Fungal cellulase production and applications

Thesis Submitted to AcSIR for the Award of the Degree of

DOCTOR OF PHILOSOPHY

In

Biological Sciences

Ayman Salih Omer Idris

AcSIR enrollment number: 10BB13A39008

Under the guidance of

Dr. Rajeev K. Sukumaran & Prof. Ashok Pandey

Microbial Processes and Technology Division

CSIR – National Institute for Interdisciplinary Science and Technology

Thiruvananthapuram 695019, INDIA

22 January 2018

राष्ट्रीय अंतर्विषयी विज्ञान तथा प्रौद्योगिकी संस्थान
NATIONAL INSTITUTE FOR INTERDISCIPLINARY SCIENCE AND TECHNOLOGY
वैज्ञानिक तथा औद्योगिक अनुसंधान परिषद्
इंडस्ट्रियल इस्टेट पी. ओ, पाप्पनंकोड, तिरुवनंतपुरम, भारत - 695 019
Council of Scientific and Industrial Research
Industrial Estate P.O., Pappanamcode,Thiruvananthapuram, India-695 019

Dr Rajeev K Sukumaran, MSc, PhD, PGDBi
Senior Scientist & Head Biofuels and Biorefineries Section
Tele: +91 471 2515368 | Email – rajeevs@niist.res.in

22 January 2018

DECLARATION

This is to certify that the work incorporated in this Ph.D. thesis entitled Fungal **cellulase production and applications** submitted by Mr. Ayman Salih Omer Idris to Academy of Scientific and Innovative Research (AcSIR) in fulfillment of the requirements for the award of the Degree of Ph.D in Biological Science (Reg. 10BB13A39008) embodies original research work under the supervision of myself and Prof. Ashok Pandey. I further certify that this work has not been submitted to any other University or Institution in part or full, for the award of any degree or diploma. Research material obtained from other sources has been duly acknowledged in the thesis. Any text, illustration, table etc., used in the thesis from other sources, have been duly cited and acknowledged.

Dr Rajeev K Sukumaran

डॉ. राजीव कुमार सुकुमारन एम.एससी, पीएच.डी.
Dr. RAJEEV K. SUKUMARAN M.Sc., Ph.D.
वैज्ञानिक, माइक्रोबियल प्रक्रिया तथा प्रौद्योगिकी प्रभाग
Scientist, Microbial Processes & Technology Division
सी एस आई आर- राष्ट्रीय अंतर्विषयी विज्ञान तथा प्रौद्योगिकी संस्थान
CSIR-National Institute for Interdisciplinary Science and Technology
तिरुवनन्तपुरम/Thiruvananthapuram-695 019

CSIR- Indian Institute of Toxicology Research

Vishvigyan Bhavan, 31 Mahatma Gandhi Marg
Lucknow-226 001, Uttar Pradesh, India

Ashok Pandey, *PhD, FBRS, FNASc, FRSB, FIOBB, FISEES, FAMI*
Distinguished Scientist

Tel:+91-522-221-7646
Email: ashok.pandey1@iitr.res.in;
ashokpandey1956@gmail.com

January 22, 2018

CERTIFICATE

This is to certify that the work embodied in the thesis entitled "**Fungal cellulase production and applications**" has been carried out by Mr. Ayman Salih Omer Idris (Reg. 10BB13A39008) under my supervision at the Centre for Biofuels, Microbial Processes and Technology Division of CSIR – National Institute for Interdisciplinary Science and Technology, Thiruvananthapuram, and the same has not been submitted elsewhere for a degree or diploma.

Sincerely,

Ashok Pandey
Thesis Co - Supervisor

Formerly- Chief Scientist and Head, Biotechnology Division, CSIR-National Institute for Interdisciplinary Science and Technology, Trivandrum-695 019, India; Eminent Scientist, CIAB, Mohali, Punjab
Editor-in-chief: Bioresource Technology- Elsevier, UK; Honorary Executive Advisor: Journal of Water Sustainability, Australia; Honorary Executive Advisor: Journal of Energy and Environmental Sustainability; Subject Editor: Proceedings of National Academy of Sciences (India): Part B- Biological Sciences

Thiruvananthapuram
22 January 2018

DECLARATION

I hereby declare that the work presented in this thesis entitled "**Fungal cellulase production and applications**" is original work done by me under the supervision of Dr. Rajeev Kumar Sukumaran and Prof. Ashok Pandey, at the Centre for Biofuels, Biotechnology Division of CSIR – National Institute for Interdisciplinary Science and Technology, Thiruvananthapuram, India. I also declare that this work did not form part of any dissertation submitted for the award of any degree, diploma, associateship, or any other title or recognition from any University/Institution.

Ayman Salih Omer Idris

Acknowledgements

Firstly, I would like to express my sincere gratitude to my advisor Dr. Rajeev K. Sukumaran for the continuous support of my Ph.D study, for his patience, motivation, and immense knowledge. His guidance helped me in all the time of research and writing of this thesis. I could not have imagined having a better advisor and mentor for my Ph.D study.

With a deep sense of gratitude, I acknowledge my research co-supervisor, Prof. Ashok Pandey, Distinguished Scientist, CSIR-Indian Institute of Toxicology Research, and our former HOD. He provided me an opportunity to join Biotechnology Team as Ph.D fellow. Without his support, I could have never completed the research. I thank him for being a caring and amazing person.

Besides my advisors, I would like to thank my Doctoral Advisory Committee members: Dr. K Madhavan Nampoothiri, Dr. Binod Parameswaran, and Dr. Luxmi Varma, for their insightful comments and encouragement.

My sincere thanks also go to Dr. A. Ajayaghosh our present director, and Dr. Suresh Das our former director, who have provided me an opportunity to work at CSIR-NIIST for doing my Ph.D and for all necessary support that they have extended.

This research was partially supported by the Third World Academy for Science and Department of Biotechnology, Government of India, (TWAS-DBT) in the form of Fellowship and flight ticket.

I need to take this opportunity to be grateful to all scientists and technical staff at the Microbial Processes and Technology Division of CSIR-NIIST, for assistance in my experiments, valuable support and comments during my research.

I am thankful to my colleagues Rajasree, Nishant, Aravind, Leya, Meena, and Sabeela, who provided their unconditional support and expertise that greatly assisted me during the research.

I am also grateful to Dr. Anil, Dr. Amith, and Dr. Leena for helping me with HPLC analyses and interpretation of data.

I have to express appreciation and thanks to my colleagues Anand, Valan, Raja, Ram Kumar, Vivek, Rahul, Prajeesh, Godan, Anju, Devi, Haseena, Athira, Meera, Reshma, Sujitha, Ashwati A, and Ashwati U for support during the course of this research.

Last but not the least; I would like to thank my family: my mother, brothers and sister for supporting me spiritually throughout my life in general. I am also grateful to my wife Fathia and kids (Aseel, Salih, and Mohammed) for making my life meaningful and enjoyable, and providing all support during my Ph.D and always.

List of publications

SCI Journals

Ayman Salih Omer Idris, Ashok Pandey, Rajeev K. Sukumaran. 2016. Production of endoglucanase from *Trichoderma reesei* RUT C30 and its application in de-inking of printed office waste paper. Biologia. 71(3): 265-271.

Ayman Salih Omer Idris, Ashok Pandey, S.S. Raod, Rajeev K. Sukumaran. 2017. Cellulase production through solid-state tray fermentation, and its use for bioethanol from sorghum stover. Bioresource Technology. 242(10): 265-271.

Book Chapter

Rajeev K. Sukumaran, Vani Sankar, Aravind Madhavan, Meena Sankar, Vaisakhi Satheesh, **Ayman Salih Omer Idris**, and Ummalyama Sabeela Beevi (2015). Enzyme Technologies: Current and Emerging Technologies for Development of Novel Enzyme Catalysts, in: M. Chandrasekaran (ed.) Enzymes in food & beverage processing, CRC press, Florida, USA, pp:39-66.

Conference Presentation/Posters

Ayman Salih Omer Idris, Rajeev Kumar Sukumaran, Ashok Pandey. Production of endoglucanase from *Trichoderma reesei* and its application in deinking, poster presented in international conference on New Horizons in Biotechnology, Nov 22-25, 2015, Trivandrum. India.

Ayman Salih Omer Idris, Rajeev Kumar Sukumaran, Ashok Pandey. Cellulase production using *Trichoderma reesei* in solid state fermentation and its application in saccharification of alkali pre-treated sorghum stover, poster presented in International Conference on Current Trends in Biotechnology, Dec 08-10, 2016, Vellore, India.

Table of Contents

Chapter 1

Introduction and Review of Literature

Chapter 1 – Introduction and Review of Literature

1.1. General Introduction

1.1.1. Cellulase Enzymes

Cellulases are enzymes that hydrolyze β -1, 4 linkages in polymeric cellulose and liberate glucose units. Cellulases are mainly classified into three groups, endo-(1, 4)-*β*-D-glucanase (EC 3.2.1.4), exo-(1, 4)-*β*-D-glucanase (EC 3.2.1.91), and *β*-glucosidases (EC 3.2.1.21). The catalytic modules of cellulases belong in glyosyl hydrolase (GH) family and have been classified into various groups based on their amino acid sequences and crystal structures. Enzymes in GH families cleave glycoside bonds by using acid–base catalysis. The hydrolysis is achieved by two catalytic residues of the enzyme in the active site region: a general acid (proton donor) and a nucleophile/base (Davies and Henrissat, 1995). The spatial position of these catalytic residues varies and depending on that, hydrolysis occurs via retention or inversion mechanism. The different classes of cellulases are given below.

1.1.1.1. Endoglucanase

Endoglucanases or endo 1, 4-β-D-glucan glucanohydrolases (EC 3.2.1.4) are enzymes which randomly act on the cellulose polymer producing nicks in the amorphous regions of cellulose (endo initiating) to expose the reducing and non reducing ends by cleaving the endo β-1,4 linkages between adjacent glucose units. They are generally measured by detecting the reducing groups released from the soluble carboxymethylcellulose (CMC) substrate (Sheehan and Himmel, 1999). Endoglucanases are classically considered as the initiators of cellulose hydrolysis by the cellulase complex, since their action is essential for exposing the reducing and non reducing ends in the cellulose polymer; essential for the action of exoglucanases/cellobiohydrolases. Recent view on endoglucanases also proposes its role to help cellobiohydrolases to overcome blockage at amorphous regions of cellulose (Payne et al., 2015). Endoglucanases are represented in several glycosyl hydrolase (GH) families and in the model organism *T. reesei*, there are six endoglucanases represented in families GH5, GH7, GH12, GH45 and GH74 (Kubicek et al., 2012)

1.1.1.2. Exoglucanases

Exoglucanases or exo cellulases are of two types namely cellulose 1, 4-β-D-cellobiosidase (reducing end) EC 3.2.1.176 (cellobiohydrolase I/CBHI) and cellulose 1, 4-β-D-cellobiosidase (non reducing end) EC 3.2.1.91 (cellobiohydrolase II/CBHII). These enzymes attack the available reducing or non-reducing free ends or the ends generated by the action of EGs to liberate cellobiose units. While CBHI attacks the reducing ends of the chain, CBHII attacks the non-reducing ends (Cantarel et al., 2009). Current view on exoglucanases does not consider them as having exclusive exoglucanase action, but as exo glucanases with endo initiating action (Kurasin and Valjamae, 2011). In the model organism *T. reesei*, cellobiohydrolases are represented in glycosyl hydrolases families GH6 and GH7.

1.1.1.3. β- glucosidases

β-glucosidases or cellobiases (EC 3.2.1.21) are enzymes that catalyze the hydrolysis of terminal, non-reducing β- -D-glucosyl residues with release of β- -D-glucose (Leah et al., 1995). β-glucosidases (BGLs) catalyze the final reaction in cellulose hydrolysis namely the hydrolysis of cellobiose to two molecules of glucose and is responsible for the regulation of the cellulolytic cascade through its own feedback inhibition by its reaction product glucose. Most of the microbial BGLs employed in biomass hydrolysis belong to GH family 3, while they can be found in families 1, 3, 9, 30 and 116 (Teugjas and Valjamae, 2013). BGL action is considered as a critical step in cellulose hydrolysis since the substrate of BGL – cellobiose is a strong inhibitor of CBHs and its hydrolysis is essential to overcome product inhibition of the exoglucanases. Since glucose accumulation can lead to BGL inhibition which in turn leads to CBH inhibition through accumulation of cellobiose, the regulation of cellulase production in response to the hydrolysis of cellulose is of critical importance in most of the organisms producing these enzymes. In several cases the BGLs are also inhibited by their substrate, believed to be caused by the transglycosylation reaction capable of being performed by these enzymes (Bohlin et al., 2013).

1.1.1.4. Other cellulolytic enzymes and accessory proteins

It was long been recognized that the hydrolysis of the dense crystalline lattices of cellulose has to be mechanically disrupted for access of the hydrolytic enzymes and the role of a "swelling factor" which was non hydrolytic was proposed as early as in 1950 (Reese et al., 1950). "Swollenin" a protein with sequence similarity to plant expansions was described in *T reesei* by Saloheimo et al., (2002). It was believed that swollenin and similar non hydrolytic swollenin like proteins act like a zipper opening up the cross linking of cellulose microfibrils just like plant expansins (Arantes and Saddler, 2010). It was also proposed originally that these proteins lack hydrolytic activity since only negligible quantities of sugar release was observed with their independent action, while they enhanced hydrolysis of cellulosic substrates (Gourley et al., 2012). The mechanism of promoting cellulose breakdown was speculated to be through a non-hydrolytic weakening of hydrogen bonding (Jager et al., 2011, Gourley et al., 2012). However, the most recent works have indicated that the protein does have hydrolytic activity and shows a unique mode of action with similarities to the action of both endoglucanases and exoglucanases (Andberg et al., 2015). Apart from swollenin, the "disrupting" or "amorphogenesis inducing" class of biomass degrading proteins include expansins, bacterial expansin like proteins, fungal expansin like proteins, loosenin etc (Arantes and Saddler, 2010; Gourley et al., 2013).

Revolutionary changes in the conventional cellulose deconstruction paradigm have emerged with the discovery of a class of enzymes that share conserved structural features binding a metal ion and following a hitherto un-described oxidative mechanism (Vaaje-Kolstad, 2010). These types of enzymes which are now considered ubiquitous have been termed as Lytic Polysaccharide Mono Oxygenases (LPMOs). The most important feature of these enzymes is their ability to attack the highly crystalline regions of cellulose where EGs are unable to bind productively. Thus they are able to synergize with glycosyl hydrolases, likely as endo-acting enzymes that act directly on the surface of crystalline cellulose. It is now known that LPMOs require a reducing agent and molecular oxygen and a copper ion in the active

site (Payne et al., 2015). The electron donor can also be a co-secreted enzyme like cellobiose dehydrogenase (CDH), the only known example of a secreted flavocytochrome (Dimarogona et al, 2012)

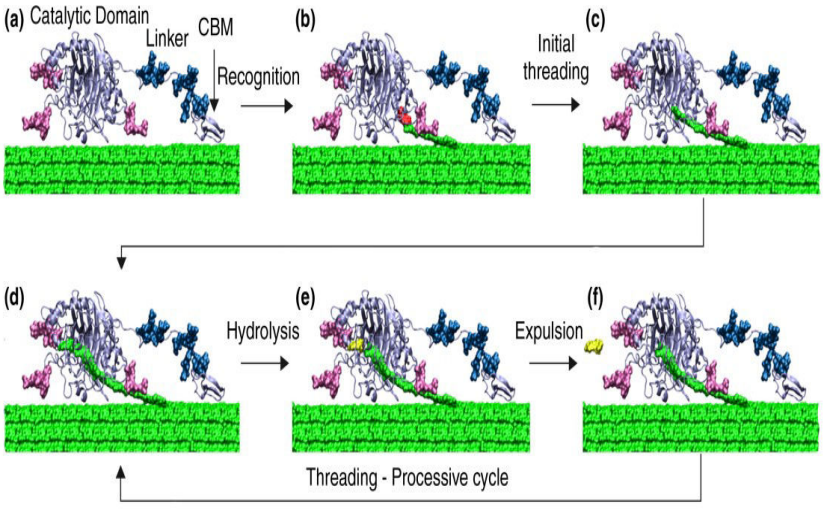
1.1.2. Mechanism cellulose degradation by cellulases

The complex structure of cellulose made of parallel unbranched D-glucopyranose units linked by β-1,4-glycosidic bonds that form highly organized crystalline microfibrils through inter and intramolecular hydrogen bonds and Van der Waals forces. There are regions of disorder, where these bonds are broken, in the arrangement of glucan chains along the cellulose microfibrils which are called the amorphous regions. The cellulases work at the solid liquid interfaces and a high concentration of catalytic units is required for efficient hydrolysis of the polymer. The typical three domain structure of endo-and exoglucanases ensures this by having a cellulose binding domain which can anchor the enzyme onto cellulose. Most of the endo and exo glucanases are processive enzymes which catalyze consecutive reactions without release of their substrate. The active site of a cellulase consists of multiple binding sites for glucose units, which helps the enzyme to remain bound to the substrate after a catalytic cycle and thereby work processively. The modular structure of cellulases helps in processivity and to keep the catalytic domain near the substrate (Teeri et al., 1998).

Generally, exo-glucanases have a tunnel-shaped active site. After recognition of a free cellulose chain end, the exo-glucanases threads the chain into the active site region in the catalytic domain (CD) of the enzyme to form a catalytically active complex (CAC). In glycosyl hydrolases, enzymatic hydrolysis of the glycosidic bond usually takes place via general acid/base catalysis, which requires two critical residues: a proton donor (HA) and a nucleophile/base (B-). This catalytic activity is provided by two aspartic- or glutamic acid residues. Hydrolysis occurs following a retaining or an inverting mechanism and in both cases, the acid-base (HA) protonates the leaving glycosidic oxygen with the formation of a partial positive charge on the C1 carbon (Davies and Henrissat, 1995). The processive cycle is continued with multiple events of hydrolysis before finally dissociating from the chain and

reinitiating the processive cycle at a new site (Payne et al., 2015). It is now known that cellulose hydrolysis by the exo-glucanases proceeds by movement of the enzyme through the cellulose surface while the glucan chain being threaded to the active site tunnel and is being hydrolyzed (Igarashi et al., 2009). Processively acting exo-glucanase molecules can get stalled at amorphous regions of cellulose and this leads to a diminished hydrolysis rate (Praestgaard et al., 2011). In the case of endo-glucanases, the processive cycle is different in that the chain threading and product expulsion are omitted. The binding site of endo-glucanase are more open, forming a cleft or groove instead of a tunnel which allows chain acquisition without threading, the enzyme bind to the middle of the substrate chain and cleave it. The renewed concept on role of endo-glucanases is that they are not only acting to generate reducing and non-reducing ends and thus helping exo-glucanases to attach, but also to help exo-gluanases dissociate from the cellulose chain when they encounter amorphous regions during their processive action (Jalak et al., 2012).

Figure 1.1. Mechanism of processive cellulose hydrolysis by *T. reesei* cellobiohydrolase (Cel7A)



a) Enzyme binding to cellulose b) recognition of the reducing end of a glucan chain c) initial threading of the glucan chain into the catalytic tunnel d) formation of CAC by threading e) processive hydrolytic cycle showing product formation (cellobiose shown in yellow) f) product expulsion

a) Reproduced from Beckham et al., (2011), with permission from Elsevier

In light of the above findings, the roles of different cellulases are now interpreted differently. Endo-glucanases and Lytic Polysaccharide Monooxygenases (LPMOs) do the endo-initiation in the amorphous and crystalline regions of cellulose respectively by breaking down the glycosidic bonds. The liberated reducing and non reducing ends are attacked respectively by the exo-glucanases (CBH1 and CBH2) which acts in a processive fashion to liberate cellobiose units and the cellobiose units are eventually cleaved to glucose by the beta glucosidases.

1.1.3. Microbial production of cellulases

Cellulases are synthesized by a large diversity of microorganisms during their growth on cellulosic materials. The industrial production of cellulases is mainly from microbial sources, bacteria and fungi. These microorganisms for cellulases can be diverse in its habitat - aerobic, anaerobic, mesophilic or thermophilic. The mechanism of cellulose degradation by aerobic bacteria is similar to that of aerobic fungi. Cellulosomes located on the cell surface of anaerobic bacteria operate on a different system. Most commonly studied cellulolytic organisms include: Fungal species - *Trichoderma, Humicola, Penicillium, Aspergillus*; Bacteria – *Bacilli, Pseudomonads, Cellulomonas;* and Actinomycetes – *Streptomyces, Actinomucor, and Streptomyces* (Sukumaran et al., 2005). The ability to secrete large amounts of extracellular protein is characteristic of certain fungi and such strains are most suited for production of higher levels of extracellular cellulases. While several fungi can metabolize cellulose as an energy source, only few strains are capable of secreting a complex of cellulase enzymes, which could have practical application in the enzymatic hydrolysis of cellulose. One of the most extensively studied fungi is *Trichoderma reesei*, which converts native as well as derived cellulose to glucose (Schulein et al., 1988). Besides *T. reesei,* other fungi like *Humicola, Penicillium* and *Aspergillus* have the ability to yield high levels of extracellular cellulases (Hayashida et al., 1988; Jorgensen et al., 2003; Ong et al., 2004). Cellulases from *Aspergillus* and *Trichoderma* of

fungal origin and *Bacillus* and *Paenibacillus* of bacterial origin are potentially used for food industry applications.

Cellulases are widely used for various industrial purposes. The major industries using cellulases are textile industry, pulp and paper industry, detergent industry, animal feed and food industry (Sukumaran et al., 2005). Now cellulases contribute a significant share of the world's industrial enzyme market. Production of cost effective cellulase preparations for industrial application is the major challenge in industry. There has been a significant improvement in production technologies that has brought down the cost of enzymes. Apparently, the production of cellulase enzymes is a complex process which has to take care of fungal growth parameters, ideal conditions for the optimal enzyme production and balance the costs involved. The present study looks in to various production strategies for improving the cellulase production in *T. reesei* RUT C-30 and its applications in different processes. The major objectives of the study are given below.

1.1.4. Objectives of the study

- Optimization of endoglucanase production by *T. reesei* RUT C-30 in Solid State Fermentation (SSF).
- Optimization of endoglucanase production by *T. reesei* RUT C-30 in Submerged Fermentation (SmF).
- Evaluation of *T. reesei* cellulase for biomass (Sorghum Stover) hydrolysis.
- Application of endoglucanase for enzymatic deinking of waste paper.
- Studies on endoglucanase for enzymatic production of cellulose nanoparticles.
- Application of *T.reesei* cellulase for synthesis through trans-glycosylation activity.

1.2. Review of Literature

1.2.1. Fungal cellulase

Filamentous fungi, especially the ascomycetes fungi *Trichoderma reesei* and *Aspergillus niger* or their derivative strains are used by most enzyme companies like Novozymes, Genencor (DuPont), and Iogen for commercial production of cellulases with the exception of Dyadic which uses engineered strains of *Chrysoporium lucknowense* (Zhang and Zhang, 2013). Most of the *Trichoderma reesei* strains currently in use for production of cellulases have been derived from the original isolate *Trichoderma* sp strain QM6a isolated by the US Army Research Laboratories at Natick over 70 years ago. The strain was improved through random mutagenesis experiments at the Natick laboratory and Rutgers University with resulted in the strain RUT C-30 with over 20 fold improvement in cellulase activity. This strain is still the prototype cellulase hyper-producer available in public domain (Bischof et al., 2016). The titers of extracellular cellulase on cellulase inducing carbon source –lactose can reach 30g/L as demonstrated by Durand et al (1988). A direct comparison of the levels of cellulase production by different organisms is complicated by the different strategies of production employed, different substrates and inducers employed and in some cases by the different units used for expressing their activity. Several such comparisons have been made which have mostly concentrated on the published information and information on the cellulase yields from studies in public domain may be obtained from these reports (Sukumaran et al., 2005; Mathew et al., 2008; Chandel et al., 2012; Hansen et al., 2015; Cunha et al., 2016). The highest cellulase activities (as filter paper units) have been recorded at about 10-15 FPUs/g substrate under solid state fermentation (SSF) and about 13 FPUs/ml for submerged fermentation (Hansen et al., 2015).

1.2.2. Regulation of cellulase production

Cellulases are inducible enzymes and the regulation of cellulase production is finely controlled by activation and repression mechanisms. In *T. reesei*, genes are co-ordinately regulated (Ilmen et al., 1997). The production of cellulolytic

enzymes is induced only in presence of the substrate, and is repressed when easily utilizable sugars are available. Natural inducers of cellulase systems have been proposed as early as 1962 (Mandels et al., 1962), and the disaccharide sophorose is since then considered to be the most probable inducer of at least the *Trichoderma* cellulase system. It is proposed that the inducer is generated by the trans-glycosylation activity of basally expressed β-glucosidase (Vaheri et al., 1979). Cellobiose, δ-cellobiose-1-5 lactone and other oxidized products of cellulose hydrolysis can also act as inducers of cellulose (Lynd et al., 2002). Lactose is another known inducer of cellulases and it is utilized in commercial production of the enzyme owing to economic considerations. Though the mechanism of lactose induction is not fully understood, it is believed that the intracellular galactose -1- phosphate levels might control the signalling. Glucose repression of cellulase system overrides its induction, and de-repression is believed to occur by an induction mechanism mediated by trans-glycosylation of glucose (Sternberg D & Mandels, 1979).

