	2	6	30
A3	RB	7.0	**V3**	T80	2	6	30
A4	RB	7.0	YE	**V4**	2	6	30
A5	RB	7.0	YE	T80	**V5**	6	30
A6	RB	7.0	YE	T80	6	**V6**	30
A7	RB	7.0	YE	T80	6	8	**V7**

Incubation temperature

The effect of incubation temperature on the production of endoglucanases and cellobiohydrolases by the test fungi was studied by incubating the inoculated flasks at different temperatures maintaining from 26 to 42°C (Table 3.2, V7; Conditions: Table 3.3, A7 and Table 3.4, B7).

Table 3.4 Culture conditions for the enzyme production for the Alternaria gaisen PVYA 11 NFCCI-3629

Exp. Code	Carbon source	pH	Nitrogen source	Surfactant	Inoculum size, discs	Incubation days, days	Temperature, °C
B1	**V1**	6.0	AS	T80	2	6	30
B2	RB	**V2**	AS	T80	2	6	30
B3	RB	7.0	**V3**	T80	2	6	30
B4	RB	7.0	YE	**V4**	2	6	30
B5	RB	7.0	YE	T80	**V5**	6	30
B6	RB	7.0	YE	T80	4	**V6**	30
B7	RB	7.0	YE	T80	4	6	**V7**

3.2.5.5 Production and Purification of endoglucanase enzymes

Enzyme production was carried out in 2 L fermentor (BE Marubishi) containing 750 mL M-1 medium. The culture was incubated at 32°C on a rotary shaker at 200 rpm. At regular intervals, the samples were withdrawn. The mycelium was removed by centrifugation at 6000 rpm. This enzyme preparation after 10 days of incubation was used for measurement of CMCase, cellobiohydrolase and filter paper activities. Results given are the mean of at least duplicate experiments. Different enzyme activities were measured as per the procedures given previously in section 3.2.1.

Ten days old *Pycnoporus sanguineus* and *Alternaria gaisen* fermented cultures were used for endoglucanase purification. The culture supernatants were initially filtered through cheese cloth to remove mycelial debris, if any. The filtrates were centrifuged (9,000 g for 30 min. at 4°C).

Ammonium sulphate fractionation

Solid ammonium sulphate was added to the culture filtrates (250 mL) to 40% saturation and was precipitated; the resulting precipitates were discarded and supernatants were collected. Again the supernatants were added with calculated amount of ammonium sulphate and kept on stirring. The protein precipitates were collected by centrifugation (10,000 g for 30 min. at 4°C), dissolved in sodium acetate buffer (100mM, pH 5.0), and dialyzed (dialysis membrane: Sigma–Aldrich; 12,000 molecular weight cutoff filter) against 10mM sodium acetate buffer (pH 5.0). The dialyzed proteins were then concentrated by lyophilization and used in further purification steps.

Size exclusion chromatography

The precipitated and dialyzed protein was loaded onto a Sephadex G-200 column (Sigma) and fractionated. Proteins were eluted with the same buffer and 2 to 3 mL fractions were collected. The protein content of each fraction was determined by measuring the absorbance at 280 nm *(Varian DU 50 spectrophotometer).* Endoglucanase-positive fractions were determined as described previously *(Manvalan et al. 2011)*, pooled, concentrated, dialyzed against sodium acetate buffer (10mM, pH 5.0), and used for further purification on a Q Sepharose column (Sigma) that was pre-equilibrated with sodium acetate buffer (10mM, pH 5.0) and fractionated. Unbound proteins were washed out with sodium acetate buffer (10mM, pH 5.0) and the bound proteins were eluted in the same buffer with a linear gradient of NaCl (0–0.5M). Then 2 to 3 mL fractions were collected and tested for CMCase activity. Active endoglucanase-rich fractions were pooled, concentrated, dialyzed against sodium acetate buffer (10mM, pH 5.0). All the fractions were again checked for endo-β-1,4glucanase activity. Fractions showing endo-β-1, 4-glucanase activity were collected and stored at -20°C until further use before molecular weight estimation on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Note: G 2000SW Sephadex G-200 column (Sigma) with dimensions 30 x 7.5mm I.D., pore size 125 A, recommended for sample M.W. 5-100 KDa was used in the purification steps. High Load ™ Q Sepharose High Performance column having 26 mm bed diameter, 10 cm bed height, 53 mL bed volume, 13 mL/min. maximum flow was used. 100ml enzyme/protein sample was used in the study.

3.2.5.6 Characterization of purified endoglucanase enzymes

For an industrial feasible product, the characterization of these enzymes requires proper knowledge of optimum pH and temperature as well as their stability because different activities viz. CMCase, CBH, FPase and Xylanase activities are subjected to change with varying pH or temperature.

Molecular weight determination on SDS – PAGE

Molecular weight of purified endoglucanase was estimated by its migration in 10% SDS polyacrylamide gel as described by *Laemmli 1970*. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out with 5% (w/v) stacking gel and 10% (w/v) resolving gel at 20 mA in Genei's mini-electrophoresis unit (Bangalore Genei India Pvt. Ltd.).

Electrophoresis was performed at room temperature at 60 V till samples migrate into resolving gel and then at 80 V till the dye front reaches towards end of the resolving gel. Along with the protein samples appropriate molecular weight marker was run on gel (Medium range molecular weight marker, Genei, Merck, India). The molecular weight markers used included β-galactosidase (120 kDa), Bovine Serum Albumin (91 kDa), Serum Albumin (66 kDa), Glutamic dehydrogenase (56 kDa), Ovalbumin (48 kDa) Carbonic anhydrase (34 kDa) and Myoglobin

(26 kDa). After electrophoresis, the protein bands were stained by 0.25% Coomassie Brilliant Blue R-250 in methanol: acetic acid: water (40:10:50) and destained in the same solution without dye. The plot of log molecular weight versus relative mobilities (Rf) of standard proteins and endoglucanase was prepared and the molecular mass of the purified endoglucanase was determined by calculating the relative mobility based on a protein molecular weight marker.

Effect of pH on enzyme activity and stability

To determine the optimum pH of the enzyme, the purified enzymes obtained from both the fungal strains were incubated in different buffer range from 5 to 9 pH. Citrate buffer was used for pH 5.0 to 6.0 and phosphate buffer for pH 6.0 to 9.0. The CMCase and CBH activities were determined as described previously in section 3.2.1.

To determine the pH stability of the enzyme, the purified enzymes obtained from both the fungal strains were incubated in optimum pH buffer at temperature 45°C after the incubation of 1 to 6 h (at 1 h interval), excluding the assay time (*i.e.* 30 min. for CMCase test, and 10 min. for CBH test). The residual CMCase and CBH activities were then measured.

Effect of temperature on enzyme activity and stability

For determining the effect of temperature on endoglucanase enzyme, enzyme activities were estimated in a temperature range of 30 to 70°C. The temperature stability of the enzyme was detected by incubating suitably diluted enzyme at 30 to 70°C for different time intervals. The samples were removed periodically and assayed for different enzyme activities under standard assay conditions.

In order to assess the temperature stability of the enzymes, the crude enzyme preparations obtained from both the fungal strains were incubated at 45°C, under the above-mentioned conditions for determination of residual CMCase and CBH activities.

Effect of metal ions on enzyme activity

The effect of different metal ions on enzyme activity by the test fungi was studied by taking Mg^{+2}, Ca^{+2}, Na^{+1}, Zn^{+2}, Cu^{+2}, as $MgCl_2.4H_2O$, $CaCl_2.2H_2O$, NaCl, $ZnSO_4.7H_2O$, $CuSO_4.5H_2O$ salts. These were mixed in the NSS in concentrations of 0.2M each.

CHAPTER 4

DRAINAGE IMPROVEMENT BY COMMERCIAL CELLULASE ENZYMES

This chapter covers the evaluation of different commercial enzymes for improvement in pulp drainage and their impact on pulp and paper properties. The increased solubilization of amorphous cellulose mediated by endoglucanase rich, Fibercare D treatments consequently improved the drainability of recycled pulp-NP, recycled pulp-WP and wheat straw pulp-WS in the range of 15.5 to 20.7%, along with better paper properties such as tensile index and smoothness.

4.1 Characterization of pulps as received from mills

The recycled pulp-NP, recycled pulp-WP and wheat straw pulp-WS were characterized for several pulp and paper properties mentioned in Table 4.1. Fines content in recycled pulp-NP, recycled pulp-WP and wheat straw pulp-WS was observed to be 33.5, 42.0 and 36.5%, respectively. Wheat straw pulp was noticed to have a better tensile strength when compared to recycled pulps.

4.2 Enzyme characterization

Different activities (CMCase, Cellobiohydrolase, Filter paper and Xylanase) of enzyme samples were evaluated by respective methods given in the material and methods section.

pH dependency

The pH dependency of different activities (CMCase, Cellobiohydrolase and FPase) for all four commercial enzymes was determined (Figure 4.1a, b, c). The optimum pH for endoglucanase activity in Texzyme M, Fibercare R and Fibercare D was 7, although the endoglucanase activity of Fibrezyme LBR was comparable at 6 and 7 pH. It was observed that the endoglucanase activity of all enzyme products was reduced on further increasing the pH to 8.0 (Figure 4.1c). All the four enzymes displayed the maximum cellobiohydrolase (CBH) activity at pH 6.

Endoglucanase activity

As shown in Figure 4.1b, the enzyme Fibercare D exhibited the highest CMCase i.e. endoglucanase activity (384 IU/mL) at pH 7.0, required for the hydrolysis of amorphous cellulose. Fibercare R exhibited the second highest endoglucanase activity (209 IU/mL) followed by Texzyme M (177 IU/mL) and Fibrezyme LBR (115 IU/mL) enzymes (Figure 4.1b).

Cellobiohydrolase CBH activity

Fibercare R had the highest CBH activity (197 IU/mL). In spite of having the highest endoglucanase activity, Fibercare D displayed the lowest CBH activity (24 IU/mL) as compared to other enzymes (Figure 4.1a). Fibrezyme LBR and Texzyme M showed moderate cellobiohydrolase activities i.e. 146 IU/mL and 124 IU/mL, respectively (Figure 4.1a).

Table 4.1 Characterization of recycled (old newsprint, writing printing grade) and wheat straw pulps

Parameter, unit	Recycled pulp-NP	Recycled pulp-WP	Wheat straw pulp-WS
Fines content, %	33.5 [0.9]	42.0 [4.1]	36.5 [3.4]
SR number, °	36 [0.5]	34 [1.0]	33 [1.0]
CSF, mL	350 [05]	372 [05]	390 [10]
Ash, %	6.3 [0.4]	3.9 [0.3]	8.2 [0.2]
Zeta potential, mV	-16.8 [1.1]	-21.9 [0.7]	-34.1 [1.6]
Viscosity, cP	4.8 [0.4]	5.1 [0.5]	7.4 [0.5]
WRV, %	120 [12]	140 [09]	165 [09]
Drainage time, s	41.1 [1.0]	39.2 [0.9]	43.2 [1.5]
Apparent density, g/cm^3	0.65 [0.10]	0.71 [0.18]	0.80 [0.20]
Tensile index, N.m/g	21.4 [0.8]	31.9 [1.1]	41.9 [1.4]
Tear index, mN.m^2/g	6.5 [0.4]	6.7 [0.4]	5.4 [0.4]
Burst index, kN/g	1.5 [0.1]	2.6 [0.2]	2.9 [0.2]
Air resistance, s/100 mL	9.5 [0.6]	12.3 [1.1]	19.7 [1.1]
Bendtsen roughness, mL/min.	204 [11]	253 [19]	102 [14]
Double fold, no.	8 [05]	11 [07]	39 [15]

Digits in parenthesis indicate the standard deviation

Filter paper activity

Fibercare D, followed by Fibercare R was observed to exhibit maximum filter paper activity at pH 6.0 (14.5 and 23.7 IU/mL, respectively) when compared to other enzymes (Figure 4.1a).

Xylanase activity

Different cellulase enzymes were also analyzed for presence of xylanase activities *(Bailey et al. 1992)*. All the cellulase enzymes were observed to contain xylanase activities. Fibercare D and Fibrezyme LBR were found to have relatively greater xylanase enzyme component , when compared to Texzyme M and Fibercare R . The xylanase activities observed in the cellulase enzymes were on higher side due the method used in the current study.

Based on different cellulase activities, Fibercare R was observed to be a mixture of both cellobiohydrolase and endoglucanase components whereas, Fibercare D was a monocomponent cellulase preparation containing predominantly endoglucanase activity.

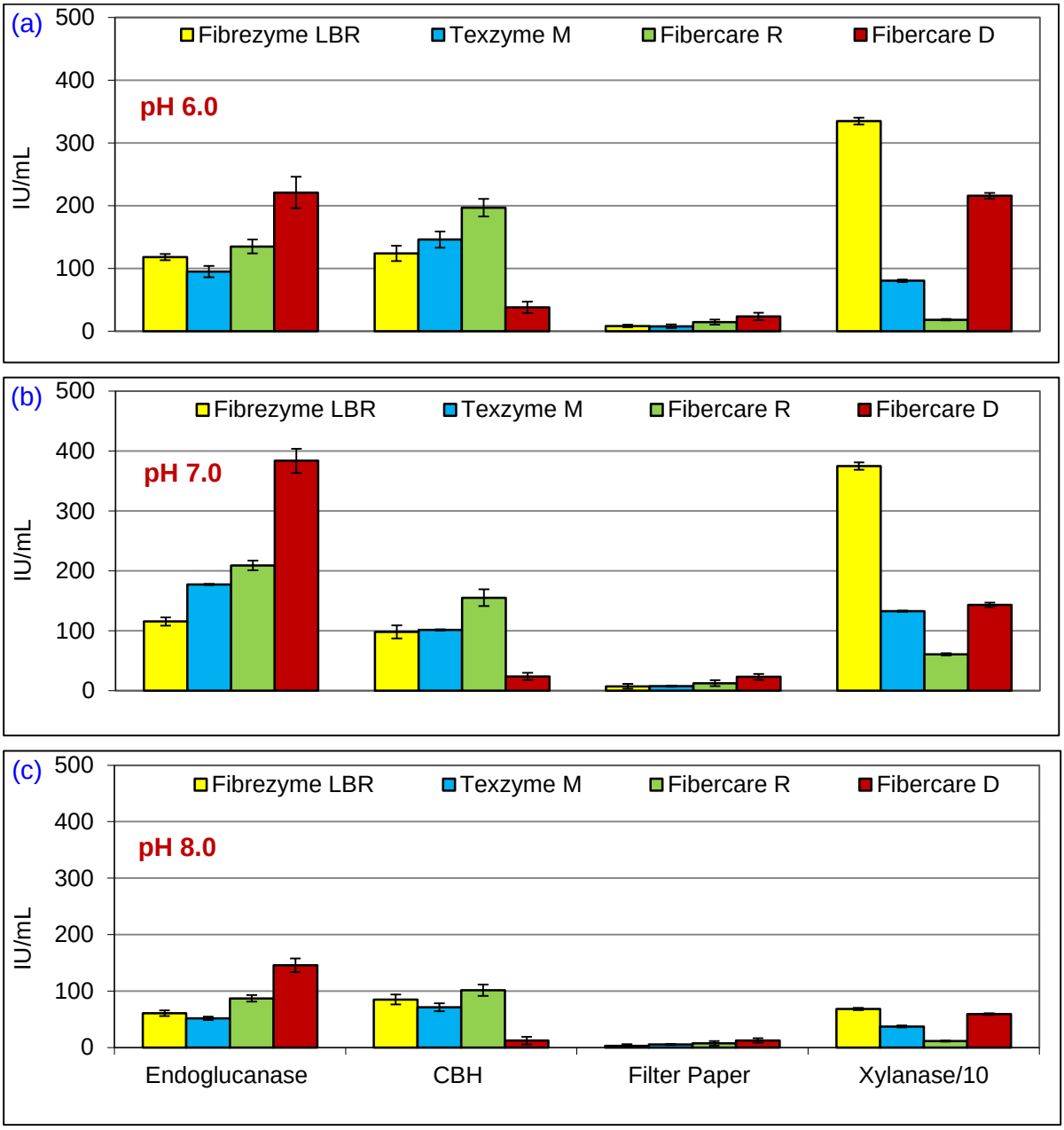


Figure 4.1 Various enzyme activities (endoglucanase, cellobiohydrolase, filter paper and xylanase) of different commercial enzymes at temperature 45˚C and (a) pH 6.0 (b) pH 7.0 (c) pH 8.0, xylanase activity taken as value/10

Reducing sugars

In order to determine the extent of enzymatic hydrolysis, the reducing sugars (as glucose equivalents) in the filtrates of untreated and enzyme treated different pulps were measured using dinitrosalicylic acid (DNS) assay *(Miller 1959)*. The plots depicting the reducing sugar production as a function of enzyme dose for different pulps are shown in Figure 4.2 a, b, c. Fibercare D and Fibercare R enzymes were observed to generate more amount of reducing sugars which represented the hydrolysis of cellulosic fines. Fibercare R exhibited the highest

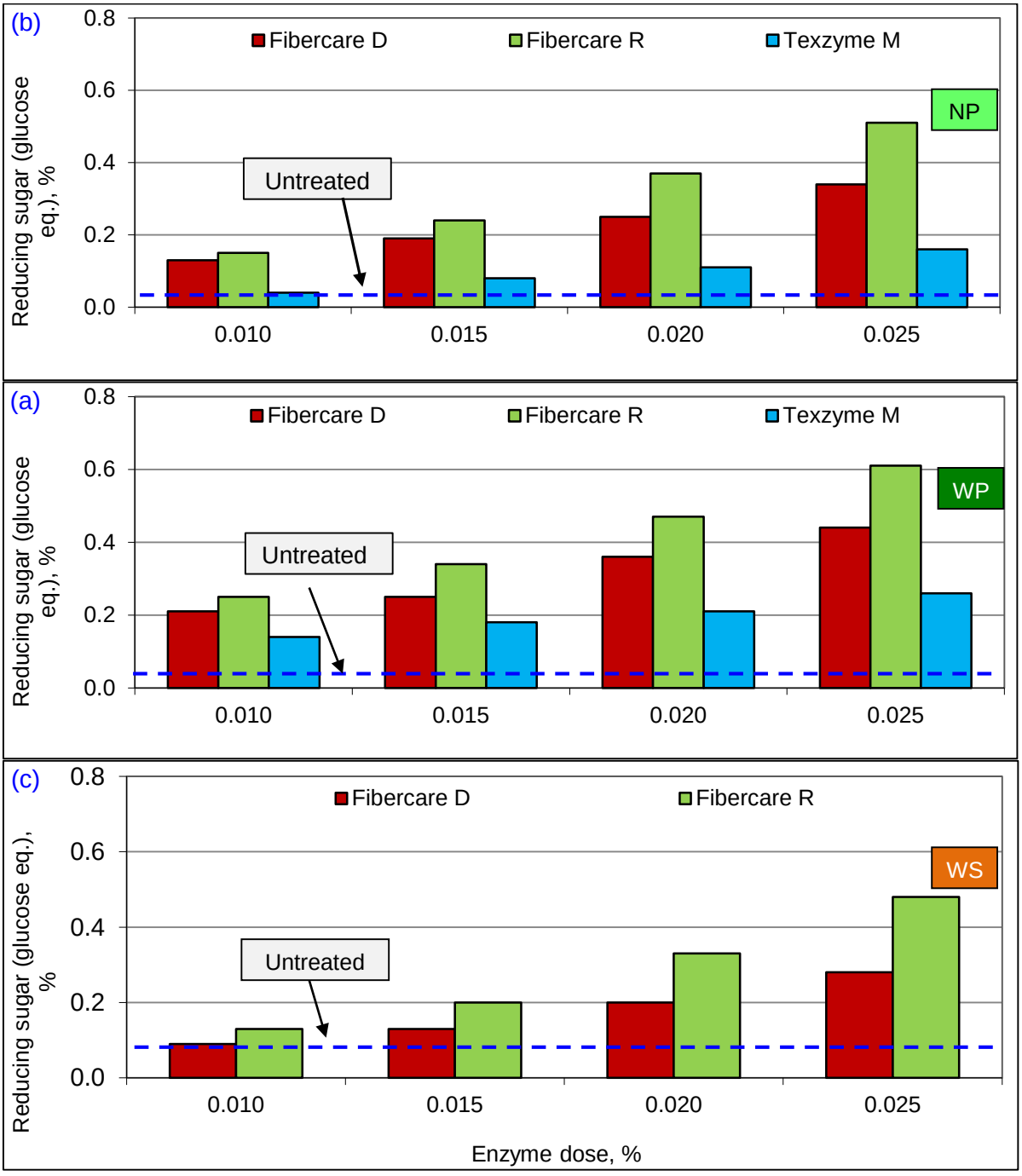


Figure 4.2 Reducing sugar production (equivalent to glucose) as a function of enzyme dose at 4% consistency and 30 min. treatment time (a) recycled pulp-NP (b) recycled pulp-WP (c) wheat straw pulp-WS

production of reducing sugars (as glucose equivalents) in the filtrates of treated pulps due to presence of high cellobiohydrolase component, known to convert the cellulose to cellobiose. The cellobiose might get converted to glucose analyzed as reducing sugar.

4.3 Effect of enzyme treatments on pulp properties

The recycled pulp-NP, recycled pulp-WP and wheat straw pulp-WS were treated with various concentrations of commercial enzymes ranging from 0.010 to 0.025% on o.d. pulp for 30 min. The parameters of enzymatic treatments (pulp consistency, temperature and pH) were adjusted to the industrial conditions mentioned in the materials and methods section. The enzymes were screened out as per their respective effects on pulp drainage and strength properties. Table 4.2 shows different enzymes evaluated on different pulps.

Table 4.2 Different commercial enzymes evaluated on different pulps

	Recycled pulp-NP	Recycled pulp-WP	Wheat straw pulp-WS
Fibercare D	√	√	√
Fibcercare R	√	√	√
Texbio M	√	√	--
Fibrezyme LBR	√	--	--

Pulp Freeness

Pulp freeness, a measure of pulp drainability, was determined by Canadian Standard Freeness (CSF) test. Enzymatic treatments of recycled pulp-NP, recycled pulp-WP and wheat straw pulp-WS and the measurements of Canadian Standard Freeness were performed with sufficient number of parallels in order to detect the changes in drainage accurately.

Recycled pulp -NP

As shown in Figure 4.3a, the highest increase in the CSF value, from 350 (untreated) to 400 mL, was observed with endoglucanase rich, Fibercare D showing that it was the most effective enzyme in making the pulp fibres more free from water than the other enzymes used in this study. Fibercare R was also found to be effective in increasing the CSF values from 350 to 380 mL at 0.025% dose (Figure 4.3a) but the effect was less significant when compared to Fibercare D. The reason of freeness increase might be the presence of endoglucanase enzyme activities in sufficient quantities. On the other hand, Texzyme M and Fibrezyme LBR were found to be less effective in increasing the freeness of recycled pulp-NP (Figure 4.3a).

Recycled pulp -WP

In case of endoglucanase rich product, Fibercare D treated pulps; significant increase in CSF values from 372 to 425 mL was observed (Figure 4.3b). Fibercare R having moderate endoglucanase activity was also found to be effective in increasing the CSF values from 372 to 405 mL at 0.025% dose (Figure 4.3b). The presence of endoglucanase enzyme activities in lower quantities might be the reason of freeness increase (Figure 4.3b). On the other hand, Texzyme M was found to be less effective in increasing the pulp freeness.

Wheat straw pulp-WS

Significant increase in CSF values from 390 mL (untreated) to 440 mL was observed in wheat straw pulps treated with endoglucanase rich product, Fibercare D (Figure 4.3c). Fibercare R having moderate endoglucanase activity was found to be less effective in increasing the CSF values from 390 to 405 mL at 0.025% dose (Figure 4.3c).

Pulp drainability

Recycled pulp -NP

The drainage time of untreated recycled pulp-NP was 41.1 sec. Endoglucanase rich product; Fibercare D displayed the highest reduction in drainage time (11.5 to 24.3%) in comparison to other enzymes at various doses (Figure 4.4a, 4.5a). Cellulase mix Fibercare R was also effective in reducing the drainage time by 8.7 to 13.5% at various doses. Cellulase mix Texzyme M and Fibrezyme LBR having lower endoglucanase activities were not found much suitable for reducing the drainage time (Figure 4.4a, 4.5a). It can be postulated that the presence of endoglucanase activity is a prerequisite for improvement in drainage of recycled fibres. Endoglucanases enhance dewatering of recycled fibres by hydrolyzing the amorphous hydrophilic cellulose which is the main constituent of the fines. It can also be speculated that due to the high specific surface area of the fines, the attack of endoglucanases was specific towards this fraction of the pulp or in other words that amorphous cellulose is easier to hydrolyze by endoglucanases when compared to crystalline cellulose.

Recycled pulp -WP

The drainage time of untreated recycled pulp-WP was 39.5 sec. Endoglucanase rich product, Fibercare D displayed significant improvement in pulp drainage ranging from 14.5 to 22.7% at various doses (Figure 4.4b, 4.5b). Cellulase mix Fibercare R was also found effective in improving the pulp drainage ranging from 8.2 to 16.6% at various doses (Figure 4.4b, 4.5b). Texzyme M having lower endoglucanase and higher cellobiohydrolase activities was not found suitable in improving the pulp drainage.

Wheat straw pulp-WS

The drainage time of untreated recycled pulp-WS was 43.5 sec. Significant improvement in drainage was observed in wheat straw pulp-WS treated with endoglucanase rich product, Fibercare D, at various doses, ranging from 12.5 to 17.6% (Figure 4.4c, 4.5c). Cellulase mix Fibercare R was found less effective in improving the pulp drainage ranging from 8.1 to 10.2% at various doses (Figure 4.4c, 4.5c).

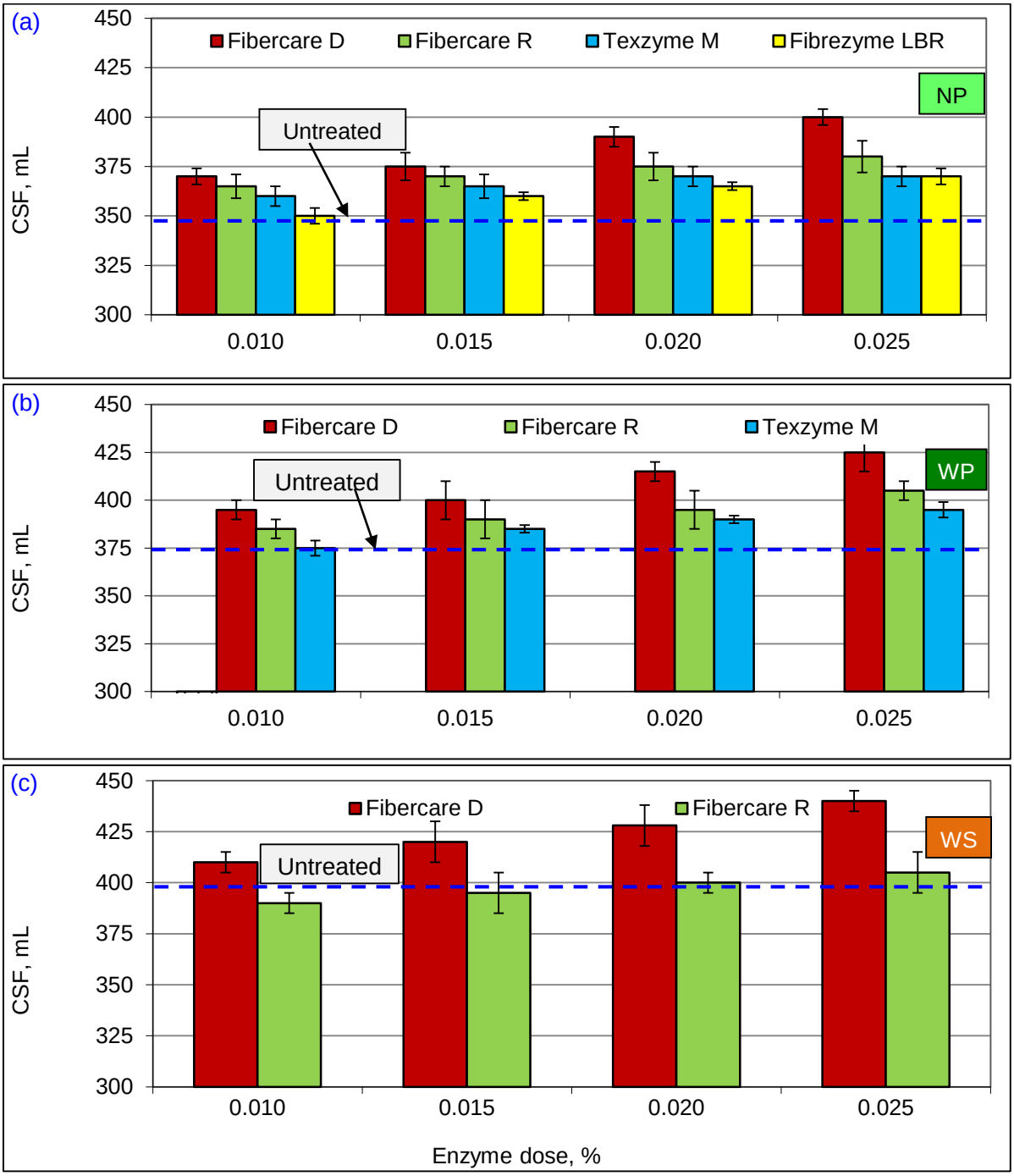


Figure 4.3 Effect of different enzymes at various doses on CSF of (a) recycled pulp-NP (b) recycled pulp-WP (c) wheat straw pulp-WS

It is in agreement with the published data that the endoglucanases significantly improve recycled pulp drainage *(Oksanen et al. 2000, Stork et al. 1995, Dienes et al. 2004)*.

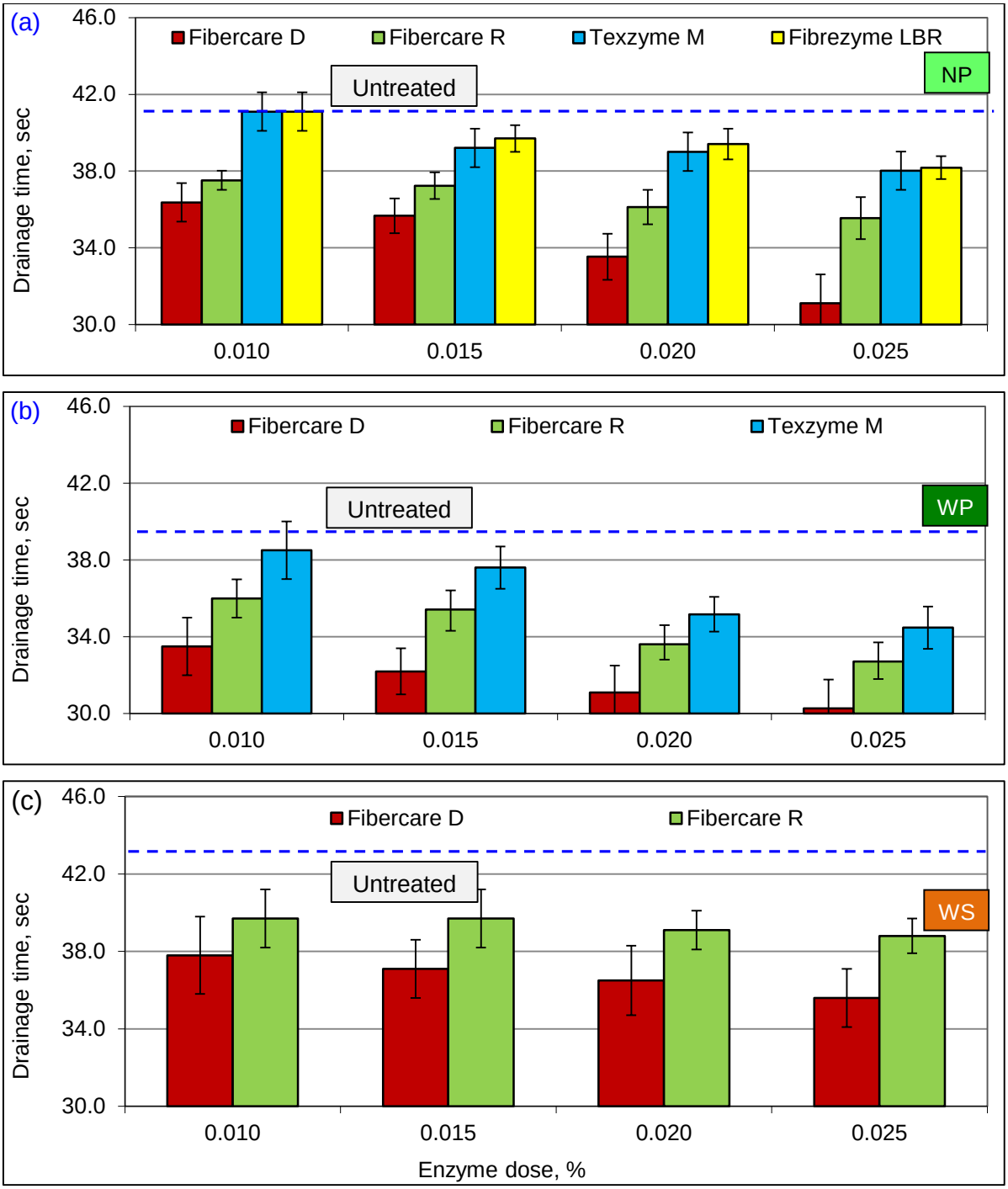


Figure 4.4 Effect of different enzymes at various doses on drainage time of (a) recycled pulp-NP (b) recycled pulp-WP (c) wheat straw pulp-WS

Significant increase in drainage rate along with improvement in smoothness and tensile index using endoglucanase enzymes was also reported by *Oksanen et al. (2009)*. Since the amorphous cellulosic fines are held responsible for the deteriorated drainability of recycled pulps, decreasing the amount of excess fines could be the key solution for improving drainage. The fact that the endoglucanase (EG) rich product appeared so promising for promoting drainage, especially at low levels of treatment, suggests that cleavage of nano-scale fines and fibrils into shorter bits may be of dominant importance. These nano-scale ultra fines after enzymatic hydrolysis would be less prone to block the water channels in wet web of paper.

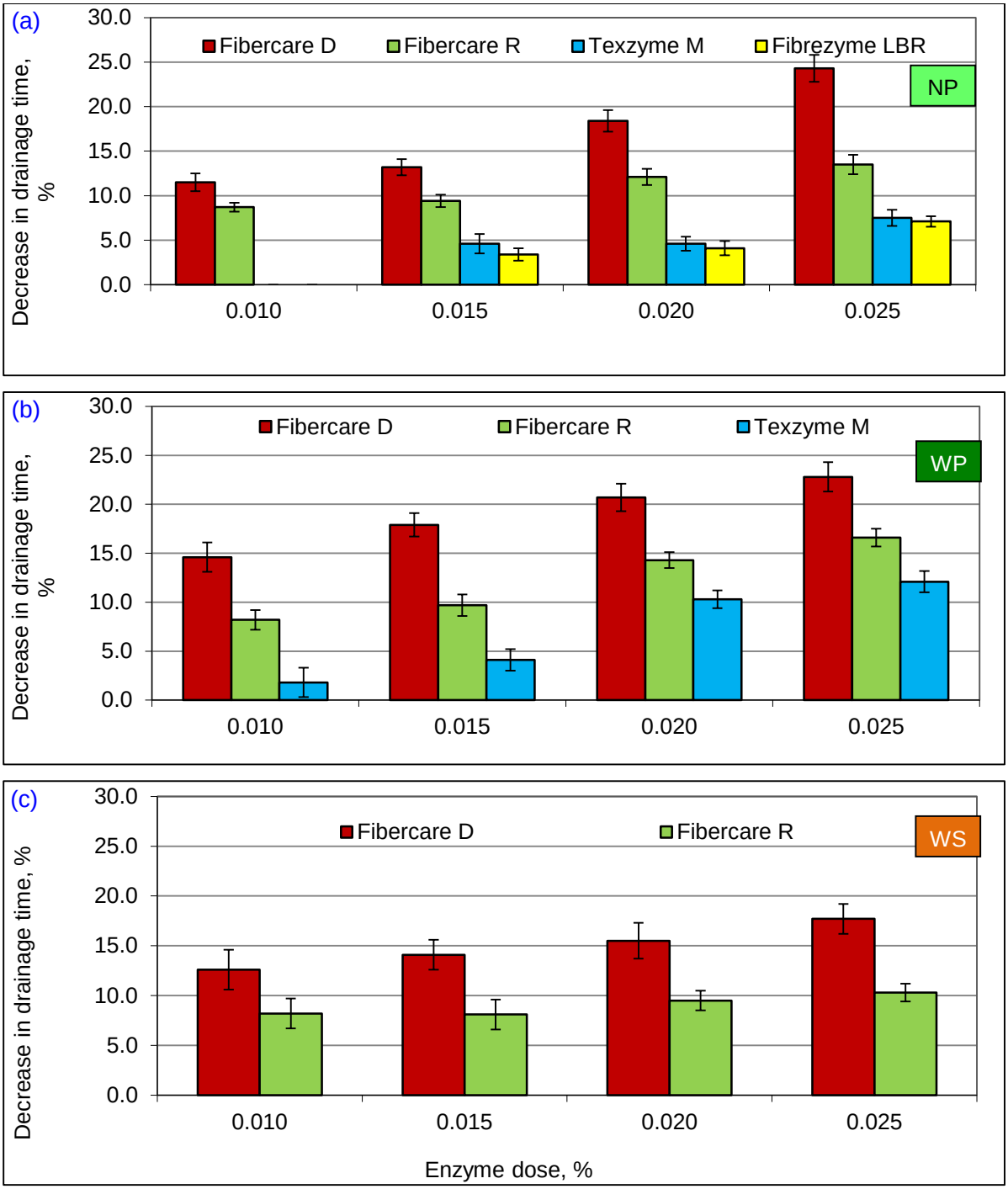


Figure 4.5 Effect of different enzymes at various doses on drainage improvement of (a) recycled pulp-NP (b) recycled pulp-WP (c) wheat straw pulp-WS

Water retention value

Recycled pulp -NP

Effect of enzymatic treatment was also investigated on the water retention value (WRV) as well. As shown in Figure 4.6a, an increase in WRV was observed in all enzyme treated pulps. Significant improvement of about 33% in WRV values was observed in pulps treated with endoglucanase rich product, Fibercare D. Fibercare R also exhibited an increase in WRV by 8 to 18% due to presence of endoglucanase component (Figure 4.6a).

Recycled pulp -WP

Similar trend of increased water retention value in enzyme treated recycled pulps-WP was also observed. The results obtained showed that pulps treated with endoglucanase rich, Fibercare D enzyme retained more water (higher WRV by 7 to 28% at different doses) compared to untreated pulp (Figure 4.6b). An increase in WRV by 4 to 11% was observed in pulps treated with Fibercare R.

