STUDIES ON CELLULASE PRODUCTION FOR ENZYME-ASSISTED REFINING OF PULP

Ph.D. THESIS

by

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DEPARTMENT OF PAPER TECHNOLOGY
INDIAN INSTITUTE OF TECHNOLOGY ROORKEE
ROORKEE – 247 667 (INDIA)
JUNE, 2014

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A THESIS

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

of

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in

PAPER TECHNOLOGY

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CANDIDATE'S DECLARATION

I hereby certify that the work which is being presented in this thesis entitled " **STUDIES ON CELLULASE PRODUCTION FOR ENZYME-ASSISTED REFINING OF PULP"**, 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 August, 2008 to June, 2014 under the supervision of Dr. Nishi Kant Bhardwaj, Ex-Assistant Professor, Department of Paper Technology, now, Deputy Director, Avantha Centre for Industrial Research and Development, Yamuna Nagar and Dr. Bijan Choudhury, Associate Professor, Department of Biotechnology, Indian Institute of Technology Roorkee, Roorkee.

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

(Rashmi Singh)

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

(Bijan Choudhury)
Supervisor

(Nishi Kant Bhardwaj)
Supervisor

Date:

The Ph.D. Viva-Voce Examination of **Ms. Rashmi Singh**, Research Scholar, has been held on……

Signature of Supervisor (s) **Chairman, SRC** **Signature of External Examiner**

Head of the Department/Chairman, ODC

Abstract

The present thesis envisages the biotechnological approach for the reduction in refining energy requirement during papermaking process. Refining, one of the crucial step for development of paper strength properties, consumes a large proportion of total electrical energy required by the paper mills. Therefore, there is a need to decrease refining energy demand. Energy reduction is required globally which is directly related to total production cost and profitability. Thus, in the present research work, a biotechnological solution for refining energy reduction is investigated for energy conservation along with pulp/paper quality improvement. Cellulase is one of the most demanded industrial enzymes. Out of its three components viz. exoglucanase, endoglucanase and β-glucosidase, endoglucanase has been paid more attention with a key role in refining behavior (Chapter 1 and 2).

This research work includes the isolation and screening of cellulase producing fungi. Subsequently, optimization for cellulase production was carried out under solid state fermentation using agro-based materials. Thus obtained crude cellulase was characterized for optimum pH and temperature with their stabilities. In the present research work, optimization studies for pre-treatment of bleached mixed hardwood pulp at different enzyme dosages and reaction times were performed keeping in view the industrial process conditions for temperature, pH and consistency. The enzyme treated pulps were subjected to refining in laboratory PFI mill to achieve a fixed value of freeness. After refining, both untreated as well as enzyme treated pulps were analyzed for reducing sugars content, fines content, water retention value and viscosity of pulps. The handsheets were prepared and the impact of different enzyme treatments on paper strength properties was studied. Fibrillation and inter-fiber bonding were observed through scanning electron microscope images of pulp handsheets. Crude fungal enzymes were evaluated for their potential use in reduction of refining energy. Furthermore, commercial enzymes (CA-endoglucanase and CB-cellulase+xylanase) were also tested for comparative evaluation of refining efficiency with laboratory produced fungal enzymes (Chapter 3).

Newly isolated cellulase producing fungal strains were identified as *Rhizopus microsporus* (UPM0810n/RS1-NFCCI 2927) and *Penicillium oxalicum* (UPS1010B/RS2-NFCCI 2926) isolated from soil and wood samples, respectively. The finding from this work favors that solid state fermentations can be used for cellulase production by using agro-wastes. Among various carbon sources (agro-wastes) tested for cellulase production wheat bran was observed to be the best carbon source for both of the isolated fungal strains. Particle size of substrate was found to have a little

effect on cellulase production. For *R. microsporus* mesh size 36 and for *P. oxalicum* mesh size 44 were observed to give maximum cellulase production. An incubation temperature of 38 °C was found to be optimum for *R. microsporus* and 34 °C for *P. oxalicum*. Endoglucanase production peaked to maximum after 5 days for *R. microsporus* and 4 days for *P. oxalicum*. Total cellulase production peaked to maximum after 6 days for *R. microsporus* and 4 days for *P. oxalicum*. Optimized inoculum size was 6 discs for *R. microsporus* and 8 discs for *P. oxalicum*. An initial pH 6.5 was found to be optimum for *R. microsporus* and 5.5 for *P. oxalicum* for endoglucanase production. Moisture content of 1:3.5 (Solid:Nutrient salt solution ratio) was observed to be the optimum for *R. microsporus* and 1:3 for *P. oxalicum*. Nitrogen sources were found to affect biomass and cellulase production. For *R. microsporus* soya peptone-2 g/L (organic nitrogen source), ammonium nitrate-0.03 M (inorganic nitrogen source) and mustard cake+wheat bran in 1:3 ratios were best (agro-waste). However, for better production ammonium nitrate was chosen among three best sources. For *P. oxalicum* yeast extract-4 g/L (organic nitrogen source), ammonium nitrate-0.03 M (inorganic nitrogen source) and soyabean hulls+wheat bran in 1:3 ratios (agro-waste) were best. Soyabean hull was chosen as best nitrogen source for *P. oxalicum*. Among various surfactants studied Tween 80 gave the best cellulase production for both *R. microsporus* and *P. oxalicum* (Chapter 4a).

After optimization, cellulase production was found to be enhanced for both the fungi on agro-wastes by upto two to three fold. Crude enzyme of *R. microsporus* was found to be active in a wide range of pH 4.0-9.0 (optimum 5.5-6.0) and temperature, 40-60 °C (optimum at 55 °C). Crude enzyme of *P. oxalicum* has been observed to have broad pH range from 3.0 to 8.0 (optimum pH 4.5-5) and temperature, 40-60 °C (optimum 50-55 °C) (Chapter 4a).

Fungal enzymes have shown the potential for refining of bleached mixed hardwood pulp. Crude enzyme can be used directly for treatment of pulp before refining under optimized conditions to get improved pulp/paper quality in addition to energy conservation. An enzyme dosage of 0.08 IU/g OD pulp for reaction time 1 h was found to be favorable for laboratory produced enzyme of *R. microsporus* (LA). At this optimized condition of pulp pre-treatment, PFI revolutions were decreased by 32% than those for control pulp. Paper properties improved for tensile index by 25%, burst index by 22% and tear index reduced by 9% in comparison to control pulp. For laboratory produced enzyme of *P. oxalicum* (LB) an enzyme dosage of 0.05 IU/g OD pulp for reaction time 1.5 h was the best. At this optimized range PFI revolutions were decreased by 28% compared to control pulp.

Paper properties improved for tensile index by 22%, burst index by 20% and tear index reduced by 8% in comparison to control pulp (Chapter 4b).

Refining efficiency of commercial enzymes was also tested for comparative evaluation of refining potential between laboratory produced enzyme and commercial enzymes. For commercial enzyme, CA 0.07 IU/g OD pulp and 1.5 h pre-treatment time was found to be optimum. At this optimized range PFI revolutions were decreased by 25% than control pulp. Paper properties improved for tensile index by 21%, burst index by 19% and tear index reduced by 10% compared to control pulp. For commercial enzyme, CB 0.06 IU/g OD pulp and 2 h reaction time was optimal for refining application. At this optimized range PFI revolutions were decreased by 20% than control pulp. Paper properties improved for tensile index by 15%, burst index by 13% and tear index reduced by 14% than control pulp. These fungal enzymes were found to be active in application conditions (temperature, pH and consistency) and showed interesting results for enzyme-assisted refining of pulp (Chapter 4b).

There was a refining energy reduction (based on PFI mill revolutions) for LA by 7% and 3% than revolutions for CA and LB, respectively. Tensile index was improved by 4% for LA and 1% for LB; burst index was improved by 3% for LA and 1% for LB; Tear index was reduced slightly less by 1% for LA and LB, when compared to CA and 4% to CB (Chapter 4b).

These fungal enzymes were found to be active in application conditions (temperature, pH, consistency). Overall, i*n the present research work, a successful reduction in refining energy is achieved with a positive impact on pulp/paper quality.* As the results for refining efficiency showed better results for fungal enzyme in crude form than CB and comparable results commercial enzyme made of monocomponent (CA), it can be concluded that these enzymes are efficient in refining of bleached hardwood pulp, eliminating a need to purify enzyme. Also, the work for fungal enzyme produced by the specific genera of *Rhizopus* and *Penicillium* for Indian pulp conditions is first time performed for refining application. Hence, these fungal enzymes can be used as effective refining agents, beneficial to pulp and paper industry (Chapter 5).

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(Rashmi Singh)

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Abbreviations

AA	Ammonium acetate
AC	Ammonium chloride
AN	Ammonium nitrate
AS	Ammonium sulphate
BE	Beef extract
BG	Sugarcane bagasse
C/N	Carbon to nitrogen ratio
CA	Commercial enzyme A
CB	Commercial enzyme A
CB	Corncob
CC	Coconut coir
CED	Cupriethylenediamine
CMC	Carboxymethyl cellulose
CMCase	Carboxymethyl cellulase activity
CO_2	Carbon dioxide
CS	Castor seeds
CSF	Canadian standard freeness
cy	Consistency
DNS	3, 5 dinitrosalicylic acid
FPase	Filter paper activity
FPU	Filter paper units
g/T	Gram per tonne
GC	Gram (pulse) seed coat
gds	Gram dry substrate
GS	Groundnut shell
gsm	Grammage
ITS	Internal transcribing spacer
IU	International units
LA	Laboratory enzyme A (Crude enzyme of *Rhizopus microspores)*
LB	Laboratory enzyme B (Crude enzyme of *Penicillium oxalicum)*
LS	Red lentil (masoor) seed coat
ME	Malt extract
MHW	Mixed hardwood
MP	Meat peptone
MS	Mustard seeds
MyP	Mycological peptone
NFCCI	National fungal culture collection centre of India
NSS	Nutrient salt solution
o.d.	Optical density
OD	Oven-dry
P	Primary layer
PP	Proteose peptone
RH	Rice husk

RS	Rice straw
S (S1, S2, S3)	Secondary layer
SB	Soyabean hulls
SD	Standard deviation
SEM	Scanning electron microscope
SM	Soya bean meal
SmF	Submerged state fermentation
SN	Sodium nitrate
SP	Soya peptone
SSF	Solid state fermentation
T	Temperature
TP	Tryptone
UA	Urea
US	Unscreened
UV-Vis	Ultra violet-visible
WB	Wheat bran
WD	Wood saw dust
WRV	Water retention value
WS	Wheat straw
YE	Yeast extract

Chapter 1
Introduction

Paper industry is one of the most energy intensive industries in the world (Abdelaziz *et al.,* 2011). Out of the total electrical energy required by a paper mill, a large part (approximate 30%) is consumed in refining step of papermaking process (Lecourt *et al.,* 2010). Refining is the mechanical treatment of fibers that provide desirable characteristics essential to make paper with some mechanical strength. The main effects of refining on fiber are fibrillation development, fines formation, fiber shortening or cutting, curling or straightening etc. (Hagemeyer *et al*., 1992). Thus, this process is important in providing strength to paper sheets by promoting the bonding potential among fibers. Strength is desired to withstand both the converting operation and the end use of the product. Refining can be performed in PFI mill or Valley beater in laboratories and disc or conical refiners in paper mills.

The ultimate need for paper industry is to reduce huge energy demand. The consumption of electrical energy has increased many folds with the pace of development, and now energy is a scarce and costly commodity. With the depletion in available energy and the high-energy cost, it is necessary to take a step for energy conservation in pulp and paper industry. Any approach that can save the energy in refining process will be beneficial for global pulp and paper industry.

Attempts are being made to reduce refining energy demand in the past three decades. Popular concepts proposed in this regard include advances in process control and machinery, like experimenting with regulating devices (feed rate and refiner feed) (Floden, 1979), controlled power supply to the refiner and the mass flow rate of stock (Burkitt *et al.,* 1980), steam balancing methods (Kuisma, 2002), changes in refiner plate design (Kure *et al*., 2000) or use of abrasive materials in refiner plates (Somboon *et al.,* 2007). Other approaches include changes in refining consistency or intensity (Miles *et al*., 1991; Alami *et al*., 1997), use of chemicals like alkali or acids (Engstarnd *et al*., 1991; Walter *et al.,* 2009) and products like starch, carboxymethyl cellulose etc. (Schellhamer *et al*., 1991; Schellhamer *et al.,* 1992; Cheng *et al*., 2013). All these technologies can be effective but are expensive and in some cases difficult to manage at an industrial level (Lecourt *et al.,* 2010a).

In recent years 'Biotechnology' has emerged as a powerful tool in various applications for paper industry (Wong and Mansfield, 1999; Bajpai, 2011). Some interesting applications are biopulping

(Singh *et al.*, 2010), biodelignification (Gupta *et al*., 2012; Saini *et al*., 2011; Saini *et al*., 2013), biobleaching (Choudhury *et al.*, 2006; Sanghai *et al*., 2009; Dhiman *et al*., 2009), biodeinking (Pathak *et al*., 2010; Lee *et al*., 2013; Lee *et al*., 2013a; Soni *et al*., 2010; Ibarra *et al*., 2012), fiber modifications (Bhardwaj *et al.*, 1996; Yang *et al*., 2007; Yang *et al*., 2008), drainage improvement (Taleb and Maximino 2007), effluent treatments (Sahoo *et al*., 2005) and biofilm control (Torres *et al*., 2011). Biotechnology is the integration of natural and engineering sciences. It is defined as the use of living organisms, or their products, for human benefit (or for benefit of human surroundings) like making a product or solve a problem (Pele and Carmen, 2012). The scope of biotechnology is very broad covering plants, animals, microbes in agricultural, veterinary, environmental, food/beverages, medical, industrial fields etc. A valuable biotechnological approach for industrial sector is the use of 'Enzymes'. These are protein molecules that act as catalysts speeding up the chemical reactions under a narrow range of conditions. There are different classes of enzymes depending on the reaction catalyzed: oxidoreductase, transferase, hydrolase, lyase, isomerase and ligase (Wolfgang, 2007).

Spurred by the recent advances in technologies, one of the steps forward in making good quality paper with energy conservation is the introduction of 'Enzyme-assisted refining'**.** Use of enzymes at a pre-refining stage can give a gentle and targeted refining with the benefits of energy reduction and improved paper properties. Hydrolytic enzymes, like cellulase and hemicellulase have gained popularity for energy reduction. Cellulase has attracted more attention and endoglucanase component of cellulase system is found to have a key role in refining behavior (Kamaya, 1996; Clark *et al*., 1997). Currently, researchers are focusing more on cellulase-assisted refining (Gil *et al*., 2009; Lecourt *et al*., 2010a; Yang *et al*., 2011; Liu and Hu, 2012; Garcia-Ubasart *et al*., 2013) than other enzymes.

Besides pulp and paper industry, cellulase has importance in textile industry, laundry detergents, food/feed processing and biofuels production. As there is an increasing demand for cellulase all over the world, *continuous efforts are being made by scientists to find new* cellulase producing microbes with novel properties. Therefore, ***one of the aims*** *of the present research work is production of cellulase effective in refining application.*

Cellulase production can be achieved using microorganisms such as bacteria (e.g. *Clostridium, Ruminococcus*), actinomycetes (e.g. *Streptomyces, Thermoactinomycetes*) and fungi (e.g. *Trichoderma, Aspergillus*). Recent evidence suggests that filamentous fungi are of main industrial

importance especially those belonging to zygomycetes family (Ferreira *et al*., 2012 and Singhania *et al*., 2010). These are known to produce hydrolytic enzymes with high extracellular activity. *The technique available for enzyme production from microorganisms is 'fermentation'.* It may be carried out in solid as well as in submerged state, either in continuous or fed-batch modes. Fermentation carried out without free water is known as solid state fermentation (SSF) whereas other type with high liquid content is known as submerged state fermentation (SmF). SSF has many advantages over SmF (Holker *et al*., 2004) and is of special interest in processes using crude fermented product like enzyme (Tengerdy, 1998; Pandey *et al.,* 2000). SmF is exploited in industries because of advantages of better monitoring and handling, but now advancement is also being made for SSF, which resulted in high titers of enzyme production (Singhania *et al*., 2010). SSF is predominantly suitable for various biotechnological applications using agro-industrial wastes such as paper mill sludge, sugarcane bagasse, wheat bran, rice bran, rice straw, wheat straw, kinnow pulp, apple pomac etc. (Dhillon *et al*., 2011; Maheshwari *et al*., 1994; Adsul *et al*., 2004; Adsul *et al*., 2009; Annamalai *et al*., 2013; Jatinder *et al*., 2006; Kumar *et al*., 2013; Oberoi *et al*., 2010; Dhillon *et al*., 2012 , Dhillon *et al*., 2012a; Sandhu *et al*., 2013; Karimi *et al*., 2006; Shrivastava *et al*., 2012; Dhillon *et al*., 2013) . These materials are nutrient rich cheap substrates often used in enzyme production. Thus, SSF is regarded as *the most economic method* for microbial cellulase production (Jecu, 2000; Holker and Lenz, 2005; Lee *et al*., 2013b) using fungi. Secretion of extracellular enzyme makes fungi to be grown easily on economically feasible and cheap agro-wastes. Thus, it can carry out fermentation and enzyme production.

The idea behind the use of enzyme-assisted refining is mainly inspired by the increasing interest in energy conservation for pulp/paper industry warranting a search for potent enzymes to be used in refining. Only a few studies have been performed on enzyme application (in purified or crude form) in refining of pulp focusing enzyme production from both the bacterial and fungal genera. Microorganisms in wild or recombinant form are employed to produce enzyme which can assist in refining of different grades of pulp (Wong and Saddler, 1992; Oksanen *et al*., 2000; Michalopoulos *et al*., 2005; Laothanachareon *et al*., 2010; Ko *et al*., 2010; Zhang *et al*., 2013). To gain benefits of saving energy and achieve quality product, there is a need to study the process parameters for the particular type of fibers and enzymes.

Hence, the ***first step towards this research work*** was to search for potent fungal strains for cellulase production capable to grow actively under SSF conditions on agro-wastes. To achieve this objective,

the work was started with the isolation and screening of fungi from natural habitats like soil, decaying wood, and pulp/paper mill sites etc. for cellulase production. *It was found in the preliminary study of this work that the agro-based materials obtained from wheat, rice, sugarcane, corn, coconut, wood etc. can be used as substrate for enzyme production.*

For using these materials as medium component, the material should have some desired properties: it should provide a good solid support along with nutrients supply, easily accessible, must aid in microbial growth and must be porous enough for proper aeration. By using materials as above, one can fulfill the requirements of carbon source but it is desired to study for other important nutrients also such as nitrogen source. *Thus,* ***another aim*** *is to optimize medium components for enhanced cellulase production.* In this work, optimization of medium components for nitrogen source was done based on natural materials in addition to synthetic (organic and inorganic) sources. Fungal growth and enzyme production is expected to achieve the best in SSF conditions, as it resembles with their natural habitat. The use of agro-based materials is one of the present trends in enzyme production by fungi, because they can act as an inert matrix offering good nutrient base. The use of agro-waste residues is an economically feasible option as it also solves the problem of waste disposal, which may otherwise cause environmental pollution (Singhania *et al*., 2009). Continuing optimization of different agro-waste materials as substrates, different parameters including nutritional and environmental etc. were studied followed by enzyme production and characterization.

Though, cellulase has main role in refining energy reduction but it also seems to reduce important paper strength properties such as tensile index (Oksanen *et al.,* 1997; Seo *et al.*, 2000). Here, the ultimate question that comes in mind is: "*whether the use of hydrolytic enzyme can bring benefits of energy reduction without diminishing paper properties or some improvement can be achieved?"*

To satisfy this query, optimization studies were performed for enzyme-assisted refining to obtain a best balance of energy savings and paper strength properties for mixed hardwood pulp (MHW). Work is performed on hardwood fiber because of its ease of availability, as in India, hardwood pulp is used in manufacturing most popular grade of paper i.e. writing/printing paper in majority of paper mills (Walker, 2006; Sharma *et al*., 2010).

Refining studies were carried out using PFI mill preferably over Valley beater. It requires less amount of pulp sample in a refining experiment than Valley beater. Besides this, it has advantage of reflecting actual mill refining energy requirements (Lumianinen, 2000; Ko *et al.*, 2010). Furthermore, PFI mill revolutions can be easily customized and because of its operation at 10%

consistency, it avoids interference from fluid to refining mechanics (Cheng *et al.*, 2013). Therefore, in this work PFI mill was used to investigate the refining performance for commercial as well as laboratory produced crude fungal enzymes.

The present research work is undertaken with the following objectives:

1. To produce and characterize the enzyme (cellulase) by the selected fungal strains.
   (i) Isolation, screening and identification of cellulase producing fungi
   (ii) Optimization of different nutritional and environmental factors affecting cellulase production under SSF conditions
   (iii) Production and characterization of crude enzyme
2. To determine the effect of obtained crude enzymes on the refining operation taking MHW pulp after optimization of the process variables that affect pulp/paper properties
3. To determine the effect of commercial enzymes on the refining operation taking MHW pulp and after optimization of the process variables that affect pulp/paper properties
4. To compare the data obtained from application of crude enzymes produced by isolated fungal strains (produced in laboratory) and commercial enzymes for enzymatic refining with that obtained from conventional mechanical refining
5. To investigate the fiber morphology after treatment on pulps with crude enzymes produced by isolated fungal strains (produced in laboratory) and commercial enzymes

The work has been organized as follows:

Chapter 1: Introduces about refining and its importance in papermaking. The present scenario of available solutions to reduce refining energy requirements is discussed. Further, it also deals with biotechnological approach of *'Enzyme-assisted refining'* as an effective solution with emphasis on enzyme production from agro-waste materials.

Chapter 2: Highlights the basic idea behind biotechnological approach for refining. The ultimate focus of this chapter lies in enzyme production and application of enzymes in refining.

Chapter 3: Descriptions have been given for all the materials, methods, standard techniques or protocols used in the present work.

Chapter 4: Details about results and discussions.

Section a: It deals with the isolation, screening and identification of potent cellulase producing fungal strains. Optimization studies performed for the factors affecting extracellular enzyme production such as nutritional and environmental etc. It discusses results with reference to the reported literature. Finally, description of enzyme production and characterization is presented.

Section b: This part focuses on application of enzyme before refining by pre-treatment of pulps and details of study performed for energy reduction and pulp/paper quality evaluation. Laboratory produced crude fungal enzymes and commercial enzymes are explored for refining efficiency. A comparative evaluation of refining performance among the laboratory produced crude fungal extracts and commercial enzymes is done.

Chapter 5: This chapter concludes the work and also depicts future perspectives.

Chapter 2

Review of literature

Paper is network of cellulosic fibers. It may be defined as felted sheet of fibers formed on a fine screen from a fiber-water suspension or pulp slurry.

The general steps in papermaking process are as follows (Hagemeyer *et al*., 1992):

Figure 2.1. General steps in a papermaking process

(i) *Availability of cellulosic fibers:* It can be obtained from softwoods (e.g. Spruce, Aspen), hardwoods (e.g. Eucalyptus, Poplar) or non-woods (e.g. Grasses-Bamboo, Kenaf) etc. Debarking and chipping is done to form small wood chips.

(ii) *Pulping process:* Wood chips are cooked with chemicals for fiber separation, the cooked fibrous material thus obtained is known as pulp.

(iii) *Bleaching process:* It turns color of pulp white by removal of lignin content from fibers.

(iv) *Refining process:* It makes pulp fibers to be acceptable for papermaking by their morphological development.

(v) *Sheet formation:* Pulp is subjected to sheet formation.

(vi) *Pressing:* Excess water from sheet formed is drained in this step.

(vii) *Drying:* In this section the wet sheets are dried.

2.1. Refining

It is a process of fiber treatment implying both of the mechanical and hydraulic forces and/or mechanical crushing or abrasion caused by beating equipment in an environment of polar penetrating liquid, water (Clark, 1985). If paper is made from pulp with fibers in unrefined condition, the sheet formed will be bulky with very weak web and hence, paper strength properties will be low. Thus, step of refining is subjected to fibers which results in mechanical alteration of fiber to make them readily acceptable for papermaking. It is responsible to form strong paper web by surface modifications of fibers, thus providing strength to paper sheets (Bhardwaj *et al*., 1996). Refining is required for both the end use of the product and the converting operation.

In paper mills, refining operation may be performed using disc or conical refiners whereas in laboratories it is performed in PFI mill or Valley beaters. It is found that most of the laboratory scale studies used PFI mill for refining studies. It may be because sample requirement of both the equipments is very different. The sample requirement for PFI mill is 30 g OD pulp and for Valley beater it is 360 g OD pulp (Standard TAPPI methods T 248 sp-00 & T 200 sp-01). In a PFI mill pulp held inside beater housing is treated mechanically between a roll with bars and smooth walled beater housing, both rotating in same direction but at different peripheral speeds. During this mechanical operation of refining, fibers are treated by repeated compression and shearing forces that bring about changes in fiber properties as described below in section 2.1.1. Freeness testing is the most common test run by papermakers to know the extent of refining for pulp based on drainage properties.

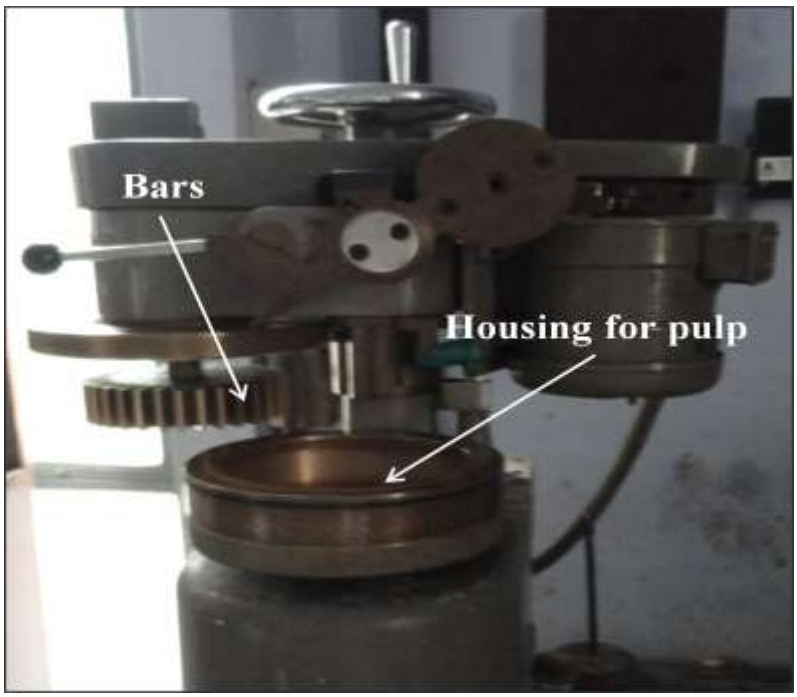


Figure 2.2. A laboratory beating equipment PFI mill

2.1.1. Effects of refining on fiber properties

Structurally, a fiber is composed of cellulose and hemicellulose layers embedded in lignin matrix. It is arranged in layers form as shown in fig. 2.3.

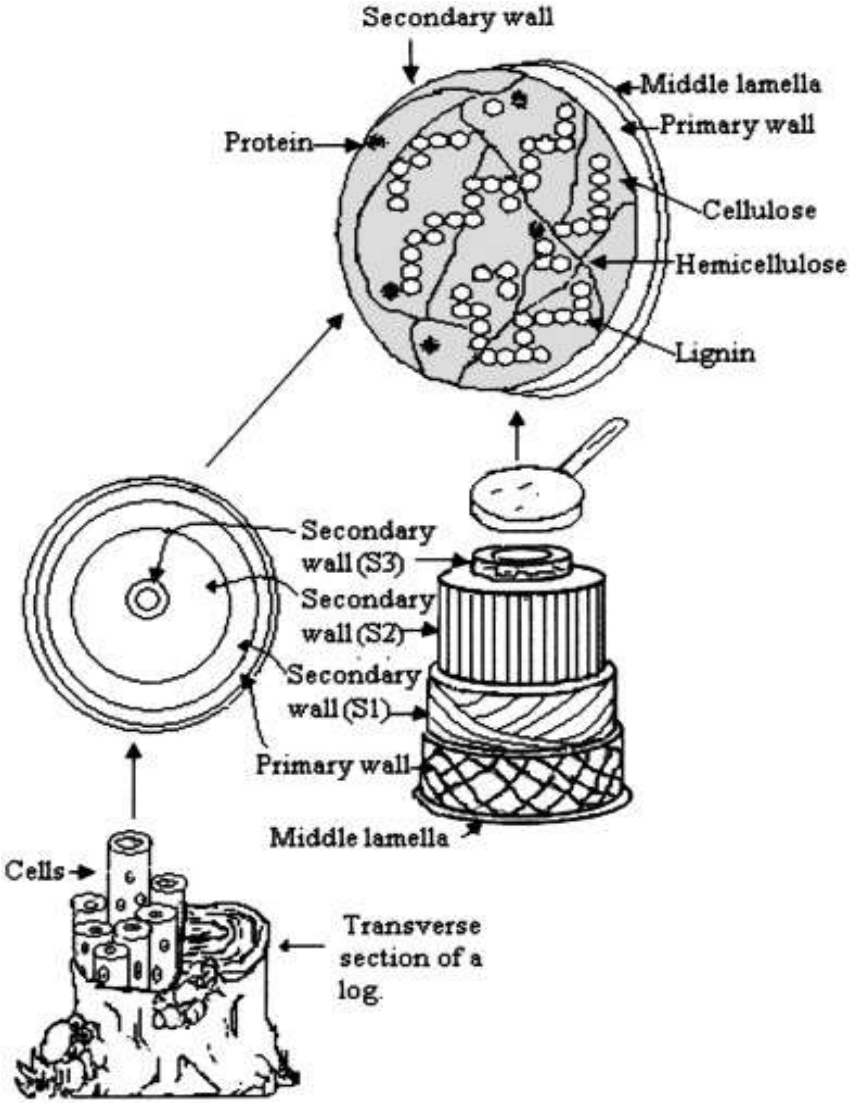


Figure 2.3 Fiber wall layers in a typical wood fiber (adapted from Sanchez, 2009)

The outer P layer is known as primary while inner S layers (S1, S2 & S3) as secondary layers. Out of all, the prime layer of importance in papermaking is S2 layer which has high cellulosic content and is responsible for individual strength of a fiber.

The main contribution towards refining action mechanism on fiber and its effects was given by Page (1989), who provided an excellent review in this regard. The common effects that occur on fiber are as follows:

(i) **Delamination:** Removal of surface covering around fiber i.e. lamina (Page, 1989).

(ii) **Fibrillation:** Emergence of thread like structure 'fibrils'-external and internal fibrillation (Strachan, 1932).

- *External fibrillation*- Outer layers (P and S1) are removed, exposing fibrils of inner S2 layer, which can participate in inter-fiber bonding.
- *Internal fibrillation*- Occurs in inner surface of fiber with delamination, enhancing water holding capacity of fiber.

(iii) **Increased fiber flexibility:** One of the main effects of refining is an increase in flexibility of fiber (Beadle, 1908).

(iv) **Fines formation:** Formation of small sized materials (that pass through 200 mesh size screen) from depth of fiber wall also known as secondary fines (Mancebo and Krokoska, 1985).

(v) **Other modifications:** Includes fiber shortening/cutting, curling/straightening. Shape change from tubular or round form to somewhat flat structure promoting an enhancement in fiber surface area (Beadle 1908, Page *et al.*, 1985).

All together, these effects of refining on fiber properties affect inter-fiber bonding and paper network, and thus the paper strength properties.

2.1.2. High energy demand for pulp refining

Pulp paper industry ranks fourth among most energy intensive industries (Abdelaziz *et al.*, 2011). This industry is a large energy consumer which accounts for almost half of the production cost of paper (Ozalp and Hyman, 2006; Hong *et al.*, 2011). Energy is required in paper mill in the form of heat and electricity in various papermaking operations (Chen *et al.*, 2012). The process of refining requires a large part of electrical energy, which constitutes around 20-30% of the total energy consumed (Lecourt *et al.*, 2010). This corresponds to about 18-25% of the total manufacturing cost (Bhardwaj *et al.*, 1996; Bajapi *et al.*, 2006).

2.2. Use of enzymes in pulp paper industry

There is an intense need to find an alternative, which can help in reducing energy demand in refining, use of enzyme, seeks to address finding the alternative. Currently, enzymes are used in various field of papermaking. A diagrammatic representation is shown below indicating enzymes used in full scale operation, pilot scale and at laboratory level (Fig. 2.4).

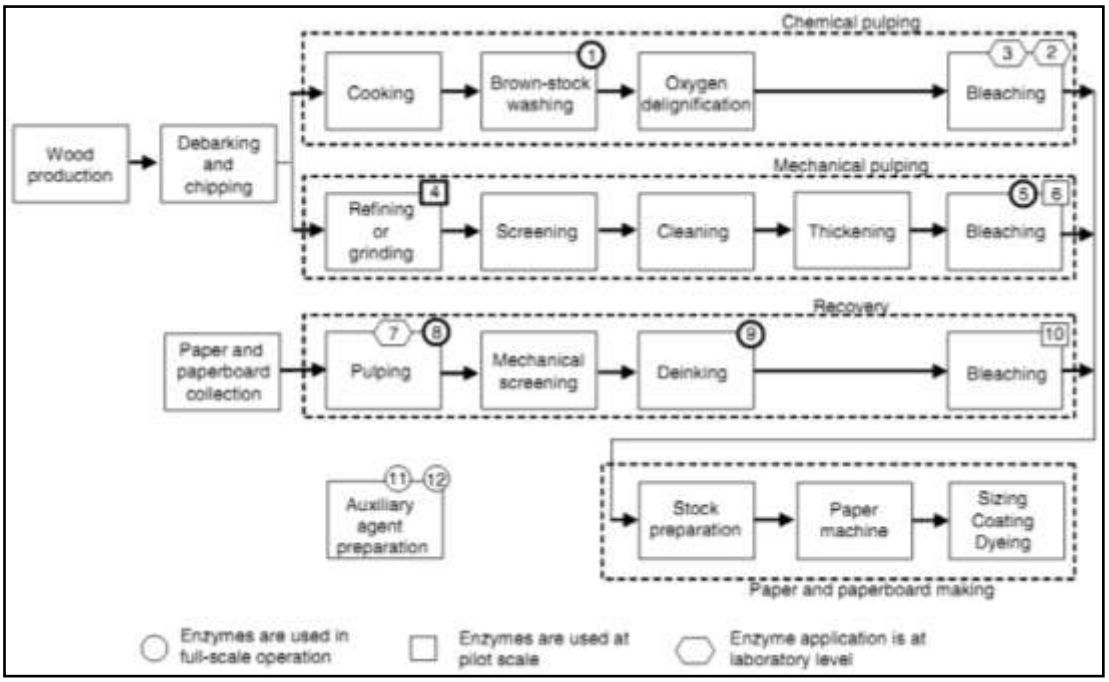


Figure 2.4. Main processes in the pulp and paper industry with indication of enzyme application (adapted from Skals *et al*., 2008)

2.2.1. Enzyme

Enzymes are complex protein molecules that act as catalysts. The activity of enzyme depends on the chemical and physical environment (i.e. temperature, pH etc.), enzyme dosage and reaction time, the type and concentration of the substrate. An enzyme (E) molecule has a highly specific binding site or active site to which its substrate (S) binds to produce enzyme-substrate complex (ES). The reaction proceeds at the binding site to produce the products (P), which remains associated with the enzyme-product complex (EP). The product is then liberated, and the enzyme molecules are freed in an active state to initiate another round of catalysis as depicted in the following equation (Wolfgang, 2007).

$$E+S \longleftrightarrow ES \longleftrightarrow EP \longleftrightarrow E+P$$

2.2.2. Enzymes used in refining

Enzymes can be added at a pre-refining stage by optimizing different process variables like temperature, pH, pulp consistency, enzyme dosage and reaction time for particular type of fiber/pulp used. However, reports are also available for energy reduction by biopulping approach but because of the main drawback of longer reaction time and contamination problem by growth of other unwanted microorganisms, associated with it a pre-treatment stage before refining is preferred for enzyme action on fiber (Singh *et al*., 2010; Torres *et al*., 2012).

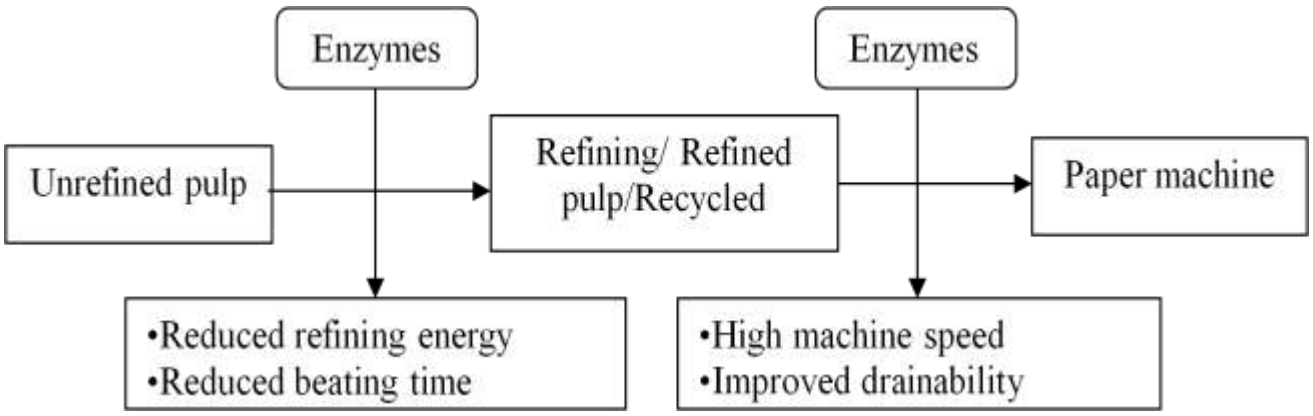


Figure 2.5. The basic model of enzyme-assisted refining

Several enzymes are reported in literature with effect on refining like cellulase, hemicellulase/xylanase, proteinase, laccase, manganese peroxidase, amylase, pectinase etc. (Mansfield *et al.,* 1999; Wong *et al.,* 2000; Sigoillot *et al.*, 2001; Spiridon *et al*., 2003; Meza *et al*., 2006; Surranakki *et al*., 2000; Torres *et al*., 2012). The most dynamically investigated enzyme is cellulase and it stands to be a good choice for refining application. A detailed account for cellulase is given below:

Cellulase

Cellulase is a multicomponent enzyme that acts on its substrate cellulose by hydrolysis of β 1, 4-glycosidic linkage (Young and Akhtar, 1998). The different components for cellulase system are:

- Exoglucanase: acts on the end of cellulose chains by cutting bonds on the end of fiber.
- Endoglucanase: act in the middle of cellulase chain causing hydrolysis of the accessible cellulose.
- β-glucosidase: releases glucose units from cellobiose dimers.