The promoter region of cellulases harbour binding sites for the *CREI* catabolite repressor protein as well as sites for the transcriptional activators including Activator of Cellulase Expression protein II (*ACE II*), besides CCAAT sequence, which binds general transcriptional activator complexes designated as '*HAP*' proteins. *ACEII* binds to the promoters of *cbh1* in *T .reesei*, and is believed to control the expression of *cbh1, cbh2, egl1,* and *egl2*. *Ace1* gene also produces a transcription factor similar to *ACEII* and has binding sites in *cbh1* promoter, but it acts as a repressor of cellulase gene expression. Glucose repression of cellulase is supposed to be mediated through carbon catabolite repressor protein *CRE1* in *T.reesei*. The promoter regions of *cbh1, cbh2, eg1* and *eg2* genes of *T. reesei* has *CRE1* binding sites indicating fine control of these genes by carbon catabolite repression. At least seven transcription factors have been identified to be involved in regulation of cellulases gene expression in *T. reesei* and studied in detail. These include five positive regulators Xyr1, Ace2, Ace 3, BglR and Hap2/3/5 complex and the negative regulators Ace1 and Cre1 (Kubicek et al., 2009).

1.2.3. Strategies for cellulase production

Commercial production of cellulases typically employs the submerged fermentation (SmF) strategy while solid state fermentation (SSF) processes are also used by companies (Sukumaran et al., 2005). Each method has its own advantages and disadvantages and the question of a best method is rather irrelevant, since the method adopted would depend on the organism employed, substrate used, growth conditions, existing infrastructure if there, need for purification etc among several other factors. While SSF is claimed to be the cheaper method for microbial enzyme production by many (eg. Tengerdy et al., 1996; Castilho et al., 2000, Viniegra-González et al., 2003; Krishna, 2005), there are also reports that states the contrary (Nakeeran et al., 2012). Also it should be noted that the enzymes elaborated by same organism can be different in SmF and SSF (Hansen et al., 2015) and the cost of production also needs to be read along with the efficiency of the enzyme in catalyzing the desired reaction. In this context, the production costs are tightly connected to the productivity of the enzyme producing strain and the final activity yield. The hydrolytic efficiency of the multienzyme complex depends on both the properties of individual enzyme and synergies between them and their ratio in the cocktail (Gusakov et al., 2014).

1.2.3.1. *Solid State Fermentation (SSF)*

Solid state fermentation is defined as the fermentation with near absence of free water but substrate moistened to support growth of microorganisms (Pandey, 2003). The substrate can either be a natural organic material like wheat bran or an inert support material impregnated or coated with the liquid growth medium (Ooijkaas et al., 2000). SSF processes are closer to the natural conditions of growth of most of the filamentous fungi and are hence expected to be ideal for production of enzymes that are required for plant cell wall degradation. There have been a large number of studies on solid state fermentation for cellulase production reported in literature. Even earlier studies like those by Chahal (1985) have demonstrated the higher yields of enzyme in SSF compared to SmF and there have been reports which indicated significant cost reductions for SSF compared to SmF (Tengerdy et al., 1996).

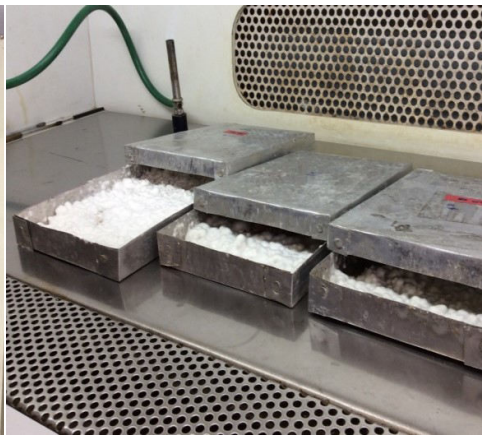
Solid state fermentation is typically performed by inoculating a natural substrate (eg wheat bran) moistened/impregnated with the suitable medium that carries essential nutrients – especially nitrogen source since in majority of the cases the substrate itself acts as the carbon source. Additional carbon source may be added for enhancing the growth rate or the final fungal biomass achieved. Since cellulases are inducible enzymes, an inducer like pure cellulose or a small molecule inducer like lactose /cellobiose may also be added for enhanced production. One of the advantages of SSF for cellulase production is the possibility of using a lignocellulosic substrate for fermentation, which will serve as a carbon source as well as the inducer (Nigam & Singh, 1996). The usage of raw material same as the feedstock to be used in a biorefinery for cellulases production has the added advantage of a more appropriate enzyme cocktail being elaborated by the organism, and this approach has been tested successfully by many (Sukumaran et al., 2009b; Roslan et al., 2011; Maeda et al., 2013; Pirota et al., 2014). It should be noted that the choice of carbon source has a significant impact on cellulase production under SSF, since differential induction of cellulases in response to the type of carbon sources used is a common phenomena in cellulase production by fungi.

The major advantages proposed for solid state fermentation include 1) higher enzyme production associated with higher biomass 2) lower product inhibition 3) lower protease activity 4) ability to use water insoluble substrates as C and N sources which allows use of cheap and abundant lignocellulosic biomass and 5) concentrated enzyme (Viniegra-González et al., 2003; Holker and Lenz, 2005). It has also been shown that SSF offers a situation of lowered catabolite repression in comparison to SmF (Díaz-Godínez et al., 2001). The immobility of typical SSF cultures can be an advantage and disadvantage at the same time. Since this replicates a natural growth environment for filamentous fungi, without any shear (as would be encountered in agitated SmF cultures), there is better growth and lesser autolysis due to hyphal rupture. At the same time, this also result in mass and heat transfer limitations. As the fermentation proceeds on an organic substrate like wheat bran, the substrate is decomposed leading to collapse of pores and aggregation of

particles which lead to less oxygen supply to cells and lower heat dissipation both of which affects the growth and enzyme production by fungi (Chen, 2013). The classical setup used for solid state fermentation for large scale enzyme production is tray reactors, though other designs like drum reactors and packed bed reactors are also employed. A typical tray reactor set up includes several trays of optimal size kept in racks inside a climate controlled chamber (Fig 1.2). The trays may be constructed of stainless steel, aluminum, wood or even plastic and may or may not have lids. The trays and lids in some setups are perforated to allow aeration and heat dissipation. The rooms are maintained at controlled temperature and humidity and often with forced circulation of HEPA filtered air (though this may not be essential). This configuration is easy to set up and easily scalable with low labour intensity.

Fig 7: Solid State Fermentation Facility (Koji) Room
(CSIR-NIIST, Trivandrum, India)

Koji Room (SSF chamber) showing steel racks for keeping trays and climate control system



Trays showing fully grown *Penicillium janthinellum* culture ready for harvest (for cellulase production)

The mixing and heat transfer issues associated with stationary tray fermenters can be addressed through use of rotating drum type reactors which is either mixed continuously or intermittently and with or without forced aeration. These types of reactors were patented by the French company Lyven and the Indian company Biocon and have been in use for commercial enzyme production. There are also several other type of reactor designs in use while tray reactors are the dominant types used in commercial SSF.

One of the major advantages of SSF is the possibility of using the fermented substrate as such in wet or dry form as the enzyme (Zuang et al., 2007; Singhania et al., 2015). The elimination of the downstream processing step can result in significant cost reductions, but has other disadvantages like carryover of the spores/mycelia to the biomass hydrolysis step (leading to contamination and lower productivity) and the increase in solids loading in the hydrolysis reactor leading to inefficient mixing. Apparently, the choice of direct use of fermented material as enzyme or after extraction and removal of the fungal spores/mycelia depends on the conditions of hydrolysis. The latter, if performed at higher temperatures can kill the mycelia and may prevent spore germination. Major disadvantages of SSF for cellulase production include the common disadvantages of SSF, which are the challenges in product purification, inability for complete automation and online monitoring of cell growth and enzyme production, providing heat and mass transfer, difficulty in mixing, necessity to keep the moisture content optimum, increased possibility for contamination (since SSF systems are not fully closed systems) etc. Moreover, heterogeneity and batch variations in solid substrates can have serious impact on reproducibility. Also there are issues with scalability, with tray reactors occupying large foot print compared to similar capacity SmF systems. Nevertheless, for production of enzymes like that used for biomass hydrolysis where purity is not a major concern, SSF systems might hold promise, since the enzymes produced on same substrates to be used as feedstock for biofuels can yield enzymes which are more appropriate for the job, and at higher concentrations. The methodology also has the process advantages of lower water and energy consumption, reduced waste stream, lesser capital infrastructure and the ability to use semiskilled labor (Zhuang et al., 2007). Recent reviews on the application of SSF technology for biomass hydrolyzing enzymes may be found in Yoon et al (2014), Farinas (2015) and in Behera and Ray (2016).

1.2.3.2. Submerged Fermentation (SmF)

Submerged fermentation is by far the most commonly used technique for production of industrial enzymes which also includes cellulases. SmF is

preferred by the industry since it allows easy online monitoring of the process parameters and allows their control (Hansen et al., 2015). Heat and mass transfer are better in SmF, since the cells are dispersed in a very conductive aqueous environment and also due to the fact that the system allows for regulation of temperature with external heating/cooling besides having efficient mechanisms for mixing. Biomass hydrolyzing enzymes from the common industrially employed cellulase producers (eg *Trichoderma reesei*) are inducible and the best activities are reported when grown in media containing cellulose. Untreated lignocellulosic substrates are generally found to support lesser enzyme yields compared top pure cellulose forms like Solka Floc or Avicell (Mathew et al., 2008). A range of different cellulosic materials has been tried as substrates for cellulase production and this includes pure celluloses (Hendy et al., 1982), paper pulp (Zuang et al., 2007), corn cob residue (Liming and Xueliang, 2004), sugar cane bagasse (Pereira et al., 2013), and even dairy manure (Wen et al., 2005). Most of these natural substrates are capable of inducing the cellulase systems in fungi often at par with known inducers or sometimes even better (Mathew et al., 2008). The choice of raw materials for cellulase production can influence both the yields as well as the final compositions of the cellulase preparation, which in turn will also affect the cost of enzyme preparations. Agro residues like corn cobs, rice or wheat straws, bagasse etc are cheap raw materials that can induce cellulase production due to their content of cellulose and hemicellulose polymers. However, they might need pretreatment for delignification before being used in cellulase production media. In a biorefinery context, especially for onsite enzyme production, this would not pose a big problem since the same feedstock that goes for biofuel production can serve as carbon source/inducer for cellulase production.

Media used for cellulase production have typically followed a composition after the original *T.reesei* medium used by Mandels and Weber (1969), but with modifications as appropriate for the strain /organism. Typically for *T.reesei* the fermentations are carried out in an acidic pH range with the optimal temperature being in the range of 25-30 °C (Mathew et al., 2008). Besides the carbon source, the choice as well as the concentration of

nitrogen sources is known to affect cellulase production and a high C/N ratio is proposed to be conductive for enhanced cellulase production by *T.reesei* (Liming and Xueliang, 2004). Stirred tank reactors are commonly employed for SmF production of cellulases which has the advantages of better mixing and aeration. However, fungal fermentation can present very unique problems associated with the control of their morphology. The morphology of fungi in SmF may vary based on various factors including the type of organism, inoculum density, pH, presence of surface active agents, agitation rates etc and careful selection of parameters might be essential to maintain the optimal fungal morphology. In typical submerged fermentations, fungi may grow in pelleted form or it may be dispersed as mycelial mats or aggregates. The latter form is often a serious limitation since the aggregates result in mass transfer limitations and cell damage due to shear. Also the mycelia may get entangled in the baffles of the reactor and can get accumulated on the fermenter wall wherever there is limited turbulence. The aggregates or mats can become really large disallowing any penetration of substrates or oxygen into the core, leading to death of cells in the centre of the aggregates. The pellets are highly entangled dense masses of hyphae which can assume sizes between few hundred micrometers to several millimeters (Domingues et al., 2000). Pelleted growth is generally preferred in fungal fermentations since it allows better mass and oxygen transfer due to an even distribution and enhanced surface area. Optimal morphologies in fermentations are dependent on operational conditions and knowledge of these can aid in enhancing the productivities in SmF (Cui et al., 1998). It has been observed that higher inoculum densities can lead to smaller pellet size translating to higher protein secretion and higher filter paper activity (Domingues et al., 2000). Similarly, in the same study it was observed that the presence of surfactant inhibited pellet formation by *T.reesei* RUT C-30. In another study which related cellulase productivity to fungal morphology, it was found that the buffers and pH conditions that promoted compact pellet formation resulted in enhanced enzyme yield (Ferreira et al., 2009). A pH of around 4.8 and 100mM succinate buffer supported maximum cellulase yield by *T. reesei* RUT C-30 in this study (Ferreira et al., 2009).

High productivities and yields of cellulases at industrial scale require that the fermentation is conducted under carbon flux limitation under either fed batch or continuous mode (Jourdier et al., 2012). Pure forms of cellulose, lignocellulosic substrates like pretreated plant biomass materials, or soluble carbon sources that can induce cellulases like lactose are the common carbon sources used in commercial scale production of cellulases. The typical processes can be batch, fed batch or continuous. Lactose is used as the carbon source/inducer in commercial production of cellulases employing *Trichoderma;* the disaccharide being the most affordable among highly potent soluble inducers. While cultivation of the fungus in cellulose/lignocellulose is cheaper, control of glucose concentration becomes a major limitation At low concentrations of (ligno) cellulose, glucose production might be too slow to meet metabolic needs of active growth and enzyme production, while at high concentrations, a higher rate of glucose generation compared to its consumption can result in catabolite repression (England et al., 2010). Apparently to maintain the conditions of carbon flux limitation, fed batch or continuous mode becomes helpful (Jourdier et al., 2013). In fact, most of the commercial production of cellulases employs the fed batch strategy where a soluble inducer like lactose or a cellulosic substrate is carefully dosed into the fermentation medium at appropriate intervals. The use of insoluble inducers/carbon sources like pure cellulose, paper pulp or any lignocellulosic substrate pose the additional challenge of mixing the medium in the production reactors. This is a very serious limitation when batch process has to be employed and often the production strategy has to ensure a size reduction treatment before the insoluble substrate is fed to the reactor (Shin et al., 2000; Liming & Xueliang, 2004). However, this can increase the production cost due to the need for pulverization of substrate. Here again, a fed batch process may help to maintain sufficient mixing, since the concentration of insoluble substrate can be kept minimum and fed as per demand (Shibuya et al., 2015). The use of a soluble carbon source in lieu of (ligno) cellulose has obvious advantages as this will allow better mixing, aeration and greater control over the fermentation process. Also the fermentation would no longer be dependent on the hydrolysis of lignocellulose (Allen and Mortensen, 1981). While sophorose is the best inducer of cellulase

in *Trichoderma reesei,* it is also the most expensive and difficult to manufacture. Recently, Danisco Inc. has patented a process using concentrated glucose, containing appreciable quantities of sophorose as carbon source/inducer for cellulase production using *T. reesei* (England et al., 2015). Here the inducing mixture of sugars was generated through transglycosylation activity employing whole cellulase preparations from *T. reesei*.

The choice of method for cellulase production depends on the end application and the cost evaluations also need to consider the efficacy of the enzyme preparation on the chosen lignocellulosic substrate. While SSF and SmF has their own advantage and disadvantages, it is not prudent to state one method is better than the other, since the choice is often made depending on the context of application, rather than on the mere advantages of a particular method.

1.2.4. Applications of Cellulase

Microbial cellulases find applications in a variety of industries where cellulases of varying degrees of purity are desired. Current market for cellulases is projected at 20% of the total world enzyme market. With the rapidly increasing demand for cellulases in biofuel industry, the projected cellulase market size is 9 billion dollars and it is speculated that cellulase will be largest industrial enzyme compared to market size for all the industrial enzymes (Zhang and Zhang, 2013).

1.2.4.1. *Food Industry*

In food industry, cellulases are used in extraction and clarification of fruit and vegetable juices, production of fruit nectars and purees, for extraction of olive oil (Galante et al, 1998b). In the beer industry exogenous glucanases are added to improve the malting of barley (Pajunen, 1986), and in wine industry, better maceration and colour extraction is achieved by use of exogenous hemicellulases and glucanases (Galante *et al* 1998b). Animal feed industry is another major beneficiary of the use of enzymes, and cellulases

constitute a major fraction of the enzymes used in animal feed processing. Enzyme preparations containing high levels of cellulase, hemicellulase and pectinase have been used to improve the nutritive quality of forages (Graham and Balnave, 1995; Kung et al., 1997).

1.2.4.2. *Pulp and Paper Industry*

In the pulp and paper industry, cellulases and hemicellulases has been employed for biomechanical pulping for modification of the coarse mechanical pulp and hand sheet strength properties (Akhtar, 1994, Pere et al, 1996), de-inking of recycled fibers (Prasad et al, 1993) and for improving drainage and runnability of paper mills (Pere et al, 1996). The use of enzymes in wood pulping considerably reduces the energy requirement resulting in a commercially attractive process. Bio characterization of pulp fibers is another application where microbial cellulases are employed (Buchert et al., 1995; Teleman et al., 1995). De-inking of waste paper is an important application of cellulases where a preparation with lesser exoglucanase content is desired. More than 70% of the mixed office waste paper consistenof uncoated papers that are printed with copy and laser printer toners, which are difficult to remove by conventional alkaline deinking processes (Heise et al, 1996). Most applications proposed so far use cellulases and hemicellulases to release the ink from the fiber surface by partial hydrolysis of carbohydrate molecules (Kuhad et al, 2011). Cellulases are highly efficient in removing toners from office waste paper (Jeffries et al, 1995). Cellulase decreases the interaction of toner and ink particles with the fibers. Primary role of cellulases in de inking involves separating ink-fiber agglomerates and dislodging or separating the ink and fibrous materials in response to mechanical action during disintegration (Kenealy and Jeffries, 2003). Although enzymatic deinking can lower the need for deinking chemicals and reduce the adverse environmental impacts of the paper industry, the excessive use of enzymes can result in significant hydrolysis of the fines, which reduces the bondability of the fibers (Karnis, 1995) Since exoglucanases result in accelerated degradation of cellulose fibers to the monomeric sugars, a preparation containing lesser endo-glucanases are often desired.

1.2.4.3. *Textile Industry*

A major consumer of commercial cellulases is the textile and laundry industry where the enzyme is used to various ends. Due to their ability in modifying the cellulosic fibers in a controlled and desired fashion so as to improve the quality of fabrics, cellulases have become the third largest group of enzymes used in the industry since their introduction only since a decade (Bhat 2000). Cellulases are used in the bio-stoning of denim garments replacing the use of pumice stones which were traditionally employed in the industry. Neutral cellulases especially from *Humicola insolescens* is used mostly in the biostoning process to prevent back staining (Galante et al, 1998a). Another important application of cellulases in the textile industry is for the bio-polishing of fabric. Cellulases are utilized for digesting off the small fibre ends protruding from the fabric resulting in a better finish (Galante et al, 1998a). The cellulase preparations capable of modifying the structure of cellulose fibrils are added to laundry detergents to improve the colour brightness, hand feel and dirt removal from cotton and cotton blend garments. Also, the degradation of microfibrils by cellulase softens the garment and removes dirt particles trapped in the microfibril network. This is currently accomplished by adding a commercial cellulase preparation from *H. insolens*, active under mild alkaline conditions (pH 8.5–9.0), and at temperatures over 50 ^{0}C in washing powders (Uhlig, 1998). Although, the amount of cellulase added represents approximately 0.4% of the total detergent cost, it is considered rather expensive and hence, alternative cellulase preparations are required to attract the worldwide laundry market.

1.2.4.4. *Biofuel*

Perhaps the most important application currently being investigated is the utilization of lignocellulosic wastes for the production of biofuel. A potential application of cellulase is the conversion of cellulosic materials to glucose and other fermentable sugars which in turn can be used as microbial substrates for the production of single cell proteins or a variety of fermentation products like ethanol (Mishra, 1980). Organisms with cellulase systems and

capable of converting biomass to alcohol directly is already reported in literature (eg Deshpande, *et al* 1983). Nevertheless it is also noted that that none of the systems described are effective alone to yield a commercially viable process. The effort to develop efficient technologies for biofuel production, significant efforts have been directed towards the identification of efficient cellulase systems and process conditions, besides efforts directed at the biochemical and genetic improvement of the existing organisms tried in the process. The use of pure enzymes in the conversion of biomass to ethanol or to fermentation products is prohibitive at present due to cost limitations. Overall, cellulosic biomass is an attractive resource that can serve as substrate for the production of value added metabolites and cellulases as such. Reviews describing the applications of microbial cellulases can be found in Bhat *et al* (2000) and Tengerdy and Szakacs 2003.

Chapter 2

Materials and Methods

Chapter 2. Materials and Methods

2.1. Microorganism and culture conditions

The microorganism used in this study was a filamentous fungus *Trichoderma reesei* RUT C-30 was kind gift from Prof. George Szakacs of the Technical University of Budapest, Hungary. The fungus was maintained as spore suspension in distilled water for long-term storage and on Potato Dextrose Agar (PDA) at 4˚C for working stocks.

2.2. Medium for enzyme production

The medium used for production of cellulase enzyme was a basal mineral salt solution according to Mandels and Weber (1969) with the following composition.

Table 0.1 Composition of Mandel and Weber Medium.

Component	Conc. (g/L)	Trace element	Conc. (g/L)
$(NH_4)_2 SO_4$	1.4	$FeSO_4.7H_2O$	0.05
K_2HPO_4	2.0	$MnSO_4.7H_2O$	0.0016
Urea	0.3	$ZnSO_4.7H_2O$	0.0014
$CaCl_2.2H_2O$	0.3	$CoCl_2.6H_2O$	0.002
$MgSO_4 .7H_2O$	0.3		
Peptone	0.75		
Yeast Extract	0.25		
Tween 80	1.0		
NaCl	0.5		

2.3. Preparation of spore inoculum

Spore suspensions were prepared in sterile saline containing 0.05% Tween 80. Three to 5ml of the sterile saline was added into fully sporulated slants (7day old) and was pipetted up and down gently to dislodge the spores into the liquid. The spore suspension thus obtained was aspirated into sterile vials, counted using a hemocytometer under phase contrast microscope and was adjusted to desired number using sterile saline.

2.4. Enzyme production

Enzyme production was carried out through submerged fermentation (SmF) or solid state fermentation (SSF) in 250 ml Erlenmeyer flasks as detailed below.

2.4.1. Submerged fermentation (SmF)

SmF was carried out in 250ml Erlenmeyer flasks containing 50ml pre-sterilized fermentation medium (Section 2.2) where the concentration of various carbon and nitrogen sources were varied as per the experiment design. The flasks were inoculated with 1.0 ml of a spore suspension containing approximately 1×10^7 spores/ml. The flasks were kept in an incubator shaker at 30 ± 2 °C and 200 rpm for the required time (as per the experiment design). At the end of fermentation, the culture was filtered through a nylon sieve to remove mycelia and then centrifuged at 10,000 rpm and 4 °C for 10 min to remove debris and the supernatant was used as crude enzyme preparation.

2.4.2. Solid state fermentation

Different agricultural waste residues were evaluated initially as substrate in the present study, and wheat bran was selected for production studies with supplementation of the added inducer- cellulose. Total of 5g dry weight of the

substrate of choice or wheat bran (WB) plus cellulose was weighed into 250-ml Erlenmeyer flasks and was moistened with the mineral salt medium (section 2.2) to obtain the appropriate initial moisture content. The pH of the medium was adjusted to 4.8 with 1 N HCl or 1 N NaOH wherever required. The flasks were then sterilized by autoclaving. The moistened solid substrate was inoculated with 1.0 ml of the spore suspension containing approximately 1×10^7 spores/ml or the appropriate volume as per the experiment design. The flasks were incubated at 30 ± 2 °C for the required duration as per the experiment design. Enzyme extraction was performed using 50 mM citrate buffer (pH 4.8). Fifty milliliter of the buffer was added to each flask containing 5g of moldy bran and was extracted by shaking on an incubator shaker (200 rpm, 30 °C, 60 min). The slurry was filtered using a nylon mesh to remove debris and the filtrate was centrifuged at 7000 rpm for 10 min at 4 °C to remove fine suspended particles. The supernatant was taken as the crude enzyme, and the enzyme activity of the enzyme was determined.

2.5. Analytical Methods

2.5.1. Endoglucanase assay

Endoglucanase (CMCase, endo-1, 4-β-D-glucanase; EC 3.2.1.4) activity was assayed in a total reaction volume of 1.0 ml containing 0.5 ml of diluted enzyme and 0.5 ml of 2.0 % (w/v) CMC solution in citrate buffer (50 mM, pH 4.8). This reaction mixture was incubated at 50 °C for 30 min. Dinitrosalicylic acid (DNS) reagent (3.0 ml) was added to the reaction mixture and boiled in a vigorously boiling water bath for 5 min. Reaction mixture was diluted 10× by adding distilled water and the absorbance was measured at 540 nm. Appropriate blanks were also included which lacked either enzyme or substrate. Standards prepared with varying glucose concentrations were run in parallel to calculate the glucose release. CMCase activity was calculated and defined as outlined by Ghose (1987), and was expressed as IU/ml.

2.5.2. Filter Paper assay

Total cellulase activity was measured using the filter paper assay according to the IUPAC method (Ghose, 1987). A rolled Whatman® #1 filter paper strip of dimension 1.0 x 6 cm (~50 mg) was used as substrate. The filter paper strips were saturated with 0.5 ml of Na-citrate buffer (0.05 M, pH 4.8) and 0.5 ml of an appropriately diluted (in Na-citrate buffer -0.05M, pH 4.8) enzyme was added to the tube and incubated at 50 °C for 60 minutes. Appropriate controls were also run along with the test. At the end of the incubation period, 3ml of DNS reagent was added and boiled for 5 min after which it was diluted appropriately and absorbance was measured at 540nm. The concentration of glucose released by different dilutions of the enzyme was determined by comparing against a standard curve constructed similarly with known concentrations of glucose. Filter Paper Activity (FPA) was calculated following the concept that 0.37 FPU of enzyme will liberate 2 mg of glucose under the above assay conditions and was expressed as Filter Paper Units (FPUs).