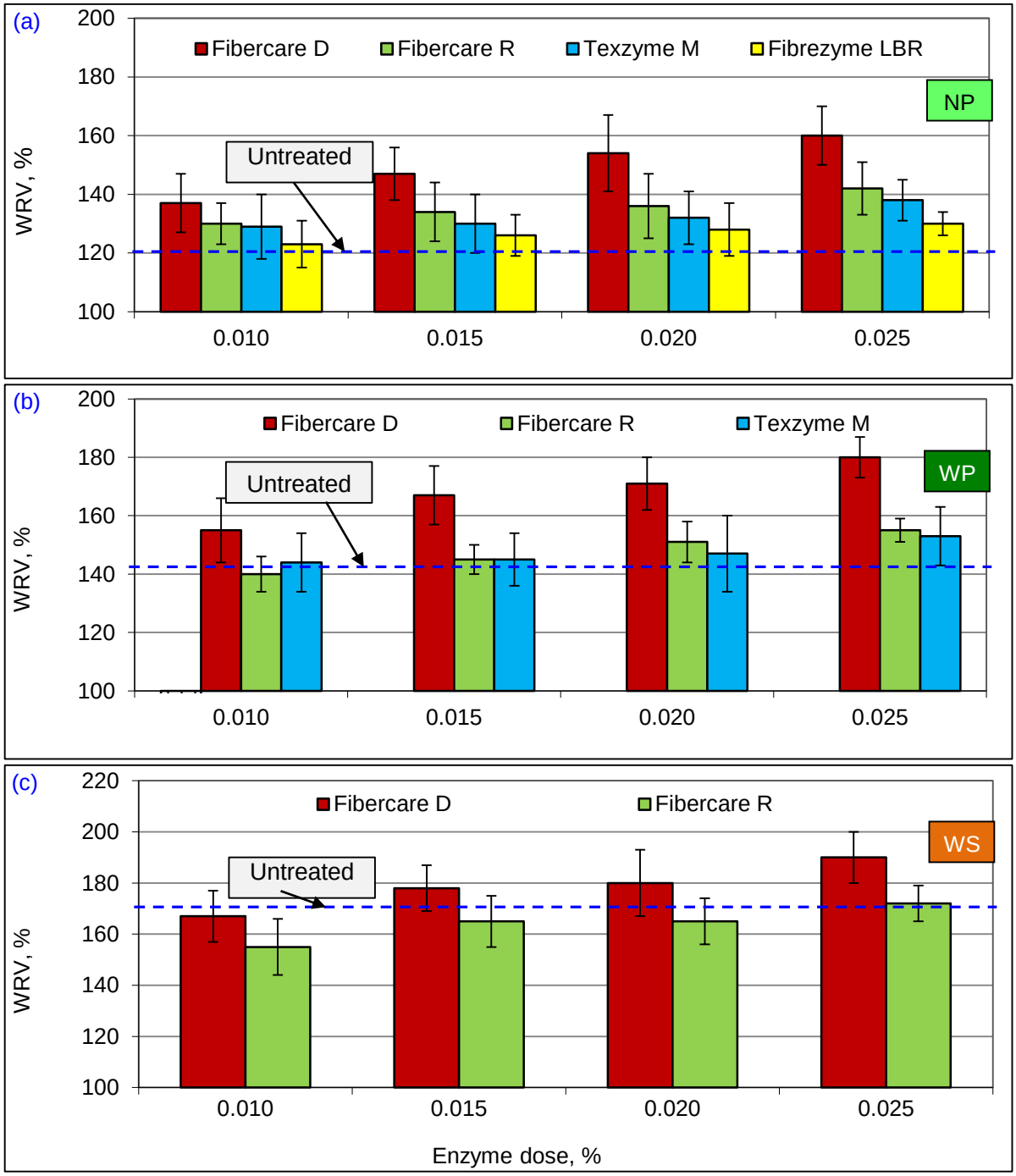


Figure 4.6 Effect of different enzymes at various doses on water retention value of (a) recycled pulp-NP (b) recycled pulp-WP (c) wheat straw pulp-WS

Wheat straw pulp-WS

It was observed that wheat straw pulps treated with Fibercare D retained more water (higher WRV by 15%) compared to control pulp (Figure 4.6c). On the other hand, the effect of Fibercare R on water retention value was negligible.

The reason of higher WRV in case of endoglucanase treated pulps might be the hydrolysis of lower molecular weight carbohydrates and partial deterioration of crystalline structure of cellulose, initiating the formation of more hydrogen bonds between cellulose and water molecules. *Dienes et al. (2004)* also demonstrated that endoglucanase improved the WRV by more than 16%, while cellulase complex deteriorated the WRV by 6%.

Pulp viscosity

Analysis of pulp viscosity was based on TAPPI standard test method T230 om-99. The pulp viscosity was somewhat reduced in all enzyme treated pulp samples.

Recycled pulp -NP

As shown in Figure 4.7a, the enzymatic treatment reduced the pulp viscosity. The highest drop in pulp viscosity was observed with Fibercare R. The possible reason for the same was the high amount of enzyme components i.e. endoglucanase and cellobiohydrolase in Fibercare R. The viscosity drop upto 0.020% dose was not significant in pulp treated with endoglucanase Fibercare D.

Recycled pulp -NP

All enzyme treated pulp samples exhibited the reduction in pulp viscosity. But, the reduction in pulp viscosity was again more prominent in Fibercare R treated samples (Figure 4.7b). The reason may be high amount of endoglucanase and cellobiohydrolase components in Fibercare R.

Wheat straw pulp-WS

Similar trend of significant reduction in pulp viscosity in Fibercare R treated samples was observed (Figure 4.7c).

It has already been reported that exoglucanases (cellobiohydrolases) can achieve complete, although slow, solubilization of cellulose crystals even without the help of endoglucanases. In the presence of endoglucanases, the rate of hydrolysis of crystalline cellulose by CBH increases drastically because of an endo-exo synergy between two classes of the enzymes *(Henrissat et al. 1985).*This might be the reason of more reduction in viscosity in CBH Fibercare R treated pulps.

Fines content

The fibre fines content analyzed through Bauer McNett fibre classification of fibres was increased by 13% on treatment of recycled pulps-NP with Fibercare R although Fibercare D

treated pulp showed up to 25% decrease in fines content (Figure 4.8a). In case of recycled pulps-WP, similar findings i.e. increase in fines content in pulps treated with Fibercare R (17%) and decrease in fines content in pulps treated with Fibercare D (20%) were observed (Figure 4.8b). The fines content in untreated recycled-NP and recycled pulp-WP were 34 and 42%, respectively. Texzyme M also displayed 10% increase in fines content.

Similar findings i.e. decrease in fines content in pulps treated with Fibercare D (20%) and increase in fines content in pulps treated with Fibercare R (12%) were also observed in wheat straw pulps. The fines content in untreated wheat straw pulps was 37% (Figure 4.8c).

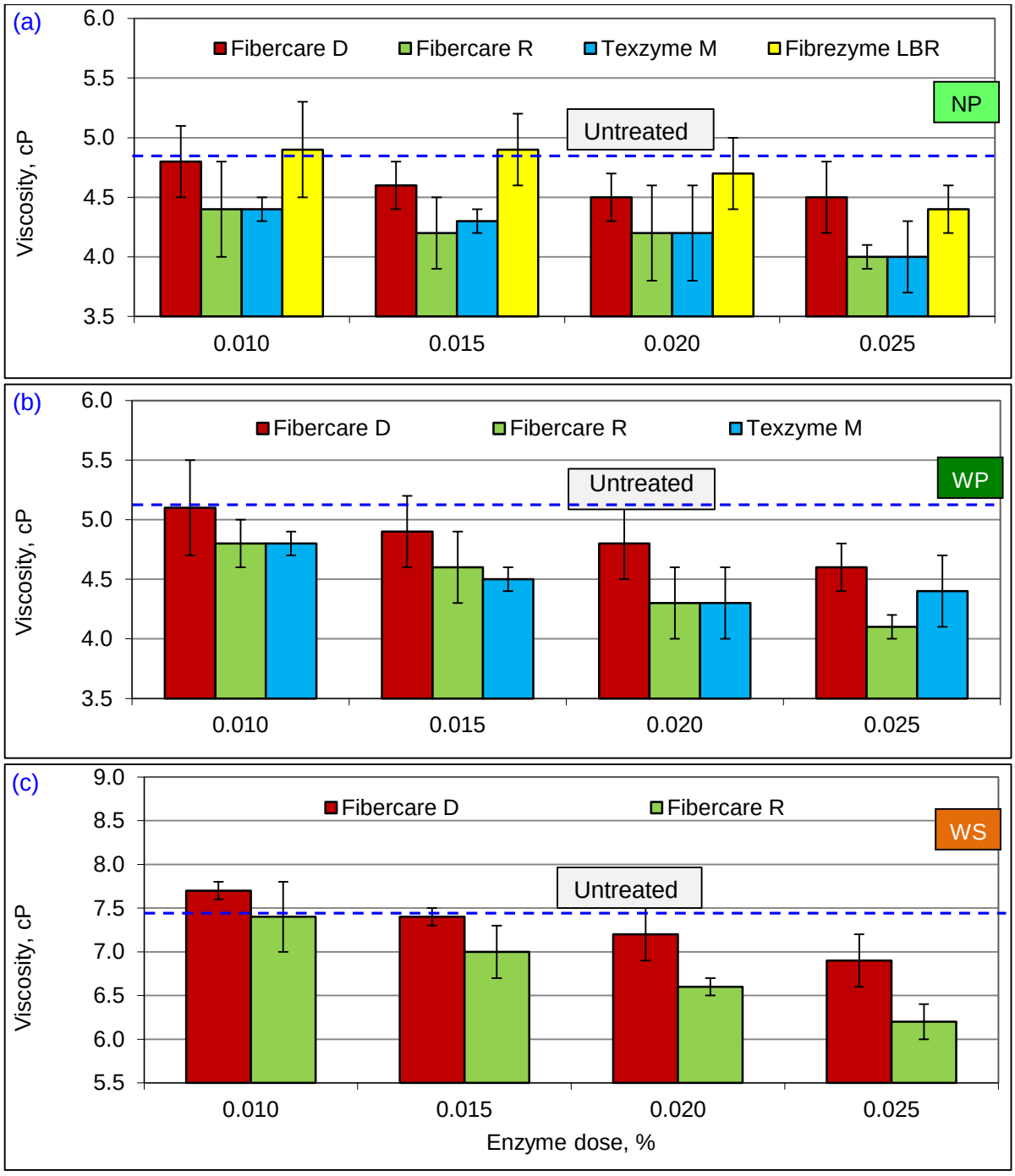


Figure 4.7 Effect of different enzymes at various doses on viscosity of (a) recycled pulp-NP (b) recycled pulp-WP (c) wheat straw pulp-WS

Fibre length distribution

The effect of two enzymes, Fibercare R and Fibercare D, on the fibre length distribution of recycled pulp-NP, recycled pulp-NP and wheat straw pulp-WS was also analyzed using L&W fibre morphology tester. As shown in Figure 4.9 a, b the proportion of longer fibres (greater than 1 mm) was increased and that of shorter fibres was decreased on treatment of pulp with Fibercare D. It was due to the effect of endoglucanase content of Fibercare D on fibre fines. The proportion of fibres greater than 1 mm was 29, 21 and 38% in untreated, treated with Fibercare R and Fibercare D recycled pulps-NP, respectively (Figure 4.9a). In case of

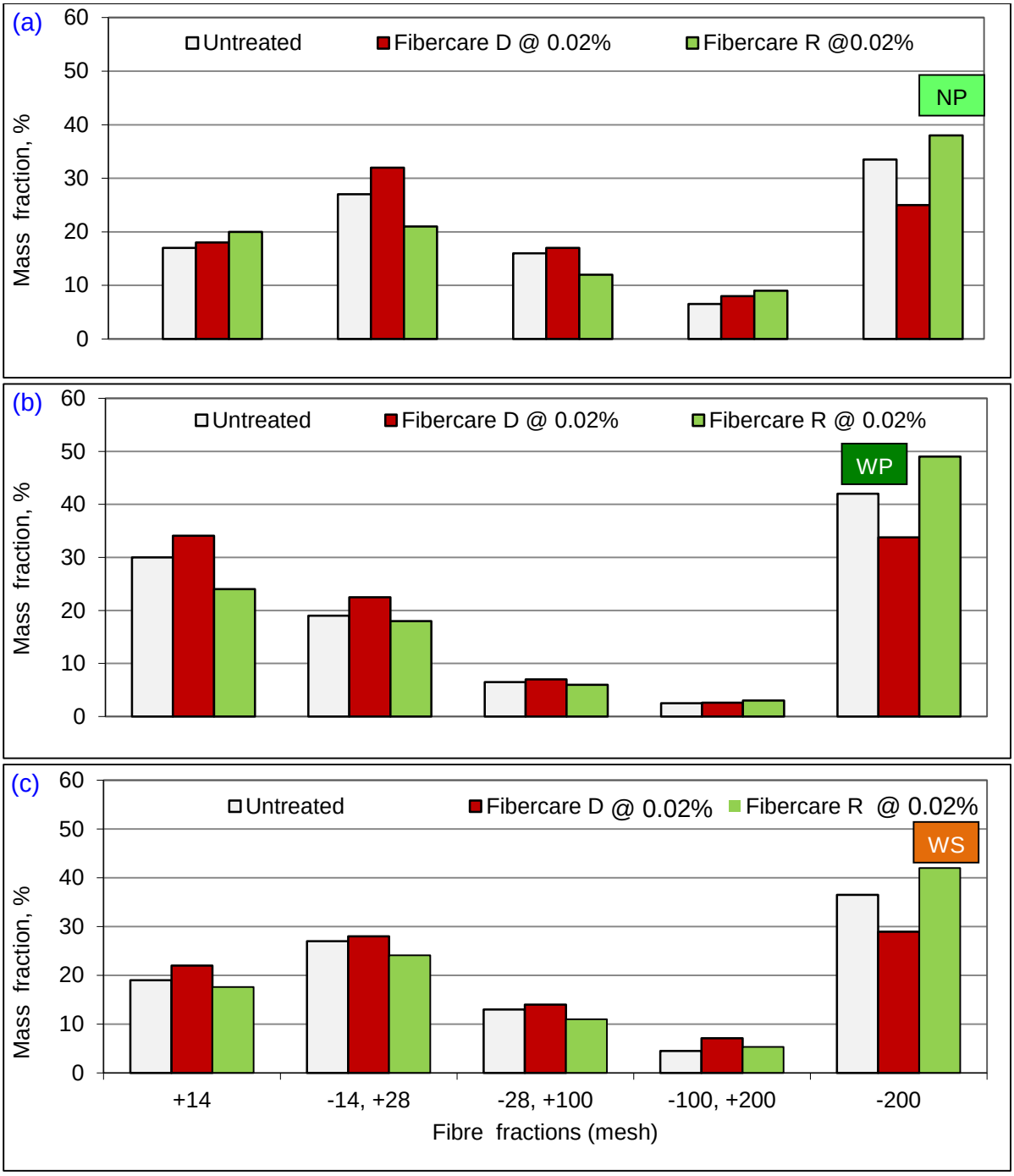


Figure 4.8 Bauer–McNett classification of untreated and enzyme treated (a) recycled pulp-NP (b) recycled pulp-WP (c) wheat straw pulp-WS

recycled pulp-WP, proportion of fibres greater than 1 mm was 24, 17.5 and 33% for untreated, treated with Fibercare R and Fibercare D (Figure 4.9b).

These results indicated that the cellulase mix Fibercare R having both endoglucanase and cellobiohydrolase activities, increased the fines content in different pulps and the proportion of fibres greater than 1 mm was reduced. Similar finding of reduction in fines content and improvement in fibre length distribution was also reported by *Dienes et al. (2004).*

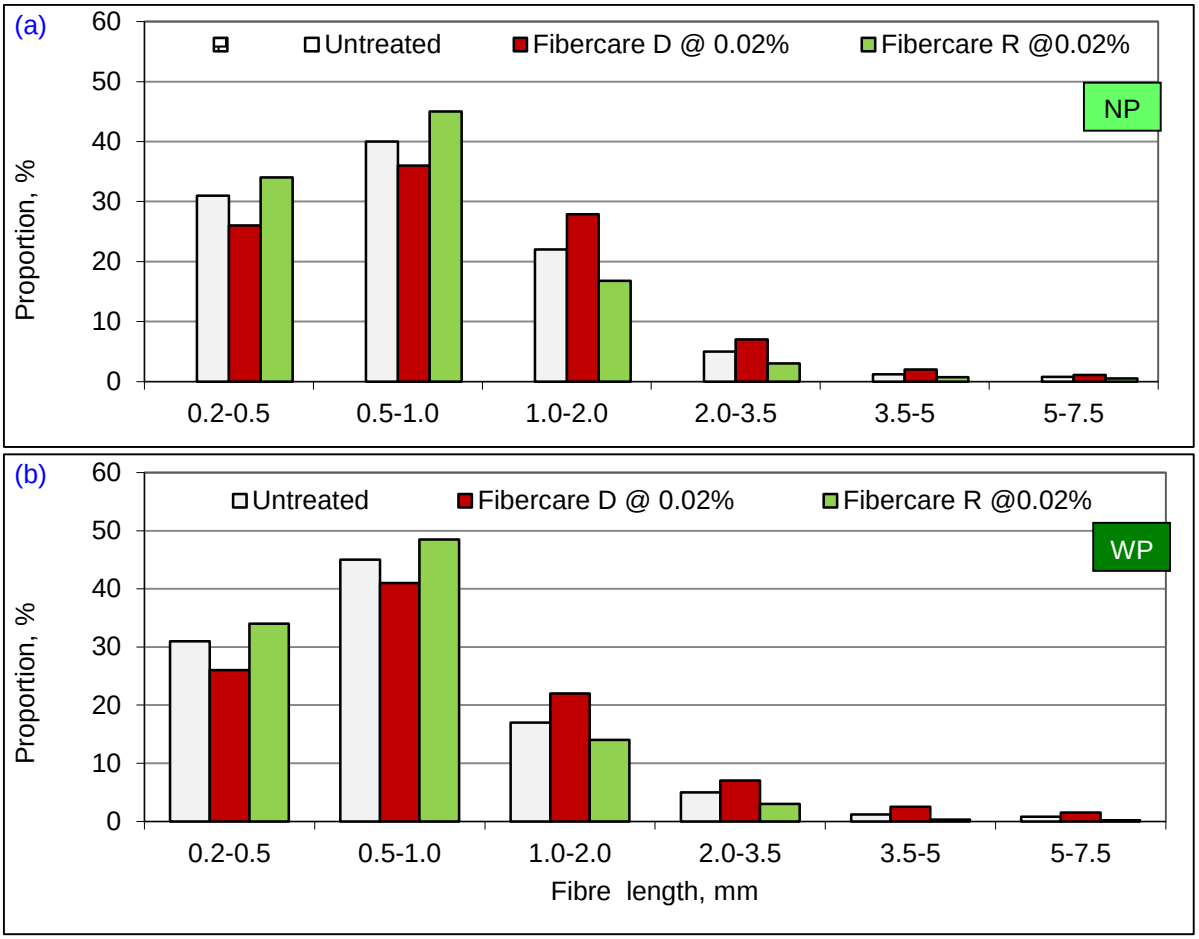


Figure 4.9 Fibre length distribution of untreated and enzyme treated (a) recycled pulp-NP (b) recycled pulp-WP

X ray diffraction studies of untreated and enzyme treated pulps

Cellulose consists of both crystalline and amorphous fractions and if enzymes preferentially attack amorphous regions then one can expect crystallinity to increase over the course of hydrolysis. The similar trend was observed in crystallinity of untreated and enzyme treated pulp samples. Figure 4.10, 4.11, 4.12 shows typical X–ray diffraction intensity profiles of the untreated and enzyme hydrolyzed recycled pulp-NP, recycled pulp-WP and wheat straw pulp-WS, respectively.

Recycled pulp-NP

Fibercare D having predominantly endoglucanase component displayed lower amorphous peaks when compared with untreated fibres (Figure 4.10) and crystallinity index was observed to increase from 80.2 to 85.5%. While in case of Fibercare R treated pulp samples, the crystallinity index was observed to decrease to 77.3%, in spite of also having endoglucanase activity in addition to CBH activity (Table 4.3).

Recycled pulp -WP

Pulp treated with endoglucanase Fibercare D displayed lower amorphous peaks if compared to untreated sample (Figure 4.11) and crystallinity index was increased from 84.2 to 90.1%. The crystallinity index was decreased to 81.4% in Fibercare R treated pulp samples, although Fibercare R contained endoglucanase activity as well (Table 4.3).

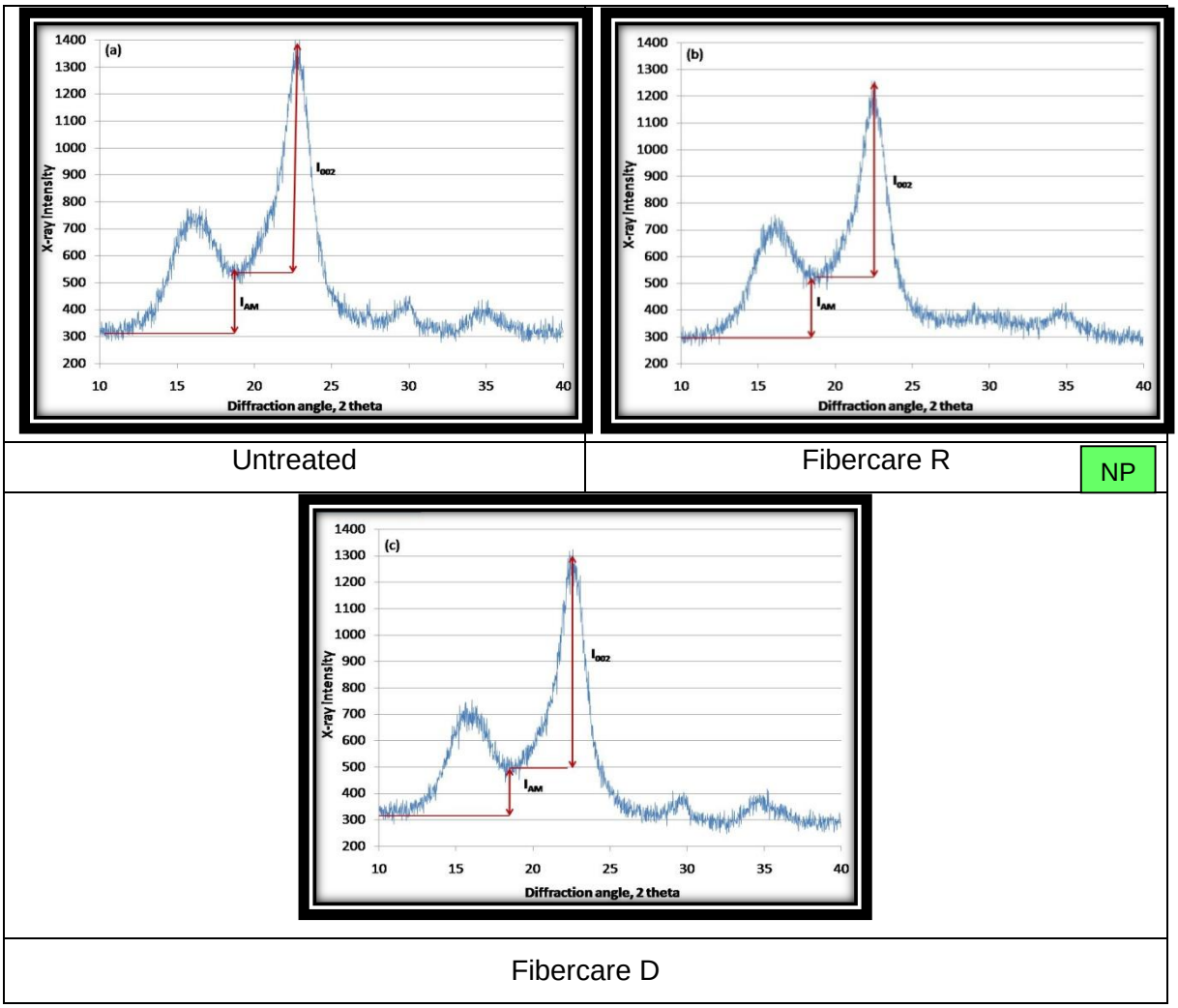


Figure 4.10 Effect of different enzymes on crystallinity of recycled pulp-NP (a) untreated (b) treated with Fibercare R having high cellobiohydrolase and moderate EG activity (c) treated with endoglucanase Fibercare D

Wheat straw pulp-WS

Similar trend i.e. lower amorphous peaks and increase in crystallinity index from 81.4 to 89.2% was observed in wheat straw pulps treated with endoglucanase Fibercare D (Figure 4.12). While in case of cellulase mix, Fibercare R treated pulp samples; the crystallinity index was observed to decrease to 80.0%, in spite of having endoglucanase activity (Table 4.3).

The increase in the crystallinity index is good evidence that the amorphous portion of the cellulose i.e. fines fraction was more readily and quickly hydrolyzed by endoglucanase than the crystalline portion. These nano-scale ultra fines fractions generated by enzymatic hydrolysis might have lost during the sample preparation for X-ray analysis. It was also reported by *Cao and Tan (2005)* that the number of small crystallites decreases during enzymatic hydrolysis by endoglucanases.

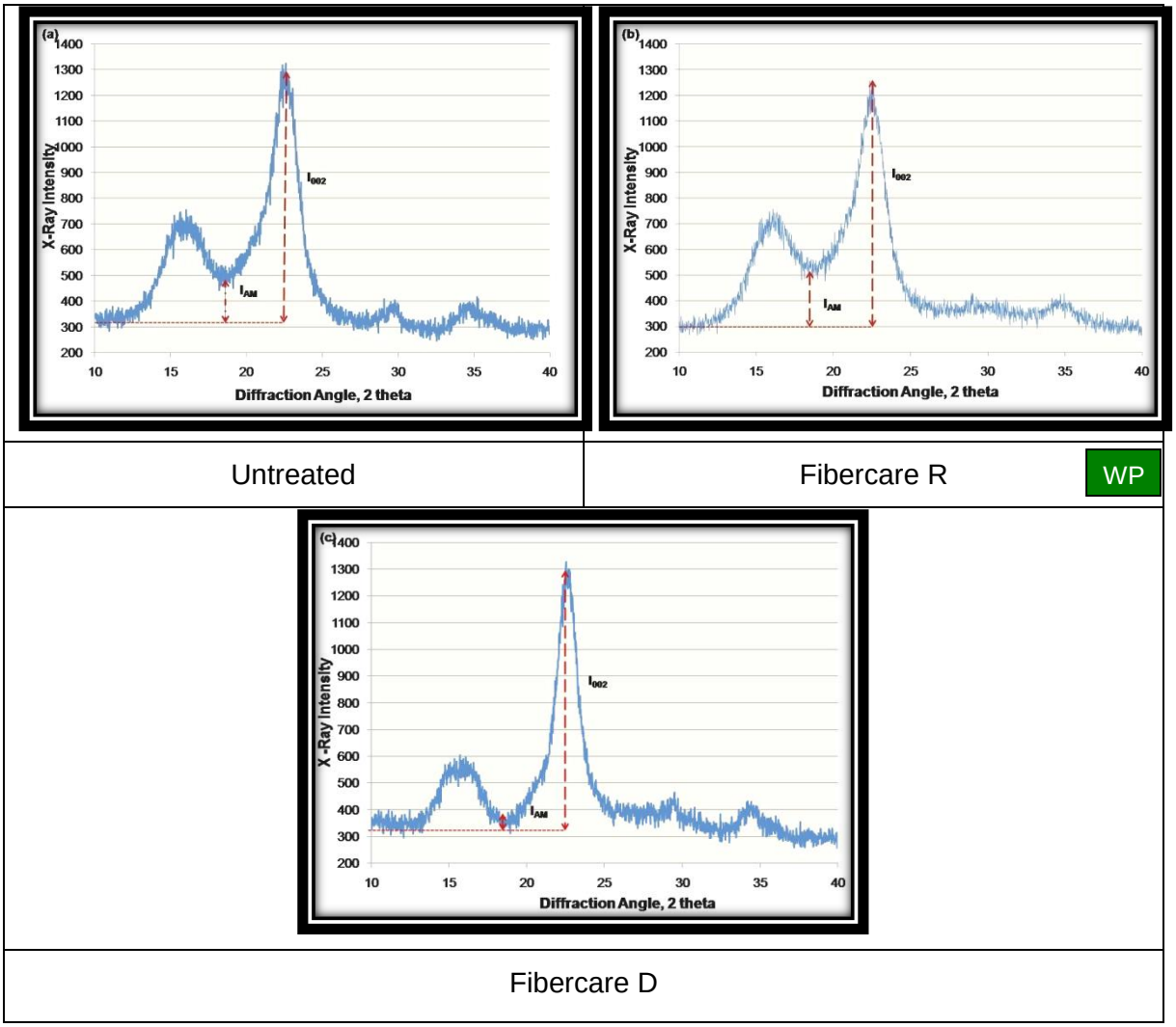


Figure 4.11 Effect of different enzymes on crystallinity of recycled pulp-WP (a) untreated (b) treated with Fibercare R having high cellobiohydrolase and moderate EG activity (c) treated with Endoglucanase Fibercare D

X-ray diffraction studies also supported the finding that endoglucanases even at a lower dose can increase the pulp drainage by hydrolyzing the most accessible parts of cellulose

i.e. ultra fines containing amorphous cellulose and other dissolved colloidal substances and hemicellulose present in the fibers. Similar findings have also been reported earlier by *Valtschev et al. (2001)*. Some conflicting results are also reported in the literature. *Sinitsyn et al. (1989)* found that crystallinity initially increases with time, then decreases and finally levels off.

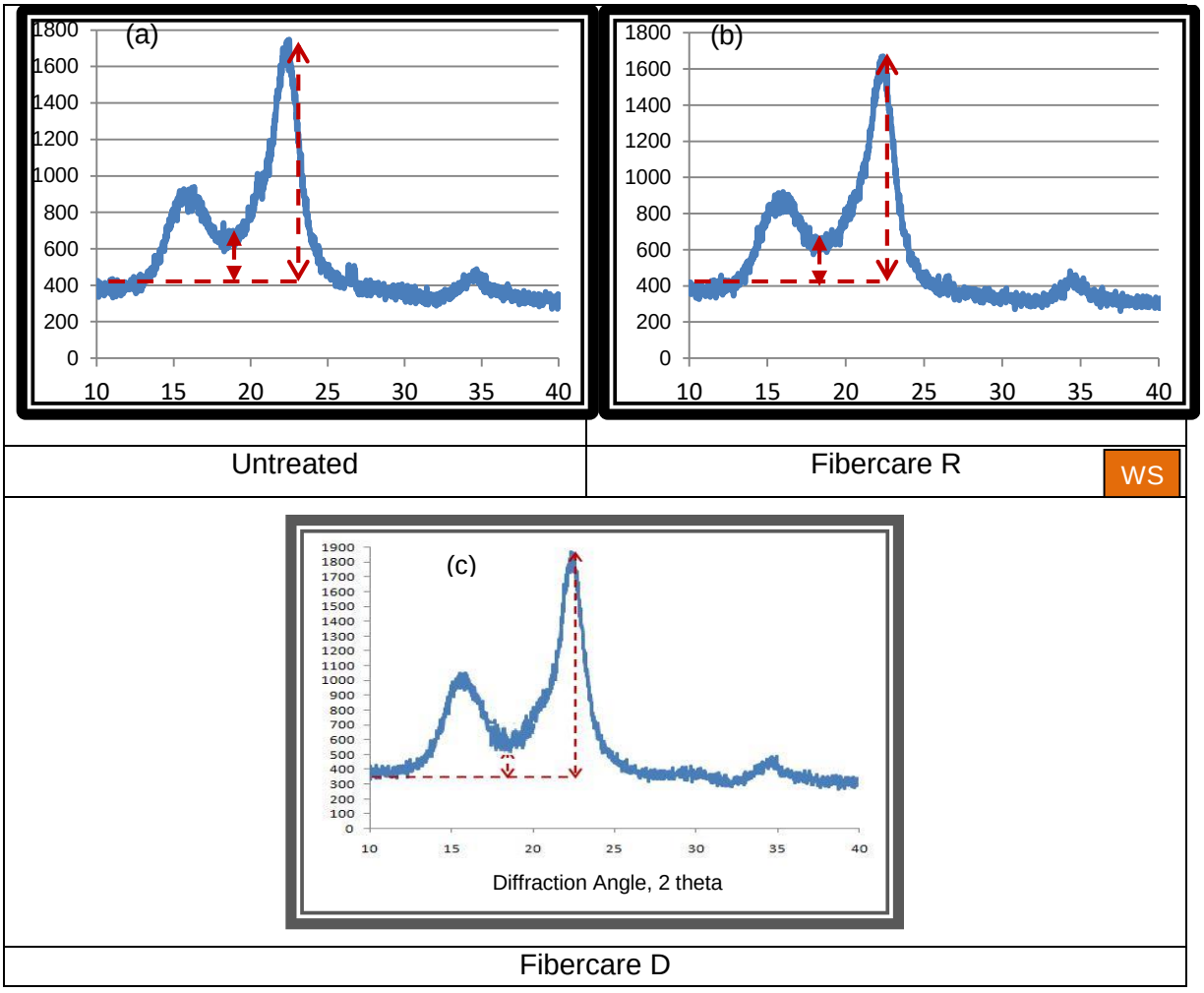


Figure 4.12 Effect of different enzymes on crystallinity of wheat straw pulps-WS (a) untreated (b) treated with Fibercare R having high cellobiohydrolase and moderate EG activity (c) treated with endoglucanase Fibercare D

Table 4.3.Crystallinity index % of different recycled pulps with different enzyme treatments

Recycled pulp	Untreated	Fibercae R	Fibercare D
NP	80.2	77.3	85.5
WP	84.2	81.4	90.1
WS	81.4	80.0	89.2

Scanning electron microscopic studies of untreated and enzyme treated pulps

In order to differentiate between the action of cellobiohydrolase and endoglucanase components on amorphous and crystalline cellulose/ cell wall degradation/ fibre collapse, the enzyme treatments were done at a higher dose of 0.5%. The untreated recycled pulp-NP, recycled pulp-WP and wheat straw pulp fibres displayed minimal amounts of fibrillation that would contribute to inter fibre bonding (Figure 4.13, 4.14, 4.15). There were limited examples of fibrils interconnecting adjacent fibers.

SEM images of fibers treated with high dose of Fibercare R were visually quite different (Figure 4.13, 4.14, 4.15). The damage to the fibers was more prominent when compared to untreated and endoglucanase treated samples. It may be because of the presence of high cellobiohydrolase component which is reported to act on crystalline cellulose. It has also been reported by *Suchy et al. (2009)* that CBH treatment affected the strength of individual fibres. In addition to fibre dislocations, weak spots as a result of localized hydrolytic action, disruptions in the form of cracks/holes on the fibre surface were also occasionally observed on fibres treated with high CBH dose.

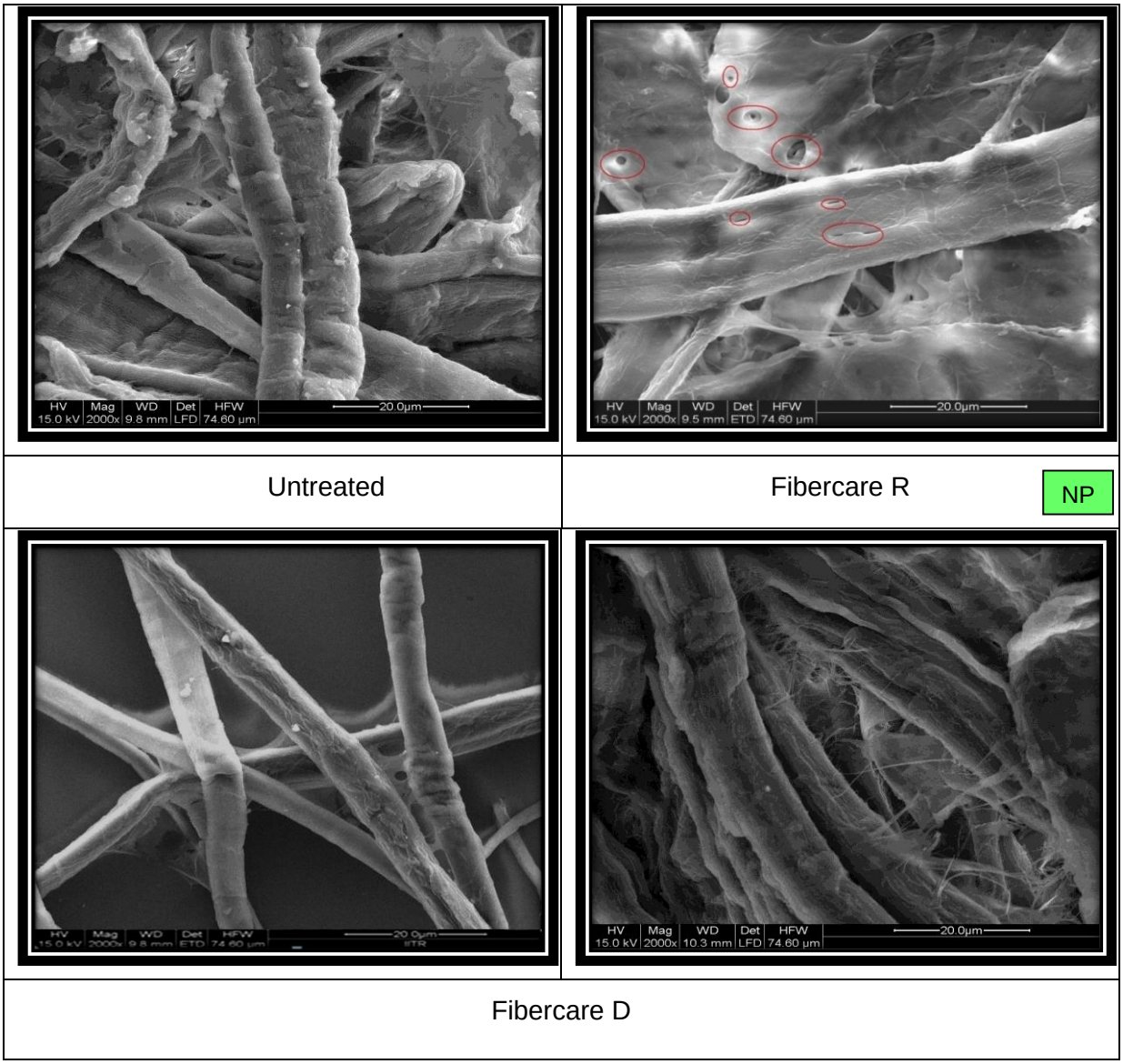


Figure 4.13 SEM micrographs (at 15kV & 2000X) of untreated and enzyme treated recycled pulp-NP

In case of samples treated with endoglucanase preparation, the adjacent fibers appear to be connected by relatively large patches of outer cell wall material (Figure 4.13, 4.14, 4.15). Treatment with the endoglucanase enzyme at higher dose resulted in extensive fibre wall peeling, fibre collapse and increased fibre flexibility. The high degree of fibre collapse contributed to an increase in relative bonded area (RBA) which may be the reason of increased

inter fibre bonding, better strength properties, decreased fibre–water interactions and improvement in pulp drainage. Electron microscopic analysis showed that ultra fine particles with large specific surface area were lower compared to untreated sample.

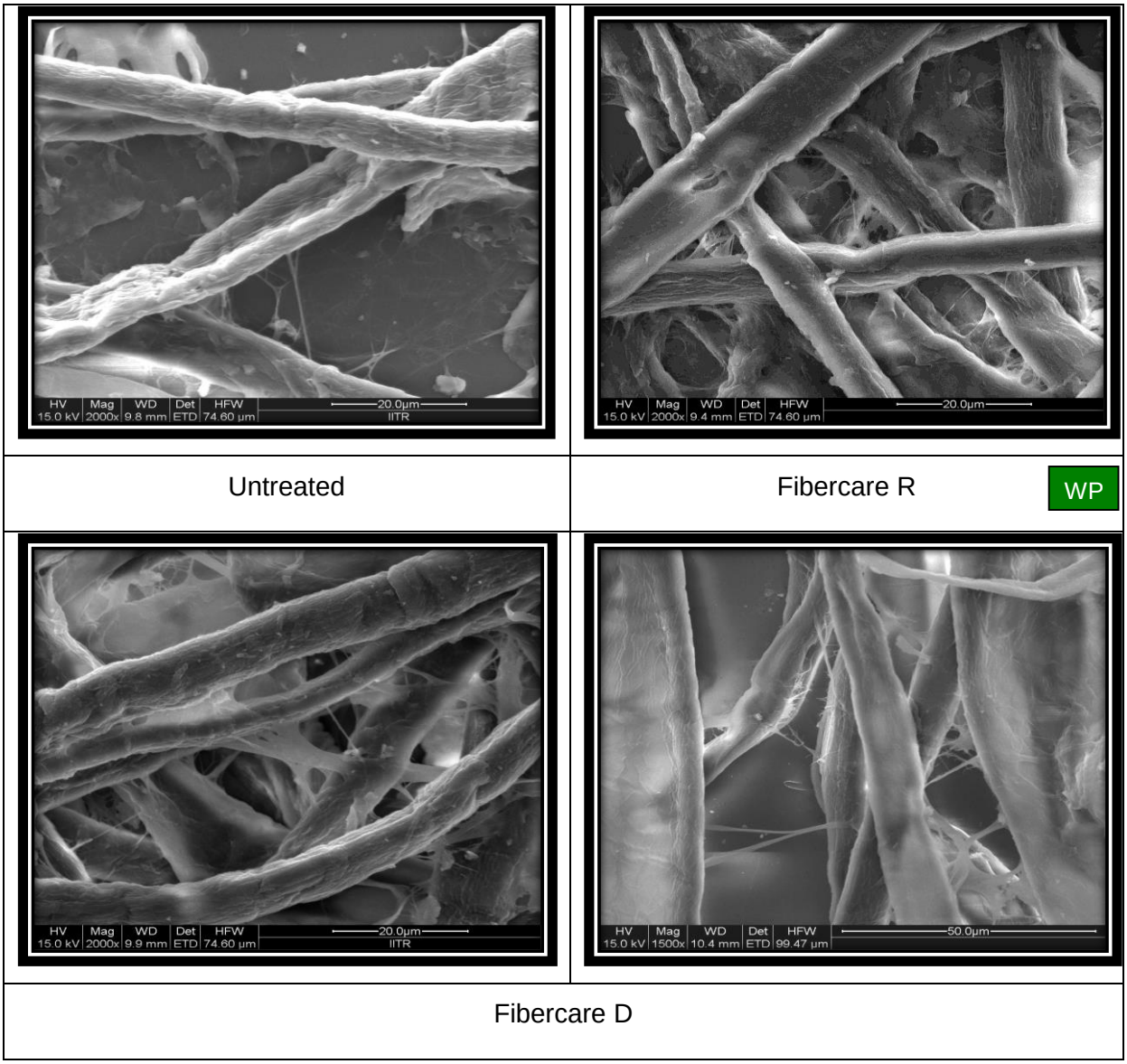


Figure 4.14 SEM micrographs (at 15kV & 2000X) of untreated and enzyme treated recycled pulps-WP

4.4 Effect of enzyme treatments on paper properties

Effect of different enzymes on various paper properties of different pulps viz. tensile, tear, smoothness, sheet density was also analyzed.