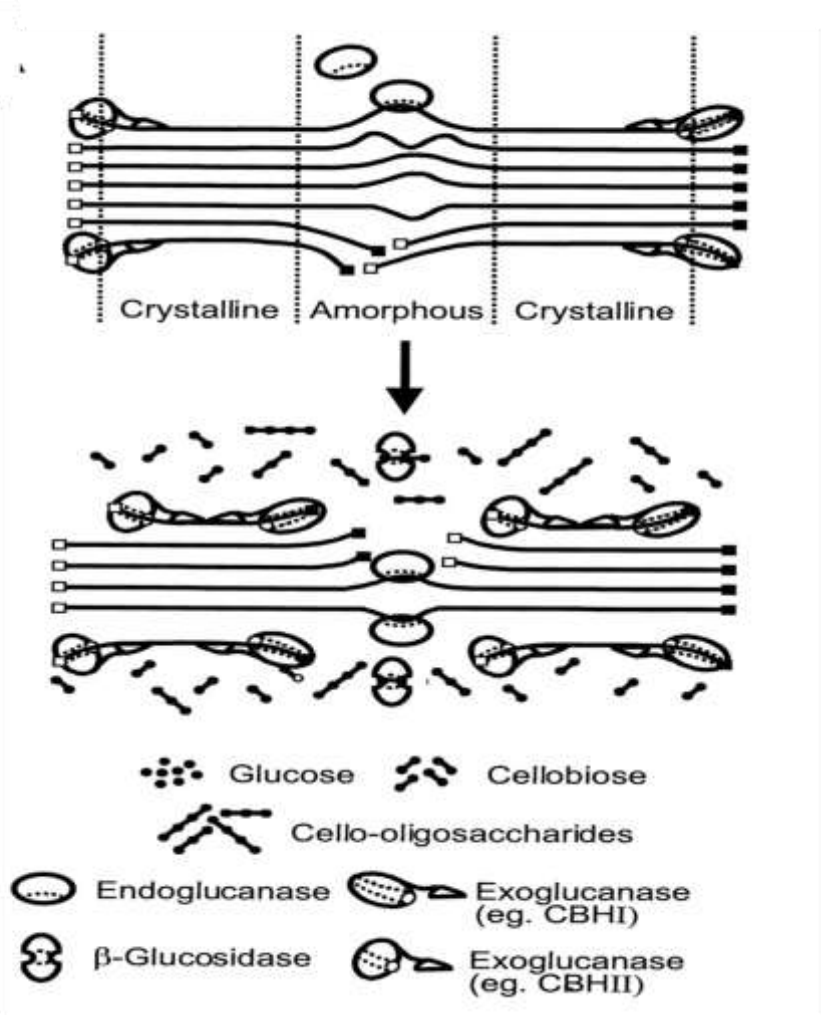


Figure 2.6. A diagrammatic representation of cellulase action on cellulose chain. Endoglucanase acts inside of cellulose chain; exoglucanase cuts ends of the fiber and β-glucosidase release glucose units from cellulobiose dimer (adapted from Lynd *et al.*, 2002).

Studies were performed to explain the cellulase system of both the fungal and bacterial genera (Jermyn, 1952; Coughlan, 1985; Tomme *et al.*, 1995; Wilson 2011). Fungal cellulases are quite simpler than the bacterial system. In case of fungal cellulase, it have two separate domains: a catalytic domain (CD) and another is cellulose binding module (CBM, comprised of approximate 35 amino acids), joined by a short polylinker region (rich in serine and threonine) to the catalytic domain at the N-terminal (Kuhad *et al.*, 2011).

Enzymes are used in paper industry from a long time but gained popularity in last two decades. In 1942, a patent claimed that hemicellulase enzymes from *Bacillus* and *Aspergillus* species aid refining and hydration of pulp fibers (Diehm, 1942). Another patent claimed the use of cellulase enzyme

from *Aspergillus niger* separates and fibrillates pulp (Bolaski, 1962). Yerkes (1968), patented for cellulase enzymes from a white rot fungus to reduce beating or refining time. The interest for cellulase and associated mechanism of enzyme action on cellulose was developed (Lee *et al.*, 1983). After that Noe *et al.*, 1986 reported external fibrillation of bleached chemical pulps and reduced energy demand in the papermaking process by xylanase. The authors concluded that because of enzyme action, removal of xylan may occur and thus, it resulted in decreased fiber intrinsic strength, while an increased bonding ability between fibers is due to increased fiber flexibility.

As cellulase acts directly on the fiber layer of interest (i.e. S2 layer). Most of the cited literature focused cellulase for refining application using fungal, bacterial and commercial enzymes for different pulp grades (Pere *et al.*, 1995; Cochaux and Daveni, 1996; Clark *et al*., 1997; Blanco *et al*., 1998; Yonghua *et al.*, 1997; Seo *et al*., 2000; Surranakki *et al*., 2000; Pastor *et al.*, 2001; Garcia *et al.*, 2002; Esteghlalian *et al.*, 2002; Cadena *et al*., 2010; Lecourt 2010a; Ko *et al*., 2010; Liu and Hu 2011; Laothanachareon *et al*., 2011; Zhang and Hu, 2011; Liu and Hu 2012; Zhang *et al.*, 2013).

Revisions were made several times for role of biotechnology in pulp and paper industry including refining/beating process (Jurasek and Paice 1988; Eriksson, 1990; Vikkari *et al*., 1991; Jeffries 1992; Wong and Saddler, 1992; Kirk and Jefferies, 1996; Eriksson *et al.*, 1998; Bajpai, 1999; Bhat, 2000; Kenealy and Jefferies 2003; Skals *et al.*, 2008; Singh and Bhardwaj 2010; Bajpai, 2011; Torres *et al*., 2012; Bajpai, 2013; Nigam, 2013).

2.3. Impact of enzyme-assisted refining on:

2.3.1. Energy consumption

Earlier it was reported that enzymes can be beneficial in reducing energy requirement at mill scale for various pulp grades (Friermuth *et al*., 1994; Caram *et al*., 1996). Pere *et al*., 1996, reported that the cellulase component cellobiohydrolase I, from *Trichoderma reesei* can be helpful in reducing energy consumption, apparently as the result of selective action on crystalline cellulose. Contrary to that, endoglucanase was reported to be main component for assistance in refining of pulp (Kamaya 1996; Clark *et al*., 1997). Several studies were performed using cellulase/xylanase or their mixtures for refining energy reduction in last decade (Bajpai *et al*., 2006; Tripathi *et al*., 2008; Ahmad *et al*., 2006; Gil *et al*., 2009; Yang *et al*., 2011) showing variable results depending on pulp and enzyme composition used.

2.3.2. Pulp/paper properties

In a previous study Nomura (1985) reported that cellulase can facilitate pulp fibrillation without strength loss. Mansfield *et al.* (1997) used cellulase and xylanase for investigating the nature of changes in fiber and observed the substantial reduction in pore volume of the fibers. They reported that enzyme treatment can erode the surfaces of fibers. In another study, endoxylanases treatment on CTMP spruce fibers resulted in improved surface area of the fibers by fibrillation (Lorenzo *et al.*, 2009). A few studies have also been performed taking recycled fibers (Pommier *et al.,* 1990; Stork *et al*., 1995; Eriksson, 1990). Janardhan and Sain (2006) used fungal enzyme for refining and found significant impact on the defibrillation characteristics of the bleached kraft pulp of northern black spruce fibers.

Enzymes produce a better fibrillation, so paper properties that depend on fibril content are generally affected. Employing the enzymatic approach significant improvement in sheet density, smoothness, burst and tensile indices can be obtained.

2.4. Advantages or benefits of enzyme-assisted refining

Since refining requires significant energy input as well as capital investment for equipment, facilitating the refining process could provide numerous benefits as follows:

2.4.1. Reduction in electrical energy

By applying enzymes, mills can reduce their pulp refining energy requirement. Enzymes are expected to give more benefits to those mills, which are not having captive power generation and/or limited by refining capacity.

2.4.2. Drainage improvement

Many researchers have explored the use of enzyme treatment for increasing pulp freeness. The enzymatic attack involves peeling mechanisms which removes fibrils and leaves the fibrils less hydrophilic and easier to drain. The increase in drainage may also attribute to the cleaving of cellulose on the surface of fines.

2.4.3. Reduction in steam consumption

An improved drainability i.e. better dewatering by using enzymes, so there is decreased steam load for paper drying and reduced steam consumption.

2.4.4. Improvement in paper properties

Enzymes produce a better fibrillation, so paper properties that depend on fibril content are improved e.g tensile strength, burst strength etc.

2.4.5. Better machine runnability

Efficient drainage of pulp furnishes on the wire of paper machines is desired to maximize machine speed. Improved drainage results in shorter time for drying period so paper machine runnability increases and results in enhanced productivity.

2.4.6. Cleaner recycled water

Enzymes partially eliminate fine fibrils and colloidal materials, mainly those contained in the white water loop due to longer contact time with the residual enzyme activities in water. This produces cleaner recycled water, with optimum fine and fibrils content (Bajpai *et al.*, 2006). Enzymatic treatments are useful in cleaning the process water within the mill. Enzymes partially hydrolyze cellulose debris to low molecular weight saccharides that are easily biodegraded in the wastewater treatment system.

2.5. Challenges for enzyme-assisted refining of pulp

Pulp viscosity decreases when cellulases cleave cellulose chains lowering the degree of cellulose polymerization and destroying the fibre integrity. The main challenge in using enzymes to enhance fibre bonding is to increase fibrillation and without pulp/paper quality deterioration. Though promising results have been achieved, yet more intensive studies on enzyme-assisted pulp refining are required to make the process cost effective for easy adoption by paper mills.

2.6. Cellulase production by fungi

2.6.1. Important cellulase producers

The main cellulase producers among fungi are white rot (e.g. *Phanaerochaete, Trametes, Agaricus, Sporotrichum*), soft rot (*Aspergillus, Fusarium, Trichoderma, Pencillium*) and brown rot (*Fomitopsis, Coniophora, Lanzites, Tyromyces*) (Kuhad *et al*., 2011). One of the most promising candidates for extracellular enzyme production is fungal members belonging to zygomycetes family with industrial importance (Singhania *et al*., 2010; Ferreria *et al.*, 2013). The most preferred choice is Phycomycetes (e.g. *Rhizopus, Mucor*), Ascomycetes (e.g. *Aspergillus, Penicillium*) and Basidiomycetes (e.g. white-rot) (Pandey *et al*., 2000).

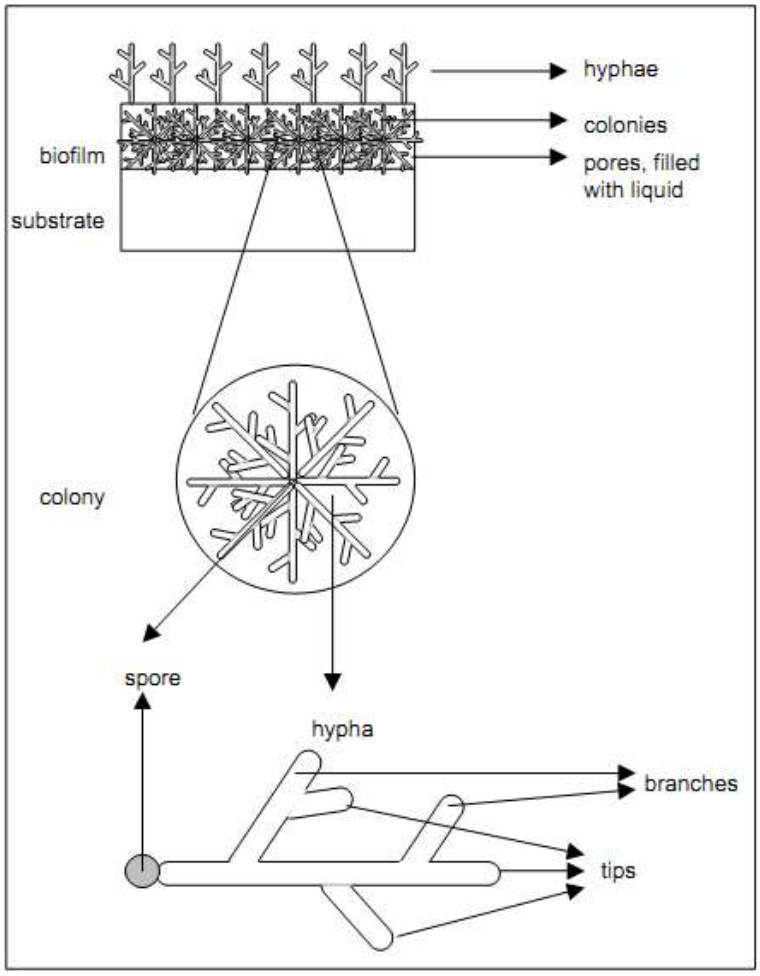


Figure 2.7. Fungal mat formation and mycelial penetration into the medium in SSF (adapted from Rahardjo, 2005)

Filamentous fungi have the ability to grow well on agro-industrial residues because of enough turgid pressure on the tip of their mycelium which helps in easier penetration to access nutrients from raw material used (Raimbault, 1998). With the germination of spore tubular hyphae emerges and elongates towards tip, branches are formed along the hyphae forming a 3-dimensional network known as mycelium. This morphology of fungi helps in easier colonization and penetration on the solid substrate (Fig. 2.7). Therefore, the waste products can be converted to value added products with the utilization of agro-industrial residues as cheap raw material, substrate. For this SSF can be employed, as it resembles with the natural habitat of fungi. (Tengerdy and Szakacs 2003; Singhania *et al*., 2009).

2.6.2. Techniques involved in enzyme production

Process of fermentation is employed for production of enzymes finding industrial importance including cellulase. There are two kinds of fermentation processes: solid state fermentation, SSF (with lesser moisture content) and submerged or liquid state fermentation, SmF (with higher moisture content).

On industrial scale Smf is employed widely but due to numerous advantages of SSF over SmF (Table 2.1) it has gained importance. Several efforts are being made with emphasis on SSF in last two decades. It is reported to be developed on commercial level in various countries such as Japan, India, USA and France (Singh *et al*., 2008).

Table 2.1. Comparison of SSF and SmF type of fermentations (Raimbault, 1998; Mitchell *et al*., 2000; Chen and Xu 2004; Holker *et al*., 2004; Ali and Zulkali, 2011; Hongzhang 2013)

S.No.	SSF	SmF
1.	There is no free water	There is free-flowing water
2.	Medium is simple and unrefined. It may contain all nutrients necessary for growth or only require wetting with a mineral solution	Medium is expensive as require highly processed ingredients
3.	Microbes absorb nutrients from the solid substrate and a nutrient concentration gradient exists	Microbes absorb nutrients from the liquid medium, no concentration gradient exists
4.	There is less chances of contamination due to low water availability	It is relatively high

5.	The culture system consists of gas, liquid as well as solid phase, and gas phase is the continuous phase	The culture system consist of liquid only and liquid is the continuous phase
6.	Energy consumption is low, as the required oxygen is supplied from the gas phase	It requires dissolved oxygen so energy consumption is high
7.	Media are in concentrated form so small bioreactors can be used	It is in diluted form and therefore requires large volume bioreactors
8.	Product yield are usually higher	It is low
9.	Mixing is difficult and growth of microbes thus may be restricted by nutrient diffusion	Mixing is easy, microbes grow easily
10.	Removal of metabolic heat is quite difficult	It is easily control
11.	Heterogeneity	Homogeneity
12.	Process control can be difficult	It is easily monitored/handled
13.	Microorganism takes nutrients by adsorption or penetration on substrate	Uniformly distributes in the culture system
14.	Downstream processing (DSP) is simpler since products are in concentrated form	It requires removal of large volume of water and expensive
15.	Large volume of liquid waste are not produced	It is produced relatively higher
16.	Growth kinetics and transport phenomena are characterized poorly	Information is available to guide for bioreactor design and operation
17.	Low water activity	It is high
18.	Low raw material cost	It is high
19.	Easy product separation	It is relatively difficult
20.	High volume productivity	Low volume productivity
21.	No anti -foam chemicals required	Addition of anti-foam chemical is necessary
22.	Lower sterility demands	High sterility demands
23.	Simulation of natural environment	It does not simulate with natural environment

2.6.2.1. SSF process-general introduction

After commencing of sporulation, the fungal hyphae spread over the solid substrate and develops a mycelial mat. Some hyphae protrude aerially in gaseous phase (aerial hyphae), while some tries to peneterate through the voids spaces between the solid substrate filled with liquid (peneterative hyphae). The metabolic activity related with the microbe used usually occurs near the substrate surface and substrate pores. Due to the hydrolytic action of enzyme produced by mycelium, the macromolecules/polymers are broken up into smaller units or monomers which are taken up as nutrients by the microbe. In this type of fermentation O_2 is consumed and formation of other molecules like CO_2 and H_2O occurs along with some biochemical products of interest.

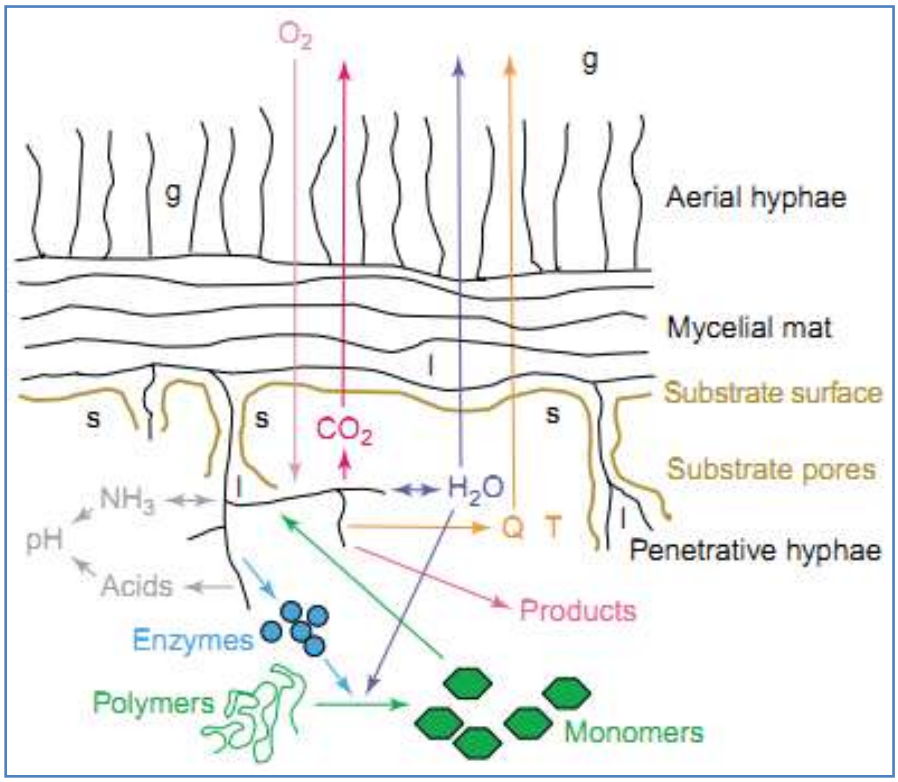


Figure 2.8. Schematic representation of the process that occur during SSF (adapted from Holker and Lenj, 2005)

Thus, diffusion of O_2 occurs in the deeper layers of mycelial mat and CO_2 diffuses out of this mat to the gaseous phase. Development of heat (Q) leads to increment in temperature. This is removed in SSF process by mechanism of conduction or evaporation. H_2O plays an important role in heat removal, and is required for microbial growth by water uptake mechanism (consumption) for hydrolysis reaction. It is affected by microbial respiration too, as it is produced during respiration. Due to the fermentation process the ammonia (NH_3) or acids formed can affect local pH and thus the

formation of product. The product of interest formed can be absorbed onto the solid substrate or in voids, can be obtained by leaching or extraction. Thus, several factors influence SSF process and product formation (Holker and Lenj, 2005).

2.6.2.2. Factors affecting SSF

The first contribution on scientific information on SSF was given by Hesseltine (1977). Based on different parameters of biological, nutritional and environmental conditions the factors affecting SSF process are as follows (Mitchell *et al*., 2000; Prabhakar *et al.,* 2005; Sermanni and Tiso, 2008; Ali and Zulkali, 2011).

2.6.2.2.i. Biological

Septic conditions- It is required to avoid any chances of contamination by growth of other undesired/polluting microorganisms.

Microbiological inoculum- Its type as well as amount influence the growth rate. For filamentous fungi both the vegetative mycelium and spore inoculum may be used. A low amount results in insufficient biomass and product formation whereas high amount results in depletion of nutrients.

Biomass uniformity- It is significant to maintain uniform biochemical reactions all over the biomass.

Morphology of the microorganism- The microorganism must be strong enough in order to assimilate complex/variable mixture of nutrients by penetration into the solid substrates and capable of adherence to mechanical resistance.

2.6.2.2.ii. Nutritional

C/N requirement- For cellular biomass formation both the carbon and nitrogen are important components. Carbon source requirement is fulfilled mainly by raw materials used in SSF, it may provide some nitrogen content too, or nitrogen component is added separately in liquid form with other micronutrients for better growth and product formation. A number of substrates have been used in SSF for cellulase production (Table 2.2.).

Properties of the solid matrix- The matrix should have wide surface area and porosity in SSF; it affects oxygen transfer, as SSF is aerobic process with gas phase as continuous phase. Also, particle

size is an important factor as a very small particle size interferes in microbial respiration and large size offers only a limited surface for microbial attack.

Table 2.2. Composition of various agro-wastes/ lignocellulosic residues used as substrates in SSF

Lignocellulosic residue	**Cellulose (%)**	**Hemicellulose (%)**	**Lignin (%)**	**Reference**
Sugarcane bagasse	44.7	36.9	11.0	Song and Wei, 2010
Wheat straw	31.5	25.2	15.9	Nabarlatz *et al*., 2007
Corn cob	34.45	28.23	16.03	Kumar *et al*., 2010
Rice straw	39	27	12	Karimi *et al*., 2006
Rice husk	36.7	20.0	21.3	Garrote *et al*., 2007
Barley straw	30.8	25.3	16.7	Nabarlatz *et al*., 2007
Barley husk	21.4	36.6	19.2	Parajo *et al*., 2004
Almond shells	26.8	32.5	27.4	Nabarlatz *et al*., 2007
Olive stones	29-34	21-27	20-25	Rodríguez *et al*., 2008
Soya stalks	34.5	24.8	19.8	Nigam *et al*., 2009
Sunflower stalks	42.1	29.7	13.4	Nigam *et al*., 2009
Wheat bran	7.57	31.19	4.06	Sanghai *et al*., 2008
Ground nut shells	35.7	18.7	30.2	Raju *et al*., 2012
Coconut coir	47.7	25.9	17.8	Raju *et al*., 2012
Hemp	70-74	17-22	3.7-5.7	Raju *et al*., 2012
Kenaf	31-39	21.5	15-19	Raju *et al*., 2012
Soyabean hulls	33.49	17.15	9.88	Brijwani *et al*., 2010
Coffee pulp	35	46.3	18.8	Pere *et al*., 2005
Hardwood stem	40-55	24-40	18-25	Sanchez, 2009
Softwood stem	45-50	25-35	25-35	Sanchez, 2009
Bamboo	26-43	15-26	21-31	Sanchez, 2009

2.6.2.2.iii. Environmental

Water activity and moisture content- This may influence biodegradation, biosynthesis and metabolites secretion.

Temperature and heat transfer- This affects bioprocess characteristics, spore germination, cell growth and efficiency of the process.

pH - It affects microbial development and enzyme activities.

Aeration and agitation- It determines O_2 and CO_2 concentration inside the bioreactor.

Table 2.3. Cellulase production by fungi under SSF and bioreactor used

Microorganism (fungi)	**Substrate**	**Fermenter type**	**References**
Trichoderma harzianum T2008	Oil palm empty fruit bunches	Rotary drum	Alam *et al*., 2009
Hypocrea jecorina	Apple pomace	Multi-layer packed bed solid-state bioreactor	Sun *et al*., 2010
Aspergillus niger	Rice straw	Tray (Erlenmeyer flask)	Dhillon *et al*., 2011
Aspergillus niger	Wheat Bran	Instrumented lab-scale bioreactor	Farinas *et al*., 2011
Penicillium verruculosum	empty palm fruit bunch fiber	Solid state bioreactor	Kim and Kim, 2012
Trichoderma viride	Sugarcane bagasse	Rotating fibrous bed bioreactor	Lan *et al*., 2013
Penicillium sp.	Oil palm empty fruit bunch	Rotary drum bioreactor	Kim *et al*., 2014

2.6.3. Challenges in SSF

The main challenge in SSF is scale up. Therefore, attempts are being made in this regard for advancement in SSF with approach of biochemical engineering (Singhania *et al*., 2009, Singhania *et al.*, 2010, Ali and Zulaki 2011, Farinas *et al*., 2011 Thomas *et al.*, 2013). Currently more research is focused on bioreactor design for better handling and on-line monitoring of important process parameters such temperature and pH.

In recent years, to overcome these challenges researchers are focusing on bioreactor design. A list is given in table 2.3 for cellulase production.

Chapter 3

Materials and methods

3.1. Materials

3.1.1. Chemicals

All the chemicals including media components and analytical reagents were procured from different companies as given in table (See Appendix).

3.1.2. Agro-wastes

The agro-wastes were selected as carbon source were corncob (CB), groundnut shell (GS), coconut coir (CC), sugarcane bagasse (BG), rice husk (RH), rice straw (RS), wheat bran (WB), wheat straw (WS) and wood saw dust (WD).

Plant based materials like mustard seeds (MS), gram (pulse) seed coat (GC), red lentil (masoor) seed coat (LS), castor seeds (CS) and soyabean hulls (SB) were taken as natural nitrogen source. All these carbon and nitrogen sources were collected directly from the fields or local market of Saharanpur, U. P., India.

3.1.3. Microbiological tools, glasswares and plasticwares

The microbiological tools used for inoculation viz. cork borer, forceps, needles and loops were purchased from Hi- media, India. Cotton plugs were hand made using sterilized cotton.

Glasswares such as blue cap bottles, Petri plates, beakers, conical flasks, test tubes, screw capped bottles and measuring cylinders were procured from Hi-media, Borosil and Rankem, India and plasticwares like beakers, microtips, centrifuge tubes, measuring cylinders, vials etc. were obtained from Axiva and Hi-media, India.

3.1.4. Pulp

Conventionally bleached hardwood chemical pulp (MHW) was procured from a leading paper mill in North India. It was composed of eucalyptus and poplar in the ratio 70:30 and found to have CSF value 590±10 mL.

3.1.5. Commercial enzymes

Commercial enzymes were obtained from Novozymes, Bangalore, India. The details are given below in table 3.1.

Table 3.1. Characteristics of commercial enzymes

Particulars	**Commercial Enzymes**	
	CA	**CB**
Trade name	Fibercare R	Biorefine L+
Microbial source	*Aspergillus* species	Not given
Components	Endoglucanase	Cellulase+Xylanase
pH	4.5-7.5	4.5-7.5
Temperature (ºC)	35-50 ºC	40-55 ºC
Dose recommended	100-150 g/T OD pulp	100-150 g/T OD pulp
Reaction time	½-2 h	½-2 h

3.2. Experimental methods

It is partitioned in general practices, analytical methods, cellulase production and its application in pulp refining. To achieve the specific goals the work was done as described below:

3.2.1. General practices

From isolation, screening/selection of fungal strains to optimization and mass production of cellulase, the general practices followed were as under:

3.2.1.1. Pre-treatment of agro-waste materials

The above mentioned carbon sources (section 3.1.2) were washed twice or thrice with warm water to remove impurities (soil and dust particles, starch materials and other residues) and then dried in sunlight. Thus, obtained dried lignocellulosic carbon sources except WD were grinded in a laboratory grinder. It was passed through 100 mesh size screen and the fractions so obtained on screen were stored in airtight polyethylene bags for further use.

3.2.1.2. Sterilization of glasswares, plasticwares, cotton plugs and inoculating tools

Glasswares were washed with detergent and thoroughly rinsed with running tap water. These were then autoclaved at 15 psi (pounds per square inch) for 20 min followed by drying in oven at 60 ºC for 1-2 h. Plasticwares and cotton plugs used for experimental work were sterilized by autoclaving at 15 psi for 20 min. Inoculating tools e.g. cork borer, forceps, needle, loop etc. were sterilized by flaming method.

3.2.1.3. Disinfection of inoculation room and inoculation chamber

Inoculation chamber and room were disinfected properly on weekly basis by fumigation method. For fumigation purpose, cotton piece was spread on the bottom part of Petri plate and adequate amount (one teaspoon) of $KMnO_4$ was kept on it. It was followed by pouring formalin (40% formaldehyde solution) over $KMnO_4$ to wet the cotton completely. The fumes coming out in the air possess the microcidal effect that kills microbial spores in the surroundings. Disinfection of inoculation chamber before and after inoculation was done by wiping it with moist cotton dipped in 70% ethyl alcohol followed by exposing it to ultra-violet (UV) radiation for 15 min.

3.2.2. Analytical methods

As enzyme application was restricted to match with industrial conditions for pre-treatment of pulp at pH 7.0 and temperature 45 ºC, studies were performed at this pH and temperature.

3.2.2.1. Estimation of cellulase activity

Cellulase is a multicomponent enzyme, the activities of different components were determined as follows:

3.2.2.1.i. Endoglucanase activity

The endoglucanase activity or carboxymethyl cellulase activity (CMCase) was determined by measuring the release of reducing sugars using carboxymethyl cellulose (CMC) as the substrate (Ghose, 1987). The amount of reducing sugars in the reaction mixture was measured by using the 3, 5 dinitrosalicylic (DNS) acid reagent method described by Miller, 1959.

0.5 mL of enzyme diluted in 0.05 M phosphate buffer of pH 7.0 was taken in a 30 mL screw capped universal tube and heated up to 45 ºC. After that 0.5 mL of substrate solution (2% w/v CMC in 0.05

M potassium phosphate buffer of pH 7.0) were added to the enzyme containing tube and well mixed by shaking. Appropriate controls, in which substrate and/or enzyme preparation had been omitted, were also included. The assay mixture was incubated at 45 ºC for 30 min in a water bath. 3.0 mL of DNS was added to the reaction mixture (1 mL) after the completion of incubation. These assay mixtures were then boiled for exactly 5.0 min in a vigorously boiling water bath. All the reaction mixtures of enzyme, enzyme blank and reference (containing buffer in place of enzyme) were boiled together in the water bath followed by quick cooling in cold water bath. After cooling, 20 mL of distilled water was added in all types of reaction mixtures and after proper mixing, absorbance as optical density (o.d.) was measured at 540 nm using a UV-Vis spectrophotometer (UV-Vis Spectrophotometer, Systronics 118). The amount of reducing sugars was calculated by translating the absorbance of sample into glucose by using a glucose standard curve prepared from D-glucose. The activity of endoglucanase was calculated as International units (IU) equivalent to the amount of enzyme which liberates 1µ mol glucose/min of the reaction mixture under standard conditions. It was expressed in IU/gds (International units per gram dry substrate).

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

3.2.2.1.ii. Total cellulase activity

For estimation of total cellulase activity of enzyme preparation, FPase (Filter paper activity) assay was conducted (Ghose, 1987). 1.0 mL of 0.05 M potassium phosphate buffer of pH 7.0 was taken into 30 mL screw capped universal tubes. 0.5 mL of enzyme, diluted in 0.05 M potassium phosphate buffer of pH 7.0 was added to the test tube and heated up to 45 ºC. After that filter paper strip (Whatman No.1 filter paper) of approx. 50 mg was added in buffer enzyme mixture and incubated at 45 ºC for 60 min in a water bath. Controls were routinely included in which enzyme or substrate was omitted and treated similarly. To stop the reaction, 3.0 mL of DNS was added in the reaction mixture. After the completion of incubation, these reaction mixtures were boiled for exactly 5.0 min in a vigorously boiling water bath. All the reaction mixtures of enzyme, enzyme blank and reference (containing buffer in place of enzyme) were boiled together in the water bath followed by quick cooling in cold water bath. 20 mL of distilled water was added after cooling, in all types of reaction mixtures and after proper mixing, absorbance was measured at wavelength 540 nm using a UV-Vis spectrophotometer. The amount of reducing sugars was calculated by translating the absorbance of sample into glucose by using a glucose standard curve. The activity of FPase was calculated as IU equivalent to the amount of enzyme which liberates 1µ mol glucose/min of the reaction mixture under standard conditions. It was expressed in FPU/gds (filter paper units per gram dry substrate).

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

3.2.2.1.iii. β-glucosidase activity

This activity was determined by method reported by Wood and Bhat, (1988) using p-nitrophenyl β-D glucoside as substrate. For this assay, 1 mL of substrate and 1.8 mL of potassium phosphate buffer (0.05 M, pH 7.0) were taken in a test tube. This tube was equilibrated to 45 °C in a water bath. To this diluted substrate, 200 µL diluted enzyme solution was added. The reaction mixture/contents were mixed properly and incubated at same temperature for 30 min time period. To stop this reaction 4 mL of glycine buffer (0.4 M, pH 10.8) was added to the tube. The p-nitrophenyl liberated was measured at wavelength 430 nm. The enzyme and reagent blanks were also taken as in above methods.

The values thus obtained for absorbance were translated to micromoles of nitrophenol using standard graph. The activity was calculated in IU equivalent to amount of enzyme required to release 1µ mol p-nitrophenol per min under the conditions of the assay. It was expressed in IU/gds.

$$\beta - \text{glucosidase production (IU/gds)} = \frac{\beta - \text{glucosidase activity (IU/mL) x Volume of enzyme extracted (mL)}}{\text{weight of substrate (g)}}$$

3.2.2.2. Biomass estimation

Glucosamine content was determined in order to estimate fungal biomass (Blix, 1948). It was done by performing assay with Ehrlich's reagent to determine the N-acetyl glucosamine content released by the acid hydrolysis of the chitin, present in cell wall of fungi. For this, 0.5 g (dry wt. basis) of fermented matter was mixed with concentrated H_2SO_4 (2 mL) and the reaction mixture was kept for 24 h at room temperature (30 °C). This mixture was diluted with distilled water (50 mL), autoclaved (15 psi for 1 h), neutralized with 1 N NaOH and made to 100 mL with distilled water. The solution (1 mL) was mixed with 1 mL acetyl acetone reagent (freshly prepared by adding 1mL acetyl acetone in 50 mL 0.5 N Na_2CO_3) and incubated in a boiling water bath for 20 min. Then, it was cooled at room temperature. After cooling, 6 mL ethanol was added followed by the addition of 1 mL Ehrlich reagent (2.67 g p-dimethyl amino benzaldehyde in 1:1 mixture of 50 mL ethanol and 50 mL concentrated HCl). It was incubated at 65 °C for 10 min. After cooling, the optical density of the reaction mixture was read at wavelength 530 nm against the reagent blank. Glucosamine (Sigma) was used as the standard. The results were expressed as mg glucosamine/gds.

3.2.3. Fungal cellulase production

For cellulase production, the work was done in following sequence (Fig 3.1):

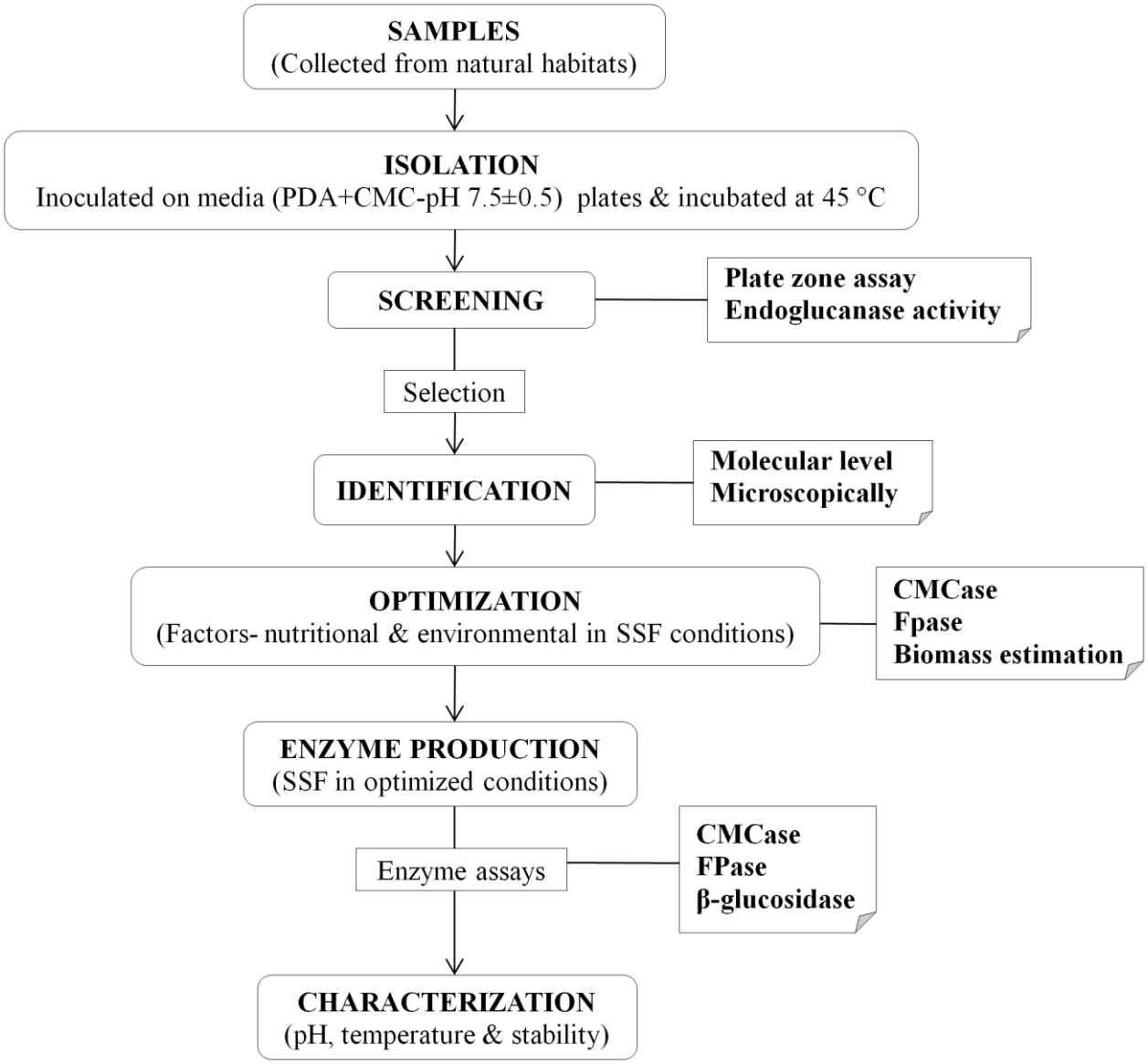


Figure 3.1. Protocol followed for fungal cellulase production

3.2.3.1. Isolation of cellulase producing fungi

With the objective to isolate cellulase producing fungi a total of 156 samples including 78 decaying wood and fruiting bodies, 32 humus, 40 soil and 6 from pulp/paper mill sites (log, effluent etc.) were collected from various places in Chandigarh, Rajasthan, Uttarakhand, Uttar Pradesh, Jharkhand and Haryana located in India.

Medium preparation and sample inoculation was done as follows:

(i) The medium for processing of samples was composed of potato dextrose agar (PDA) 3.5% and CMC 0.2% with antibacterial agent chloramphenicol and streptomycin (50 µg/mL each, added before pouring of media in Petri plates) to avoid any bacterial contamination (Vu *et al.*, 2009; Jadon *et al*., 2013). The initial pH of medium was adjusted to 7.5±0.5.

(ii) For the isolation of fungi from decaying wood, samples were kept in 9 cm diameter glass Petri plates damp chambers (Petri plates with moist blotting paper inside). These Petri plates were incubated at 37±2 ºC in incubator and the growth was observed every day. To maintain the moisture, plates were moistened with sterile tap water carefully to avoid any free water for better growth of fungi. The fungal species, in the form of mycelia growth, spores or fruiting bodies were purified by aseptically transferring these on fresh medium plates.

(iii) Fungi were isolated from the soil and humus samples by serial dilution method. For this, 1 g of sample was suspended in 10 mL sterile distilled water in a test tube. It was vortexed for atleast 5 min and serial dilutions were made upto 10^{-6} . By using a spreader 1 mL aliquot of various dilutions was added on sterile Petri plates.

All the plates were incubated at 45±2 ºC in an incubator. It was observed for fungal growth. The single colonies were transferred from mother plate to fresh medium ((PDA+CMC) plates. Thus, purified strains were processed for next step of screening.

3.2.3.2. Screening of potential cellulase producing fungi for refining application

Highly selective and standard procedures were followed for selection of fungal strains. The aim of screening was to select two isolates exhibiting the highest cellulase activity. It was done as follows:

3.2.3.2.1. Primary Screening

CMC-agar medium with 2% CMC and 1% agar (pH 7.5±0.5) was prepared in 9 cm Petri plates. In this Petri plate, a well of around 6 mm was cut in center with the help of a sterilized cork borer. To this well fungal disc was inoculated. This disc was obtained from 6 days old pure mother culture grown on PDA+CMC medium using sterilized cork borer of same size i.e. 6 mm. The plates thus inoculated were incubated at 45±2 °C until substantial growth was recorded. These Petri plates were flooded with Congo red solution (0.5% w/v Congo red in distilled water. After 15 min, this red dye was discarded and the plates were destained with 1M NaCl solution (Teather and Wood, 1982). The plates were then observed with naked eye for appearance of clear zones/halos around the fungal cultures against the red background to check if the fungal enzymes had utilized the substrate CMC.