2.5.3. Sugar estimation

Total reducing sugars were estimated using DNS method (Miller, 1959) and glucose estimations were performed by HPLC following NREL protocols (Sluiter et al., 2008). Phenomenx® Rezex RNM carbohydrate HPLC column was used for the analysis and a refractive index detector was used for detection. The analysis was performed at 55 °C column temperature and 40 °C detector cell temperature. The flow was 0.6 mL/min using 0.01 N H_2SO_4 as mobile phase. Glucose standard used was HPLC grade D-Glucose from Sigma-Aldrich India.

2.6. Chromosomal DNA isolation from the fungus

Fungal spores (1 $\times 10^6$ spores) were inoculated into 50 ml Potato Dextrose Broth in 250 ml Erlenmeyer flasks and were incubated at room temperature (28 ± 2 °C)

for 48 h. One gram wet-weight of the mycelium was frozen by addition of liquid nitrogen and was ground to a fine powder. It was suspended in 10 ml of Lysis buffer (250 mM NaCl, 25 mM EDTA, 0.5 % w/v CTAB and 200 mM Tris–HCl, pH 8.5). The suspension was incubated at 65 °C for 30 min with occasional gentle mixing. After centrifugation at 13,000 rpm for 15 min (4 ºC), the supernatant was transferred to a new tube and polysaccharides and proteins were precipitated by adding equal volume of Phenol: Chloroform: Isoamyl Alchohol (25:24:1). Clear aqueous fraction was transferred with a wide bore nuclease free pipette tip into a clean nuclease free centrifuge tube. DNA was precipitated by adding 2/3 volumes of chilled Iso-propanol to the aqueous fraction. The solution was gently mixed by inversion, placed at -20 ºC for 20 min and DNA was recovered by centrifugation at 13000 rpm for 15 min (4 ºC). The DNA precipitate was washed twice with 70% ethanol and was allowed to air dry after which it was resuspended in 10 mM Tris-EDTA buffer (pH 8.0). RNAase was added to a final concentration of 10 µg/ml at 37 °C (Doyle and Doyle, 1987). The DNA was purified again using phenol: chloroform: isoamyl alchohol precipitation method. After a final wash with ethanol, the DNA was allowed to air dry and was resuspended in 10 mM Tris-EDTA buffer (pH 8.0). For long term storage DNA was kept at -20 °C.

2.7. Agarose Gel Electrophoresis

Agarose Gel electrophoresis of DNA was conducted in a Biorad Horizontal Gel electrophoresis apparatus. 1% Agarose gel was used for PCR Amplicons and 0.8% was used for Genomic DNA. Ethidium bromide was included in the gel for fluorescent visualization of DNA fragments under UV light. On every gel 0.5µg of 1kb DNA ladder (Thermo Scientific, USA) was run as a molecular weight marker for determination of the approximate size of DNA fragments

2.8. Studies on applications of the enzyme

The endoglucanase produced using *T. reesei* RUT C-30 was evaluated for potential applications in the generation of nanocellulose and for glucosylation of L-Ascorbic acid.

2.8.1. Nanocellulose production

Enzymatic production of Nanocellulose was evaluated using alkali pretreated cotton stalk (CS) biomass.

2.8.1.1. Alkali Pretreatment and Bleaching of CS Biomass

Alkali treatment was conducted using 6.0 % NaOH solution at 121 °C for 60 min. Alkali treated fibers were subjected to 2.0 % $NaClO_2$ bleaching in acetate buffer at 80 °C for 60 min. The fibers were strained, washed and the bleaching was performed as above for one more time. The resulting fibers were strained using a nylon filter and washed with distilled water until it reached neutral pH.

2.8.1.2. Nanocellulose production

Digestions for Nanocellulose (NC) production were performed in deionzied water due to the flocculation of Nanocellulose in buffer. The experiment used 300 mg of alkali pretreated and bleached cotton stalk (APBCS) in 30 mL of solution incubated with a total enzyme loading of 2.0 FPU per gram of glucan in a 150 mL hydrolysis flask. Incubations were performed at 50 °C shaker incubator; Samples were collected at 12, 24, and 48 h and consisted of a “kill series” where individual tubes were sacrificed for the analyses. Yields of NC were determined gravimetrically after differential centrifugation to separate NC from unconverted bulk cellulose. Samples collected at each time point were sonicated for 25 min with an amplitude setting of 75 on a sonicator. Following sonication, the

suspension was centrifuged at 3000g for 10 min in a swinging bucket rotor. The supernatant was decanted into a new pre-weighed Falcon tube and the pellet was resuspended in 30 mL of deionized water. This was repeated twice, yielding three supernatant tubes and one final pellet for each sample. Aliquots (2.0 mL) of supernatant were taken for imaging and dynamic light scattering (DLS) from the final supernatant; whereas 100 µL samples of all three supernatants were taken for sugar analysis by HPLC (Section 2.5.3) followed by centrifugation. The combined supernatants were lyophilized and NC yield was calculated from the resultant masses after correcting for free sugar content. To determine the progress of glucan conversion, 1000 µL aliquots of the well-mixed slurries were taken at 12, 24, and 48h. Samples were filtered through Pall Acrodisc™ Nylon 0.2 µm syringe filters (Pall, Port Washington, NY) and refrigerated until HPLC analysis. Glucan conversion was calculated by adding the total glucose and cellobiose yields (both glucose and cellobiose were converted to glucan equivalent) for each hydrolysis time point.

2.8.2. Glucosylation of L-Ascorbic acid and HPLC analysis

Transglycosylation of L-Ascorbic Acid (L-AA) was performed in 1.5 mL Eppendorf tubes using 100 mM sodium citrate buffer (pH 4.5), containing 2.6 mM Thiourea as a reducing agent using different enzymes (*T.reesei* cellulase, β-glucosidase (BGL) from *Aspergillus niger*, BGL from *Aspergillus unguis*, and Commercial acid cellulase (Zytex, India) in a total volume of 1.0 ml. Cellobiose (0.3M) was used as a donor substrate. L-Ascorbic acid (0.2M) was used as the acceptor substrate. The ratio of acceptor to donor substrate was varied as part of the reaction optimization. Temperature was also varied in the range of 30−50 °C. Reaction was started by adding 50 µL of the respective enzyme.

2.8.2.1. TLC analysis

Thin layer chromatography was done using modified carbohydrate separation method, chloroform: methanol: acetic acid: water 15:6:4:2 was used as mobile phase and UV detector was used to determinate the separation of Ascorbic Acid, and Ascorbic Acid 2 Gluconate (AA2G).

2.8.2.2. HPLC analysis

HPLC analyses were performed using an organic acid column (Phenomenex, India) at 35 °C was use. Products were detected using a UV detector at 243 nm. An isocratic elution with 20 mM H_2SO_4 in water was used. A constant flow-rate of 0.4 ml/min was applied. Samples of enzyme-catalyzed reactions, zero hour reaction mixture, L-AA acid, reaction mixture without enzyme, and cellobiose was run separately and also as a mixture to confirm production of AA2G.

Chapter 3

Production of endoglucanase by Solid State Fermentation

Chapter 3. Production of endoglucanase by Solid State Fermentation

3.1. Introduction

One of the major contributors to the cost of lignocellulosic biofuel is the cost of enzymes needed for hydrolyzing the biomass to fermentable sugars. Worldwide there is active R&D to bring down the cost of cellulases required for biomass hydrolysis, which encompasses multiple approaches including development of hyper- producing strains, improvement of the existing enzymes by engineering them and optimization of strategies for the cost effective of enzyme production. Process improvement for enhancing enzyme production is an important strategy since this can produce results within the shortest span, often taking production levels to the maximum attainable by wild type organisms. Industrial enzymes are primarily produced by two methods: Submerged fermentation (SMF) and Solid State Fermentation (SSF). While each process has its own merits and de-merits, SSF is often considered more cost efficient owing to the use of cheaper substrates, lesser infrastructure, requirement of only semiskilled manpower, a concentrated product stream and less technical intensiveness (Reimbault, 1998).

Solid-state fermentation (SSF) is defined as the microbial cultivation process in the absence or near absence of free water in the substrate (Pandey *et al*., 1992). However, there must be enough moisture to support microbial growth (Pandey *et al*., 1996). In nature, bacteria and filamentous fungi grow attached on solid substrates, such as rocks, wood, seeds, stems, roots and leaves of plants freely or in symbiotic associations with plants. Several complex natural substrates are degraded by these microorganisms with the aid of their extracellular enzymes, allowing them to utilize these compounds as carbon or nitrogen sources. Extracellular enzymes are considered important from the industrial viewpoint as they allow easy extraction and downstream processing (Hölker *et al*., 2004). Diverse group of enzymes have been produced by submerged fermentation techniques on large scale at the industrial level. However, the generation and

application of these groups of enzymes produced by solid state fermentations are comparatively rare.

Gas phase in SSF is affected by the molecular size and shape, and nature of the substrate. Availability of spaces between the particles in SSF ensures the supply of oxygen to growing microorganisms. This will improve the enzyme production for aerobic organisms that are commonly employed for industrial production. Use of naturally occurring waste materials/residues such as wheat bran, rice bran, saw dust etc , provides increased surface area for the microbes to colonize and also several nutrient requirements are satisfied without their external supplementation; hence increasing the yield during solid-state fermentation (Hölker *et al*., 2004). However, the growth of microbes in natural substrates is generally very slow as the carbon sources are often presented in complex forms, which needs to be degraded before assimilation. This limitation is often overcome by the addition of substances such as carbon and nitrogen sources, regulators ions and vitamins, where needed. Application of mechanical and chemical pretreatment of the raw substrate may decrease the growth due to release of toxic byproducts. Therefore, pretreatments when needed; should not induce any structural changes that could lead to the alteration of the natural physicochemical properties for their effective use as solid substrates in SSF (Pandey 1992, Pandey *et al*., 1996; Parekh *et al*., 2000). A scale-up of solid-state processes seems to be difficult due to the generally known problems of heat transfer, non- homogeneous media and difficulties with aeration. During cultivation, these problems may be amplified by the shear sensitivity of the microbe (Smits et al., 1996; Reimbault, 1998).

Another important aspect in the large scale production of enzymes or any other microbial metabolite by SSF is the process development. Process development involves the optimization of several variables/process parameters. The traditional approach such optimizations have been the ‘one-factor at a time’ method. In this, one parameter is varied while all the others are held constant and optimum level of this variable is determined. Incorporating this, the second

optimum level of the second variable is determined. This is repeated to find the optimal levels of all variable. This strategy is cumbersome and times consuming and often inaccurate in cases where the combination effects of variables are dominant.

Alternatively statistical experiment design methods can be employed which include fractional factorial designs when a large number of parameters are to be screened and response surface methods (RSM) to optimize a few significant variables. Plackett-Burman design (Plackett and Burman, 1946) is a common example of fractional design used to identify the most significant variables and is preferred when one needs to screen a large number of factors. Response surface methodology or RSM on the other hand is a collection of statistical techniques that are useful for modeling and analysis of problems in which a response is influenced by several variables and the objective is to optimize this response. Box-Behnken design (Box and Behnken, 1960) is a common example of RSM design.

Apparently, there have been several attempts on producing cellulase employing solid state fermentation targeting significant cost reductions. Agro residues as solid substrates have been successfully employed in our lab (CSIR-NIIST) as elsewhere. In several cases, instead on growing the enzyme producers on pure celluloses like Avicell® or SolkaFloc® , supplementing very small quantities of the pure celluloses into the cheap agro residue used as main substrate can give comparable enzyme yields at a comparatively cheaper cost (Singhania et al, 2009). The type of substrate used and culture conditions can affect the expression and secretion of cellulases and the composition of the cellulase cocktail secreted by *T. reesei* (Peciulyte et al, 2014). The current study was undertaken to optimize the culture parameters under SSF so that a cellulase cocktail enriched in endo glucanases may be obtained *from T. reesei* RUT C-30. Since the *T. reesei* culture was retrieved from a long term preserved stock, its identity was reconfirmed by sequencing of ITS region.

3.2 . Materials and Methods

3.2.1. Reconfirmation of the culture identity

While the *T. reesei* RUT C-30 culture was originally received as freeze dried sample from Technical University of Budapest (courtesy- Prof. George Szakacs), The culture was being subcultured in PDA slants and preserved as spore suspensions for long term in -20 °C. The culture was retrieved from one of the long term preserved stocks and hence its identify was reconfirmed by rRNA sequence analyses before commencing the work.

3.2.1.1. Chromosomal DNA isolation from fungal strains

Chromosomal DNA from the fungus was isolated as per the protocol stated in section 2.6. The DNA precipitate was washed twice with 70 % ethanol allowed to air dry and was resuspended in 10 mM Tris-EDTA buffer (pH 8.0).

3.2.2.2. PCR Amplification and analysis of 18S rRNA gene

A portion of the 18S rRNA gene was amplified from the genomic DNA by polymerase chain reaction (PCR) using the universal primers NS1, NS4, NS3, NS8 (White et al. 1990). PCR reactions contained 0.5 units of Taq DNA polymerase, 1× Taq buffer, 200 µM of each dNTPs, 2.0 µM MgSO4 (All from ThermoFischer Scientific, USA), 0.2 µg genomic DNA, and 0.5 µM forward and reverse primers. Reaction conditions for PCR amplification were an initial 94 °C for 3 min, followed by 30 cycles of 94 °C for 30 sec, 55 °C for 30 sec and 72 °C for 1 min; and a final extension step at 72 °C for 10 min. A ThermoFischer Scientific- ProFlex Gradient PCR system was used for the amplification. PCR products were separated by electrophoresis on a 1 % agarose gel and products

were visualized in long range UV trans-illumination for documentation. Identity of the sequence assembly was established by BLAST analysis (Altschul et al. 1990).

3.2.2.3. ITS amplification and analysis

PCR amplification of Internal Transcribed Spacer region (ITS) of rDNA was carried out using universal eukaryotic ITS primers. The primers used for ITS amplification is given in Table 3.1. The forward primer binds to 3' end of 18S rDNA and the reverse primer binds to 5' end of 28S rDNA (Inglis and Tigano 2006). Conditions provided for PCR amplification were similar as above (section 3.2.2.3), and sequence analysis was performed using BLAST.

Table 3.1. ITS primer

Primer name	Primer sequence(5'-3')	Reference
ITS1F	TCCGTAGGTGAACCTGCGG	(White et al 1990)
ITS4R	TCCTCCGCTTATTGATATGC	(White et al 1990)

3.2.2. Enzyme production

Different agricultural waste residues were evaluated initially as substrate in the present study, and wheat bran was selected for production studies with supplementation of the added inducer-cellulose. Total of 5g dry weight of the substrate of choice or wheat bran plus cellulose was weighed into 250-ml Erlenmeyer flasks and was moistened with mineral salt medium (Mandels and Weber 1969) to obtain the appropriate initial moisture content. The flasks were then sterilized by autoclaving. The pH of the medium was adjusted to 4.8 with 1N HCl or 1N NaOH wherever required. The flasks were inoculated with 1.0 ml of the spore suspension containing approximately 1×10^7 spores/ml or the

appropriate volume as per the experiment design and the experiments were performed and enzyme extracted as outlined under section 2.4.2.

3.2.3. Enzyme assays

3.2.3.1. *Carboxy Methyl Cellulase (CMCase) activity*

Endoglucanase (CMCase, endo-1, 4-β-D-glucanase; EC 3.2.1.4) activity was assayed as per the IUPAC guidelines (Ghose, 1987) as outlined under section 2.5.1, and was expressed as international units per gram dry substrate (IU/gDS)

3.2.3.2. Filter Paper Activity Assay

Total cellulase activity was measured using the filter paper assay according to IUPAC (Ghose, 1987) as specified under section 2.5.2, and Filter Paper Activity (FPA) was calculated following the concept that 0.37 FPU of enzyme will liberate 2mg of glucose under the above assay conditions and was expressed as Filter Paper Units (FPUs) per gram dry substrate (FPUs/gDS).

3.2.4. Optimization of cellulase production

Different carbon sources and others variables were screened in a one factor at a time approach initially, and these were incorporated in the medium for further optimization using a two stage statistical experimental design strategy. In the first stage, a fractional factorial design was used to screen 10 process variables to identify the most significant ones affecting endoglucanase (measured as CMCase activity) production and in the second stage, a response surface Box Behnken design (Box and Behnken, 1960) was used to optimize the levels of these selected variables.

3.2.4.1. Screening of variables affecting production

The screening of important variable affecting cellulase production was performed using a Plackett and Burman design (Plackett and Burman, 1946), where 11 variables were evaluated at 2 levels. The actual factors tested and their levels are given in Table 3.3. Experimental runs were performed according to the design and the response (CMCase activity) was recorded. A factorial model was fitted for the main effects using Design Expert® software (Stat-ease Corp, USA). The effects of individual parameters on CMCase production was calculated by the following equation (Eqn.1)

$$\varepsilon = (\Sigma\mu_{+} - \Sigma\,\mu_{-})/n \qquad (1)$$

Where ε is the effect of parameter under study and "μ_{+}" and "μ_{-}"are responses (CMCase activity) of trials at which the parameter was at its higher and lower levels respectively and "n" is the total number of trials. Analysis of variance (ANOVA) was performed on the data to determine the significance of fitted model and to test the significance of the effect of individual parameters on CMCase production. The most significant parameters affecting CMCase production were identified.

3.2.4.2. *Optimization of Process variables by RSM design*

The SSF production of endoglucanase on combination of wheat bran and cellulose is influenced by various factors including media components and environmental parameters. Cellulose concentration, Initial moisture content, and inoculum size were identified as significant parameters affecting endoglucanase production by an initial screening of parameters. The levels of these variables were optimized for enhancing the endoglucanase yield using a response surface Box– Behnken experiment design (Box and Behnken 1960). The design matrix with 17 experimental runs is shown in Table 3.4. The variables selected for

optimization, i.e., Initial moisture content, cellulose content, and inoculum size, were coded as X_1, X_2, and X_3, respectively. A second-order polynomial (Eq. 2) was used to represent the response as a function of the tested variables

$$Y = \beta_0 + \sum_{i=1}^{n} \beta_i X_i + \sum_{i=1}^{n} \beta_{ii} X_i^2 + \sum_{i=1}^{n} \beta_{ij} X_i X_j \quad (2)$$

Where, Y is the measured response (CMCase yield), β, βi, and βii are the regression coefficients, and X_1–X_3 are the factors under study. Regression analysis and estimation of the coefficients were performed using Design Expert ® software (State Corp, USA). The contributions of individual parameters and their quadratic and interaction effects on cellulase production were determined.

3.2.5. Tray fermentation for scaled up cellulase production

Solid state fermentation on production scales are usually performed in trays and inside climate controlled chambers called Koji rooms. Initial evaluation of the larger scale production of enzymes through SSF using the *T. reesei* culture was attempted in 50g capacity trays in the Pilot scale SSF facility (Koji Room) of CSIR NIIST with relative humidity and temperature maintained at 80% and 30 ± 2 °C respectively. Fifty grams of total dry substrate containing 20% cellulose was mixed well and moistened with the production medium according to the conditions optimized in flask cultivation. Inoculation was carried out with spore inoculum prepared with the optimized concentration and fermentation was carried out till 14 days when mycelial growth was visible and sporulation was set in; which were the conditions when maximum cellulase production was observed in flask fermentation. Enzyme extraction and assay was performed as previously described.

3.3. Results and Discussion

3.3.1. Molecular identification using 18S rRNA sequence analysis

PCR amplification of the 18S rRNA region yielded a 519 bp sequence which was showed 99 % similarity (*Table* 3.2) with 18S rRNA sequence from other strain of *Trichoderma reesei* reconfirming the identity of the stock culture.

Partial sequence of the ITS region from *T.reesei* RUT C-30

```
ACGTTACCAATCTGTTGYCTCGGCGGGATTCTCTGCCCCGGGCGCGTCGCAGCCCCGGA
TCCCATGGCGCCCGCCGGAGGACCAACTCAAACTCTTTTTTCTCTCCGTCGCGGCTTCC
GTCGCGGCTCTGTTTTACCTTTGCTCYGAGCCTTTCTCGGCGACCCTAGCGGGCGTYTS
GAAAATGAATCAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGC
AGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAAC
GCACAYTGCGCCCGCCAGTATTCTGGCGGGCATGCCTGTCCGAGCGTCATTTCAACCCT
CGAACCCCTCCGGGGGGTCGGCGTTGGGGATCGGCCCCTCACCGGGCCGCCCCCGAAAT
ACAGTGGCGGTCTCGCCGCAGCCTCTCCTGCGCAGTAGTTTGCACACTCGCACCGGGAG
CGCGGCGCGGCCACAGCCGTAGAAACACCCCAAACTCTGAAATGTG
```

Table 3.2. Sequences with significant similarity to *Trichoderma reesei* ITS region identified by BLAST analysis

No	Sequence Description	Max Score	Total score	Query cover	E value	Ident	Accession
1	Trichoderma reesei strain CHR3P2F3 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1 and 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence	931	931	100%	0	99%	KT580962.1
2	Trichoderma reesei strain BHR2P2F3 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1 and 5.8S ribosomal RNA gene, complete sequence; and internal	931	931	100%	0	99%	KT580954.1

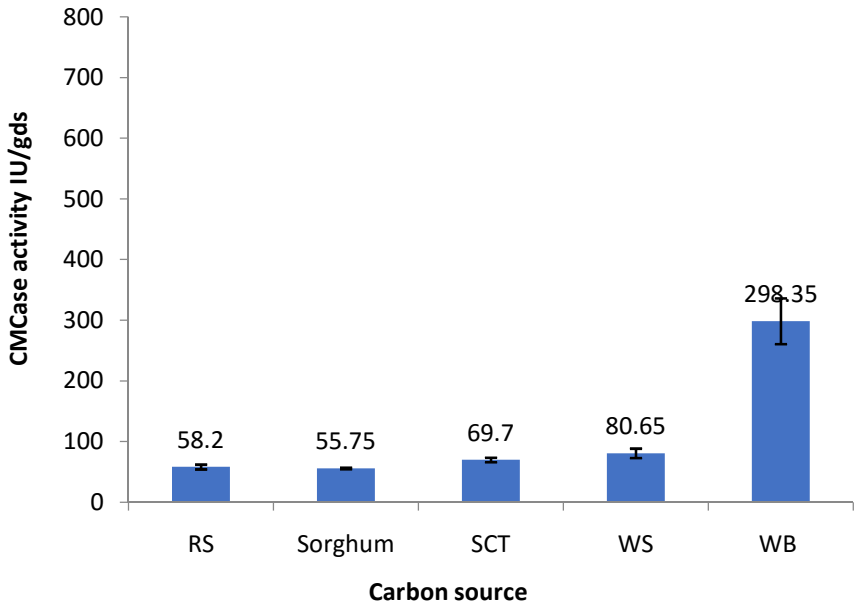
	transcribed spacer 2, partial sequence						
3	Trichoderma reesei isolate Tr-02 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence	931	931	100%	0	99%	KF294851.1
4	Trichoderma reesei isolate Tr-01 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1 and 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence	931	931	100%	0	99%	KF294850.1
5	Hypocrea jecorina strain GXNN4056 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	931	931	100%	0	99%	JQ040380.1
6	Hypocrea koningii strain NTLF11 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	931	931	100%	0	99%	HQ245158.1

7	Trichoderma reesei isolate CPTrZC-04 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence	929	929	99%	0	99%	MG687493.1
8	Trichoderma reesei strain SC46a small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1 and 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence	929	929	99%	0	99%	MG519514.1
9	Trichoderma saturnisporum strain SPH-2010-9-132 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	929	929	99%	0	99%	KY025558.1
10	Trichoderma reesei strain 10-56 TD1 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	929	929	99%	0	99%	KY025557.1

3.3.2. Selection of carbon sources for CMCase production

Cellulase production by the *Trichoderma reesei* RUT C-30 under solid state fermentation was evaluated using the agricultural residues rice straw, sorghum straw, sugar cane trash, wheat straw, and wheat bran as carbon sources. Among these crude carbon sources, wheat bran could induce the maximum cellulase (CMCase) activity of 298.35 IU/gDS. While the lignocellulosic substrates did induce cellulase activity, the enzyme yields were considerably lower compared to wheat bran as the substrate (Figure 3.1). Wheat bran (WB) is a cheap carbon source routinely used as substrate for SSF and is rich in several vitamins and other nutrients in addition to sugars and sugar polymers, and this could be the reason for its better performance as it may enhance cell growth and metabolism. WB was selected as the carbon source for all further experiments.