Tensile index

Recycled pulp -NP

As shown in Figure 4.16a, the tensile index of paper sheets was increased on increasing the dose of enzymes. It was applicable to all enzyme products. The highest increase was observed with endoglucanase rich, Fibercare D enzyme. The tensile index of the untreated pulp sheets was 21.4 N.m/g which was increased to 25.7 N.m/g, showing about 20% increase

in tensile index, on treatment using Fibercare D (0.020% dose). It was due to the increase in longer fibre fraction, fibrillation and inter-fibre bonding. The second highest improvement in tensile index of 13% was observed in Fibercare R treated pulps. Presence of endoglucanase

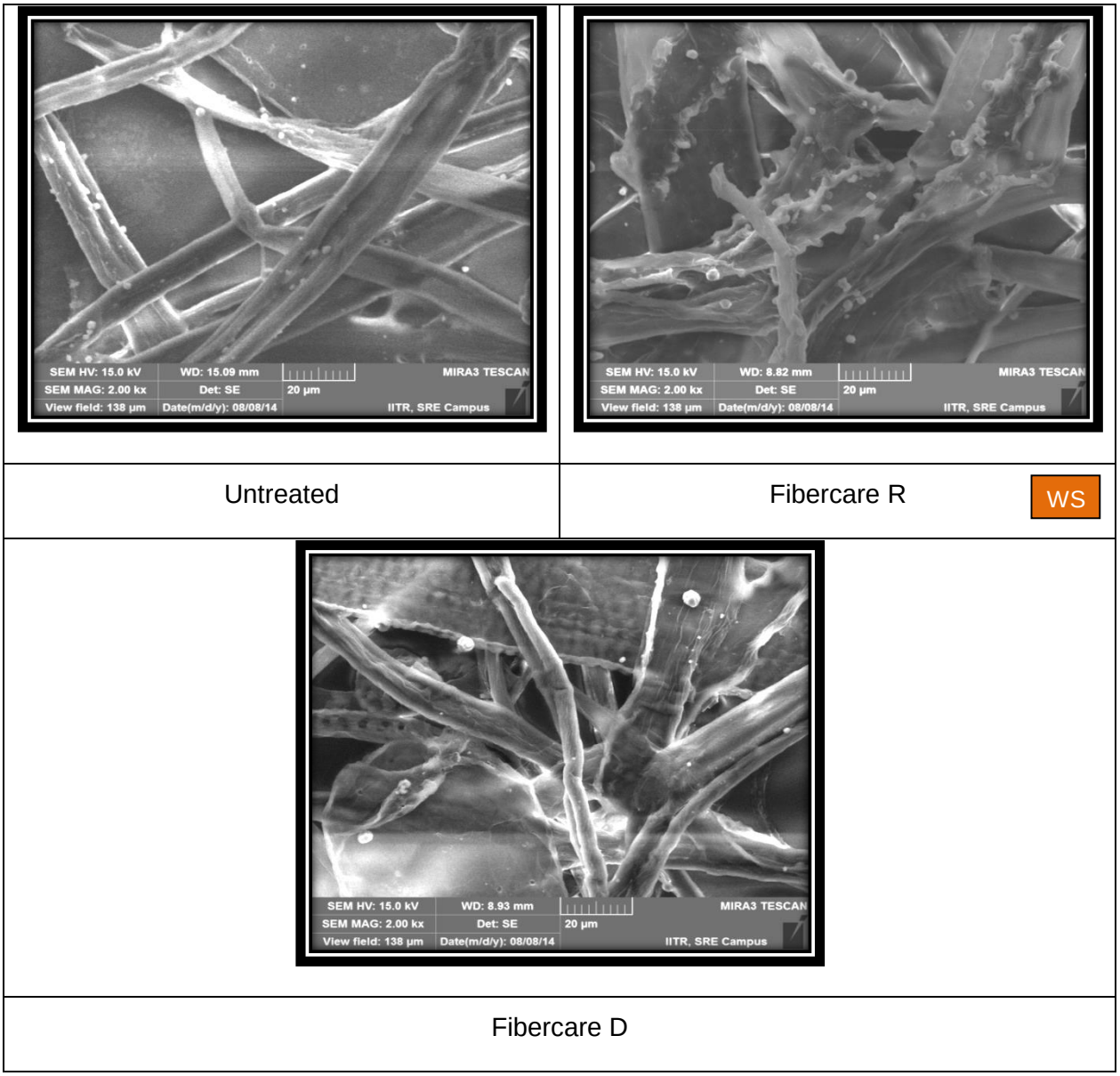


Figure 4.15 SEM micrographs (at 15kV & 2000X) of untreated and enzyme treated wheat straw pulps-WS

component in sufficient quantity in Fibercare R may be the reason for improvement in tensile index. Texzyme M and Fibrezyme LBR showed less impact on tensile index of paper sheets (Figure 4.16a).

Recycled pulp -WP

Endoglucanase rich Fibercare D at a dose of 0.020% showed significant increase of about 13% in tensile index (Figure 4.16b). No change in tensile index was observed in pulps treated with Fibercare R and Texzyme M.

Wheat straw pulp-WS

As shown in Figures 4.16c, the tensile index of paper sheets was increased on increasing the dose of endoglucanase rich, Fibercare D. The tensile index of the untreated wheat straw pulp sheets was 41.9 N.m/g. 9% increase in tensile index (45.6 N.m/g) was observed in pulps treated with endoglucanase Fibercare D at 0.020% dose. On the other hand, no significant improvement in the tensile index was observed in pulps treated with Fibercare R.

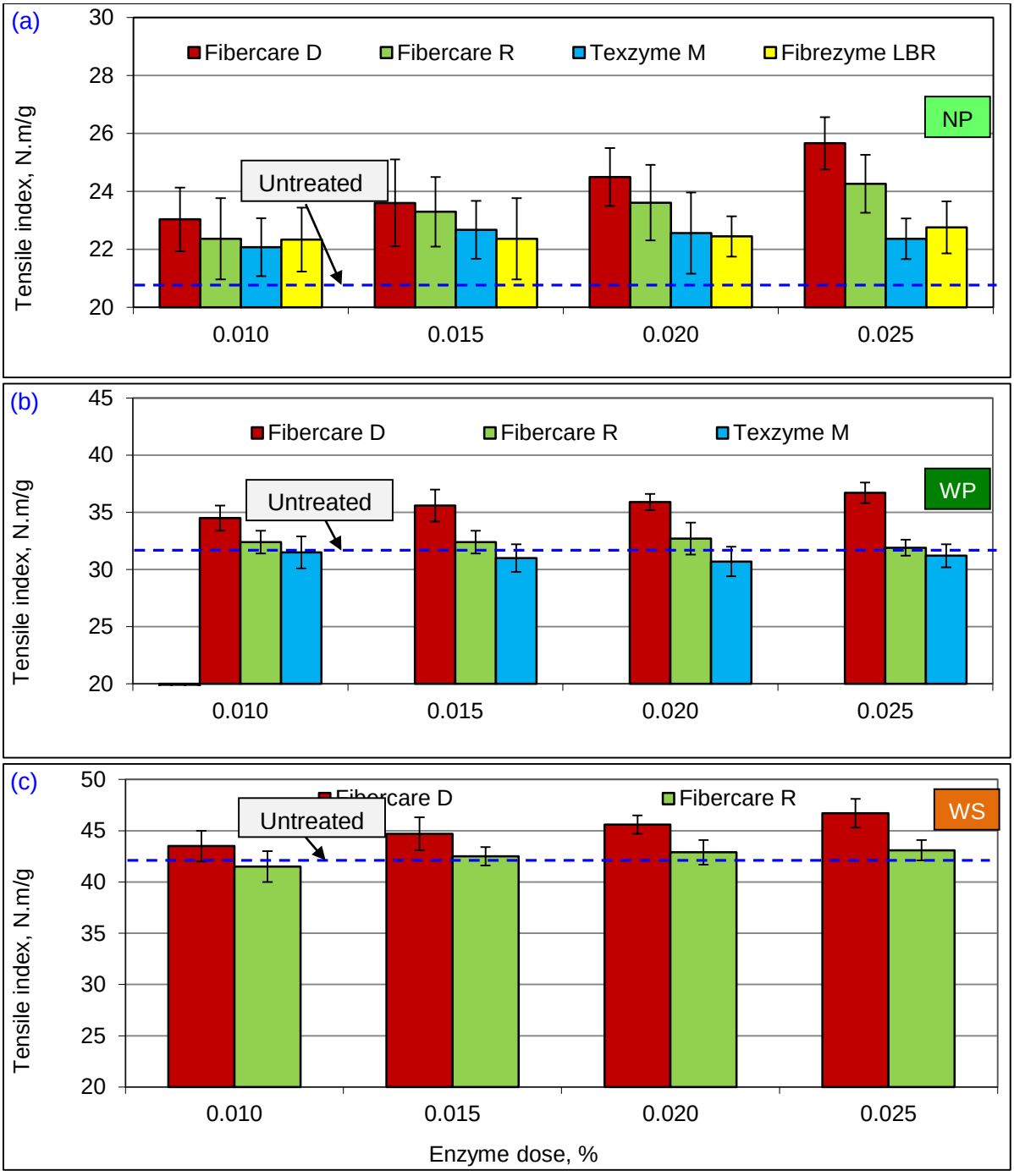


Figure 4.16 Effect of different enzymes at various doses on tensile index of (a) recycled pulp-NP (b) recycled pulp-WP (c) wheat straw pulp-WS

It was reported elsewhere that the endoglucanase treatment of pulp showed significant increase in tensile index of paper *(Valchev and Bikov 2011)*. Drainability and strength

properties especially tensile were reported to be increased when the enzymatic treatment was applied to the pulp without industrial refining, whereas no improvement was observed for pulp with industrial refining *(Maximino et al. 2013).* However, enzymatic improvement of refining efficiency achieved at laboratory scale (sometimes spectacular) has been mostly correlated with decline in paper properties *(García et al. 2002*). The enzymes are reported to modify the interfacial properties of fibre, increasing the water affinity which, in turn, changes the technical properties of pulp and paper, such as drainability and strength *(Pala et al. 2002).*

Tear index

Recycled pulp -NP

Except Fiberzyme LBR, which had no impact on tear index, all other enzymes exhibited a decrease in tear index (Figure 4.17a). The tear index of the untreated sheets was 6.5 mN.m^2/g which dropped to the highest level using Texzyme M (about 12% drop). The tear index was reduced by 6% in pulps treated with Fibercare D at maximum dose of 0.025%. As the Fibercare R dose was increased beyond a certain level, say 0.020%, the combined effect of cellobiohydrolase and endoglucanase reduced the tear strength significantly (Figure 4.17a). Similar results showing reduction in tear and burst indices in enzyme treated pulps were reported in the literature *(Valchev and Bikov 2011).*

Recycled pulp -WP

Common trend of decrease in tear index was observed in all sheets prepared from enzyme treated pulps (Figure 4.17b). But in case of endoglucanse rich, Fibercare D, the decrease in tear was less. Whereas in case of Fibercare R and Texzyme M, at a higher dose of 0.02% and 0.025%, there was a significant decrease in tear strength. The combined effect of cellobiohydrolase and endoglucanase components in Fibercare and Texzyme M are supposed to affect the tear strength in a negative manner.

Wheat straw pulp-WS

Both the enzyme products decreased the tear index of the paper sheet (Figure 4.17c). The tear index values of the untreated wheat straw pulp sheets were 5.35 mN.m^2/g, which dropped to the highest level using Fibercare R (about 25% drop).The reason might be due to increased hydrolysis/solubilization of crystalline cellulose by CBH in presence of EG. Drop in tear index values in endoglucanase Fibercare D treated pulps, up to 0.02% dose, was not significant (Figure 4.17c).

Henrissat et al. (1985) also reported the increased hydrolysis of crystalline cellulose by cellobiohydrolase. In the literature also, it has been reported by *Valchev and Bikov (2011)* that the effect of endoglucanase treatment on the pulp strength properties showed significant increase in tensile index while the tear index and the burst index got decreased.

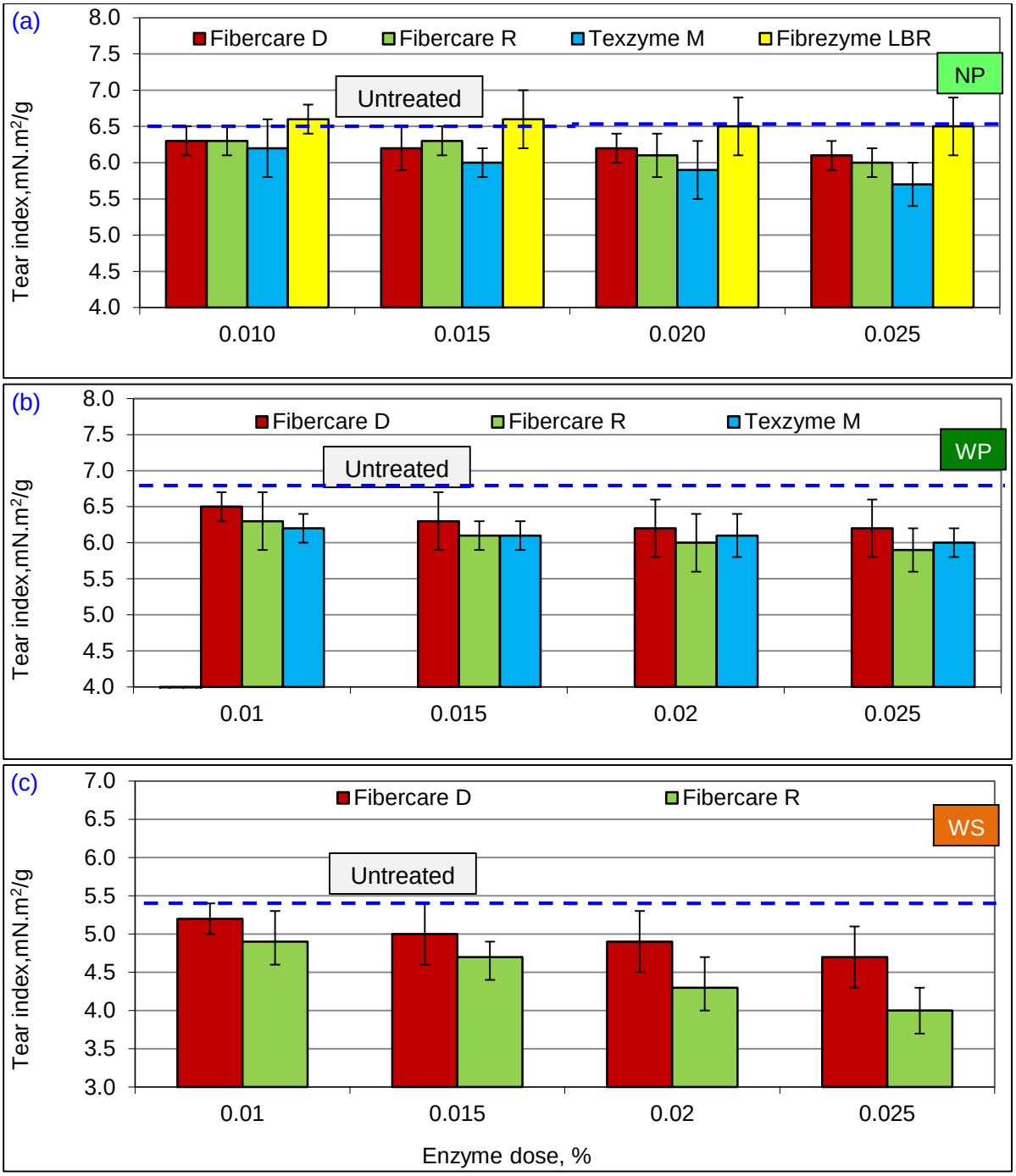


Figure 4.17 Effect of different enzymes at various doses on tear index of (a) recycled pulp-NP (b) recycled pulp-WP (c) wheat straw pulp-WS

Smoothness

Recycled pulp -NP

Similar to the effectiveness of Fibercare D on tensile index, significant improvement in smoothness by 44%, shown as reduction in Bendtsen roughness value from 200 to 112 mL/min., was also noticed in case of endoglucanase Fibercare D treated pulps (Figure 4.18a). The reduction in roughness of paper using highest dose of Texzyme M, and Fibercare R was

about 24 and 34%, respectively (Figure 4.18a). In this case also, Fibrezyme LBR did not show any improvement in paper smoothness.

Recycled pulp -WP

Significant increase in smoothness by 43%, showed as reduction in Bendtsen roughness value from 250 to 142 mL/min., was noticed in case of endoglucanase Fibercare D treated pulps. Reduction in roughness of paper of about 23 and 28% was observed using Texzyme M and Fibercare R, respectively at 0.025% enzyme dose (Figure 4.18b).

Wheat straw pulp-WS

Significant improvement of 45% in paper smoothness was noticed in case of endoglucanase Fibercare D treated pulps (Figure 4.18c). On the other hand, Fibercare R was observed to increase the smoothness by 32%.

Probably the endoglucanase action at low enzyme dose contributed to an improvement of the paper structure independent of degradation process of dissolved and colloidal substances. Significant improvement in drainage rate along with improvement in smoothness and tensile index using endoglucanase enzyme was also reported by *Jackson et al. (1993).*

Apparent sheet density

Recycled pulp -NP

A plot depicting the apparent sheet density vs. tensile strength was plotted in Figure 4.19a. At the same apparent density of the paper sheets the tensile index was higher with Fibercare D as compared to Fibercare R clearly showing the higher densification in the sheets with the former enzyme product.

Recycled pulp-WP

Figure 4.19b depicts the tensile strength vs apparent sheet density clearly showing higher densification in the sheets. The apparent density for the untreated pulp was 0.658 g/cm^3. The apparent density varied from 0.662 to 0.688 g/cm^{3}and from 0.680 to 0.732 g/cm^3 for paper samples produced from Fibercare R and endoglucanase Fibercare D treated pulps, respectively.

Wheat straw pulp-WS

Similarly, in case of untreated and enzyme treated wheat straw pulp-WS, apparent sheet density vs. tensile strength plot was plotted in Figure 4.19c. At the same apparent density of the paper sheets, the tensile index was higher with endoglucanase Fibercare D as compared to cellulase mix Fibercare R, clearly showing higher densification and increased fibre flexibility in the sheets.

The apparent sheet density increase would be one of the primary effects of the enzyme treatment possibly due to increased fibre flexibility *(Maximino et al. 2011).* This higher

densification in the sheets might be the cause of increase in tensile strength and decrease in tear values.

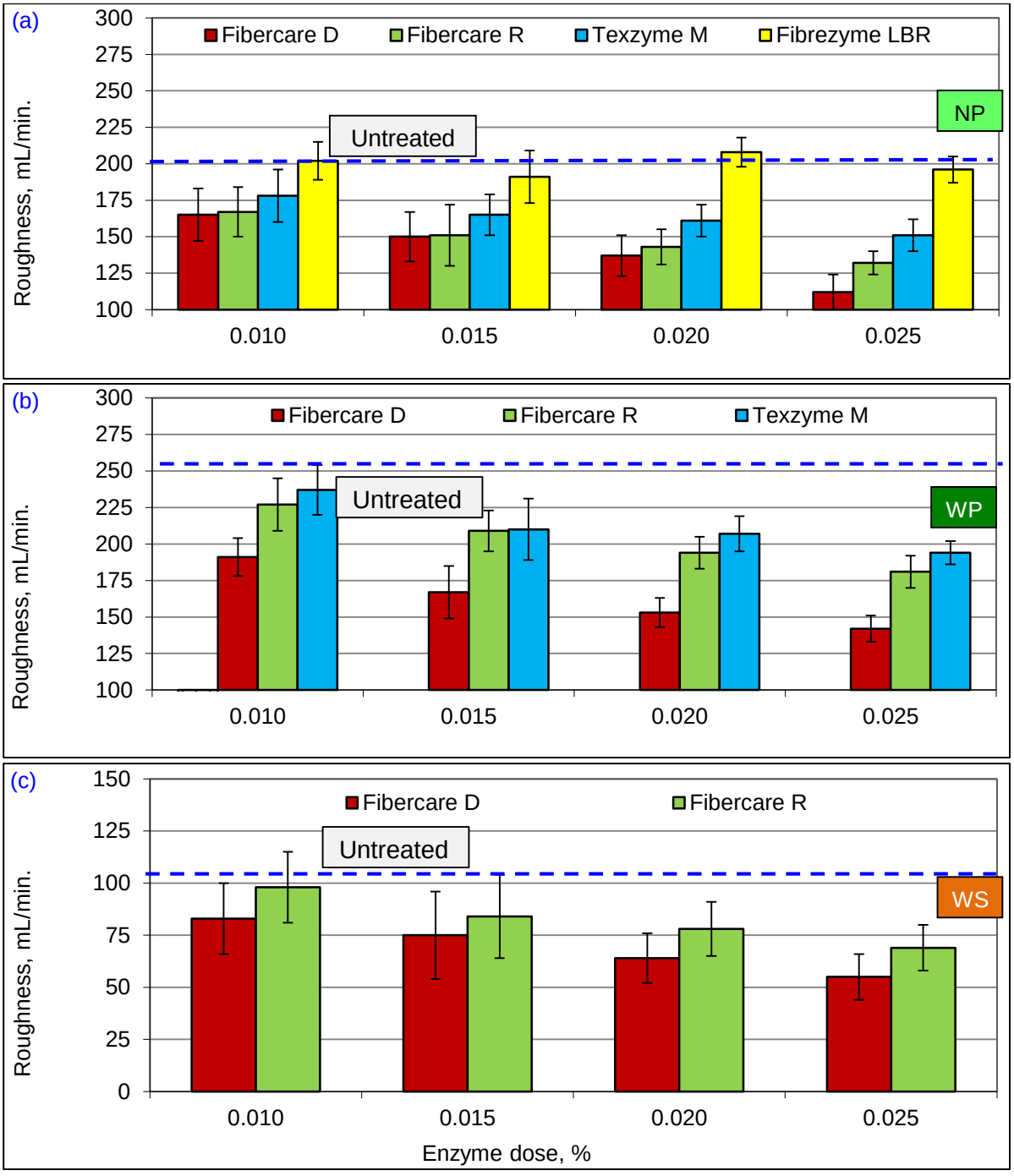


Figure 4.18 Effect of different enzymes at various doses on roughness of (a) recycled pulp-NP (b) recycled pulp-WP (c) wheat straw pulp-WS

4.5 Effect of enzyme treatments on pulp and paper properties (at same EG activity level)

The plots depicting the effect of different enzymes at same EG activity level i.e. 0.08 IU/g of oven dry pulp, on pulp and paper properties of different pulps were plotted in Figure 4.20 and 4.21, respectively. Significant reduction in drainage time and increase in pulp freeness was observed in endoglucanase Fibercare D treated pulps (Figure 4.20a, b, c). It was clearly

demonstrated that Fibrezyme LBR, Texzyme M and Fibercare D exhibited a deterrant effect on the pulp and paper properties at same EG activity levels but at higher dose levels (with higher CBH activities) on w/w basis when compared to endoglucanase Fibercare D. Significant reduction in tensile and tear strength (Figure 4.21a, b, c) as well as in pulp viscosity (Figure 4.21a, b, c) was observed due to the presence of high cellobiohydrolase component, reported to act on crystallline cellulose.

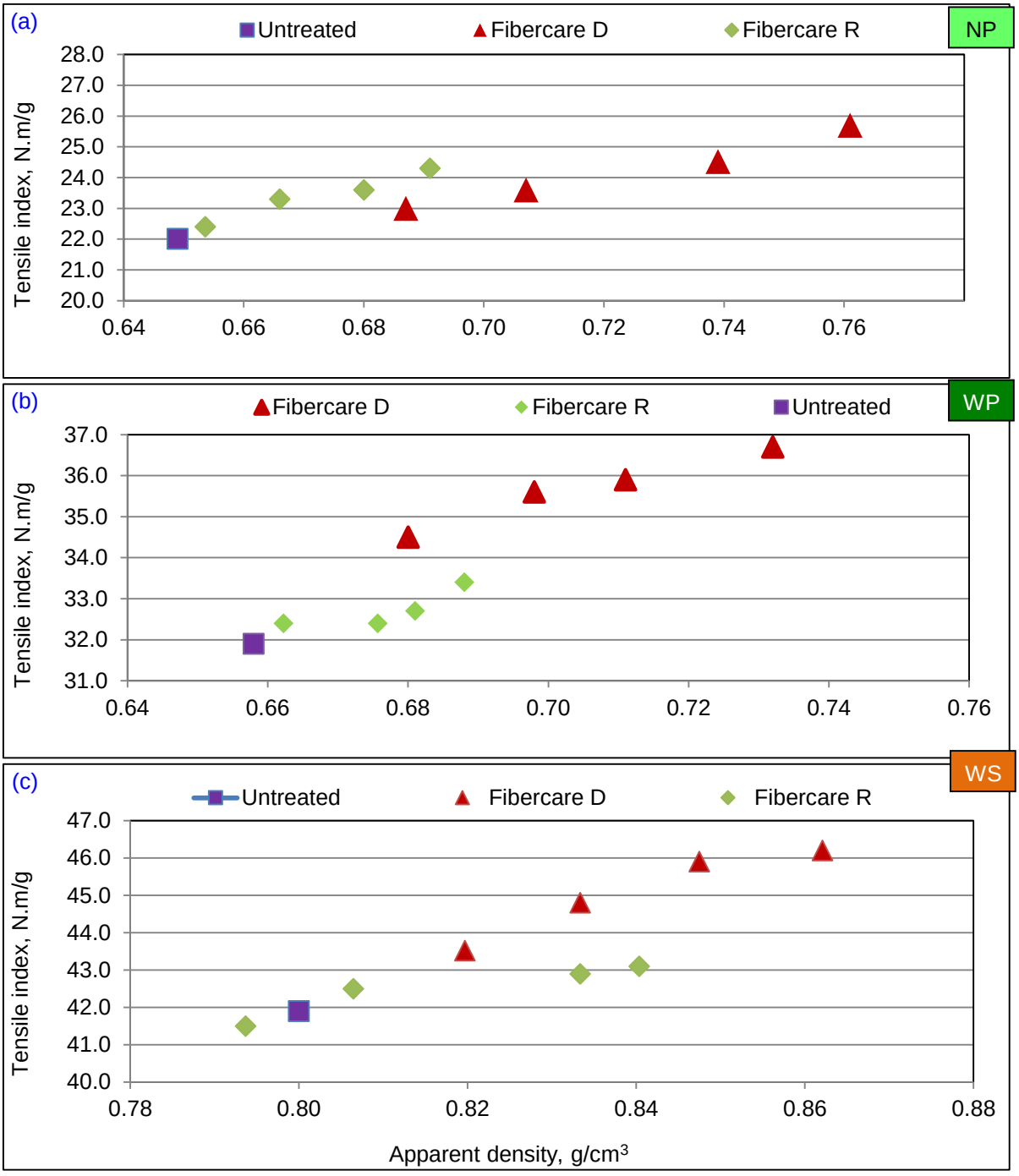


Figure 4.19 Evolution of tensile index and apparent density of sheet in enzyme treated (a) recycled pulp-NP (b) recycled pulp-WP (c) wheat straw pulp-WS

On the other hand, endoglucanase Fibercare D treatment exhibited a significant increase in tensile strength without any significant loss in pulp viscosity and tear strength. Significant

improvement in smoothness, shown as reduction in Bendtsen roughness value in Figure 4.21a, b, c was also noticed in case of endoglucanase Fibercare D treated recycled-NP, recycled-WP and wheat straw pulp-WS, respectively.

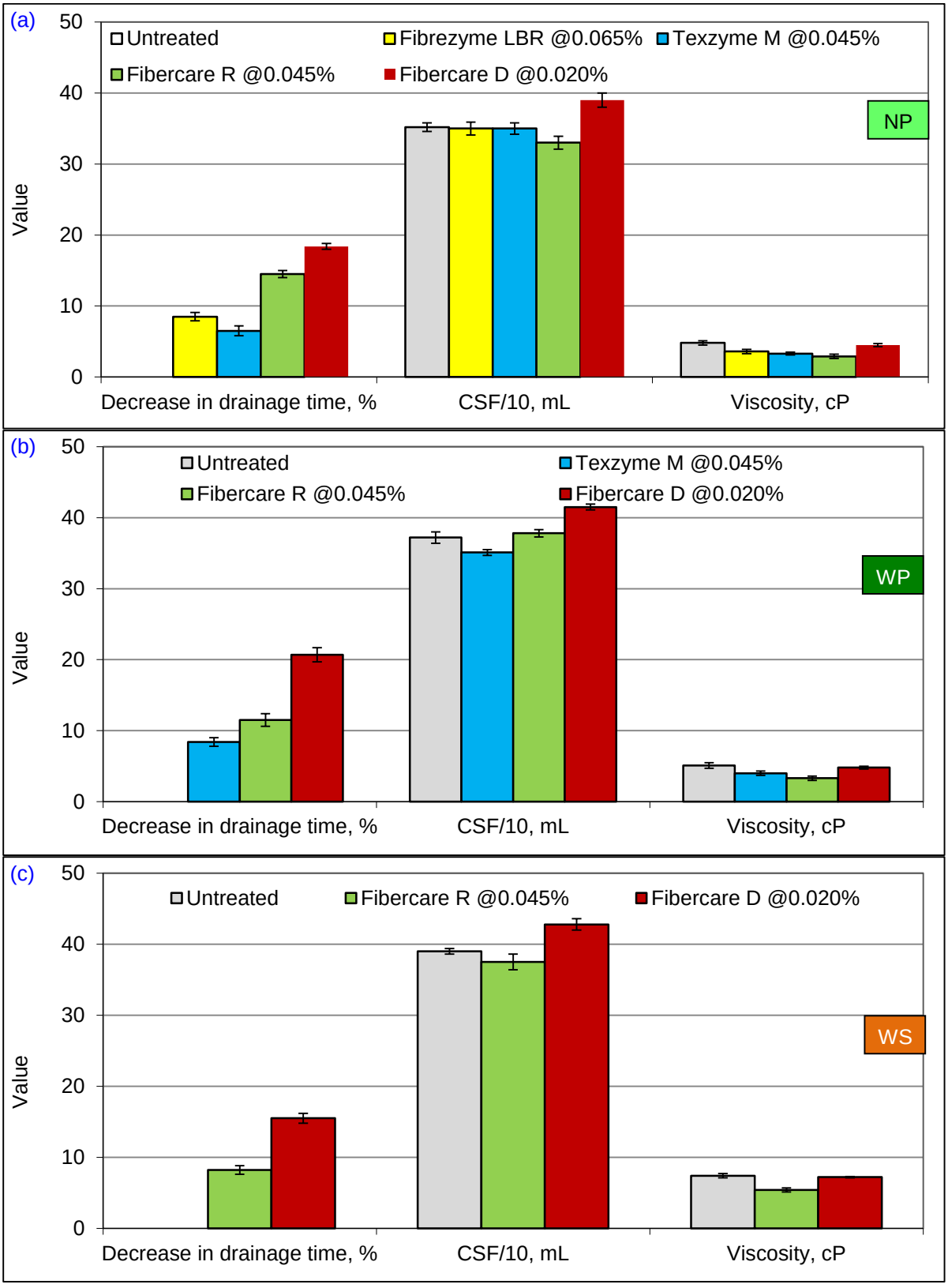


Figure 4.20 Effect of different enzymes (at same endoglucanase, EG activity levels i.e. 0.08 IU/g of oven dry) on pulp properties of (a) recycled pulp-NP (b) recycled pulp-WP (c) wheat straw pulp-WS, CSF value taken as Value/10

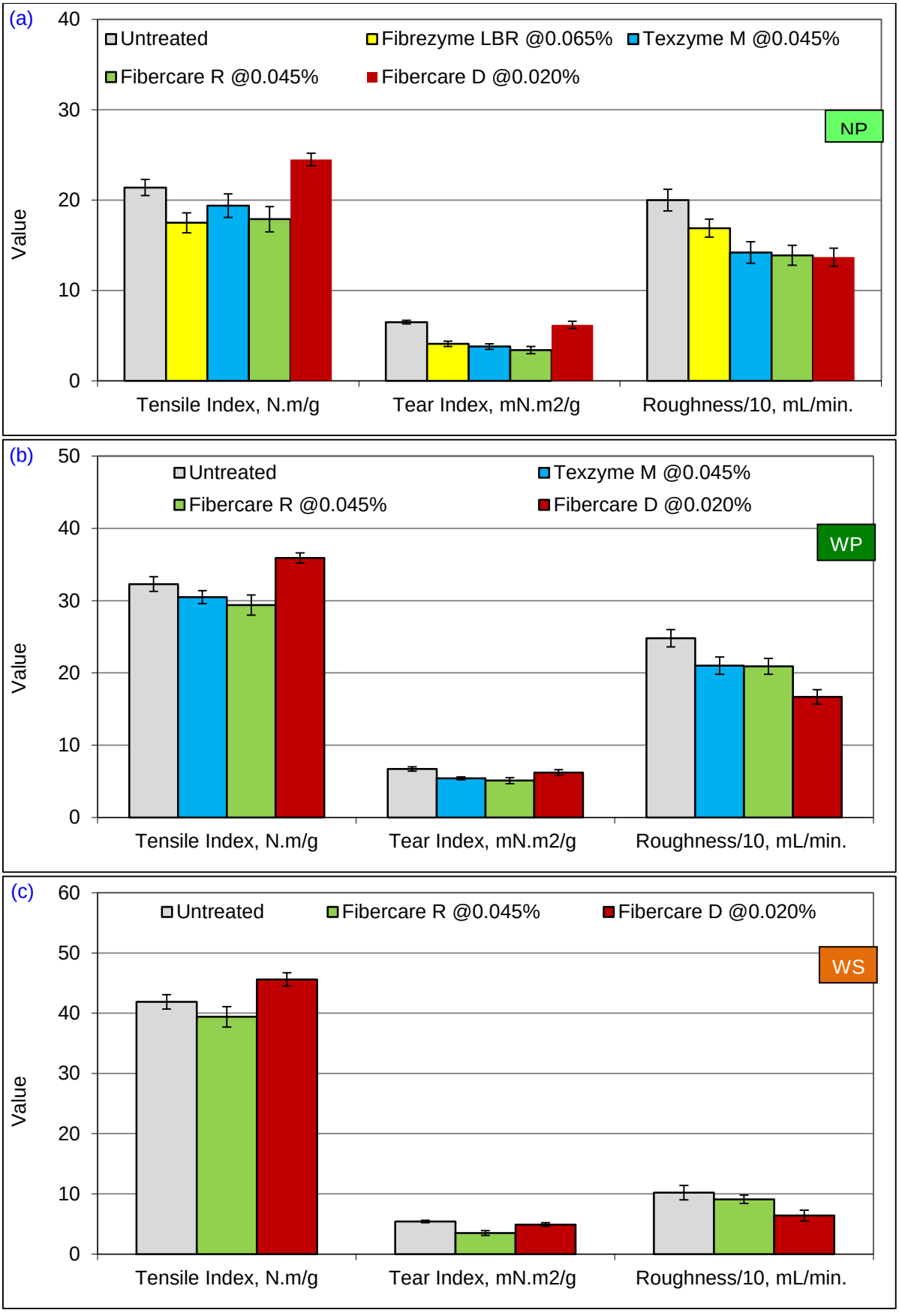


Figure 4.21 Effect of different enzymes (at same endoglucanase, EG activity levels i.e. 0.08 IU/g of oven dry) on paper properties of (a) recycled pulp-NP (b) recycled pulp-WP (c) wheat straw pulp-WS, roughness value taken as Value/10

In the literature also, it has been reported by *Valchev and Bikov (2011)* that the effect of endoglucanase treatment on the pulp strength properties shows insignificant increase in tensile index, while the tear index and the burst index got decreased. It has also been reported by *Pala et al. (2002)* that recycled fibres can be upgraded through treatments of cellulases.

Therefore, it can be postulated that, when cellulolytic enzymes are to be used for partial hydrolysis of cellulose chains for improvement in drainage and thus to form a better recycled fibre structure, it is important to find the balance between two opposite directions. On one hand, by the hydrolysis of ultra fines, increased dewatering rates are obtained. On the other hand, enough fines have to be left in the pulp in order to obtain optimal inter fibre bonding, which is required for good strength properties of the end product. Moreover, enzyme action should not result in excessive hydrolysis, as this means loss of weight and thus production. This study reflects that using purified endoglucanase enzymes, specific regions of the cellulose fibers can be attacked and the desired part of the pulp could be modified in a controlled manner to get expected benefits.

4.6 Salient findings

- Based on different cellulase activities, Fibercare R was observed to be a mixture of both cellobiohydrolase and endoglucanase components whereas, Fibercare D was a monocomponent cellulase preparation containing predominantly endoglucanase activity.
- Fibercare R was observed to contain maximum cellobiohydrolase activity.
- Other two commercial enzymes Texzyme M and Fibrezyme LBR were also observed to be a mixture of different cellulase components.
- The optimum pH for endoglucanase activity in Texzyme M, Fibercare R and Fibercare D was 7, although the endoglucanase activity of Fibrezyme LBR was comparable at 6 and 7 pH.
- Fibercare D and Fibercare R enzymes were observed to generate more amount of reducing sugars which represented the hydrolysis of cellulosic fines.
- Significant improvement of pulp dewatering of about 11 to 24%, 15 to 23% and 15 to 20% was observed in Fibercare D treated recycled pulp-NP, recycled pulp-WP and wheat straw pulp-WS, respectively.
- Cellulase mix Fibercare R having high cellobiohydrolase activity as well as moderate endoglucanase activity (CMCase) was found less effective in improving the pulp drainage.
- Increase in tensile index and smoothness was also observed with endoglucanase rich, Fibercare D treatments.
- Higher WRV in case of endoglucanase rich, Fibercare D treated pulps was observed due to the hydrolysis of lower molecular weight carbohydrates and partial deterioration

of crystalline structure of cellulose, initiating the formation of more hydrogen bonds between cellulose and water molecules.

- Endoglucanase rich, Fibercare D enzyme was observed to increase the drainage up to a dose of 0.02% without any negative impact on paper strength.
- When the dose of endoglucanase enzyme was increased beyond 0.02%, there was an improvement in pulp drainage, but tear strength and viscosity got decreased.
- Significant reduction in pulp viscosity and tear index was observed in pulps treated with Fibercare R.
- Decrease in fines content in pulps treated with Fibercare D and increase in fines content in pulps treated with Fibercare R was observed.
- Proportion of longer fibres (greater than 1 mm) was increased and that of shorter fibres was decreased on treatment of pulp with Fibercare D.
- Lower amorphous peaks and significant increase in crystallinity index was observed in recycled and wheat straw pulps treated with endoglucanase rich, Fibercare D enzyme.

CHAPTER 5

Drainage improvement by combined treatment of commercial cellulases and polymeric drainage aid

The interaction of various types of cellulases (Fibercare D and Fibercare R) and polymeric drainage aid for enhancing the freeness of recycled and wheat straw pulps were also explored. This chapter describes the combined effect of commercial cellulases and polymeric drainage aid for improvement in pulp drainage.

5.1 Effect of different enzymes and polymeric drainage aid on pulp properties

The recycled and wheat straw pulps were treated with various concentrations of commercial enzymes ranging from 0.01 to 0.03% on dry weight of pulp for 30 min. The parameters of enzymatic treatments (pulp consistency, temperature and pH) were adjusted to the industrial conditions mentioned before in the material and methods section. After the enzyme treatment, pulp was diluted to the consistency of 0.3% (As per Tappi standard T 205 sp-02) and polymeric drainage aid (P) Percol 47 at two dose levels i.e. 0.010 and 0.02% was added before sheet formation. The polymeric drainage aid Percol 47 is a medium to high molecular weight cationic polyacrylamide (CATPAM].