3.2.3.2.2. Secondary Screening

The best five fungal isolates, exhibiting areas of clear zones in Congo red test above (qualitative), were further subjected to determine their extracellular endoglucanase (cellulase) activity quantitatively.

3.2.3.2.2.i. Cellulolytic enzyme system production at flask level in SSF

For this screening, 5 g substrate of composition: 4 g pulp powder and 1 g CMC was used with 15 mL of nutrient salt solution (NSS) in 250 mL Erlenmeyer flasks. The requirement for moisture and micronutrients availability was fulfilled by NSS of composition: (g/L): 5 $(NH_4).SO_4$, 3 KH_2PO_4, 0.5 $MgSO_4.7H_2O$ and 0.5 $CaCl_2.H_2O$ (Toyama and Ogawa, 1977) with pH adjustment to 7.0 using dil. NaOH or H_2SO_4. The flasks were properly cotton plugged and sterilized in autoclave as mentioned above (section 3.2.1.2.) and kept for cooling at room temperature in the inoculation chamber under UV light. After cooling, flasks were inoculated with fungal inoculum (6 fungal discs of 8 mm diameter were obtained as described in section 3.2.3.2.1) from 6 days old mother culture. After incubation period of 6 days at 45 ºC, enzyme was harvested from the fermented matter produced in flasks by the respective isolates and the cellulase activities of enzyme samples from each isolate were determined.

3.2.3.2.2.ii. Harvesting and storage of enzyme

After incubation, the flasks were taken out from incubator and contents were leached out from the fermented matter. 15 mL of potassium phosphate buffer (pH 7) was added to the flask containing fermented matter for the extraction of enzyme and then filtered through cheesecloth. This filtrate was centrifuged at 10,000-Xg (Remi C-18) for 15 min at 4 ºC. The supernatant was stored at 4 ºC.

3.2.3.3. Identification of selected fungi

It was done based on molecular as well as morphological basis for both the isolates selected for further studies.

Molecular identification of fungal strains was done on internal transcribing spacer (ITS) sequencing. Both the fungal cultures were renamed as RS1 and RS2, and sent to Agharkar Research Institute (ARI), Pune, India. Cultures were submitted at National Fungal Culture Collection Centre of India (NFCCI) and requested for accession number.

Morphological study was performed by Scanning electron microscope (SEM) image analysis. For this fungal mat was fixed on a small rectangular microscopic glass slide piece using 3% (v/v) glutarldehyde, 2% (v/v) formaldehyde in ratio 4:1 for 24 h. Following the primary fixation, samples were washed thrice with double distilled water. Subsequently, the samples were dehydrated under the alcohol gradients of 30 to 100% (for 15 min upto 70% alcohol gradient and 30 min afterwards for upto 100%) and air dried. The slides thus prepared were finally examined under SEM at desired magnifications (Gabriel, 1982).

3.2.3.4. Microorganisms, their propagation and maintenance

Purified cultures were routinely subcultured on medium PDA in slant form and glycerol stocks (15% v/v) as a suspension of spores and hyphal fragments in vials and preserved at 4 °C.

3.2.3.5. Optimization strategy-factors affecting extracellular cellulase activity

This was done to determine the most favorable conditions for achieving enhanced levels of endoglucanase (cellulase) production under SSF cultivation as described above in section 3.2.3.2.2.i and 3.2.3.2.2.ii by the test fungal isolates. Mother plates required for obtaining cultures in active form to perform optimization studies were prepared on cheap medium, wheat bran agar (WBA-4%+WB-2% Agar) in Petri plates. Optimization for enhanced production of cellulase was done following classical one variable at a time approach. In this approach culture conditions were optimized by changing one independent variable at a time while keeping the other variables constant.

In all the experiments, cellulase activity (CMCase, FPase) was measured. Biomass was determined during optimization studies (incubation days, nitrogen source and carbon source). All activities for cellulase such as CMCase, FPase, and β-glucosidase were determined for finally produced enzyme obtained under optimized conditions.

3.2.3.5.1. Agro-wastes as carbon source

Natural carbon substrates such as CB, WS, WB, RS, RH, SB, GS, CC and WD were used singly on 5g dry wt. basis. The NSS prepared was adjusted to initial pH 7.0. Flasks were prepared in a manner as described previously and inoculated with 6 fully mycelia covered agar plugs (fungal discs) of 8 mm diameter for both the fungi studied. After 6 days of incubation at temperature 45 °C all the flasks were taken out of incubator. Harvesting of enzyme was done and checked for cellulase activity and protein concentration.

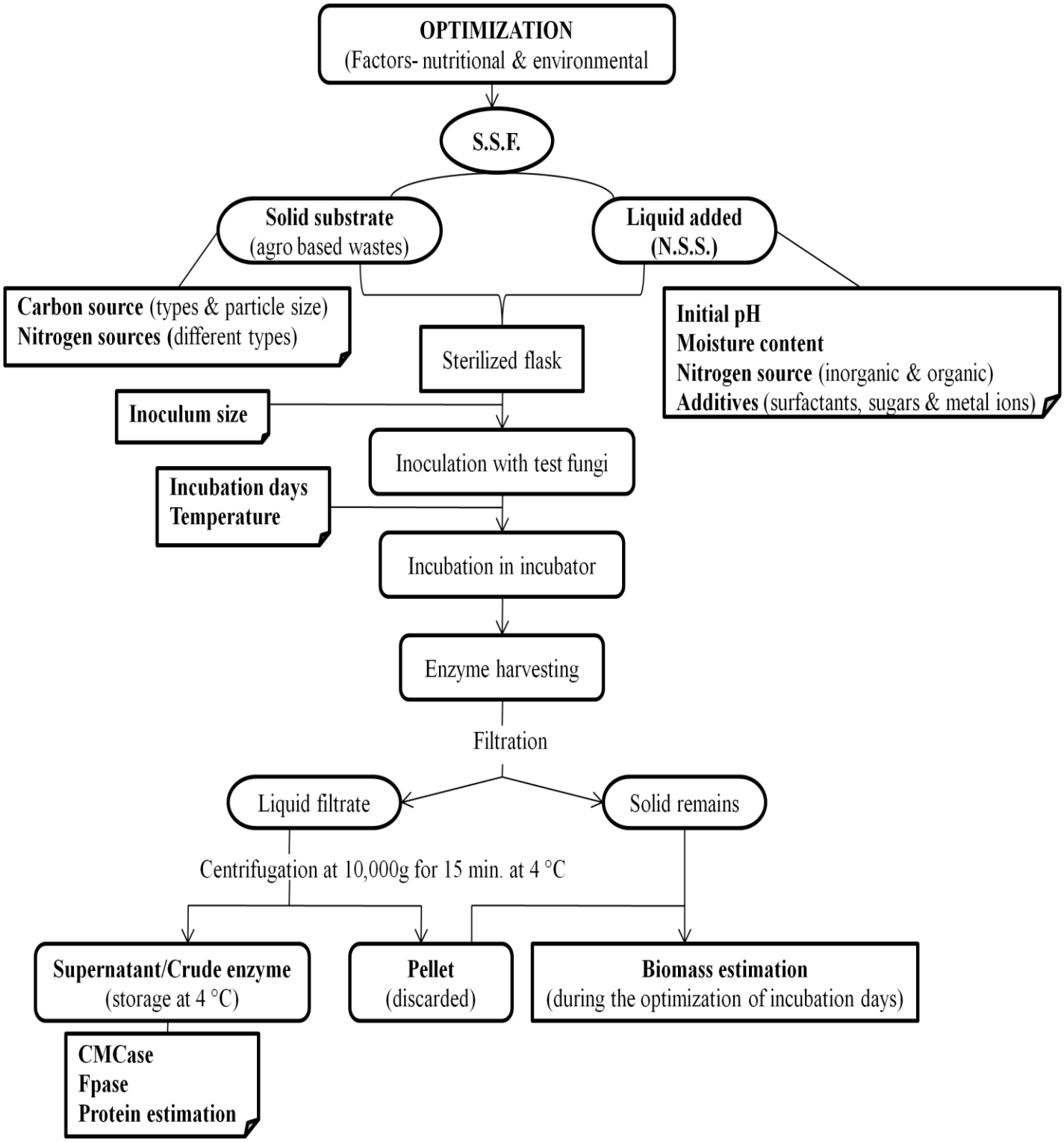


Figure 3.2. Optimization of various factors affecting extracellular cellulase production

Table 3.2. Factors affecting extracellular cellulase production

PARAMETERS	VARIABLES
Agro-waste	Corncob (CC), Wheat straw (WS), Wheat bran (WB), Rice straw (RS), Rice husk (RH), Sugarcane bagasse (BG), Groundnut shell (GS), Coconut coir (CC) & Wood saw dust (WD)
Carbon sources Combinations	Best three carbon sources for each test fungi in ratio 1:1, 1:3 & 3:1, respectively
Particle size	Mesh size- 14, 22, 22, 30, 36, 44, 52, 60, 72, 100 & as such particles Unscreened (US)
Temperature	26 to 50 °C (at 4 °C interval)
Incubation days	1, 2, 3, 4, 5, 6, 7, 9, 11, 13, 15
Inoculum Size	2-10 fully mycelia covered discs (2, 4, 6, 8 & 10)
Initial pH	3.0 to 11.0 (at interval of 0.5)
Moisture ratio	1:2.5, 1:3, 1:3.5, 1:4 & 1:4.5
Nitrogen source	Organic (1, 2, 3, and 4g/L of each): Tryptone (TP), Meat peptone (MP), Soya peptone (SP), Soya bean meal (SM), Soya peptone (SP), Malt extract (ME), Proteose peptone (PP), Yeast extract (YE), Mycological peptone (MyP), Beef extract (BE)
	Inorganic **(**0.01M to 0.04M of each): Sodium nitrate (SN), Ammonium sulphate (AS), Urea (UA), Ammonium nitrate (AN), Ammonium acetate (AA) & Ammonium chloride (AC)
	Agro-waste (total weight basis), Carbon source+ Nitrogen source 1:1, 1:3 & 3:1): Mustard cake (MC), Gram seed coat (GC), Soya Bean Hull (SB) Castor Seed (CS) & Masoor Seed Coat (MS)
Surfactants	Tween 20, Tween 40, Tween 60, Tween 80 & Triton X100 (0.1v/v% basis)

3.2.3.5.2. Combinations of carbon sources

The best three carbon sources obtained in above step were added in combinations of 1:1, 1:3 and 3:1. All the conditions were same as above.

3.2.3.5.3. Particle size

To see the effect of particle size, the raw material was screened with different screens of 14, 22, 30, 36, 44, 52, 60, 72 and 100-mesh size. One set of WB was kept as such i.e. unscreened (US) and used as a control.

3.2.3.5.4. Incubation temperature

The effect of incubation temperatures on the production of cellulase by the test fungi was studied. All the experimental conditions were same as above with optimized particle size except for temperature, which was varied in temperature range 26 to 50 °C at an interval of 4 °C. After 6 days enzyme was harvested to measure enzyme activities.

3.2.3.5.5. Incubation period

This parameter was optimized by taking incubation days range 1-15 days. For this, 11 flasks were prepared to carry out fermentation under optimized conditions as found from the results in above steps for both the isolated fungi. Out of theses flasks, a single flask was taken out from incubator at a time on a regular interval of one day from day 1 to 7, then at interval of 2 days from day 7 to 15

3.2.3.5.6. Inoculum size

The inoculum size was optimized by preparing the inoculum on WBA plate containing test fungi by using sterile cork borer of 8 mm size. The wheat bran containing fermentation media inoculated with 2, 4, 6, 8 and 10 discs of fungal culture aseptically. After inoculation, the flasks were incubated in incubator at optimized temperatures.

3.2.3.5.7. Initial pH

To study the effect of initial pH, the pH of the NSS was varied from 3.0 to 11.0 by using diluted $NaOH/H_2SO_4$ separately, with the help of pH meter. It was added in flask containing solid substrate and experiment was proceeded in optimized conditions obtained as in above experiments.

3.2.3.5.8. Initial moisture level

The effect of moisture level on cellulase production by the isolated fungi was tested by varying the ratio of solid substrate amount to NSS volume from 1:2.5 to 1:4.5.

3.2.3.5.9. Nitrogen sources

To study this parameter various nitrogen sources such as organic, inorganic and agro-wastes were incorporated into the fermentation medium.
Note: There was not any other nitrogen source in original medium.

3.2.3.5.9.1. Organic

The effect of complex soluble nitrogen sources on cellulase production by the test organisms was studied by taking Tryptone (TP), Meat peptone (MP), Soya peptone (SP), Soya bean meal (SM), Soya peptone (SP), Malt extract (ME), Proteose peptone (PP), Yeast extract (YE), Mycological peptone (MyP) and Beef extract (BE) as complex nitrogen source in the NSS with different concentrations (varying from 1 to 4 g/L of each). All other optimized parameters were same as obtained from previous studies.

3.2.3.5.9.2. Inorganic

The effect of soluble inorganic nitrogen sources on cellulase production by the test fungal strains was studied by taking Sodium nitrate (SN), Ammonium sulphate (AS), Urea (UA) and Ammonium nitrate (AN) as insoluble soluble nitrogen source in the NSS at concentrations calculated to supply equivalent amounts of nitrogen (varying from 0.01M to 0.04M of each).

3.2.3.5.9.3. Agro-wastes

The effects of insoluble natural nitrogen sources obtained from agro-wastes on cellulase production for both the test fungi were studied. The sources were Mustard cake (MC), Gram seed coat (GC), Soya bean hull (SB), Castor seeds (CS) and Masoor seed coat (MS).

For testing a single nitrogen source, it was added with respective optimized carbon source for both the fungi in different ratios of 1:1, 1:3 and 3:1 in a manner so that the total weight of the raw material/solid bed was 5g (dry weight basis).

3.2.3.5.10. Surfactants

Different surfactants Tween 20, Tween 40, Tween 60, Tween 80 and Triton X100 were used in NSS (0.1% v/v) .

3.2.3.6. Mass production of crude cellulase for refining

After optimization of test variables, mass productions of cellulase from both the isolated fungi were carried out under optimized conditions of SSF. Erlenmeyer flasks of 1 L capacity were used for the mass production of enzyme. 20 g of WB (carbon source) was taken in each flask and optimized NSS of desired initial pH were added. These flasks were inoculated with the test fungi and incubated at desired temperatures. After incubation period of optimized duration enzyme was harvested and centrifuged.

3.2.3.7. Characterization of crude enzyme produced by the test fungi under optimized conditions of SSF

The effect of pH and temperature on the CMCase and FPase were studied to obtain the optimum conditions and its working efficiency.

3.2.3.7.1. Effect of pH on CMCase, FPase and enzyme stability

For determining pH optima a wide pH range of different buffers were used in range from 3-11 pH (at the difference of 0.5). Four different buffers (0.05 M) were used to maintain different pH levels. Citrate buffer was used for pH 3.0-6.0, phosphate buffer for pH 6.5-9.0, and glycine-NaOH buffer for pH 9.5-11.0. The enzyme activities were determined as described previously.

pH stability was checked by incubating the enzyme in buffer (0.05M) of pH optimum as well as 7.0 for 0, 0.5, 1, 2, 3 and 4 h at 45 °C excluding the assay time (30 min for CMCase test and 60 min for FPase test). The residual CMCase and FPase activities were then measured by the standard assay procedure as discussed earlier.

3.2.3.7.2. Effect of temperature on CMCase, FPase and enzyme stability

For optimum temperature determination the temperature range was varied from 20 to 70 °C (5 °C interval). Crude enzyme supernatant was pre-incubated at 45 to 60 °C for various times (0, 0.5, 1, 2, 3 and 4 h) to determine the thermal stability (pH optimum and 7.0). Enzyme activities were determined as previously described.

3.2.3.8. Statistical analysis

All cultures and analyses were replicated three times and the results are mean±standard deviation

(SD) of the values.

3.2.4. Enzyme-assisted refining of pulp

In this section application part of enzyme is described. The standard protocol was followed for refining studies and pulp/paper properties determination. A schematic representation is shown below (Fig. 3.3):

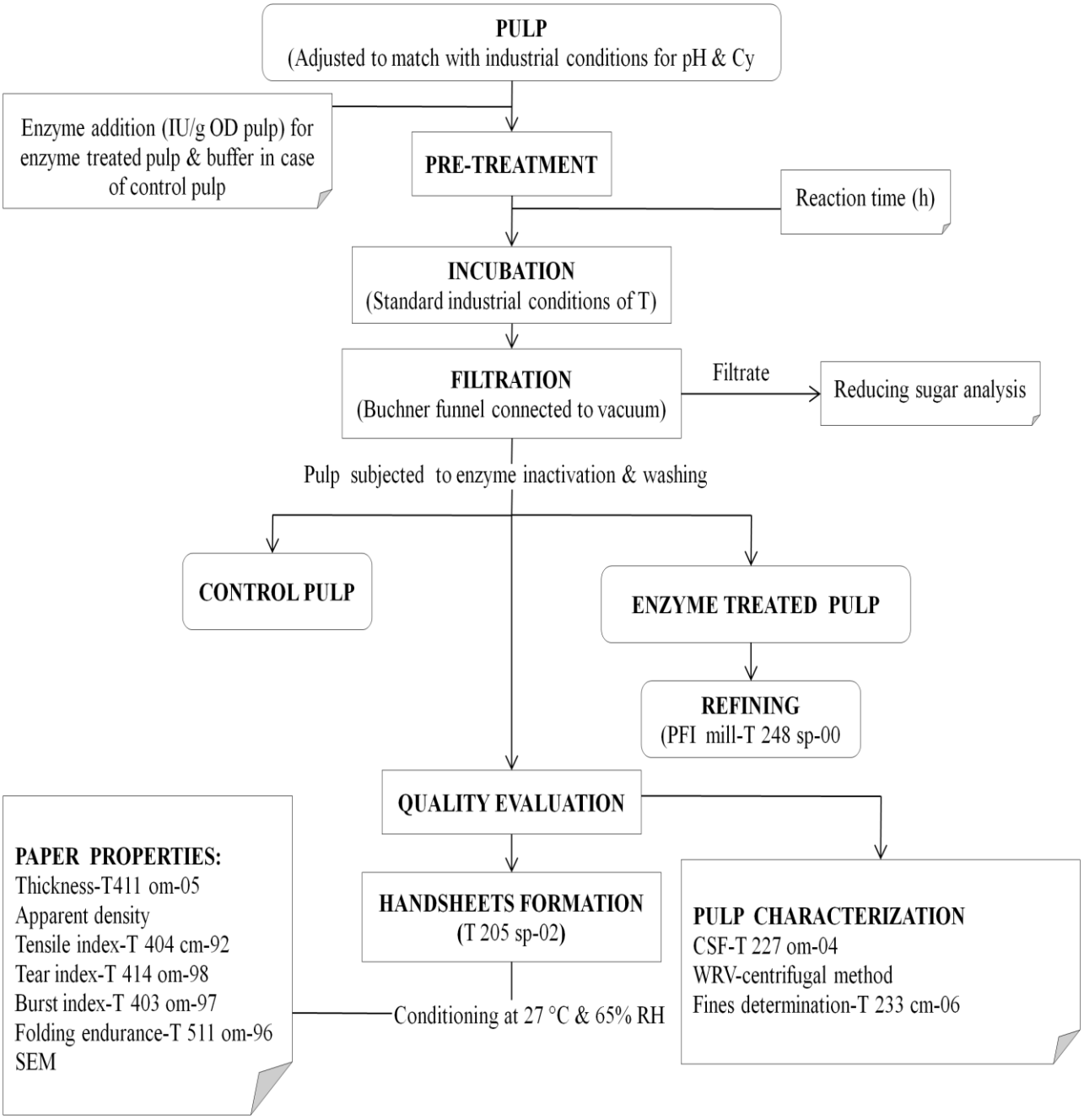


Figure 3.3. Protocol followed to perform refining and pulp/paper quality evaluation

3.2.4.1. Preparation and pre-treatment of pulp

It started with moisture content (%) determination of pulp using standard TAPPI test method 412 om-02. For a single trial approximate 35 g OD pulp was taken. It was adjusted to achieve consistency (cy) of 5% and pH 7.0 with dilution of pulp slurry with water and addition of diluted H_2SO_4. For pre-treatment pulp held in polythene bag was kept in a thermostatic water bath for pre-incubation at temperature 45 °C. After preparation and conditioning treatment on pulp was conducted as follows:

For all the commercial as well as laboratory produced fungal enzymes experiments were performed in different ranges of enzyme dosages and reaction times. Addition of enzyme was done on CMCase activity basis. The dosage range for commercial enzyme 'CA' and laboratory produced crude fungal extracts from *P. oxalicum* 'LB' was 0.01, 0.03, 0.05, 0.07 and 0.09 IU/g OD pulp. For commercial enzyme 'CB' and laboratory produced crude fungal extracts from *R. microsporu*s 'LA' treatments were performed in dosage range 0.02, 0.04, 0.06, 0.08 and 0.10 IU/g OD pulp. For a single experiment, the pre-incubated pulp was mixed properly with the enzyme to ensure its uniform distribution. After proper mixing of enzyme and pulp, pulp bag was subjected to incubation at temperature 45 °C, which was maintained throughout the experiment for respective time duration. The reaction times studied were 1, 1.5 and 2 h. For pulps to be taken as controls, buffer (pH 7.0) was used in place of enzyme.

Once pre-treatment was completed, enzyme reaction was terminated by submitting the pulp to boiling water for 10 min, so that enzyme could be deactivated. The whole pulp was transferred for filtration. It was filtered in a Buchner funnel using a laboratory vacuum pump, so that the residual filtrate could be collected. Thus, obtained filtrate was recirculated to avoid any loss of fines and after that finally washed thoroughly.

3.2.4.2. Reducing sugar analysis

The residual filtrate collected after pre-treatment was used to determine reducing sugar content released. After the completion of experiment, approximate 5 mL of filtrate was taken and kept immediately in the boiling water bath to stop any enzyme activities. 1 mL of this filtrate was added with 3 mL of DNS in a 30 mL screw capped bottle. Then, sugar content was estimated by DNS method as described previously. Reference blank contained 1 mL of water in place of filtrate.

3.2.4.3. Refining and handsheets formation

Enzyme treated and control pulps were refined in PFI mill (Ham-Jern, Norway) according to Tappi test method T 248 sp-00. For this 30 g OD pulp per run was taken and adjusted for standard 10% consistency required to beat pulp in PFI mill. These were beaten to an extent so that pulp CSF reached 300±10 mL. CSF is measured as the volume of excess water when the pulp is drained at standard conditions of testing freeness. It was determined using CSF tester (Lorentzen & Wettres, Sweden) as per Tappi test method T 227 om-04. This test required 3 g OD pulp sample for a single test.

Eight handsheets per run of grammage (gsm) 60±2 were made on a British handsheets former unit according to Tappi test method T 205 sp-02.

3.2.4.4. Paper testing

The handsheets thus prepared were tested in accordance with standard methods as given in Table 3.3 below. Apparent density (g/cm^3) was calculated by dividing gsm (g/m^2) to thickness (µm) values. Folding endurance was calculated as Log_{10} (no. of double folds).

Table 3.3. Quality evaluation using standard methods of testing for various paper properties

Paper properties	**Units**	**TAPPI-method**	**Instruments used and their make**
Thickness	µm	T-411 om-05	Micrometer (Aktiebolaget Lorentzen & Wettres, Stockholm Sweden)
Tensile index	N.m/g	T- 404 cm-92	Electro hydraulic tensile tester (same as above)
Tear index	$mN.m^2/g$	T- 414 om-98	Elmendorf tear tester (same as above)
Burst index	$kPa.m^2/g$	T- 403 om-97	Mullen burst tester (same as above)
Double fold	No.	T- 511 om-96	Kohler Mullin double fold tester (same as above)

3.2.4.5. Water retention value (WRV) determination

Pulps were characterized for water absorption by fiber through water retention value determination by a centrifugal method using centrifuge (Sigma, Germany). For this 1.5 g OD sample was centrifuged at 3000-Xg for 20 min and then weighed before and after drying. WRV was calculated as follows:

$$\text{WRV}\,(\%) \;=\; \frac{\text{m1} - \text{m2}}{\text{m2}} \text{X}\; 100$$

where, m1 is the weight of wet pulp after centrifugation and m2 is weight of dry pulp.

3.2.4.6. Fines content determination

The Bauer McNett fiber classifier (Universal Engineering Corporation, India) was used to determine fines content according to Tappi test method T 233 cm- 06. 10 g OD pulp was taken for a single run. Fines fraction passed through mesh screen <200 was collected and considered as fines component obtained in the experiment.

3.2.4.7. Scanning electron microscopy (SEM)

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

3.2.4.8. Viscosity measurements

Pulp viscosity which reflects degree of polymerization of cellulose chain was determined before and after pre-treatment by standard method ISO/FDIS 5351:2009 (E) measuring limiting viscosity number in cupriethylenediamine (CED) solution. For this fresh CED solution was prepared according to standard protocol described in above mentioned method.

3.2.5. Statistical analysis

All the experiments were carried out in triplicates. Ten measurements for handsheets were taken to calculate mean value and SD at a confidence level of 95%.

Chapter 4a
Fungal cellulase production

This work is focused on the production of endoglucanase, a component of cellulase from isolated fungal strains with the objective of using enzyme at application conditions i.e. 45 °C temperature (T) and 7.0 pH for pulp refining purpose. The mentioned temperature and pH were chosen because the pulp to be pre-treated with enzyme was obtained after conventional bleaching. At this stage the pulp temperature and pH falls nearly in the selected range in mill conditions.

Screening of microorganisms, evaluation of their nutritional and environmental requirements as well as selection of suitable substrates for the desired enzyme production are some of the crucial steps in development of economically feasible process/technology (Aristidou and Penttila, 2000). A detailed account is given below for fungal cellulase production to be used in enzyme-assisted refining of bleached mixed hardwood (MHW) pulp:

4a.1. Isolation and screening of cellulase producing fungi

A total of twenty fungal strains (Fig. 4a.1.i) were isolated on PDA medium enriched with CMC (pH-7.5±0.5, T-45±2 °C) from samples of natural habitat by serial dilution method for soil samples and point inoculation method for wood samples (described in section 3.2.3.1). Pure cultures were obtained by subculturing several times on fresh medium plates. These isolates exhibited clear zones of variable area around the fungal cultures, when screened for cellulase production by Congo red plate assay technique (Table 4a.1.1). Because of diffusion of endoglucanase in the medium, the solubilization of CMC occurred by the hydrolytic action of enzyme. Due to this hydrolysis, halo was produced around the cellulase positive strains as viewed by Congo red staining method. The principle behind this technique is that the dye remains attached only in those portions where there is β-1, 4-D glucanohydrolase bond (Lamb and Toy, 2005), where the bonds were broken by cellulolytic action at other portions, it appears as clear zone.

The best five cellulase positive isolates (Fig. 4a.1.ii) were screened based on the diameter of clear halo zones (>2.1 cm) and were further subjected to SSF conditions for cellulase production. The aim was to select two isolates that would show the highest endoglucanase activities. Two strains isolates were selected to study the refining potential of crude enzyme produced by these fungi. It may be

possible that either single or both the strains can be successful for required application in pulp refining.

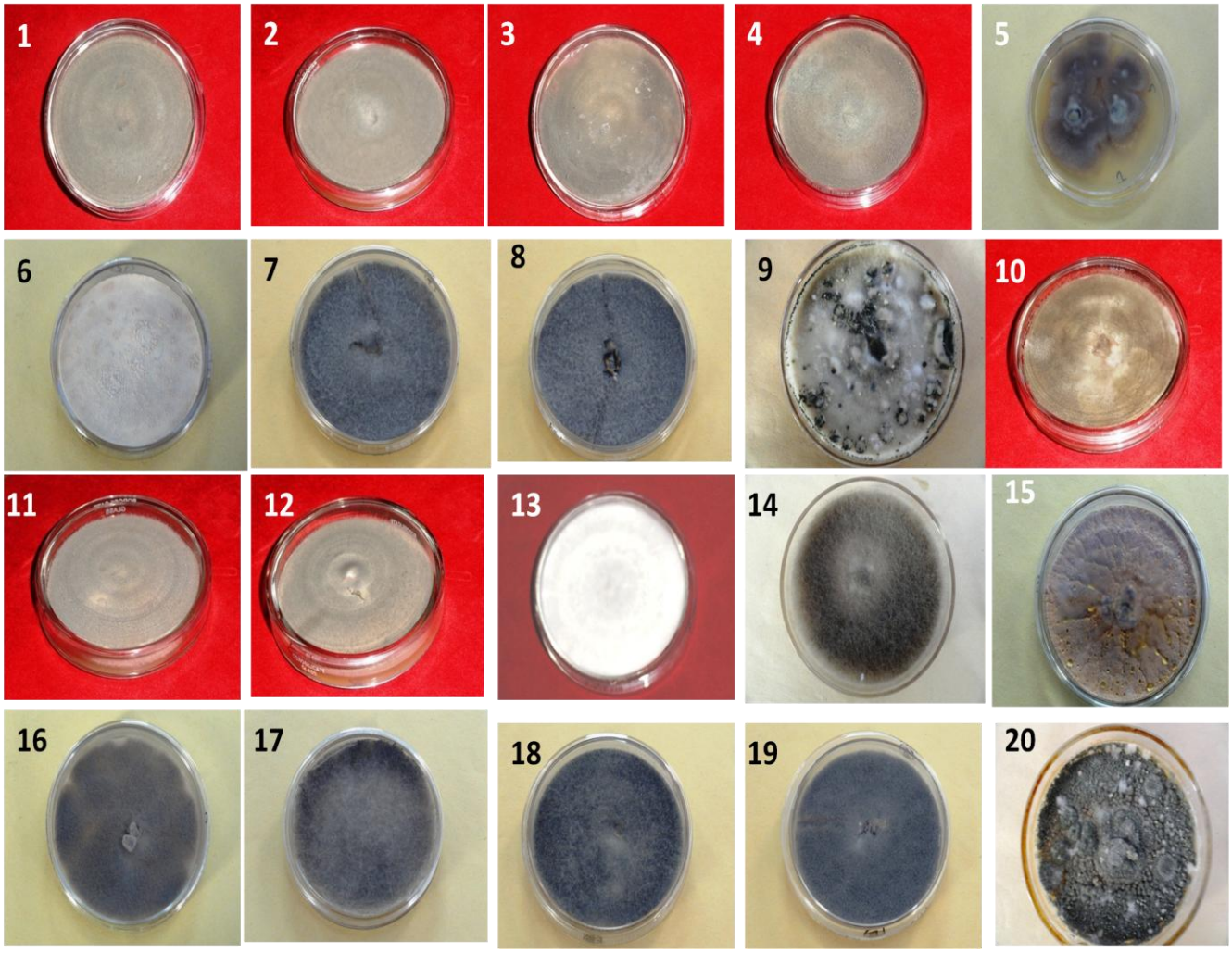


Figure 4a.1.i. Total isolates obtained from samples of natural habitat on PDA+CMC medium (pH 7.0±0.5) at 45±2 °C

Most of the industrially important fungi produce cellulase after an incubation period range of 4-6 days (Kang *et al*., 2005; Gautam *et al*., 2011; Jaafaru, 2013), therefore, in secondary screening enzyme was harvested from all the isolates after 6 days of incubation. Here, 6 days of incubation was selected to take into account the commercial point of view. Further, it can be stated from the results that endoglucanase activities were higher in selected isolates as compared to filter paper activities.

Table 4a.1.1. Isolation of cellulase producing fungi and primary screening by plate assay technique (Congo red staining method)

S. No.	Strain no.	Source	Location	Characteristics of isolates on medium (PDA+CMC) plates	Zone assay (diameter)
1.	PUC0809M	Wood	Chandigarh, Panjab	Velvety, brown mycelia and brown spores	++
2.	RAJ0809c	Soil	Jaipur, Rajasthan	Velvety, brownish-gray mycelia and spores	+++
3.	RAJ0809v	Soil	Jaipur, Rajasthan	Furry, brown mycelia with gray spores	+
4.	UKH1009o	Humus	Haridwar, Uttarakhand	Velvety, light brown colored mycelia and spores	+
5.	HAC0909T	Wood	Chandigarh, Haryana	Yeasty, Creamy-white color	+++
6.	RAJ0909d	Soil	Jodhpur , Rajasthan	Powdery, peach color	+++
7.	RAJ0909V	Wood	Jodhpur , Rajasthan	Furry, white- gray mycelia and black spores	++
8.	**HAC0909h**	**Fruiting body**	**Chandigarh, Haryana**	**Furry, black dark gray mycelia and spores**	**++++**
9.	**HAC0909f**	**Soil**	**Chandigarh, Haryana**	**Furry, green white color**	**++++**
10.	UPM0810L	Wood	Meerut, Uttar Pradesh	Furry, brownish white mycelia, brown spores	++
11.	UPS0910iv	Paper industry effluent (soil site)	Saharanpur, Uttar Pradesh	Velvety, brown mycelia and brown spores	++
12.	UPM0810j	Humus	Meerut, Uttar Pradesh	Velvety, light brown mycelia and spores	++
13.	UPM0810K	Wood	Meerut, Uttar Pradesh	Furry, white- mycelia and spores	+++
14.	**UPM0810n**	**Soil**	**Meerut, Uttar Pradesh**	**Furry, dark brown-gray mycelia, dark gray spores**	**++++**
15.	**UPS0810ii**	**Rotten pulp**	**Saharanpur, Uttar Pradesh**	**Powdery, orange colored with yellow exudates**	**++++**
16.	UPS0910e	Decomposing manure	Saharanpur, Uttar Pradesh	Furry, dark brown mycelia and spores	+
17.	JHB1010G	Wood	Bokaro, Jharkhand	Furry, white- gray mycelia and black spores	+++
18.	JHB1010D	Plant debris	Bokaro, Jharkhand	Furry, black dark gray mycelia and spores	+++
19.	JHB1010U	Wood	Bokaro, Jharkhand	Furry, black dark gray	+++
20.	**UPS1010B**	**Wood**	**Saharanpur, Uttar Pradesh**	**Velvety, powdery dark green color**	**++++**

Conditions: Media, PDA+CMC (3.5% & 0.2%); pH-7.0±0.5; Incubation temperature (T)-45 ^{o}C
Zone diameter (cm): +, very poor activity (<0.6 cm); ++, poor activity (0.7-1.3 cm); +++, good activity (1.3-2.0 cm); ++++ very good activity (>2.1 cm)

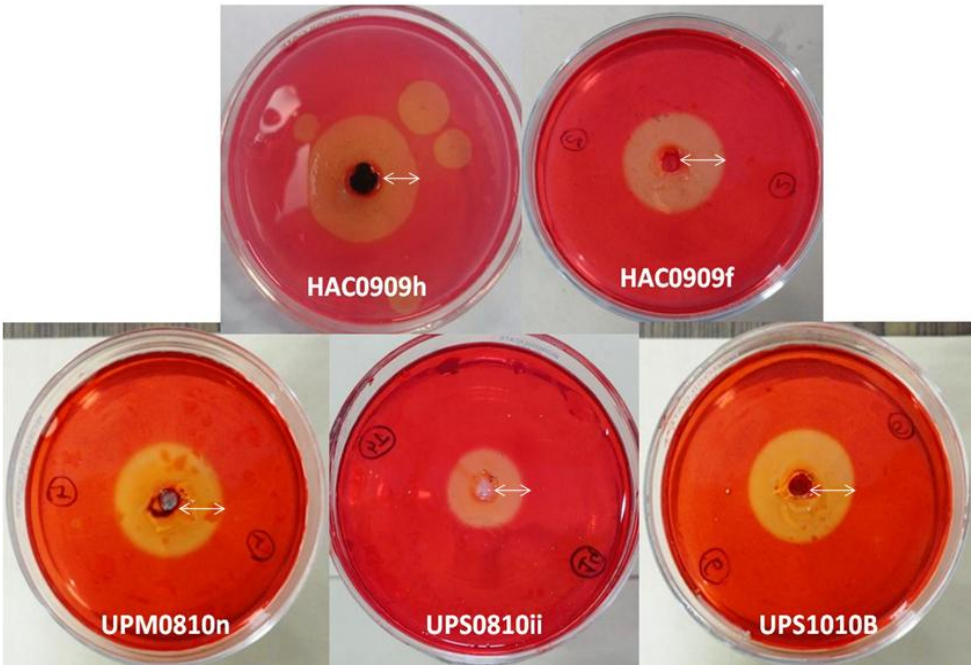


Figure 4a.1.ii. Plate assay test using Congo red dye by incubating fungal disc (6mm diameter from 6 days old pure mother culture) in center of CMC-agar medium (2% CMC and 1% agar, pH 7.0±0.5), best five isolates are shown here

Table 4a.1.2. Secondary screening for cellulase producing fungi by fermentation technique (SSF)

S. No.	**Isolates**	**Cellulase profile**	
		CMCase (IU/mL)	FPase (IU/mL)
1.	HAC0909h	0.32±0.04	0.19±0.03
2.	HAC0909f	0.25±0.04	0.18±0.06
3.	**UPM0810n**	**0.42±0.03**	0.21±0.09
4.	UPS0810ii	0.35±0.05	0.29±0.06
5.	**UPS1010B**	**0.45±0.05**	0.19±0.07

Fermentation conditions:	**Assay conditions:**
Substrate: NSS-1:3	Temperature-45 °C
Initial pH-7.0	pH-7.0
Temperature -45 °C	
Incubation time-6 days	

± Standard deviation (SD) from mean

4a.2. Identification of selected isolates

The selected isolates UPM0810n and UPS1010B were identified at molecular level by internal transcribed spacer (ITS) sequencing (See Appendix). ITS sequencing is the most useful technique for

molecular systematics at the species level (White *et al*., 1990). This was done by NFCCI on the basis of ~550 bp (base pairs) sequencing of the genomic DNA. The results showed 99% sequence similarity with *Rhizopus microsporus* for UPM0810n (RS1) and *Penicillium oxalicum* for UPS1010B (RS2). The cultures were deposited at NFCCI, Pune, India with accession no. NFCCI 2927 for strain UPM0810n (*R. microsporus*) and NFCCI 2927 for strain UPS1010B (*P. oxalicum*). SEM analysis was done at different magnifications for isolates as shown below (Fig. 4a.2.i--ii). It showed structures with characteristic features of *Rhizopus* for UPM0810n and *Penicillium* UPS1010B, respectively (Carlile *et al*., 2001; Bastos, 2004; Gilman and Joseph, 1998).