Figure 3.1. CMCase production on different agriculture residues/ waste



(RS –Rice straw, SCT-Sugar Cane Trash, WS- Wheat Straw, WB –Wheat bran)

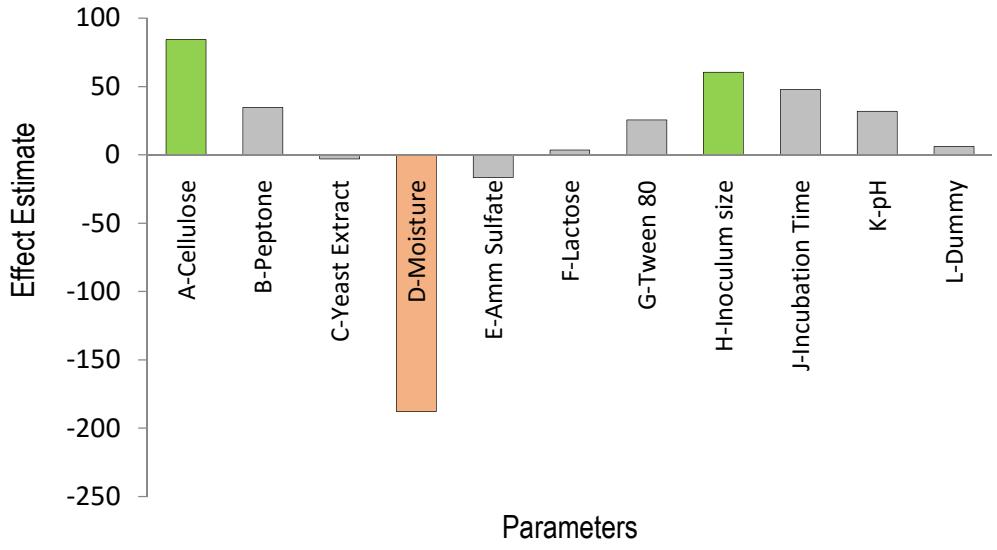
3.3.3. Optimization of cellulase production

Screening experiments performed using the Placket and Burman design showed indicated considerable variation in CMCase production ranging from 237.67 IU/gds to 547.28 IU/gds (Table 3.3), indicating the role of various parameter combinations in differentially inducing enzyme production in *T.reesei*. The effects of various parameters on CMCase production was estimated using equation 1 and the Pareto chart showing the effect estimates are presented in Figure (3.2). The factors with highest effects were initial moisture content (-ve), cellulose concentration (+ve), and inoculum size (+ve). Cellulases are inducible enzymes and production of the enzyme by *T. reesei* depends on the induction mediated by the lignocellulosic substrate under natural conditions. Pure cellulose is a strong inducer of cellulases and apparently a dominant factor of cellulase production (Bischof et al., 2013).

Table 3.3. Placket and Burman Design matrix for screening of process variables

Run #	Cellulose (g/g)	Peptone (g/L)	Yeast Extract (g/L)	Moisture (%)	Amm Sulfate (a/L)	Lactose (g/L)	Tween 80 (g/L)	Inoculum size (spores/g)	Time (Days)	pH	Dummy	Enzyme Activity (IU/gDS)
1	0.80	3	5.0	65	2	20	0.25	2.0E+06	7	5.5	-1	260.59
2	0.20	3	7.5	55	2	20	1.00	2.0E+06	7	4.8	1	360.95
3	0.80	0	7.5	65	1	20	1.00	1.0E+07	7	4.8	-1	293.69
5	0.20	0	7.5	55	2	20	0.25	1.0E+07	9	5.5	-1	434.69
6	0.20	0	5.0	65	1	20	1.00	2.0E+06	9	5.5	1	237.67
4	0.20	3	5.0	65	2	0	1.00	1.0E+07	9	4.8	-1	274.53
7	0.80	0	5.0	55	2	0	1.00	1.0E+07	7	5.5	1	502.13
8	0.80	3	5.0	55	1	20	0.25	1.0E+07	9	4.8	1	547.28
9	0.80	3	7.5	55	1	0	1.00	2.0E+06	9	5.5	-1	531.88
10	0.20	3	7.5	65	1	0	0.25	1.0E+07	7	5.5	1	252.94
11	0.80	0	7.5	65	2	0	0.25	2.0E+06	9	4.8	1	241.03
12	0.20	0	5.0	55	1	0	0.25	2.0E+06	7	4.8	-1	310.55

Figure 3.2. Pareto chart showing effect of different process variables on CMCase production



Cellulose was supplemented in the production medium so as to have strong induction of the enzymes and the results indicate that this approach was successful. Increase in moisture content had a negative influence on cellulase production, and the results confirmed to our previous observations that *Trichoderma reesei* produce more cellulase at low moisture contents under SSF (Singhania et al., 2007). *T reesei* is a highly aerobic culture and higher moisture content would have prevented proper aeration through formation of substrate aggregates limiting oxygen transfer. Increase in inoculum concentration within tested range resulted in enhanced activities which might be attributed to higher growth rates achieved initially. Since initial moisture content, cellulose concentration and inoculum size was the most significant parameters that were identified by Placket and Burman Design; their levels were further optimized using a Box Behnken design (BBD).

The Box–Behnken design, which was used for optimization of cellulase (CMCase) production, contained 17 experimental runs in a single block, where each of the three parameters (cellulose concentration, moisture content and

inoculum size) was evaluated at 3 levels. The maximum response (959.53 IU/gDS) was obtained in run number 9, and in general, runs with middle levels of parameters gave higher enzyme production compared to other combinations (Table 3.4).

Table 3.4. Box Behnken Design matrix for optimization of CMCase production by *T. reesei*

Run No	X_1 Cellulose (g/5g)	X_2 Inoculum Density (Spores/g)	X_3 Moisture (%)	CMCase (IU/gDS)
1	0.8	3.00E+06	55.0	733.06
2	1.0	3.00E+06	57.5	940.95
3	1.0	3.00E+06	57.5	954.71
4	1.0	3.00E+06	57.5	956.02
5	1.2	3.00E+06	60.0	774.00
6	1.2	5.00E+06	57.5	830.16
7	1.2	1.00E+06	57.5	778.29
8	1.2	3.00E+06	55.0	774.00
9	1.0	3.00E+06	57.5	959.53
10	0.8	1.00E+06	57.5	755.23
11	1.0	5.00E+06	55.0	878.29
12	0.8	3.00E+06	60.0	723.13
13	1.0	5.00E+06	60.0	756.62
14	1.0	1.00E+06	55.0	857.18
15	0.8	5.00E+06	57.5	795.42
16	1.0	1.00E+06	60.0	794.73
17	1.0	3.00E+06	57.5	827.38

The data was analyzed by multiple regression analysis using the software Design Expert® v8.00, and the regression coefficients were determined. A second-order polynomial equation (Eq. 4) was derived to represent the cellulase production as a function of the independent variables tested.

$$Y = 927.718 + 18.70X_1 + 9.38X_2 - 24.25X_3 - 104.3{X_1}^2 - 33.64{X_2}^2 - 72.37{X_3}^2 + 2.92X_1X_2 + 2.48X_1X_3 - 14.80X_2X_3 \quad (4)$$

Where, Y is the predicted response, and X_1, X_2, and X_3 are coded values of cellulose content, inoculation size and moisture content, respectively.

The quadratic regression model adopted was significant as suggested by the Analysis of Variance (ANOVA) which indicated computed F value of 3.7821and a P value lower than 0.05 (Table 3.5). The coefficient of variation was low (6.15 %) suggesting higher reliability of the experiment. The R^2 value obtained was 0.8294, indicating that 82.94% of the sample variation is attributed to the factors and only 17.06% can occur due to chance. Table 3.5 also gives the P values of each of the parameters and their quadratic and interaction terms. ${X_1}^2$, ${X_3}^2$ were found to be significant model terms based on their low p-values which were lower than 0.05. There were no statistically significant interactions between the parameters. Response surface curves were plotted to view the interaction effects of variables and for identifying the optimal levels of each parameter for obtaining maximum yield.

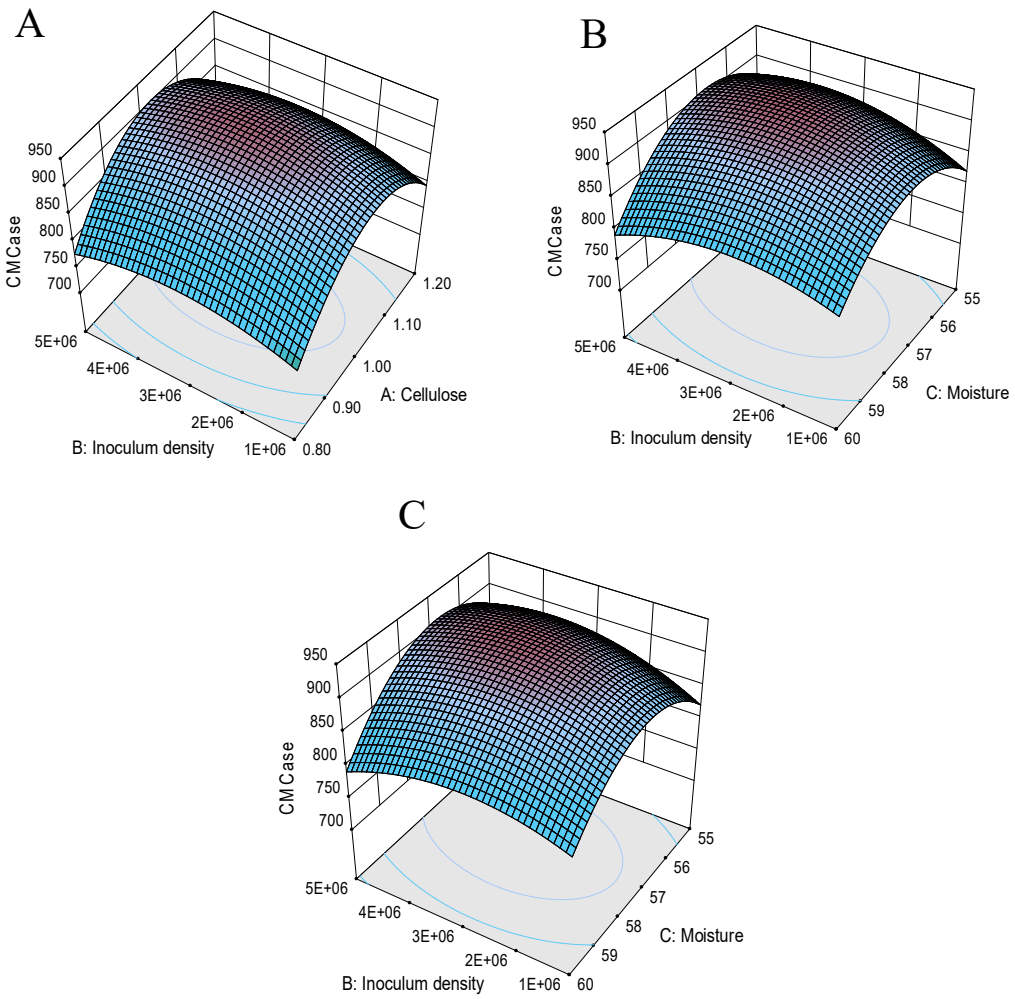
Table 3-5. ANOVA for Response Surface Quadratic Model

Source	Sum of Squares	DF	Mean of Square	F Value	p-value Prob > F
Model	88543.35	9	9838.15	3.78	0.0466
A-Cellulose	2797.89	1	2797.89	1.08	0.3342
B-Inoculum Density	704.25	1	704.25	0.27	0.6189
C-Moisture	4706.93	1	4706.93	1.81	0.2205
AB	34.11	1	34.11	0.01	0.9121
AC	24.65	1	24.65	0.01	0.9252
BC	876.75	1	876.75	0.34	0.5797
A^2	45804.39	1	45804.39	17.61	0.0041
B^2	4765.62	1	4765.62	1.83	0.2180
C^2	22052.43	1	22052.43	8.48	0.0226

Figure 3.3 represents the response surfaces obtained for the interaction effects of tested variables. Increase in cellulose concentration had a positive impact on cellulase production up to a concentration of about 1.0-1.1 g/5g (20-22% w/w) of total substrate beyond which it decreased (Figure 3.3A). Probably this concentration may indicate the threshold maximum attainable with the given biomass growth under the tested SSF conditions. Increase in cellulose concomitantly decreases the wheat bran content in total substrate and this could affect the amount of fungal biomass achieved, since wheat bran can be used more easily by the organism. Similar to cellulose concentration, inoculum density also had a positive effect till ~3.5 $\times 10^6$ spores/g concentration beyond which it diminished gradually. Decrease in enzyme yield at higher spore density may be attributed to the increased growth and biomass accumulation where majority of the resources are used for growth and not for cellulase production. Analyses of the response curve for moisture against inoculum density showed the same trend for inoculum concentration with the optimal concentration remaining between 3.0 $\times 10^6$ and 4 $\times 10^6$ spores/g dry substrate. The ideal moisture content was found to be between 55-57 % (Figure 3.3B). The parameters cellulose concentration and moisture content acted totally independently as indicated by the data in Figure 3.3C. The optimal cellulose concentration was between 1.0-1.1 g/5g and the moisture content between 55-57 %. Incidentally, the maximum activity of ~950 IU/gDS was obtained at near middle levels of each of the parameters indicating that the choice of levels have been very appropriate to arrive at the optimal concentration. Many factors such as moisture content, cellulose content, inoculum size, oxygen transfer and concentrations of nutrients can significantly affect microbial growth and product formation under SSF, and the selection the optimum level for these parameters can lead to increase in enzyme production under SSF. *Trichoderma reesei* produced 298.35 IU/gds SSF on wheat bran in the basal medium and under the optimal combination of parameters the enzyme titers had gone up 3.2 fold higher to 959.53 IU/gDS. This demonstrated that SSF can be used effectively for the production of cellulases from agroindustrial

byproducts/residues. In this study, initial moisture content had the largest influence on production of CMCase. *T. reesei* have been cultivated in different moisture ranges from 37.56% to 80% for production of the enzyme (Kilikian et al., 2014; Singhania et al., 2007). In the current study, 57 % was found to be optimal for enzyme production. Under the optimal conditions of moisture content, cellulose concentration and inoculum size, the ideal incubation time was found to be 9.0 days. Filter paper activity was determined for the enzyme produced under optimal conditions and it was found to be 22.37 FPUs/gDS.

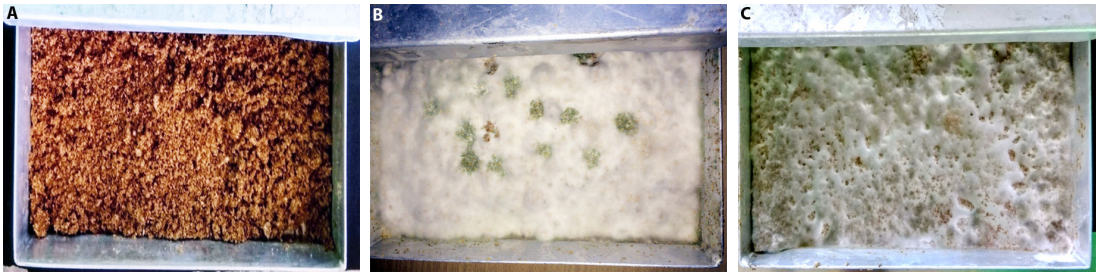
Figure 3.3 Response surface plots showing the interaction effects of process variables on production of cellulase by *T. reesei.*



3.3.3. Cellulase production in pilot scale trays

Cultivation of the fungus in pilot scale trials resulted in metabolic heat generation and it was observed that the trays were warm and the mycelial growth was lesser in the centre of trays. Enzyme extraction and assay indicated that the yield as almost half (457.27 ± 24.7 IUs/gDS) of what was obtained in flasks, though there was no contamination. It is speculated that the heat and oxygen transfer could be limiting and more studies and optimization in trays have to be performed to obtain the same levels of production as obtained in flasks. Nevertheless, it seems promising since the production is still higher than base level and even after performing the experiments in a semi sterile atmosphere, there was no contamination and about 450 IUs/gDS of enzyme was obtained from the system, indicating scope for large scale production of the enzyme using SSF.

Figure 3.4. Growth of *T. reesei* in pilot scale SSF trays



Start of incubation 9 days 14 days

3.4. Conclusion

Solid State Fermentation strategy was used for *in-situ* production of cellulase employing *T. reesei* and by optimizing process variables a 3.2 fold increase in CMCase yield was attained. The process was successfully demonstrated at pilot scale in tray fermenter system albeit with lower production compared to flask experiments.

Chapter 4

Production of endoglucanase by Submerged Fermentation

Chapter 4. Production of Endoglucanase by Submerged Fermentation

4.1. Introduction

Submerged Fermentation (SmF) or Liquid Fermentation (LF) utilizes free liquid substrates, such as broths. The enzymes are secreted into the fermentation broth. The substrates are utilized quite rapidly and growth is faster compared to SSF. This fermentation technique is best suited for microorganisms that require high levels of moisture for growth.

Submerged fermentation is highly preferable for enzyme production as large scale production is much more easier in SmF than SSF, due to its inherent advantages of better sterility, heat and mass transfer, easiness in process monitoring and automation etc., though it is more technically intensive with higher levels of sophistication required (Reimbault, 1998). Pure cellulose or different lignocellulosic biomass materials may be used as substrates for cellulase production, though the use of insoluble substrates like cellulose (microcrystalline cellulose, Avicell®, SolkaFloc® etc) or lignocellulosic biomass residues may be challenging to be used in higher scales due to problems with mixing and mass transfer (Kubicek et al, 2009). Water soluble substrates are preferred for cellulase enzyme production due to reasons stated above with lactose being commonly used as a carbon source since it is cheaper and water soluble (Jun, 2009; Kubicek et al, 2009). Along with the major carbon source, often cellulase production systems use inducers of cellulase system like sophorose (Kubicek et al, 2009). While sophorose is a highly potent cellulase inducer, it is very costly to be used in large scale production. Cellobiose also induce cellulase enzyme but lesser than sophorose.

Environmental factors such as pH, temperature, oxygen levels, water activity, and concentrations of nutrients and products; all significantly affect microbial growth and product formation. In submerged cultures, environmental control is relatively easy due to the homogeneity of the broth, and distribution of microbial cells, the nutrient solution, and the products in the liquid phase

(Alberton, 2004). Better control of environmental factors makes the SmF easier to perform in a large scale production.

4.2. Materials and Methods

4.2.1. Optimization of endoglucanase production

Initially different carbon sources and nitrogen sources were screened in a "*one factor at a time*" approach and these were incorporated in the medium for further optimization using a two stage statistical experimental design strategy. In the first stage a fractional factorial design was used to screen 11 process variables to identify the most significant ones affecting CMCase production and in the second stage a response surface design was used to optimize the levels of these selected variables.

4.2.1.1. *Plackett and Burman design*

The experimental matrix for the fractional factorial screening design was a Plackett and Burman (1946) design where 11 variables were evaluated at 2 levels. The actual factors tested and their levels are given in Table 4.2. Experimental runs were performed according to the design, and the response (enzyme activity) was recorded. A factorial model was fitted for the main effects using Design Expert® software (Statease Corp, USA). The effects of individual parameters on CMCAse production was calculated by the following equation (Eqn. 4.1)

$$\varepsilon = (\Sigma\mu_{+} - \Sigma\,\mu_{-})/n \qquad (4.1)$$

Where ε is the effect of parameter under study and "μ_{+}" and "μ_{-}"are responses (CMCase activity) of trials at which the parameter was at its higher and lower levels respectively and "n" is the total number of trials. Analysis of variance (ANOVA) was performed on the data to determine the significance of fitted model and to test the significance of the effect of individual parameters on

CMCase production. The most significant parameters affecting CMCase production were identified.

4.2.2. Central Composite Design (CCD)

The significant parameters identified by the Placket and Burman design were optimized using a response surface methodology (RSM). Specifically, a Central Composite Design (CCD) was used for this study, where the effect of the significant variables was studied at 5 different levels (-2, -1, 0, +1, +2) (Castillo, 2007). The design matrix with 13 experimental runs in a single block where the midpoint was replicated 5 times is shown in Table 4.3. The behavior of the system was modeled by a second order polynomial equation. The model equation used for the analysis is given below (Eqn. 4.2)

$$Y = \beta_0 + \sum_{i=1}^{n} \beta_i X_i + \sum_{i=1}^{n} \beta_{ii} X_i^2 + \sum_{i=1}^{n} \sum_{j=1}^{n} \beta_{ij} X_i X_j + \in \qquad (4\text{-}2)$$

Where, Y is the predicted response; β_0 is the offset term; β_i is the linear effect; β_{ii} is the squared effect, β_{ij} is the interaction effect, X_i and X_j are coded terms for independent variables under study and ε is the error factor. Regression analysis and estimation of the coefficients were performed and the three dimensional response surfaces were generated using Design Expert®. The ideal levels and combinations of parameters were identified by optimization functions in the Design Expert software, and experiments were run at these levels for validation of the model.

4.3. Results and discussion

4.3.1. Selection of carbon and nitrogen sources for CMCase production

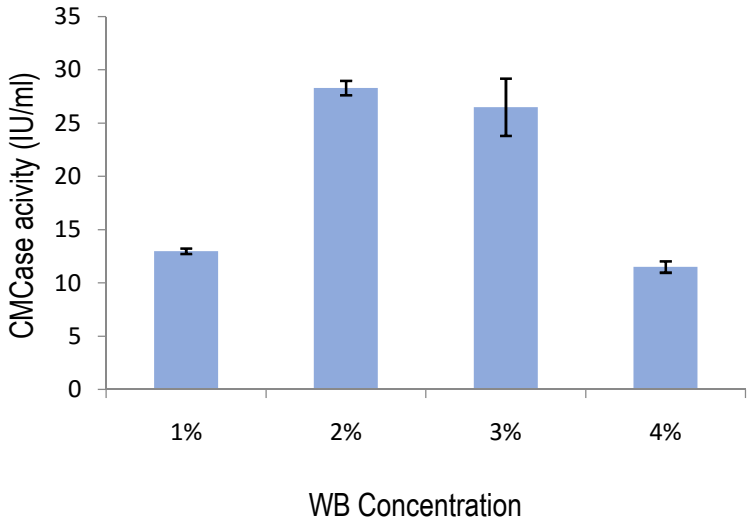
The enzyme production by the *Trichoderma reesei* RUT C-30 was evaluated using the carbon sources- cellulose, microcrystalline cellulose, wheat bran, wheat straw, and sugar cane trash at 1.0 % (w/v) level. Apparently, cellulose and microcrystalline cellulose could induce endoglucanase synthesis to the maximal levels (21.1 and 25.6 IU/ml respectively). Wheat Bran (10.5 IU/ml) was also a good raw material for endoglucanase production compared to wheat straw (5.9 IU/ml), and sugar cane trash (0.21 IU/ml) (Table 4.1).

Table 4.1. Enzyme production in different carbon sources

Carbon source	CMCase (IU/ml)
Cellulose	21.05 ± 0.8
Microcrystalline Cellulose	25.60 ± 0.5
Wheat bran	10.49 ± 2.4
Wheat straw	5.86 ± 0.4
Sugar Cane Trash	0.21 ± 0.1

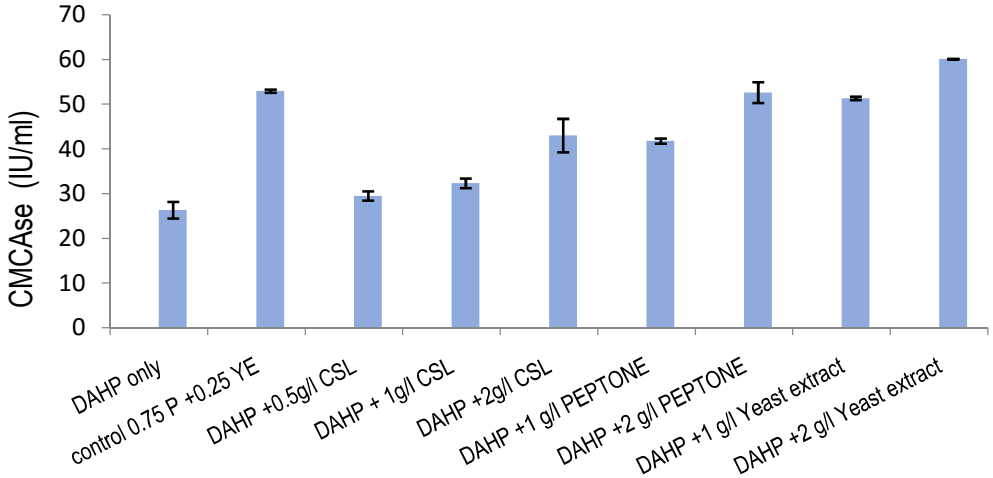
However, no growth was detected with rice straw as carbon source. While pure celluloses supported maximum enzyme yields, Wheat bran (WB) could support nearly half of the enzyme yields obtained on using pure celluloses. Since wheat bran is a cheap carbon source, it was selected as the carbon source in production medium for further optimization. Studies performed with different concentrations of WB indicated that the maximum enzyme yield of 28.3 IU/ml was obtained at 2% concentration (Fig 4.1).

Figure 4.1. Enzyme production at different wheat bran concentrations



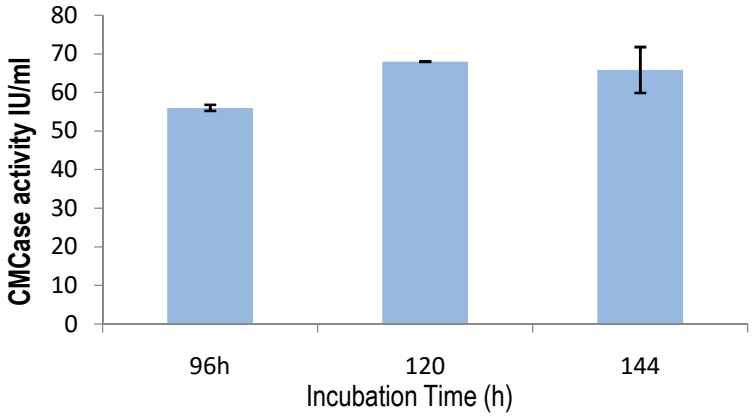
It has been demonstrated in *Trichoderma reesei* that the nitrogen demand for cellulase production can be fulfilled by the use of inorganic nitrogen sources. Ilmen *et al* (1979) showed that peptone in the medium does not exert an inducing effect on cellulase, and it just increases the growth of *T.reesei* strain QM9414, subsequently elevating cellulase levels. However, it is a common practice to add peptone or some other organic nitrogen source to enzyme production media to improve cellulase yields (Krishna et al., 2000; Jun et al., 2009). Here supplementation of di- ammonium hydrogen phosphate (DAHP) either alone as an inorganic nitrogen source or in combination with corn steep liquor (CSL), peptone or yeast extract each at 1 or 2 g/l concentrations were tried as nitrogen sources. Results shown in Figure 4.2 indicated that DAHP alone was not as effective as a N_2 source compared to DAHP supplemented with CSL, peptone or yeast extract. Maximal enzyme yields were obtained on using DAHP supplemented with 2g/l yeast extract. Complex organic nitrogen sources like peptone or yeast extract can also supplement essential nutrients, vitamins, cofactors, amino acids etc. and this could be the reason for enhanced growth and enzyme production with their supplementation.