Pulp freeness

Enzymatic and polymeric treatments of recycled and wheat straw pulps and the measurements of CSF were performed with sufficient number of parallels in order to detect the changes in drainage accurately.

Recycled pulp-NP

As shown in Figure 5.1a, the highest increase in the CSF value, from 440 to 535 mL, was observed with endoglucanase Fibercare D showing it to be the most effective enzyme in making the pulp fibres more free from water. Similar results and discussions have already been reported in the Chapter 4. Polymeric drainage aid, Percol 47 also showed a significant increase in CSF values from 440 to 540 and 580 mL at 0.010 and 0.02% dose, respectively.

When Percol 47 at 0.01% dose was added after the Fibercare D (0.02 and 0.03% dose) treatment, the CSF was further increased to 560 and 620 mL respectively. Addition of Percol 47 at higher dose of 0.02% after Fibercare D treatment did not show further increase in CSF values. On the other hand, addition of Percol 47 after Fibercare R (0.02%) treatment was observed to show less increase in pulp freeness i.e. 460 and 510 at 0.01 and 0.02% Percol 47 dose, respectively (Figure 5.1a).

Recycled pulp-WP

In case of endoglucanase Fibercare D treated recycled pulp-WP samples; significant increase in CSF values ranging from 442 (untreated) to 521 mL was observed (Figure 5.1b). Percol 47 also showed a significant increase in CSF values from 442 (untreated) to 490 and 525 mL at 0.01 and 0.02% dose, respectively. Addition of Percol 47 at 0.01 and 0.02% dose, after Fibercare D (0.02%) treatment, further increased the CSF values to 558 mL and 542 mL, respectively. The increase in CSF values after the combined Fibercare R and Percol 47 treatment was less when compared to Fibercare D and Percol 47 treatments (Figure 5.1b).

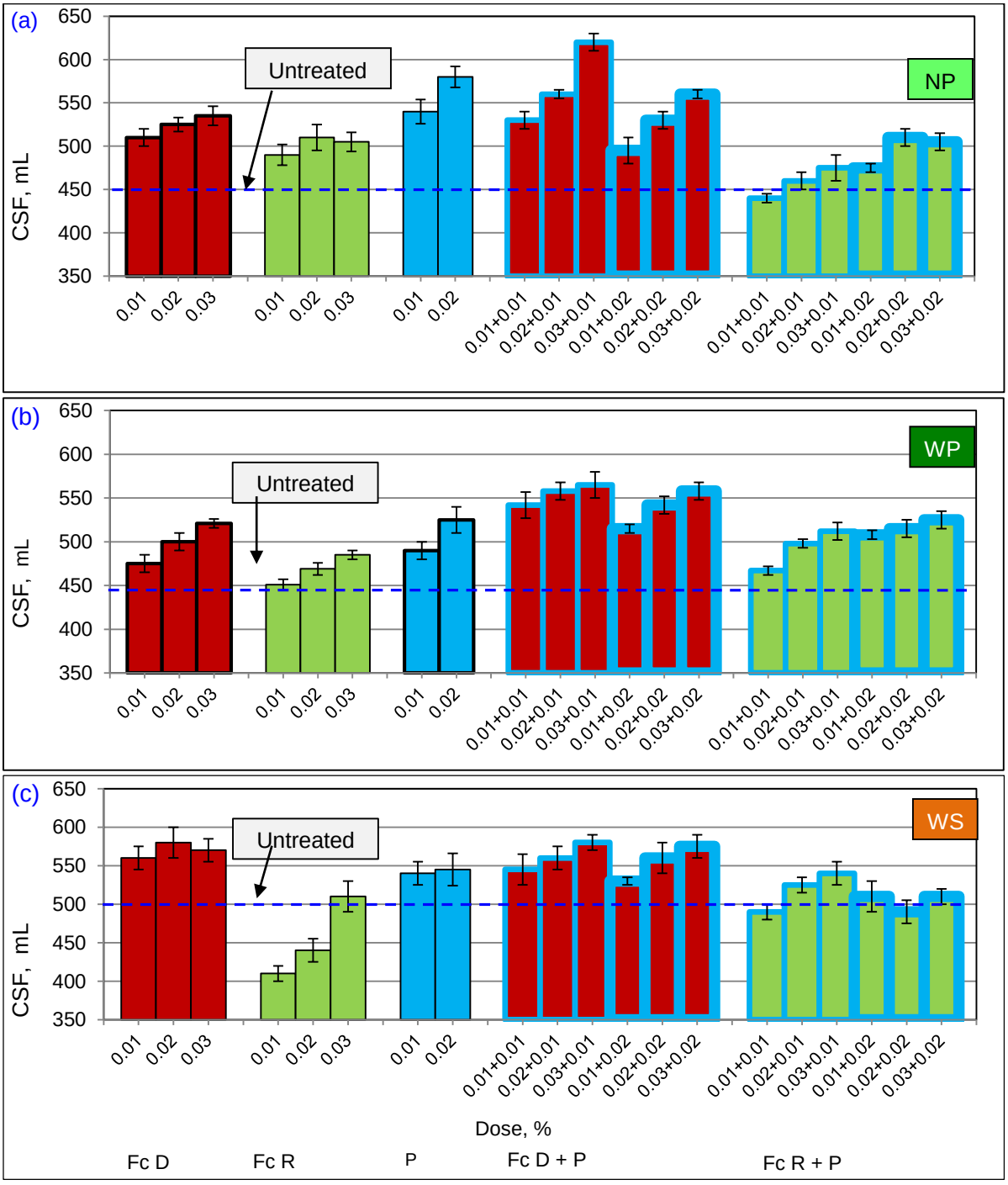


Figure 5.1 Effect of different enzymes and polymeric drainage aid at various doses on CSF of (a) recycled pulp-NP (b) recycled pulp-WP (c) wheat straw pulp-WS

Wheat straw pulp-WS

Similar trend of increased pulp freeness after the Fibercare D and Percol 47 treatment at various doses was observed in wheat straw pulp (Figure 5.1c). Addition of Percol 47 at 0.01 and 0.02% dose, after Fibercare D (0.02% dose) treatment, was observed to further increase the CSF values to 560 mL.

Pulp drainability

Recycled pulp-NP

As shown in Figure 5.2a, 5.3a, Enzyme Fibercare D displayed the highest reduction in drainage time (10.7 to 25.0%) at various doses. Fibercare R having high cellobiohydrolase activity as well as moderate endoglucanase activity was also effective in reducing the drainage time by 3.6 to 14.3% at various doses. Percol 47 alone, exhibited improvement in pulp drainage by 14.3 and 25.0% at 0.01 and 0.02% dose, respectively. When polymeric drainage aid Percol 47 at a dose of 0.01 and 0.02% was added after the Fibercare D (0.02% dose) treatment, the drainage time was further reduced by 28.5%. Reduction in drainage time after the sequential addition of Fibercare R and Percol 47 was less compared to that of Fibercare D and Percol 47 treatment (Figure 5.2a, 5.3a).

Recycled pulp-WP

Endoglucanase enzyme Fibercare D displayed significant improvement in pulp drainage ranging from 14.3 to 25.0% at various doses. Cellulase mix Fibercare R was also found effective in improving the pulp drainage ranging from 7.1 to 17.9% at various doses. Percol 47 alone was observed to improve the pulp drainage by 17.9 and 25.0% at 0.01 and 0.02% dose, respectively. Further addition of Percol 47 at 0.01 and 0.02% dose after Fibercare D (0.02% dose) treatment improved the drainage by 28.6 and 32.1%, respectively. Fibercare R treatment followed by Percol 47 addition was not found effective in drainage improvement (Figure 5.2b, 5.3b).

Wheat straw pulp-WS

Endoglucanase Fibercare D at various doses displayed significant improvement in pulp drainage wheat straw pulps, ranging from 12 to 24%. Significant improvement of 24 and 26% in pulp drainage was observed when Percol 47 at 0.01 and 0.01% dose, respectively was added after the Fibercare D (0.02% dose) treatment. Fibercare R with polymeric drainage aid Percol 47 did not show any notable results (Figure 5.2c, 5.3c).

Water retention value

Recycled pulp-NP

Effect of sequential enzyme and polymer treatment was also investigated on the WRV as well. As shown in Figure 5.4a, an increase in WRV was observed in all enzyme and polymer treated

pulps. The highest WRV was obtained using Fibercare D due to the hydrolysis of lower molecular weight carbohydrates and partial deterioration of crystalline structure of cellulose. The hydrolysis might have initiated the formation of more hydrogen bonds between cellulose and water molecules.

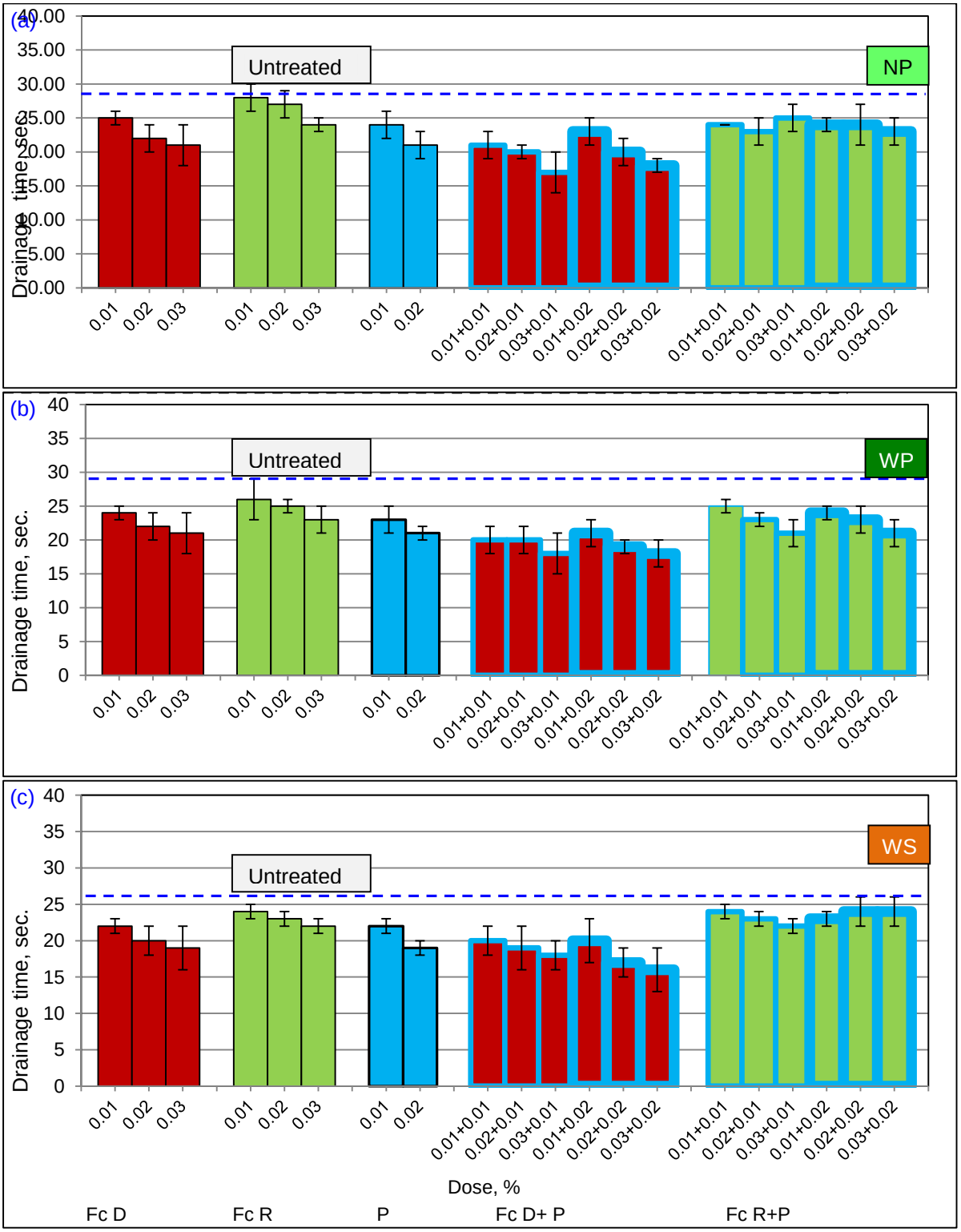


Figure 5.2 Effect of different enzymes and polymeric drainage aid at various doses on drainage time of (a) recycled pulp-NP (b) recycled pulp-WP (c) wheat straw pulp-WS

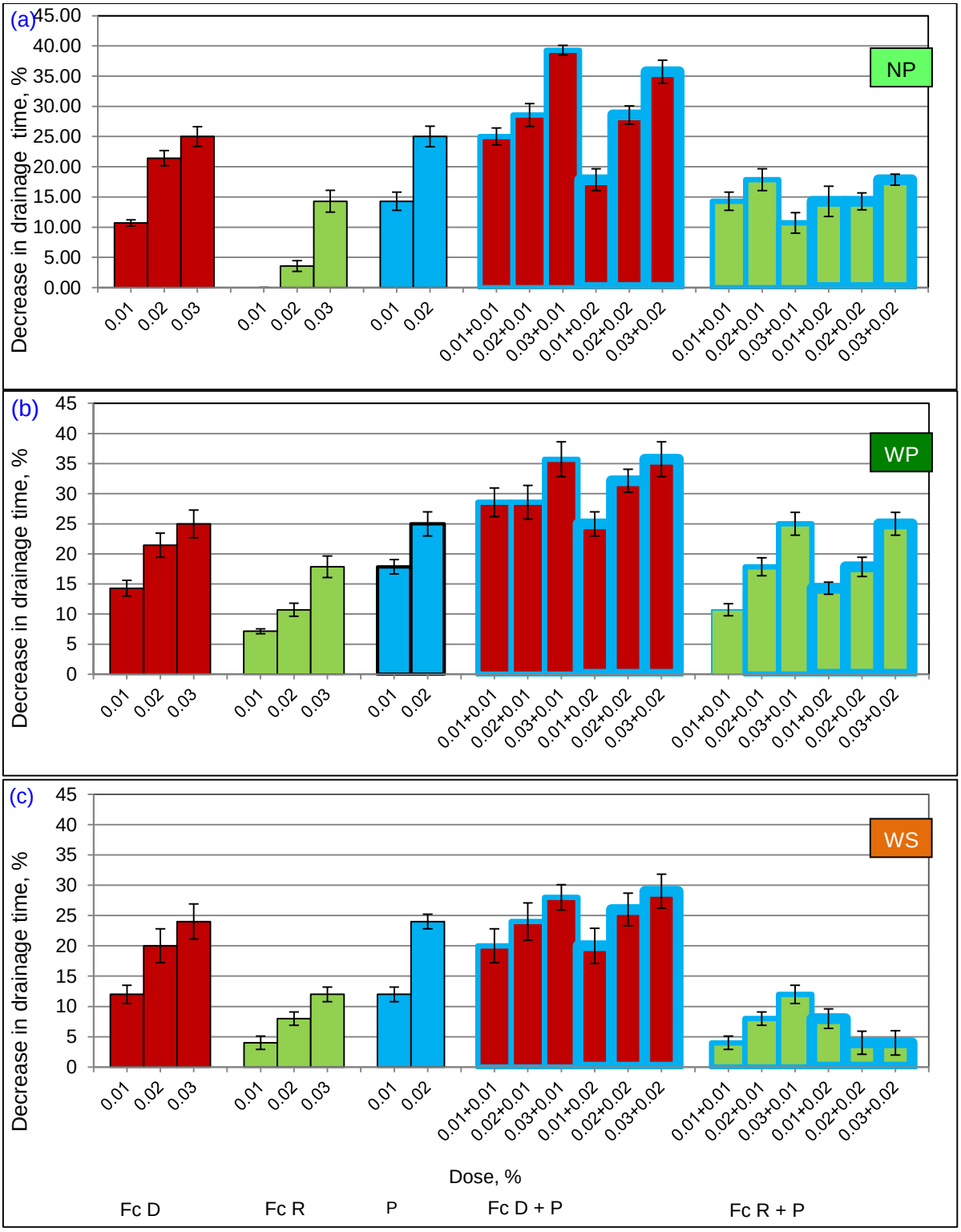


Figure 5.3 Effect of different enzymes and polymeric drainage aid at various doses on drainability of (a) recycled pulp-NP (b) recycled pulp-WP (c) wheat straw pulp-WS

When polymeric drainage aid Percol 47 was added after the Fibercare D (0.02%) treatment, the water retention value was again increased from 90 to 116 and 111 at 0.01 and 0.02% doses, respectively (Figure 5.4a). Slight increase in water retention value was observed in Fibercare R treated pulp with and without Percol 47 treatment. Percol 47 alone was also

observed to increase the WRV to some extent but not significant when compared to enzyme and polymer treatment.

Recycled pulp-WP

Similar trend of increased water retention value with enzyme treated pulps was also observed in recycled-WP recycled pulps. The results obtained showed that pulps treated with endoglucanase Fibercare D retain more water (higher WRV by 37%) compared to control pulp (Figure 5.4b). On the other hand, further addition of Percol 47 after Fibercare D treatment decreased the water retention value by 16%. No significant change in water retention value was observed in Fibercare R treated pulp with and without Percol 47 treatment. Percol 47 alone displayed a decrease in WRV.

Wheat straw pulp-WS

It was observed that wheat straw pulps treated with endoglucanase Fibercare D with and without Percol 47 treatment retain more water (higher WRV by 22 to 27%) compared to control pulp (Figure 5.4c). No significant change in WRV was observed in case of Percol 47 treated pulps. Fibercare R with Percol 47 was not found suitable to increase the water retention value.

Endoglucanase-rich product, Fibercare D even at low levels of treatment, was supposed to cleave fines and fibrils into shorter bits. These fines after enzymatic hydrolysis were less prone to block the water channels in wet web of paper and pulp drainage was improved in Fibercare D treated recycled (NP, WP) and wheat straw pulps with and without Percol 47 treatment. Our results were in agreement with *Sarkar et al. 1995,* whereas, they have reported significant improvements in paper machine runnability by applying enzymes plus a polymer. Several CATPAM are reported to serve as catalysts and to improve the efficiency of both amylase and cellulase *(Reye et al. 2009).*

Fines content

The fibre fines content analyzed through Bauer McNett fibre classification was observed to increase by 5.8% on treatment of recycled pulps-NP with Fibercare R whereas Fibercare D treated pulp showed decrease in fines content (16%). Sequential treatment of Fibercare D and Percol 47 also displayed the reduction in fines content by 18%. On the other hand, Percol 47 addition after Fibercare R treatment displayed an increase in fines content by 7.5%. In case of recycled pulps-WP, similar findings i.e. increase in fines content in pulps treated with Fibercare R (16%) and decrease in fines content in pulps treated with Fibercare D (23%) were observed. Reduction in fines content by 18.4% was observed in pulps after combined Percol 47 and Fibercare D treatments. The fines content in untreated recycled pulp-NP and recycled pulp-WP were 36.0 and 31.5%, respectively (Figure 5.5 a, b).

Similar findings i.e. decrease in fines content in pulps treated with Fibercare D (19%) and increase in fines content in pulps treated with Fibercare R (8%) were also observed in wheat straw pulps. Reduction in fines content after Percol 47 addition in Fibercare D treated pulps was also observed in wheat straw pulps. The fines content in untreated wheat straw pulps was 34.8%. Whereas, Percol 47 addition in Fibercare R treated pulps displayed an increase in fines content by 10% (Figure 5.5 c).

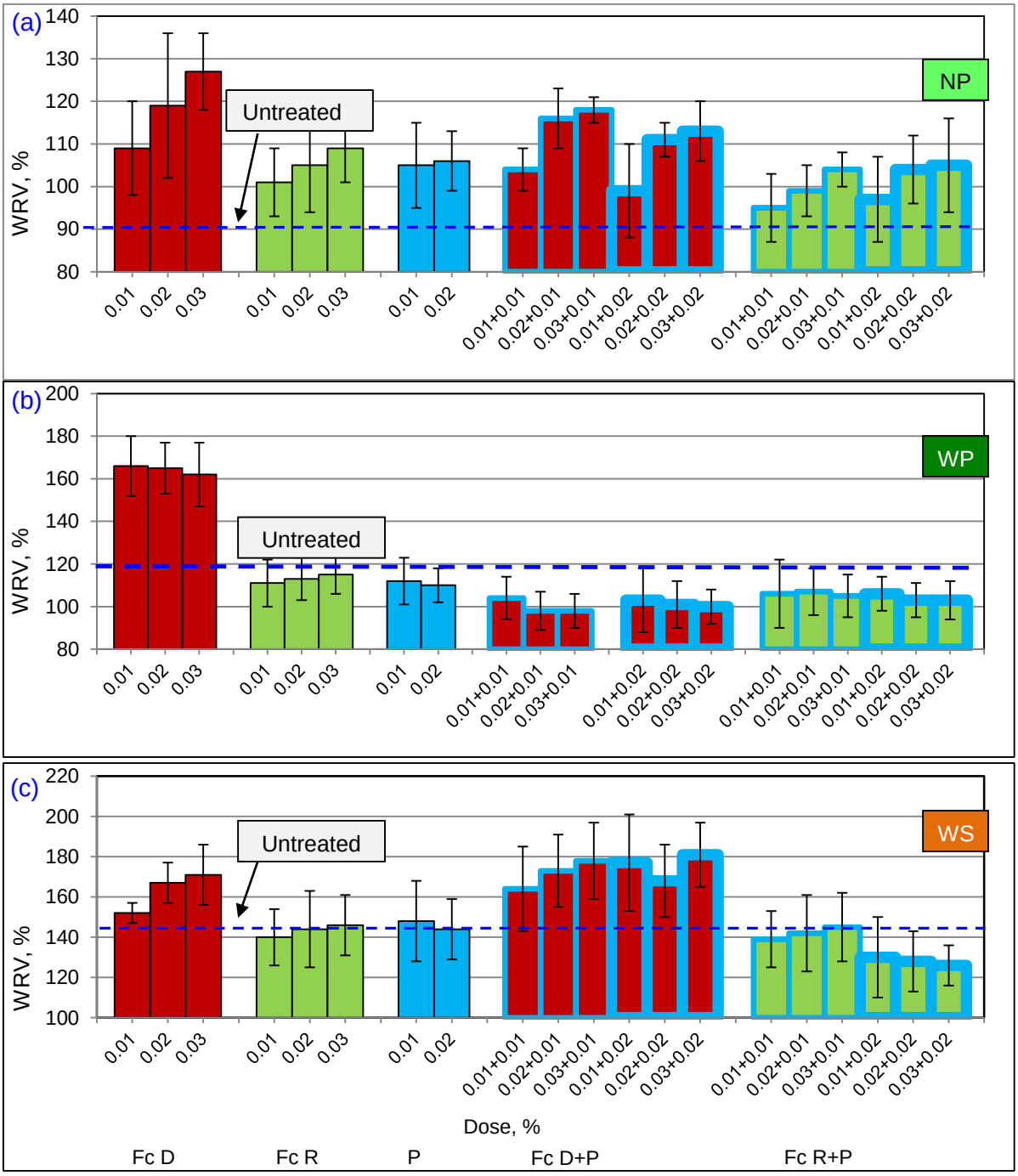


Figure 5.4 Effect of different enzymes and polymeric drainage aid at various doses on water retention value of (a) recycled pulp-NP (b) recycled pulp-WP (c) wheat straw pulp-WS

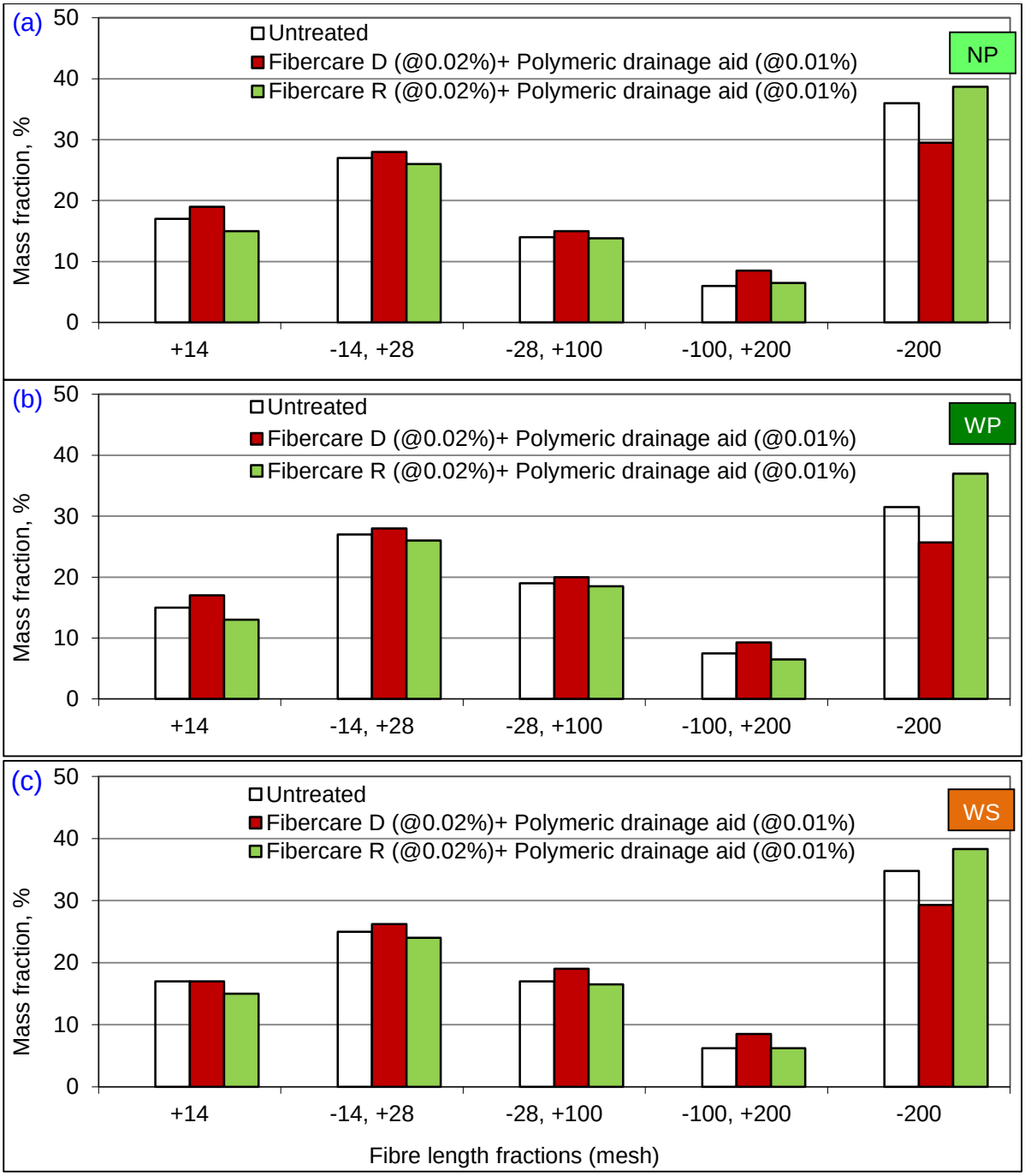


Figure 5.5 Effect of different enzymes and polymeric drainage aid at various doses on fines content of (a) recycled pulp-NP (b) recycled pulp-WP (c) wheat straw pulp-WS

Scanning electron microscopic studies

FE-SEM studies of recycled-NP, recycled-WP and wheat straw-WS pulps treated with both enzymes and polymeric drainage aid does not show any notable results (Figure 5.6a, b, c). Although, over flocculation and poor sheet formation was observed in paper sheets prepared after addition of polymeric drainage aid in untreated pulps.

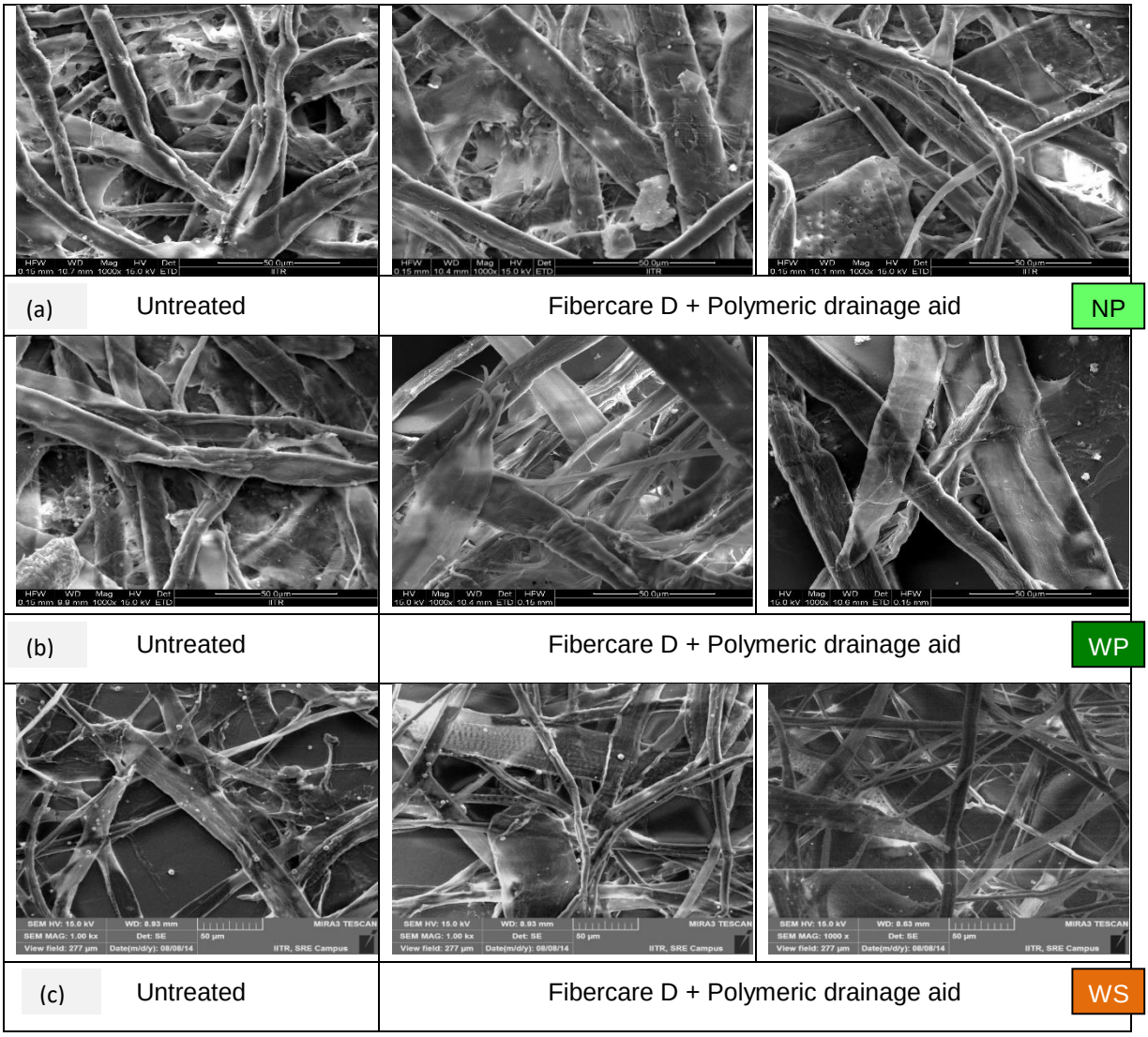


Figure 5.6 SEM micrographs (at 15kV and 1000X) of untreated and enzyme + polymeric drainage aid treated recycled pulp-NP, recycled pulp-WP and wheat straw pulp-WS

5.2 Effect of different enzymes and polymeric drainage aid on paper properties

Tensile index

Recycled pulp-NP

As shown in Figure 5.7a, the tensile index of paper sheets was increased on increasing the dose of Fibercare D. The tensile index of the untreated pulp sheets was 15.4 N.m/g which was increased to 17.4 N.m/g, showing about 13% increase in tensile index, on treatment using Fibercare D. It was due to the increase in longer fibre fraction, fibrillation and inter-fibre bonding. Addition of Percol 47 alone did not enhibit any change in tensile index. When polymeric drainage aid Percol 47 at 0.01 and 0.02% dose was added after the Fibercare D (0.02%) treatment, the tensile index was observed to be 17.5 and 17.4, respectively. No significant change in tensile strength was observed in Fibercare R treated pulps, with and without addition of Percol 47.

Recycled pulp-WP

There was a common trend of increase in tensile index in sheets prepared from enzyme treated pulps. But significant increase in tensile index of about 19% was observed in pulps treated with Fibercare D (Figure 5.7b). Similar results showing no change in tensile index were observed in pulps after addition of Percol 47 alone. Addition of Percol 47 at 0.01 and 0.02% dose after Fibercare D (0.020% dose) treatment was observed to increase the tensile index by 16 and18%, respectively. The increase in tensile index in Fibercare R and Percol 47 treated pulps were less significant as compared to Fibercare D and Percol 47 treated pulps (Figure 5.7b).

Wheat straw pulp-WS

As shown in Figures 5.7c, increase in tensile index from 40.6 to 45.0 N.m/g in pulps treated with endoglucanase Fibercare D was observed. The tensile index of the untreated wheat straw pulp sheets was 40.6 N.m/g and tensile index was increased by 11%. Further addition of Percol 47 at 0.01% dose after Fibercare D (0.020% optimum dose) treatment increased the tensile index by 15%. On the other hand, reduction in tensile index was observed in Fibercare R treated pulp with and without Percol 47 addition (Figure 5.7c).

Tear index

Recycled pulp-NP

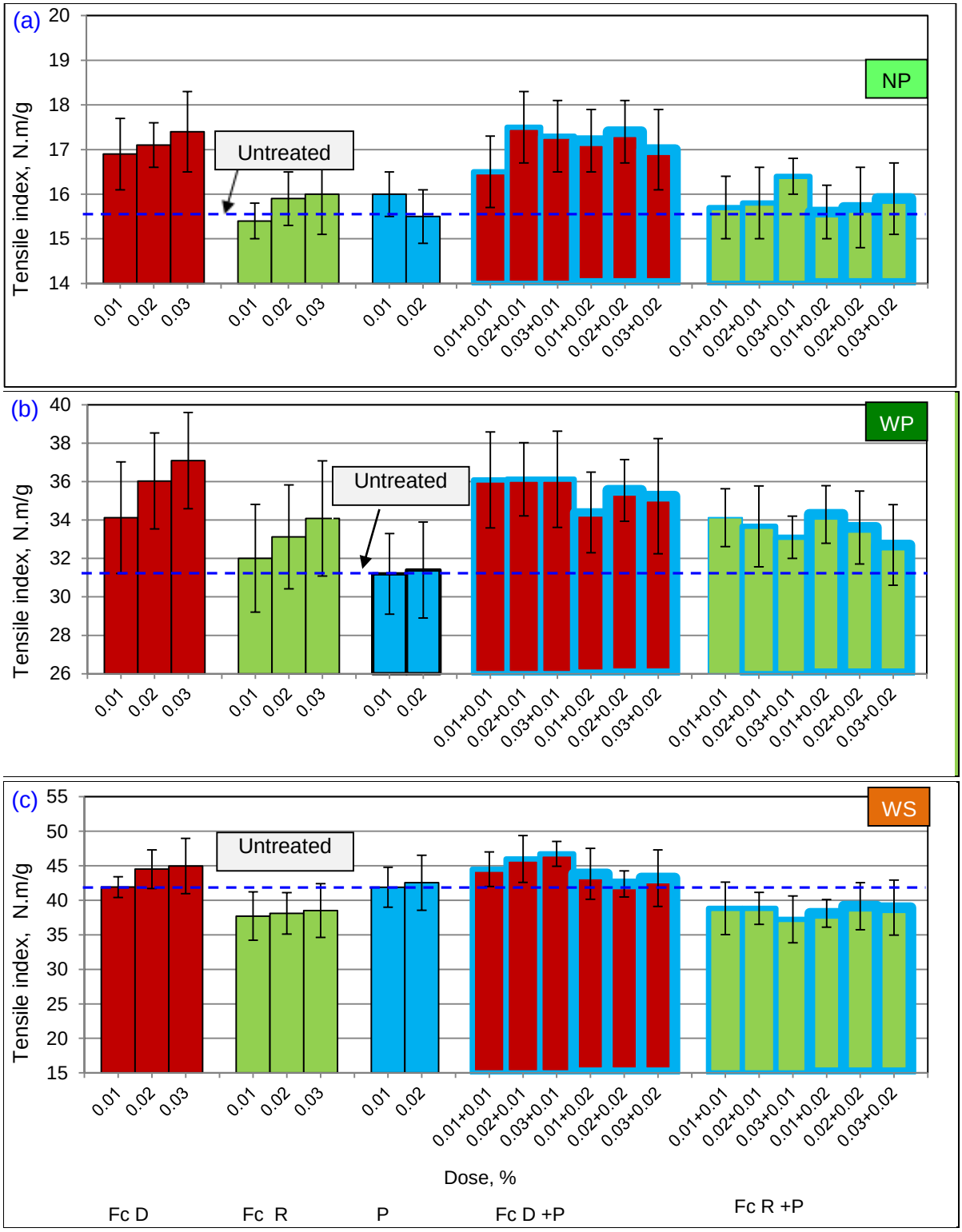
Both enzyme products Fibercare R and Fibercare D decreased the tear index of the paper sheet with increase in dose. The drop in tear index using the highest dose of Fibercare R and Fibercare D was 4 and 8%, respectively. Further addition of Percol 47 in pulps treated with Fibercare R and Fibercare D also exhibited the similar trend of decrease in tear index. But the decrease in tear index (4% drop) was not significant upto 0.02% dose of Fibercare D (Figure 5.8a).

Recycled pulp-WP

No significant change in tear index was observed in pulps treated with Fibercare D with and without Percol 47 addition (Figure 5.8b). Whereas, Fibercare R displayed reduction in tear index at increasing dose levels. Addition of Percol 47 to Fibercare R treated pulps displayed significant reduction in tear index as the dose was increased.

Wheat straw pulp-WS

Both the enzyme products with and without Percol 47 decreased the tear index of the paper sheet. The drop in tear index in pulps treated with Fibercare D with and without Percol 47 was between 6-9%. On the other hand, Fibercare R displayed more reduction in tear index with and without Percol 47 addition (Figure 5.8c).



ZFigure 5.7 Effect of different enzymes and polymeric drainage aid at various doses on tensile index of (a) recycled pulp-NP (b) recycled pulp-WP (c) wheat straw pulp-WS

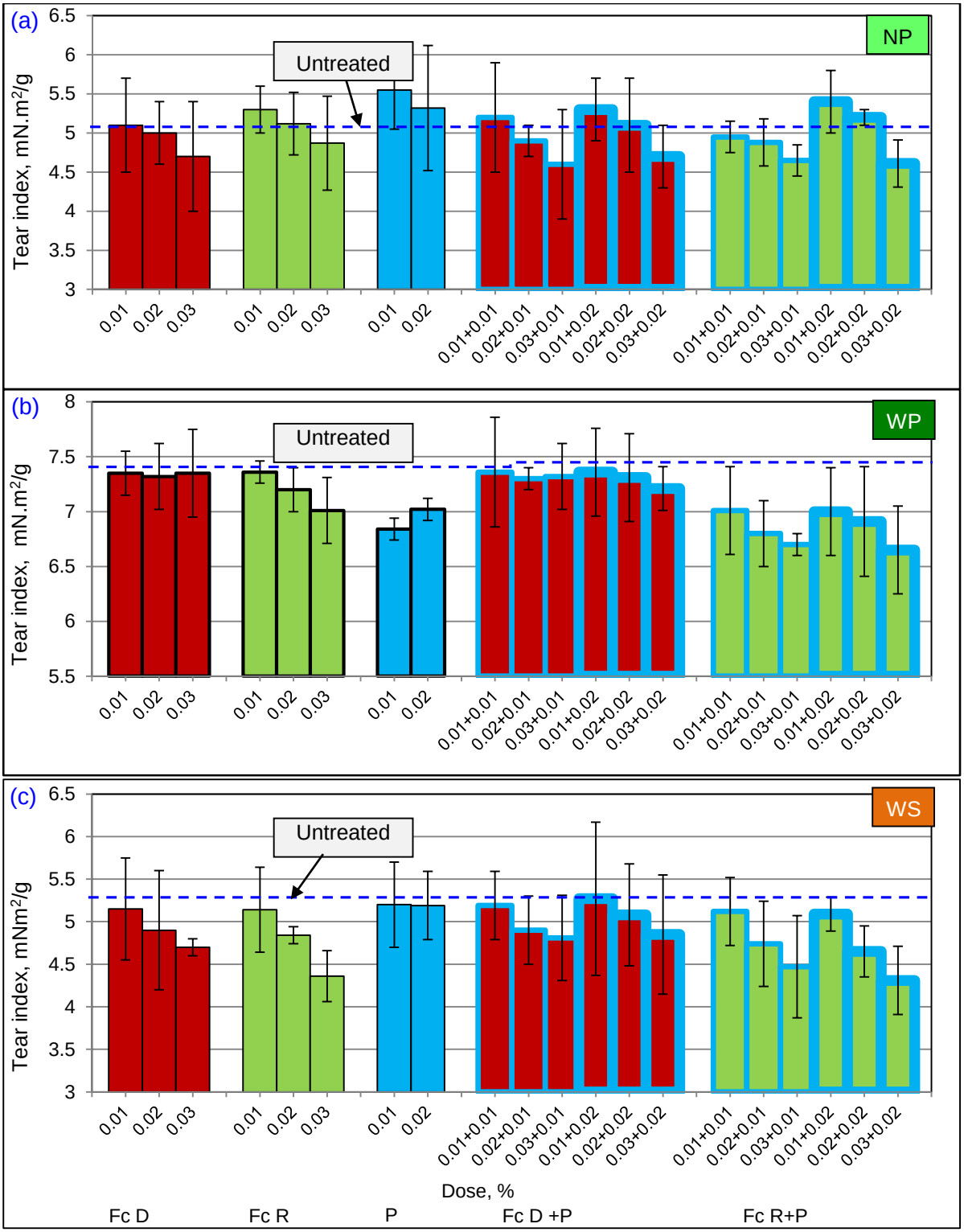


Figure 5.8 Effect of different enzymes and polymeric drainage aid at various doses on tear index of (a) recycled pulp-NP (b) recycled pulp-WP (c) wheat straw pulp-WS

Formation index

The effect of enzyme and polymer treatment on sheet formation was also analyzed. Different recycled (NP, WP) and wheat straw pulps exhibited similar trends.