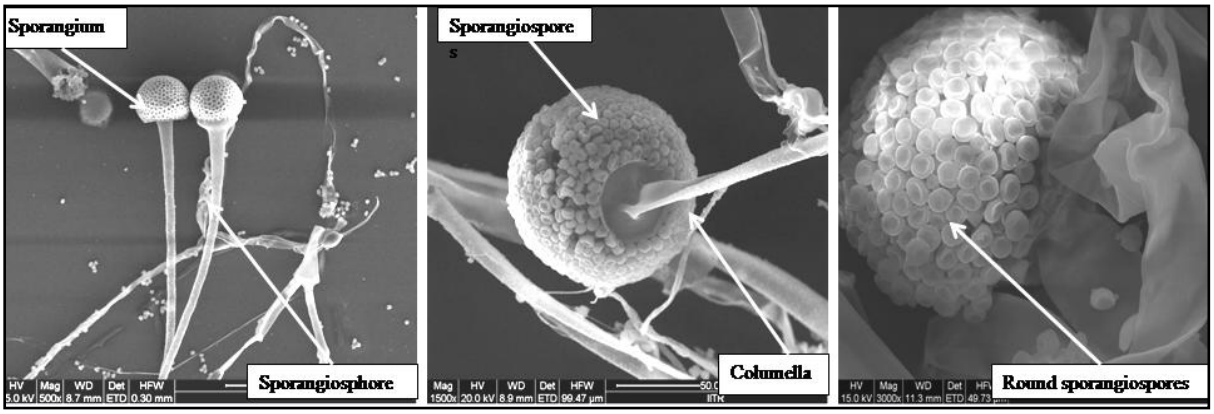


Figure 4a.2.i. SEM analysis of UPM0810n showing features for *Rhizopus* with sporangium bearing sporangiospores

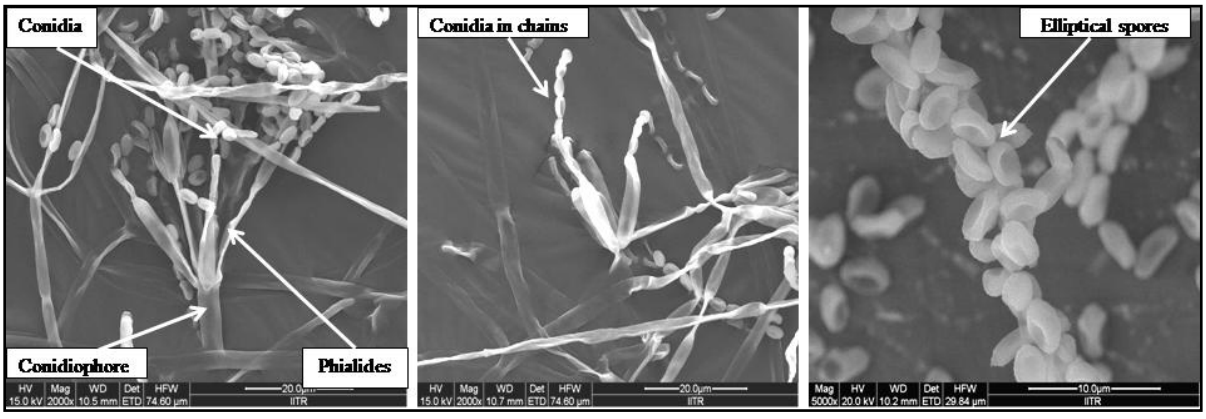


Figure 4a.2.ii. SEM analysis of UPS1010B (NFCCI 2926) showing features for *Penicillium* bearing conidia in chains arrangement on conidiophores

4a.3. Optimization for cellulase production

The selected fungal strains *R. microsporus* and *P. oxalicum* are reported to be able to grow at a high temperature but below 55 ^{0}C (Dolatabadi *et al*., 2014, Johri *et al*., 1999). These fungal strains are good cellulase producers with high enzyme activities (Dolatabadi *et al*., 2014, Celestino *et al*., 2006, Khokhar *et al*., 2012; Lelaie *et al*., 2013).

Several important environmental and nutritional factors were studied to see the effect on endoglucanase (cellulase) production. The details are as under:

4a.3.1. Effects of different agro-wastes as carbon source on fungal cellulase production

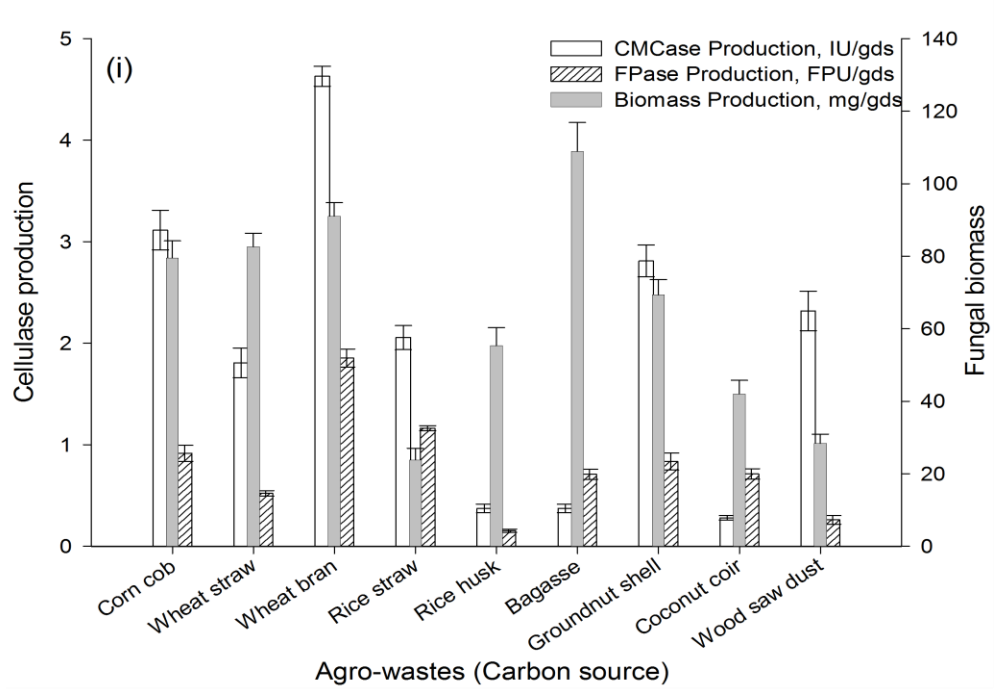
Carbon source plays a vital role in the cellular metabolism and cellulase production. Lignocellulosic agro-wastes have been reported to offer a cheap substrate (carbon source) for microbial growth and enzyme production. The various raw materials tested as carbon source were corncob (CB), wheat straw (WS), wheat bran (WB), rice straw (RS), rice husk (RH), sugarcane bagasse (BG), groundnut shell (GS), coconut coir (CC) and wood saw dust (WD).

In case of fungi *R. microsporus*, the best three substrates showing higher endoglucanase productions were WB (4.62±0.09 IU/gds- International unit per gram dry substrate), CB (3.11±0.19 IU/gds) and GS (2.80±0.15 IU/gds) as shown in figure 4a.3.1.i.

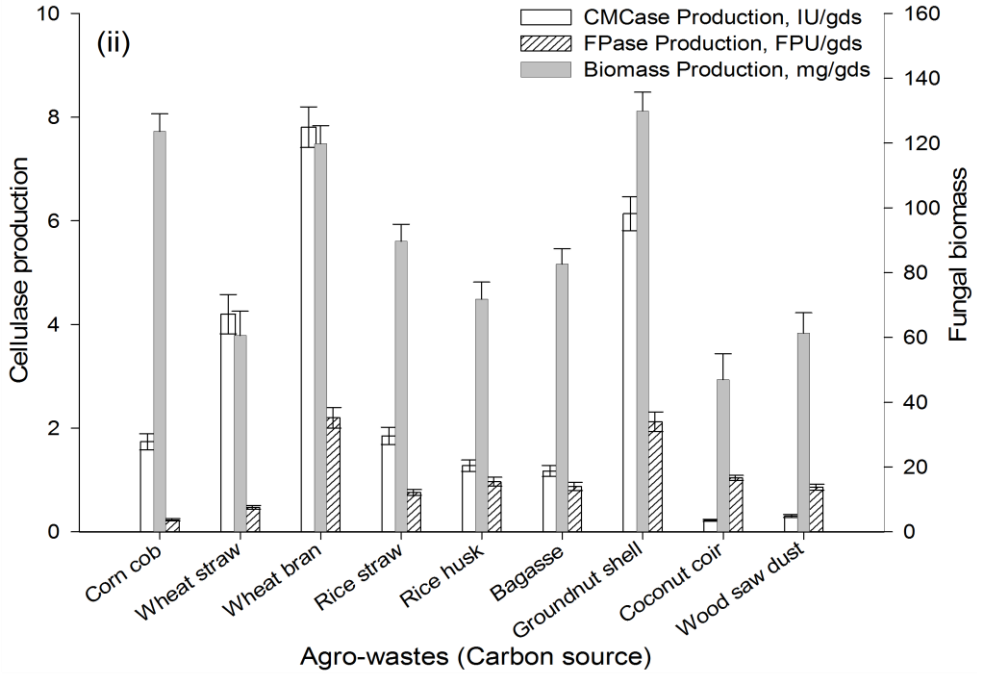
For *P. oxalicum* the best three substrates for endoglucanase productions were WB (7.80±0.39 IU/gds), GS (6.13±0.32 IU/gds) and WS (4.19±0.38 IU/gds) in comparison to other substrates studied (Fig. 4a.3.1.ii). WB was found to be of prime importance with best endoglucanase activities for both the isolated fungi.

Total cellulase production was found to be maximum using WB for *R. microsporus* with enzyme production 1.85±0.08 FPU/gds followed by RS, 1.16±0.02 FPU/gds and CB, 0.91±0.7 FPU/gds (Fig. 4a.3.1.i. For *P. oxalicum* WB and GS shown total cellulase production in similar range with production values 2.19±0.19 and 2.12±0.19 FPU/gds, respectively, followed by CC, 1.03±0.04 FPU/gds (Fig. 4a.3.1.ii.).

The variation in the production may be due to compositional variation in different agro-wastes used as substrate (i.e., amount of cellulose, hemicellulose and lignin) and nutrient availability (Sherief *et al.*, 2010). For all the substrates biomass content was different, no correlation was found between the biomass and enzyme production. This may depend on the difference in substrate composition and fungal utilization. For *R. microsporus* the substrates BG, WB, CB, GS and for *P. oxalicum* CB, WB and GS supported the fungal biomass growth whereas CC, WD, RH, RS resulted in comparatively less biomass content than the other substrates used. It was observed that BG supported higher biomass production for *R. microsporus* and it was better with GS for *P. oxalicum*.



Conditions (R. microsporus):
Substrate-5g, NSS-1:3, Initial pH-7.0, Inoculum size- 6 discs, Incubation T-45 ^{0}C, Incubation period-6 days



Conditions (P. oxalicum):
Substrate-5g, NSS-1:3, Initial pH-7.0, Inoculum size- 6 discs, Incubation T-45^{o}C, Incubation period-6 days

Figure.4a.3.1. Effect of different agro-wastes as carbon source on fungal cellulase production (i) *R. microsporus*, (ii) *P. oxalicum*

In SSF process, the solid substrate supplies the nutrients to the microorganism and filamentous fungi can utilize the available nutrients due to the capability of penetration into these porous substrates (Ramachandran *et al*., 2004). In contrast, the microbial growth may be retarded with a highly rigid

substrate (e.g. CC, WD) resulting into reduced enzyme production. Therefore, a favorable balance is required between starvation and an excess of energy for high enzyme production (Esterbauer *et al.*, 1991).

WB has been reported to have a good nitrogen and carbon content with low lignin and silica (Sanghi *et al*., 2008). It offers a free space for microbial growth even in high moist conditions by providing a large surface area (Archana and Satyanarayana, 1997). WB acted as a good solid support for microbial growth and production of enzymes. Earlier work reported WB as most suitable substrate among various agro-wastes (WB, groundnut fodder, rice bran and saw dust) tested for endoglucanase production (Chandra *et al*., 2007). It is also reported to be a substrate of choice for exoglucanase production by *Aspergillus niger* (Chandra and Reddy, 2013) in addition to β-endoglucanse production (Chandra *et al*., 2010).

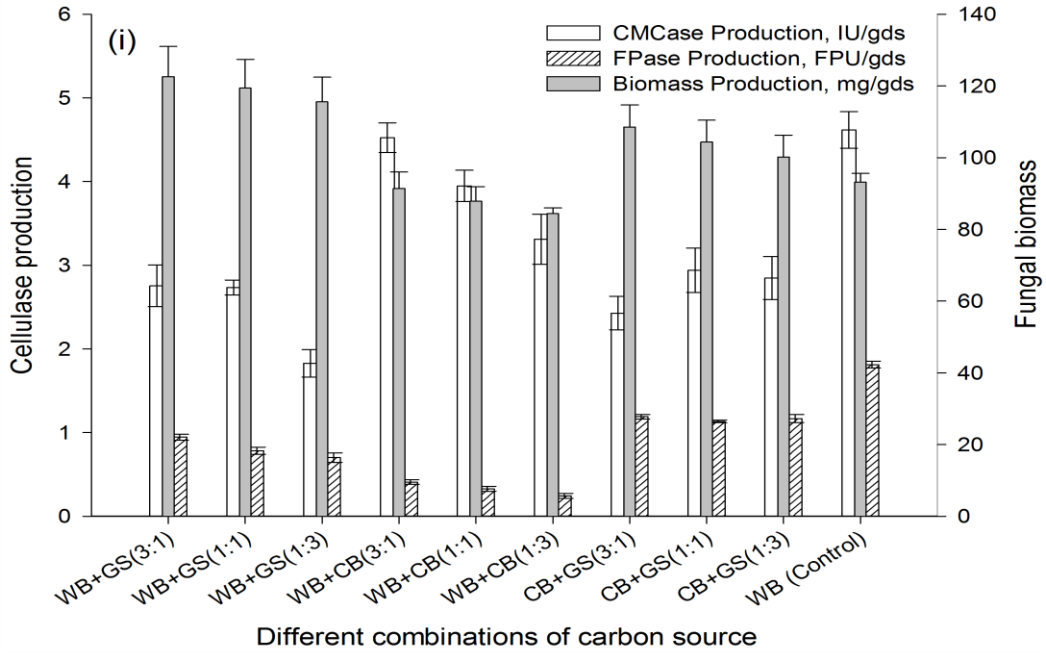
4a.3.2. Effect of different combinations of agro-wastes carbon source on fungal cellulase production

Some researchers have used WB in combinations with other substrates to evaluate the beneficial effect of mixed substrate, which is imparted due to nutritional differences, and particle property of different substrates (Deschamps *et al*., 1985, Sherief *et al*., 2010). For both the isolated fungi, fermentation was performed in different combinations of best three substrates (Fig. 4a.3.2) for respective fungal strains.

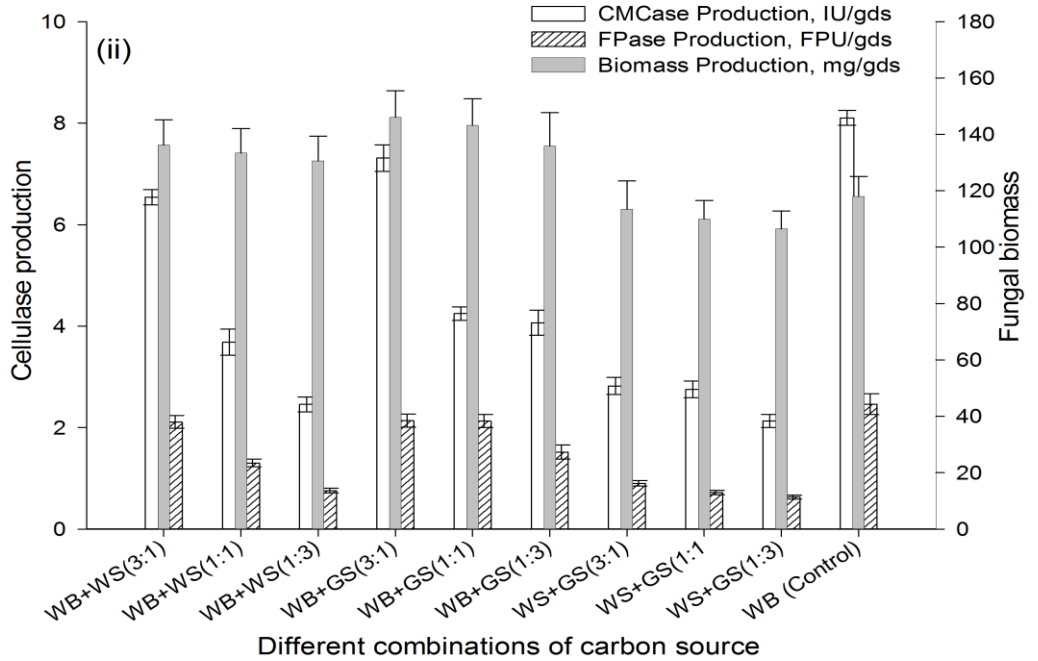
The variation in cellulase yields, with different substrates is not mainly owing to the sugar composition and lignin content of these materials but probably due to the nature of cellulose and hemicellulose, presence of activators in the substrates, surface and pore size of the substrate particles (Senior *et al*., 1989). Oxygen transfer and heat transfers may play an important role within the mycelia and substrate particles (Kaur *et al*., 2011).

As observed GS mixed with WB supported higher biomass production for both the isolated fungi but endoglucanase production was comparatively less than the single substrate (WB) used. Biomass production depends on fungal growth related with solid bed properties like bed height, composition, nutritional availability, porosity and homogeneity/heterogeneity. WB when used singly showed high endoglucanase production by both the isolated fungi.

A similar result for WB showing higher cellulase production than other agro-wastes was also observed by Pathak *et al*., 2014.



Conditions (R. microsporus):
Substrate-5g, NSS-1:3, Initial pH-7.0, Inoculum size- 6 discs, Incubation T-45°C, Incubation period-6 days
WB- Wheat bran, GS- Groundnut shells, CB- Corn cob



Conditions (P. oxalicum):
Substrate-5g, NSS-1:3, Initial pH-7.0, Inoculum size- 6 discs, Incubation T-45°C, Incubation period-6 days
WB- Wheat bran, WS- Wheat straw, GS- Groundnut shells

Figure 4a.3.2. Effect of different agro-wastes carbon source in different combinations on fungal cellulase production (i) *R. microsporus*, (ii) *P. oxalicum*

Contrary to this, Atev *et al*., 1987 reported that for enzyme production combined substrates can be better than a single substrate. However, in this study a mono substrate i.e. WB was found to be best substrate for both the fungal isolates. WB is produced worldwide in large quantities as a by-product of the wheat milling industry. It can be a promising renewable substrate for biotechnological processes to be used for enzyme production.

The use of pure substrate as a carbon source (such as CMC, cellulose and xylan) have been shown to be uneconomical, while the agro-wastes offer cost effective substrate for large-scale production of cellulase through SSF. The induction of particular enzyme mainly depends on substrate composition but cost as well as availability is also a deciding factor for commercialization in SSF (Aristidou and Penttila, 2000). Agro-wastes are about 10 times cheaper substrates than the primary cellulosic materials (Doppelbauer *et al*., 1987, Esterbauer *et al*., 1991).

4a.3.3. Effect of particle size on fungal cellulase production

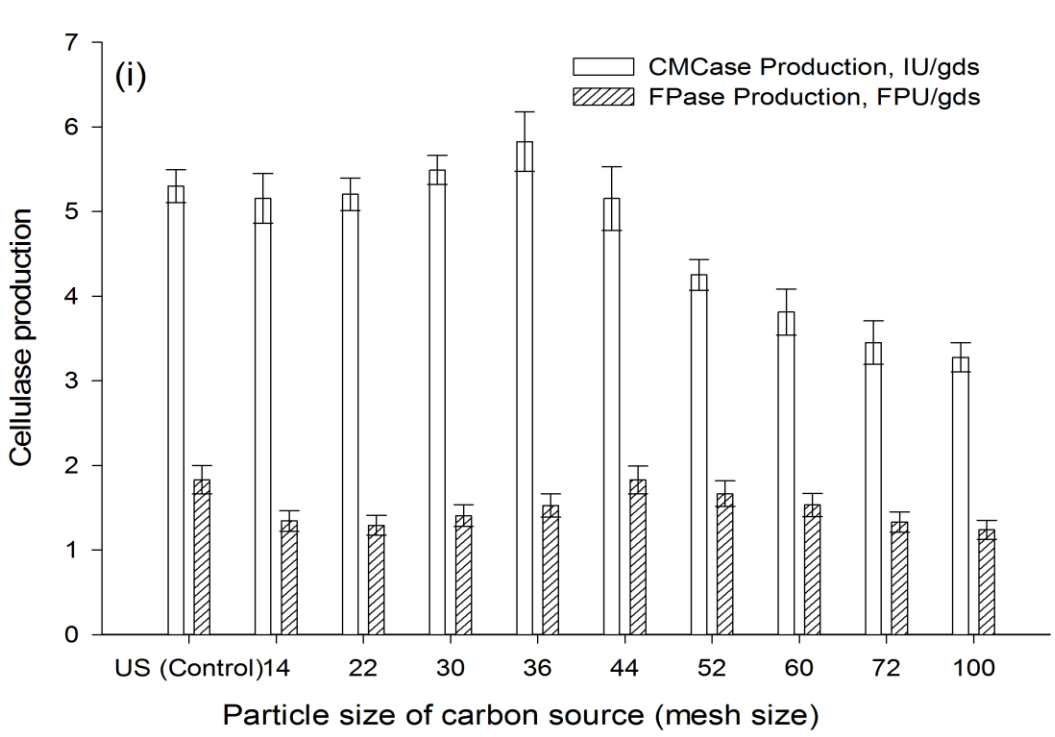
The particle size of a substrate to be used for SSF affects microbial growth and enzyme production by interfering with oxygen transfer (Pandey *et al*., 2000) and thus, the fermentation process. This parameter need to be optimized for a fermentation process.

Particle size results showed marginal difference among US and screened material. For *R. microsporus* using WB as substrate, it was observed that higher mesh size had pronounced effect on cellulase production and activity was found to be declined on increasing mesh size (52-100). It showed better endoglucanase production on mesh size of 36 with endoglucanase production 5.82±0.35 IU/gds and total cellulase production 1.52±0.13 FPU/gds (Fig. 4a.3.3.i).

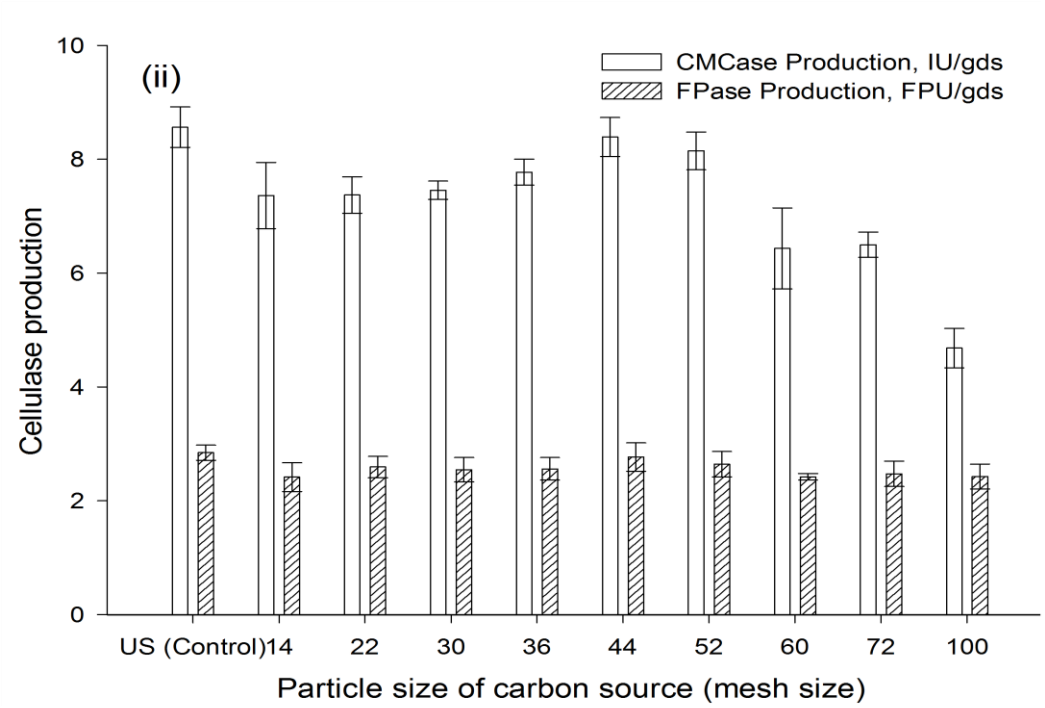
For *P. oxalicum* with increasing mesh size (>44), endoglucanase production was found to be decreased. A high endoglucanase production was observed on mesh size range 44 with value of 8.39±0.34 IU/gds for endoglucanase, at the same particle size total cellulase production was 2.76±0.24 FPU/gds (Fig. 4a.3.3.ii). The results for *P. oxalicum* was comparable for unscreened (US) particle size, with endoglucanase production of 8.56±0.35 IU/gds and total cellulase production 2.84±0.13 FPU/gds. Therefore, for *R. microsporus* further studies were carried out on optimized range and for *P. oxalicum* on US particles.

In an earlier work, *Penicillium* species was observed to produce maximal activity in particle size range of 500-600 µm (30-36 mesh size) for WB (Sindhu *et al*., 2011). For other substrates like WS,

mesh size range 40, promoted xylanase production using *Aspergillus ochraceus* (Biswas *et al*., 1987). Reports are also available showing endoglucanase production in a wide particle size range



Conditions (R. microsporus):
WB-5g, NSS-1:3, Initial pH-7.0, Inoculum size- 6 discs, Incubation T-45°C, Incubation period-6 days



Conditions (P. oxalicum):
WB-5g, NSS-1:3, Initial pH-7.0, Inoculum size- 6 discs, Incubation T-45°C, Incubation period-6 days

Figure 4a.3.3. Effect of particle size on fungal cellulase production (i) *R. microsporus*, (ii) *P. oxalicum*

(200-500 µm) for horticultural waste powder using *Trichoderma ressei* (Xin and Geng, 2010). A difference in particle size may induce different component of enzyme e.g. Bahrin *et al.,* (2011) found maximum CMCase activity for particle size range 0.84 to 1.0 mm and for FPase and β-glucosidase particle size 0.42 to 0.60 mm using *Botryosphaeria sp*. on oil palm empty fruit bunch as a substrate.

The factors, surface area to volume ratio of the particle and packing density are related to particle size of the substrate used. This affects void spaces between the particles which are directly related to aeration. Small particles may come closer together restricting aeration and diffusion of oxygen in the medium.

The exposed surface area of cellulose can be accessible to microbe used for cultivation, and is more important than the actual amount of cellulose present (Fan *et al*., 1982). Generally, low particle sizes (400µm) results in larger specific surface area but low porosity. Due to the inverse correlation of porosity and surface area, most researchers claimed that 400 µm (equivalent to 36 mesh size) substrate sized particles contribute to the optimum fungal growth and cellulase production (Krishna *et al*., 1999; Tao *et al*., 1997). It was found that for very fine particles cellulase production was lowered down, this is because of agglomeration of substrate particles resulting in poor microbial respiration. Large particle size is responsible for increased surface area and the bulk density of the raw materials (Tao *et al*., 1997).

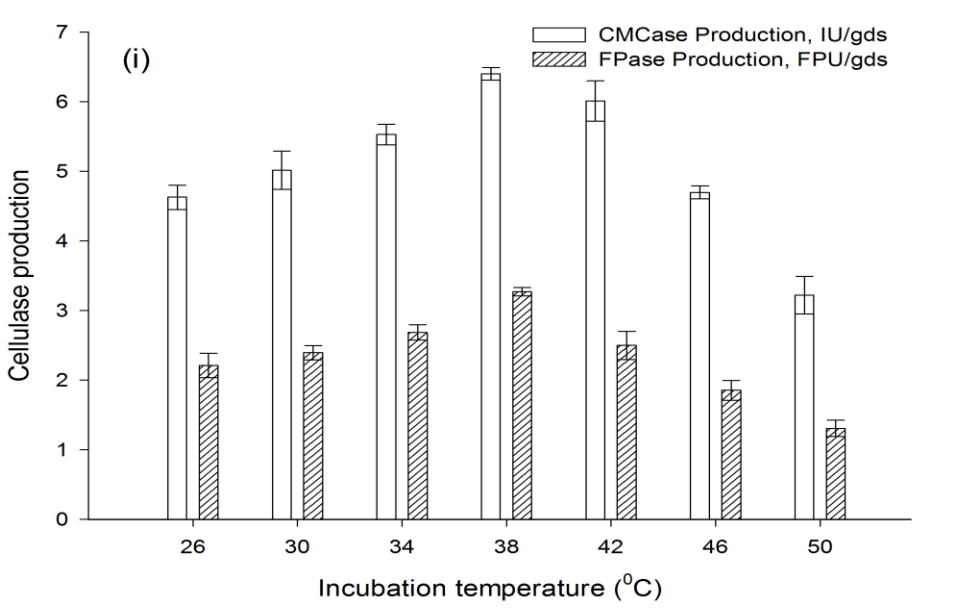
4a.3.4. Effect of temperature on fungal cellulase production

Temperature is one of the crucial factors, which affect both the cell growth and enzymes or metabolites production. Every microbe has some optimum temperature on which its secretary machinery works at the maximum.

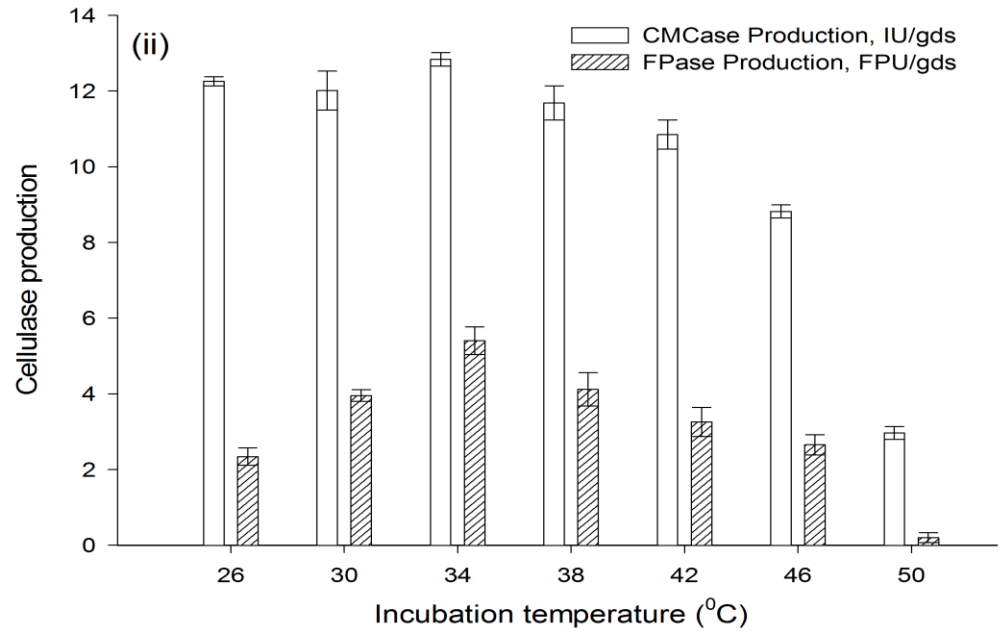
For *R. microsporus,* endoglucanase production was observed to increase from 26-38 °C temperature, with further increase in temperature it was declined. Temperature, 38 °C was observed to be best for *R. microsporus* with endoglucanase production of 6.39±0.08 IU/gds, at this temperature total cellulase production value was 3.26±0.06 FPU/gds (Fig. 4a.3.4 i). This range of temperature was also recorded favorable for *R. oryzae* in a previous work (Kannakar and Ray, 2010).

For isolate *P. oxalicum,* endoglucanase production was found to be increased marginally on increasing temperature from 26-34 °C, but it was reduced further at a higher temperature range (38-

50 °C). A temperature range of 34 °C was found to be optimum for endoglucanase production with value 12.83±0.18 IU/gds and total cellulase production 5.40±0.36 FPU/gds (Fig. 4a.3.4.ii).



Conditions (R. microsporus):
WB-5g, particle size- 36 mesh, NSS-1:3, Initial pH-7.0, Inoculum size- 6 discs, Incubation period-6 days



Conditions (P. oxalicum):
WB-5g, particle size- US, NSS-1:3, Initial pH-7.0, Inoculum size- 6 discs, Incubation period-6 days

Figure 4a.3.4. Effect of temperature on fungal cellulase production (i) *R. microsporus*, (ii) *P. oxalicum*

Work reported by other authors (Gautam *et al*., 2011) supports that mostly fungal species produce enzyme in a mesophilic range. The selected fungi were screened at temperature 45 °C but optimal temperature at a lower range supports the fungal growth temperature and enzyme production temperature can be different (Xin and Geng, 2010). At temperature of 46 °C and above, cellulase production decreased significantly. At a temperature other than optimum, enzyme production was less, which may be due to reduced mycelial growth (Sindhu *et al*., 2011).

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

4a.3.5. Effect of incubation period on fungal cellulase production

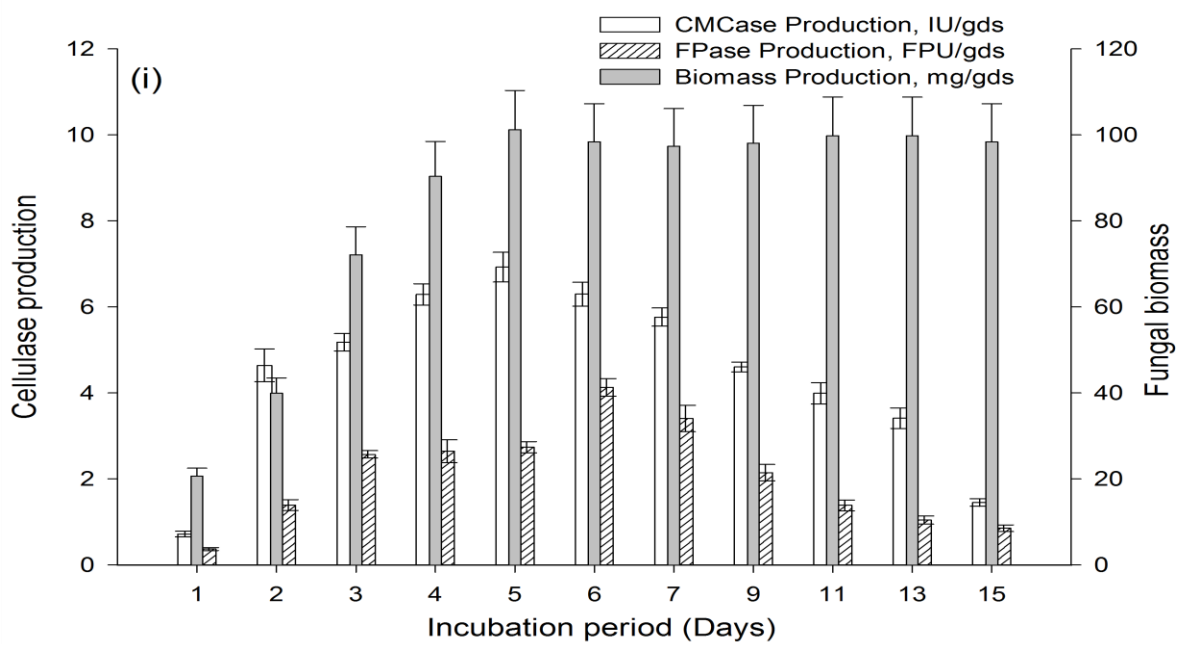
With the introduction of microorganism into the substrate medium, it starts to prepare itself for effective utilization of substrate by absorption of nutrients from the surroundings. Time of incubation may vary with the particular microorganism type and substrate to be used.

During initial period of incubation, the microorganism was in acclimatizing stage and therefore, no recordable fungal growth was observed for both the fungal isolates, as verified by a low value for biomass/glucosamine content (Fig. 4a.3.5.i & ii).

The endoglucanase production was peaked to maximum for *R. microsporus* after 5 days (6.92±0.34 IU/gds) and 4 days for *P. oxalicum* strain (14.06±0.27 IU/gds), respectively. However, total cellulase production was found to be maximum on day 6^{th} for *R. microsporus* with production value of 4.12±0.20 FPU/gds (Fig. 4a.3.5i), it was maximum on day 4^{th} for *P. oxalicum* with production value of 6.12±0.39 FPU/gds (Fig. 4a.3.5ii). Higher incubation results in significant decrease in enzyme production which may be due to nutrients depletion or pH variation in the medium or any cellular metabolism causing enzyme denaturation (Xin and Geng, 2010). Biomass content did not increase further, may be due to starvation conditions and unavailability of nutrients in the medium.

Potent fungal cellulase producers *Aspergillus, Trichoderma, Penicillium* etc. are also reported to produce cellulase within 3-6 days (Gautam *et al*., 2011; Camassola and Dillon, 2007).

Mycelial growth is an indication of substrate utilization. The probable reason for declination in cellulase production after attaining a maximum value may be catabolite repression. With increased incubation time, there is delay in harvesting of enzyme and thus, there occurs accumulation of hydrolysis products such as glucose and cellobiose, which may inhibit cellulase-system activity and could adversely affect the rate of cellulase production (Esterbauer *et al*., 1991, Montenecourt and Eveleigh 1977, Rocky and Hamidi 2010, Xin and Geng 2010).



Conditions (R. microsporus):
WB-5g, Particle size- 36 mesh, NSS-1:3, Initial pH-7.0, Inoculum size- 6 discs, Incubation T-38°C

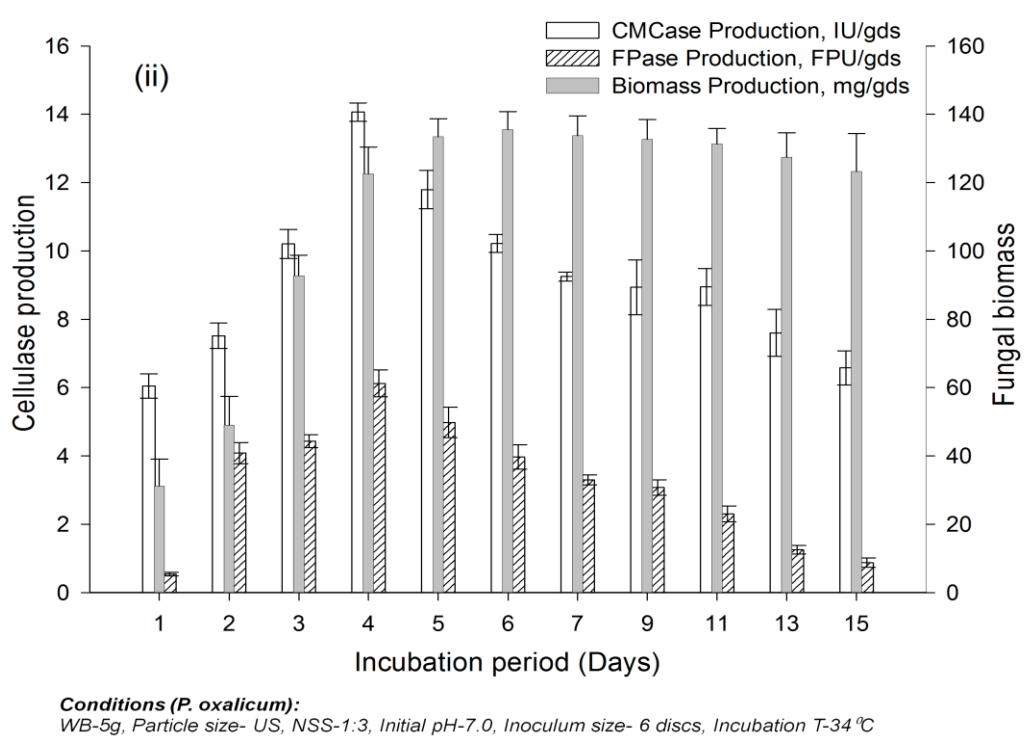
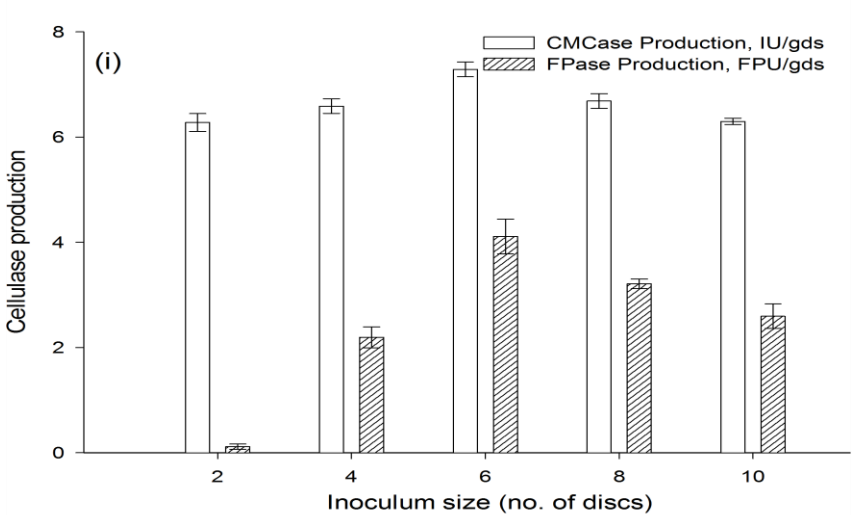


Figure 4a.3.5. Effect of incubation period on fungal cellulase production (i) *R. microsporus*, (ii) *P. oxalicum*

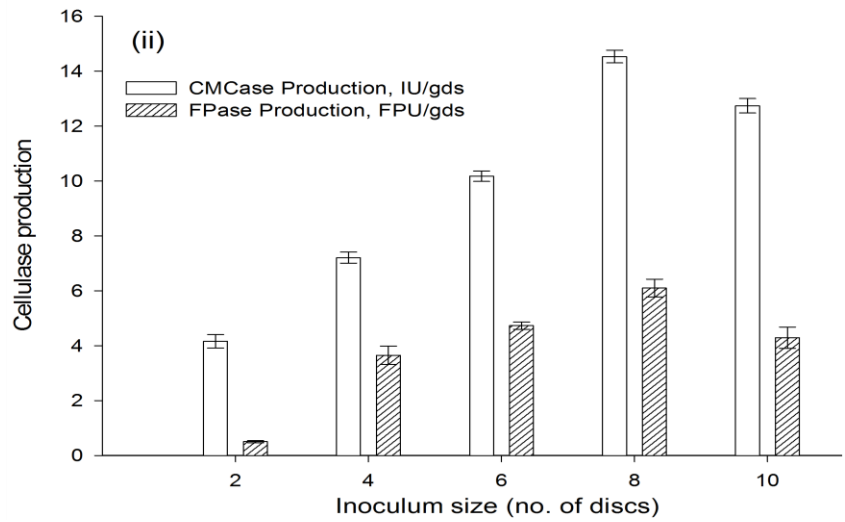
4a.3.6. Effect of inoculum size on fungal cellulase production

For fermentation process involving fungi, either vegetative mycelium or spores may be used as inoculum. However, spore inoculum has the disadvantage of an extended lag phase requirement for germination and extracellular enzyme induction (Babel *et al.*, 2007).