Figure 4.1. Endoglucanase production under various combinations of organic and inorganic nitrogen sources



Since cellulose as carbon source seemed to be a dominating factor for enzyme production, it was decided to include minimal amount of cellulose along with wheat bran and to optimize the medium components and production parameters using statistical experiment designs. To determine the best sampling time, enzyme production studies were conducted in a medium containing 1.5% WB, 0.5% cellulose, 0.2 % yeast extract and 10 mM DAHP incubated for 96, 120 and 144h. CMCase yields obtained for 96, 120 and 144h were 56, 68 and 66 IUs/ml respectively (Figure 4.3). Since 120h incubation gave maximal enzyme yield, the incubation time was fixed as 120h for the factorial screening experiments.

Figure 4.3. Enzyme production at different durations of incubation



4.3.2. Optimization of CMCase production

4.3.2.1. *Screening of parameters affecting CMCase production using Placket and Burman design*

Results of the Placket and Burman experiments showed wide variation in CMCase production ranging from 32.5 IU/ml to 75.5 IU/ml (Table 4.2), indicating the importance of optimizing the production parameters for enhancing enzyme production.

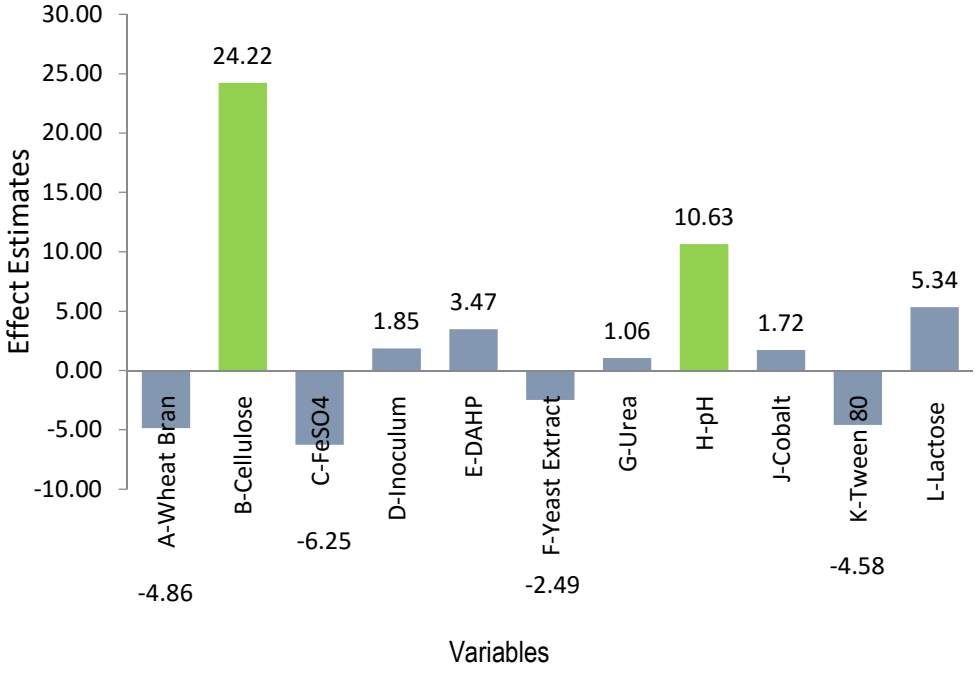
Table 4.2. Placket-Burman Design matrix with Responses

Run No.	A-Wheat bran (g/l)	B- Cellulose (g/l)	C-FeSO4 (mg/L)	D-Inoculum Conc. (spores/ml)	E- DAHP (g/L)	F-Yeast extract (g/l)	G-Urea (g/L)	H- pH	J- Co Cl_2 (mg/L)	K-Tween 80 (g/L)	L-Lactose (g/L)	Y- CMCase (IU/ml)	STDEV (±)
1	15	10	5	5.0×10^7	2.1	2	0.3	7	20	0.5	1	75.50	1.66
2	25	10	5	1.0×10^7	1.4	4	0.1	7	20	0.5	2	67.11	1.21
3	15	10	7.5	1.0×10^7	2.1	4	0.3	5	2	0.5	2	57.88	1.92
4	15	5	5	5.0×10^7	1.4	4	0.3	5	20	1.0	2	35.44	2.37
5	15	5	5	1.0×10^7	1.4	2	0.1	5	2	0.5	1	32.54	4.37
6	25	5	7.5	5.0×10^7	1.4	4	0.3	7	2	0.5	1	32.49	0.56
7	15	5	7.5	1.0×10^7	2.1	4	0.1	7	20	1.0	1	35.04	0.92
8	25	5	7.5	5.0×10^7	2.1	2	0.1	5	20	0.5	2	33.81	2.21
9	15	10	7.5	5.0×10^7	1.4	2	0.1	7	2	1.0	2	63.75	3.54
10	25	10	5	5.0×10^7	2.1	4	0.1	5	2	1.0	1	50.16	5.44
11	25	10	7.5	1.0×10^7	1.4	2	0.3	5	20	1.0	1	43.85	4.45
12	25	5	5	1.0×10^7	2.1	2	0.3	7	2	1.0	2	43.60	2.12

The effects of various parameters on CMCase production was estimated using equation 1 and the Pareto chart showing the effect estimates are presented in Figure 4.4. The magnitude and direction of effects gives the importance of the variables. Negative values for effect estimate indicate that increase in level of the

concerned parameter will result in a decrease in yield. The factors with highest effects were cellulose concentration and pH of the medium. Interestingly, in presence of cellulose, an increase in WB concentration was shown to influence the CMCase production negatively.

Figure 4.4. Effect of parameters on enzyme activity.



ANOVA of the results also confirmed the importance of cellulose concentration and pH with p values of 0.002 and 0.024 respectively. Cellulose is a strong inducer of cellulases and apparently the dominant influence of cellulose do not come as a surprise. However, the positive influence of increased pH is indeed interesting, since in several cases the optimal pH of cellulase production and activity has been reported in the acidic range. It has been shown that depending on the nature of the carbon source used to induce the cellulase production in *Trichoderma* strains, the optimal pH for maximum cellulose yield might be different. Ryu and Mandels (1980) had reported that a pH range of 3.0 to 4.0 was optimal for pure cellulose as carbon source, but a higher initial pH was recommended for lignocelluloses. While the pH of production medium drops

from the typical 4.8 to as low as 2.5 after 2 days due to acid formation, it was also shown that the decrease of medium pH from 3.0 to 2.4 resulted in a loss of 50% of filter paper activity (Sternberg, 1976). Since cellulose concentration and medium pH was the most significant parameters that were identified by Placket and Burman Design, their levels were further optimized using a response surface central composite design (CCD).

Table 4.3. ANOVA for the Factorial Model

Source	Sum of Squares	DF	Mean Square	F Value	Prob > F
Model	2099.49	2	1049.74	22.84	0.0003
B-Cellulose/ Cellulosic material	1760.23	1	1760.23	38.29	0.0002
H-pH	339.25	1	339.25	7.38	0.0237
Residual	413.74	9	45.97		
Corr. Total	2513.22	11			

4.3.3. Optimization of the significant parameters using CCD

Two critical parameters that were found to have the maximum effect on the CMCase production was taken up for further studies to optimize their levels using response surface methodology. The levels of wheat bran, DAHP, Yeast Extract, Lactose etc were fixed at the level that gave the maximum production of CMCase (Run No.1 in Table 4.2) and the levels of the significant parameters identified by the factorial design i.e – cellulose concentration and pH were optimized using the CCD. The Central Composite Design and the experimental and predicted responses obtained for CMCase production by *T. reesei* was shown in Table 4.4

Table 4.4. Central composite Design matrix for SmF production of CMCase with responses

Run	X1 Cellulose (g/L)	X2 pH	CMCase	STDV
1	8.0	30.0	49.93	6.65
2	6.0	30.0	57.36	5.96
3	8.4	20.0	66.21	8.25
4	7.0	20.0	83.95	9.71
5	6.0	10.0	57.78	10.34
6	7.0	20.0	77.75	4.24
7	7.0	34.1	56.49	3.20
8	7.0	20.0	82.41	12.18
9	7.0	5.9	27.74	7.07
10	7.0	20.0	79.52	8.37
11	8.0	10.0	21.37	0.57
12	5.6	20.0	48.53	6.72
13	7.0	20.0	80.73	8.89

The data was analyzed by multiple regression analysis and a second order polynomial equation (Eqn. 4.3) was derived to represent the CMCase production as a function of the independent variables tested.

$$Y = 80.87 - 2.35X_1 + 8.6X_2 + 7.24X_1X_2 - 12.53X_1^2 - 20.16X_1^2 \quad (4.3)$$

Where Y = predicted response (CMCase yield), X_1, X_2 are coded values of cellulose concentration and pH respectively.

Testing of the model was performed by the Fisher's statistical test for the analysis of variance (ANOVA) using Design Expert software. ANOVA of the quadratic regression model suggested that the model was significant with a computed F

value of 9.34 and a p value of 0.005. The regression coefficient (R^2) was 0.870 which indicated that 87.0% experimental data fitted with expected data in model. The p values for individual model terms indicated that pH, and the quadratic effects of both cellulose concentration and pH was significant (p values of 0.040, 0.011 and 0.001 respectively). The interaction between cellulose concentration and pH was not significant (Table 4.5).

Table 4.5. ANOVA for the Response Surface quadratic model

Source	Sum of Squares	DF	Mean Square	F Value	Prob > F
Model	4368	5	874	9.34	0.005
A-Cellulose	44	1	44	0.47	0.513
B-pH	591	1	591	6.32	0.040
AB	210	1	210	2.24	0.178
A^2	1093	1	1093	11.68	0.011
B^2	2828	1	2828	30.22	0.001
Residual	655	7	94		
Lack of Fit	631	3	210	35.90	0.002
Pure Error	23	4	6		
Cor Total	5022	12			

Nevertheless, response surface curve was plotted to understand the level of interaction of variables and for identifying the optimal levels of each parameter for attaining maximal CMCase yield. Figure 4.5 represent the response surface for the interaction of cellulose concentration and pH. It was observed that the enzyme production increased with increase in cellulose concentration from 10g/l to about 20g/l and thereafter decreased at all the pH tried. Similarly, pH effect was also independent and the CMCase activity increased with increase in pH till about 7.0 and then decreased with further increase of pH. Numerical optimization function in Design Expert® software was used to optimize the levels of these parameters and the optimized condition was a cellulose concentration of 19.7g/l and a pH of

7.2. Under these conditions, the predicted CMCase yield was 82 IU/ml. Replicated experiments performed under this optimized conditions gave an enzyme yield of 83.63 ± 1.86 IU/ml validating the optimization. A comparison of the productivities of CMCase from *T.reesei* under different conditions showed that the CMCase productivity obtained in the current study is one of the highest reported (Table 4.6).

Figure 4.5. Interaction effects of cellulose and medium pH represented as response surfaces

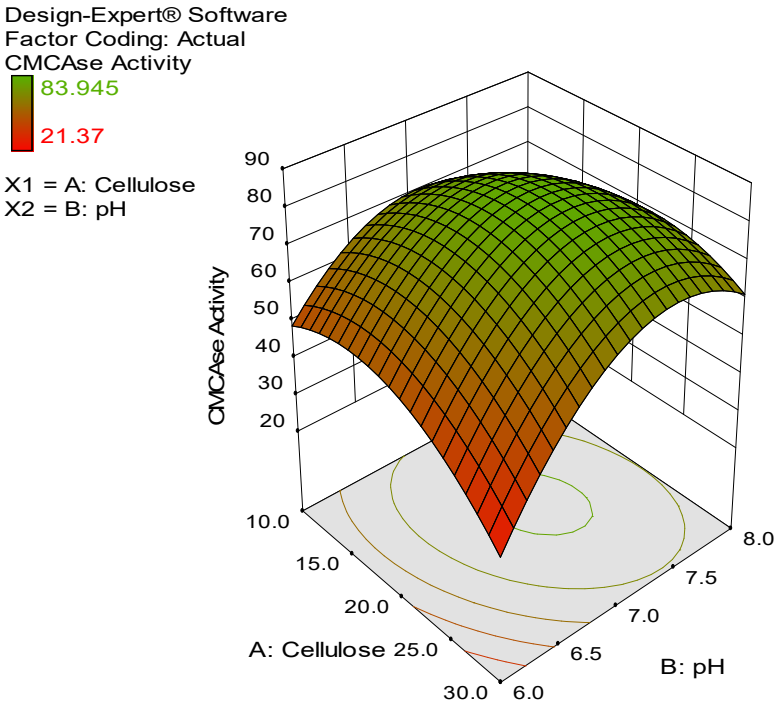


Table 4.6. Comparison of CMCase production by *T reesei* RUT C-30 in this study with other reported work

Trichoderma reesei strain	Production (IU/ml)	Productivity (IU/L.h)	Incubation Time (Days)	Conditions	Reference
RUT C-30	16.00	94.11	7	Pretreated Corn stover	Juha et al. 2005
RUT C-30	12.6	75	7	30g/l Solid (pulp and paper sludge)	Lai *et al*, 2016
PTr2 Isolate	120	833.33	6	5g/l carboxymethylcellulose	Prabavathy *et al*. 2006
RUT C-30	18.2	252.77	3	BM (1%) and wheat bran (1%),	Jun, 2009
NU-6 UV mutant	54.2	752.77	3	BM+YC (1%) + lactose (1%)	Jun, 2009
RUT C-30	12.22	84.86	6	Dairy manure with minimum salt media contain KH_2PO_4, tween-80 and $CoCl_2$ only	Wen *et al*, 2005
RUT C-30	150	446.42	14	6% (w/v) roll-milled cotton in a 10L fermenter	Ryu & Mandels, 1980)
RUT C-30	81.64	680.33	5	pH 7.2 1.9% Cellulose+1.5% Wheat Bran	This study

4.4. Conclusion

By using single parameter optimizations, the combination of wheat bran and cellulose was selected as carbon source for enhanced endoglucanase production using *T. reesei* RUT C-30. Supplementation of 2g/l of yeast extract and 10mM di-ammonium hydrogen phosphate instead of ammonium sulfate, peptone and yeast extract as nitrogen sources resulted in an improved CMCase yield. Placket-Burman experiments identified cellulose concentration and pH as the most significant variables that significantly enhanced CMCase activity. Optimization of these parameters using a central composite design led to a final CMCase yield of 83.63 ± 1.86 IU/ml and FPU of 2.58 ± 0.2 IU/ml. While the enzyme activity is not the highest, productivity was higher compared to several previous reports.

Chapter 5

Application of the Endoglucanase enriched cellulase in biomass hydrolysis and paper deinking

Chapter 5. Application of the Endoglucanase enriched cellulase in biomass hydrolysis and paper deinking

5.1. Introduction

The enzymatic saccharification of plant biomass is the most efficient method for lignocellulose breakdown to sugars, though the cost of this operation is still one of the major bottlenecks in commercialization of second generation alcohol. Cellulase cost reduction remains the top agenda of establishments involved in bioethanol research, and several methods including onsite production, use of cheap raw materials for production of the enzyme, and use of enzyme in crude concentrated form without purification are being investigated to further reduce the cost of biomass hydrolyzing enzymes – primarily cellulases. Cellulases are a group of enzymes including endo and exoglucanases and beta glucosidases that act synergistically and are necessary for the efficient hydrolysis of cellulose to soluble oligosaccharides (Beckham et al., 2010; Kubicek et al., 2009).

Sweet sorghum is an ideal food cum bioenergy crop that can produce grain, stalk sugar, forage as well as large amounts of biomass in the order of 10-20 dry tons/ha/3.5 months (Gonsalves, 2006; Rao et al, 2007). Since it require less water compared to sugarcane and since the crop duration is less, cultivation of sorghum offers an interesting opportunity for better utilization of available arable land. With an annual surplus availability of ~1.6 million metric tons (MMT) in India, sorghum biomass is a formidable feedstock resource for second generation ethanol and very less work has been done on this feedstock towards its conversion to ethanol (Pandey et al, 2009). Also there is very less information on the enzyme cocktails suitable for optimal sorghum biomass hydrolysis. Hence, the present investigation was undertaken for *in-situ* production of cellulases using *Trichoderma reesei* utilizing cheap raw material under solid state fermentation and the enzyme was used for hydrolysis of pretreated sorghum stover to generate fermentable sugars and their fermentation to produce bioethanol.

Recycled paper is an important raw material for the paper and pulp industry. Utilization of these secondary fibers is important in the environmental

point of view to reduce deforestation and the use of recycled paper has assumed significant proportions all over the world. Deinking of printed paper is an important step in the recycling process for white grade papers. In the traditional deinking process, large quantities of chemicals are used (Shrinath et al., 1991), which makes the method expensive, environmentally damaging, and also increases the release of contaminants. Enzymes can reduce the demand of chemicals and would also lower the process costs and the environmental impact (Thomas, 1994; Bajpai and Bajpai 1998). The aim of this study was therefore to use enzyme produced on cheap carbon source (wheat bran and cellulose) and the evaluation of the so produced enzyme for deinking of waste office paper.

5.2. Materials and Methods

5.2.1. Enzyme evaluation for hydrolysis of sorghum stover and bioethanol production

5.2.1.1. *Raw material, pre-processing and composition analyses*

Sorghum stover (SS) samples were kindly provided by the Indian Institute of Millets Research, Hyderabad. The samples were air-dried and were milled in knife mill to a particle size less than 2mm and were stored in air tight containers until used. Moisture content was estimated prior to use using an infrared moisture analysis balance (AND, Japan). Milled SS biomass was pretreated by dilute acid or alkali using previously optimized conditions and used a 10% biomass loading in either case. In the case of acid pretreatment, 1.5 % H_2SO_4 was used for 60min at 121 °C in an autoclave and in the case of alkali, 2% NaOH was used for 60min at 121 °C in an autoclave. After cooling, the biomass was washed with tap water until the pH became neutral. The wet biomass after solid liquid separation using a nylon mesh was air dried and used for enzymatic saccharification experiments. Compositional analyses of the untreated and pretreated sorghum stover was carried out based on the NREL protocol (Sluiter et al., 2008a) and Scanning

electron microscopy of the samples were performed as described in Kuttiraja et al, (2013).

5.2.1.2. *Enzyme production and concentration*

For obtaining a concentrated enzyme (for application studies), enzyme production was carried out by SSF and extracted using buffer as outlined in chapter 3. For concentration of the crude enzyme preparation, two volumes of chilled acetone (-20 °C) was slowly added to the pre-cooled extract (~4-8 °C) with constant stirring. The mixture was kept at ~8°C for 12 h in a cold room, followed by centrifugation at 8000 rpm for 15 min at 4 °C. The supernatant was decanted and pellet was allowed to air dry at room temperature (28 ± 2 °C) to remove residual acetone. The pellets were then dissolved in minimal volume of 50mM citrate buffer (pH 4.8), and were used for further studies. The beta glucosidase (BGL) used was from *Aspergillus niger,* produced in the lab as described in Singhania et al., (2011).

5.2.1.3. Enzyme assays

Filter paper assay and endoglucanase assay was done following Ghose, (1987) as outlined under section 2.5.1 and 2.5.2. BGL assay was conducted as described in Rajasree et al., (2013) using p-nitro phenyl-β-D glucopyranoside (pNPG) as substrate. The reaction mixture which consisted of 1 ml of citrate buffer (0.05 M, pH 4.8), 0.5 ml of enzyme sample and 0.5 ml of 10 mM pNPG was incubated at 40 °C for 15 min. Reaction was terminated by adding 2 ml of 0.2 M Na_2CO_3. The absorbance of p-nitro phenol (pNP) released was measured at 400nm. One unit enzyme activity was defined as the amount of enzyme required releasing 1 uM of pNP per minute and was expressed as IU/ml.

5.2.1.4. *Enzymatic hydrolysis and ethanol fermentation*

Enzymatic saccharification of biomass was performed at 10% biomass loading by incubating 2g of pretreated biomass with the crude *T. reesei* cellulase and beta glucosidase (BGL) produced under the optimal conditions as described above (section 5.2.1.2). Cellulase was used at a concentration of 20FPUs/g with or without β-glucosidase (BGL) supplementation at a concentration of 50 IU/g or 100 IU/g biomass. Hydrolysis was performed in 150ml screw capped flasks in a reaction volume of 20ml at 50°C, and with 100rpm agitation in a water bath shaker to a maximum duration of 48h. After hydrolysis, the un-hydrolyzed debris was separated by filtration and concentrated by vacuum evaporation. The concentrated filtrate was filter sterilized and was inoculated at 10% v/v using a 12h old seed culture of *Saccharomyces cerevisiae* cultivated in YPD broth (Himedia, India). Incubation was carried out for 24h in stopper flasks at room temperature under static condition. Samples (1.0ml) were withdrawn at 0, 12, and 24h; were centrifuged for 10min at 4 °C and supernatant was filtered and analyzed for ethanol by gas chromatography as described previously (Kuttiraja et al, 2013). Total reducing sugars were estimated in hydrolysates by DNS method (Miller, 1959) and glucose estimations were performed by HPLC following NREL protocols (Sluiter et al., 2008b). Sugar concenrtaions were expressed as mg/g of biomass. Hydrolysis efficiencies were calculated according to the following formula

$$\in = \left(\frac{concenration\ of\ sugar\ released}{(cellulose\ conc. \times\ 1.11) + (hemicellulose\ conc. \times 1.12)}\right) \times 100$$

Where " ∈" is the hydrolysis efficiency in percentage and the concentration of sugar released is the total sugar release in the hydrolysate measured in mg/g of biomass. Cellulose and hemicellulose concentrations are expressed in g/g biomass and 1.11 and 1.12 are the standard conversion factors for calculating the amount of monomeric sugars released on hydrolysis of the respective sugar polymers.

5.2.2. Deinking of printed office waste paper using endoglucanase enzyme

Modified method of Vyas & Lachke (2003) was employed for de-inking studies using printed A4 size pages from a Laser printer. The papers were printed with black ink, covering 100% printable area on the both sides of the paper. The sheets were cut into 1× 6 cm strips (50mg) and were incubated in citrate buffer (0.05M, pH 4.8) with crude enzyme from *T.reesei* at 50 °C for 2h. A control with heat denatured enzyme was also performed in parallel. All experiments were performed in triplicates. The deinking was measured by recording the absorbance of released color in the filtrate at 596nm. Microscopic images (100× magnification) of the treated and control paper strips were taken using a stereo microscope.

5.3. Results and discussion

5.3.1. Enzymatic digestion of sorghum stover biomass for bioethanol production

5.3.1.1. *Enzyme concentration*

Crude enzyme prepared by SSF under optimized conditions as detailed under section 5.2.1.2 was concentrated by acetone precipitation which resulted in the recovery of 68.4 % of cellulase and 86.9% of the CMCase activity (Table 5.1)

Table 5.1. Enzyme concentration and recovery by acetone precipitation

Crude Enzyme Type	Activity (Units)	Concentrated enzyme	Recovery (%)
Cellulase	2.86 (FPU/ml)	32.62	68.43
CMCAse	102.7 (IU/ml)	1488.07	86.93

5.3.1.2. *Pretreatment*

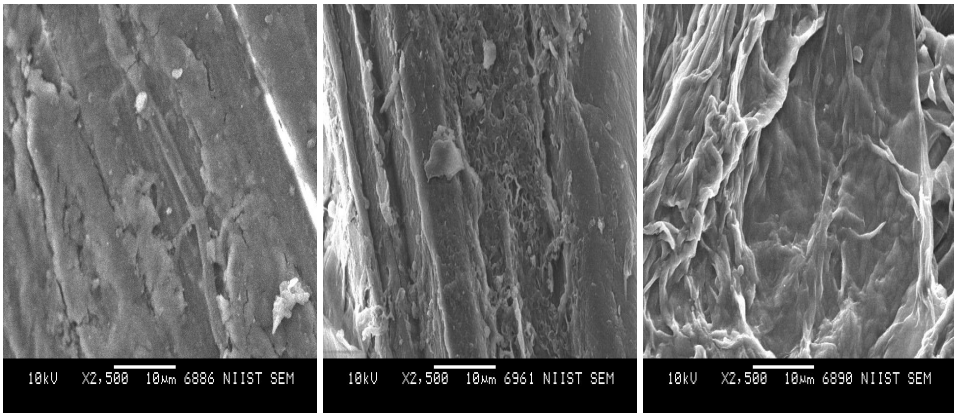
Chemical composition of biomass varies with respect to several parameters and depending on the method of pretreatment. Also the composition of the biomass is essential to estimate the efficiencies of hydrolysis and for calculation of the potential yield of bioethanol. The composition of biomass was analyzed experimentally for the native sample as well as for dilute acid and alkali pretreated sorghum stover. As expected, dilute acid pretreatment removed more of hemicellulose by its break down while alkali pretreatment removed more of lignin (Table 5.2).