Recycled pulp-NP

There was a significant improvement in formation of handsheets prepared from recycled (NP) pulp treated with Fibercare D. The formation index was increased from 133 to 195. On the other hand, formation was severely affected in case of handsheets prepared after the addition of polymeric drainage aid Percol 47. The formation index got reduced to 118 and 91 at 0.01 and 0.02% Percol 47 dose, respectively. When both Fibercare D and Percol 47 were used for further improvement in drainage, sheet formation was not much affected even at a higher dose (0.02%) of Percol 47(Figure 5.9a).

Recycled pulp-WP

Similar trend of improvement in formation index in handsheets prepared from Fibercare D treated pulps were observed in recycled pulps-WP. The sheets prepared from pulps after addition of polymeric drainage aid, Percol 47 alone exhibited poor formation and appearance. After the Percol addition at 0.01 and 0.02% dose in Fibercare D treated pulps, the prepared handsheets displayed 6% and 28% reduction in formation index values, respectively (Figure 5.9b).

Wheat straw pulp-WS

Both enzymes Fibercare D as well as Fibercare R displayed an increase of 37% and 25% in formation index values, respectively. Significant drop in formation index values was observed with addition of only polymeric drainage aid. When polymeric drainage aid, Percol 47 was added in endoglucanase Fibercare D and Fibercare R treated pulps, formation values were in line with the untreated pulps (Figure 5.9c).

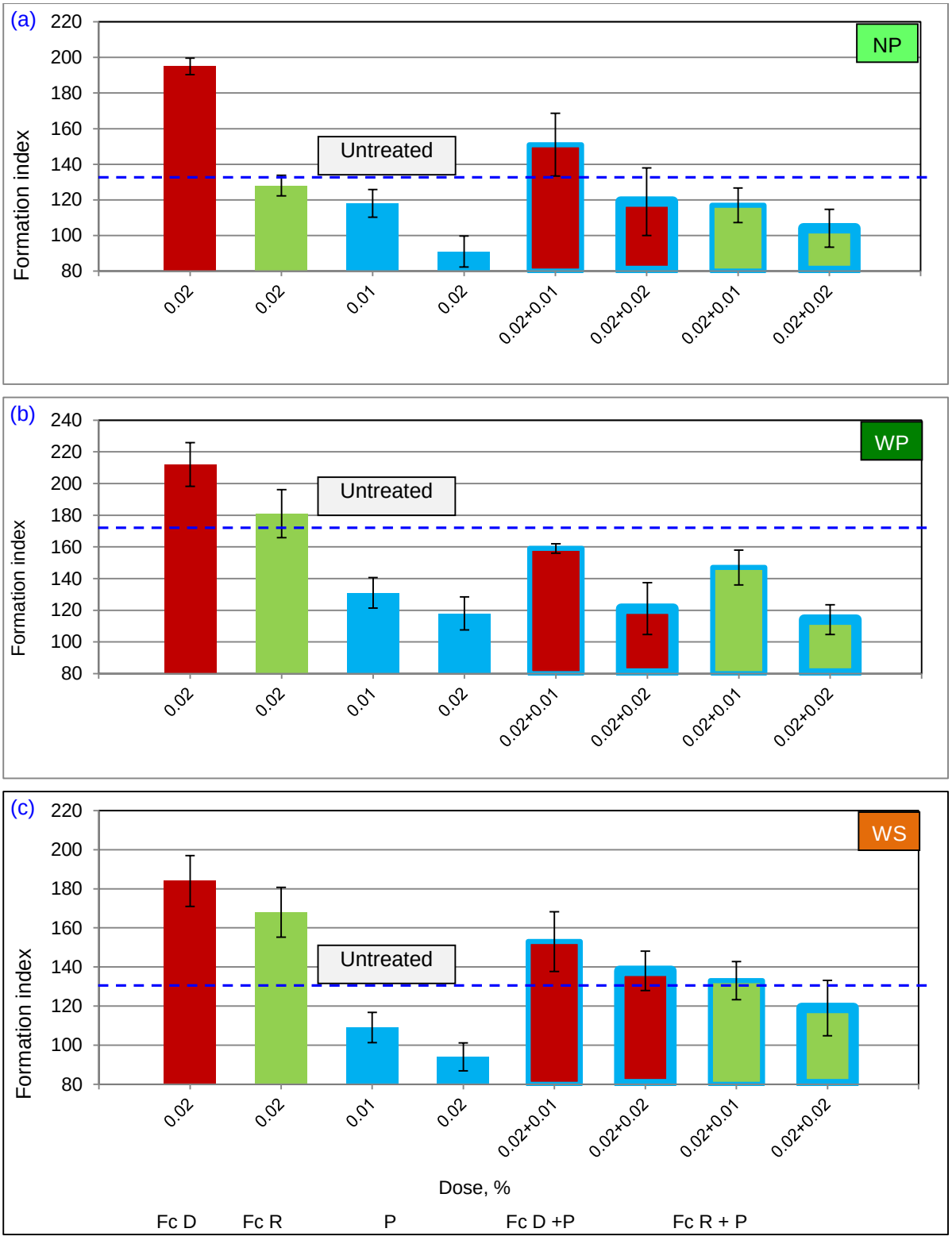


Figure 5.9 Effect of different enzymes and polymeric drainage aid at various doses on formation index of (a) recycled pulp-NP (b) recycled pulp-WP (c) wheat straw pulp-WS

5.3 Salient findings

- Polymer treatment alone at various doses was found to increase the drainage of different pulps in the range of 12 to 25%, but formation was adversely affected due to over flocculation.
- Significant improvement in pulp dewatering by 25 to 39% was observed after polymer addition at 0.01% dose in endoglucanase rich, Fibercare D (various doses) treated recycled pulp-NP.
- In Fibercare D (various doses) treated recycled pulp-WP, pulp drainage was improved by 29 to 36% after polymer addition at 0.01% dose.
- In wheat straw pulp-WS, polymer addition at 0.01% dose after Fibercare D treatments at various doses further improved the pulp drainage by 20 to 28%.
- Improvement in pulp drainage exhibited by polymer addition at 0.02% dose in Fibercare D treated pulps was less compared to polymer addition at 0.01% dose.
- Endoglucanase rich Fibercare D enzyme, in combination with polymeric drainage aid at optimized dose levels, was observed to further enhance the drainage of recycled and wheat straw pulps by 25-28%, without any negative effect on sheet formation and other strength properties.

CHAPTER 6

Lab scale production of endoglucanase enzymes and their application for drainage improvement of pulps

This chapter describes the isolation and screening of two cellulase producing fungus i.e. *Pycnoporus sanguineus* PVYA07 NFCCI-3628 and *Alternaria gaisen* (PVYA11 NFCCI-3629). It also covers the lab scale production and purification of isolated endoglucanase enzymes and subsequent evaluation of for improvement in pulp drainage.

6.1 Lab scale production of endoglucanase enzyme

6.1.1 Isolation and screening of fungi for the production of cellulase enzymes

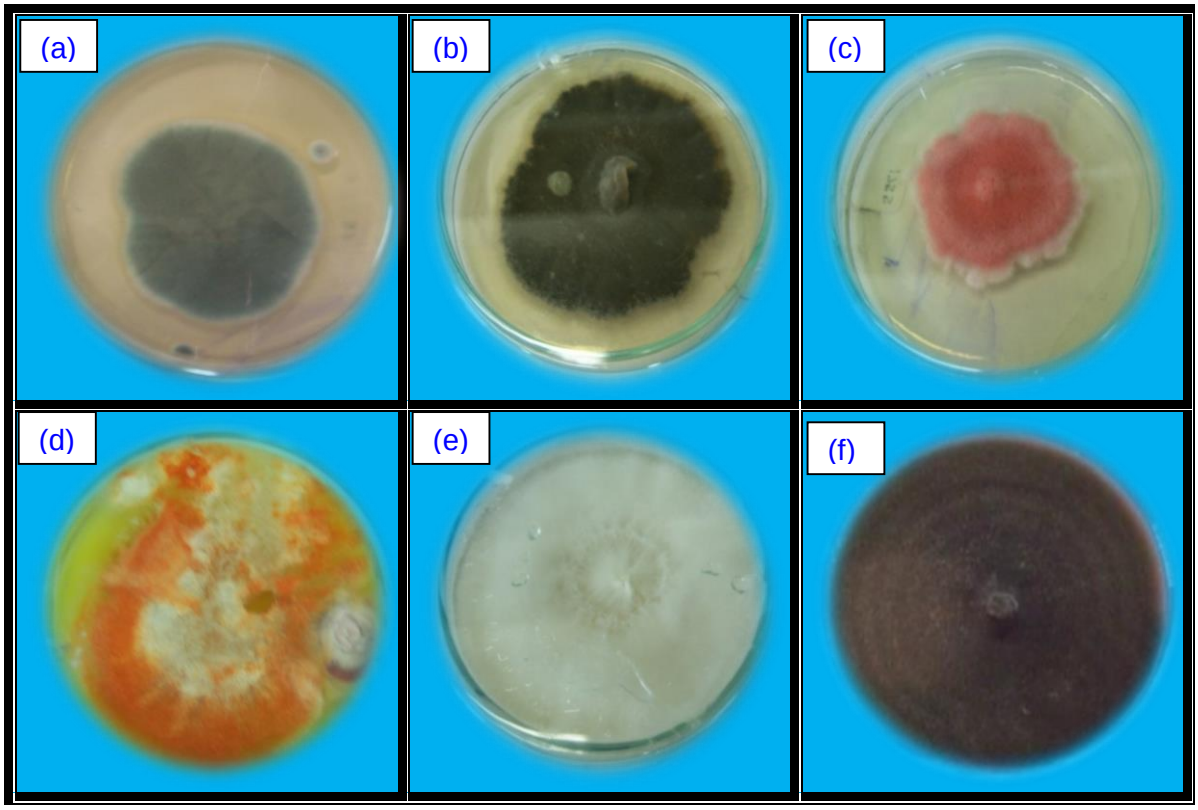
Out of the 96 samples (62 decaying wood and 34 humus) collected from Yamuna Nagar, Meerut and Saharanpur, 54 different (appearance basis) fungal strains (35 from wood and 19 from humus) were isolated (Table 6.1). Eighteen strains, which showed a clearing zone around the colonies, were screened as potential alkaline cellulase producing fungi (Figure 6.1 and Table 6.1). The said 18 fungal strains denoted as PVYA03, PVMT06, PVMT08, PVYA07, PVYA09, PVYA11, PVMT10, PVSR04, PVYA13, PVSR05, PVMT12, PVMT13, PVYA17, PVMT14, PVSR07, PVYA20, PVYA21, PVYA23 (Figure 6.1) and confirmed to be cellulase producing by primary screening (congo red) (Figure 6.2 and Table 6.1) were used in the further studies. The said strains were grown on M-1 medium *(Reese and Mandels 1963)* supplemented with malt extract as a carbon source (Table 3.1) and analyzed for their cellulase (CMCase) producing ability. The enzymes from the fermented matter were harvested on 3 to 8th day after achieving full growth (depends on growth rate of isolates) and the cellulase activities of enzyme samples from each isolate were determined (Table 6.1). The aim of screening was to select isolates exhibiting the highest CMCase activity. Two fungal isolates PVYA 07 and PVYA 11 exhibited highest endoglucanase activity of 4.6±0.9 and 5.12±0.5 IU/mL, respectively. Cellulose hydrolyzing bacteria isolated from rhinoceros dung have been reported to be tested for clear zone formation around the colonies on the agar plates containing the medium amended with carboxy methyl cellulose as a sole carbon source *(Singh et al. 2013).*

The two selected fungal isolates, PVYA 07 AND PVYA 11 selected by primary (congo red) and secondary screening (CMCase activities) were sent to National Fungal Culture Collection of India (NFCCI), Pune, India, for further identification. The cultures were identified as *Trametes sanguinea* and *Alternaria gaisen* based on sequencing of the genomic DNA, and were deposited at NFCCI, Pune, India with accession numbers PVYA07 NFCCI-3628 and PVYA07 NFCCI-3629, respectively. Currently *Trametes sanguinea* is known as *Pycnoporus sanguineus,* also mentioned in the identification report *(Annexure 1).* The detailed report

containing the details of fungi identified, accession numbers, results of molecular identification, top five hits upon BLAST analysis is given in *Annexure 1*. After the molecular identification, the images of the two fungal strains were found to be similar in appearance, to the respective images of the two strains available on internet (Figure 6.3, 6.4).

Pynoporus sanguineus (L.) Murrill is a basidiomycete and an efficient producer of polyphenol oxidase that acts on variety of aromatic hydrogen donors. It has been reported for fermentation of agro-industrial waste, decolourization of Kraft effluent, and in various dyes *(Valeriano et al. 2007).* Despite the limited references on this fungus, there is a growing trend to employ it in biotechnological processes.

Alternaria species has long been reported for their cellulolytic potential *(Logan and Siehr 1966).* It has also been proved that some of the species of *Alternaria* induce plant invasion by elaborating the cellulolytic enzymes and genes encoding endoglucanases from this organism have been characterized *(Eshel et al. 2002).* The hydrolytic potential of an indigenous strain MS28 of *Alternaria* has been reported by *Sohail et al. (2009). Alternaria gaisen Nagano* belongs to Ascomycetes (anamorph). Japanese pear (*Pyrus pyrifolia*) is considered to be the main host of *A. gaisen*. *A. gaisen* has been reported in very few countries, while *A. alternata* is extremely widespread in most parts of the world. *A. gaisen* is able to survive in adverse conditions due to resting bodies (microsclerotia) or resting spores (chlamydospores) in the soil. Under favourable conditions (warm and moist), conidial masses are produced on leaf debris, from which they are disseminated by wind and rain *(Alternaria gaisen)*. According to *Nishimura et al. (1978)*, *A. gaisen* is a *forma specialis* of *A. alternata*.



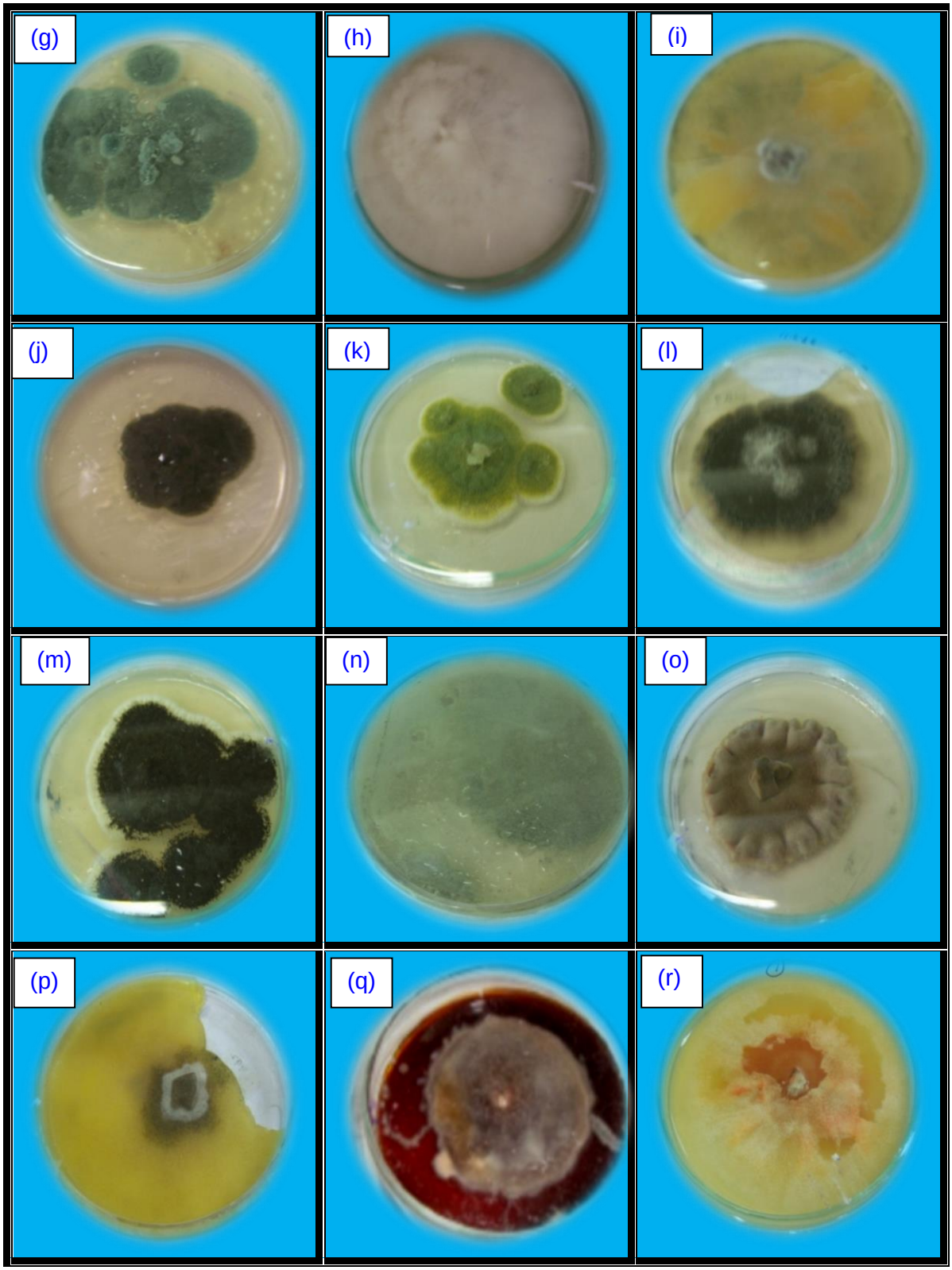
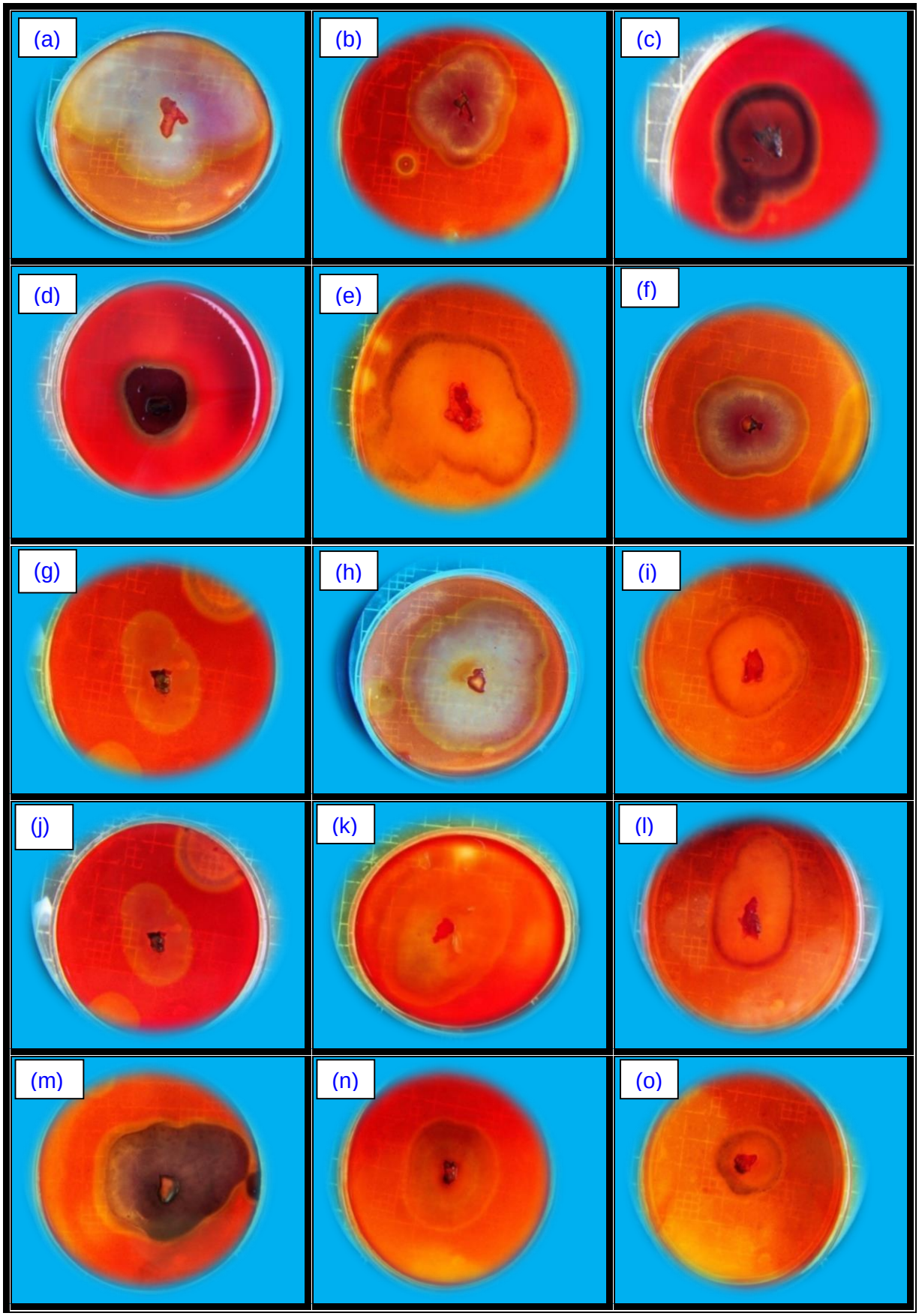


Fig. 6.1 Growth of different fungal strains (a) PVYA03 (b) PVMT06 (c) PVMT08 (d) PVYA07 (e) PVYA09 (f) PVYA011 (g) PVMT10 (h) PVSR04 (i) PVYA13 (j) PVSR05 (k) PVMT12 (l) PVMT13 (m) PVYA17 (n) PVMT14 (o) PVSR07 (p) PVYA20 (q) PVYA21 (r) PVYA23


(a)
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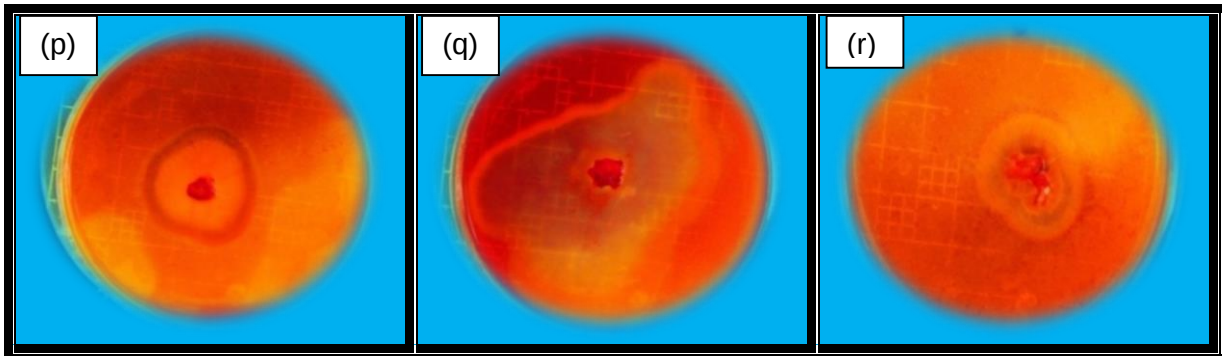

Fig. 6.2 Congo red screening of different fungal strains for cellulase enzyme production (a) PVYA03 (b) PVMT06 (c) PVMT08 (d) PVYA07 (e) PVYA09 (f) PVYA011 (g) PVMT10 (h) PVSR04 (i) PVYA13 (j) PVSR05 (k) PVMT12 (l) PVMT13 (m) PVYA17 (n) PVMT14 (o) PVSR07 (p) PVYA20 (q) PVYA21 (r) PVYA23

Table 6.1 Primary and secondary screening of cellulase producers isolated from different sources

	S. No	Code No.	Location	CMCase (IU/mL)		S. No.	Code No.	Location	CMCase (IU/mL)
Wood sample	1	PVMT01	Meerut	-	Wood sample	28	PVMT11	Meerut	-
	2	PVMT02	Meerut	-		29	PVSR05	Saharanpur	+[1.71]
	3	PVYA01	Yamuna Nagar	-		30	PVYA14	Yamuna Nagar	-
	4	PVYA02	Yamuna Nagar	-		31	PVMT12	Meerut	+[2.43]
	5	PVMT03	Meerut	-		32	PVSR06	Saharanpur	-
	6	PVMT04	Meerut	-		33	PVYA15	Yamuna Nagar	-
	7	PVYA03	Yamuna Nagar	+[2.45]		34	PVMT13	Meerut	+[1.97]
Humus sample	8	PVSR01	Saharanpur	-		35	PVYA16	Yamuna Nagar	-
	9	PVSR02	Saharanpur	-		36	PVYA17	Yamuna Nagar	+[0.98]
	10	PVMT05	Meerut	-	Humus sample	37	PVMT14	Meerut	+[0.83]
	11	PVMT06	Meerut	+[2.94]		38	PVYA18	Yamuna Nagar	-
	12	PVMT07	Meerut	-		39	PVSR07	Saharanpur	+[1.17]
	13	PVYA04	Yamuna Nagar	-		40	PVYA19	Yamuna Nagar	-
	14	PVMT08	Meerut	+[1.34]		41	PVMT15	Meerut	-
	15	PVYA05	Yamuna Nagar	-		42	PVSR08	Saharanpur	-
Wood sample	16	PVYA06	Yamuna Nagar	-	Wood sample	43	PVMT16	Meerut	-
	17	**PVYA07**	Yamuna Nagar	**+[4.60]**		44	PVYA20	Yamuna Nagar	+[1.74]
	18	PVYA08	Yamuna Nagar	-		45	PVMT17	Meerut	-
	19	PVYA09	Yamuna Nagar	+[2.31]		46	PVMT18	Meerut	-
	20	PVMT09	Meerut	-		47	PVYA21	Yamuna Nagar	+[1.63]
	21	PVYA10	Yamuna Nagar	-		48	PVSR09	Saharanpur	-
	22	**PVYA11**	Yamuna Nagar	**+[5.12]**		49	PVYA22	Yamuna Nagar	-
	23	PVSR03	Saharanpur	-	Humus sample	50	PVSR10	Saharanpur	-
	24	PVMT10	Meerut	+[1.71]		51	PVSR11	Saharanpur	-
	25	PVYA12	Yamuna Nagar	-		52	PVYA23	Yamuna Nagar	+[2.28]
	26	PVSR04	Saharanpur	+[2.30]		53	PVYA24	Yamuna Nagar	-
	27	PVYA13	Yamuna Nagar	+[1.92]		54	PVYA25	Yamuna Nagar	-

+ sign showed 'producer strains' and – sign showed 'non-producer strains',
The values in paranthesis show the CMCase activity

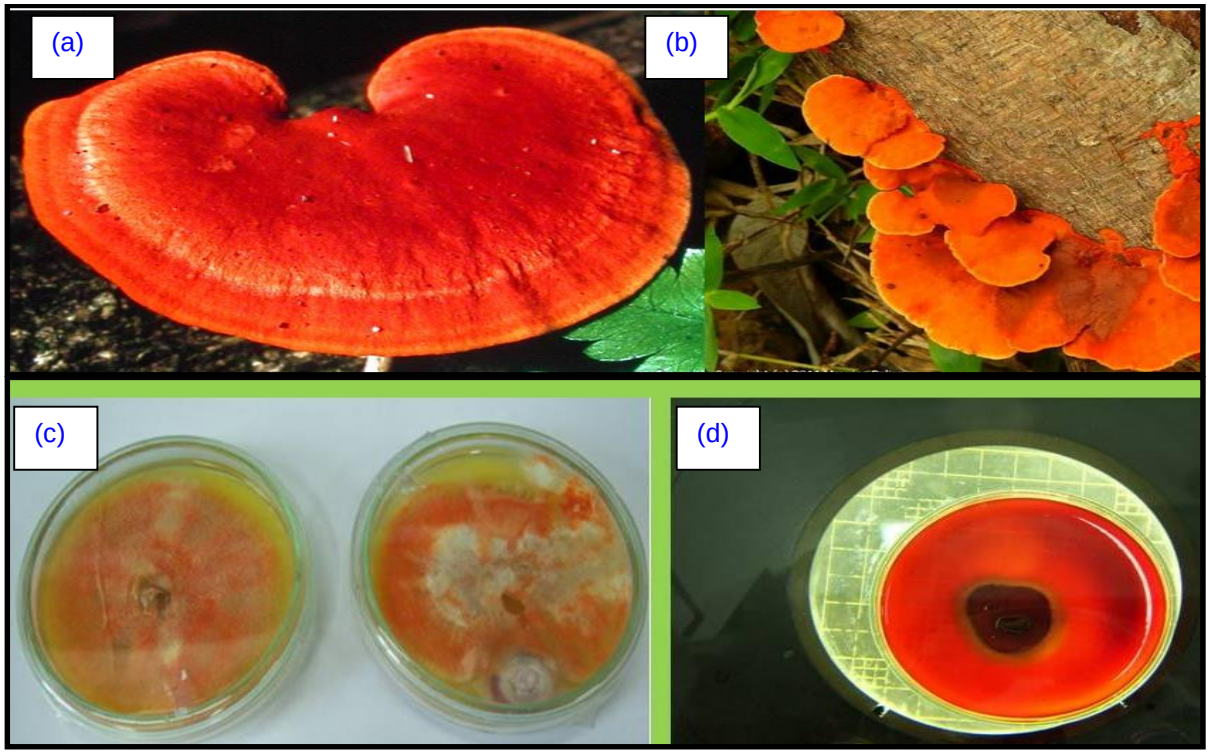

Fig. 6.3 Images of Pycnoporus sanguineus
(a) http://www.diark.org/diark/species_list?species_id=5149
(b) (http://www.mycodb.fr/fiche.php?genre=Pycnoporus&espece=sanguineus)
(c) Pycnoporus sanguineus (research work)
(d) Zone of clearance produced by Pycnoporus sanguineus (research work)

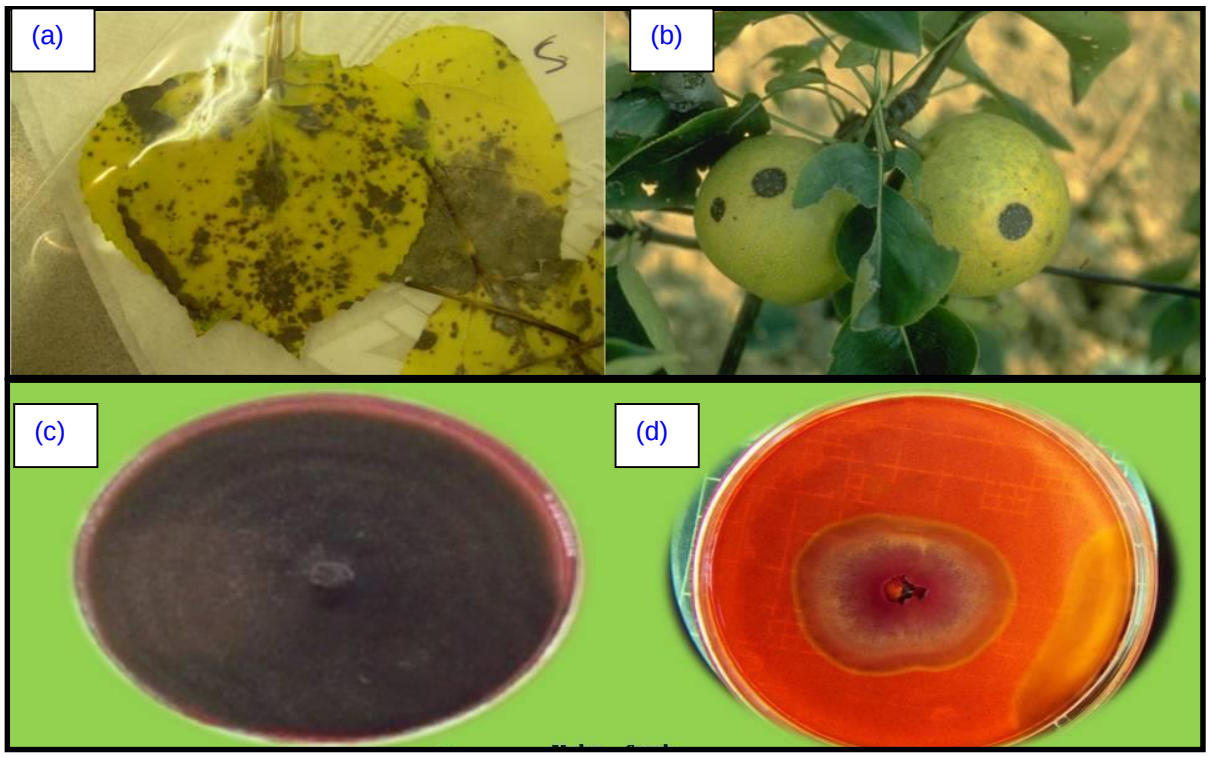

Fig. 6.4 Images of Alternaria gaisen
(a) https://it.wikipedia.org/wiki/Alternaria
(b) https://gd.eppo.int/taxon/ALTEKI/photos
(c) Alternaria gaisen (research work)
(d) Zone of clearance produced by Alternaria gaisen (research work)

6.1.2 Effect of different culture conditions on enzyme production

Fungal growth is dependent on several physical and chemical parameters such as cellulosic substrate, inoculum size, incubation period, pH, temperature, nitrogen source, surfactant, substrate induction and specificity. Effect of different culture conditions on the growth and enzyme production by two selected fungal strains was analyzed separately as mentioned in the Material and Methods section.

Effect of cellulosic substrate

P. sanguineus PVYA07 NFCCI-3628

The highest amount of cellulase activities were induced on refined cellulosic substrate such as Avicel PH 101 (7.8±1.1 IU/mL) and carboxy methyl cellulose (7.0±0.9 IU/mL) in *P. sanguineus* PVYA07 NFCCI-3628. The enzymes were also produced on agricultural residues like bagasse pith and rice bran. However, enzyme yields obtained were quite moderate when compared to refined cellulosic substrates (Figure 6.5a). Similar trend of increasing CBH enzyme activities in medium supplemented with Avicel (1.8±0.2 IU/mL) and CMC (1.5±0.3 IU/mL) was observed.

A. gaisen PVYA11 NFCCI-3629

Similar results showing increased enzyme activities by supplementation of efined cellulosic substrates in the growth medium as carbon source was observed in case of *A. gaisen*. Highest CMCase activity of 8.1±0.8 and 6.5±1.0 IU/mL was achieved in the growth medium supplemented with CMC and Avicel, respectively. Although other cellulosic substrates, baggase pith (BP) and rice bran also proved to be good and cheaper source for endoglucanase production, but the activities were comparatively on lower side (4.6±0.4 and 5.4±0.3 IU/mL, respectively) (Figure 6.5b). Rice bran was chosen as a cellulosic substrate and various other culture conditions like different inoculum size, incubation period, pH, temperature, nitrogen source and surfactant were further optimized to achieve maximum endoglucanase enzyme production.

In most organisms, cellulase production is reported to be induced by low molecular weight compounds such as cellobiose and CMC and repressed in presence of readily metabolisable carbon sources such as glucose *(Lynd et al., 2002).* Other researchers have also reported that refined cellulosic substrates such as Solka floc *(Hayward et al. 2000),* Avicel *(Aiello et al. 1996)* are better substrates for cellulase production than agricultural residues. This may be attributed to higher lignin content in agricultural residues affecting the cellulase production. *(Aiello et al. 1996, Bigelow and Wyman 2002). Deka et al. (2011) and Akhtar et al. (2015)* also reported that CMC (1.8%), peptone (0.8%), and yeast extract (0.48%) affect the enzyme production in a positive fashion. Cellulase synthesis in *Alternaria* species was reported to be increased by ~10 folds in the presence of cellulose and repressed in the presence of glucose

(*Jahangeer et al. 2005*). CMC as an efficient carbon source for the growth and enzyme production by *Alternaria sp*. MS28 was also reported by *Sohail et al. (2011).*

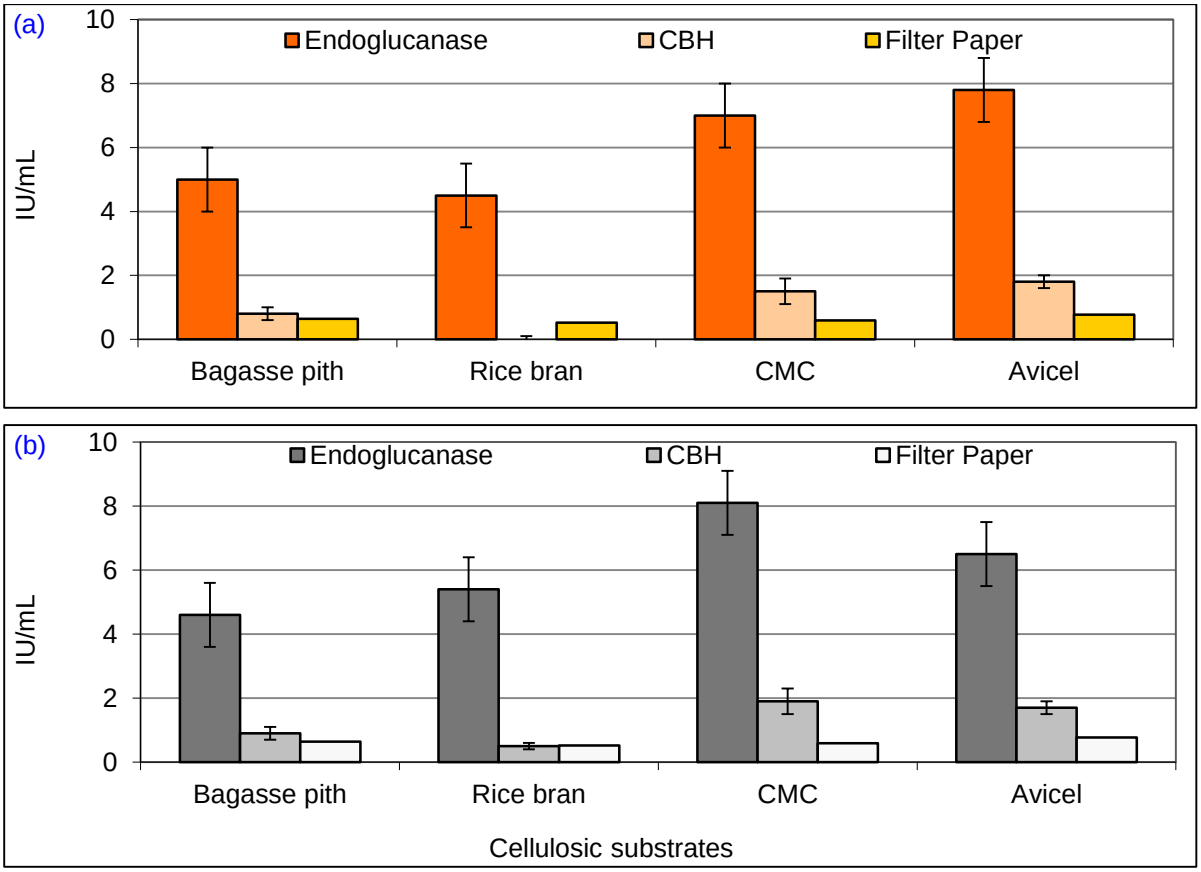

Fig.6.5 Effect of different cellulosic substrates on enzyme production (a) P. sanguineus (b) A. gaisen

Similar findings demonstrating more endoglucanase production with CMC supplementation as carbon source were also reported by *Vyas and Lachke (2003)*. Significant production of the cellulase enzyme by white rot fungi, *Phlebia gigantea* in the presence of CMC, Avicel and celobiose was reported by *Niranjane (2006).* Wheat bran, containing good amount of cellulose, hemicellulose and nitrogen source was also reported to be an efficient nutrient source for the growth of fungi as well as enzyme production *(Babu and Satyanarayana 1996).*

Effect of initial pH

The production of hydrolytic enzymes is greatly affected by the hydrogen ion concentration, pH of the medium. The optimum pH for fungal cellulases and crude protein production varies from species to species, though in most cases, the optimum pH ranges from pH 3.0 to 7.0 *(Garg and Neelakantan 1981).*

P. sanguineus PVYA07 NFCCI-3628

In the present research work, *P. sanguineus* PVYA07 NFCCI-3628 exhibited the highest CMCase and CBH activity i.e. 7.1±0.5 and 2.3±0.2 IU/mL, respectively at pH 7.0. Although, optimum growth was observed around pH 7.0, the selected fungal cultures produced high

amounts of enzyme at pH 8.0 (80% relative activity) and 9.0 (60% relative activity) confirming the alkalotolerant nature of the fungal isolate (Figure 6.6a).