Inoculum size results shown marginal difference for *R. microsporus* and it was significant for *P. oxalicum*. The endoglucanase production increased slightly with inoculum size range 2-6 discs for *R. microsporus* and then decreased a little with high level of inoculum i.e. 8-10 discs (Fig. 4a.3.6.i). In case of *P. oxalicum* endoglucanase production increased significantly from 2-8 discs then decreased



Conditions (R. microsporus):
WB-5g, Particle size- 36 mesh, NSS-1:3, Initial pH-7.0, Incubation period-5 days, Incubation T-38 ºC



Conditions (P. oxalicum):
WB-5g, Particle size- US, NSS-1:3, Initial pH-7.0, Incubation period-4 days, Incubation T-34 ºC

Figure 4a.3.6. Effect of inoculum size on fungal cellulase production (i) *R. microsporus*, (ii) *P. oxalicum*

slightly at a high inoculum i.e. 10 discs (Fig. 4a.3.6.ii). Optimum inoculum level was found to be 6 discs for *R. microsporus* with endoglucanase production of 7.28±0.13 IU/gds and total cellulase production 4.11 ±0.32 FPU/gds. For *P. oxalicum* optimal level of inoculum observed was 8 discs with endoglucanase production value of 14.53±0.22 IU/gds and total cellulase production 6.09±0.32 FPU/gds, respectively.

Several studies employed fungal disc method for cellulase production using fungal inoculum ranging 2-8 discs (Kleman-Leyer and Kirk, 1994; Deswal *et al.*, 2011; Jadon *et al*., 2013). A low inoculums size retards the proliferation of biomass by affecting the initiation rate for microbial growth and thus

long lag phase. Thus, substrate degradation becomes slower and affects metabolite production. A higher inoculum size than the optimum may promote over growth of microbes, it may deplete the nutrients available in the medium and thereby, decrease enzyme production (Pandey *et al*, 2000; Ahmed *et al*. 2012).

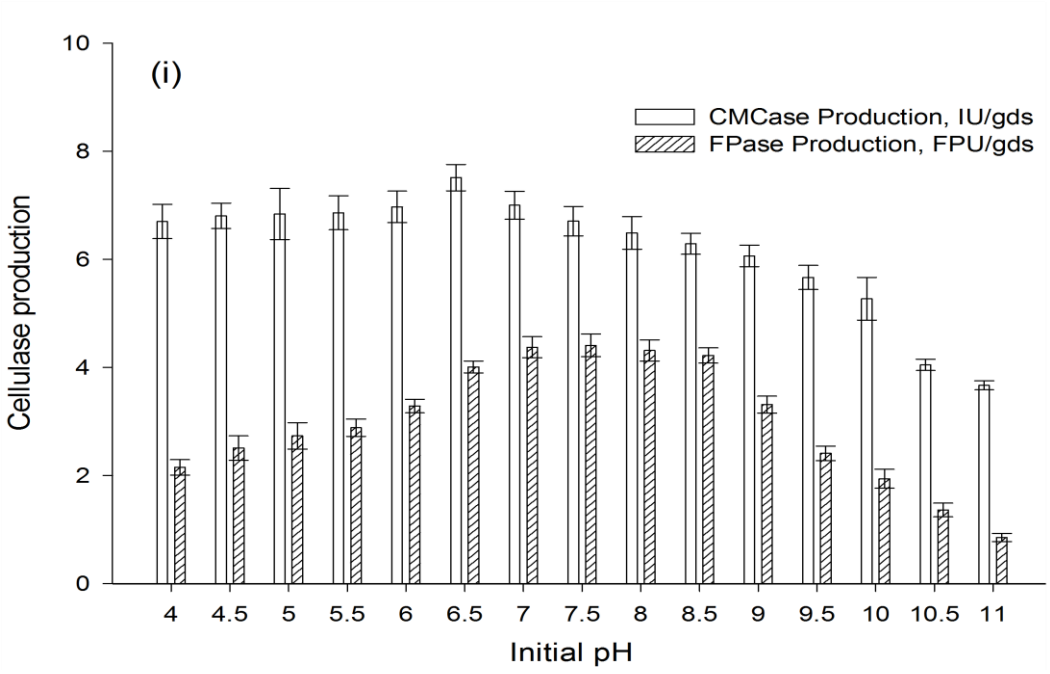
4a.3.7. Effect of initial pH on fungal cellulase production

pH of medium is another critical factor influencing enzyme production. Filamentous fungi can grow in wide pH range from 2.0-9.0 (Gowthaman *et al*., 2001).

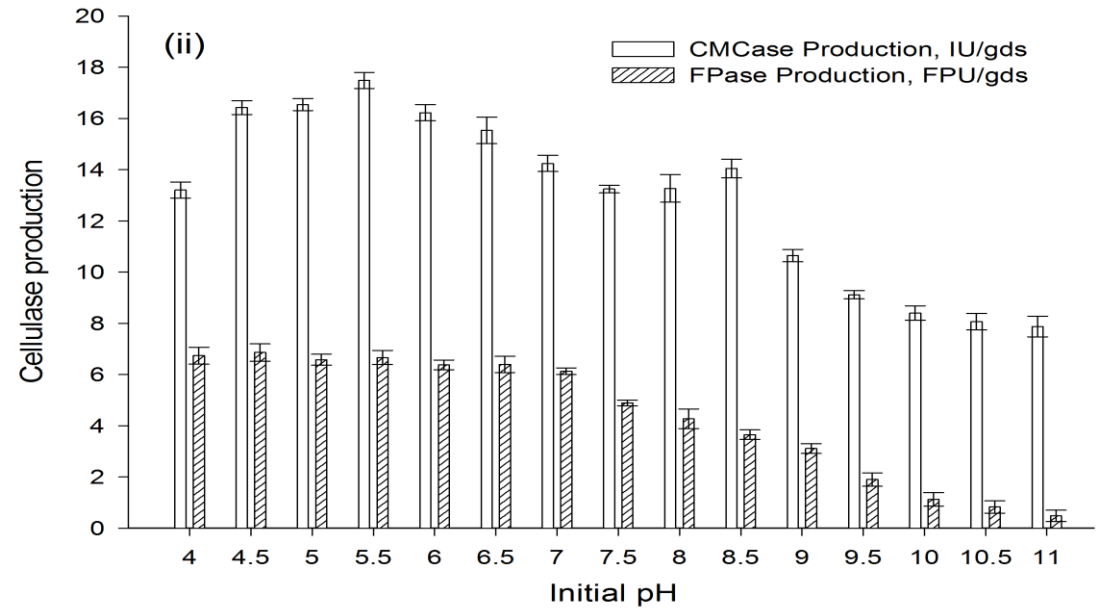
In this experiment, it was observed that in case of *R. microsporus* endoglucanase production was increased marginally in pH range 4.0-6.5, and then it decreased in alkaline range. Thus, optimal initial pH value was found to be 6.5 for *R. microsporus* (7.51±0.24 IU/gds for endoglucanase & 4.00±0.11 FPU/gds for total cellulase production). This level of pH was also found to be optimum by Karnnakar and Ray, 2010.

For *P. oxalicum* the same trend observed with increased endoglucanase production in acidic range but further decrease in alkaline range. Optimal initial pH 5.5 promoted endoglucanase production with 17.48±0.31 IU/gds value, at this pH total cellulase production was 6.66±0.27 FPU/gds (Fig. 4a.3.7.). An initial pH of 5.5 was also reported for maximum endoglucanase production (Zhang *et al*., 2009).

pH lower or higher than optimum affects enzyme production. The pH of the medium is an important parameter for enzyme production. Microorganisms may need to adapt their function in order to cope up with a change in hydrogen ion concentration. If this change is too abrupt, the response of microbes might lag behind or overshoot. Apart from affecting cell membrane permeability, pH may also determine the solubility of some components of the medium because of ionic nature of amino



Conditions (R. microsporus):
WB-5g, Particle size- 36 mesh, NSS-1:3, Inoculum size-6 discs, Incubation period-5 days, Incubation T-38 ℃



Conditions (P. oxalicum):
WB-5g, Particle size- US, NSS-1:3, Inoculum size-8 discs, Incubation period-4 days, Incubation T-34 ℃

Figure 4a.3.7. Effect of initial pH on fungal cellulase production (i) *R. microsporus*, (ii) *P. oxalicum*

and carboxylic groups present in proteins. Any change in these groups will affect the catalytic ability of enzymes and metabolic processes (Poorna and Prema 2007, Kaur *et al*. 2011). Thus, a modification in the pH might also cause some micronutrients to precipitate and become impossible to be assimilated (Sanchez *et al*., 1997). The permeability of cells as well as stability of enzyme gets

affected with the change in the pH value of fermentation medium (Ahmed *et al*., 2012). Therefore, an optimum pH during fermentation will avoid the possibility of denaturation at lower and higher pH.

4a.3.8. Effect of initial moisture content on fungal cellulase production

Moisture content in SSF plays a crucial role because it influences cell growth, biosynthesis and enzyme secretion. In a SSF process, this parameter depends on the nature of substrate, microbial requirements and the type of end-product (Kalogeris *et al*., 2003).

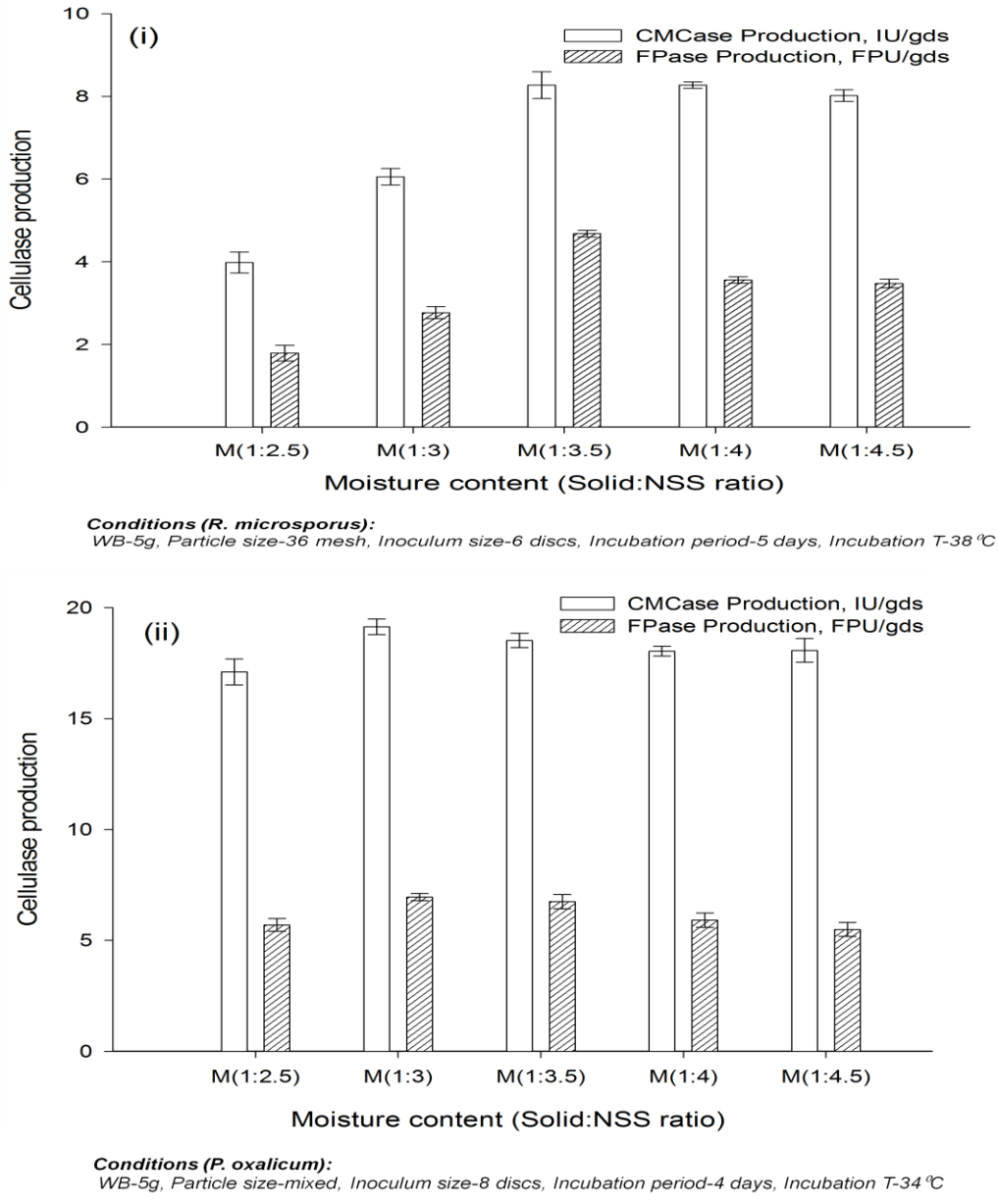


Figure 4a.3.8. Effect of initial moisture content on fungal cellulase production (i) *R. microsporus*, (ii) *P. oxalicum*

For both the isolated fungi, a very low and very high range of initial moisture content slightly reduced endoglucanase production. However, it was observed that initial moisture content ranging 1:3.5 was optimum for strain *R. microsporus* with endoglucanase production of 8.26±0.32 IU/gds and total cellulase production 4.67±0.07 FPU/gds (Fig. 4a.3.8.i). For strain *P. oxalicum* initial moisture content 1:3 given maximum endoglucanase production (19.13±0.35 IU/gds) and total cellulase production with value of 6.94±0.16 FPU/gds (Fig. 4a.3.8.ii). Using WB as substrate in SSF conditions Deswal *et al*., (2011) reported maximum endoglucanase production for *Fomitopsis* at 1:3.5 and Pathak *et al*., (2014) for *Trichoderma harzianum* at 1:3, substrate:initial moisture ratio.

Availability of moisture depends on the water-binding capacity of substrate. This interferes with oxygen transfer affecting both growth and metabolism of fungi. Moisture content relates with aeration and diffusion of both oxygen and nutrients. Low moisture may reduce the swelling capacity of substrate and minimize microbial growth. High moisture content results in swelling of substrate, thereby facilitating better utilization of the substrate by the microorganism (Pandey *et al*., 2000). A reduction in enzyme yield at high moisture level may be due to reduction in inter particle space, decreased porosity, gummy texture, alteration in WB particle structure and impaired oxygen transfer (Babel *et al*., 2007).

4a.3.9. Effect of nitrogen source on fungal cellulase production

Nitrogen source is known to be sensitive for cellulase production (Chaabouni *et al*. 1995, Kachlishvili *et al*. 2006; Soni *et al*. 2010). The ratio of C/N is most crucial for a particular process to obtain specific production (Brijwani *et al*. 2010). Enzyme production is influenced by precursors of protein synthesis. In this study, organic, inorganic and plant based nitrogen sources were tested for enzyme production by both the fungal isolates.

Among various organic nitrogen sources studied, soya peptone (SP, 2 g/L) was found to be the best organic nitrogen source for *R. microsporus* with endoglucanase production of 12.15±0.74 IU/gds and total cellulase production 5.56±0.41 FPU/gds (fig. 4a.3.9a.-i). It was followed by beef extract (BE, 1 g/L), which produced endoglucanase 11.79±0.20 IU/gds and total cellulase, 4.49±0.27 FPU/gds and tryptone (TP, 4 g/L) with 11.33±0.17 IU/gds for endoglucanase and 5.32±0.32 FPU/gds for total cellulase. Biomass content was high for all concentrations of proteose peptone (PP) and yeast extract (YE), it was low for meat peptone (MP) using *R. microsporus*. With *P. oxalicum* biomass production was maximum using yeast extract (YE). No major difference was observed with all other substrates.

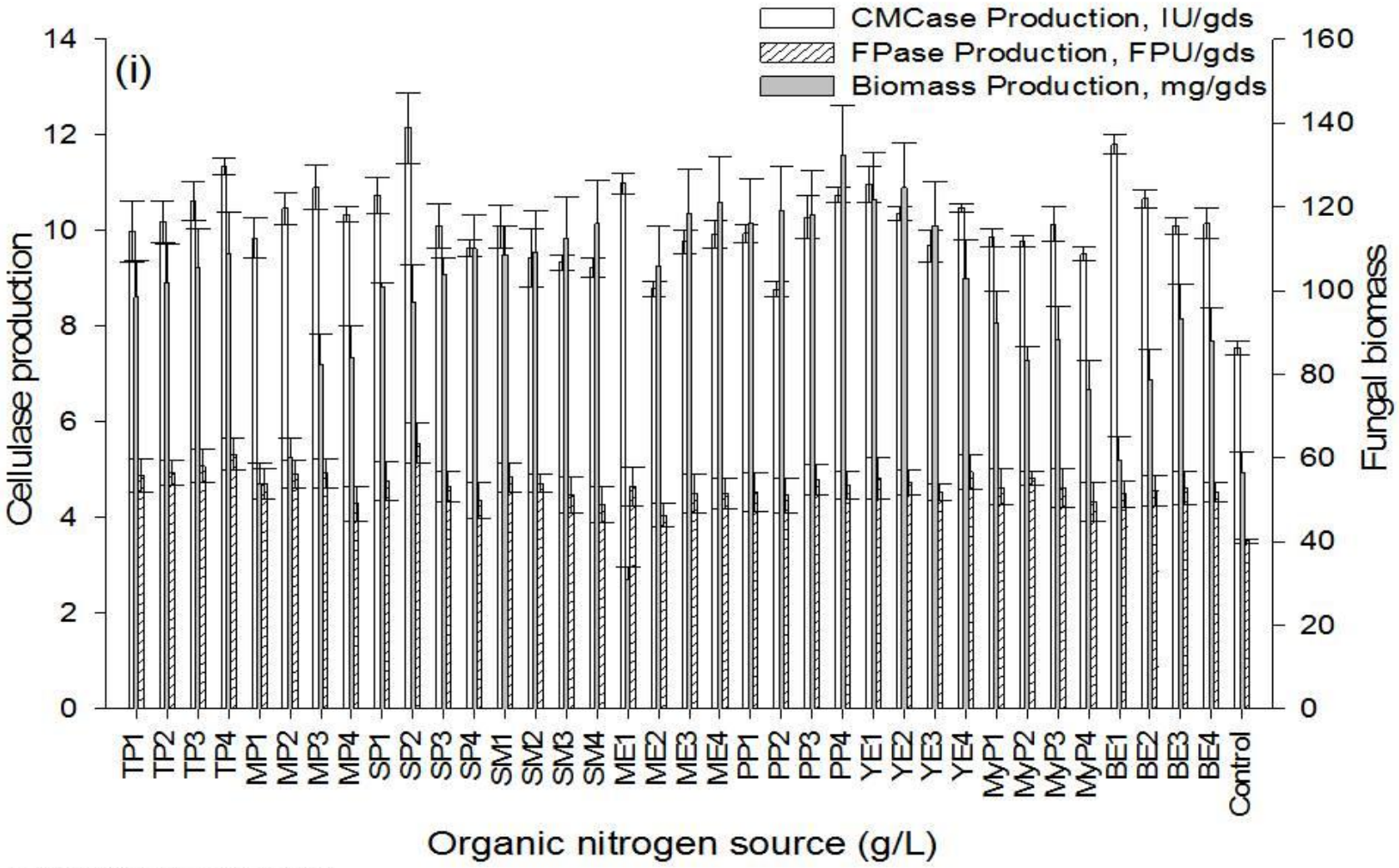
For *P. oxalicum*, yeast extract (YE, 4 g/L) was resulted in the maximum endoglucanase production with value of 22.81±0.19 IU/gds and total cellulase, 6.79±0.30 FPU/gds (Fig. 4a.3.9a.-ii). It was followed by tryptone (TP, 4 g/L) and beef extract (BE, 3 g/L). The enzyme production value was 22.74±0.23 IU/gds for endoglucanase and 6.85±0.47 for total cellulase using TP, 4 g/L. It was 22.01±.69 IU/gds for endoglucanase and 6.11±0.59 FPU/gds for total cellulase using BE, 3 g/L.

Peptones are nitrogen rich and easily soluble nutrient material for fungal growth. Other researchers have reported different nitrogen sources for cellulase production like peptone and yeast extract using *Cellulomonas fimi* (Ali *et al*., 2013), beef extract for *Aspergillus fumigatus* (Soni *et al.*, 2010), soya peptone for *Trichoderma lignorum* (Baig, 2005) and tryptone for *Aspergillus* and *Trichoderma* sp. (Das *et al*., 2011) as organic nitrogen sources.

In general, the yields of hydrolytic enzymes are increased by supplementation of medium with an additional nitrogen source (Kachlishvili *et al*. 2006). The production of enzymes is increased with the nitrogen content because the fungal biomass increased with the level of nitrogen concentration and excess nitrogen in the media suppressed growth (Qinnghe *et al*. 2004). The differences in production on various complex organic nitrogen sources may be due to their varying contents of amino acids, peptides, vitamins, trace elements and inducers (Lan *et al*. 1998). These bio-molecules can be absorbed directly by mycelia.

In this study, inorganic nitrogen source showed slightly lower production than organic nitrogen sources. However, some studies reported inorganic nitrogen source resulted in improved enzyme production compared to organic nitrogen source (Kalogeris *et al*., 2003). For both the isolated fungi ammonium nitrate (AN) gave maximum endoglucanase production with values of 10.66±0.18 IU/gds for *R. microsporus* and 22.49±0.40 for *P. oxalicum* at same level of 0.03M concentration of nitrogen source (figure 4a.3.9-b.). Total cellulase production values using same source were 4.04±0.26 FPU/gds for *R. microsporus* and 8.49±0.40 FPU/gds for *P. oxalicum*. In case of *R. microsporus*, biomass content was found to be promoted using nitrates and sulphates and it retarded using chloride or acetate. Biomass formation did not affect greatly using inorganic nitrogen sources for *P. oxalicum.*

Ammonium nitrate was also reported to enhance cellulase production by *Rhizopus oligosporus* (Vattem and Shetty, 2002) and for *P. oxalicum* (Zhang *et al*., 2009) in a previous study. This was most probably due to the role played by ammonia as it may be transported into the cell, which triggered the synthesis of protein and cellulase (Spiridonov and Wilson, 1998). Apart from that some reports has supported that ammonium sulfate facilitated cellulase production in many fungus such as



Conditions (R. microsporus):

WB-5g, Particle size-36 mesh, Inoculum size-6 discs, Incubation period-5 days, Incubation T-38°C, Initial pH- 6.5, Initial moisture-1:3.5
TP-Tryptone, MP- Meat peptone, SP- Soya peptone, SM- Soya bean meal, ME- Malt extract, PP- Proteose peptone, YE- Yeast extract, MyP- Mycological peptone, BE- Beef extract

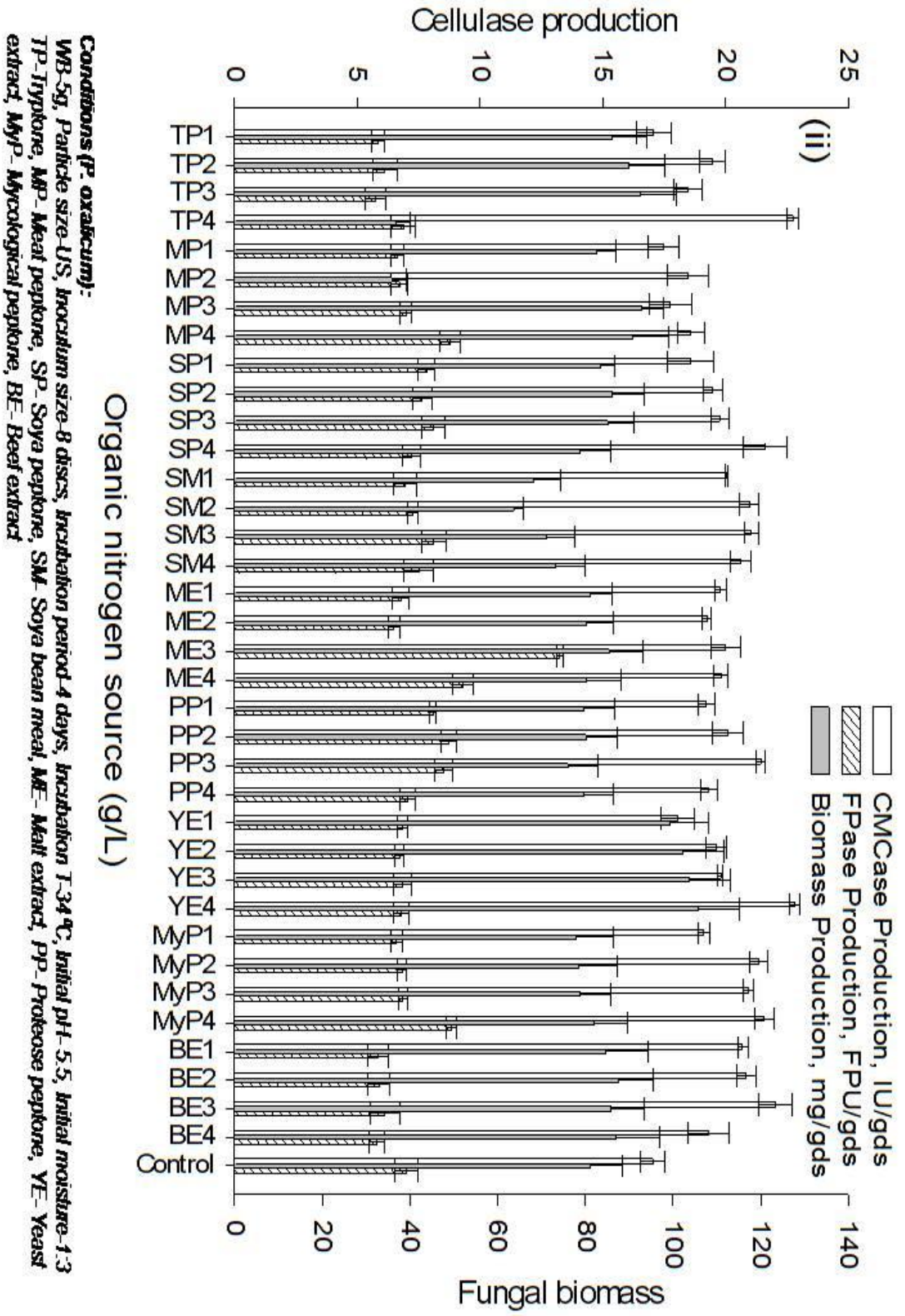
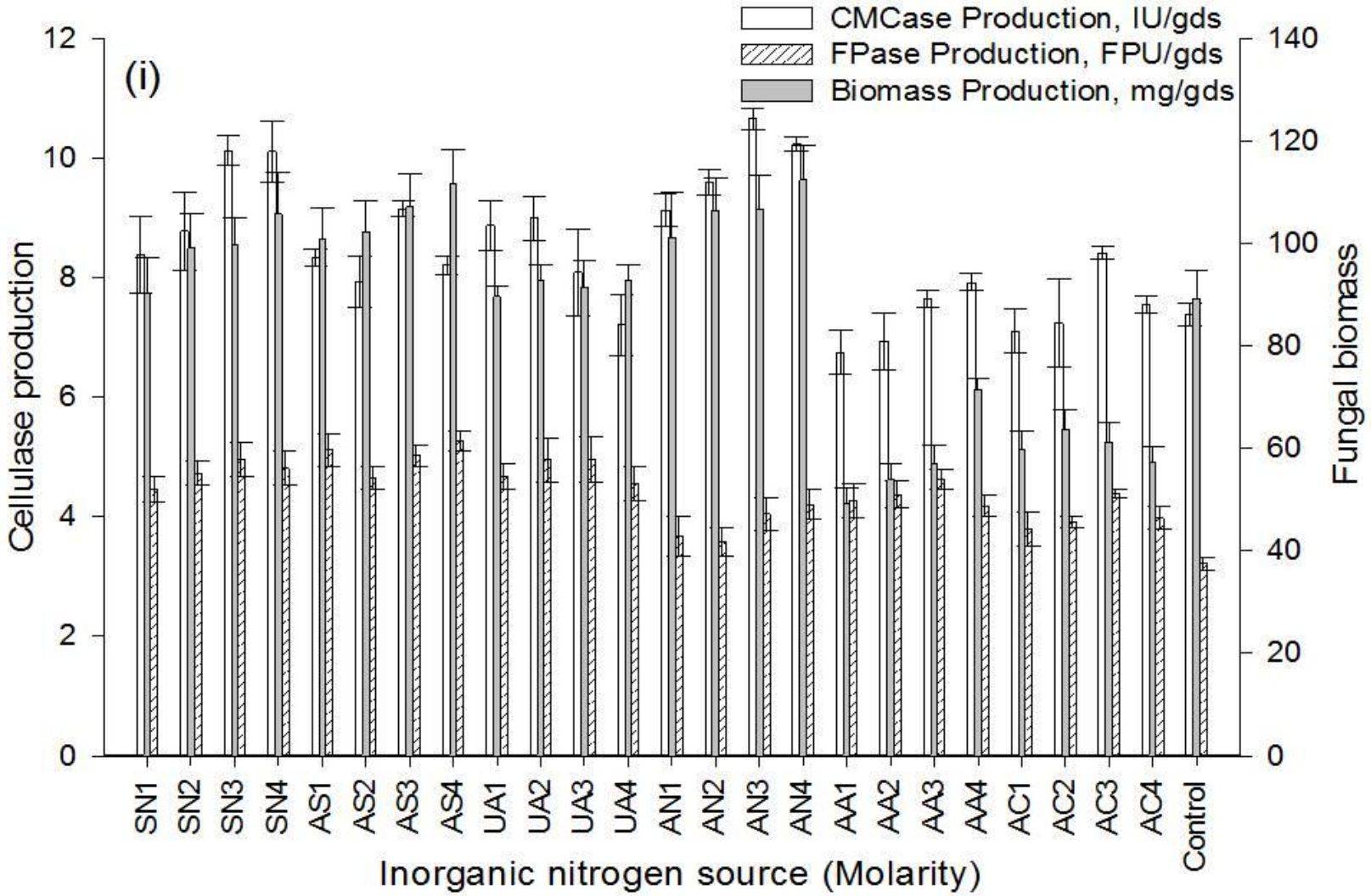


Figure 4a.3.9a. Effects of organic nitrogen sources on fungal cellulase production (i) *R. microsporus*, (ii) *P. oxalicum*



Conditions (R. microsporus):

WB-5g, Particle size-36 mesh, Inoculum size-6 discs, Incubation period-5 days, Incubation T-38°C, Initial pH- 6.5, Initial moisture-1:3.5
AN-Ammonium nitrate, AA- Ammonium acetate, AC- Ammonium chloride, SN- Sodium nitrate, AS- Ammonium sulphate, UA- Urea
1,2,3,4 indicates molar concentrations: 0.01, 0.02, 0.03 and 0.04, respectively

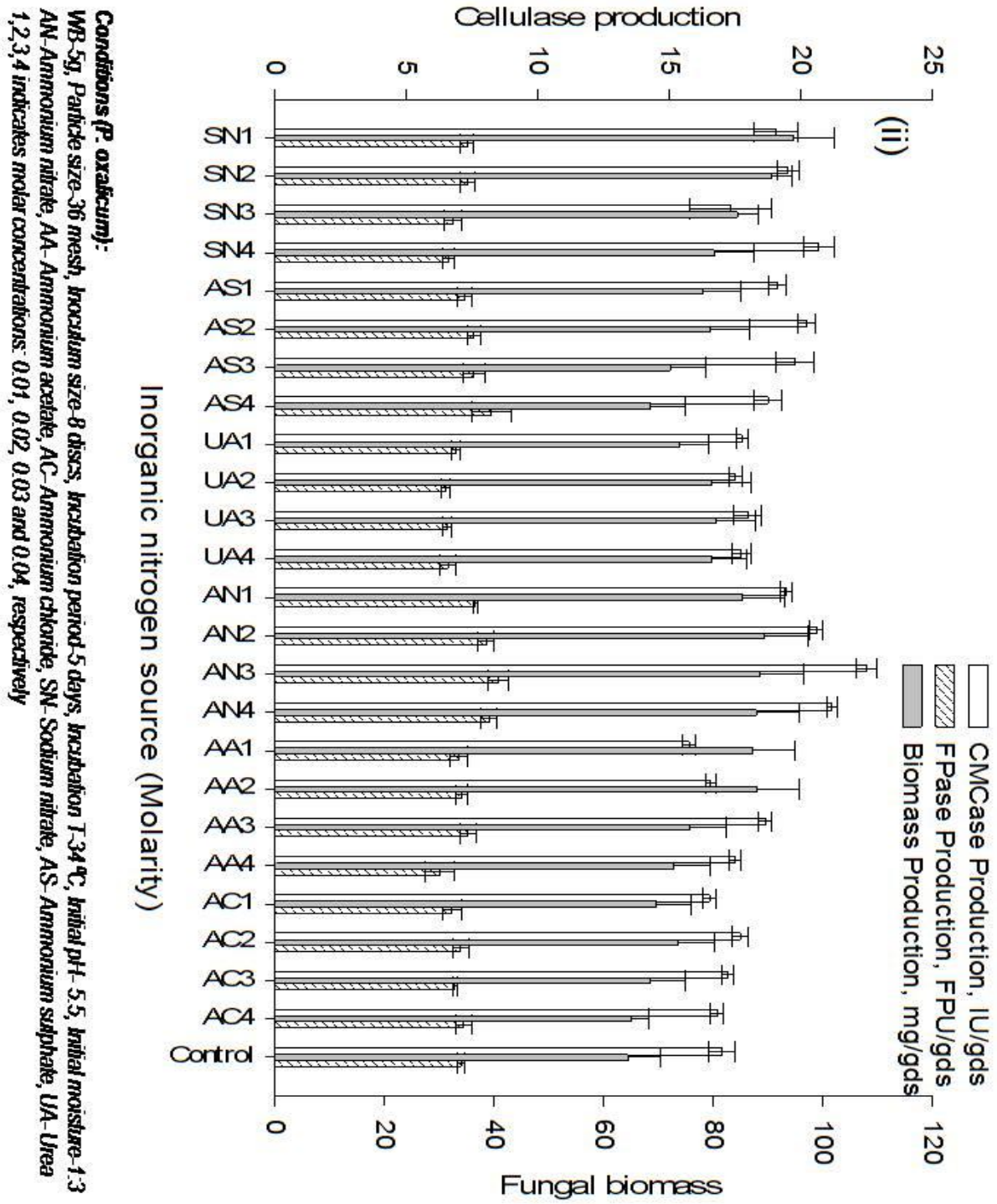
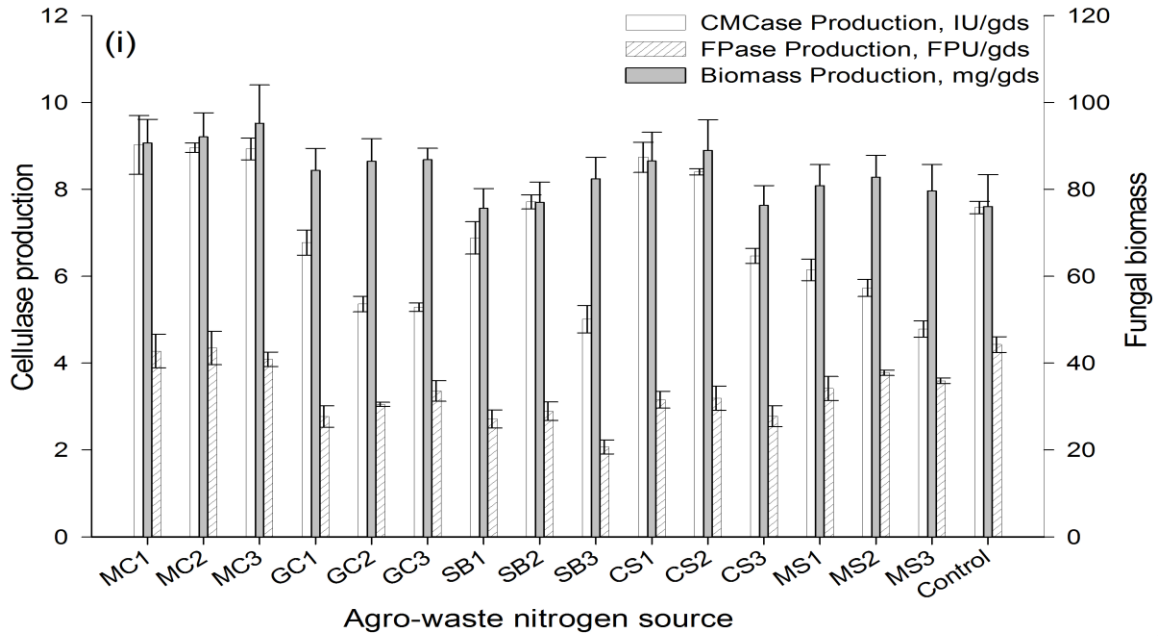


Figure 4a.3.9b. Effect of inorganic nitrogen sources on fungal cellulase production (i) *R. microsporus*, (ii) *P. oxalicum*

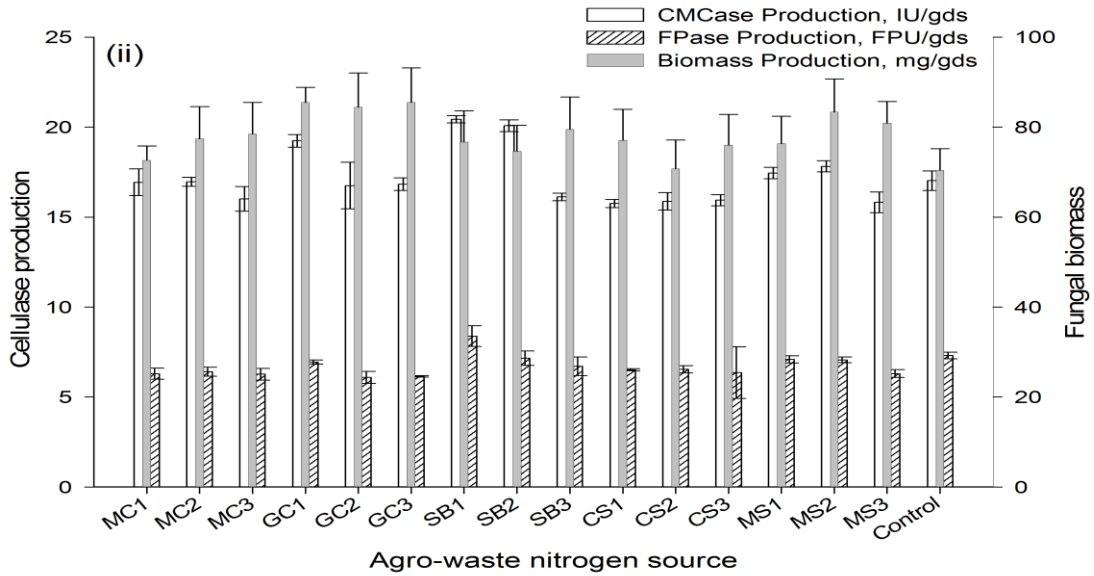
Penicilium funiculosum, Myrothecium sp., Chaetomium cellulolyticum, T. reesei, A. niger, A. terreus (Fadel, 2000) and *Rhizopus oryzae* (Fadel, 2000; Karmakar and Ray, 2010) in both SSF and SmF

process. According to Stewart and Parry (1981), cellulase production is always high when ammonium salts are used.