Table 5.2. Composition analysis of native and pretreated sorghum stover

Pretreatment	Cellulose	Hemicellulose	Lignin
None (Native)	39.58	20.15	21.72
1.5% Acid	50.40	9.20	25.85
2% NaOH	57.16	27.16	7.36

While the improvement in cellulose content was similar with all the different pretreatments (From 40 % in Native to ~50 % in the pretreated samples), the major differences were in the removal of lignin and hemicellulose. While acid pretreatment actually resulted in an increased final percentage of lignin, there was a considerable hemicellulose removal with this treatment. On the contrary, alkali removed more of lignin and the final lignin percentage was only 7.36%. These results were also complemented by SEM analyses of the differently pretreated samples which indicated that alkali pretreatment resulted in a more disrupted surface morphology of the sorghum stover, allowing for better enzyme access (Figure 5.1).

Figure 5.1. Scanning Electron Micrographs showing changes in surface morphology on pretreatment of sorghum stover



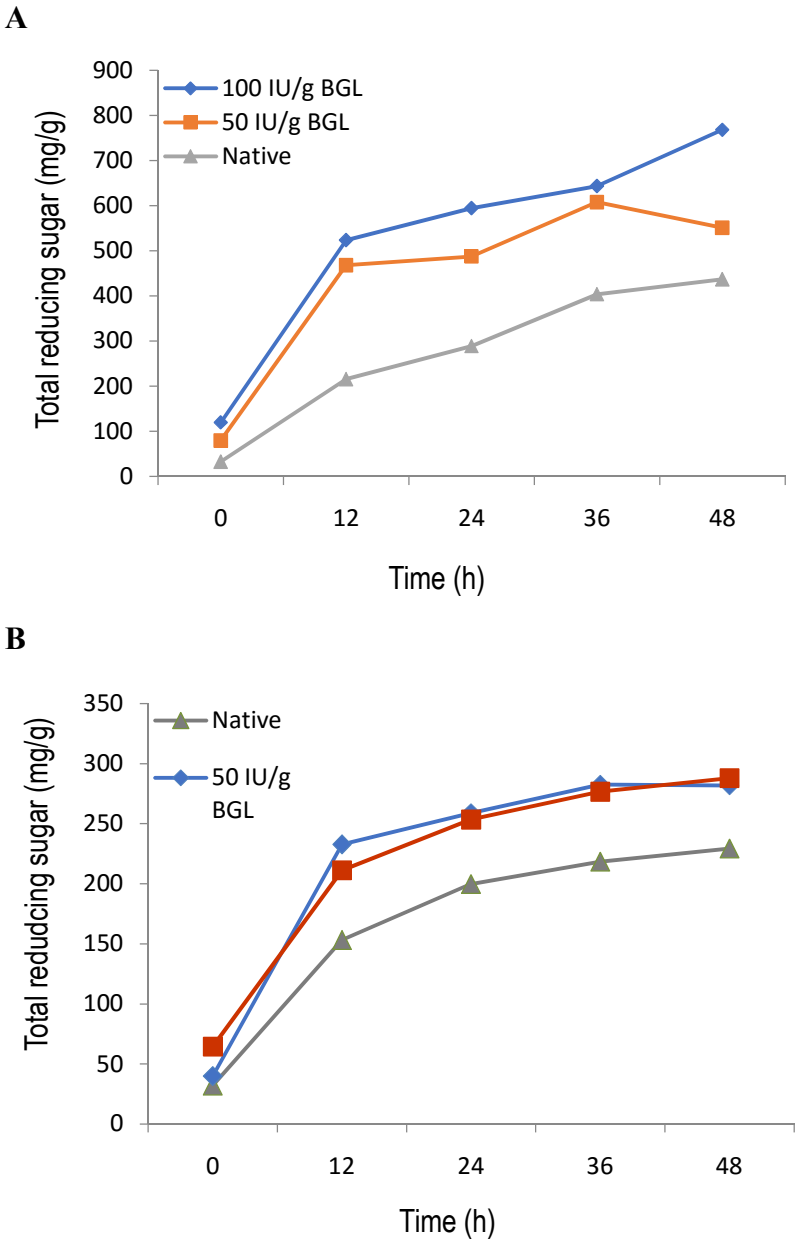
A) Native SS B) Acid pretreated SS C) Alkali pretreated SS

5.3.1.3. *Hydrolysis of sorghum stover using in-situ produced T. reesei cellulase*

The efficiency of the SSF produced *T.reesei* cellulase for hydrolysis of pretreated sorghum stover was evaluated with and without supplementation of *in-house* produced *Aspergillus niger* BGL at 50 or 100 IU/g biomass concentration. Cellulase was used at 20FPUs/g biomass in all the hydrolysis experiments. Figure 5.2 A & B indicate the sugar yields obtained for hydrolysis employing the *T. reesei* cellulase for acid and alkali pretreated sorghum stover respectively. Hydrolytic efficiencies were lower for acid pretreated biomass compared to alkali pretreated in all the enzyme combinations attempted. The conversion efficiency was only about 25% of theoretical maximum, in the case of native unblended *T. reesei* cellulase in the case of acid pretreated SS. With addition of either 50 or 100 IUs/g of BGL, the hydrolytic efficiency improved to 30 % of theoretical maximum. The situation was totally different in the case of alkali pretreated biomass. The native *T. reesei* cellulase had an efficiency of 47% whereas with supplementation of 50 or 100 IUs/g BGL with the cellulase, the efficiencies improved significantly to almost 60% and 82 % respectively, indicating excellent synergy between the enzymes. Apparently, the type of pretreatment had considerably influenced the hydrolysis efficiencies, since the acid pretreated material was hydrolyzed poorly. This is speculated to be due to the poor removal of lignin in dilute acid pretreatment and its re-deposition on the biomass preventing enzyme access a phenomenon which is already reported (Selig et al, 2007). With a better pretreated sample, the hydrolysis efficiencies were superior and with BGL supplementation it improved to 82 %. *T. reesei* is known to produce very less of BGL enzymes and BGL blending is known to improve its hydrolytic potential (Chir et al, 2011, Hu et al, 2011). Apparently, with BGL blending, the SSF produced *T. reesei* cellulase is an effective way for improving its hydrolytic efficiency. With the results, it becomes clear that the strategy works well with sorghum biomass as well. The result is compatible with previous report mention that majority of the commercial cellulase are *Trichoderma reesei*

cellulases which contain high activities of exo and endo glucanases, but very low titers of beta glucosidases that leads to increased accumulation of cellobiose (Duff and Murray, 1996; Nieves et al., 1998). This limitation is overcome by addition of extraneous beta glucosidases to cellulases.

Figure 5.2. Hydrolysis of sorghum stover biomass by native and BGL blended *T. reesei* cellulase

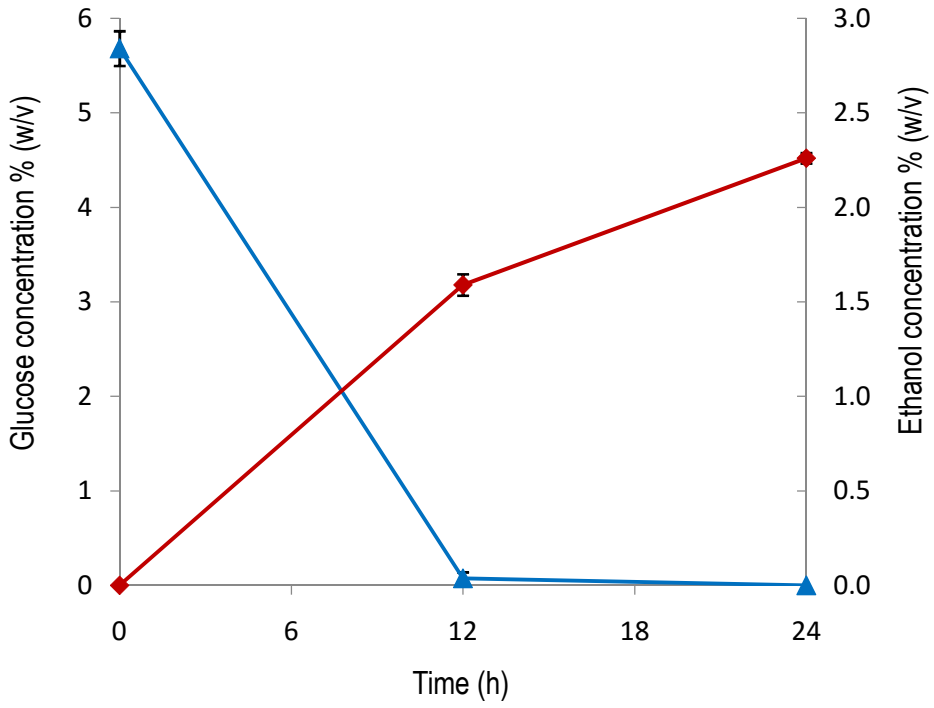


A) Enzymatic hydrolysis of acid pretreated SS
B) Enzymatic hydrolysis of alkali pretreated SS

5.3.1.4. *Fermentation of the hydrolysate to ethanol*

Ethanol production from the enzymatic hydrolysate of alkali pretreated sorghum stover was studied using a lab strain of *Saccharomyces cerevisiae*. The hydrolysate generated using *T. reesei* cellulase supplemented with 100 IU/g of *A. niger* BGL contained 56.67 g/L of glucose after concentration by vacuum evaporation. Almost entire glucose was consumed in about 12h duration and the maximal ethanol yield of 22.2g/L was obtained in 24h which corresponded to 76.8% conversion efficiency (Fig 5.3). This proved that the hydrolysate is efficiently fermented by a standard yeast strain.

Figure 5.3. Bioethanol production from sorghum stover hydrolysate



5.3.2. Deinking of printed office waste paper

Deinking experiments were performed under three different enzyme loadings and the samples were compared for optical density as well as microscopic images in order to evaluate the efficacy of the *T. reesei* CMCase preparation on deinking. The deinking process resulted in increase in ink removal as compared to control samples which were treated with denatured enzyme. Clearly, the increase in ink removal was increased with increase in enzyme loading (Figure 5.4). Microscopic images confirmed the result and showed clearing of ink patches in larger area with increased enzyme loads (Figure 5.5). Several studies have found that during enzymatic deinking, the cellulases act preferentially on the fines and microfibrils protruding out from the surfaces. This enzymatic action increases the freeness of the pulp (Jeffries et al, 1994; Gubitz et al, 1998)

Figure 5.4 Deinking Performance of *T. reesei* CMCase on printed office waste paper

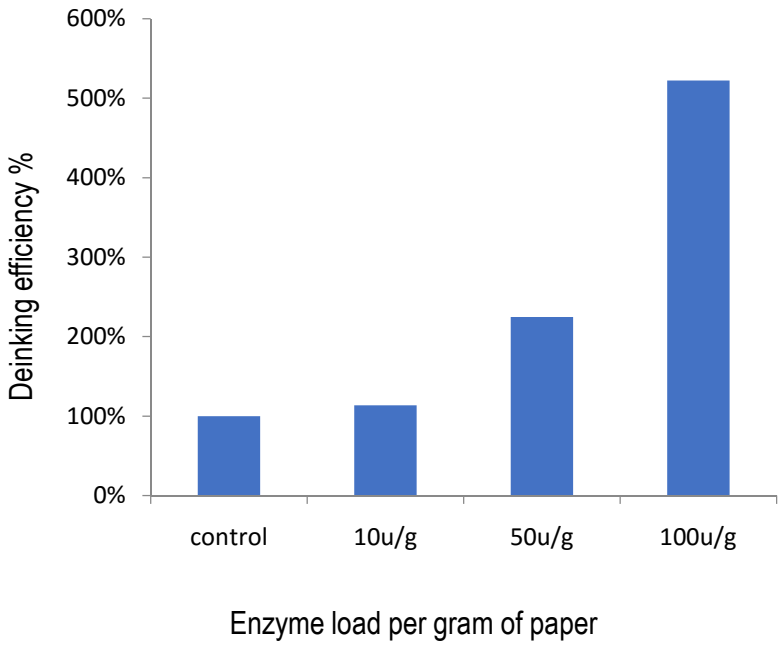
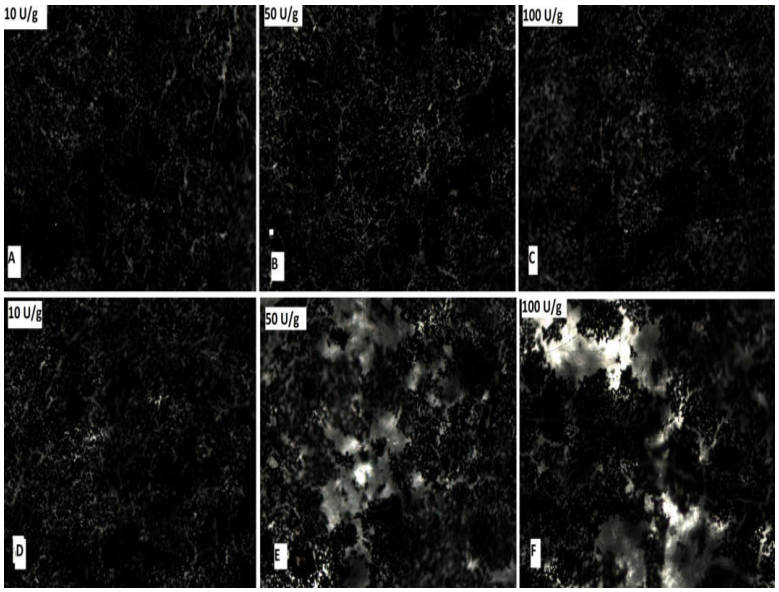


Figure 5.5. Microscopic images showing deinking of printed paper using *T. reesei* CMCase



A, B, C: Treatment controls 10, 50 and 100µg/g of heat denatured enzyme respectively
D, E, F: Deinking of paper using 10, 50 and 100µg/g of CMCase respectively

5.4. Conclusion

Applicability of the *T. reesei* CMCase produced through SSF using cheap carbon source for biomass hydrolysis and for de-inking office waste paper was successfully demonstrated. Sorghum stover was digested efficiently by the crude concentrated enzyme preparation resulting in a sugar solution which was fermented with ~77% efficiency. The enzyme performed better on BGL: supplementation confirming previous reports on relative lack of BGL in the *T reesei* arsenal of biomass hydrolyzing enzymes, and also indicating the necessity of supplementing such heterologous accessory enzymes for developing efficient cocktails. The crude enzyme helped in the deinking of office waste paper where the efficiencies improved on a dose dependent manner. Use of such cheap enzymes for deinking can eliminate/reduce the need of harmful chemicals traditionally used for the purpose and can help in bringing down the cost of recycling paper by reducing the energy cost as these reactions happen at more ambient conditions compared to traditional de-inking processes.

Chapter 6

Enzyme applications in the synthesis of nanocellulose and Ascorbic Acid 2 Glycoside (AA2G)

Chapter 6. Enzyme applications in the synthesis of nanocellulose and Ascorbic Acid 2 Glycoside (AA2G)

6.1. Introduction

Nanocellulose (NC) is rapidly attracting more interest as an impressive class of renewable nano-biomaterials with widespread uses and can be produced from biomass at industrial scales. These materials can be produced from virtually any cellulosic feedstock, from hardwoods, to grasses, or algae (Wang et al., 2012; Meng et al., 2015; Feng et al., 2015). NC can be generally grouped into two classes of nanomaterials, consisting of cellulose nanocrystals (CNCs) and cellulose nano-fibers (CNFs), although considerable ambiguity surrounds the specific definition of these materials, Nanocrystals are described as highly crystalline microfibril fragments with diameters ranging from ~3 to 10 nm and lengths from 50 to 500 nm. Cellulose nanofibers are generally described as cellulose microfibrils or bundles with diameters ranging from a few to several hundred nanometers and lengths up to several microns, depending on the source and production method (Moon et al., 2011). The properties of these materials, including high mechanical strength, biodegradability, biocompatibility and relatively low cost compared to many other classes of nanomaterials makes them desirable in several applications (Brinchi et al., 2013 ; Zhu et al., 2016).

The production of NC is typically done by applying mechanical force to de-lignified cellulosic feedstock or by chemo-mechanical processing (Chen et al., 2015; Wang et al., 2014). The production process determine if the NC consists of CNFs, CNCs, or mixtures of both. Mechanical refining alone is sufficient to produce CNFs (Zimmermann et al., 2010), whereas addition of other enzymatic or chemical treatments, such as 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO) oxidation can reduce the energy requirements and enhance fibrillation of cellulose bundles (Qing et al., 2013; Syverud et al., 2011). The most common method for CNCs production is controlled acid hydrolysis, which is often performed in tandem with mechanical refining (Brinchi et al., 2013). Sulfuric acid is the most commonly used hydrolytic reagent due to the charged sulfate groups introduced

by esterification of surface hydroxyl groups during the hydrolysis step. These non-native sulfates help stabilize the resultant CNCs in aqueous suspensions (Beck-Candanedo et al., 2005).

Enzymatic hydrolysis provides an ideal concept to integrate production of nanocellulose and biofuels from biomass. In the traditional process, hydrolytic enzymes with specific functionalities work in concert to efficiently hydrolyze the carbohydrate polymer to produce a monosaccharide that is well-suited for downstream fermentative processing. Additional advantages to this process are afforded by the specificity of enzymes, which target certain regions or functional groups of carbohydrate substrates with high selectivity compare to chemical catalysts (Himmel et al., 2007).

The physiological roles of ascorbic acid are well known. It is involved in collagen biosynthesis, as a biological antioxidant, and a catalyst of oxidation-reduction reactions of the Fe ion in cytochrome C (Myer and Kumar, 1984). Ascorbic acid is widely used in the pharmaceutical and cosmetic industries as well as a preservative, because of its antioxidant activity (Mead and Finamore, 1969). However, ascorbic acid is extremely unstable when exposed to light, oxygen, heat, and metal ions and aqueous solutions are unstable even at normal pH and 37 °C (Kumano et al, 1998). Various trials to modify the structure have been made to improve the stability and alter its physical properties. Stable derivatives evaluate include L-Ascorbic acid -2-sulfate (AA2S), L-ascorbic acid-2-phosphate (AA2P) etc., though 2-O-ά-D-Glucopyranosyl-L- ascorbic acid or (AA2G) is probably the most widely accepted derivative due its beneficial biological properties. AA2G has been the most investigated form of stabilized ascorbic acid and is currently used in cosmetics and as a food additive. The ά -glucoside is highly stable to acidic and various oxidative conditions. Following oral administration it is hydrolyzed by ά -glucosidases in the digestive organs (saliva, intestinal digestive juices, and the small intestinal tract) to generate active ascorbic acid. It is also hydrolyzed by enzymes present in the epithelial cell.

While the synthesis of AA2G can be performed chemically, the popular route is enzymatic through the use of α glucosidases or glucano transferases

(Muto et al, 1990; Eibaid et al, 2014). Cyclodextrin glucano transferase (CGTAse) has been mostly employed for synthesis of AA2G, which uses whether cyclodextrin α or β as the substrates. CGTase transfer the transfer the glucose unit to C2 in ascorbic acid via transglycosylation from glycosyl donor (cyclodextrin). Though cyclodextrins are good substrates for production of AA2G, the cost of α cyclodextrin is very high and β cyclodextrin is not very much preferred due to the low solubility (Zhang et al, 2011). CGTAse has several other important industrial applications also, and the enzyme has been drawing increasing attention recently. *Escherichia coli* has been the main host for the heterologous expression of α-CGTase. However, *E. coli* does not normally secrete proteins extracellularly. Complicated process control technologies are needed to avoid the inefficient secretory systems and inactive inclusion bodies. A number of studies have attempted to improve α-CGTase production using methods that enhance secretion. However, the yields of α-CGTase in most of these studies were below 30 U·mL– Higher yields though were achieved with fed-fermentation, had complicated fermentation processes (Zhou et al, 2012).

Similar to the 2-O-(ά-D-Glucopyranosyl) ascorbic acid, 2-O-(β-D-Glucopyranosyl) ascorbic acid, also designated as AA2G or (AA2βG) is proven to have pro-vitamin C activity and more importantly it is proven that the compound may be synthesized using enzymatic methods where relatively cheaper and easily available enzymes like cellulases and β-glucosidases (Toyada –Ono et al, 2005). Since cellulases and BGL can synthesize AA2βG and T. reesei can produce abundant and cheap cellulases it was decided to explore the enzyme from this fungus for production of AA2βG.

6.2. Materials and Methods

6.2.1. Enzymatic production of nano-cellulose

6.2.1.1. *Alkali pretreated and bleaching of cotton stalks*

Pretreatment was done followed by bleaching method described in section 2.8.1.1.

6.2.1.2. *Composition analysis of biomass*

Composition analysis of biomass was carried out as per the NREL protocols (Ehrman, 1994). Three hundred milligram of biomass was dissolved in 3 mL of 72% (v/v) sulfuric acid, mixed well using glass rod, and digested for 1h at 30°C in a temperature controlled bath (Buchi Multivapor®,Switzerland) . After 1 hour the content was transferred from the tube to a hydrolysis flask and 84ml of distilled water was added. The mixture was kept at 121 °C for 1 h in a laboratory autoclave. Small amount of the solution was filtered through 0.2 µm filter for HPLC analysis. The remaining solution and residue were retained for lignin estimation. Carbohydrate concentration in the hydrolysates were determined using Shimadzu HPLC unit equipped with Phenomenex Rezex RNM column (300 × 7.8 mm) using RI detector. This analysis was performed at 75 °C column temperature and 40 °C detector cell temperature with a flow rate of 0.6 ml/min using degassed Milli Q water as mobile phase. Glucose, xylose, galactose, mannose and arabinose (Sigma Aldrich, USA) were used as standards for HPLC analysis. Acid soluble lignin was measured by UV spectroscopy at 205 nm (Ehrman, 1996). The acid insoluble lignin and ash was estimated by oxidation method in which the sample was heated up to 575 ˚C in a muffle furnace for 3 h (Templeton and Ehrman, 1995).

6.2.1.3. *Nanocellulose production*

Bleached delignified cotton stalks were used for nanocellulose production by using enzymatic and mechanical force to generate the cellulose nanoparticles as described in section 2.8.1.2.

6.2.1.4. *Sugar estimation*

Glucose, xylose and cellobiose estimations were performed by HPLC following NREL protocols (Sluiter et al., 2008b).

6.2.1.5. *Dynamic Light Scattering*

Size distribution experiments were performed with a Zetasizer Nano-ZS (Malvern Instruments Ltd., Britain). Measurements were carried out at 25 °C in triplicate for error analysis

6.2.1.6. *Scanning Electron Microscopy*

Scanning Electron Microscopy (SEM) was performed for detailed observation of morphological features at higher resolution. For SEM analysis, the nanoparticles were coated with gold palladium using a JEOL JFC-1200 fine coater and their structures were observed using scanning electron microscope (SEM, JEOL Model JSM -5600, Japan) at 15kV and 500-5000× magnification.

6.2.2. Glycosylation of ascorbic acid

Ascorbic acid glycosylation was performed according to Gudiminchi et al., (2016) as detailed under section 2.8.2. TLC and HPLC analysis were done to confirm the product formation.

6.3. Results and Discussion

6.3.1. Nanoparticle production

6.3.1.1. *Composition analysis of bleached delignified Cotton Stalk (CS)*

Chemical composition of biomass varies depending on the method of pretreatment. Also the composition of the biomass is essential to estimate the efficiencies of the delignification process. The composition of biomass was analyzed experimentally for the native sample as well as for alkali pretreated cotton stalk and bleached biomass. As expected, alkali pretreatment removed more of lignin and bleaching removes more lignin and hemicellulose (Table 6.1).

Table 6.1. Composition analysis of native, alkali pretreated, and bleached CS

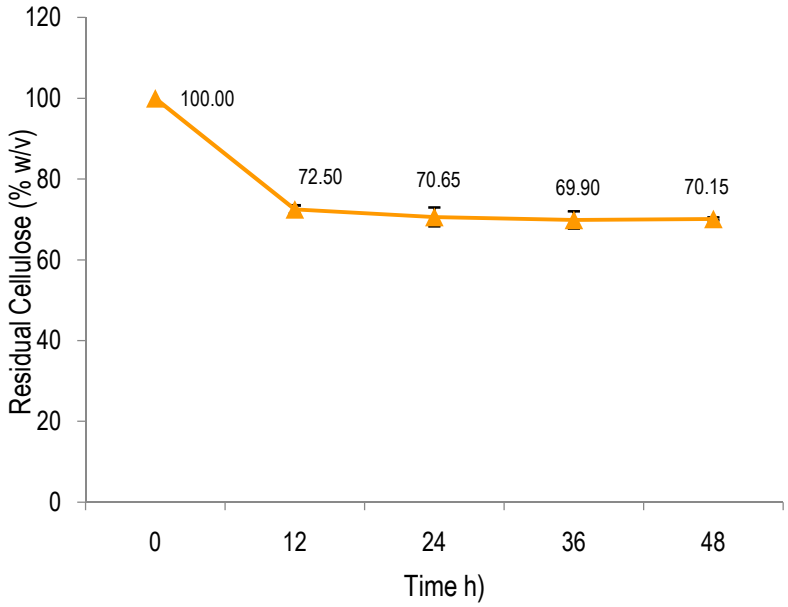
Condition	Cellulose (%)	Hemicellulose (%)	Lignin (%)
Native	50.00	20.00	30.00
Alkali pretreated 6%	54.33	23.16	18.03
Bleached	73.82	16.35	8.96

6.3.1.2. *Saccharification yields*

The resultant yields of unconverted cellulose, soluble sugars, and nanocellulose produced by enzymatic method through 48h of saccharification time are presented in Figures 6.1, 6.2, and 6.3 respectively. The unconverted cellulose continually decreased throughout the saccharification and reached the minimum at 36h. Further incubation did not have any effect on residual cellulose and it remained almost constant. The soluble sugar production (shown in Figure 6.2) which is desirable for effective biofuel production, but limiting feature for NC production

had increased till 36h after which it decreased. The NC fraction, defined as the mass fraction present in the supernatant following centrifugation less the soluble sugars, is presented in Figure 6.3. By using 2FPU/g biomass NC production of 25.98 % was obtained after 24h, which remained the same (26.02%) after 36h of incubation. However no further increase in NC yield was recorded after 36h and infact there was a decrease to 21.36% at 48h of incubation indicating that a portion of the NC was further digested to soluble sugars. The total glucan conversion was kept lower by using a lower enzyme loading than would have been used for effective conversion of polymeric cellulose to monomeric sugars since NC was the desired product.