A. gaisen PVYA11 NFCCI-3629

However, optimum pH for enzyme production was 7.0; the difference in enzyme activity at pH 6.0 was not significant. The endoglucanase enzyme from *A. gaisen* retained around 65% activity after incubation at pH 8.0 while 55% residual activity was detected at pH 9.0. In the present studies, the optimum pH for CBH production for *A. gaisen* PVYA11 NFCCI-3629 was found to be between 6.0 to 7.0 (Figure 6.6b).

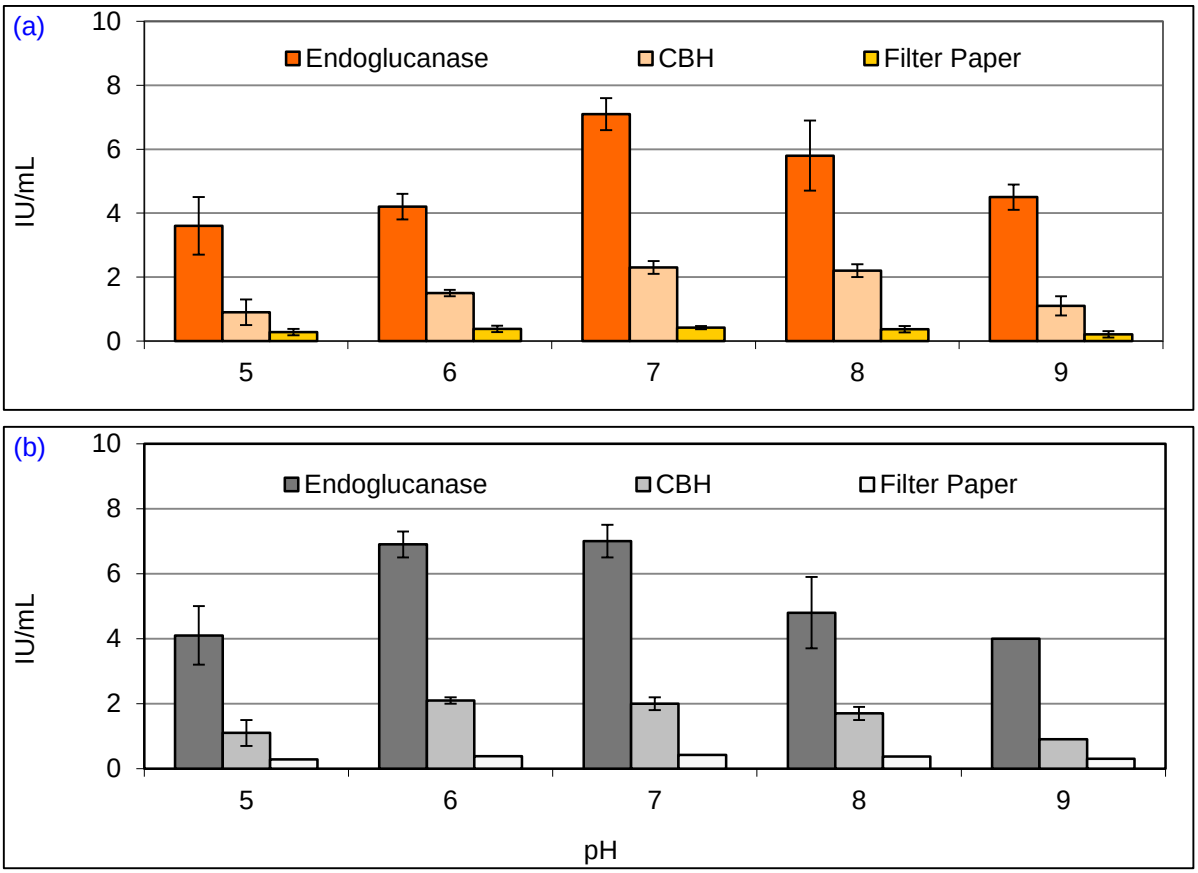

Fig.6.6 Effect of initial pH on enzyme production (a) P. sanguineus (b) A. gaisen

Our results showing pH 7.0 and 6.0 as the optimum pH for enzyme production by P. sanguineus and *A. gaisen*, respectively were in line with the literature. *P. sanguineus* was reported to optimally produce endoglucanase enzyme at pH 6.0 by *Quiroz-Castaneda et al. (2009),* but retained 80% of activity at pH 7.0, and showed more than 60% residual activity at pH 8.0. Maximum endoglucanase enzyme activities by *A. alternata, A. citri* and *Cochliobolus spicifer* were reported at pH 6.0 (*El-Said et al. 2014*). *Amir et al. (2011)* found that pH 6.0 was the optimum for cellulase production by *A. alternata* by solid state fermentation. However, in some research papers, optimum pH for endoglucanase production by *P. sanguineus* was observed to be 5.5 by *Alam et al. (2008)* and *Onofre et al. (2015).*

Effect of nitrogen source

P. sanguineus PVYA07 NFCCI-3628

Experiments to assess the effect of various nitrogen sources (at 2.0 g/L dose) on enzyme production demonstrated that there was a substantial increase in the enzyme activity when the medium is supplemented with complex nitrogen sources like yeast extract and urea (Figure 6.7a). Yeast extract was found to be the most suitable inorganic nitrogen source for both CMCase and CBH enzyme production amongst other nitrogen sources (Figure 6.7a).

A. gaisen PVYA11 NFCCI-3629

On the other hand, in case of *A. gaisen*, both yeast extract followed by urea were observed to provide maximum enzyme yields (Figure 6.7b). Other nitrogen sources, ammonium sulphate and protease peptone (PP) were less helpful in increasing the enzyme production.

Cellulase production is known to be sensitive to the nature and level of nitrogen source *(Kachlishvili et al. 2006, Soni et al. 2010). Brijwani et al. (2010)* reported the C/N ratio to be most crucial to obtain specific production. In general, the yields of hydrolytic enzymes are increased by media supplementation with an additional nitrogen source *(Kachlishvili et al. 2006).* The production of enzymes is reported to be increased with the nitrogen content up to certain levels but at the same time, excess nitrogen in the inoculum media was found to suppress the fungal growth *(Qinnghe et al. 2004).*

Effect of surfactants

P. sanguineus PVYA07 NFCCI-3628

Addition of surfactant (0.1%, v/v) was found to be essential in order to facilitate the release of cellulases in the medium. Tween 80 was found to be the best surfactant amongst other ones used in the present study. Maximum CMCase (6.9±0.4 IU/mL) and CBH (1.1±0.5 IU/mL) activities were achieved in medium supplemented with Tween 80 (Figure 6.8a).

A. gaisen PVYA11 NFCCI-3629

Similar trend of increase in CMCase and CBH activities in the growth medium, in which Tween 80 was added, was observed with *A. gaisen* fungal cultures (Figure 6.8b)

It was demonstrated by *Sukan et al. (1989)* that emulsification with Tween 80 led to higher cellulase activities. The reason might be the increased permeability of cell membranes and/or enhanced release of cell-bound enzymes.

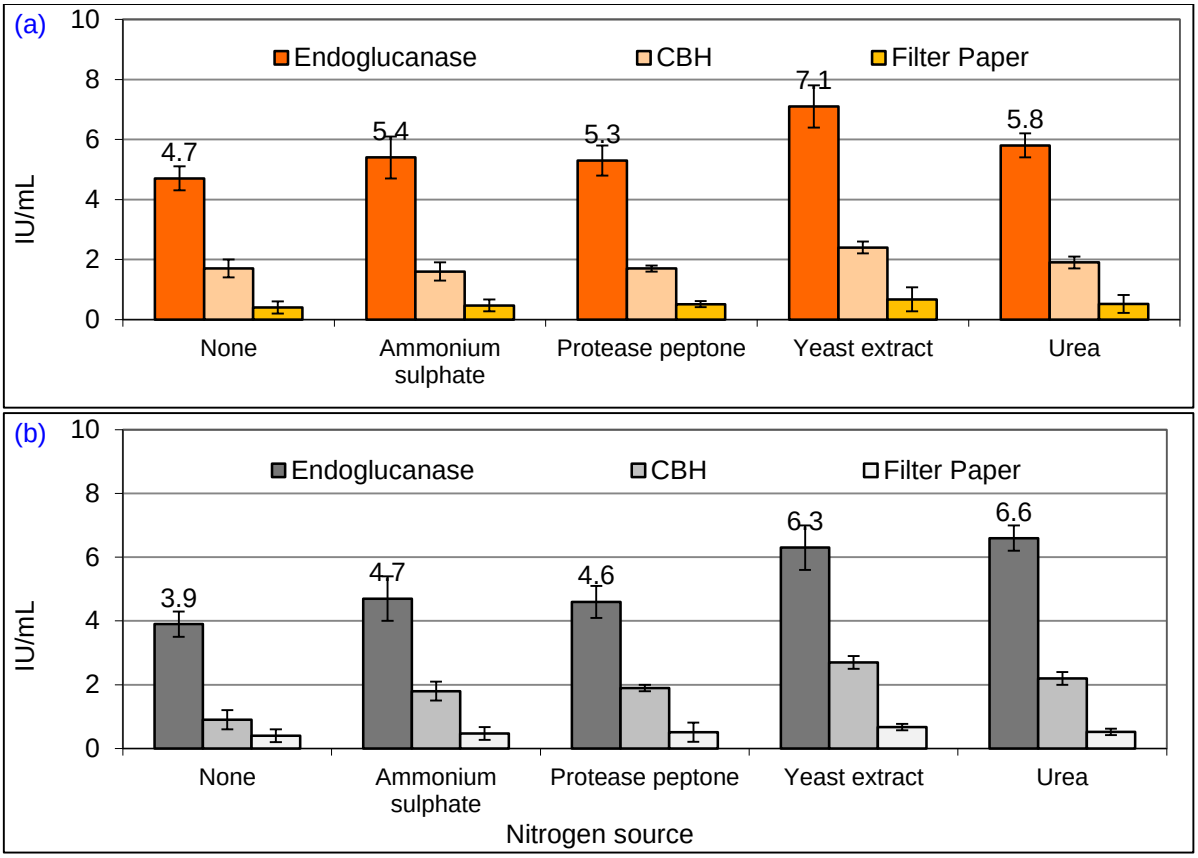

Fig.6.7 Effect of different nitrogen sources on enzyme production (a) P. sanguineus (b) A. gaisen

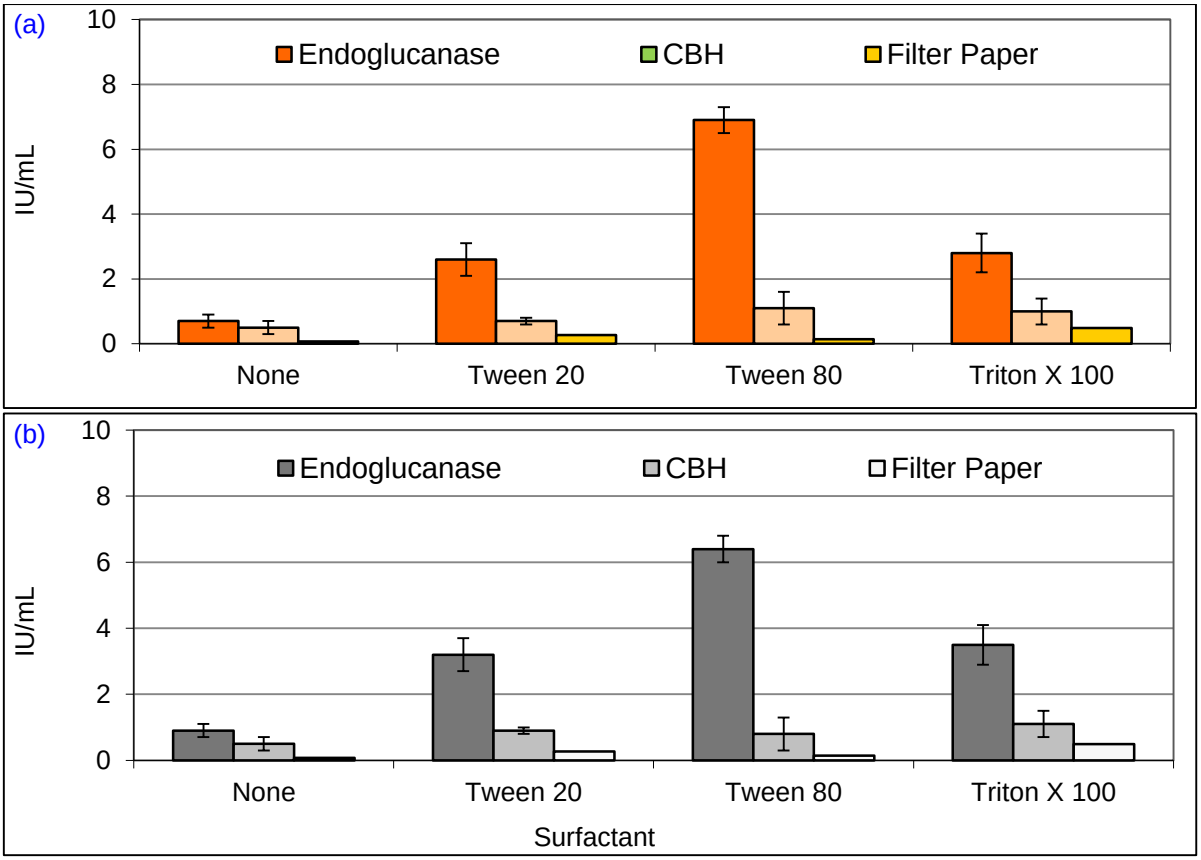

Fig.6.8 Effect of different surfactants on enzyme production (a) P. sanguineus (b) A. gaisen

Tween 80 at 0.1 and 0.2% concentrations, through increased interaction with the lipid components of cell membranes, has been reported to improve the membrane permeability for cellulases *(Ahamed and Vermette 2008).* Increased interaction with lipid components facilitates the contact between the fibrous portion of the substrate and mycelium *(Eriksson et al. 2002, Singh et al. 2007)* and subsequently the nutrient uptake and release of cell-bound enzymes *(Pardo 1996, Soni et al. 2010).*

Effect of inoculum size

P. sanguineus PVYA07 NFCCI-3628

The maximum enzyme production was observed with the inoculum size of 6 discs (for 0.5% CMC) in 8 to 9 days for *P. sanguineus*. Maximum endoglucanase and cellobiohydrolase activities i.e. 7.1±0.4 IU/mL and 2.6±0.4 IU/mL, respectively were observed after inoculation of 6 discs (Figure 6.9a).

A. gaisen PVYA11 NFCCI-3629

Maximum CMCase (6.5±0.7 IU/mL) and CBH (02.5±0.1 IU/mL) activities were observed in 5 to 6 days even at a lower inoculum size of 4 discs (Figure 6.9b).

Inoculum size is also considered to be an important factor for the enzyme production. Low inoculum level may not be sufficient to initiate the growth of microorganisms and therefore, will result in long lag phase and slow enzyme formation. High inoculum size can reduce or deplete the lag phase and promote over growth which can result in fast nutrient depletion (*Ahmed et al. 2012)*. Inoculum size (number of discs) is important to influence the enzyme production. For basidiomycetes, filamentous inoculi are mostly used to avoid longer lag phase and contamination *(Ahmed et al. 2012).*

Effect of incubation days

The effect of incubation days was also assessed from 1 to 12 d for the two fungal strains. Figure 6.10a and b shows the effect of incubation temperature on CMCase production by the two isolated fungal strains.

P. sanguineus PVYA07 NFCCI-3628

P. sanguineus PVYA07 NFCCI-3628 exhibited the maximum CMCase (7.6±0.8 IU/mL) and CBH (2.5±0.4 IU/mL) activity at 8 days incubation time. After 10 days, the production of enzymes started to decrease (Figure 6.10a).

A. gaisen PVYA11 NFCCI-3629

On the other hand, *A. gaisen* showed highest enzyme activities between 5 to 6 days incubation time. Highest CMCase activity of 7.5±0.5 IU/mL and CBH activity of 1.9±0.4 IU/mL was observed at 5 to 6 days incubation time (Figure 6.10b).

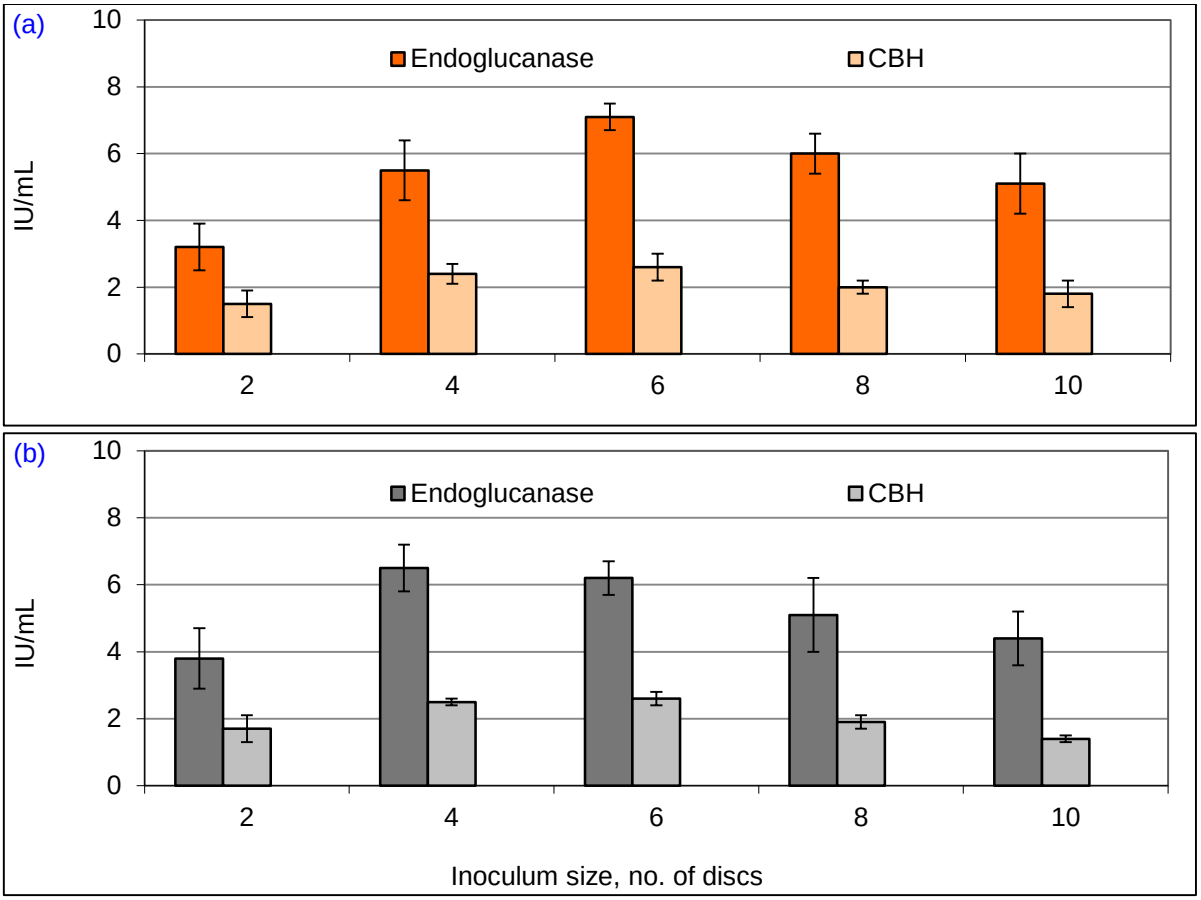

Fig.6.9 Effect of inoculum size on enzyme production (a) P. sanguineus (b) A. gaisen

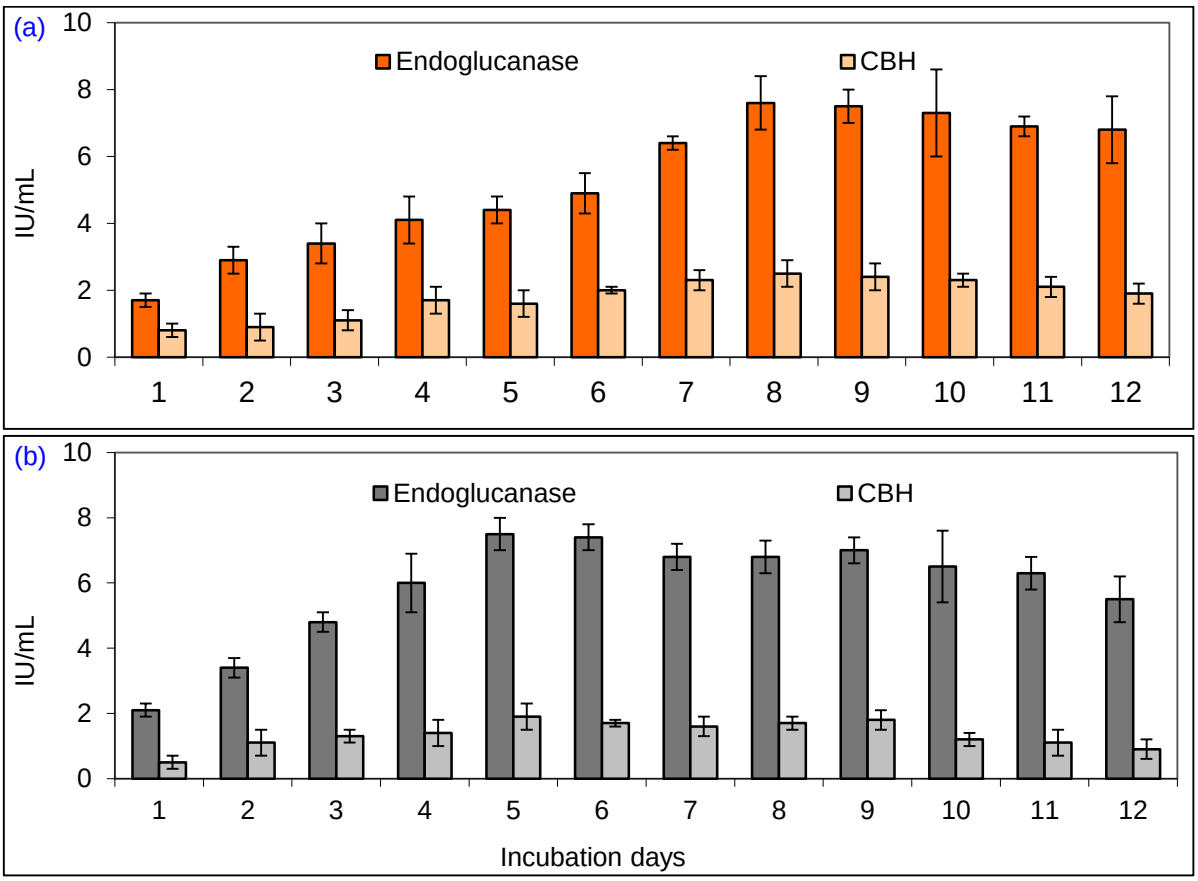

Fig.6.10 Effect of incubation days on enzyme production (a) P. sanguineus (b) A. gaisen

Similar results showing maximum enzyme production at 8 days from *P. sanguineus* was earlier reported (*Castaneda et al. 2009)*. Recently, maximum endoglucanase acitivty on 5th day was reported in *A. brassicicola* (*Deep et al. 2014)*. However, the optimum incubation period of 8 days for endoglucanase enzyme production by *Alternaria alternata* and *A. citri* was also reported by *El-Said et al. (2014).* Mycelial growth indicates substrate utilization. The possible reason for decrease in production, after attaining the maximum, may be catabolite repression to utilize the released sugars 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, Rocky and Hamidi 2010).*

Effect of incubation temperature

Temperature is one of the most important factors that influenced the secretion of fungal enzymes. The effect of incubation temperature on the production of endoglucanases and cellobiohydrolases by the test fungi was also studied by incubating the inoculated flasks at different temperatures maintaining from 26 to 42°C.

P. sanguineus PVYA07 NFCCI-3628

The optimum temperature for growth and enzyme production was observed to be 30°C for *P. sanguineus*. Up to 38°C, 90% relative activity was observed. After 38°C, sharp decrease in enzyme activities was noticed (Figure 6.11a).

A. gaisen PVYA11 NFCCI-3629

Maximum enzyme production was observed at 30°C for A. gaisen. The enzyme activities were observed to decrease at temperatures below and above 30°C (Figure 6.11b).

It should be considered that temperature close to 30°C comprise a range of temperatures in which mesophillic microbes develop. *Onofre et al. (2015)* has also reported 30°C as optimum temperature for the growth as well as production of cellulolytic enzymes by *P. sanguineus.* The results obtained in the present study were also in line with studies done by *Buswell et al. (1998)* in which they also found 30°C temperature as the ideal temperature for cellulase production by *Pycnoporus* species. *P. sanguineus* has also been reported to grow at more elevated temperatures i.e. 37°C by *Dantan-Gonzalez et al. (2008).* Most suitable temperature for growth and enzyme production in case of *Alternaria alternata* and *A. citri* was reported to be 30°C *(El-Said et al. 2014). Amir et al. (2011)* also reported that the optimum temperature for cellulase produced by *Alternaria alternata* by solid state fermentation was 35°C. *Sohail et al. (2011)* has even reported the temperature optima of 50°C for endoglucanase production by *Alternaria sp. MS28.*

Both low as well as high incubation temperature can affect the enzyme production. Low temperature will affect the substrate transport across the cells, while high temperature will result into enzyme and protein denaturation for (*Agnihotri et al. 2010, Kaur et al. 2011, Singh et al. 2009)*. Under stressed conditions (*i.e.* high temperature) the microorganisms synthesize only essential proteins for their growth and other physiological processes *(Gawande and Kamat 1999).* 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).*

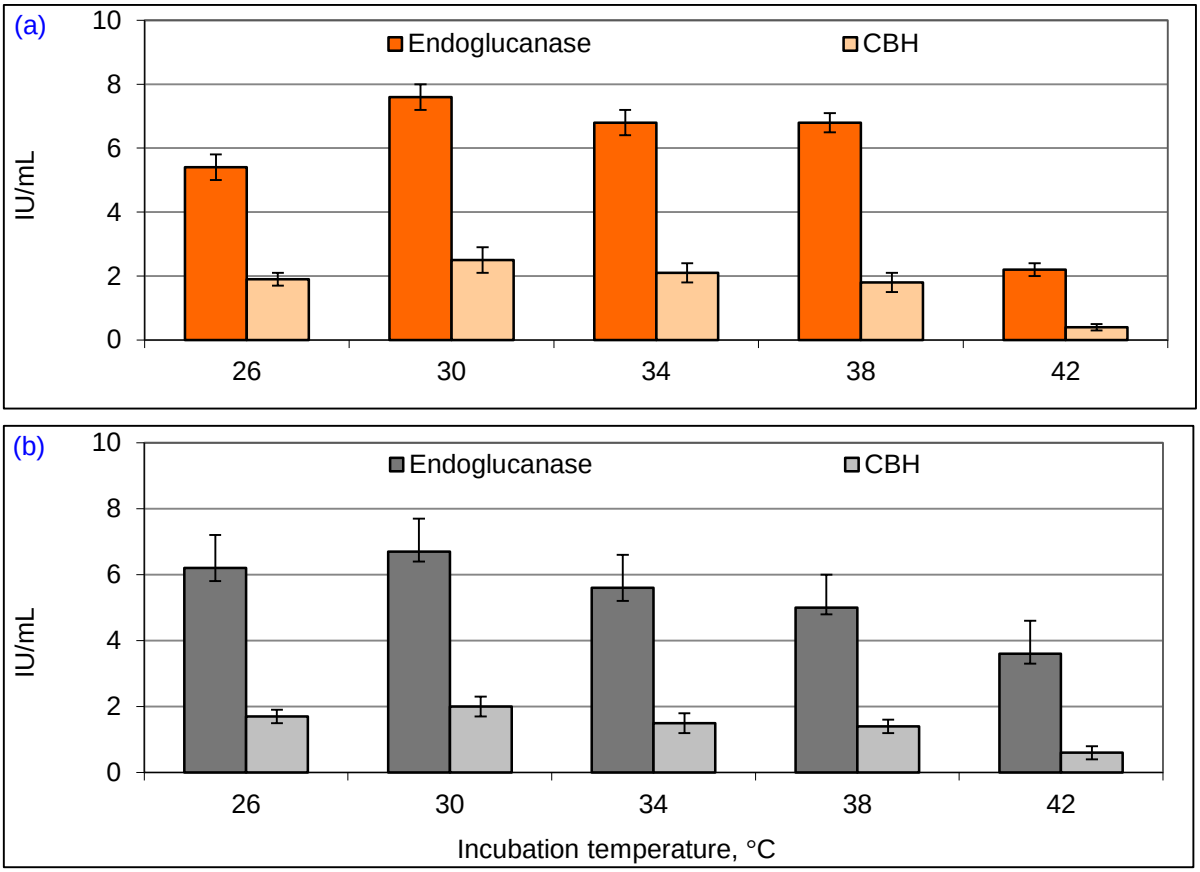

Fig.6.11 Effect of incubation temperature on enzyme production (a) P. sanguineus (b) A. gaisen

6.1.3 Purification and characterization of endoglucanase enzymes

The endoglucanase enzymes (1, 4-β-D glucan-4 glucanohydrolase (EC 3.2.1.4)) were well purified to homogeneity from the culture filtrates of *Pycnoporus sanguineus and Alternaria gaisen* via ammonium sulfate fractionation, Sephadex G-100, and Q-Sepharose column chromatography. The summary of the purification scheme for endoglucanase enzymes, obtained from *Pycnoporus sanguineus* PVYA07 NFCCI-3628 and *Alternaria gaisen* PVYA11 NFCCI-3629 is presented in Table 6.2b and 6.2c, respectively.

P. sanguineus PVYA07 NFCCI-3628

Endoglucanase enzyme from *Pycnoporus sanguineus* PVYA07 NFCCI-3628 was purified 9.7-fold from its initial culture broth with a final yield of 24%, with a specific activity of 611 IU/mg

of protein (Table 6a, b). The molecular weight of the enzyme was determined by SDS PAGE. The purified enzyme appeared as a single band on SDS-PAGE with molecular mass of approximately 48kDa (Figure 6.12a).

A. gaisen PVYA11 NFCCI-3629

Endoglucanase enzyme from *A. gaisen* was purified 10.5-folds from its initial culture broth with a final yield of 24% and specific activity of 607 IU/mg of protein (Table 6.2a, c). The molecular weight of the enzyme, determined by SDS PAGE, was found to be approximately 61 kDa in case of *A. gaisen* (Figure 6.12b).

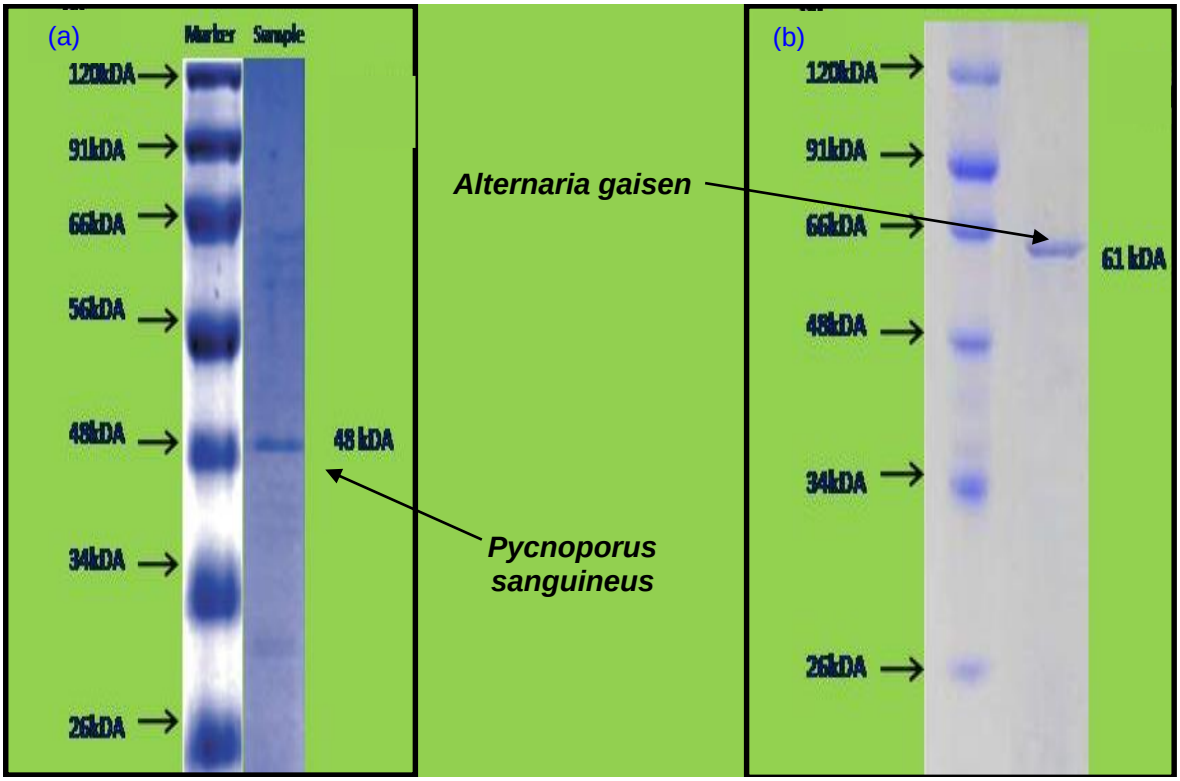

Fig. 6.12 Molecular mass determination of purified endoglucanase through SDS-PAGE (a) Pycnoporus sanguineus (b) Alternaria gaisen

The molecular weights of endoglucanases from various white-rot fungi were reported to vary from 15.5 to 65 kDa *(Manavalan et al. 2015)*. They vary between different genera, species, and strains, which might arise from different ecological origins of these mushrooms. Similar observations showing endoglucanase of 50 kDa from *Pycnoporus sanguineus* were also reported by *Quiroz-Castaneda et al. (2009). Salinas et al. (2011)* also reported endoglucanase enzyme of molecular weight, 38.6 kDa, from *Trametes versicolor.* The endoglucanase from another white rot fungi *Ganoderma lucidum* was reported to be purified by 12.5-fold via ammonium sulfate fractionation, Sephadex G-100, and Q-Sepharose column chromatography with a final yield of 15% by *Manavalan et al. (2015)*. They further reported an endoglucanase having a molecular mass of 64 kDa from *Ganoderma lucidum*.

Purification of enzyme produced from *Bacillus amyloliquefaciens* SS35 was reported to be done using ion exchange chromatography and the molecular weight of the CMCase was determined as 37 kDa *(Singh et al. 2015).* Endoglucanase enzyme from *Alternaria brassicicola* of 60 kDa was reported by *Deep et al. (2014).*

pH and temperature profile of enzymes produced from two different fungal strains

The optimum pH of the endoglucanse enzymes from *P. sanguineus* and *A. gaisen* PVYA11 NFCCI-3629 were determined by measuring their activities at different pH values ranging from 5.0 to 9.0 at an interval of 0.5. Similarly, in order to confirm the optimum temperature of the selected fungal strains, enzyme activities were determined at various temperatures ranging from 30 to 70°C.

P. sanguineus PVYA07 NFCCI-3628

Further characterization studies on endoglucanase showed that the enzymes isolated from *P. sanguineus* were active in broad pH range of 5 to 8 and temperature range of 30 to 60°C with pH and temperature optima at 7.0 and 40 to 50°C, respectively (Figure 6.13a, b and Table 6.3a, 6.4a). Testing temperature-dependent activity and thermal stability, we found that the purified endoglucanase from *P. sanguineus* showed optimum activity in sodium phosphate buffer (0.05M, pH 7.0) between 40 to 50°C and even after 6 h of incubation at optimum temperature, the decrease in enzyme activities was not significant.

A. gaisen PVYA11 NFCCI-3629

The optimum pH of the endoglucanase enzyme was observed to be 6.0, although 96% of relative activity was retained at pH 7.0 (Figure 6.13c and Table 6.3b).

The optimum temperature in case of A. gaisen was observed to be 40°C (Figure 6.13 d). However, no significant reduction in endoglucanase activities was seen up to a temperature of 55°C. The optimum temperature for CBH enzyme production was between 45 to 50°C. The thermal stability of the purified endoglucanase in sodium phosphate buffer (0.05M, pH 7.0) from *P. sanguineus* was maximal at 30 to 55°C, and it retained over 90% of relative activity; however, enzyme activity decreased abruptly beyond 55°C (Table 6.4b).

Similar findings showing cellulase activity over a wide range of temperature (30-60°C) and pH (5.0 to 9.0) were reported by *Akhtar et al. (2013).* The optimum pH and temperature for growth and enzyme production in *Alternaria brassicicola* was reported to be in the range of 6 to 7 and 35 to 40°C, respectively *(Deep et al. 2014).* Most microbial cellulases are reported to be active in acidic to neutral pH range. Alkaline active carboxymethyl cellulases from *Cephalosporium* and *Humicola* sp have been commercially exploited for the production of alkaline cellulases *(Kang and Rhee 1995)*. These endoglucanases are optimally active in a broad pH range of 6 to 10 but their thermal stability in the alkaline pH range is low. Certain strains of *Trichoderma*

sp. and *Penicillium* sp. are also reported to give high cellulase yields but their activities at alkaline pH are negligible *(Schmoll and Kubicek 2003).* The enzyme produced by alkalotolerant *Pycnoporus* and *Alternaria* strains reported in this work were active and stable under alkaline conditions indicating their potential for commercial use.