Some plant based nitrogen sources was also tested in different combinations to carbon source. Agro-



Conditions (R. microsporus):
WB-5g, Particle size-36 mesh, Inoculum size-6 discs, Incubation period-5 days, Incubation T-38 ^{0}C, Initial pH- 6.5, Initial moisture-1:3.5
MC- Mustrad cake, GC- Gram seed coat, SB- Spya bean hull, CS- Castor seeds, MS- Masoor seed coat
1,2,3 indicates Insoluble agro waste-Nitrogen:WB ratios, 1:3, 1:1 & 3:1, respectively



Conditions (P. oxalicum):
WB-5g, Particle size-US, Inoculum size-8 discs, Incubation period-4 days, Incubation T-34 ^{0}C, Initial pH- 5.5, Initial moisture-1:3
MC- Mustrad cake, GC- Gram seed coat, SB- Spya bean hull, CS- Castor seeds, MS- Masoor seed coat
1,2,3 indicates Insoluble agro waste-Nitrogen:WB ratios, 1:3, 1:1 & 3:1, respectively

Figure 4a.3.9c. Effect of natural nitrogen sources on fungal cellulase production (i) *R. microsporus*, (ii) *P. oxalicum*

wastes were taken as nitrogen source were insoluble and mixed in solid substrate WB, therefore in order to provide same solid bed properties as bed height, bed weight etc; substrate was kept constant to 5 g in all flasks. For *R. microsporus*, the enzyme production was enhanced by mustard cake (MC) but not equivalent to synthetic nitrogen sources (inorganic/organic) with endoglucanase production of 9.02±0.67 IU/gds (Fig. 4a.3.9c-i) in MC+WB composition of 1:3. This substrate was observed to have total cellulase production ranging 4.27±0.38 FPU/gds.

For *P. oxalicum* endoglucanase production was comparable using soya bean hulls with endoglucanase production range 20.42±0.20 IU/gds and total cellulase production, 8.38±0.57 FPU/gds (Fig. 4a.3.9.c-ii). No major fluctuation was observed in biomass content for both the isolated fungi using agro-wastes for all substrates.

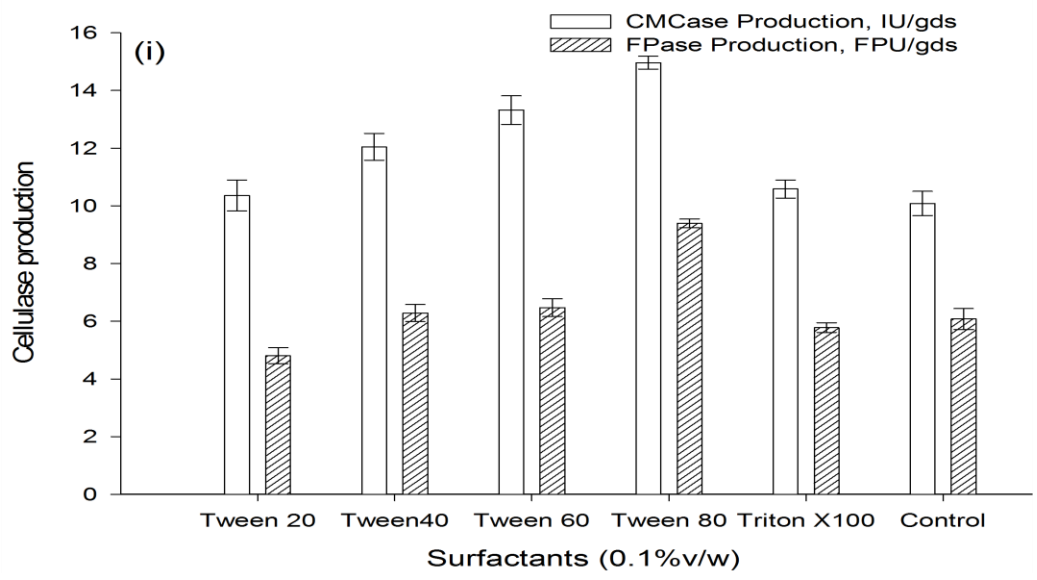
In a previous work for *Pencillium* species, mustard cake was reported to enhance endoglucanase level (Dutta *et al.*, 2008). In a co-culture study for *Trichoderma ressei* and *Aspergillus oryzae* SB mixed with WB in 4:1 was used for production of cellulolytic enzyme in SSF (Brijwani *et al*., 2010).

4a.3.10. Effect of surfactants on fungal cellulase production

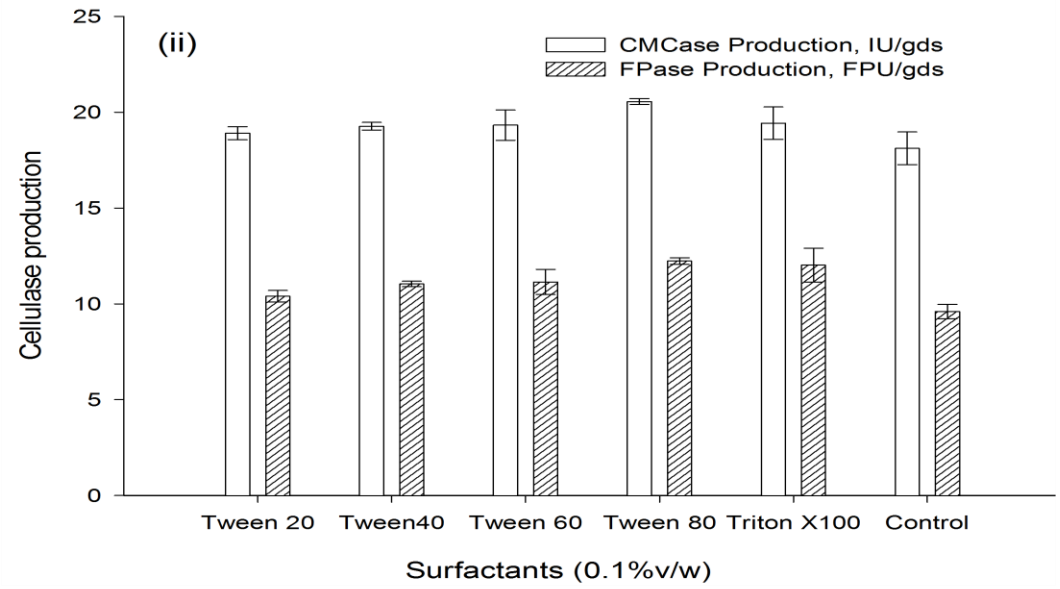
Surfactants can act as a leaching agent for enzyme. The various surfactants Tween 20, Tween 40, Tween 60, Tween 80, and Triton X100 were tested. For *R. microsporus*, Tween 20 and Trition X 100 didn't influence cellulase production. It was observed to improve slightly using Tween (40, 60 and 80). Using Tween 80 endoglucanase level reached to 14.96±0.22 IU/gds and total cellulase, 9.39±0.15 FPU/gds (Fig. 4a.3.10.i). .

For *P. oxalicum*, Tween 20 was found to have no effect on cellulase production. Using other surfactants, it showed slight improvement. Using Tween 80, cellulase production value reached to 20.55±0.15 IU/gds for endoglucanase and total cellulase, 12.23±0.17 FPU/gds. Therefore, for both the fungi, Tween 80 gave the maximum endoglucanase production (Fig. 4a.3.10).

The possible reason behind enhanced enzyme level using Tween 80 may attributes to the stimulatory effect of the non-ionic surfactant that alters the cell membranes causing increased permeability and efficient uptake of nutrient as well as promoting the release of cell-bound enzymes (Prado, 1996). The similar results were also reported by other researchers (Kuhad *et al*., 1994; Sonia and Chadha 2005; Gomes *et al*., 2006).



Conditions (R. microsporus):
WB-5g, Particle size-36 mesh, Inoculum size-6 discs, Incubation period-5 days, Incubation T-38 ℃, Initial pH- 6.5, Initial moisture-1:3.5, Nitrogen source- ammonium nitrate (0.03 M)



Conditions (P. oxalicum):
WB-5g, Particle size-US, Inoculum size-8 discs, Incubation period-4 days, Incubation T-34 ℃, Initial pH- 5.5, Initial moisture-1:3, Nitrogen source- Soya bean hull (WB+SB, 3:1)

Figure 4a.3.10. Effect of surfactants on fungal cellulase production (i) *R. microsporus*, (ii) *P. oxalicum*

Most of the researchers have used tween 80 in concentrations between 0.1 and 0.2% due to obvious positive effect of the non-ionic surfactant towards improving the permeability of the membranes for cellulases due to its interaction with the lipid components of cell membranes (Ahamed and Vermette 2008, Pardo 1996). This facilitates the contact between the fibrous portion of the substrate and

mycelium (Eriksson *et al*. 2002, Esterbauer *et al*. 1991, Reese and Maguire 1969, Singh *et al*. 2007,) and helps to uptake nutrient efficiently as well as promoting the release of cell-bound enzymes (Pardo 1996, Soni *et al*. 2010). According to Kruszewska *et al*. (1990), Tween-80 has an influence on the level of glycosylation and thus, on protein stability.

4a.4. Production and characterization of fungal cellulase after mass production

Industrial importance of an enzyme depends on its optimum temperature, pH and operational stability. Thus, in the present study optimizations of parameter for crude enzyme was carried out.

After optimization of fermentation conditions, mass production of cellulase from both the fungal strains was carried out under optimized conditions of SSF. The mass production was carried out in flasks of 1 L capacity. The enzyme thus obtained in crude form was used for refining experiments.

4a.4.i. Enzyme Production

The crude enzyme obtained after mass production was tested for various enzyme activities as under in Table 4a.2:

Table 4a.2. Enzyme profile of crude fungal enzymes after mass production under optimized conditions of SSF

Fungal strains	Endoglucanase (IU/gds)	Total cellulase (FPU/gds)	β-glucosidase (IU/gds)
R. microsporus	15.0±0.5	9.22±0.14	19±2
P. oxalicum	20.9±0.4	11.6±0.3	31±3

Assay conditions: T-45 °C, pH-7.0

± SD from mean

4a.4.ii. Optimum temperature, pH and stability

Endoglucanase and total cellulase activities for *R. microsporus* were optimally active at temperature 55 °C and pH 5.5 and 6.0, respectively (Fig. 4a.4i-a, b). This range of temperature and pH was also found to be optimum for other *Rhizopus* species for cellulase activity (Murashima *et al.*, 2002).

For *P. oxalicum* endoglucanase and total cellulase activities were found to be optimum at temperature 50 °C and 55 °C and pH 4.5 and 5.0, respectively (Fig. 4a.4.ii-a, b). Previous work on

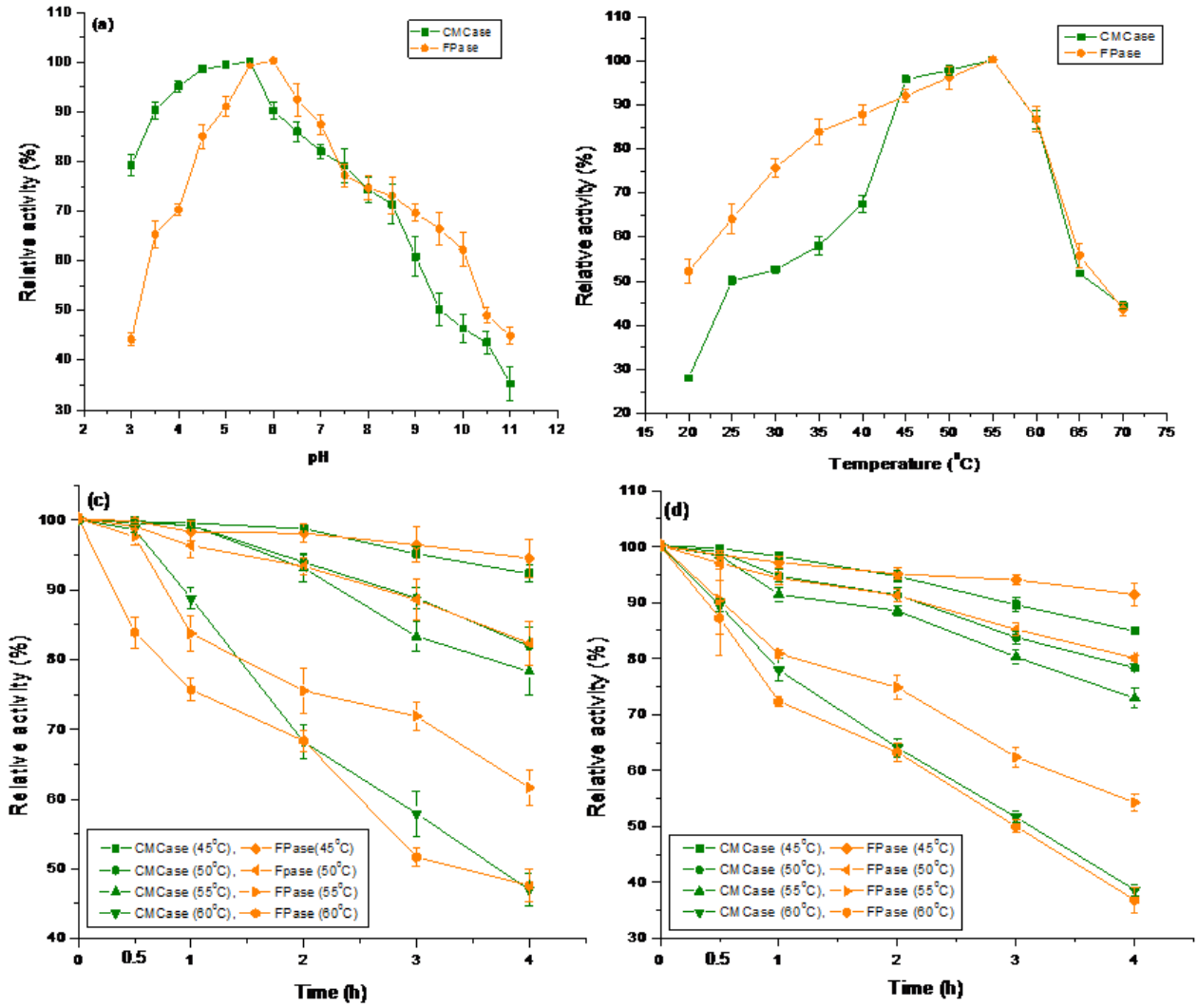


Figure 4a.4.i. pH (a) and temperature (b) optima curves of crude cellulase from *R. microsporus*, and its thermostability profile at optimum pH (c) and pH 7.0 (d) for upto 4 hours at different temperatures

Penicillium pinophilum reported endoglucanase activity in broad range pH of 4-7, with optimum pH 5.0 and temperature 50 °C (Pol *et al*., 2012).

For both the isolated fungi, it was found that the cellulase was active in a wide pH range. For *R. microsporus* more than 70% cellulase activity remained at pH 8.5, it was halved at temperature 65°C. For *P. oxalicum* cellulase activity was nearly 70% at pH 8.5, and it also reduced to its half activity at a high temperature of 65°C.

In a study performed on *Aspergillus niger* HN-2, it was found to produce cellulase with 80% activity at pH 3.0, with wide range of activity between pH 3.0 to 5.0 for FPase (Oberoi *et al*., 2014). *Aspergillus terreus* was reported to produce endoglucanase active in pH range 3.5-6.0 (around 90-

100% range), but with a sharp decline in activity at pH 7.0 (Narra *et al*., 2014). Optimum pH value at 5.5 with 80% endoglucanase activity at pH 3.5 and 50% at pH 7.0 was reported for *Penicillium citrinum*, but enzyme activity was increased again at pH 8.0 (Dutta *et al.*, 2008).

Earlier studies on cellulase production with special reference to pulp refining application showed enzyme activity with optimum pH at 5.5 and temperature 53 ^{o}C for recombinant cellulase obtained by bacterium, *Paenibacillus sp*. (Pastor *et al.*, 2001). For fungal strains used in refining optimal cellulase activity was found at optimal pH 4.0-6.0 and temperature 45 ^{o}C for *Trichoderma sp.* (Liu and Hu, 2012). Similar results were found for *Mamillisphaeria* sp. for cellulase with optimal pH value at 5.5 and temperature 50 ^{o}C (Laothanachareon *et al.*, 2011).

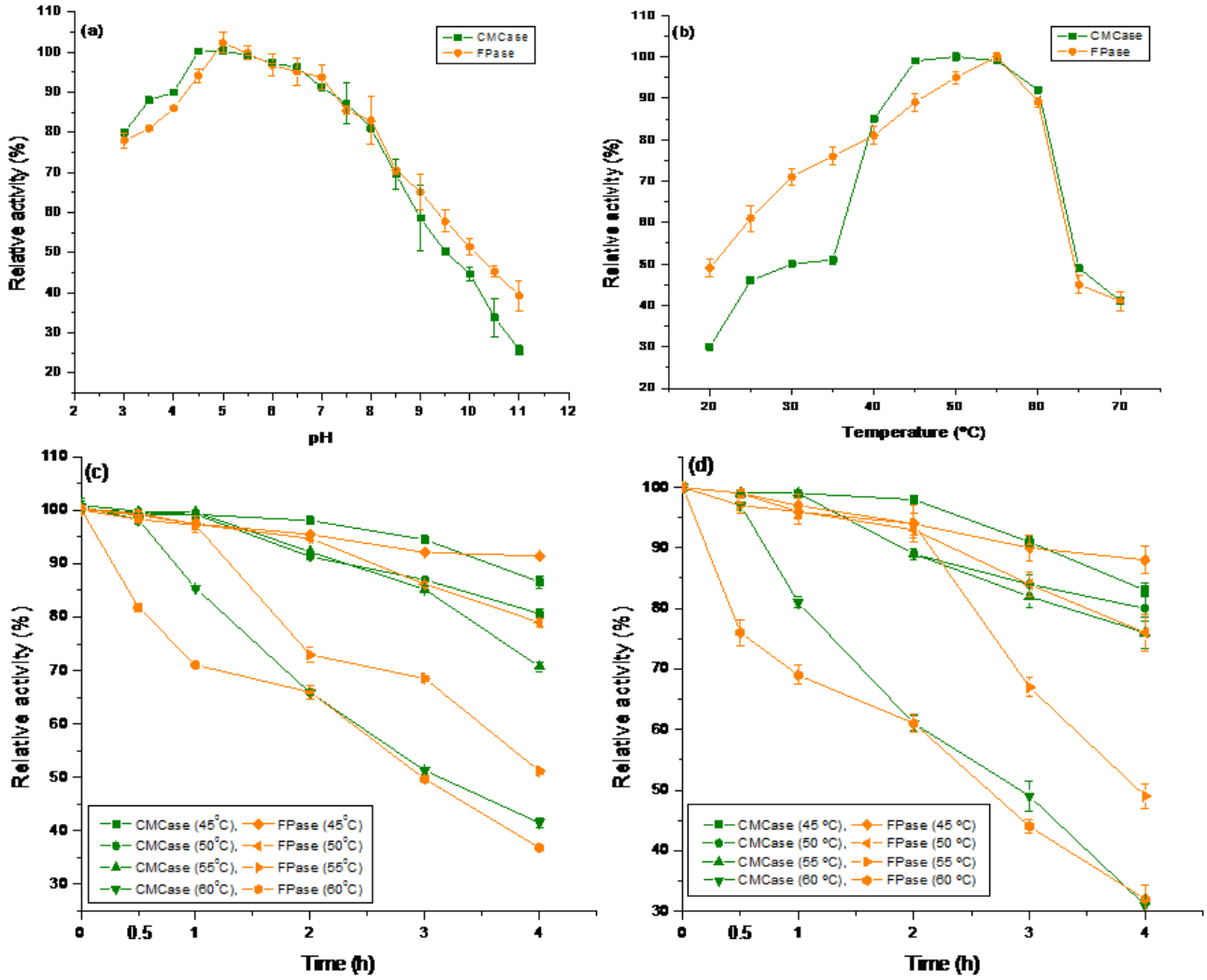


Figure 4a.4.ii. pH (a) and temperature (b) optima curves of crude cellulase from *P. oxalicum*, and its thermostability profile at optimum pH (c) and pH 7.0 (d) for upto 4 hours at different temperatures

At pH 7.0 and temperature 45 ºC (suitable conditions for refining), both the endoglucanase and total cellulase activities remain stable and it retain more than 85% of its activity upto 4 h for *R. microsporus* and 80% for *P. oxalicum*, respectively (Fig. 4a.4i c, d & Fig. 4a.4ii c, d). These stabilities for crude enzymes are good at application conditions of temperature and pH even after 4 hours i.e. more than any anticipated enzymatic reaction for refining application.

Table 4a.3. Characteristics of cellulase employed in pulp and paper industry

Microorganism used	**Enzyme type**	**Optimum T (°C)**	**Optimum pH**	**References**
Mamillispahaeria sp.	Crude	50	5.5	Larathaceron *et al*., 2010
Trichoderma sp.	Purified	45	4.0-6.0	Liu and Hu, 2012
Fusarium sp.	Purified	50	5.0	Vyas and Lachke, 2003
Ceriporiopsis subvermispora	Crude	60	4.0-5.0	Heidorne *et al*., 2006
Fomitopsis sp.	Crude	30	5.5	Deswal *et al.*, 2011
Aspergillus terreus	Crude	50-60	5.5	Marques *et al*., 2002
Trichoderma viride	Crude	50-60	5.5	-
Bacillus sp.	Purified	40	4.0	Blanco *et al.*, 1998
Paenibacillus sp.	Purified	53	5.5	Pastor *et al*., 2001
Trichoderma harzianum	Crude	55-60	5.5	Pathak *et al*., 2014

For both the isolated fungi, at optimum pH, enzyme stability was quite higher than at pH 7.0. The enzyme stability profile at various temperatures is shown in figure 4a.4 (c & d). It was observed for *R. microsporus* that endoglucanase activity remained 92% after 4 h at 45 °C, 81% after 4h at 50 °C, 78% at 55°C for 4 h incubation; this was declined to 46% at a higher temperature 60 °C after 4h. For *P. oxalicum* endoglucanase activity remained 86% at 45 °C, 80% at 50 °C, 70% at 55 °C and 41% at 60 °C after 4 h of incubation period. Purified bacterial cellulase (used for pulp refining application), obtained from recombinant *Paenibacillus campinasensis*, retains more than 90% stability at pH 6 in temperature range 30-50 °C even after 7 h of incubation (Ko *et al.*, 2010). A list of reported cellulase

characteristics, which were found to be effective in paper industry, is given in table 4a.3 showing optimum temperature and pH and it showed that they are in same range as obtained by the isolated fungal strains.

Chapter 4 b

Enzyme-assisted refining of pulp

In this section application part of enzyme in refining is described. For commercial (CA and CB) and laboratory produced fungal enzymes (LA and LB), experiments were performed in different ranges of enzyme dosages and reaction times.

4b.1. Enzymes used in refining

Addition of enzyme was done on CMCase activity basis. Characteristics of commercial enzymes are presented in Table 4b.1 and Table 4b.2.

Table 4b.1. Details of all enzymes used for refining application

Enzyme	Microbial source	Optimum pH	Optimum T (°C)
CA	*Aspergillus* species	4.5	50
CB	Not given	5.0	55
LA	*Rhizopus microsporus*	5.5	55
LB	*Penicillium oxalicum*	4.5	50

C- Commercial enzyme, L- Laboratory produced fungal enzyme (crude extract)

Table 4b.2. Characterization of all enzymes

Enzyme	CMCase activity (IU/mL)	Fpase activity (IU/mL)	β-glucosidase activity (IU/mL)
CA	222±11	11±1	21.0±2
CB	162±12	42±3	72±5
LA	3.41±0.09	2.06±0.02	4.42±0.38
LB	4.70±0.09	2.62±0.07	7.08±0.79

Enzyme assay conditions: Temperature-45 °C, pH-7.0
C- Commercial enzyme, L- Laboratory produced fungal enzyme (crude extract)

4b.2. Enzyme action on fiber

Enzyme hydrolytic efficiency of cellulosic material was expressed mostly by the quantity of sugar released from the treated matter. For all the commercial as well as laboratory produced enzymes

reducing sugars content was found to be increased with increased reaction time or increased enzyme dosage (Table 4b.3).

Table 4b.3. Enzyme action verification by reducing sugar analysis

Reaction time (h)	Enzyme dosage (IU/g OD pulp)	Reducing sugars (mg/g)			
		CA	CB	LA	LB
1	Control	0.01±0.01	-	-	-
	E1	0.03±0.02	0.04±0.02	0.02±0.02	0.04±0.02
	E2	0.10±0.01	0.18±0.02	0.13±0.01	0.14±0.01
	E3	0.15±0.01	0.21±0.02	0.17±0.01	0.18±0.03
	E4	0.19±0.02	0.30±0.01	0.23±0.02	0.26±0.02
	E5	0.27±0.01	0.37±0.02	0.29±0.01	0.32±0.01
1.5	Control	0.01±0.01	-	-	-
	E1	0.06±0.02	0.08±0.01	0.07±0.02	0.08±0.02
	E2	0.13±0.02	0.16±0.02	0.14±0.01	0.15±0.01
	E3	0.17±0.01	0.30±0.01	0.19±0.01	0.25±0.02
	E4	0.20±0.03	0.40±0.01	0.30±0.03	0.33±0.03
	E5	0.28±0.02	0.43±0.03	0.37±0.02	0.40±0.02
2	Control	0.01±0.01	-	-	-
	E1	0.10±0.01	0.15±0.01	0.13±0.01	0.12±0.02
	E2	0.14±0.02	0.23±0.01	0.18±0.02	0.19±0.02
	E3	0.17±0.01	0.36±0.02	0.21±0.01	0.29±0.02
	E4	0.23±0.03	0.42±0.01	0.36±0.03	0.39±0.01
	E5	0.28±0.02	0.47±0.02	0.42±0.02	0.44±0.01

Conditions: Enzyme dosage range E1-E5 (0.01, 0.03, 0.05, 0.07, 0.09 IU/g OD pulp for CA and LB; 0.02, 0.04, 0.06, 0.08, 0.10 IU/g OD pulp for CB and LA, respectively); pH-7.0, temperature-45 °C, consistency-5%, CSF-300±10 mL

This was due to release of glucose and reducing units from cellulosic chain. Reducing sugars content was found to be comparatively higher in case of CB than the other enzymes studied. This is because of higher enzyme activity for FPase and β-glucosidase releasing more glucose units (Lecourt *et al.*, 2010).

Cellulase hydrolyses β-1, 4 glycosidic linkages in cellulose chain. The components of cellulase system are exoglucanase, endoglucanase and β-glucosidase that act in a synergistic fashion. Exoglucanase cuts the bonds at the end of fiber by disruption in cellulose hydrogen bonding in crystalline region. Endoglucanase causes hydrolysis of the accessible cellulose in the amorphous region and β-glucosidase acts on cellobiose units to release glucose (Young and Akhtar, 1998). Other researchers have also reported similar trend of increment in reducing sugars using enzymes (Gil *et al*., 2009 and Lecourt *et al.*, 2010).

4 b.3. Effect of enzyme-assisted refining on PFI revolutions/energy consumption

To make quality paper product with desirable strength properties it is required to treat fibers mechanically. This mechanical operation brings changes in fiber surface, which develop fiber morphology. Mechanical operation of refining is energy-consuming process. PFI mill revolutions reflect actual mill refining energy demand (Ko *et al*., 2010). In this study, a pulp freeness of 300±10 mL CSF for the control pulps was achieved using 3000 PFI mill revolutions.

For different reaction times studied, it was observed that energy consumption was reduced greatly with increased reaction times (Table 4b.4). Similarly, at a higher enzyme dosage energy required to refine pulp was less as compared to lower dosage treatments. A maximum reduction in PFI revolutions of 47% was observed for CA, 42% for CB, 47% for LA and 45% for LB in comparison to respective controls. It was reported that enzyme can assist in refining but in extreme conditions of pre-treatment such as higher degree of refining, higher enzyme dosage or reaction time etc. paper strength properties may be diminished (Hoang *et al*., 2000; Gil *et al*., 2009).

Therefore, it is necessary to study the best balance between energy consumption and strength properties for any pulp to make good quality product. Similar conclusions on energy reduction were also reported for mixed tropical hardwood pulp with approximate 18% energy reduction using cellulase and hemicellulase mixtures (Bajpai *et al*., 2006) and 10-37% for eucalyptus pulp using recombinant cellulase (Ko *et al*., 2010). In another study on poplar pulp, cellulase reduced energy requirement upto 22% to achieve CSF 300 ml (Yang *et al*., 2011).

4 b.4. Effect of enzyme-assisted refining on pulp quality

Water retention values and fines content were determined for all the control as well as enzyme treated pulps

Table 4b.4. Effect of enzyme-assisted refining on PFI revolutions

Reaction time (h)	Enzyme dosage (IU/g OD pulp)	PFI mill Revolutions (Number)			
		CA	CB	LA	LB
1	Control	3000	-	-	-
	E1	2900	2900	2600	2650
	E2	2700	2800	2450	2600
	E3	2500	2600	2250	2300
	E4	2300	2450	2050	2100
	E5	2000	2150	1850	1900
1.5	Control	3000	-	-	-
	E1	2850	2850	2550	2600
	E2	2700	2750	2400	2500
	E3	2400	2500	2200	2150
	E4	2250	2100	1950	2000
	E5	1800	1850	1800	1800
2	Control	3000	-	-	-
	E1	2800	2800	2500	2550
	E2	2600	2700	2300	2400
	E3	2350	2400	2000	2100
	E4	2200	1900	1900	1900
	E5	1600	1750	1600	1650

Conditions: Enzyme dosage range E1-E5 (0.01, 0.03, 0.05, 0.07, 0.09 IU/g OD pulp for CA and LB; 0.02, 0.04, 0.06, 0.08, 0.10 IU/g OD pulp for CB and LA, respectively); pH-7.0, temperature-45 °C, consistency-5%, CSF-300±10 mL

4 b.4.1. Water retention value

Water retention value measures hydration capacity of fiber or swelling ability. It showed a dramatic increase for enzyme treated pulp than control pulps for all the enzymes studied. The WRVs was found to be further improved for extreme pre-treatment conditions of enzyme dosage and reaction time for all enzymes (Table 4b.5). This is mainly due to enzymatic modification of fiber surface properties resulting in increment of water affinity (Pala *et al*., 2002).

Addition of enzyme at a pre-refining stage may help in easier removal of outer layers and internal fibrillation. However, mechanical refining using PFI mill promoted fibrillation, both external and internal. Enzyme because of its hydrolytic nature may absorb and penetrate in the fiber wall resulting in more fibrillation. Thus, ability to hold water by fiber was increased after enzyme treatment. Fiber swelling depends on fibers charge (Bhardwaj *et al*., 2004, Bhardwaj *et al.,* 2004a) and is influenced by carboxyl groups situated in fibers/fines surface area (Bhardwaj *et al*., 2006). This is related with zeta potential and cationic demand of pulp, which is reported to be relatively higher for refined/beaten pulp than unrefined/unbeaten pulp (Bhardwaj *et al*., 2004b).

Table 4b.5. Effect of enzyme-assisted refining on water retention value

Reaction time (h)	Enzyme dosage (IU/g OD pulp)	Water retention value (%)			
		CA	CB	LA	LB
1	Control	156	-	-	-
	E1	163	161	168	168
	E2	170	169	179	173
	E3	174	172	186	179
	E4	178	176	192	180
	E5	181	179	194	185
1.5	Control	156	-	-	-
	E1	166	164	176	172
	E2	173	171	180	177
	E3	177	176	187	178
	E4	180	179	194	184
	E5	184	184	197	190
2	Control	157	-	-	-
	E1	169	168	178	174
	E2	175	172	184	180
	E3	181	179	189	185
	E4	184	181	194	188
	E5	189	189	198	191

Conditions: Enzyme dosage range E1-E5 (0.01, 0.03, 0.05, 0.07, 0.09 IU/g OD pulp for CA and LB; 0.02, 0.04, 0.06, 0.08, 0.10 IU/g OD pulp for CB and LA, respectively); pH-7.0, temperature-45 °C, consistency-5%, CSF-300±10 mL

A rise in WRV with application of enzyme for bleached eucalyptus kraft pulp was also reported by many investigators (Lecourt *et al.*, 2010; Gil *et al.*, 2009 and Pastor *et al.*, 2001).

4b.4.2. Fines content

Fines act as fillers and are important in sheet consolidation causing an increase in apparent density of sheet, which is further related to bonding potential and elastic modulus, linear slope of stress versus strain curve (Bhardwaj *et al.*, 2006). Studies for fines content showed an increase for enzyme treated pulps as compared to control pulps for all the four enzymes studied (Table 4b.6).

Table 4 b.6. Effect of enzyme-assisted refining on fines content

Reaction time (h)	Enzyme dosage (IU/g OD pulp)	Fines content (%)			
		CA	CB	LA	LB
1	Control	14.4	-	-	-
	E1	15.1	15.1	15.1	15.8
	E2	15.2	15.1	15.1	16.5
	E3	15.3	15.3	15.3	17.9
	E4	15.3	14.4	14.4	18.3
	E5	15.5	14.0	14.0	18.0
1.5	Control	14.4	-	-	-
	E1	15.2	16.	16.0	16.9
	E2	16.4	16.4	16.4	17.7
	E3	16.9	17.3	17.3	18.3
	E4	16.4	16.3	16.4	17.6
	E5	15.4	16.1	16.1	16.2
2	Control	14.4	-	-	-
	E1	16.1	16.7	16.7	17.6
	E2	16.5	17.4	17.4	18.2
	E3	17.2	18.0	18.0	18.0
	E4	16.2	16.8	16.8	17.1
	E5	15.1	15.3	15.3	15.8

Conditions: Enzyme dosage range E1-E5 (0.01, 0.03, 0.05, 0.07, 0.09 IU/g OD pulp for CA and LB; 0.02, 0.04, 0.06, 0.08, 0.10 IU/g OD pulp for CB and LA, respectively); pH-7.0, temperature-45 °C, consistency-5%, CSF-300±10 mL

It was noticed to be improved with increased enzyme dosages and reaction times. An increment in fines content is reasonable due to enzyme addition which carried refining action, causing disturbance in inner S2 layers resulting fibrillation and fines (secondary fines) generation (Vainio and Paulapuro, 2007). However, at further higher dosage and reaction time, fines content get reduced most probably due to enzyme action resulting in its disappearance due to solubilization (Jackson *et al*., 1993). This can be speculated from increased sugar content at extreme pre-treatment conditions (Table 4b.3). Here, primary fines content get reduced and secondary fines generation is not so much that it could compensate the loss of primary fines.

4b.5. Effect of enzyme-assisted refining on paper properties

Paper is made after 'refining' process. Any damage to fiber at this stage cannot be repaired elsewhere. In this work apparent density of paper handsheets was found to be increased from control 0.62 to 0.66 g/cm^3 reflecting higher fiber collapsibility and packing. This is due to greater internal as well as external fibrillation related to conformability and web consolidation. In an earlier study performed by Maximino *et al*., 2011 it was reported that an increase in density is one of the main effects of enzymatic refining and is related to fiber flexibility. The mechanism behind this is not clear but it differs from mechanical refining (Wong *et al*., 1999). Cellulase application in refining was also found to increase apparent density of handsheets marginally for Douglas fir kraft pulp with increasing enzyme dosage (Mansfield and Dicksont, 2001). Enhanced fiber flexibility and fibrillation promotes fiber-fiber bonding (Fardim and Duran, 2003). This phenomenon of inter-fiber bonding is mainly responsible for providing strength to paper sheet in addition to fines content.

4b.5.1. Effect on tensile index

Tensile index is an important strength property of paper as it represents overall sheet strength encompassing contributions from individual fiber strength and inter-fiber bonding (Page, 1969). An inter-fiber bond may be described as a zone where two fibers are close to each other by chemical bonding like hydroxyl linkage, vander-waals interaction or molecular entanglement. Tensile forces when applied to specimen paper strip it extend the bond between and within fibers. In the fiber network of paper sheet some fibers broken up and others got pulled out in rupture zone. The factor on which tensile property depends is bonding strength as well as individual fiber strength. The bonding strength is more important and dominating factor responsible for tensile strength.

For CA, tensile index was improved by 21% at enzyme dosage of 0.07 IU/g OD pulp for 1.5 h reaction time. It was improved nearly by 15% for 1 h reaction time and decreased at a higher reaction time i.e. 2 h by 5% at same dosage (Fig. 4b.1a). However, a little difference in this property was noticed at lower enzyme dosages. This seems to be important when considering the enzyme dosage/cost, reaction time and energy requirement to achieve a higher tensile index. For example, an enzyme dosage of 0.05 IU/g OD pulp for reaction time of 2 h, it was showing nearly same result for tensile index, as of 0.07 IU/g OD pulp for reaction time 1.5 h i.e. consuming more time and energy.

For CB, in all reaction times i.e. 1 h, 1.5 h and 2 h, enzyme dosage of 0.06 IU/g OD pulp was found to show good tensile index property. Although the amount of enzyme dosage was same, it was observed that fiber properties developed strongly with increasing reaction time. It may be noted here that tensile index was improved from 5% to 15% with increase in incubation time from 1 to 2 h at the same dosage of 0.06 IU/g OD pulp (Fig. 4b.1b). An increment in this property may be explained on the basis of development of fibrillation resulting in inter-fiber bonding.