Figure 6.1. Residual Cellulose at different time points of enzymatic digestion



Nanocellulose is typically produced through mechanical or chemo-mechanical processing of de-lignified cellulosic substrates (Chen et al, 2015). Here a very unique biomass type, ie cotton stalk which is abundantly available in India as surplus (as it find no other applications except use as domestic fire wood), was converted to nano-cellulose through an efficient enzymatic process. The crude enzyme preparation from *T. reesei* was able to digest the delignified CS to NC to maximum concentration of 25% in 24h.

Figure 6.2. Soluble sugar formation during synthesis of NC by enzymatic digestion

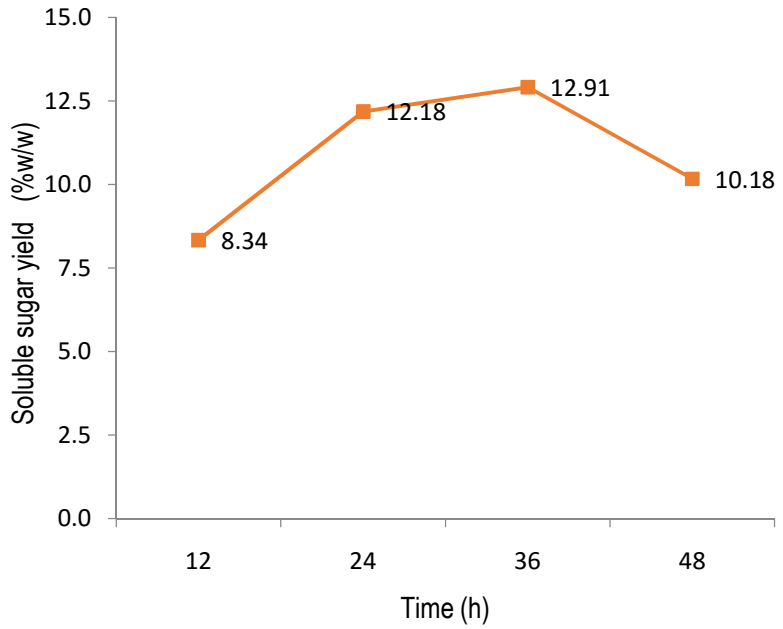
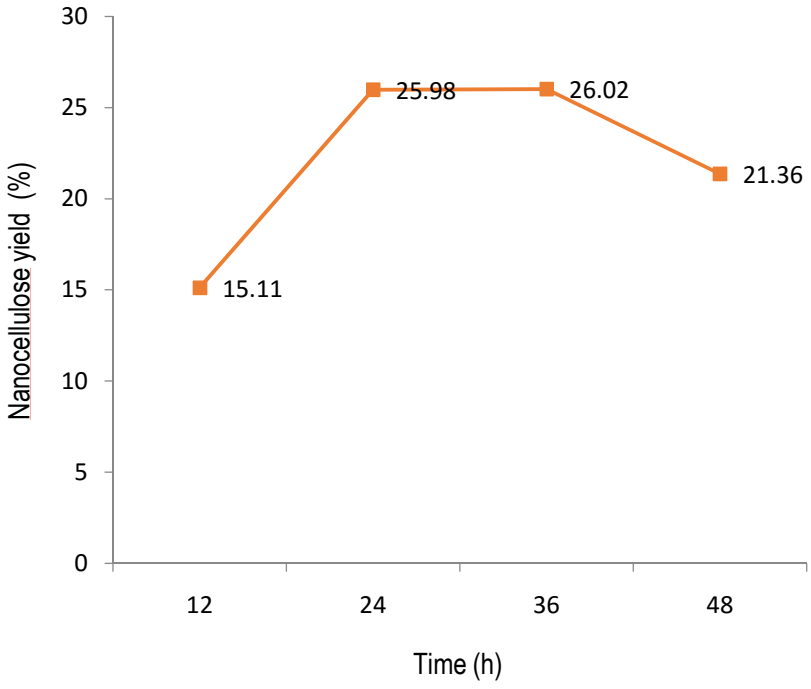


Figure 6.3. Nanocellulose formation at different incubation times during enzymatic synthesis



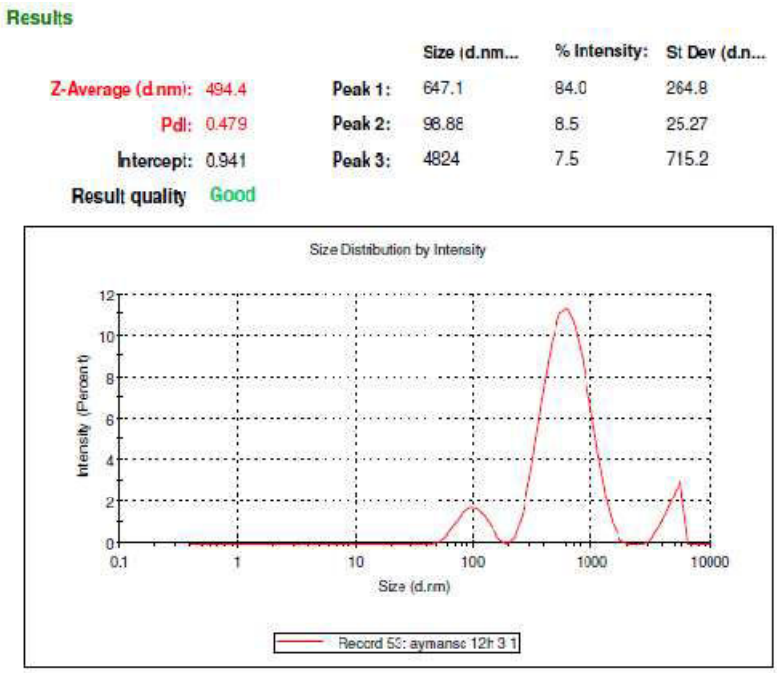
While the yield cannot be considered as exceptionally high, it needs to be noted that the enzyme preparation was a complete cellulase cocktail which was only enriched in endoglucanase and as evidenced by saccharification results, there was further digestion of nanocellulose to sugars. It is well known that *T.reesei* contains high levels of exoglucanases which in presence of endo glucanases aggressively de-polymerize cellulose (Kurasin et al, 2011). While this is good for biomass hydrolysis for sugar generation, this is not a desirable trait in the synthesis of nanocellulose and there are attempts at evaluation of different enzyme systems for NC production. In a recent study where *Caldicellulosiruptor bescii* cellulase was compared with *T. reesei* cellulase for NC production from softwood bleached kraft pulp, both enzymes was found to yield ~30% NC in 24 h, and continuation of the incubation resulted in higher yields only for the *C. besci* enzyme (Yarbrough et al, 2017). In comparison, the CMCase enriched cellulase from *T. reesei* used in this study produced a comparable 25% NC in 24h in a more difficult substrate – cotton stalk. The results do indicate scope for developing a more fractionated preparation from *T .reesei* where exoglucanase content can be reduced to obtain higher yields.

6.3.1.3. *Characterization of the Nanocellulose Saccharification Product*

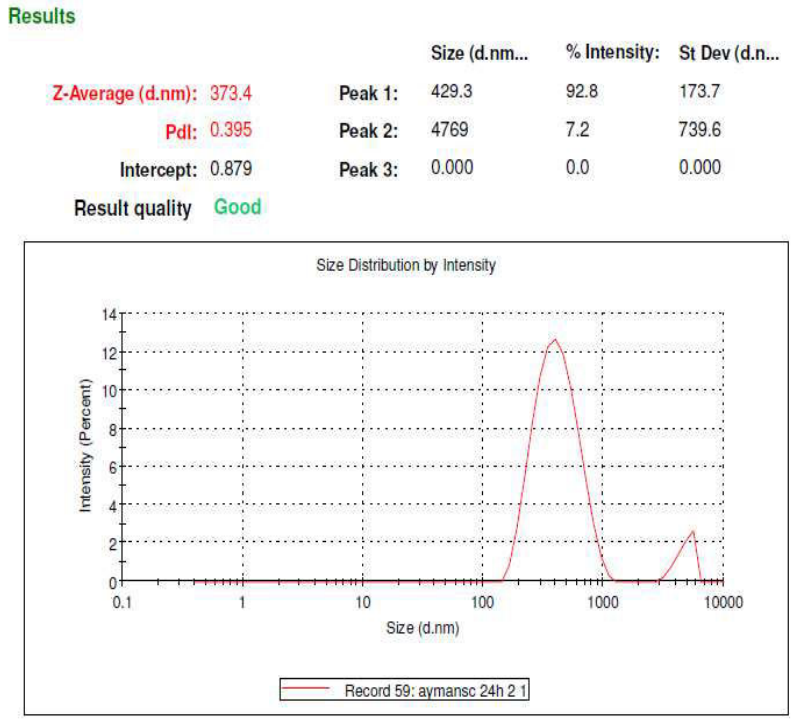
Dynamic light scattering (DLS) studies were performed in tandem with multimodal imaging on the nanocellulose fractions produced by enzyme systems at various time points to assess the size and shape distributions of the NC particles produced by saccharification. The light scattering intensity spectra for enzyme systems at 12, 24, 36, and 48 are presented in Figure 6.4.

Figure 6.4. Size distribution by dynamic light scattering of nanocellulose produced by cellulases from *T. reesei* at different time points.

A: 12h incubation



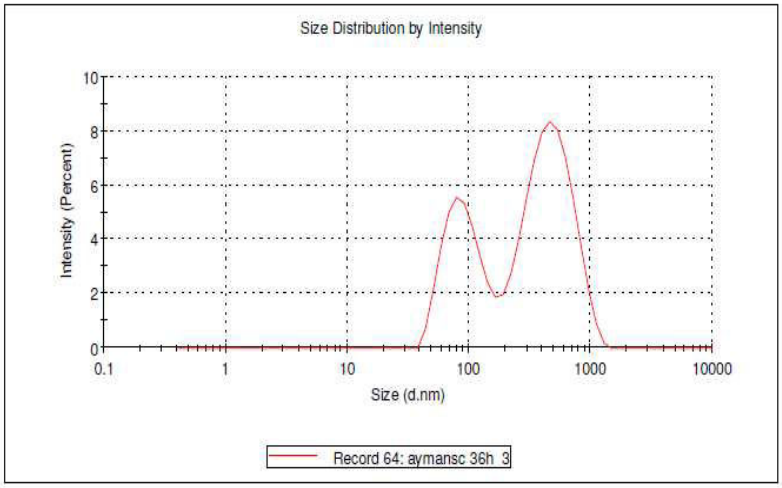
B: 24h incubation



C: 36h incubation

Results

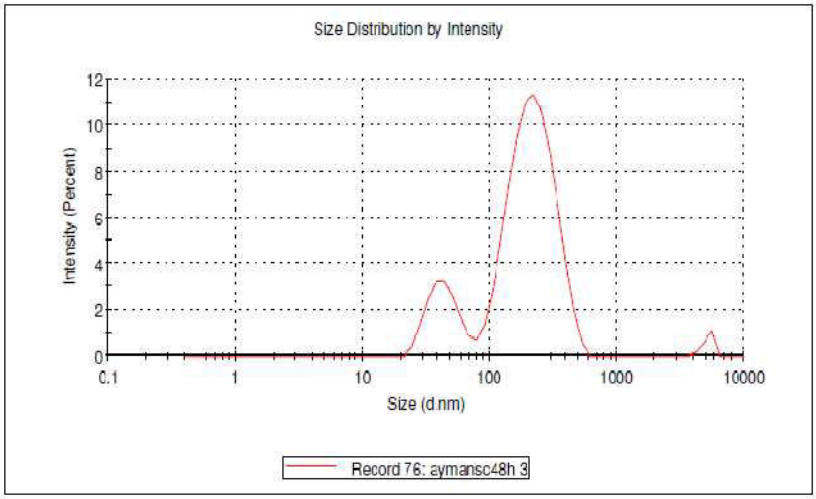
		Size (d.nm...	% Intensity:	St Dev (d.n...
Z-Average (d.nm): 174.3	Peak 1:	482.0	65.9	211.5
PdI: 0.526	Peak 2:	91.31	34.1	30.89
Intercept: 0.965	Peak 3:	0.000	0.0	0.000
Result quality Good				



D: 48h incubation

Results

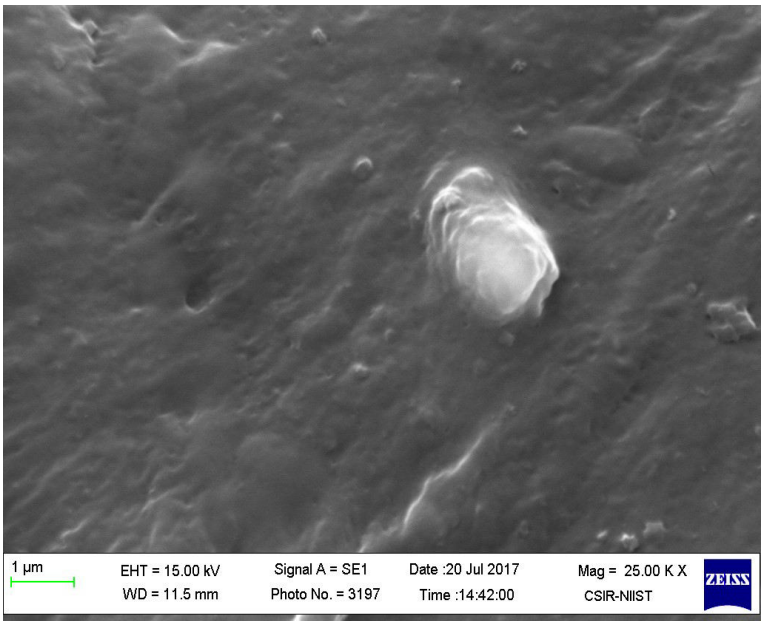
		Size (d.nm...	% Intensity:	St Dev (d.n...
Z-Average (d.nm): 139.8	Peak 1:	227.4	81.5	89.22
PdI: 0.445	Peak 2:	44.59	16.6	12.95
Intercept: 0.937	Peak 3:	5196	1.9	476.7
Result quality Good				



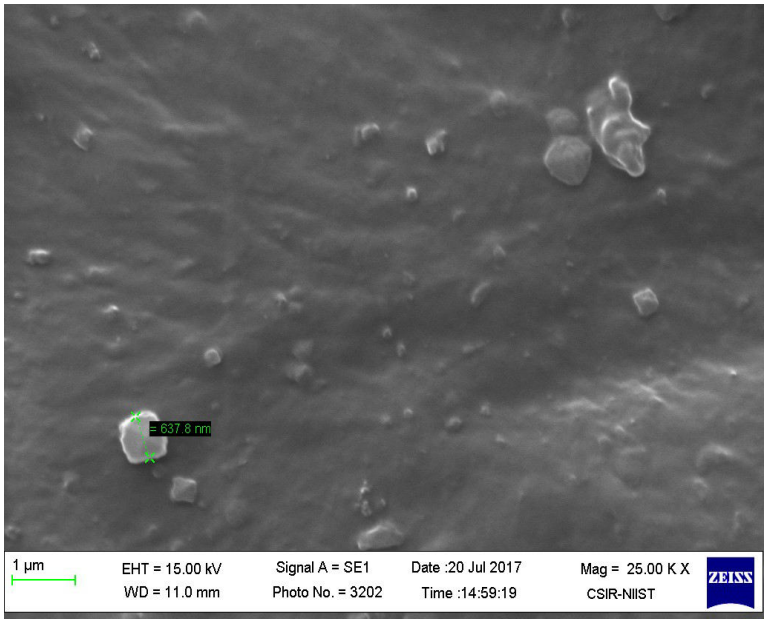
DLS analysis showed that particles sizes decreased with increasing hydrolysis time; with minimum size at 48 h of digestion with an intensity maximum at R_g of ~224 ± 84 nm. The corresponding image data for these samples (Figure 6.5) indeed reveal the presence of largely uniform nanoparticles. This may indicate that the cellulose nanoparticles characterized in this study may be treated as spheroids with largely similar minor axes, the radius of gyration increasing with increasing aspect ratio. Therefore, the observation of larger Rg values for the nanocellulose fractions produced by *T. reesei* is consistent with the assertion that the samples contain larger fractions of high aspect-ratio CNFs.

Figure 6.5. SEM analysis showing the particle size at different hydrolysis times

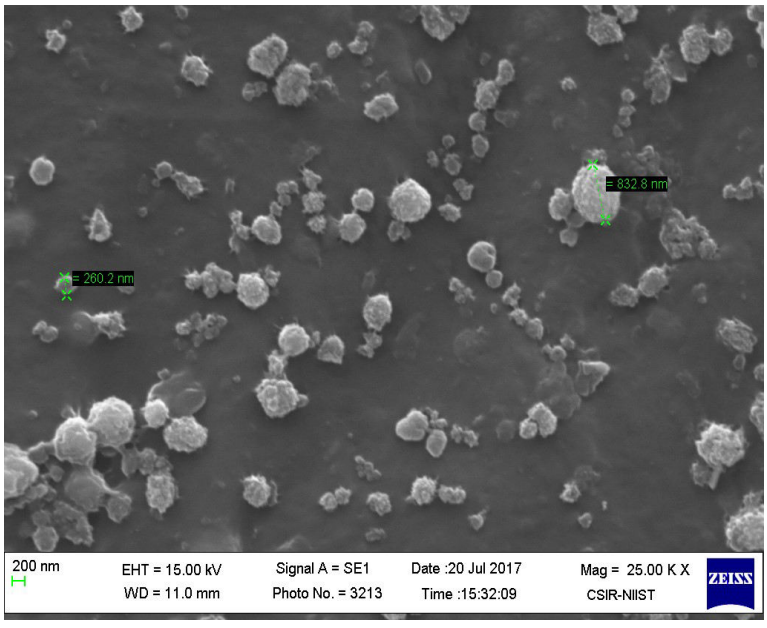
A: 12h incubation



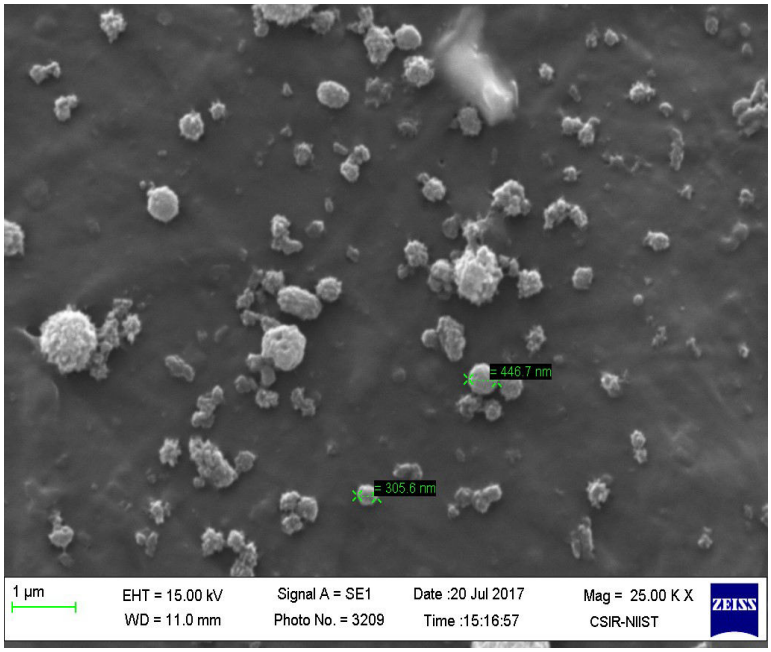
B: 24h incubation



C: 36h incubation



D: 48h incubation



6.3.2. Glycosylation of Ascorbic acid

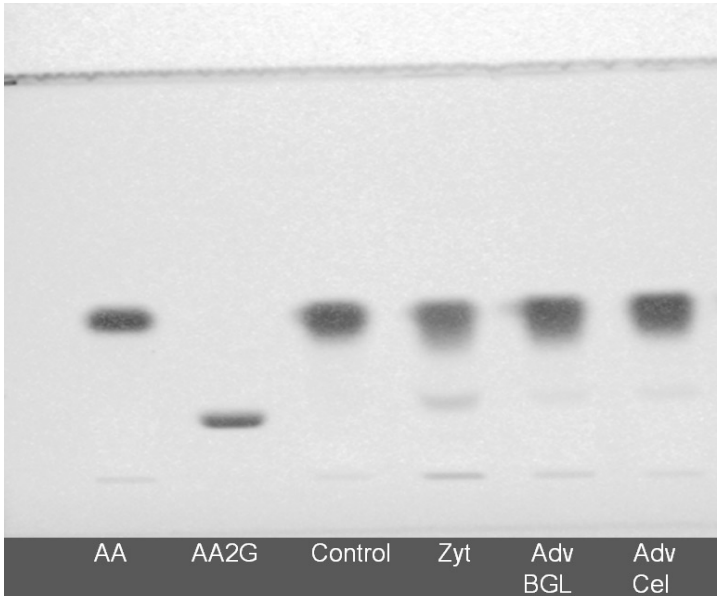
6.3.2.1. *Screening of product formation*

The production of ß-glycosylated AA by using cellobiose as substrate donor and different cellulase enzyme such as *T.reesei*, *A.niger* (BGL), and commercial cellulase, all cellulolytic enzymes showed positive result for the production of AA2G by using TLC method, though the intensity of AA2G spots were less for enzymes from *A unguis*, and *A niger* BGLs (Table 6.2). TLC plate showing AA2G production from commercial enzymes is shown in Figure 6.6.

Table 6.2. AA2G synthesis using enzyme preparations from *T reesei* and other cellulases

Enzyme	AA2βG
T.reesei	+
A.unguis	+
Commercial cellulase (Advanced Enzymes, India)	+
Commercial BGL (Advanced Enzymes, India)	+
Commercial cellulase (Zytex, India)	+
A. niger	+

Figure 6.6. TLC plate for detection the glycosylated Ascorbic Acid by using commercial enzyme.