Table 6.2a Enzyme activities in culture filtrate of P. sanguineus

Activity	***P. sanguineus***	***A. gaisen***
	IU/mL	
CMCase [Endo (1-4) β– D-glucan-glucano hydrolase	7.6 [1.1]	7.5 [1.0]
CBH	2.5 [0.8]	1.7 [0.6]
FPase (Filter paper degrading activity)	0.5	0.7

Table 6.2b Summary of purification steps of endoglucanase enzyme from P. sanguineus

Purification steps	**Total proteins, mg**	**Total activity, IU**	**Specific activity, IU/mg**	**Fold purification**	**Enzyme yield, %**
Culture filtrate	288	17600	61	1.0	100
80% $(NH4)_2$ SO_4	95	14600	154	2.4	88
Sephadex G-200	19	6210	327	5.2	37
Q-sepharose	7	4280	611	9.7	24

Table 6.2c Summary of purification steps of endoglucanase enzyme from A. gaisen

Purification steps	**Total proteins, mg**	**Total activity, IU**	**Specific activity, IU/mg**	**Fold purification**	**Enzyme yield, %**
Culture filtrate	304	17500	58	1.0	100
80% $(NH4)_2$ SO_4	101	15400	152	2.6	88
Sephadex G-200	21	6450	307	5.3	37
Q-sepharose	7	4250	607	10.5	24

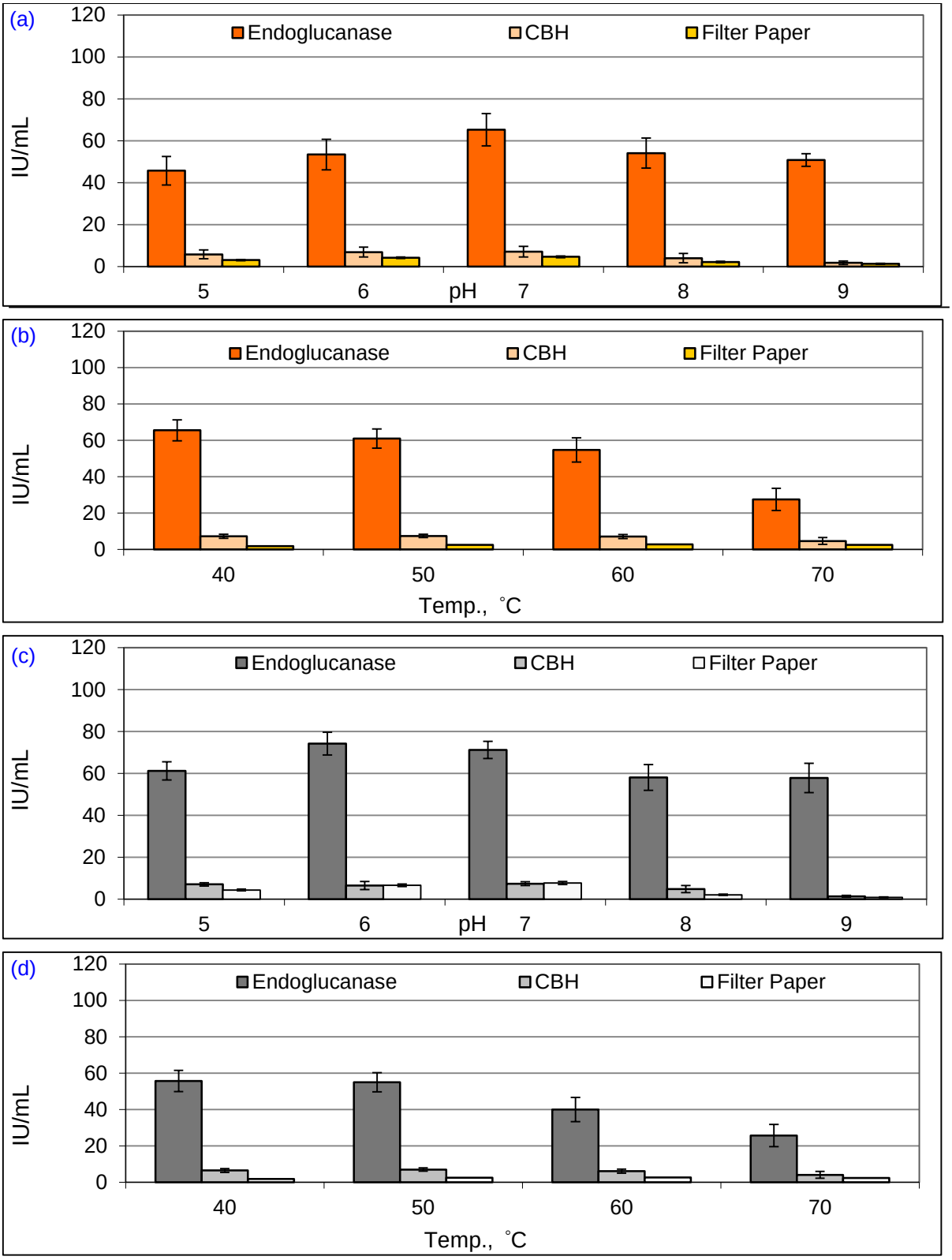

Fig. 6.13 pH and temperature profile of enzymes produced from two different fungal strains (a), (b) P. sanguineus (c), (d) A. gaisen

Table 6.3 Effect of pH on the CMCase and CBH activities of the purified enzyme and their stability at optimum pH

(a) *P. sanguineus* PVYA07 NFCCI-3628

pH of buffer	Relative activity,%		Treatment hours	Residual activity,% at pH 7.0, 45˚C	
	CMCase	CBH		CMCase	CBH
5.0	70.0	81.7	**0**	100.0	100.0
6.0	81.8	97.2	**1**	99.0	97.5
7.0	100.0	100.0	**2**	98.4	96.7
8.0	82.8	56.3	**3**	95.6	95.6
9.0	64.8	25.4	**4**	93.4	94.4
			5	92.5	93.5
			6	90.3	89.3
			24	81.6	80.0

(b) A. gaisen PVYA11 NFCCI-3629

pH of buffer	Relative activity,%		Treatment hours	Residual activity,% at pH 7.0, 45˚C	
	CMCase	CBH		CMCase	CBH
5.0	82.4	94.9	**0**	100.0	100.0
6.0	100.0	87.8	**1**	98.9	98.7
7.0	96.0	100.0	**2**	96.5	97.5
8.0	78.3	64.9	**3**	95.3	96.4
9.0	77.9	18.9	**4**	93.5	94.1
			5	92.4	94.2
			6	91.2	89.2
			24	84.1	85.5

Effect of metal ions on endoglucanase activity

Effect of presence of different metal ions in the form of salts was also studied. The endoglucanase enzymes were incubated with the respective metal ions at pH 7.0 and 45°C. An aliquot of 1mL was taken after 30 min. of incubation time for determination of CMCase activities under standard assay conditions. It was observed that $MgCl_2.4H_2O$ and $CaCl_2.2H_2O$ were more suitable for increasing the growth and enzyme production. Endoglucanse activities were observed to be inhibited at 10mM concentrations of Cu^{+2} (Table 6.5). No significant change in endoglucanase activity was noticed in presence of Zn^{+2} and Na^{+1} metal ions.

The presence of metal ions such as Na, Ca^{+2}, Mg^{+2} and Zn^{+2} were reported to positively influence the enzyme activity from *Melancarpus sp.* MTCC 3922 *(Kaur et al. 2007).* Ca^{+2}, Mg^{+2} and Mn^{+2} were reported to enhance the endoglucanase activity from *Ganoderma lucidum* by *Mananvalan et al. (2015).*

Table 6.4 Effect of incubation temperature (at pH 7.0) on the CMCase and CBH activities of the purified enzyme and their stability at optimum temperature (at pH 7.0, temperature 45°C)

(a) *P. sanguineus* PVYA07 NFCCI-3628

Incubation temperature, °C	Relative activity,%		Treatment time, h	Residual activity,% at 45°C	
	CMCase	CBH		CMCase	CBH
30	72.5	54.1	**0**	100.0	100.0
40	100.0	97.3	**1**	98.4	98.2
50	93.1	100.0	**2**	96.8	97.0
60	83.5	95.9	**3**	93.7	94.9
70	42.0	62.2	**4**	90.0	93.4
			5	89.2	92.6
			6	86.4	90.8

(b) *A. gaisen* PVYA11 NFCCI-3629

Incubation temperature, °C	Relative activity,%		Treatment time, h	Residual activity,% at 45 °C	
	CMCase	CBH		CMCase	CBH
30	81.5	72.9	**0**	100.0	100.0
40	100.0	92.9	**1**	99.1	97.5
50	98.7	100.0	**2**	97.9	95.6
60	71.8	87.1	**3**	95.4	94.5
70	46.1	58.6	**4**	91.6	92.4
			5	90.1	90.0
			6	89.4	88.5

Table 6.5 Effect of metal ions on cellulase production

Metal ions (10mM)	***P. sanguineus* PVYA11 NFCCI-3628**	***A. gaisen* PVYA11 NFCCI-3629**
	Relative CMCase activity, %	
None	100	100
$CaCl_2$, $2H_2O$	108	111
NaCl	103	101
$MgCl_2$, $4H_2O$	110	116
$ZnSO_4$, $7H_2O$	101	96
$CuSO_4$, $5H_2O$	79	81

6.2 Application of isolated fungal enzymes for drainage improvement of recycled pulps

The recycled pulps (old newsprint and writing printing grade) were treated with various concentrations of endoglucanase enzymes produced by *Pycnoporus sanguineus* and *Alternaria gaisen* ranging from 0.050 to 0.125% on dry weight of pulp for 30 min. The pulp consistency, temperature, pH, incubation time were adjusted to the industrial conditions mentioned in the materials and methods section.

In order to avoid duplicacy, only results obtained in this chapter with the purified endoglucanase enzymes from isolated fungal strains *P. sanguineus and A. gaisen* has been presented here. Detailed discussion on the effect of endoglucanase enzymes on pulp and paper properties alongwith suitable literature support has already been mentioned in Chapter 4, Section 4.4 and 4.5.

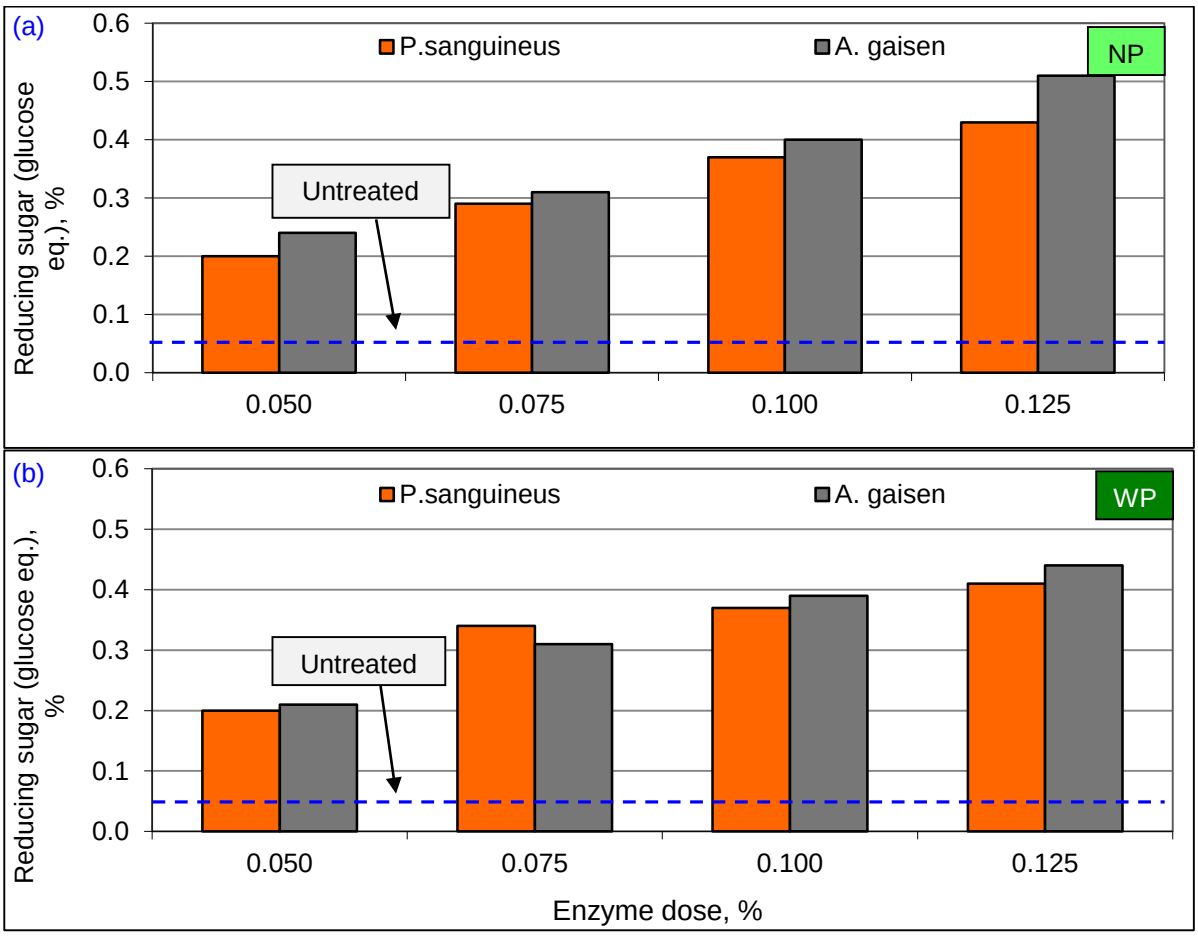

Fig. 6.14 Reducing sugar production (equivalent to glucose) as a function of enzyme dose at 4% consistency and 30 min. treatment time (a) recycled pulp-NP (b) recycled pulp-WP

The reducing sugars (as glucose equivalents) in the filtrates of untreated and enzyme treated different pulps were measured using dinitrosalicylic acid (DNS) assay *(Miller 1959)*. The plot depicting the reducing sugar production as a function of enzyme dose in different pulps were plotted in Figure 6.14 a, b. Endoglucanase enzymes from the two isolated fungal strains were

observed to generate more amount of reducing sugars when compared to untreated pulps. Sugar generation represents the hydrolysis of cellulosic fines.

6.2.1 Effect of enzyme treatments on pulp properties

Pulp freeness

Experiments for pulp freeness, determined by Canadian Standard Freeness (CSF) tests, were performed with sufficient number of parallels in order to detect the changes in drainage accurately.

Recycled pulp-NP

As shown in Figure 6.15a, the enzymes produced by *P. sanguineus* and *A. gaisen* exhibited significant increase in CSF values, from 350 to 385 mL and 395 mL, respectively.

Recycled pulp-WP

Similar trend of increase in CSF values after the treatment of enzymes produced by *P. sanguineus* and *A. gaisen* was also observed in recycled pulp-WP. CSF values were increased from initial value of 372 mL upto 400 and 415 mL in case of *P. sanguineus* and *A. gaisen*, respectively (Figure 6.15b).

Pulp drainability

Recycled pulp-NP

Endoglucanase enzyme produced by *P. sanguineus* displayed significant improvement in pulp drainage ranging from 8.0 to 20.1% at various doses (Figure 6.16a). The endoglucanase enzyme produced by *A. gaisen* was observed to be even better than *P. sanguineus* in reducing the pulp drainage time by 9.3 to 23.2% at various doses.

Recycled pulp-WP

The drainage time was observed to be reduced by 17.3 and 19.5% in recycled pulps-WP treated with endoglucanase enzymes by *P. sanguineus* and *A. gaisen*, respectively at 0.125% dose (Figure 6.16b).

Similar trend of improved pulp drainage in pulps treated with commercial enzyme Fibercare D, having predominantly endoglucanase activity, was discussed in Chapter 4. They hydrolyze the amorphous fines, thereby enhancing the dewatering of recycled pulps. These fines having high specific surface area were more usceptible to cellulase hydrolysis when compared to crystalline cellulose.

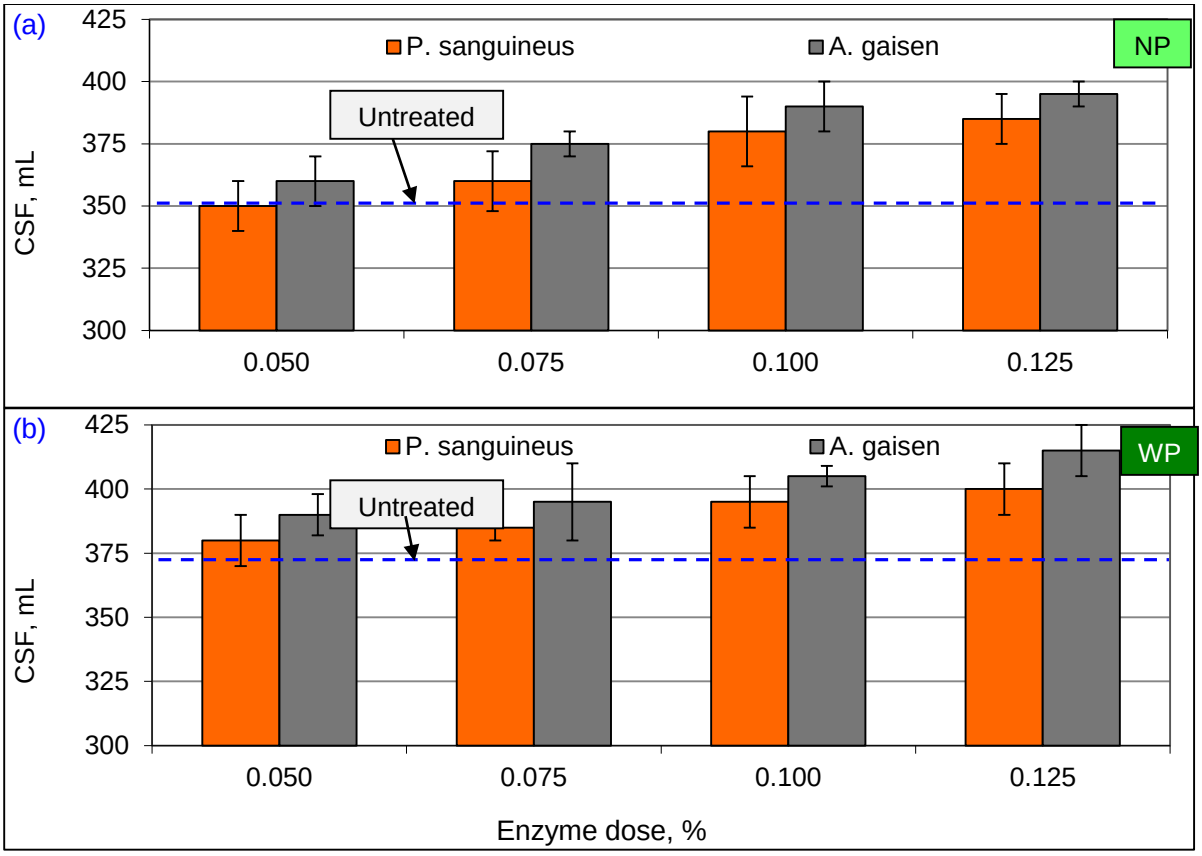

Fig. 6.15 Effect of different enzymes on CSF at various doses (a) recycled pulp-NP (b) recycled pulp-WP

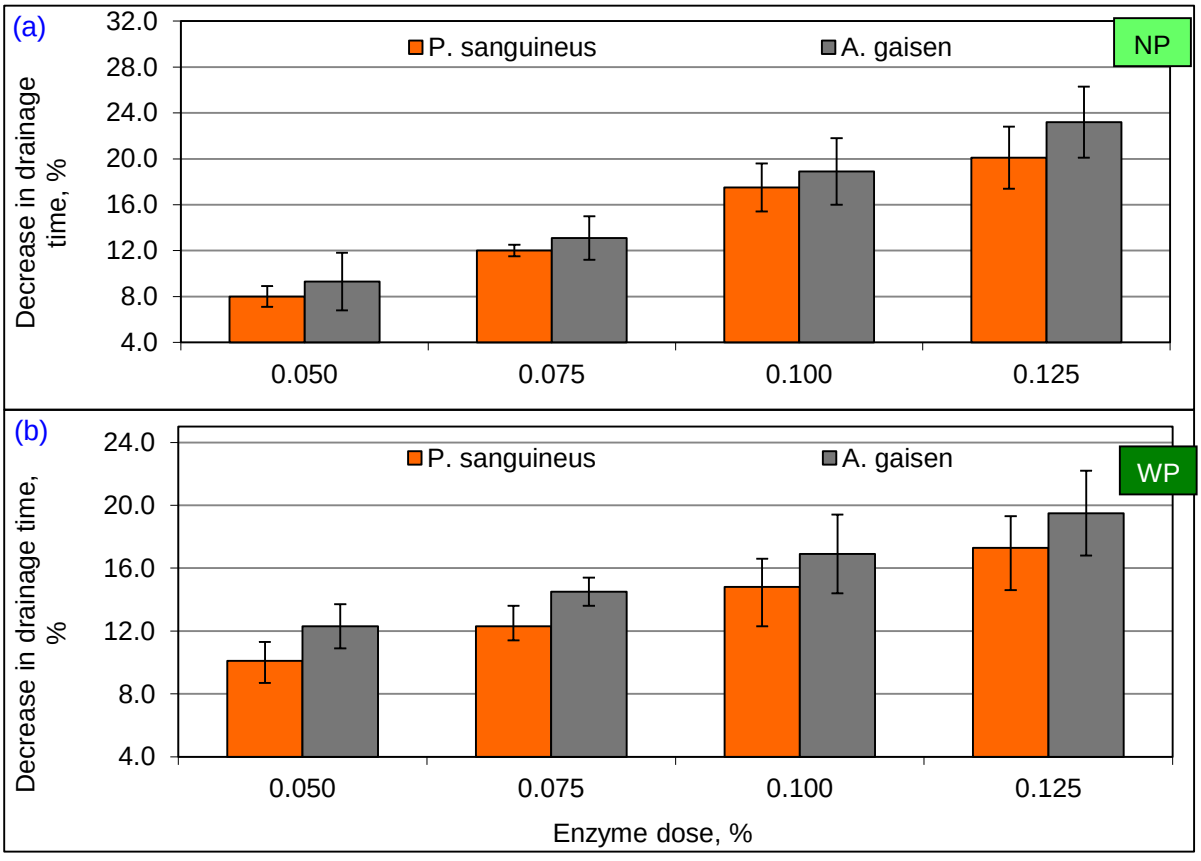

Fig. 6.16 Effect of different enzymes at various doses on decrease in drainage time (a) recycled pulp-NP (b) recycled pulp-WP

Water retention value

Recycled pulp-NP

To ascertain the effect of enzyme treatments, water retention value (WRV) was also analyzed. Pulps treated with lab produced endoglucanase enzymes retained more water i.e. higher WRV by 30% compared to untreated pulps (Figure 6.17a).

Recycled pulp-WP

Similar trend of increased water retention value in pulps treated with lab produced endoglucanase enzymes was also observed in recycled pulps-WP. Significant increase in water retention values from 140 to 180% was noticed in pulps treated with purified endoglucanase produced by *A. gaisen.* In case of purified enzyme produced by *P. sanguineus*, the increase in WRV was slightly less ranging from 140 to 170% (Figure 6.17b).

These results showing increase in water retention values by isolated fungal enzymes were in line with those obtained with commercial endoglucanase Fibercare D, discussed in Chapter 4. The reason for increased WRV may be the formation of more hydrogen bonds between cellulose and water molecules by the hydrolysis of low molecular weight carbohydrates and partial deterioration of crystalline structure of cellulose. *Dienes et al. (2004)* also reported an increase of 16% increase in WRV after cellulase treatments.

Pulp viscosity

Recycled pulp-NP

The pulp samples treated with purified endoglucanase by *P. sanguineus* displayed slight reduction in pulp viscosity. Reduction in the pulp viscosity was more prominent as the enzyme dose was increased above 0.075%. No significant reduction in viscosity was noticed in pulp treated with endoglucanase from *A. gaisen* (Figure 6.18a).

Recycled pulp-WP

The pulp samples treated with both endoglucanase enzymes exhibited reduction in pulp viscosity (Figure 6.18b). Reduction in viscosity was less significant up to 0.075% enzyme dose levels. Further increase in enzyme dose reduced the pulp viscosity by 12%.

Presence of cellobiohydrolase component as side activities in purified endoglucanase enzymes might have further increased the cellolose hydrolysis, exhibited by viscosity reductions. The hydrolysis rate of crystalline cellulose by CBH has been reported to increase drastically in presence of endoglucanases because of an endo-exo synergy between two classes of the enzymes *(Henrissat et al. 1985).*

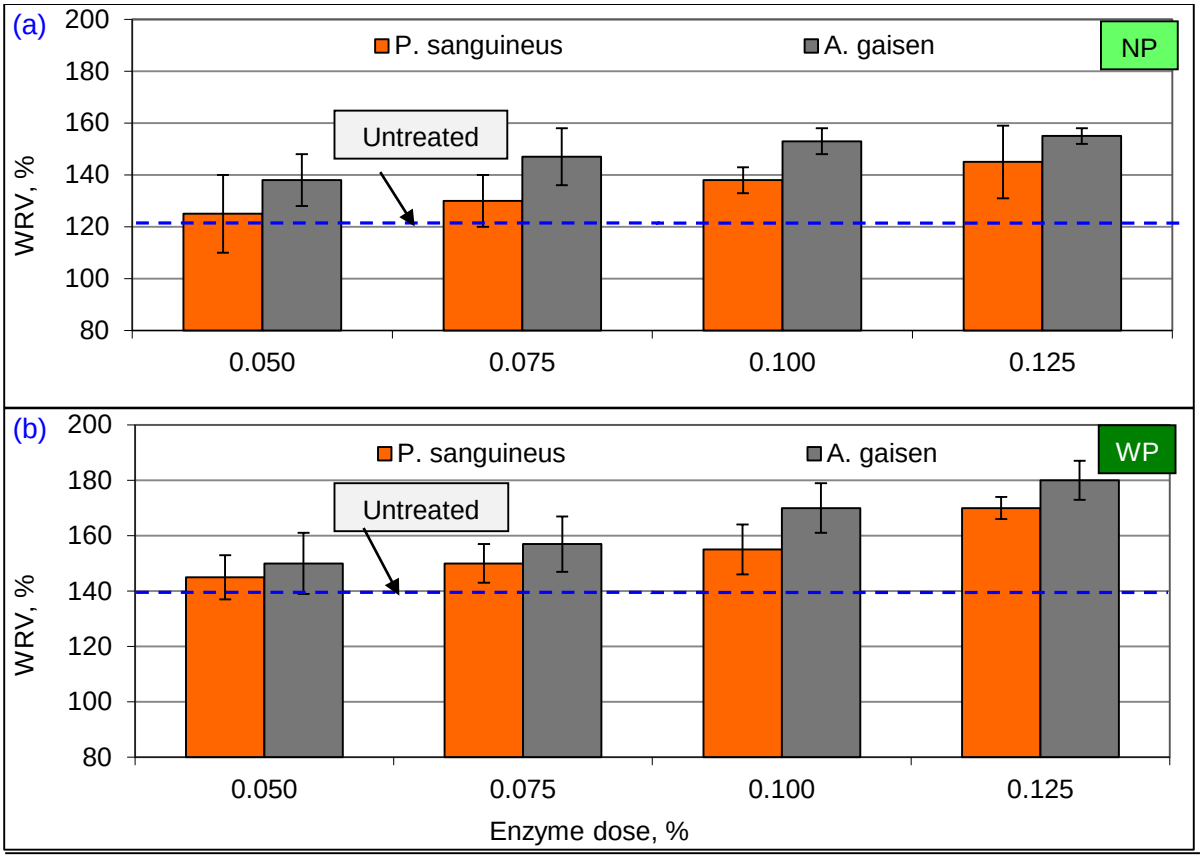

Fig. 6.17 Effect of different enzymes at various doses on water retention value (a) recycled pulp-NP (b) recycled pulp-WP

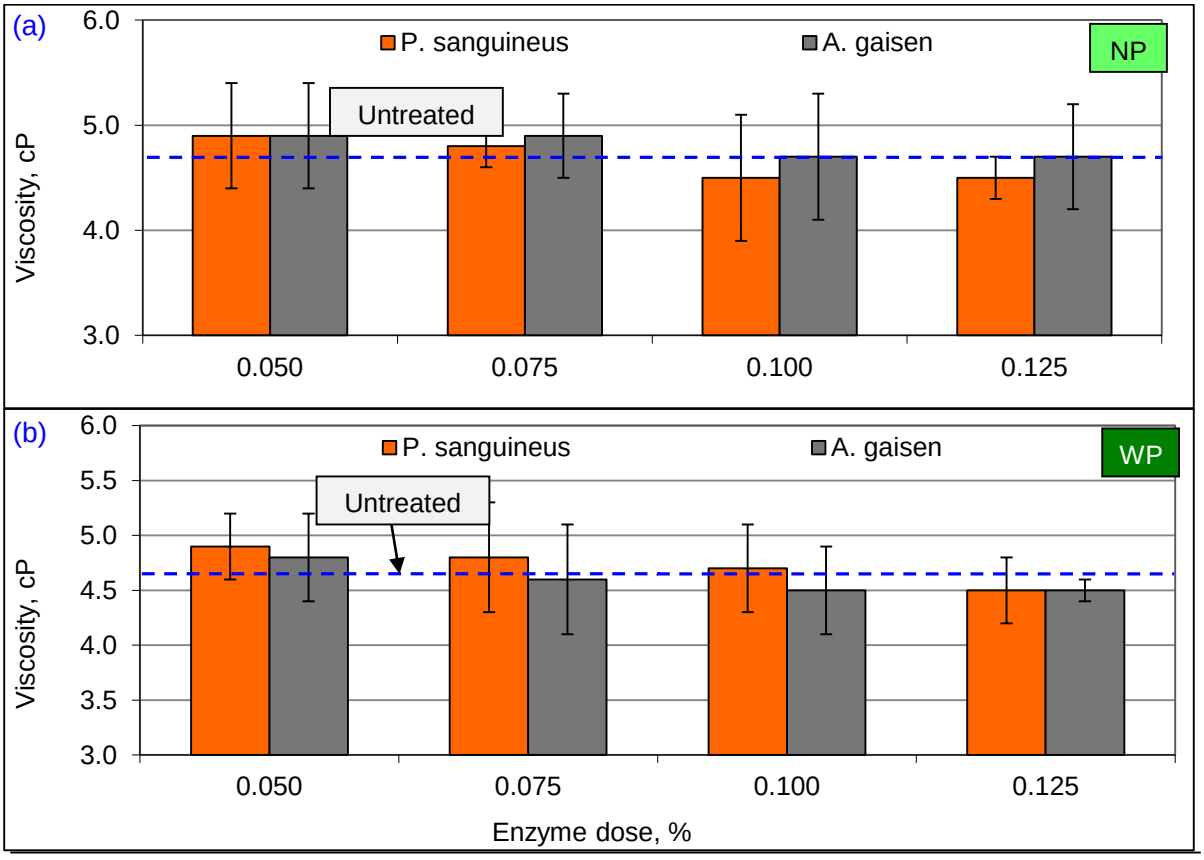

Fig. 6.18 Effect of different enzymes at various doses on viscosity of (a) recycled pulp-NP (b) recycled pulp-WP

Fines content

Bauer McNett fibre classification of recycled pulps-NP displayed 13 and 21% reduction in fines content after the endoglucanase treatment from *P. sanguineus* and *A. gaisen*, respectively (Figure 6.19a). On the other hand, in case of recycled pulps-WP, the reduction in fines content was 12 and 16% after treatment of enzymes from *P. sanguineus* and *A. gaisen*, respectively (Figure 6.19b).

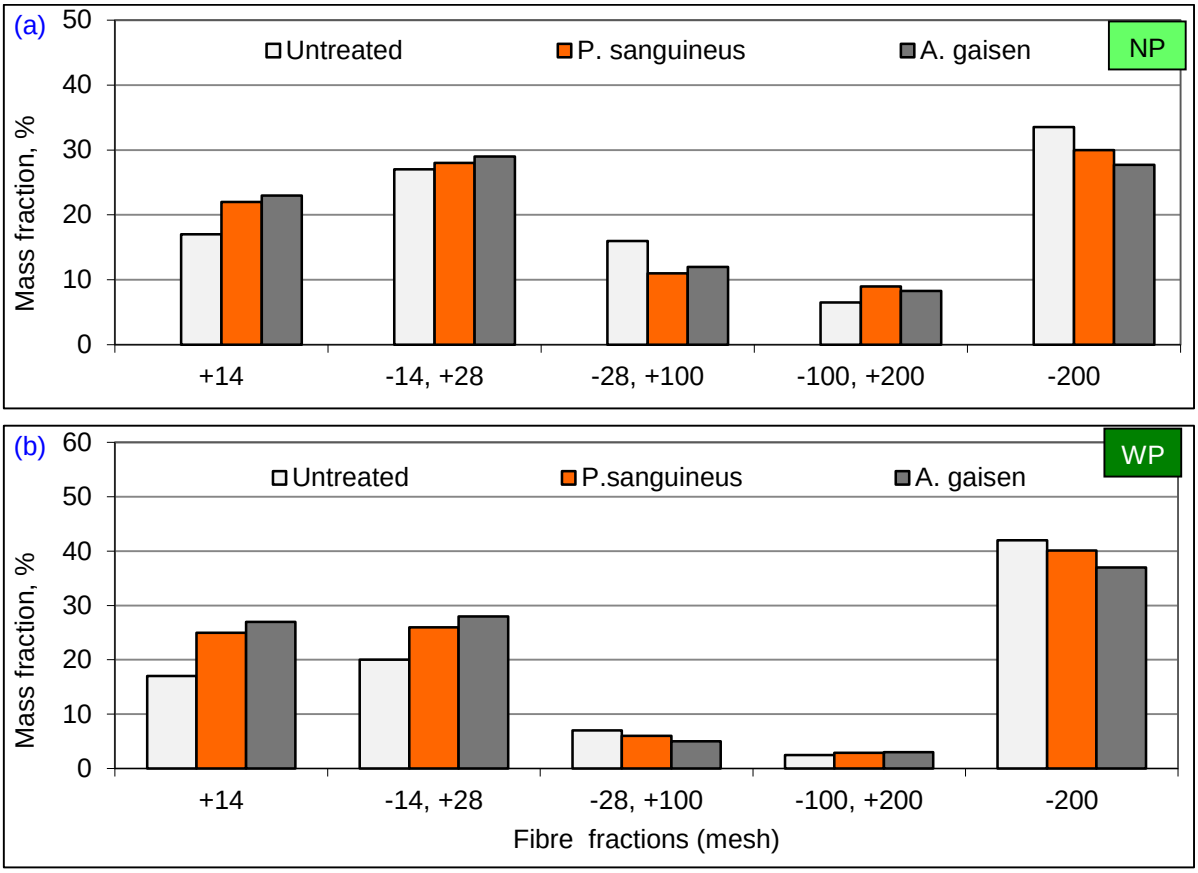

Fig. 6.19 Bauer–McNett classification of untreated and enzyme treated pulps (a) recycled pulp-NP (b) recycled pulp-WP

Fibre length distribution

The effect of purified enzymes, from *P. sanguineus* and *A. gaisen*, on the fibre length distribution of recycled pulps was also analyzed using L&W fibre morphology tester. As shown in Figure 6.20a, b the proportion of longer fibres (greater than 1 mm) was increased and that of shorter fibres was decreased on treatment of pulp with isolated fungal enzymes. Endoglucanase action on fibre fines might have lead to reduction in shorter fibres. The proportion of fibres greater than 1 mm was 29.0, 36.1 and 39.8% in case of recycled (NP) recycled pulp for untreated, treated with purified enzyme from *P. sanguineus* and *A. gaisen, respectively* (Figure 6.20a). In case of recycled pulp-WP, proportion of fibres greater than 1 mm was 24.0, 30.5 and 34.5% for untreated, treated with purified enzyme from *P. sanguineus* and *A. gaisen, respectively* (Figure 6.20b). Similar findings of reduction in fines content and

improvement in fibre length distribution in commercial endoglucanase treated pulps were discussed in length in Section 4.3.

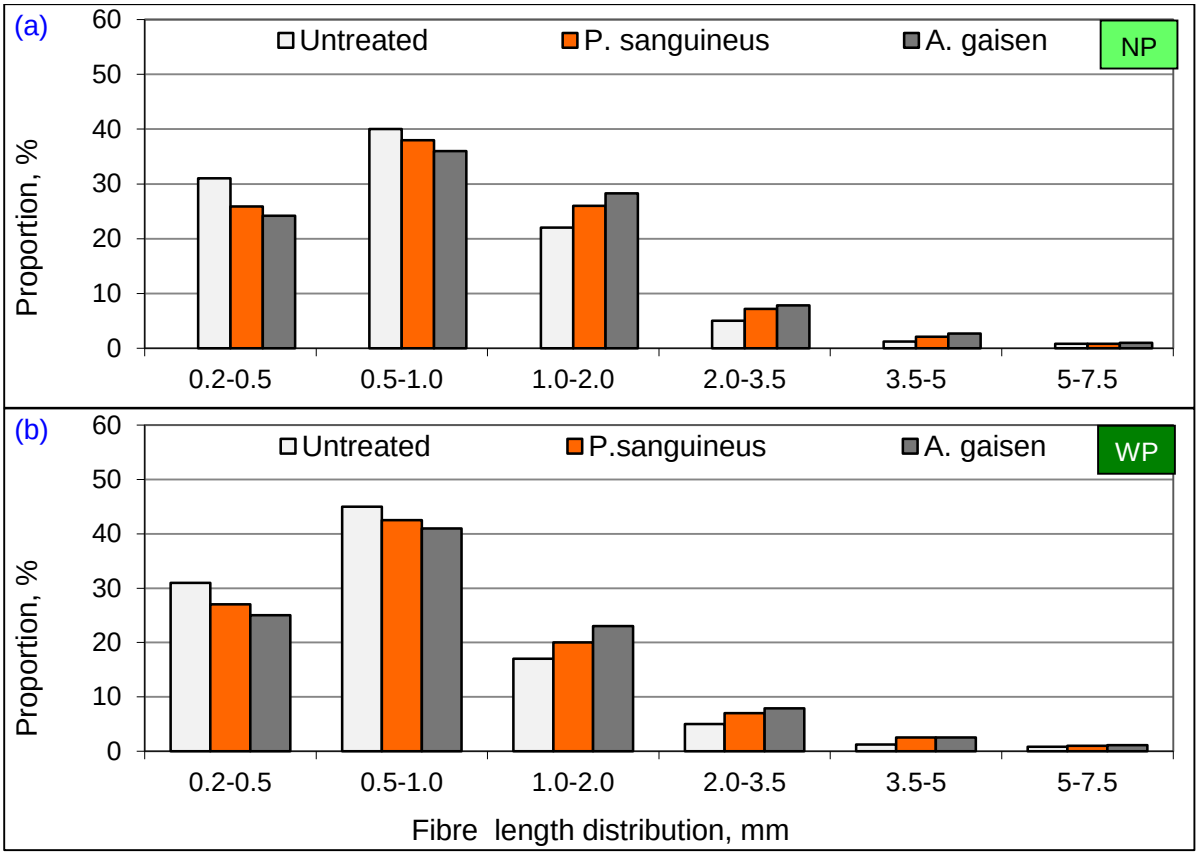

Fig. 6.20 Fibre length distribution of untreated and enzyme treated pulps (a) recycled-NP (b) recycled-WP

6.3 Effect of enzyme treatments on paper properties

Effect of enzymes produced by *P. sanguineus* and *A. gaisen* on various paper properties of recycled pulps viz. tensile, tear, sheet density and formation index was also analyzed.

Tensile index

Recycled pulp-NP

Pulps treated with purified endoglucanase produced by *P. sanguineus* showed significant increase in tensile index of about 7.5 to 15% at varying doses between 0.050 to 0.125% (Figure 6.21a). The purified endoglucanase from *A. gaisen* also exhibited an increase in tensile index by 16% (Figure 6.21a). The endoglucanase action at even low enzyme charge is expected to contribute to an improvement of the paper structure independent of degradation process of dissolved colloidal substances.

Recycled pulp-WP

Similar trend of increase in tensile index was also observed in sheets prepared from enzyme treated recycled (WP) grade recycled pulps. Significant increase of 8.5 to 12.0% in tensile index was observed in pulps treated with purified endoglucanase from *P. sanguineus*, at

varying doses of 0.075 to 0.125%. On the other hand, less improvement in tensile strength was obtained in pulps treated with endoglucanase from *A.g* (Figure 6.21b).