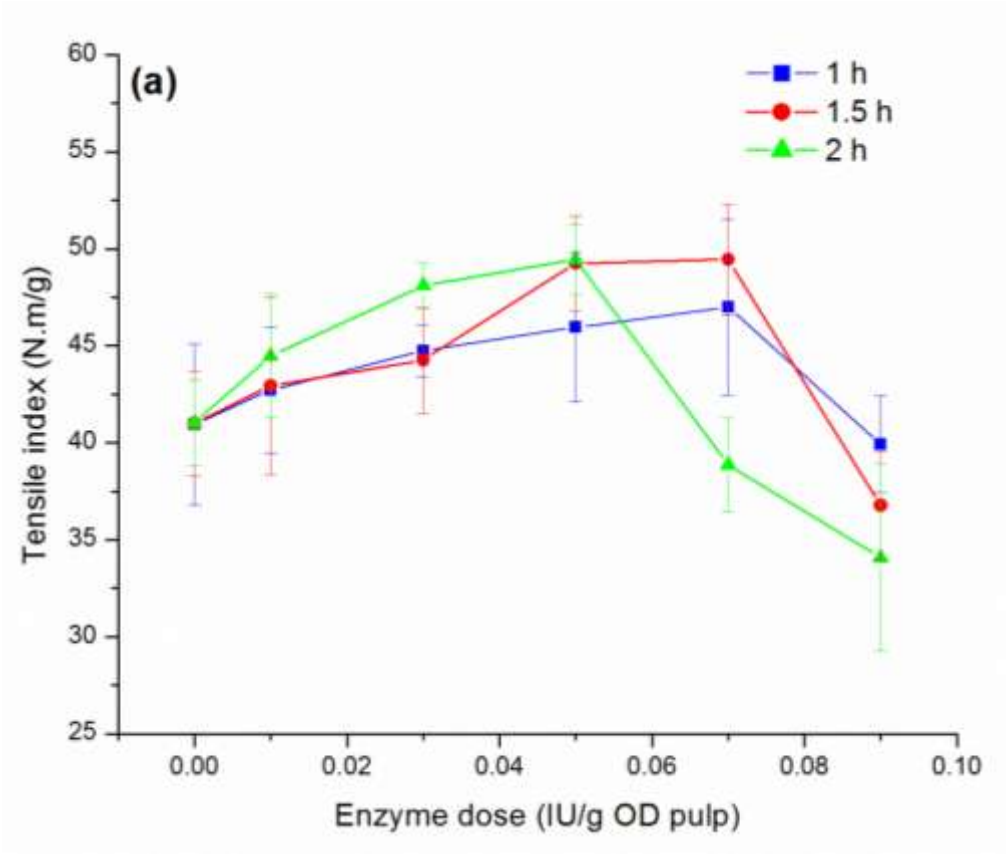


Figure 4b.1a Effect on tensile index using enzyme CA at different dosages and reaction times

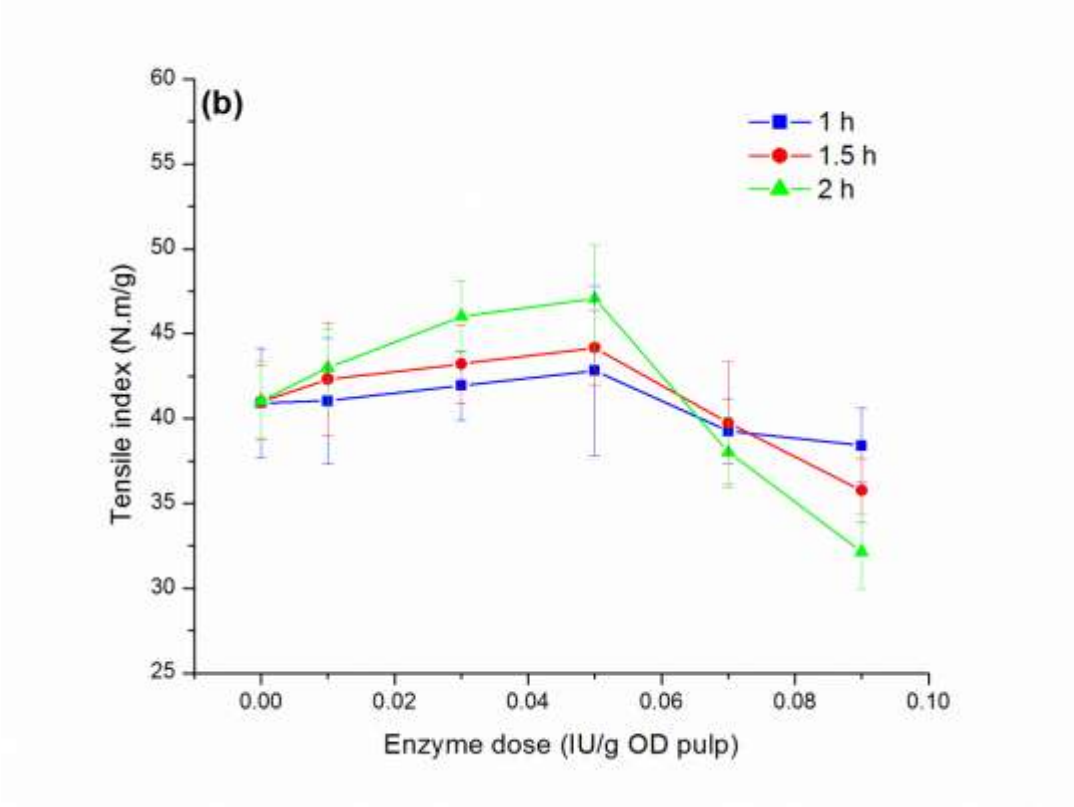


Figure 4b.1b. Effect on tensile index using enzyme CB at different dosages and reaction times

For LA, it was found to be increased by 25% at an enzyme dosage 0.08 IU/g OD for 1 h, 17% at 0.06 IU/g OD for 1.5 h and 22% at 0.04 IU/g OD for 2 h, respectively in comparison to control pulps (Fig. 4b.1c).

For LB, reaction time of 1 h showed maximum improvement of 16 % in tensile index at enzyme dosage 0.07 IU/g OD pulp. This property was found to be improved further by 22% at a comparatively lower dosage (0.05 IU/g OD pulp) and longer reaction time of 1.5 h. In the later case of extreme treatment time of 2 h, tensile index was found to be increased by 21% at a dosage of 0.05 IU/g OD pulp (Fig. 4b.1d). It was observed that the effect of enzyme on tensile index of handsheet did not affect greatly with prolonged incubation time, however it reduced slightly showing harmful effect of enzyme action on fiber. Thus, enzyme dosage of 0.05 IU/g OD pulp was chosen as best for pre-treatment of pulp.

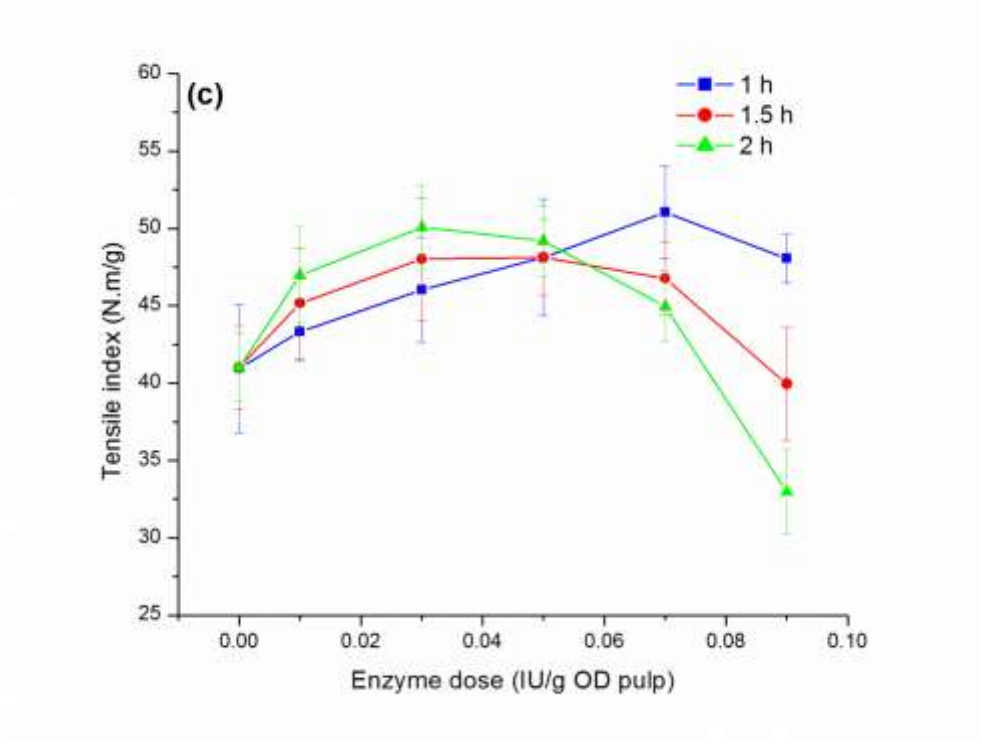


Figure 4b.1c. Effect on tensile index using enzyme LA at different dosages and reaction times

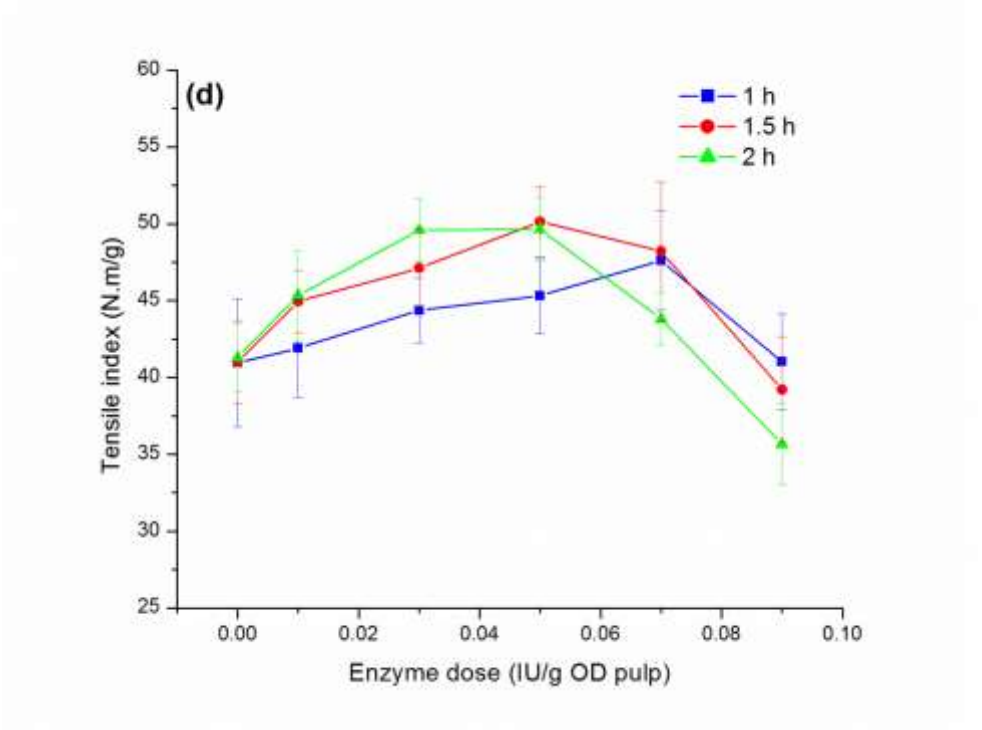


Figure 4b.1d. Effect on tensile index using enzyme, LB at different dosages and reaction times

Fibrillation favors cohesion between fibers promoting fiber-fiber bonds to occur. Therefore, the tensile force required to rupture specimen was found to be less in control pulp because of weaker inter-fiber bonding and also less fines content. This lead to an easier breaking and pulling of fibers as

compared to enzyme treated pulp, where stronger inter-fiber bonds and production of fines leads to synergistic improvement of the elongation. Fibrillation and inter-fiber bondings were observed to be more prominent in enzyme treated pulps compared to control pulp (Fig. 4b.2a-f). A similar trend of improvement in tensile index was also noticed by other authors (Bhardwaj *et al*., 1996; Bajpai *et al*., 2006; Yang *et al*., 2011). Tensile index was affected greatly at a higher dosage and time, most probably due to fiber cellulosic chain deterioration and removal of fines.

4b.5.2. SEM analysis of paper handsheets

SEM analysis of paper handsheets of optimized treatment were analyzed at different magnifications (Fig. 4b.2a-f). Control pulps had shown very less degree of fibrillation, whereas enzyme treated pulps had shown somewhat high fibrillation in comparison to control pulps.

4b.5.3 Effect on burst index

Burst index is another important paper strength property which depends on inter-fiber bonding. An improvement of burst index was noticed with enzyme pre-treatment for all the enzymes studies.
A maximum of 20% improvement was observed for burst index of CA treated pulp at an optimized dosage 0.07 IU/g OD and reaction time 1.5 h. Approximate 19% improvement was observed at the similar dosage but time of 1 h. In both treatment conditions, burst index were marginally attained but tensile strength properties developed greatly (Fig. 4b.3a)..

For CB, a rise of 12 to 13% in burst index was observed with increasing reaction time from 1 to 2 h (Fig. 4b.3b).. This increment did not vary greatly considering enzyme dosage 0.06 IU/g OD pulp and 0.08 IU/g OD pulp. It means that at a lower dosage (0.06 IU/g OD pulp) the improvement in burst index is quite similar to higher dosage of 0.08 IU/g OD pulp. At further higher dosage, burst index seems to be decreased i.e. enzyme appeared to be detrimental to strength property while energy conservation may be achieved greatly.
For LA, burst index was observed to increase by 22% at an enzyme dosage 0.08 IU/g OD for 1 h, 20% at 0.06 IU/g OD for 1.5 h and 18% at 0.04 IU/g OD for 2 h, respectively in comparison to control pulps(Fig. 4b.3c)..

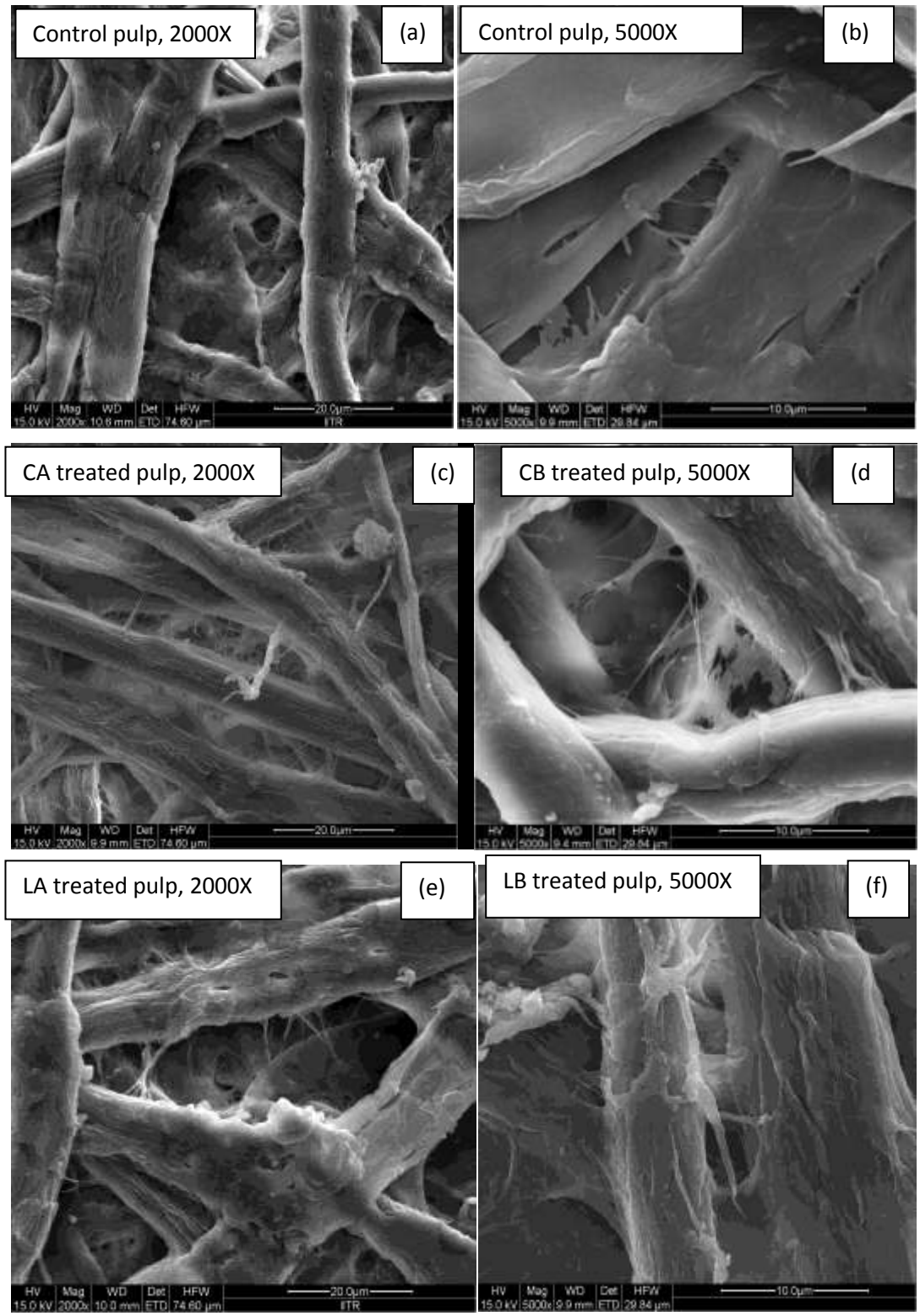


Figure 4b.2. SEM photographs showing fibrillation pattern for control pulps (a,b), CA enzyme treated pulp (c) , CB enzyme treated pulp (d), LA, enzyme treated pulp (e) and LB enzyme treated pulp (f). Control pulps are showing less fibrillation in comparison to all enzymes studied which shown relatively higher degree of fibrillation

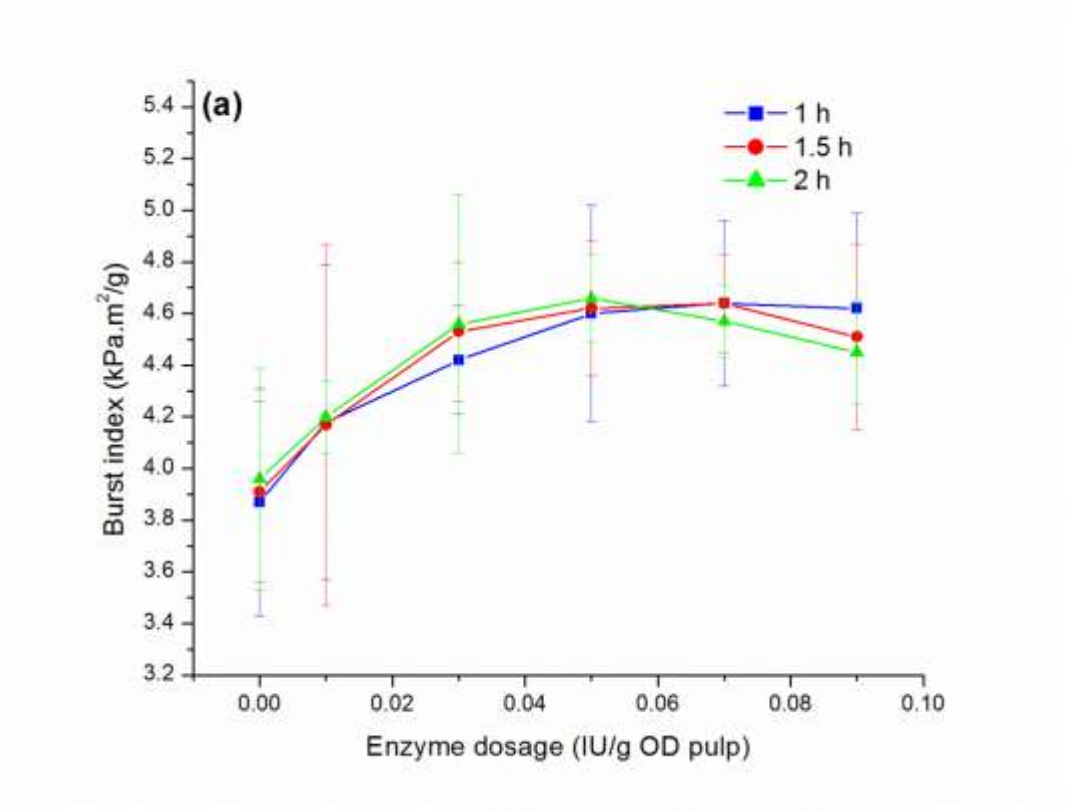


Figure 4b.3a. Effect on burst index using enzyme CA at different dosages and reaction times

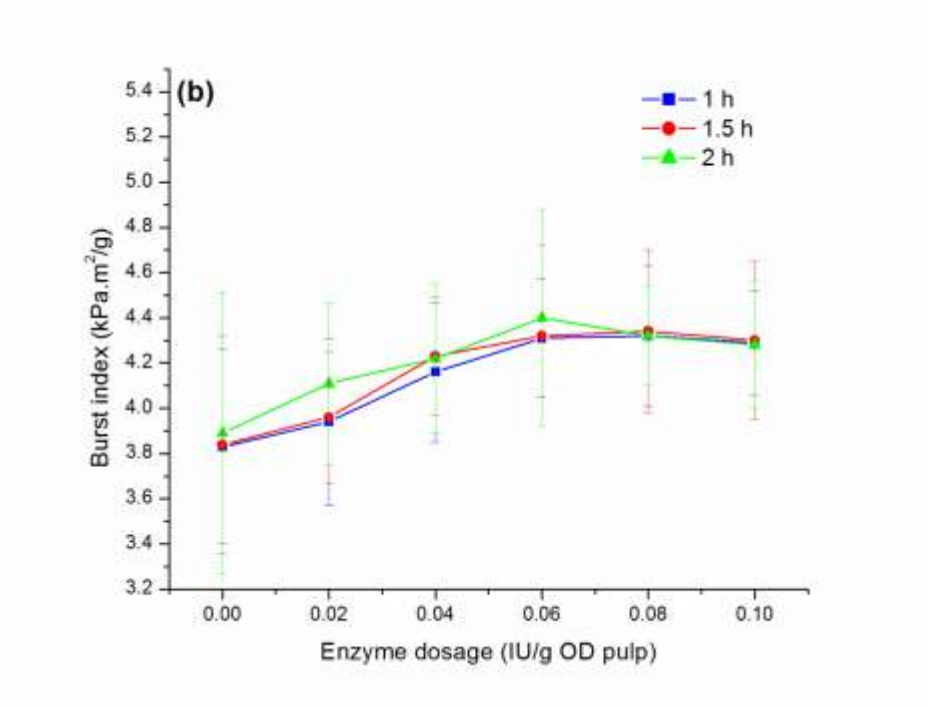


Figure 4b.3b. Effect on burst index using enzyme CB at different dosages and reaction times

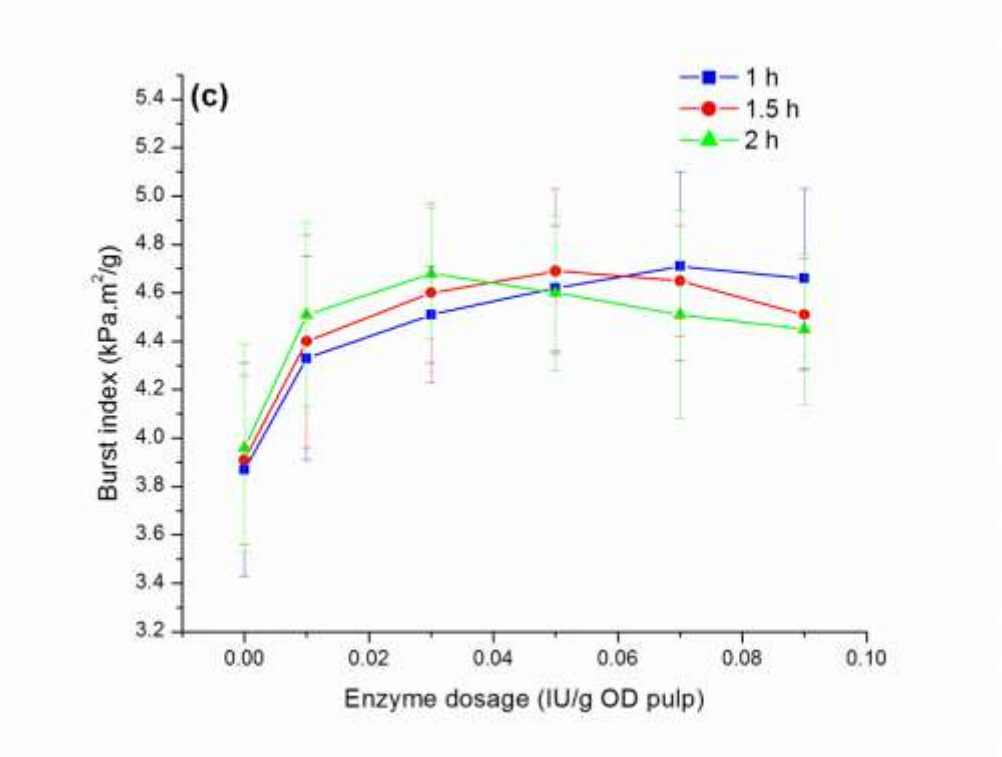


Figure 4b.3c. Effect on burst index using enzyme, LA at different dosages and reaction times

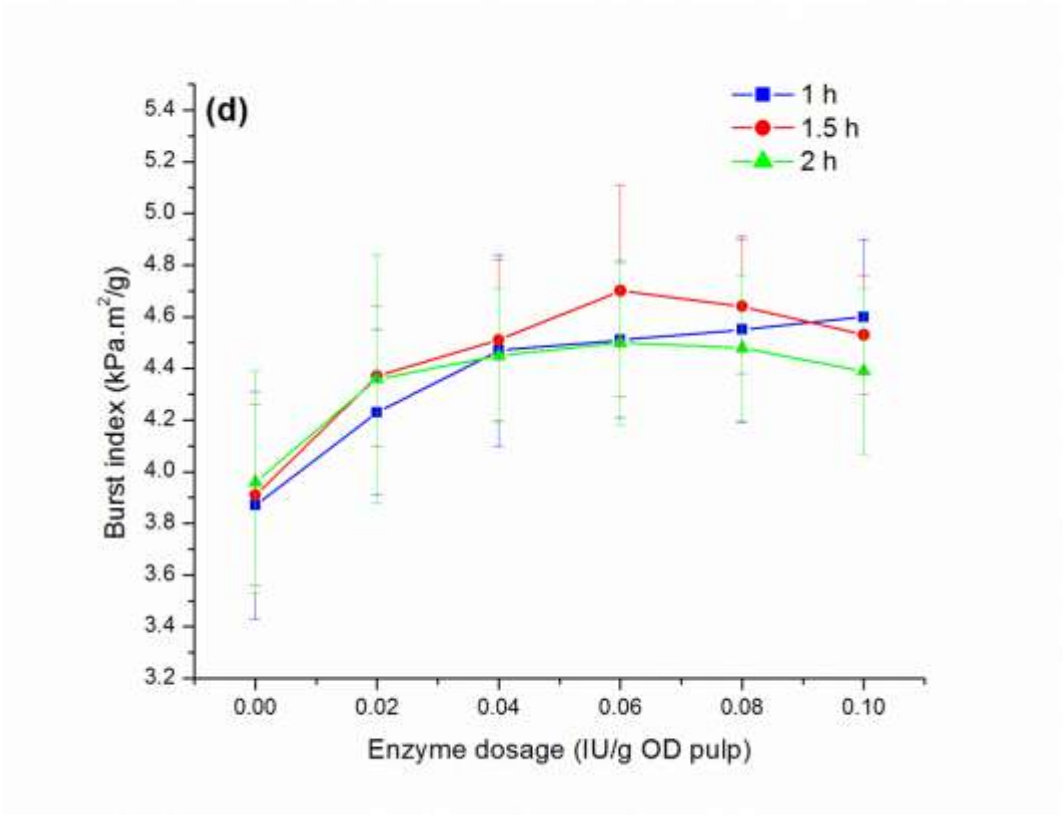


Figure 4b.3d Effect on burst index using enzyme LB at different dosages and reaction times

For LB, at 1 h reaction time and enzyme dosage of 0.07 IU/g OD pulp, burst index was improved by 18%, further it was improved to a maximum of 19% at 0.09 IU/g OD pulp for the same reaction time (Fig. 4b.3d). At longer time trials of 1.5 and 2 h burst index was observed to have a higher value at same dosage of 0.05 IU/g OD pulp. However, reaction time of 1.5 h was found to have a higher burst index improvement of 20% than in comparison to respective control. In the later case of 2 h duration, burst index improved to a maximum of 14% as compared with control pulp. Therefore, a longer treatment time may diminish strength properties (Pala *et al.*, 2002).

This property also depends on fiber-fiber bonding. Cadena *et al.*, (2010) also reported an enhanced value of this property using enzymes for eucalyptus pulp.

Refining may increase the fiber surface area of fibrillated portion, which can provide a larger surface area exposing more hydroxyl groups leading to a strong cross linkage among fibers. Formation of this linkage begins with water removal during papermaking. As the solid content of pulp increases in papermaking with liquid removal, surface tension comes in action and pulls fibers to come closer by Campbell effect (Vainio and Paulapuro, 2007). This later on forms hydrogen bonds due to availability of exposed hydroxyl groups. As enzymatic refining results in high degree of fibrillation therefore, the hydrostatic pressure required to produce rupture under standard conditions of testing was also increased, causing an increment in burst index property**.** A decrease in this property at a high enzyme dosage and reaction time may be because of fines removal and weak cross linkage among fibers.

4b.5.4. Effect on folding endurance

In continuation with strength properties, folding endurance, which reflects an elasticity and viscoelastic property of paper was also, studied (Figure 4b.4a-d). It depends on flexibility of fiber and associated with internal fibrillation of fibers. No major change was observed for this property for all the enzymes investigated. Only a minor effect was observed for LB. In the majority of the available literatures, researchers observed minor or no change in this property after the treatment with enzymes (Yang *et al.*, 2011; Cadena *et al.*, 2010).

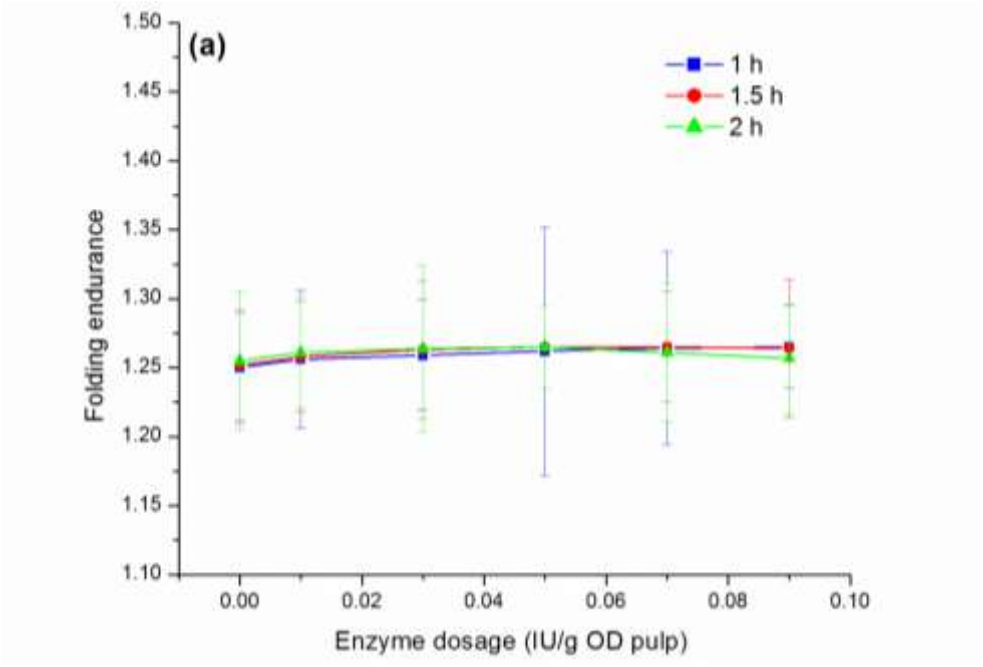


Figure 4b.4a. Effect on folding endurance using enzyme, CA at different dosages and reaction times

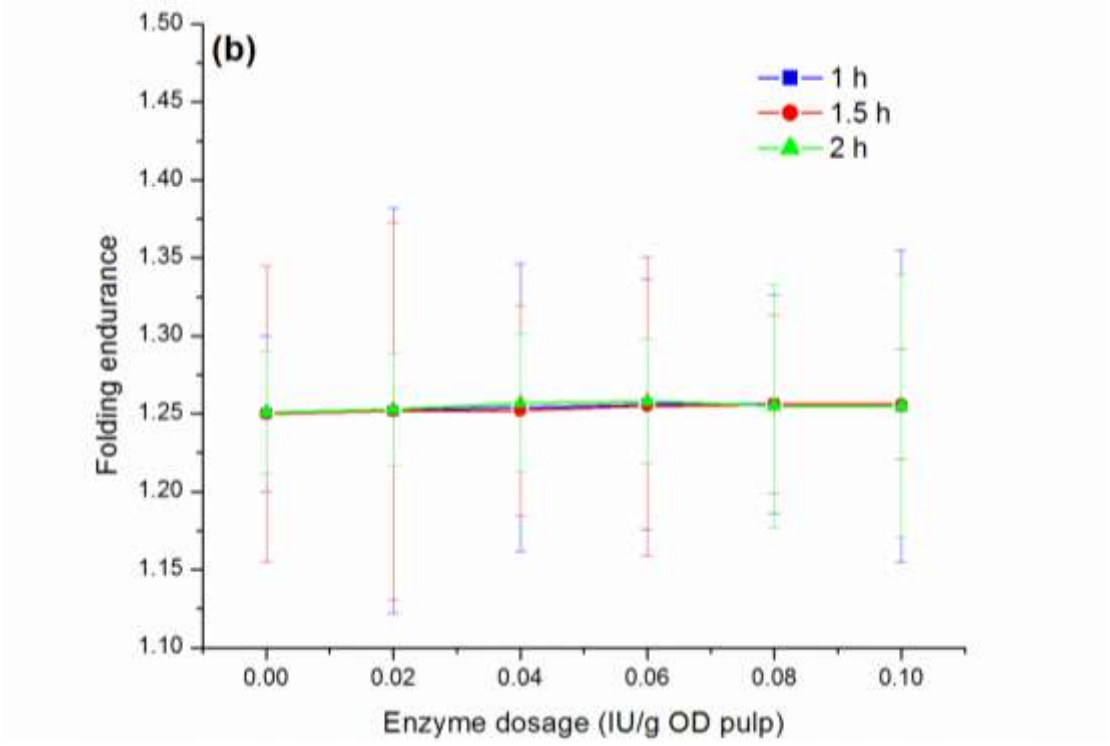


Figure 4b.4b. Effect on folding endurance using enzyme, CB at different dosages and reaction times

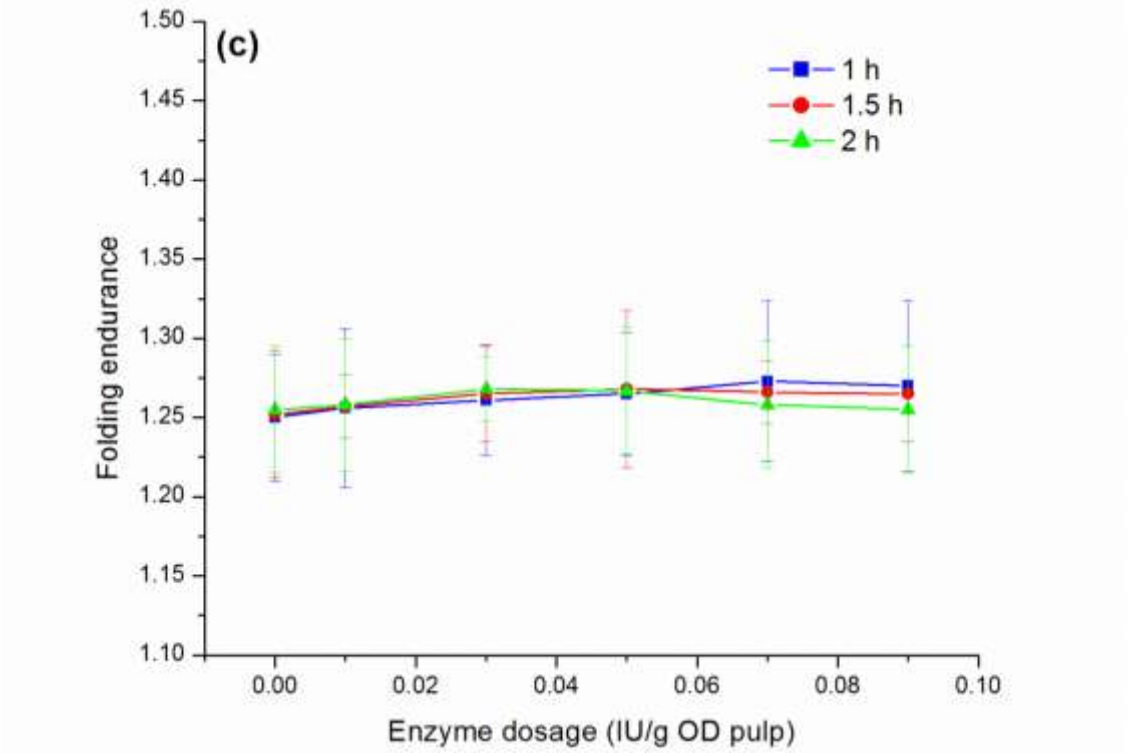


Figure 4b.4c. Effect on folding endurance using enzyme LA at different dosages and reaction times

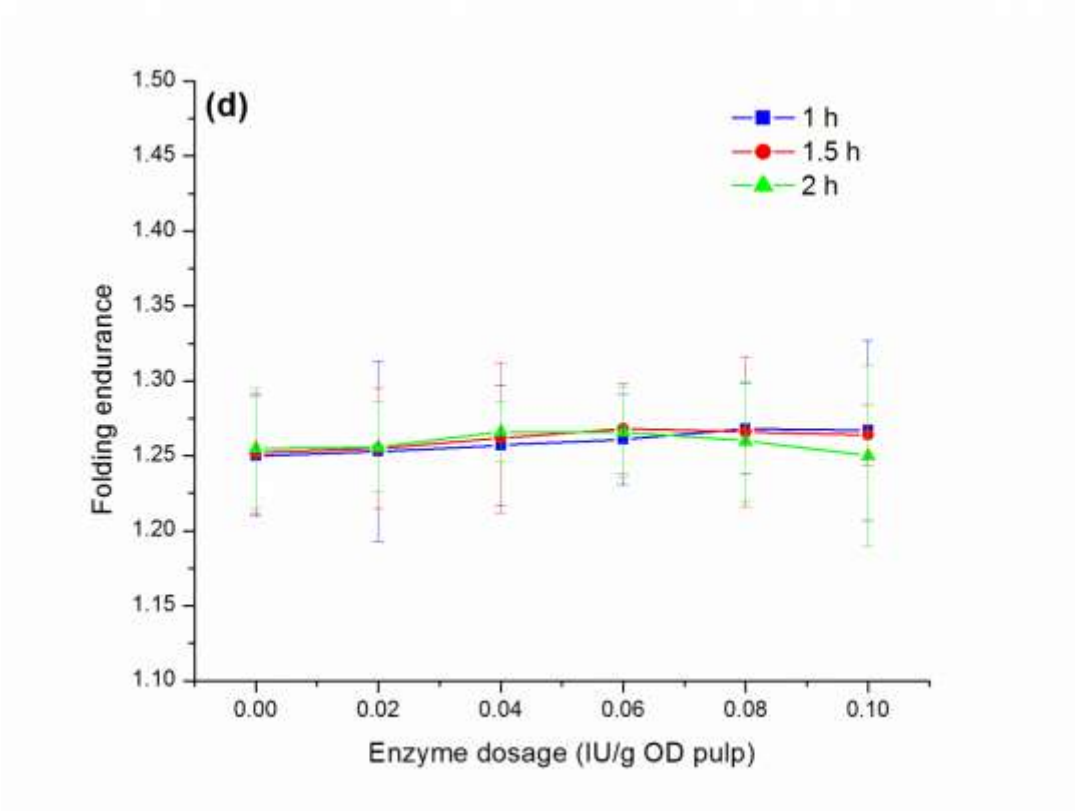


Figure 4b.4d. Effect on folding endurance using enzyme LB at different dosages and reaction times

4b.5.5. Effect on tear index

Tear index depends on individual fiber length, strength, external fibrillation and fines proportion. Tear strength measures the force perpendicular to the plane of specimen test paper to continue tear through the object after a cut is made and tear has already been started. Any mechanical operation (such as refining) will affect on fiber length by fiber shortening/cutting effect.

This property has a higher value in case of control pulp and is decreased with enzyme treatments (Fig. 4b.5a-d). In case of CA, it decreased up to 8-10% while for CB it decreased by 5-14% with enzyme treatments. For laboratory produced fungal enzymes, LA decreased tear index upto 7-10% and LB, reduced this property by 8-9% with prolonged incubation time.

It may decrease because of fiber cutting or shortening effect due to refining. A major decrease in this property using cellulase was also reported by other authors (Lecourt *et al*., 2010). Contrary to this, another enzyme preparation from Novozymes (Cartazyme) was reported to increase tear index for different pulp grade of *Pinus pinaster* (Pulp *et al*., 2003). During tear testing, force comes in play, it may stretch fibers until they break depending on individual fiber strength or may pull out fibers from paper network depending on both breakage of bonds and frictional forces. Any of these effects i.e. breaking or pulling of fibers will disturb inter-fiber bonding. The fibrillation was found to be increased with refining making fiber easier to break/ pull because of reduced individual fiber length and strength.