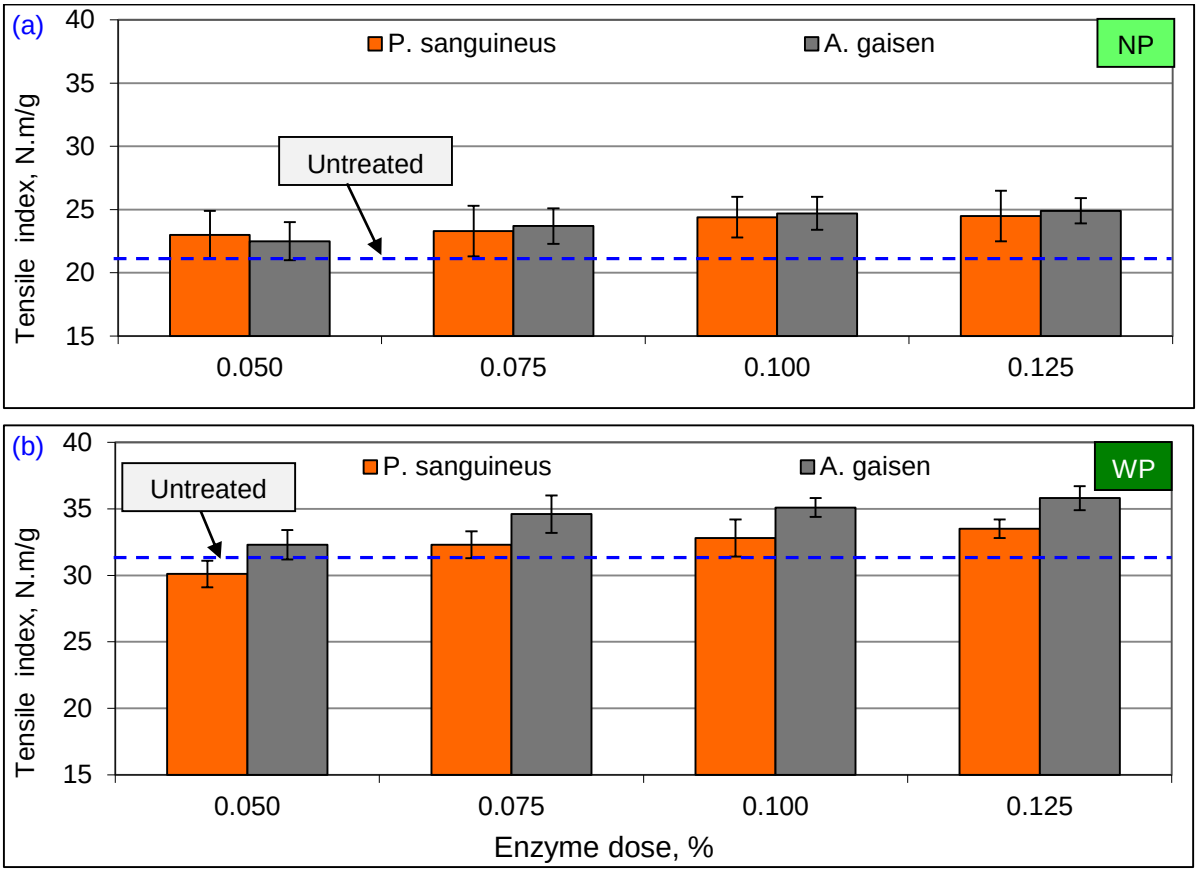

Fig. 6.21 Effect of different enzymes at various doses on tensile index of (a) recycled pulp-NP (b) recycled pulp-WP

Tear index

Recycled pulp-NP

A drop in tear index ranging from 2 to 9% was noticed in pulps treated with varying doses of endoglucanases from both fungal strains *P. sanguineus* and *A. gaisen* However, the drop in tear index was not significant (3% only) up to a dose of 0.100% of endoglucanase from *A. gaisen.* But, in case of endoglucanase from *P. sanguineus* treated pulps, 8% reduction in tear index was observed at a dose of 0.100% (Figure 6.22a).

Recycled pulp-WP

Common trend of decrease in tear index was observed in all sheets prepared from enzyme treated pulps (Figure 6.22b). Reduction of 9 to 10% in tear index values were observed in pulps treated with endoglucanase from *P. sanguineus* and *A. gaisen.* Side activities of cellobiohydrolase component in purified endoglucanse enzyme from *P. sanguineus* and *A. gaisen* is supposed to affect the tear strength in a negative manner, especially at a higher dose.

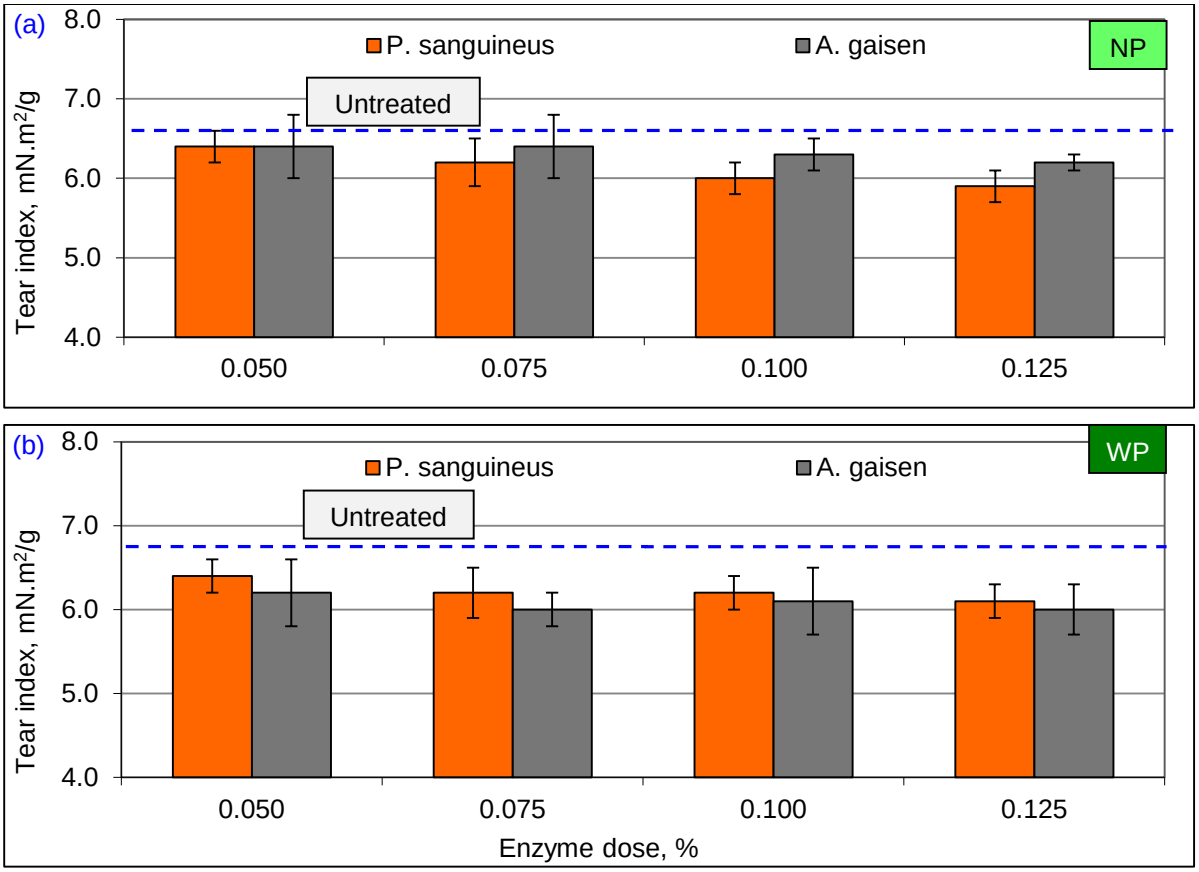

Fig. 6.22 Effect of different enzymes at various doses on tear index of (a) recycled pulp-NP (b) recycled pulp-WP

Smoothness

Recycled pulp-NP

Roughness values were observed to be reduced in all the sheets prepared from enzyme treated pulps. Significant reduction of 32% in roughness (improvement in smoothnes) was observed in sheets prepared after treatment with endoglucanase from *P. sanguineus* (Figure 6.23a). 41% reduction in roughness (improvement in smoothness) was observed in sheets prepared from endoglucanse (*A. gaisen)* treated pulps (Figure 6.23a).

Recycled pulp-WP

Similar trend of reduction in roughness or improvement in paper smoothness was observed with sheets prepared from endoglucanse treated recycled pulps-WP. A reduction of 25% in roughness i.e. improvement in smoothness was observed in sheets prepared from endoglucanse (*P. sanguineus)* treated pulps (Figure 6.23b). On the other hand, sheets prepared from endoglucanse (*A. gaisen*) treated pulps exhibited a decrease of 43% in roughness values (Figure 6.23b).

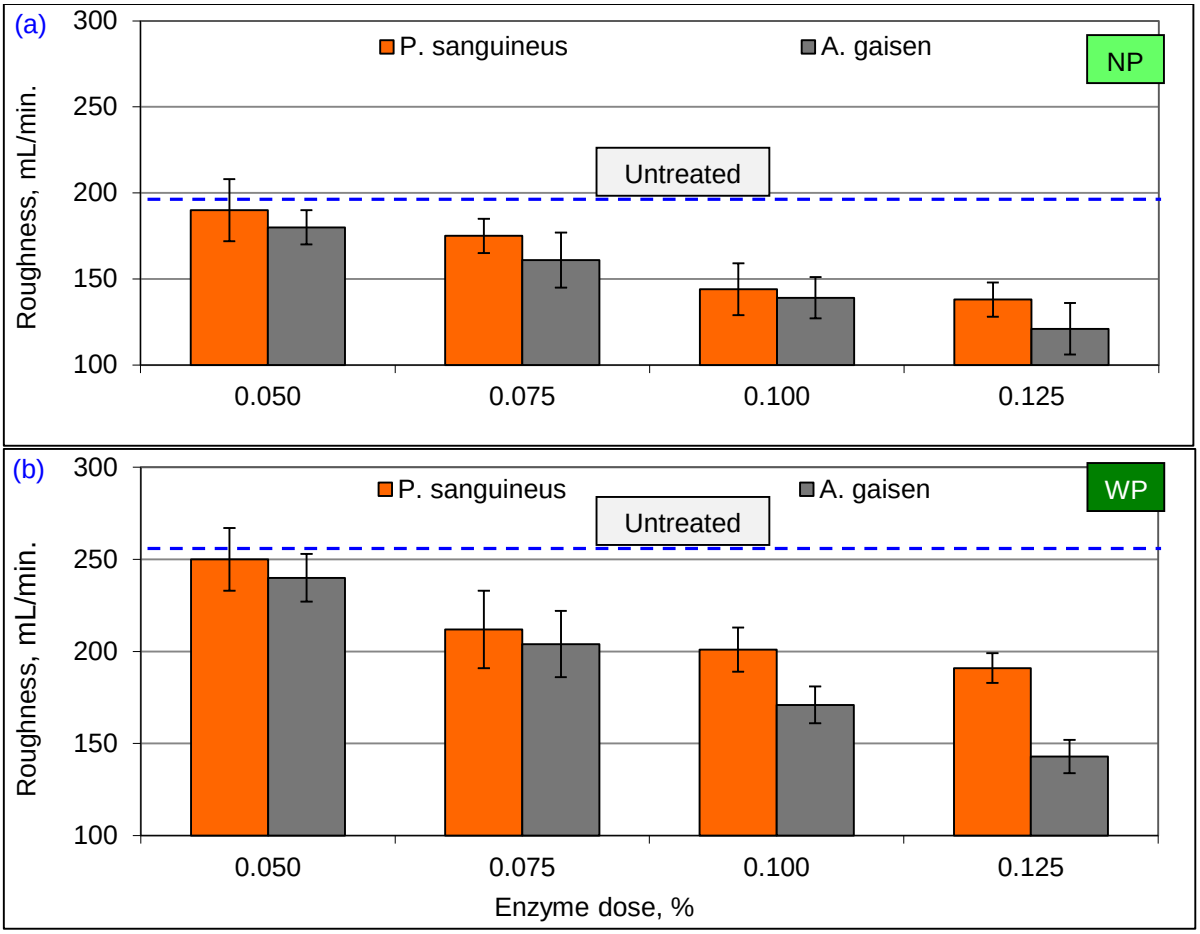

Fig. 6.23 Effect of different enzymes at various doses on roughness of (a) recycled pulp-NP (b) recycled pulp-WP

Formation index

Recycled pulp-NP

Sheets prepared from endoglucanase treated pulps exhibited a common trend of improved formation index values. Significant improvement of 31% in appearance measured in terms of formation index values was observed in sheets prepared from endoglucanase (*P. sanguineus*) treated pulps. Further improvement of 37% in sheet formation index was observed with endoglucansae (*A. gaisen*) treated pulps (Figure 6.24).

Recycled pulp-WP

Similar trend of improvement in sheet formation and appearance was observed in sheets prepared from endoglucanase treated recycled pulps-WP. Sheets prepared after the endoglucanase (*P. sanguineus*) treatment showed 19% improvement in formation. On the other hand, sheets prepared from endoglucanase (*A. gaisen)* treated pulps were 24% better than those prepared from untreated pulps (Figure 6.24).

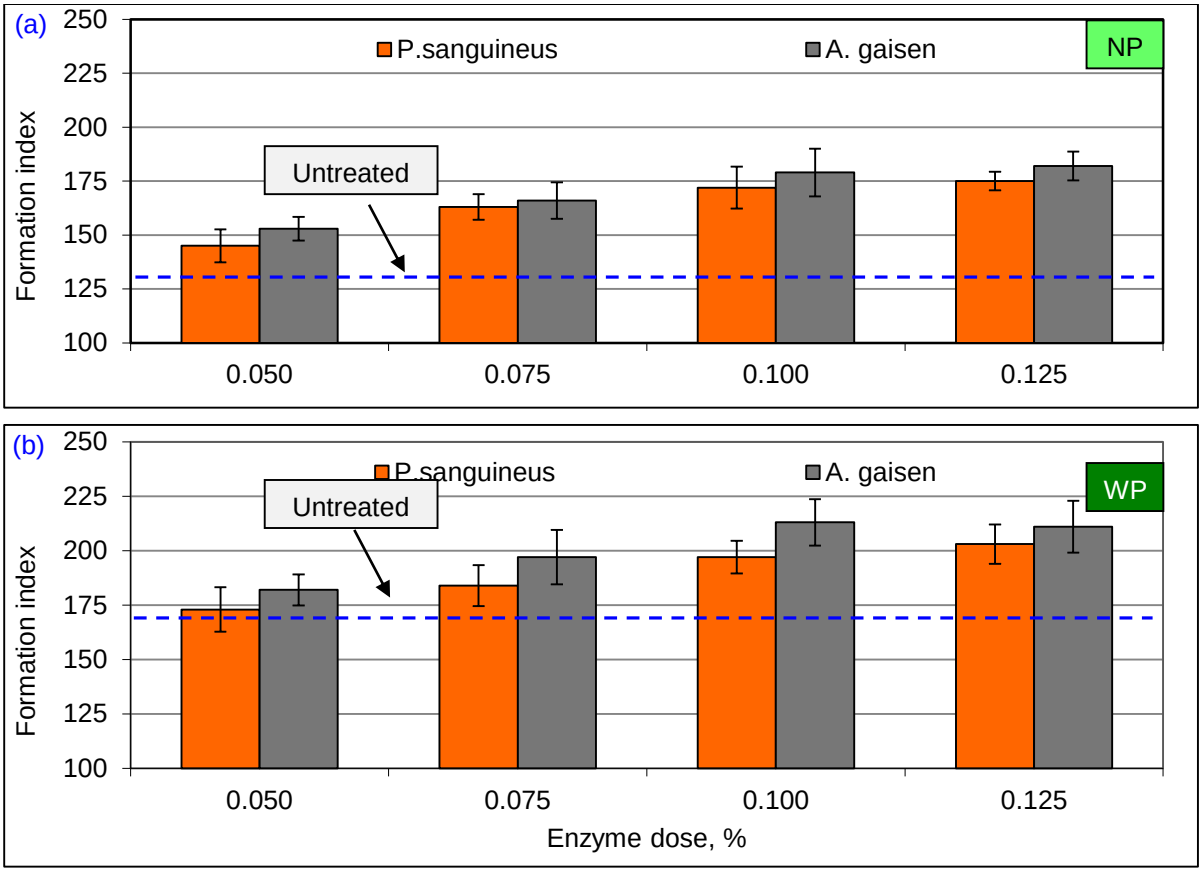

Fig. 6.24 Effect of different enzymes at various doses on formation index of (a) recycled pulp-NP (b) recycled pulp-WP

6.4 Comparison with commercial endoglucanase rich, Fibercare D enzyme (at same EG activity level)

The plots depicting the effect of different isolated and commercial enzymes at same EG activity level (0.08 IU/g of o.d. pulp) on pulp and paper properties of recycled pulp-NP and recycled pulp-WP were plotted in Figure 6.25 and 6.26, respectively.

The endoglucanase enzymes obtained during the research work were comparable to commercial endoglucanase rich, Fibercare D enzyme in decreasing the drainage time. Significant reduction in drainage time by 17.5 and 17.3% was observed in endoglucanase *(P. sanguineus)* treated recycled pulp-NP and recycled pulp-WP, respectively. Similar results showing the improvement in pulp drainage was observed in pulps treated with endoglucanase from *A. gaisen*. No deterrent effect on viscosity of endogucanase treated recycled pulp-NP was observed. However, the viscosity of endoglucanase treated recycled pulp-WP was observed to be reduced (Figure 6.25).

Significant improvement of 15% in tensile index was observed in recycled pulp-NP treated with endoglucanase from *P. sanguineus*. Although, tensile index in recycled pulp-WP, treated with endoglucanase (*P. sanguineus)* was almost similar to untreated pulps. Smoothness of the paper sheets prepared from enzyme treated recycled pulp-NP and recycled pulp-WP was

observed to be improved, as shown in Figure 6.26 as reduction in roughness. Reduction in tear values in enzyme treated pulps was not significant.

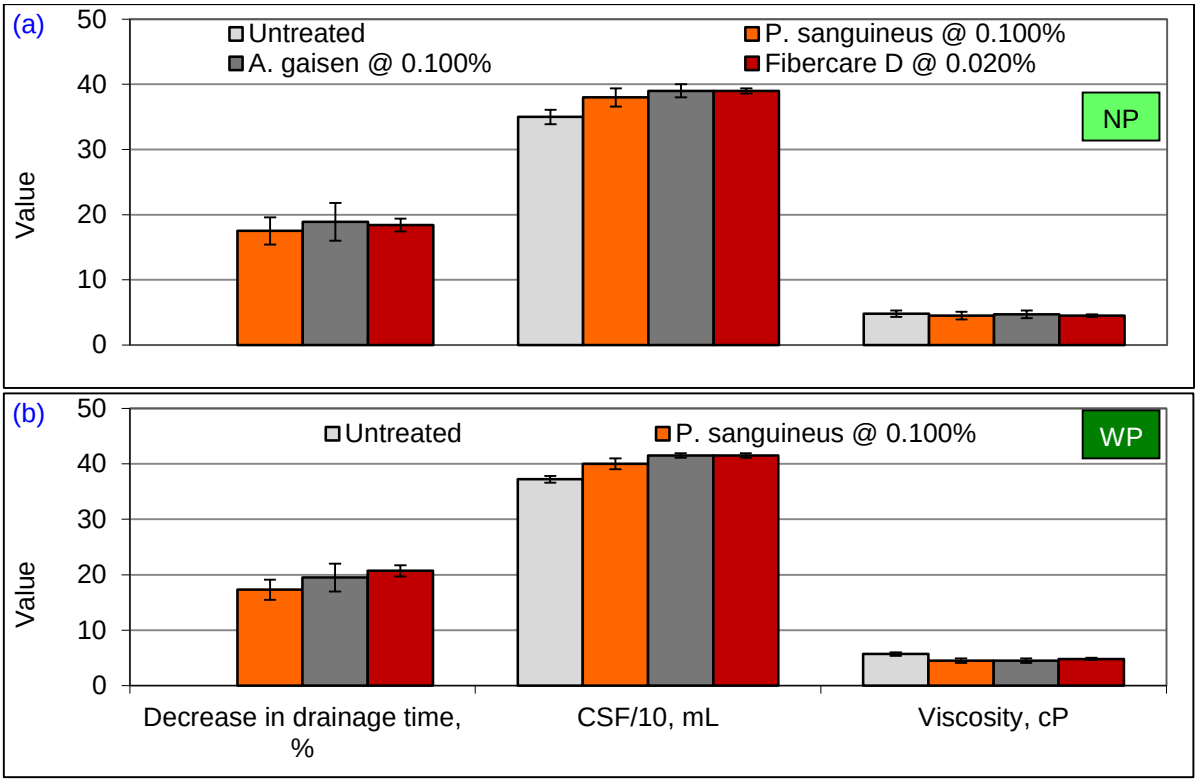

Fig. 6.25 Effect of different isolated and commercial enzymes at same endoglucanase, EG activity levels i.e. 0.08 IU/g of oven dry (a) recycled pulp-NP (b) recycled pulp-WP, CSF value taken as Value/10

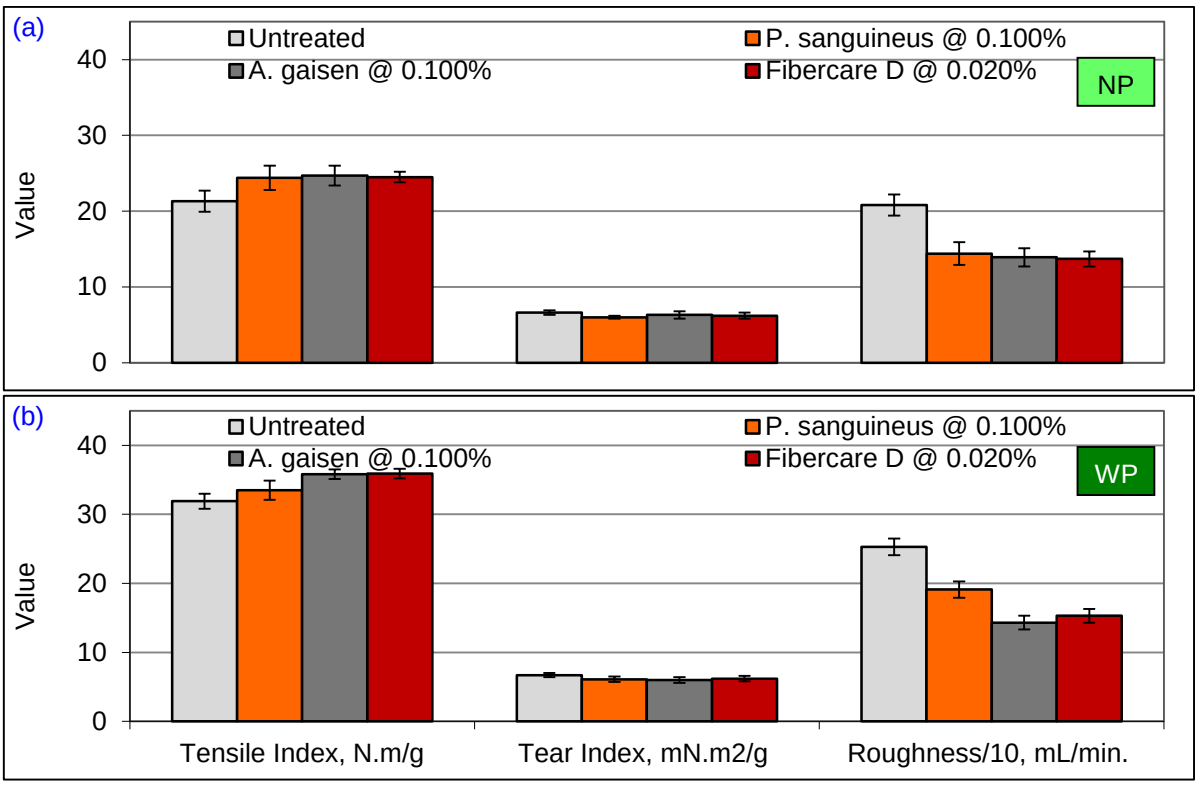

Fig. 6.26 Effect of different isolated and commercial enzymes at same endoglucanase, EG activity levels i.e. 0.08 IU/g on paper properties of oven dry (a) recycled pulp-NP (b) recycled pulp-WP, roughness value taken as Value/10

Table 6.6 Comparison with commercial enzyme (recycled pulp-NP and recycled pulp-WP)

	Recycled pulp-NP					Recycled pulp-WP			
	Untreated	**Fibercare D @ 0.020%**	***P. sanguineus* @ 0.100%**	***A. gaisen* @ 0.100%**		**Untreated**	**Fibercare D @ 0.020%**	***P. sanguineus* @ 0.100%**	***A. gaisen* @ 0.100%**
Pulp Properties									
Decrease in drainage time, %	--	18.4 [1.7]	17.5 [2.1]	18.9 [2.9]		--	20.7 [1.5]	17.3 [2.0]	19.5 [2.7]
CSF, mL	350 [5]	390 [5]	380 [14]	390 [10]		372 [10]	415 [15]	400 [1]	415 [1]
WRV, %	120 [12]	154 [12]	138 [5]	153 [5]		140 [9]	171 [9]	170 [4]	180 [4]
Viscosity, cP	4.8 [0.4]	4.5 [0.4]	4.5 [0.6]	4.7 [0.6]		5.1 [0.5]	4.8 [0.3]	4.5 [0.3]	4.5 [0.1]
Fines content ,%	27.1 [2.1]	25 [1.2]	23.6 [1.7]	21.3 [1.9]		42 [4.1]	33.8 [3.2]	37.0 [1.9]	35.3 [2.1]
Paper Properties									
Tensile index, N.m/g	21.4 [0.8]	24.5 [1.0]	24.4 [1.6]	24.7 [0.2]		31.9 [1.1]	35.9 [0.7]	33.5 [0.7]	35.8 [0.9]
Tear index, mN. m^2/g	6.5 [0.4]	6.2 [0.2]	6.0 [0.2]	6.3 [0.5]		6.7 [0.4]	6.2 [0.4]	6.1 [0.3]	6.0 [0.2]
Roughness, mL/min.	204 [11]	137 [14]	144 [15]	139 [12]		253 [19]	153 [10]	191 [08]	143 [09]
Formation index	133 [14]	195 [5]	172 [10]	179 [11]		170 [19]	212 [14]	203 [9]	211 [12]

Digits in parenthesis indicate the standard deviation

Salient findings

- Two efficient, cellulase producing fungal strains identified as *Pycnoporus sanguineus* PVYA07 NFCCI-3628 and *Alternaria gaisen* PVYA11 NFCCI-3629 were isolated from degraded wood samples.
- Purified cellulosic substrates such as carboxymethyl cellulose (CMC) and Avicel were found to be best carbon source for cellulase production by the two fungal strains.
- Rice bran also proved to be good and cheaper source for cellulase enzyme production.
- Optimum pH for maximum endoglucanase enzyme production by two isolated fungal strains was found to be between 6.0 to 7.0.
- Yeast extract amongst other nitrogen sources was found to be the most suitable inorganic nitrogen source for both CMCase and CBH enzyme production.

- Tween 80 was found to be the best surfactant amongst other ones used in the present study.
- Optimum noculum size, also considered to be an important factor for the enzyme production, was found to be 4 and 6 discs for *P. sanguineus* and *A. gaisen*, respectively.
- *P. sanguineus* and *A. gaisen* exhibited maximum enzyme production at 8 and 5 days incubation time, respectively.
- Maximum enzyme production by both fungal strains was observed to be at 30°C.
- The endoglucanase enzymes were purified to homogeneity by 9.7 and 10.5 fold from the culture filtrates of *Pycnoporus sanguineus* PVYA07 NFCCI-3628 and *Alternaria gaisen* PVYA11 NFCCI-3629, respectively.
- The molecular weight of the endoglucanases, determined by SDS PAGE, were found to be 48 and 61 kDa in case of *P. sanguineus* and *A. gaisen*, respectively.
- Further characterization studies on endoglucanases showed that the enzymes isolated from the two fungal strains were active in broad pH range of 5 to 8 and temperature range of 30 to 60°C with pH and temperature optima at 6.0 to 7.0 and 40 to 50°C, respectively.
- Significant improvement in drainage by 8.0 to 20.1% and 9.3 to 23.2% in recycled-NP and recycled-WP pulps treated with purified endoglucanases from P. sanguineus and A. gaisen respectively was achieved.
- Improvement in tensile index and smoothness was also observed in handsheets prepared from enzyme treated pulps.
- The optimum enzyme dose for improvement in pulp drainage, with no significant reduction in viscosity and tear strength, was 0.100%.
- At same EG activity levels, endoglucanase enzymes produced at lab scale by the two isolated fungal strains were found to be comparable to commercial endoglucanase rich enzyme, Fibercare D in drainage improvement.

CHAPTER 7

Conclusions and Recommendations

The present chapter concluded this thesis by summarizing the results and achievements and recommendations for future work in this area.The broad research objectives (mentioned in Chapter 1) successively attained through the research work presented within this thesis, are further discussed below.

7.1 Conclusions

The present research work demonstrates that applying specific cellulase component, i.e. endoglucanase, rather than multicomponent cellulase can be more effective in improving the pulp drainage. Several commercial cellulase enzymes are available which claim to improve the drainage of secondary fibres. These types of enzymes are to be applied after refining/ beating of the pulp, mainly to improve the dewatering. Using mixtures of cellulases can be disadvantageous for certain pulp and paper properties such as viscosity and tear strength. The study demonstrated that cellulase action is a progressive action and due to higher rate of hydrolysis of amorphous cellulose than that of crystalline cellulose, either monocomponent EG alone or endoglucanase rich enzymes at low dose are required exclusively, for drainage improvement. X-ray diffraction studies also supported the finding that endoglucanases even at a lower dose can increase the pulp drainage by hydrolyzing the most accessible parts of cellulose i.e. ultra fines containing amorphous cellulose and other dissolved colloidal substances and hemicellulose present in the fibers.

Polymer treatment alone at various doses was found to increase the drainage of different pulps in the range of 12 to 25%, but formation was adversely affected due to increased flocculation. It was observed that endoglucanase Fibercare D, in combination with polymeric drainage aid at optimized dose levels, further enhances the drainage of recycled and wheat straw pulps by 25 to 28% without any negative effect on sheet formation and other strength properties. Therefore it can be postulated that enhanced dewatering with similar or even better sheet appearance can be achieved using the combination of enzymes and polymer.

Two unique cellulase producing fungal strains, identified as *Pycnoporus sanguineus* PVYA07 NFCCI-3628 and *Alternaria gaisen* PVYA11 NFCCI-3629 have been isolated among 54 different fungal strains. Rice bran was chosen as a cheaper cellulosic substrate and various other culture conditions like different inoculum size, incubation period, pH, temperature, nitrogen source and surfactant were further optimized to achieve maximum endoglucanase enzyme production. Microbial cellulases are generally active and stable in acidic to neutral range. The enzymes obtained in the present studies were alkaline active and alkali stable

indicating their potential for industrial applications. The carried out investigations on endoglucanase treatments showed an opportunity for significant improvement in dewatering of different pulps at different enzyme doses. The fact that the endoglucanase (EG) rich preparations appeared so promising for promoting drainage, especially at low levels of treatment, suggests that cleavage of nano-scale fines and fibrils into shorter bits may be of dominant importance. These nano-scale ultra fines after enzymatic hydrolysis would be less prone to block the water channels in wet web of paper.

7.2 Recommendations for future work

This thesis includes many multidisciplinary research areas such as fermentation, enzyme production, cellulase enzyme chemistry and selective application of cellulase components for improvement in paper quality and process. In the said thesis, emphasis is given to the usage of monocomponent endoglucanase enzymes rather than the complete cellulase mix for selective and controlled hydrolysis of hydrocolloidal fines fraction. The established effect of mono component endoglucanases for improving pulp drainage can be utilized for enhancing the paper productivity (increased machine speed) and decreasing the dryer steam consumption. The research work done in this thesis has highlighted many new areas for further research to form a firm scientific and technical base. Further research efforts should be carried out in these areas:

- Research studies on evaluation of endoglucanase enzymes for drainage improvement of pulps containing fillers are recommended.
- Multivariate analysis for the optimization of different growth parameters for enzyme production and their application for drainage improvement is recommended.
- Large scale production of enzymes from these isolated fungal strains for other applications such as enzymatic deinking and reduction in refining energy of different pulps is recommended.
- Evaluation of endoglucanase impact on steam consumption in dryer section of paper machine is recommended for further research.
- Impact of endoglucanase application on paper machine speed and paper production should be analyzed.
- Endoglucanase influences on sheet drainage, level of refining, rate of use of chemical additives (especially drainage aids), stock consistencies, headbox parameters, vacuums imposed, press pressures need to be well studied.
- Evaluation of commercial utility of the endoglucanase enzymes for drainage improvement at mill scale is further recommended.

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Annexure-1

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. Piyush Kumar Verma C/o Dr. Yuvraj Singh Negi, Professor and Head , Indian Institute of Technology, Roorkee, Saharanpur Campus, Paper mill Road, Saharanpur, 247001, Uttar Pradesh

Details of Fungus identified

Sr. No.	Culture	NFCCI Accession	Identification Remarks
1	PVYA 07	**NFCCI- 3628**	*Trametes sanguirea* (L.) Lloyd*
2	PVYA 11	**NFCCI- 3629**	*Alternaria gaisen* Nagano

***Current Name:**
Pycnoporus sanguineus (L.) Murrill (http://www.indexfungorum.org/names/Names.asp)

CONDITIONS AND REMARKS:

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2. THE RESULTS HAVE BEEN OBTAINED ON CAREFUL ANALYSIS AND EXAMINATION OF THE SAMPLE ONLY AND IN THE CONDITION RECEIVED.
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Phone: 020-25653680

NFCCI/ 2014-7/AKC 2129-11/SKS/DKM

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)

MOLECULAR IDENTIFICATION REPORT

SENDER : **Dr. Yuvraj Singh Negi, Professor & Head**

INSTITUTE/ORGANIZATION : Indian Institute of Technology, Roorkee, Saharanpur Campus, Paper mill Road, Saharanpur, 247001, UP

ACKNOWLEDGEMENT CODE : **2129**

JOB TITLE : Molecular identification of the fungal isolate **PVYA07**.

PROCEDURE:

- Genomic DNA was isolated in pure form, from the culture provided by the sender.
- The **ITS region of rDNA** was successfully amplified using fungal universal primers ITS4 & ITS5.
- 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.

Results of Molecular Identification:

- The tested fungal isolate **PVYA07** showed **99%** sequence similarity with ***Trametes sanguinea***.
- Sequence analyses with NCBI accession number JX082366.1, ***Trametes sanguinea*** strain BRFM 1114 resulted in following alignment statistics.
- Alignment statistics: Query Length - 558, Score - 1003 bits(1112), Expect - 0.0, Identities - 557/558 (99%), Gaps - 0/558 (0%), Strand - Plus/Minus

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ar Road, Pune - 411 004, India, Phone : (020) 2567-8916/17/18, 2565 - 3680/4357/4106/4097/4167 Fax : (020) 2565 1542

Web : www.aripune.org E-mail : director@aripune.org

Top five hits upon BLAST analysis

Gene Bank Accession No.	Description	Max score	Query cover	E value	Identity (%)
JX082366.1	*Trametes sanguinea* strain BRFM 1114	1003	1003	0.0	99%
JN164981.1	*Trametes sanguinea* voucher CR35	1003	1003	0.0	99%
JF792517.1	*Pycnoporus coccineus* strain Thongkred 013 BCU	1003	1003	0.0	99%
FJ234196.1	*Pycnoporus sanguineus* strain CIRM-BRFM 906	1003	1003	0.0	99%
FJ234193.1	*Pycnoporus sanguineus* strain CIRM-BRFM 902	1003	1003	0.0	99%

Reference:

Altschul, S.F., Gish, W., Miller, W., Myers, E.W. & Lipman, D.J. (1990) "Basic local alignment search tool." J. Mol. Biol. 215:403-410.

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S.K. Singh

Kind Attn:

Dr. Yuvraj Singh Negi, Professor and Head
Indian Institute of Technology, Roorkee,
Saharanpur Campus, Paper mill Road, Saharanpur,
247001, UP

NFCCI/ 2014-7/AKC 2129-10/SKS/AB/DKM

महाराष्ट्र विज्ञान वर्धिनी
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MOLECULAR IDENTIFICATION REPORT

SENDER : **Dr. Yuvraj Singh Negi, Professor & Head**
INSTITUTE/ORGANIZATION : Indian Institute of Technology, Roorkee, Saharanpur Campus, Paper mill Road, Saharanpur, 247001, UP
ACKNOWLEDGEMENT CODE : **2129**
JOB TITLE : Molecular identification of the fungal isolate **PVYA11**.
PROCEDURE:

- Genomic DNA was isolated in pure form, from the culture provided by the sender.
- The **ITS region of rDNA** was successfully amplified using fungal universal primers ITS4 & ITS5.
- 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.

Results of Molecular Identification:

- The tested fungal isolate **PVYA11** showed **99%** sequence similarity with ***Alternaria gaisen***.
- Sequence analyses with NCBI accession number KJ806190.1, ***Alternaria gaisen*** culture-collection FCBP: 1354 resulted in following alignment statistics.
- Alignment statistics: Query Length - 476, Score - 843 bits (934), Expect - 0.0, Identities - 471/472 (99%), Gaps - 1/472(0%), Strand - Plus/Minus

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arkar Road, Pune - 411 004, India, Phone : (020) 2567-8916/17/18, 2565 - 3680/4357/4106/4097/4167 Fax : (020) 2565 1542
Web : www.aripune.org E-mail : director@aripune.org

Top five hits upon BLAST analysis

Gene Bank Accession No.	Description	Max score	Query cover	E value	Identity (%)
KJ160502.1	*Alternaria* sp. ASL-6	850	850	0.0	99%
KJ826508.1	*Alternaria* sp. SW3	843	843	0.0	99%
KJ806190.1	*Alternaria gaisen* culture-collection FCBP:1354	843	843	0.0	99%
JN108906.1	*Alternaria brassicae* isolate M4	843	843	0.0	99%
JN108902.1	*Alternaria brassicae* isolate U7	843	843	0.0	99%

Reference:

Altschul, S.F., Gish, W., Miller, W., Myers, E.W. & Lipman, D.J. (1990) "Basic local alignment search tool." J. Mol. Biol. 215:403-410.

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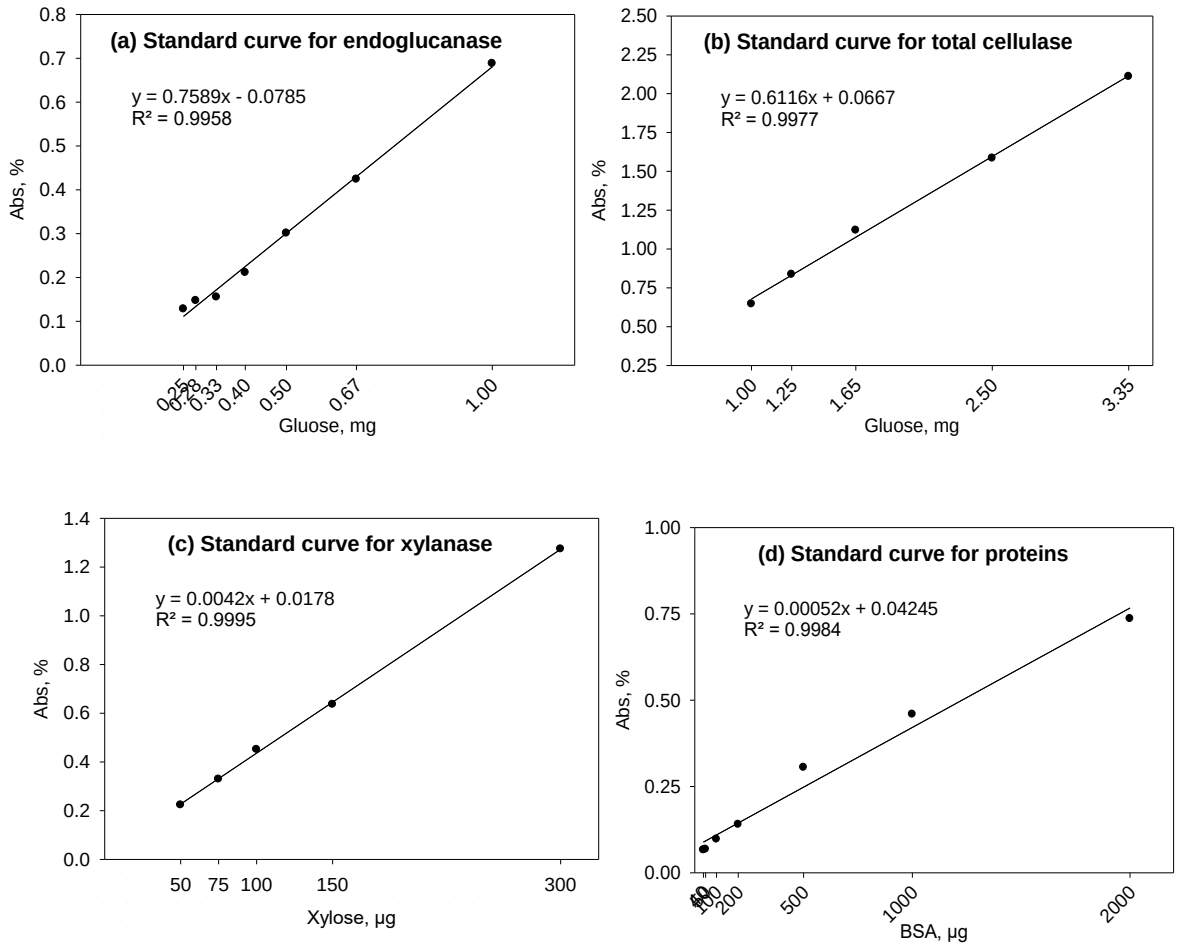
Paper mill Road, Saharanpur,
247001, UP

NFCCI/ 2014-7/AKC 2129-10/SKS/AB/DKM

Annexure-2



Standard curves used for the determination of (a) endoglucanase; (b) total cellulase; (c) xylanase; and (d) protein