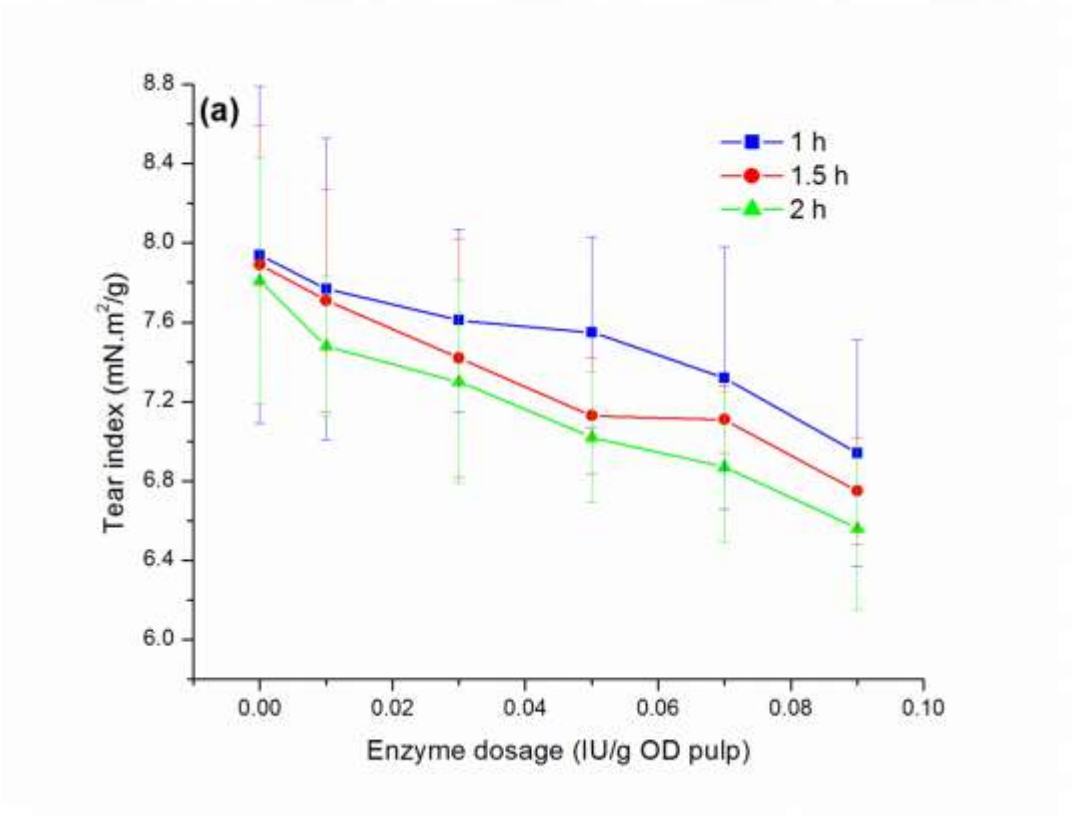


Figure 4b.5a. Effect on tear index using enzyme CA at different dosages and reaction times

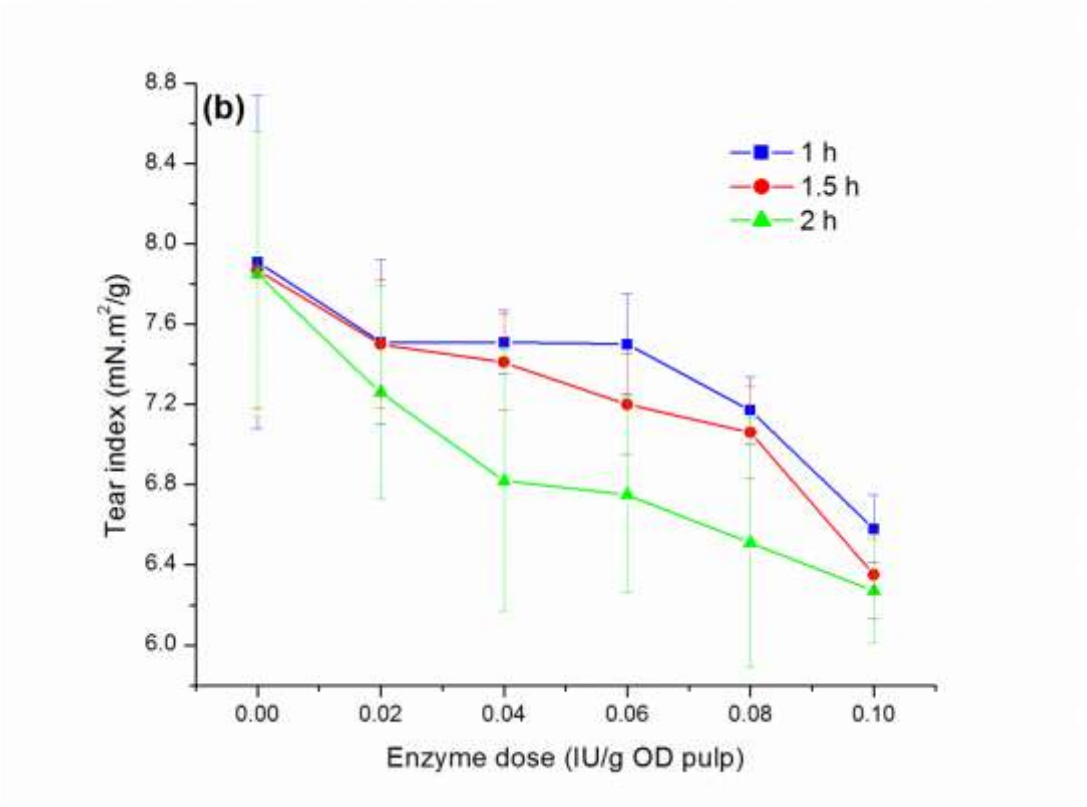


Figure 4b.5b. Effect on tear index using enzyme CB at different dosages and reaction times

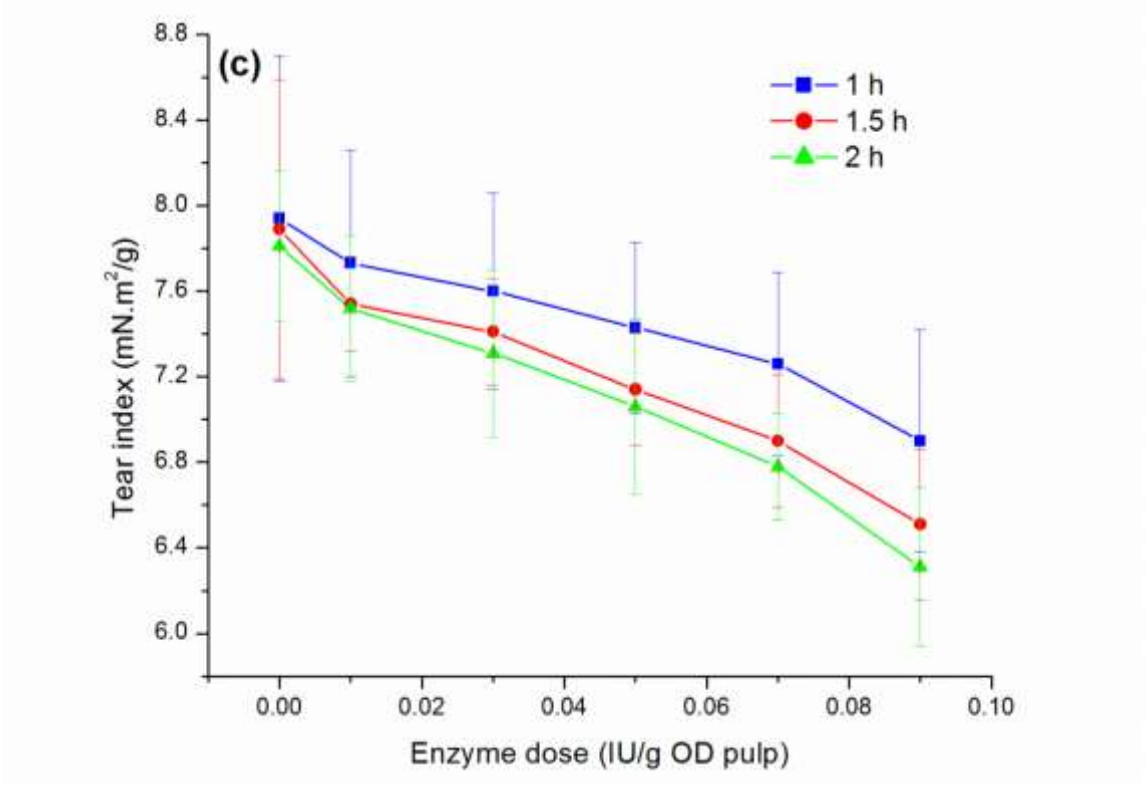


Figure 4b.5c. Effect on tear index using enzyme LA at different dosages and reaction times

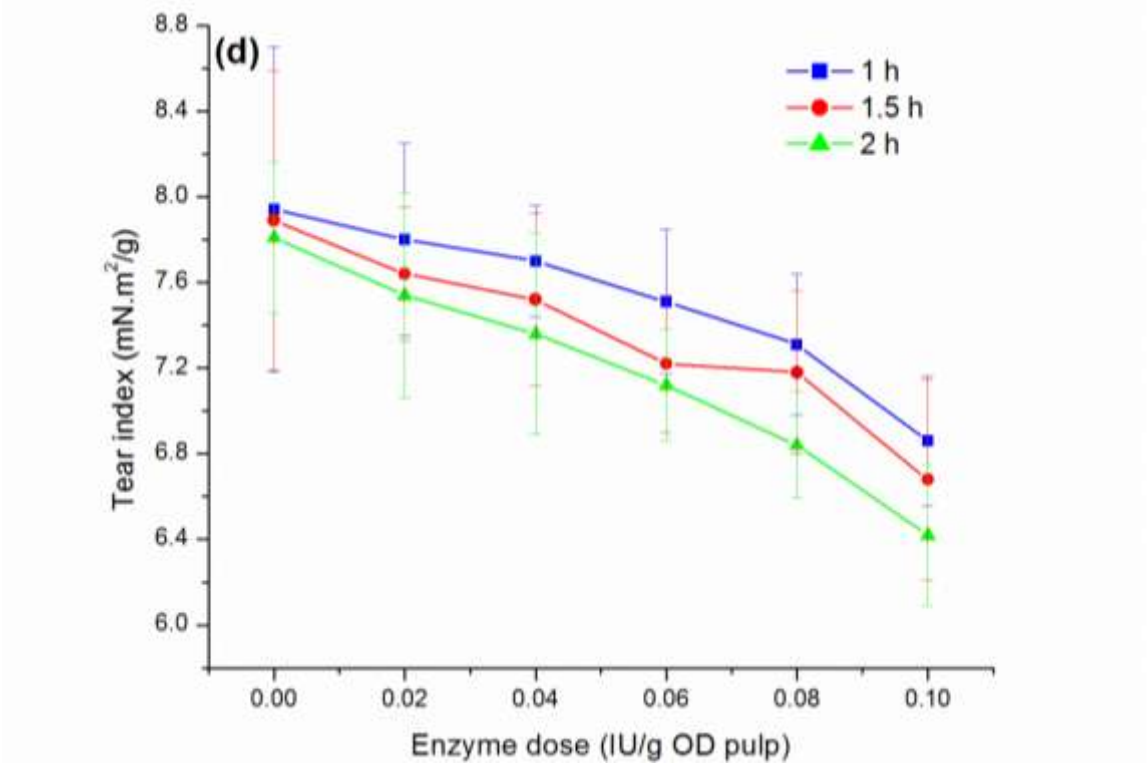


Figure 4b.5d. Effect on tear index using enzyme LB at different dosages and reaction times

4b.6. Comparative evaluation of refining potential between commercial and lab produced enzymes

For all the enzymes the best results found for enzyme-assisted refining were as follows:

1. For LA, the process parameters like enzyme dosage and reaction time were found to be favorable at 0.08 IU/g OD pulp and 1 h. At this optimized range, PFI revolutions were decreased by 32% than in comparison to control pulp. Paper properties improved for tensile index by 25%, burst index by 22%, folding endurance by 2% and tear index reduced by 9% as compared to control pulp.
2. For LB, an enzyme dosage of 0.05 IU/g OD pulp for reaction time 1.5 h was best. At this optimized range, PFI revolutions were decreased by 28% in comparison to control pulp. Paper properties improved for tensile index by 22%, burst index by 20%, folding endurance by 1% (unaffected) and tear index reduced by 8% in comparison to control pulp.
3. For CA, 0.07 IU/g OD pulp and 1.5 h pre-treatment time was optimum. At this optimized range, PFI revolutions were decreased by 25% in comparison to control pulp. Paper properties improved for tensile index by 21%, burst index by 19%, folding endurance by 1% (unaffected) and tear index reduced by 10% in comparison to control pulp.
4. For CB, 0.06 IU/g OD pulp and 2 h reaction time was optimal for refining application. At this optimized range PFI revolutions were decreased by 20% in comparison to control pulp. Paper properties improved for tensile index by 15%, burst index by 13%, folding endurance by 1% (unaffected) and tear index reduced by 14% in comparison to control.

Between CA and CB, CA was observed to show better results than CB and between laboratory produced fungal enzymes, LA was found to give best performance.

It was found that LA saved refining energy by reducing PFI revolutions by 32% as compared to commercial enzyme, which saved 25% of energy. For LA, paper properties were slightly improved for tensile index (+4%), burst index (+3%) and reduced tear index by (-1%) than compared to CA. In comparison to CB, PFI revolutions were decreased by 12%, paper strength properties were improved for tensile index by 10%, burst index 9% and reduced tear index by 9% for LA.

In case of LB, 3% energy reduction was achieved compared to CA and 8% compared to CB. Paper properties were found to be comparable for CA and better for CB. Tensile and burst index were improved 7% and reduced tear index by 5% compared to CB.

Table 4b.7. Comparison of refining efficiency between crude fungal enzymes and commercial enzymes

Properties	**Control pulp**	**Commercial enzyme, CA**	**Commercial enzyme, CB**	**Crude enzyme of *R. microsporus*, LA**	**Crude enzyme of *P. oxalicum*, LB**
Enzyme dosage (IU/g OD) pulp	0	0.07	0.06	0.08	0.05
Reaction time (h)	Variable	1.5	2	1	1.5
PFI revolutions (no.)	3000	2250 (-25%)	2400 (-20%)	2050 (-32%)	2150 (-28%)
Viscosity (cp)	11.4	8.2 (-28%)	9.0 (-21%)	9.4 (-18%)	9.0 (-21%)
Tensile Index (N.m/g)	40.95±4.14 (1h) 41.00±2.73 (1.5 h) 41.03±2.2 (2 h)	44.26±2.71 (+21%)	47.07±3.16 (+15%)	51.05±2.98 (+25%)	50.14±2.30 (+22%)
Tear index ($mN.m^2/g$)	7.94±0.76 (1 h) 7.89±0.70 (1.5 h) 7.81±0.35 (2 h)	7.42±0.60 (-10%)	6.75±0.49 (-14%)	7.26±0.43 (-9%)	7.22±0.32 (-8%)
Burst index ($kPa.m^2/g$)	3.87±0.44 (1 h) 3.91±0.35 (1.5 h) 3.96±0.43 (2 h)	4.53±0.27 (+19%)	4.40±0.48 (+13%)	4.71±0.39 (+22%)	4.70±0.41 (+20%)
Folding Endurance *(Log_{10} no. of double folds)	1.250±0.04 (1 h) 1.252±0.04 (1.5 h) 1.255±0.04 (2 h)	1.263±0.03 (+1%)	1.258±0.040 (+1%)	1.273±0.051 (+2%)	1.268±0.035 (+1%)

Values in bracket indicate percentage increase or decrease for the paper properties with respect to control pulps of same reaction time

Chapter 5

Conclusions and recommendations for future work

The noteworthy conclusions drawn from the present study and the recommendations for future work are mentioned below.

5.1. Conclusions

1. *R. microsporus*-UPM0810n (RS1) NFCCI 2927 and *P. oxalicum*-UPS1010B (RS2) NFCCI 2926 isolated from soil and wood samples, respectively are cellulase producers capable to produce enzyme under SSF conditions active in wide pH range.
2. These crude enzymes have the potential towards the efficient refining of MHW pulp (eucalyptus and poplar pulp in 70:30 ratios) of Indian origin.
3. Among all the enzymes studied (commercial and crude fungal extracts) energy reduction was achieved greater for LA followed by LB, CA and CB. Paper strength properties were higher for LA and lower for CB.
4. Paper strength properties (tensile and burst index) were improved because of generation of secondary fines and fibrillation development promoting inter-fiber bonding.
5. The laboratory produced crude fungal enzymes produced by isolated fungi have better refining efficiency than commercial enzyme, CB (crude cellulase+xylanase). It is comparable to CA (endoglucanase). Thus, the fungal enzymes LA and LB are effective in pulp refining process.

5.2. Recommendations for future work

1. Other raw material may be tested for efficient cellulase production and utilization of wastes, an important application is bioethanol formation.
2. It is recommended to study the large-scale production of these enzymes in the fermenter or bioreactor. These crude enzyme preparations may be used for other pulp grades after optimizing process parameters for a particular case, also can be employed for other applications like deinking and bleaching etc.

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Appendix

List of Publications:

Paper Publications

1. R. Singh, N. K. Bhardwaj and B. Choudhury. "An experimental study of the effect of enzymatic refining on energy consumption and paper properties for mixed hardwood pulp" APPITA- Australian pulp and paper technical association (SCI journal, Accepted manuscript)
2. R. Singh, N. K. Bhardwaj and B. Choudhury. "Cellulase-assisted refining optimization for saving electrical energy demand and pulp quality evaluation" JSIR- Journal of scientific and industrial research (SCI journal, Submitted manuscript)

Poster Presentation

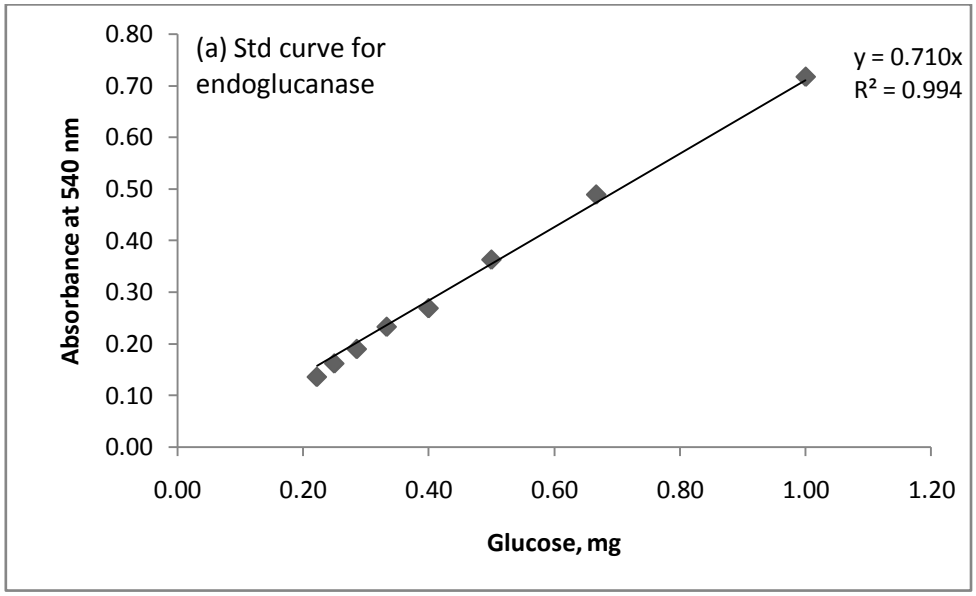
1. R. Singh, N. K. Bhardwaj and B. Choudhury. "Effect of cellulase on mixed hardwood pulp for refining energy", International conference on biotechnology and bioengineering, 6-7 july IMS, Ghaziabad.

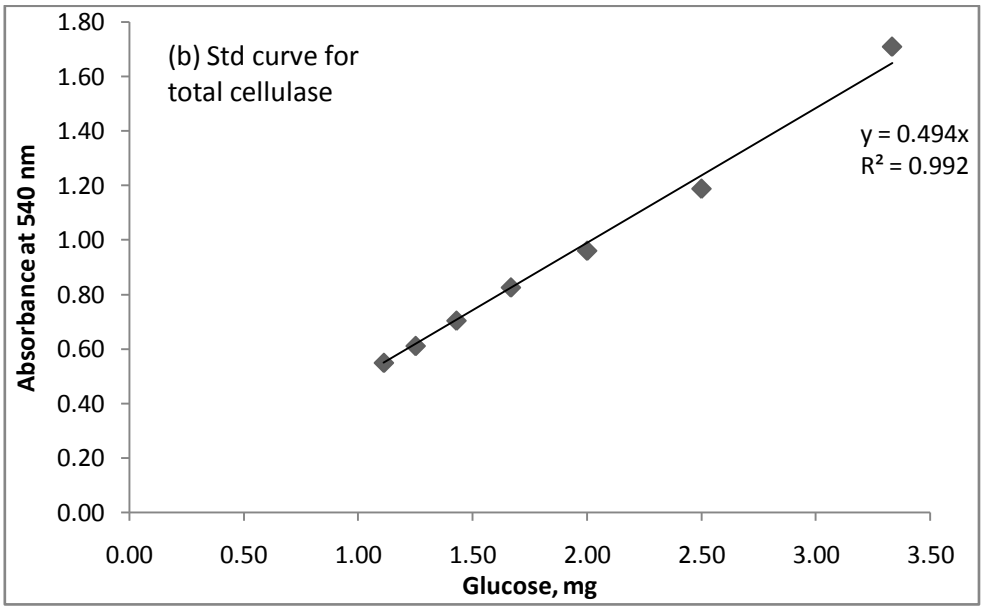
List of chemicals

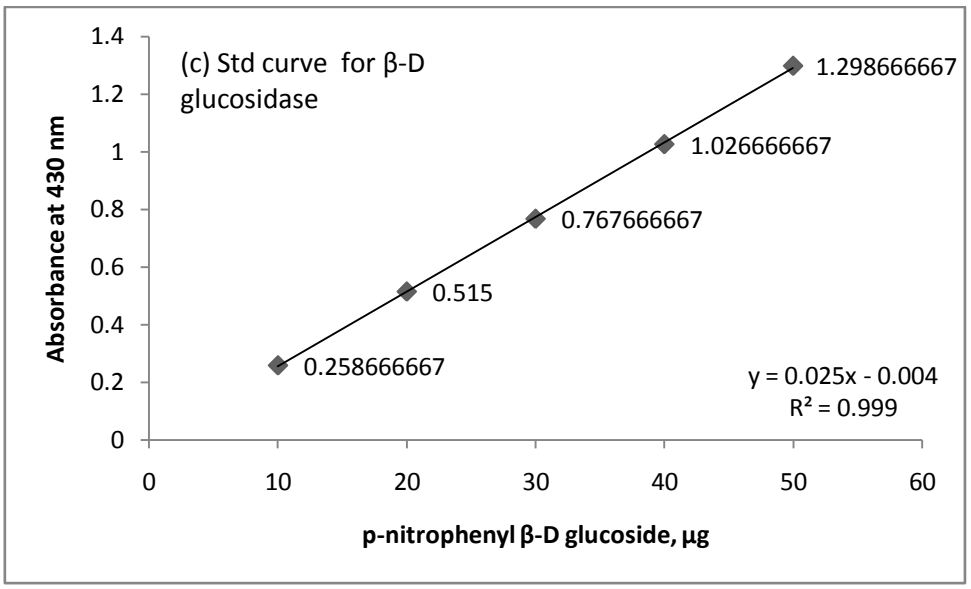
Chemicals	Company name
Potassium permanganate	Qualigens
Formaldehyde	Qualigens
Ethanol	Qualigens
Potato dextrose agar	Hi media
Conc. Hydrochloric acid	Rankem
Conc. Sulphuric acid	Rankem
Agar- Agar	Hi media
Carboxy methyl cellulose	Sigma-Aldrich
Congo red dye	Hi media
Sodium chloride	Hi media
Glycerol	Merck
Parafilm	Hi media
Glutarldehyde	SD Fine-Chem. Ltd
Whatman no. 1 filter paper	GE Healthcare UK ltd.
Potassium dihydrogen phosphate	Hi media
Potassium hydrogen phosphate	Hi media
Calcium chloride, dehydrate	Merck
Tween-80	Merck
Magnesium sulphate, heptahydrate	Merck
Ammonium sulphate	Merck
Iron sulphate heptahydrate	Merck
Manganese sulphate	Merck
Zinc sulphate	Qualigens
Cobalt chloride	Central drug house lab reagent
Sodium hydrogen phosphate	Hi media
Sodium phosphate	Hi media
Sodium hydroxide pellets	Merck
Potassium sodium tartarate tetrahydrate	Merck
3, 5, dinitro salicyclic acid	Merck

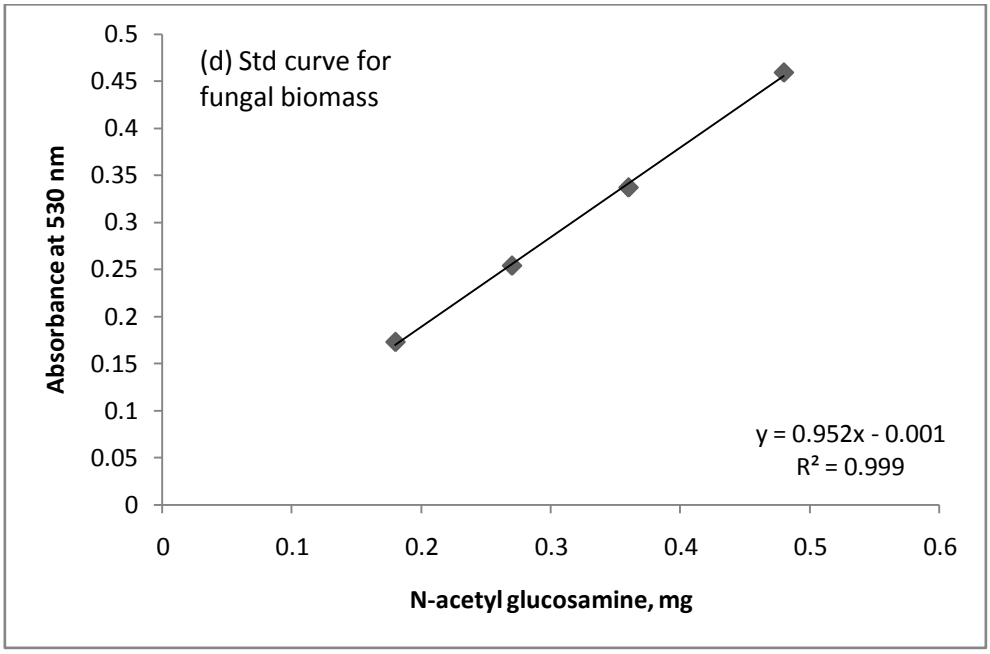
Phenol crystals	Fisher scientific, Qualigens
Sodium sulphite anhydrous excela R	Fisher scientific, Qualigens
Sucrose	Rankem
Sodium nitrate	Hi media
Potassium chloride	Hi media
Malt extract	Hi media
Mycological peptone	Hi media
Dextrose	Hi media
Peptone	Hi media
Yeast extract	Hi media
Sodium carbonate	Qualigens
Copper sulphate pentahydrate	Hi media
Bovine serum albumin	Hi media
Acetyl acetone	SRL
p- dimethyl amino benzaldehyde	Hi media
Glucosamine	Sigma- Aldrich
Ammonium Ferrous Sulphate Hexahydrate	Qualigens
Urea	Merck
Ammonium Nitrate	Merck
Ammonium Acetate	Merck
Ammonium Chloride	Hi media
Tryptone	Hi media
Meat Peptone	Hi media
Soya Peptone	Hi media
Peptone Bacteriological	Hi media
Proteose Peptone	Hi media
Beef Extract	Hi media
Tween 20	Hi media
Tween 40	Hi media
Tween 60	Hi media
Triton X100	Hi media
p-nitrophenyl β-D glucoside	Sigma

Standard curves used for the determination of (a) endoglucanase; (b) total cellulase; (c) β-D glucosidase and (d) fungal biomass






(c) Std curve for β-D glucosidase
1.298666667
1.026666667
0.767666667
0.515
0.258666667
y = 0.025x - 0.004
R² = 0.999
Absorbance at 430 nm
p-nitrophenyl β-D glucoside, µg
0
0.2
0.4
0.6
0.8
1
1.2
1.4
0
10
20
30
40
50
60


(d) Std curve for fungal biomass
y = 0.952x - 0.001
R² = 0.999
Absorbance at 530 nm
N-acetyl glucosamine, mg
0
0.05
0.1
0.15
0.2
0.25
0.3
0.35
0.4
0.45
0.5
0
0.1
0.2
0.3
0.4
0.5
0.6

MOLECULAR IDENTIFICATION REPORT

Name : Ms. Rashmi Singh

Institute/Organization : Department of Paper Technology, IIT Roorkee, Saharanpur Campus, 247001, Uttar Pradesh

Data Sheet Number : 143/1580/**16/04**/2012/R Singh/ Uttar Pradesh/-/---

Job Title : To identify the culture RS2

Procedure:

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

RESULT for Sample:

Strain 1580 RS2 showed 99% sequence similarity with genus *Penicillium* Link. (1809) species ***Penicillium oxalicum*** Currie. & Thoom. (1915). The genus currently has 304 species as mentioned in the *Dictionary of Fungi*, 10th edn. edited by Paul Kirk et al. in 2008.

STATUS OF YOUR SENT FUNGUS No. **1580 RS2** (QUERY)

Penicillium oxalicum

SEQUENCE ANALYSIS (**544 bases**) with NCBI sequence Accession JQ446378.1

```
NCBI Accession JQ446378.1/ B3-11(2)/ Penicillium oxalicum
Length=593
Score = 974 bits (527), Expect = 0.0
Identities = 539/544 (99%), Gaps = 4/544 (1%)
Strand=Plus/Plus

Query  1    AGTGAGGGCCCTCTGGGTCCAACCTCCCACCCGTGTTTATCGTACCTTGTTGCTTcggcg  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  35   AGTGAGGGCCCTCTGGGTCCAACCTCCCACCCGTGTTTATCGTACCTTGTTGCTTCGGCG  94

Query  61   gg[illegible]AAGACACA  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  95   GGCCCGCCTCACGGCCGCCGGGGGGCATCCGCCCCCGGGCCCGCGCCCGCCGAAGACACA  154

Query  121  CAAACGAACTCTTGTCTGAAGATTGTAGTCTGAGTACTTGACTAAATCAGTTAAAACTTT  180
            |||||||||||||||||||||||| ||||||||||||||||||||||||||||||||||||
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Query  241  GTGAATTGCAGAATTCAGTGAATCATCGAGTCTTTGAACGCACATTGCGCCCCCTGGTAT  300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  275  GTGAATTGCAGAATTCAGTGAATCATCGAGTCTTTGAACGCACATTGCGCCCCCTGGTAT  334

Query  301  TCCGGGGGGCATGCCTGTCCGAGCGTCATTGCTGCCCTCAAGCACGGCTTGTGTGTTGGG  360
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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Query  361  CTCTCGCCCCCCGCTTCCGGGGGGCGGGCCCGAAAGGCAGCGGCGGCACCGCGTCCGGTC  420
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Query  481  CACCATCAATCTTTAACCAGGTTGACCTCGGGATCAGGTAGGGGATACCCGCTGAACTTA  540
            |||||||||||||| |||||||||||||||| |||||||||| ||||||||||||||||
Sbjct  514  CACCATCAATCTT-AACCAGGTTGACCTCGG-ATCAGGTAGGG-ATACCCGCTGAACTTA  570

Query  541  AGCA  544
            ||||
Sbjct  571  AGCA  574
```

Sequences producing significant alignments:

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
JQ446278.1	Penicillium oxalicum isolate B3-11(2) 18S ribosomal RNA gene,	974	974	100%	0.0	99%
HE651152.1	Penicillium oxalicum genomic DNA containing 18S rRNA gene, I	974	974	100%	0.0	99%
HE651146.1	Penicillium oxalicum genomic DNA containing 18S rRNA gene, I	974	974	100%	0.0	99%
JF731233.1	Penicillium oxalicum strain M1 18S ribosomal RNA gene, partial	974	974	100%	0.0	99%
JN207294.1	Penicillium sp. P18E1 18S ribosomal RNA gene, partial sequenc	974	974	100%	0.0	99%
HM469410.1	Penicillium oxalicum strain KUC1674 18S ribosomal RNA gene, p	974	974	100%	0.0	99%
HQ832798.1	Penicillium sp. LH33 18S ribosomal RNA gene, partial sequence	974	974	100%	0.0	99%
HQ732137.1	Penicillium expansum strain G3 18S ribosomal RNA gene, partia	974	974	100%	0.0	99%
HM595499.1	Penicillium oxalicum isolate M3 internal transcribed spacer 1, p	974	974	100%	0.0	99%
GQ851781.1	Uncultured fungus clone LX042234-122-013-F04 internal trans	974	974	100%	0.0	99%
GQ851780.1	Uncultured fungus clone LX042234-122-013-E04 internal trans	974	974	100%	0.0	99%
GQ851776.1	Uncultured fungus clone L042884-122-064-D05 internal transc	974	974	100%	0.0	99%
GQ851773.1	Uncultured fungus clone L042884-122-064-C04 internal transc	974	974	100%	0.0	99%
HM991884.1	Penicillium sp. 07 SMR-2010 18S ribosomal RNA gene, partial s	974	974	100%	0.0	99%
EU057977.1	Penicillium sp. SN2-1 18S ribosomal RNA gene, partial sequenc	974	974	100%	0.0	99%
HM439951.1	Penicillium sp. JSC94 internal transcribed spacer 1, partial seq	974	974	100%	0.0	99%
HM236021.1	Penicillium oxalicum strain UASWS0381 18S ribosomal RNA gen	974	974	100%	0.0	99%
HM053477.1	Penicillium oxalicum strain RCEF4908 18S ribosomal RNA gene,	974	974	100%	0.0	99%
GU134996.1	Penicillium expansum isolate Y2-08 internal transcribed spacer	974	974	100%	0.0	99%
GU078430.1	Penicillium oxalicum isolate 68 18S ribosomal RNA gene, interna	974	974	100%	0.0	99%
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]

Additional References:

1. Peterson Rev. Iberoam. Micol. 23, 134, 2006, phylogeny.
2. Geiser et al. Mycol. 98, 1053, 2006, phylogeny.
3. Seifert et al. PNAS, USA, 104, 3901, 2007, phylogenetic barcodes.

CONDITIONS AND REMARKS:

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Kind Attn:

Ms. Rashmi Singh
Department of Paper Technology
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Uttar Pradesh – 247001

(S.K. Singh)

ARI/NFCCI/FIS/2012/Add. Reg./ Sl .No.1580/RAS/SBG

MOLECULAR IDENTIFICATION REPORT

Name : Ms. Rashmi Singh

Institute/Organization : Department of Paper Technology, IIT Roorkee, Saharanpur Campus, 247001, Uttar Pradesh

Data Sheet Number : 142/1580/**16/04**/2012/R Singh/ Uttar Pradesh/-/---

Job Title : To identify the culture RS1

Procedure:

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

RESULT for Sample:

Strain 1580 RS1 showed 99% sequence similarity with genus *Rhizopus* Ehrenb. (1821) species ***Rhizopus microsporus*** Boedijn (1859). The genus currently has 9 species as mentioned in the *Dictionary of Fungi*, 10th edn. edited by Paul Kirk et al. in 2008.

STATUS OF YOUR SENT FUNGUS No. **1580 RS1** (QUERY)

Rhizopus microsporus

SEQUENCE ANALYSIS (**543 bases**) with NCBI sequence Accession JX120676.1

```
NCBI Accession JX120676.1/ CNM-CM-2328/ Rhizopus microsporus
Length=613
Score =  976 bits (528),  Expect = 0.0
Identities = 538/542 (99%), Gaps = 4/542 (1%)
Strand=Plus/Plus

Query  4    TCATAA-TAATGTATTGGCACTTTACTGGGA-TTACTTCTCAGTATTGTTTGCTTCTATA  61
            |||||| |||||||||||||||||||||||| |||||||||||||||||||||||||||||
Sbjct  4    TCATAACTAATGTATTGGCACTTTACTGGGATTTACTTCTCAGTATTGTTTGCTTCTATA  63

Query  62   CTGTGAACCTCTGGCGATGAAGGTCGTAACTGACCTTCGGGAGAGACTCAGGACATATAG  121
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  64   CTGTGAACCTCTGGCGATGAAGGTCGTAACTGACCTTCGGGAGAGACTCAGGACATATAG  123

Query  122  GCTATAATGGGTAGGCCTGTTCTGGGGTTTGATCGATGCCAATCAGGATTACCTTTCTTC  181
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  124  GCTATAATGGGTAGGCCTGTTCTGGGGTTTGATCGATGCCAATCAGGATTACCTTTCTTC  183

Query  182  CTTTGGGAAGGAAGGTGCCTGGTACCCTTTACCATATACCATGAATTCAGAATTGAAAGT  241
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  184  CTTTGGGAAGGAAGGTGCCTGGTACCCTTTACCATATACCATGAATTCAGAATTGAAAGT  243

Query  242  ATAATATAATAACAACTTTTAACAATGGATCTCTTGGTTCTCGCATCGATGAAGAACGTA  301
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  244  ATAATATAATAACAACTTTTAACAATGGATCTCTTGGTTCTCGCATCGATGAAGAACGTA  303

Query  302  GCAAAGTGCGATAACTAGTGTGAATTGCATATTCGTGAATCATCGAGTCTTTGAACGCAG  361
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  304  GCAAAGTGCGATAACTAGTGTGAATTGCATATTCGTGAATCATCGAGTCTTTGAACGCAG  363

Query  362  CTTGCACTCTATGGATCTTCTATAGAGTACGCTTGCTTCAGTATCATAACCAACCCACAC  421
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  364  CTTGCACTCTATGGATCTTCTATAGAGTACGCTTGCTTCAGTATCATAACCAACCCACAC  423

Query  422  ATAAAATTTATTTTATGTGGTGATGGACAAGCTCGGTTAAATTTAAATTATTATACCGAT  481
            |||||||||||||||||||||||||||||||||||||||||||||| ||||||||||||
Sbjct  424  ATAAAATTTATTTTATGTGGTGATGGACAAGCTCGGTTAAATTTAA-TTATTATACCGAT  482

Query  482  TGTCTAAAATACAGCCTCTTTGTAATTTTTCATTAAATTACGAACTACCTAGCCATCGTG  541
            |||||||||||||||||||||||||||| |||||||||||||||||||||||||||||||
Sbjct  483  TGTCTAAAATACAGCCTCTTTGTAATTTT-CATTAAATTACGAACTACCTAGCCATCGTG  541

Query  542  CT  543
            ||
Sbjct  542  CT  543
```

Sequences producing significant alignments:

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
JX120676.1	Rhizopus microsporus strain CNM-CM-2328 18S ribosomal RNA	976	976	99%	0.0	99%
JX120685.1	Rhizopus microsporus strain CNM-CM-5511 18S ribosomal RNA	972	972	99%	0.0	99%
JX120683.1	Rhizopus microsporus strain CNM-CM-5226 18S ribosomal RNA	972	972	99%	0.0	99%
JX120682.1	Rhizopus microsporus strain CNM-CM-5167 18S ribosomal RNA	972	972	99%	0.0	99%
JX120681.1	Rhizopus microsporus strain CNM-CM-5165 18S ribosomal RNA	972	972	99%	0.0	99%
JX120680.1	Rhizopus microsporus strain CNM-CM-4244 18S ribosomal RNA	972	972	99%	0.0	99%
JN561271.1	Rhizopus microsporus isolate F6-03 18S ribosomal RNA gene, p	972	972	99%	0.0	99%
JN561253.1	Rhizopus microsporus isolate F2-02 18S ribosomal RNA gene, p	972	972	99%	0.0	99%
HQ285720.1	Rhizopus microsporus strain JJ-A3 18S ribosomal RNA gene, pa	972	972	99%	0.0	99%
HQ285719.1	Rhizopus microsporus strain WJ-A3 18S ribosomal RNA gene, p	972	972	99%	0.0	99%
HQ285708.1	Rhizopus microsporus strain DG-C4 18S ribosomal RNA gene, p	972	972	99%	0.0	99%
GQ502279.1	Rhizopus microsporus strain D4-1 isolate 2 18S ribosomal RNA	972	972	99%	0.0	99%
FJ854337.1	Rhizopus microsporus strain P2 18S ribosomal RNA gene, parti	972	972	99%	0.0	99%
EF151442.1	Rhizopus microsporus isolate F17 internal transcribed spacer 1	972	972	99%	0.0	99%

Additional References:

1. Nyilasi el al. Clin. Microbiol. Infect., 14, 393, 2008, molecular identification.
2. Liu et al. Sydowia, 59, 235, 2007, phylogeny.
3. Zheng et al. Sydowia, 59, 273, 2007, monograph.

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Kind Attn:

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