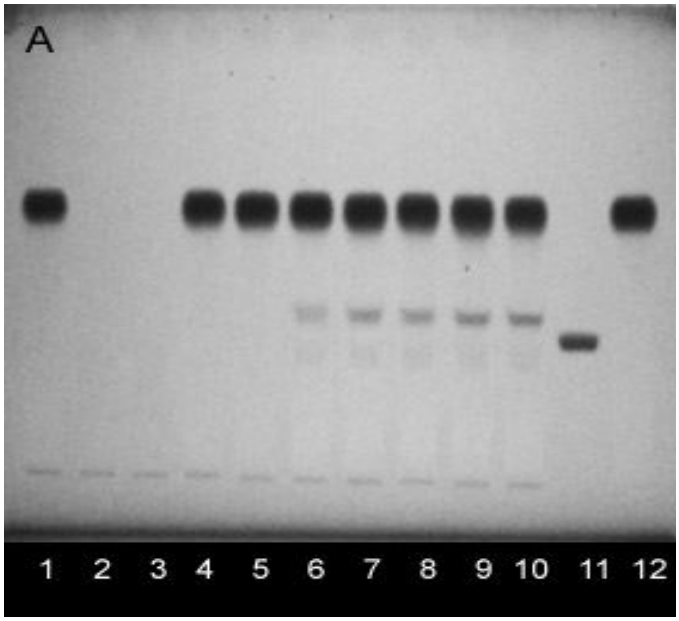
AA –Ascorbic Acid, AA2G- Ascorbic Acid 2 glucoside,
Control – Reaction mixture without enzyme, Zyt- Cellulase from Zytex India,
Adv BGL- BGL from Advanced Enzymes, India,
Adv.Cel-Cellulase from Advanced Enzymes, India

6.3.2.2. *AA2βG synthesis using T. reesei enzyme*

Since the commercially available AA2G product was of alpha form and the product formed through current enzymatic synthesis was the beta form, two mobile phases were used for resolving the plates to obtain a reliable detection. The first one contained butanol 3: acetic acid 1: and water 1 and the second one was made with chloroform 16: methanol 6: acetic acid 4: and water 2. By using the first one, the Rf value obtained was slightly different from the standard product while with the second mobile phase an R_f value similar to the standard AA2G alpha form was obtained (Figure 6.7 A & B respectively)

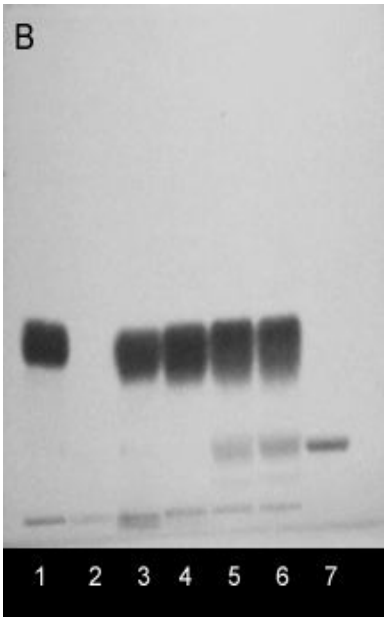
Figure 6.7. AA2G synthesized by *T reesei* cellulase at different time intervals detected by TLC

A Mobile Phase 1



1 - Ascorbic Acid +Enzyme
2 - Cellobiose+Enzyme
3 - Enzyme Control
4 - React. Mix (Denatured Enzyme)
5 - React. Mix 0h
6 - React. Mix 3h
7 - React. Mix 6h
8 - React. Mix 9h
9 - React. Mix 12h
10 - React. Mix 15h
11 - AA2G
12 - AA

B Mobile phase 2

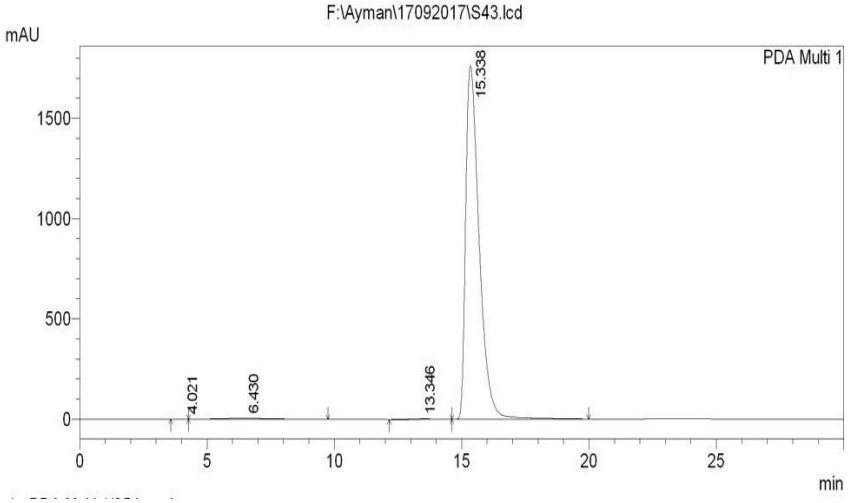


1 - Ascorbic Acid +Enzyme
2 - Cellobiose+Enzyme
3 - React. Mix (Denatured Enzyme)
4 - React. Mix 6h
5 - React. Mix 15h
6 - AA2G

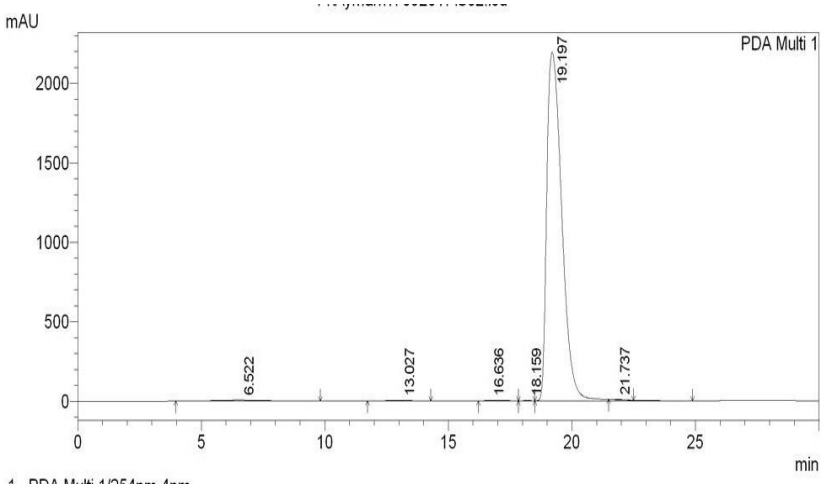
HPLC analysis confirmed the results and indicated that there is a slight difference between standard and product resolution time, probably since the standard is alpha form of AA2G (Fig 6.8).

Figure 6.8. HPLC chromatogram showing AA2G formation by transglycosylation at different time intervals

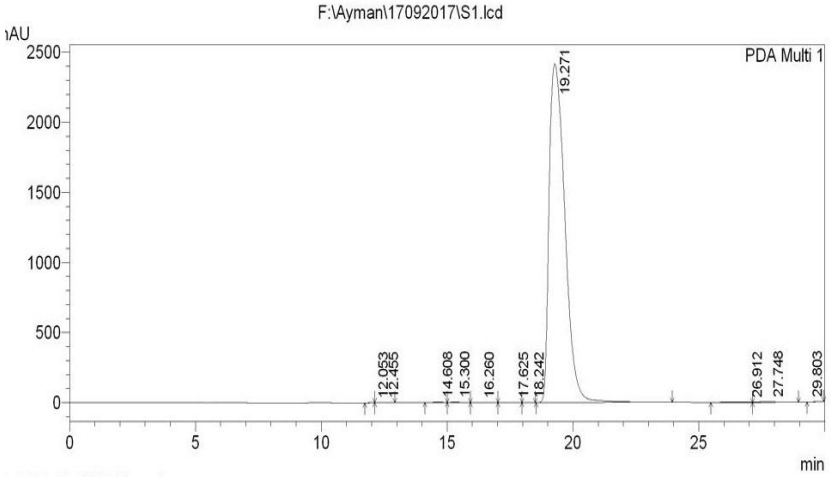
A- Standard AA2αG



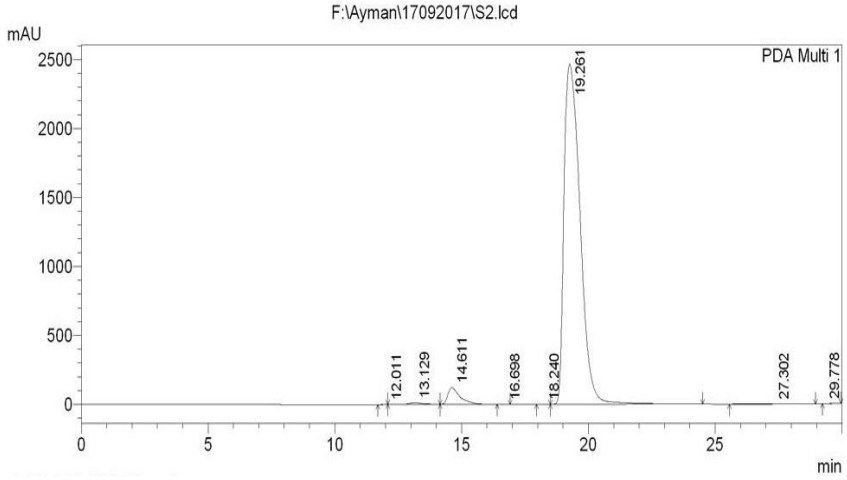
B- Ascorbic Acid



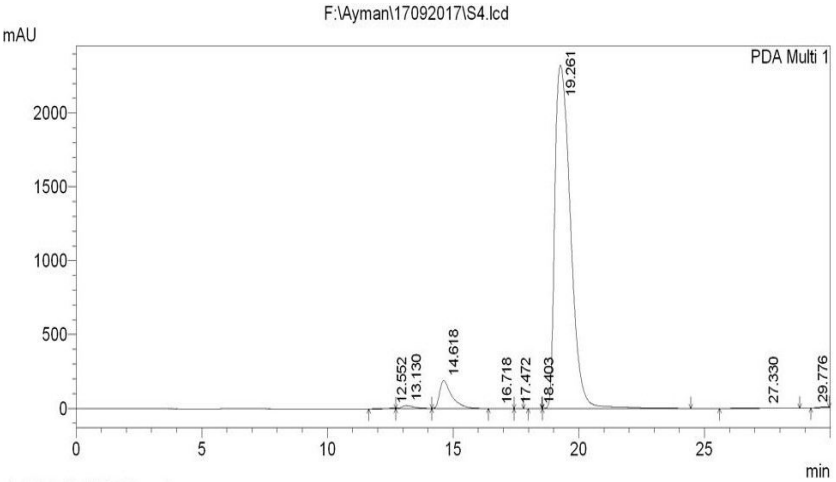
C- 0h reaction mixture



D- 3h reaction mixture

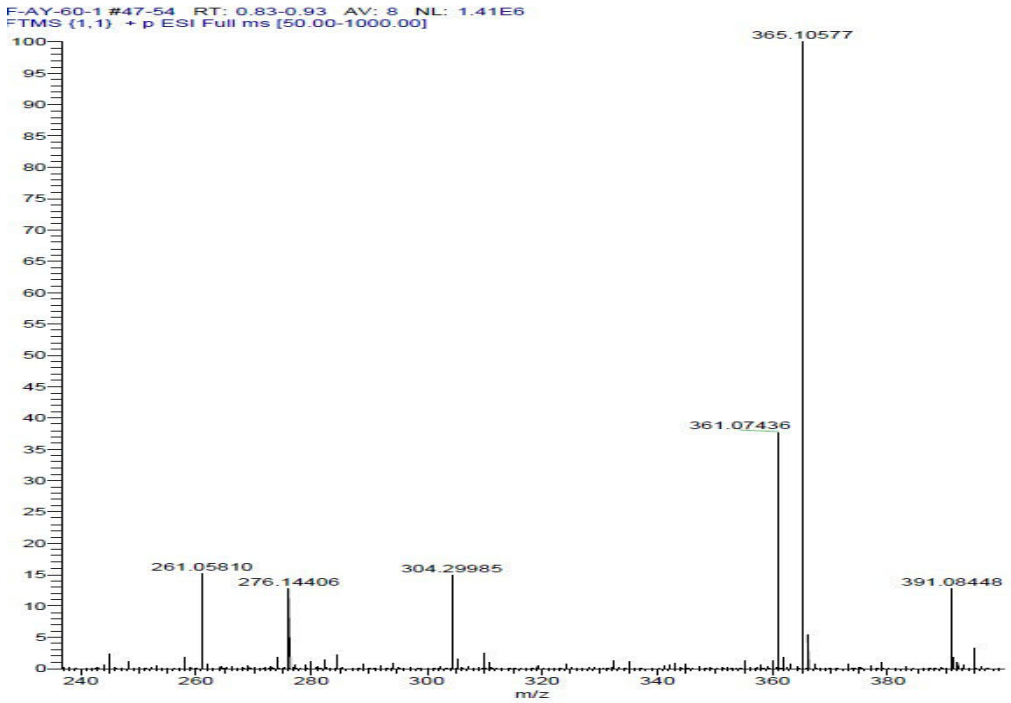


E- 9h reaction mixture



The formation of a minor peak at 14.62 min which does not correspond to AA2αG (15.34 min); only in active reaction mixtures after incubation for a minimum of 3, indicated formation of a UV active compound due to enzymatic reaction. The compound was confirmed to be AA2βG, by LC-MS analysis (out-sourced)which indicated the presence of 361MW compound corresponding to AA2βG + sodium salt and 365MW compound corresponding to cellobiose plus sodium salt (Figure 6.9)

Figure 6.9. Mass Spectrum showing AA2βG and Cellobiose



6.4. Conclusion

Cellulose nanoparticles were produced using *T. reesei* cellulase and mechanical force. Size of nanocellulose particles obtained was not uniform and increase in the incubation time lead to hydrolysis of the cellulose nanoparticles. CMCase enriched cellulase from *T. reesei* used in this study produced ~ 25% NC in 24h in a difficult to hydrolyze substrate – cotton stalk despite having a relatively higher

exoglucanase that tends to increase saccharification of cellulose to glucose. The results indicate scope for developing a more fractionated preparation from *T. reesei* where exoglucanase content can be reduced to obtain higher yields. The enzyme was also evaluated for synthesis of AA2G, a high value compound with therapeutic applications through transglycosylation reaction. Beta form of AA2G was synthesized using the *T. reesei* cellulase using cellobiose as the glucose donor. While it may be speculated that the BGL in the *T. reesei* cellulase preparation could have aided this transglycosylation reaction, conclusive proofs can be obtained only by using fractionated enzymes for the synthetic reaction. However, the fact that even a crude preparation of cellulase from *T. reesei* could catalyze the synthesis of AA2G indicates its scope for further development.

Chapter 7

Summary and Conclusion

Chapter 7. Summary and Conclusion

Cellulase is widely used in various industries and its potential share in current industrial enzyme market is rapidly increasing with the current share being 20 % of the total world enzyme market. The growth in cellulase market is driven by its demand in biofuel applications and even with accelerated development in this sector, the cost of cellulases for biofuel applications is considered high. Significant reductions in production cost needed for application of cellulases in various industrial applications, especially in biomass conversion.

Filamentous fungi, especially the ascomycetes fungi *Trichoderma reesei* is commonly used for industrial production of this enzyme. Improving the production of cellulases in native producers is an active area of research for the reduction of enzyme cost. The media components used for production and various fermentation conditions has a significant role for increasing the cellulase production. Cellulases are multi component enzymes which act in synergy, and some applications of the enzyme require preparations that do not do a complete hydrolysis of cellulose. As an example, in the case of paper and pulp industry, often cellulases are required which has no or limited exoglucanase activity. It is known that media engineering can give cellulase preparations that are enriched in a particular component like endo-glucanase and this strategy can be employed to obtain cellulases tailored for specific applications.

The present study therefore looked at various fermentation strategies and media engineering approaches for enhancing the production of cellulase enriched in endo-glucanase (CMCase) in *Trichoderma reesei* RUT C30. The application of this cellulase preparation in selected industrial were also evaluated

Since cellulases are inducible enzymes, various inducers were evaluated for the production of CMCase under solid state fermentation. A combination of cotton linter cellulose and wheat bran was optimized as best substrate/carbon source for CMCase production by *Trichoderma reesei* RUT C30. Experiments conducted on process improvement following a fractional factorial (Placket and Burman) experiment design, identified three factors -initial moisture content,

cellulose concentration, and inoculums size to be significant for cellulase production. A response surface (Box–Behnken) experiment design was used to optimize the levels of these parameters and final optimal conditions were 57% initial moisture content, 20% (w/w) cellulose concentration, and spore inoculum between 3.0×10^6 and 4×10^6 spores/g dry substrate. The maximum enzyme activity under the optimized conditions was 959.5 IU/gDS, which was 3.2 fold higher that obtained under un-optimized conditions (298.3 IU/gDS). The optimized conditions were also used for evaluating production of enzyme at a semi –pilot scale employing tray fermenters. However, the yield obtained in the tray fermenter trials were almost half of what was obtained in flasks under optimized conditions, speculated to be due to limitations in mass and heat transfer occurring on using larger quantity of substrate which prevents air diffusion.

In submerged fermentation, wheat bran and di-ammonium hydrogen phosphate (DAP) was identified as best carbon and nitrogen sources respectively for enhanced production of CMCase. The screening experiments based on statistical experimental design (Placket and Burman) design showed two significant factors (Cellulose concentration and pH) involved in CMCase production. Optimization of these factors resulted in a maximum CMCase production of ~ 84 IU/ml and total cellulase activity of ~2.6 FPUs/ml respectively. The concentration of cellulose for maximum cellulase production was optimized as 19.7 g/L. The maximum cellulase production was observed at a pH of 7.2.

The enzymes produced from *Trichoderma reesei* RUT C30 was evaluated for four important industrial applications, which included biomass hydrolysis, de-inking of office waste paper, production of nano-cellulose and synthesis of Ascorbic Acid 2 β Gluconate (AA2βG). Biomass hydrolysis was more effective when the cellulase was blended with heterologous beta glucosidase preparation from *Aspergillus niger*. The efficiency was up to 80% when 20FPU/g biomass of *T.reesei* cellulase and 100IU/g biomass of *A.niger* ß-glucosidase was used for hydrolysis. The de-inking property of crude enzyme was evaluated on printed office waste paper and the experiment showed significant ink removal. The

efficiency of process increased in a dose dependent manner. The crude CMCase enriched enzyme was evaluated for making nanocellulose. Cellulose nanoparticles were generated using enzymatic method and 25% NC production was recorded in 24h, in a highly recalcitrant substrate – cotton stalk despite having a relatively higher exoglucanase that tends to increase saccharification of cellulose to glucose. The nanoparticle size decreased with increasing incubation period (494nm in 12h to 174 nm in 36h) indicating the possibility to tune the particle size by careful manipulation of enzyme type and incubation time.

The enzyme was also proven to be useful in transglycosylation reaction for synthesis of the high value compound -Ascorbic Acid 2 β-D-Glucoside. The formation of ascorbic acid 2-ß-D-glucoside was confirmed by various procedures like TLC, HPLC and LC-MS. Beta form of AA2G was synthesized using the *T. reesei* cellulase using cellobiose as the glucose donor. While it may be speculated that the BGL in the *T. reesei* cellulase preparation could have aided this transglycosylation reaction, conclusive proofs can be obtained only by using fractionated enzymes for the synthetic reaction. However, the fact that even a crude preparation of cellulase from *T. reesei* could catalyze the synthesis of AA2G indicates its scope for further development. The studies conducted as part of this thesis have demonstrated that a cellulase enriched in CMCase can be produced effectively using cheaper media under both solid state and submerged fermentation and the so produced enzyme can have versatile applications in various fields.

References

References

Akhtar, M., 1994. Biomechanicaal pulping of aspenwood chips with three strains of Ceriporiopis subvermispora. Holzforschung. 48, 199–202

Alberton, L.R., 2004. Produção de xilanase em resíduos agroindustriais por Streptomyces viridosporus T7a e aplicação do extrato bruto em veterinária. Ph.D. Thesis, Universidade Federal do Paraná, Curitiba.

Allen, A.L., Mortensen, R.E., 1981. Production of cellulase from *Trichoderma reesei* in fed-batch fermentation from soluble carbon sources. Biotechnology and Bioengineering 23, 2641–2645. doi:10.1002/bit.260231119

Altschul, S., 1990. Basic Local Alignment Search Tool. Journal of Molecular Biology. 215, 403–410. doi:10.1006/jmbi.1990.9999.

Andberg, M.B.,Penttilä,M .,Saloheimo, M., 2015. Swollenin from *Trichoderma reesei* exhibits hydrolytic activity against cellulosic substrates with features of both endoglucanases and cellobiohydrolases. Bioresour. Technol. DOI 10.1016/j.biortech.2015.01.024

Arantes, V.,Saddler, J., 2010.Access to cellulose limits the efficiency of enzymatic hydrolysis: the role of amorphogenesis. Biotechnol. Biofuels. 3 (2010), p. 4.

Bajpai, P., and Bajpai, P.K., 1998. Deinking with enzymes: a review. Tappi J. 81, 111–117.

Beck-Candanedo, S., Roman, M., Gray, D. G., 2005. Effect of Reaction Conditions on the Properties and Behavior of Wood Cellulose Nanocrystal Suspensions. doi:10.1021/BM049300P

Beckham, G.T., Bomble, Y.J., Bayer, E.A., Himmel, M.E., Crowley, M.F., 2011. Applications of computational science for understanding enzymatic deconstruction of cellulose. Curr. Opin. Biotechnol. 22, 231–238. doi:10.1016/j.copbio.2010.11.005

Beckham, G.T., Bomble, Y.J., Matthews, J.F., Taylor, C.B., Resch, M.G., Yarbrough, J.M., Decker, S.R., Bu, L., Zhao, X., McCabe, C., Wohlert, J., Bergenstr??hle, M., Brady, J.W., Adney, W.S., Himmel, M.E., Crowley, M.F., 2010. The O-Glycosylated linker from the *Trichoderma reesei* family 7 cellulase is a flexible, disordered protein. Biophys. J. 99, 3773–3781. doi:10.1016/j.bpj.2010.10.032

Behera, S.S., Ray, R.C., 2016. Solid state fermentation for production of microbial cellulases: Recent advances and improvement strategies. Int. J. Biol. Macromol. 86, 656–669. doi:10.1016/J.IJBIOMAC.2015.10.090

Bhat, M.K., 2000. Cellulases and related enzymes in biotechnology. Biotech. Adv. 18, 355–383.

Bischof, R.H., Ramoni, J., Seiboth, B., 2016. Cellulases and beyond : the first 70 years of the enzyme producer *Trichoderma reesei*. Microb. Cell. Fact. 1–13.

doi:10.1186/s12934-016-0507-6.

Bohlin, C., Praestgaard, E., Baumann, M.J., Borch, K., Praestgaard, J., Monrad, R.N., Westh, P., 2013. A comparative study of hydrolysis and transglycosylation activities of fungal β-glucosidases. Appl. Microbiol. Biotechnol. 97, 159–169. doi:10.1007/s00253-012-3875-9

Box, G.E.P., and Behnken, D.W., 1960. Some new three level designs for the study of quantitative variable. Technometrics 2: 455-475. Technometrics 2, 455–475.

Brinchi, L., Cotana, F., Fortunati, E., Kenny, J.M., 2013. Production of nanocrystalline cellulose from lignocellulosic biomass: Technology and applications. Carbohydr. Polym. 94, 154–169. doi:10.1016/J.CARBPOL.2013.01.033

Buchert, J., Teleman, A., Harjunpaa, V., Tenkanen, M., Viikari, L., Vuorinen, T., 1995. Effect of cooking and bleaching on the structure of xylan in conventional pine kraft pulp. Tappi. J. 78,125–30.

Cantarel, B.I., Coutinho, P.M., Rancurel, C., Bernard, T., Lombard, V., Henrissat, B., 2009. The Carbohydrate-Active EnZymes database (CAZy): An expert resource for glycogenomics. Nucleic Acids Res. 37, 233–238. doi:10.1093/nar/gkn663.

Castilho, L.R., Polato, C.M.., Baruque, E.A., Sant'Anna, G.L., Freire, D.M.., 2000. Economic analysis of lipase production by Penicillium restrictum in solid-state and submerged fermentations. Biochem. Eng. J. 4, 239–247. doi:10.1016/S1369-703X(99)00052-2.

Castillo, E. Del., 2007. Process Optimization A Statistical Approach. Springer Science.New York, USA: 118-122.

Chahal, D.S., 1985. Solid-State Fermentation with *Trichoderma reesei* for Cellulase Production. Appl. Environ. Microbiol. 49, 205–10.

Chandel, A.K., Chandrasekhar, G., Silva, M.B., Silvério da Silva, S., 2012. The realm of cellulases in biorefinery development. Crit. Rev. Biotechnol. 32, 187–202. doi:10.3109/07388551.2011.595385

Chen, H., 2013. Modern Solid State Fermentation. Springer Netherlands, Dordrecht. doi:10.1007/978-94-007-6043-1

Chen, L., Wang, Q., Hirth, K., Baez, C., Agarwal, U.P., Zhu, J.Y., 2015. Tailoring the yield and characteristics of wood cellulose nanocrystals (CNC) using concentrated acid hydrolysis. Cellulose 22, 1753–1762. doi:10.1007/s10570-015-0615-1

Chir, J., Wan, C., Chou, C., Wu, A., 2011. Hydrolysis of cellulose in synergistic mixtures of beta-glucosidase and endo/exocellulase Cel9A from Thermobifida fusca. Biotechnol Lett.,33, 777-782.

Cui, Y., Ouwehand, J.N., van der Lans, R.G.J., Giuseppin, M.L., Luyben, K.C.A., 1998. Aspects of the use of complex media for submerged fermentation of Aspergillus awamori. Enzyme Microb. Technol. 23, 168–177.

doi:10.1016/S0141-0229(98)00038-6

Cunha, F. M., Vasconcellos, V. M., Florencio , C., Badino, A. C., Farinas, C. S., 2017. On-Site Production of Enzymatic Cocktails Using a Non-conventional Fermentation Method with Agro-Industrial Residues as Renewable Feedstocks. Waste and Biomass Valorization 8. doi:10.1007/s12649-016-9609-y.

Davies, G., Henrissat, B., 1995. Structures and mechanisms of glycosyl hydrolases. Structure 3, 853–859. doi:10.1016/S0969-2126(01)00220-9

Deshpande, V., SivaRaman, H. S., Rao, M., 1983. Simultaneous saccharification and fermentation of cellulose to ethanol using *Penicillium funiculosum* cellulase and free or immobilized Saccharomyces uvarum cells. Biotechnology and Bioengineering, 25, 1679–84.

Díaz-Godínez, G., Soriano-Santos, J., Augur, C., Viniegra-González, G., 2001. Exopectinases produced by *Aspergillus niger* in solid-state and submerged fermentation: a comparative study. Journal of Industrial Microbiology & Biotechnology 26, 271–275. doi:10.1038/sj.jim.7000113

Dimarogona, M., Topakas, E., Christakopoulos, P., 2012. Recalcitrant polysaccharide degradation by novel oxidative biocatalysts. Appl Microbiol Biotechnol. 97, 8455. https://doi.org/10.1007/s00253-013-5197-y.

Domingues, F.C., Queiroz, J.A., Cabral, J.M.S., Fonseca, L.P., 2000. The influence of culture conditions on mycelial structure and cellulase production by *Trichoderma reesei* Rut C-30. Enzyme Microb. Technol. 26, 394–401. doi:10.1016/S0141-0229(99)00166-0

Doyle, J., Doyle, J., 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochemistry Bulletin 19, 11-15.

D'Souza, J., Volfová, O., 1982. The effect of pH on the production of cellulases in *Aspergillus terreus*. European Journal of Applied Microbiology and Biotechnology 16, 123–125. doi:10.1007/bf00500739.

Duff, S.J.B., Murray, W.D., 1996. Bioconversion of forest products industry waste cellulosics to fuel ethanol: A review. Bioresour. Technol., 55, 1–33.

Durand, H., Clanet, M., Tiraby, G., 1988. Genetic improvement of *Trichoderma reesei* for large scale cellulase production. Enzyme Microb. Technol. 10, 341–346. doi:10.1016/0141-0229(88)90012-9

Ehrman, T., 1994. Determination of acid-soluble lignin in biomass. In: Laboratory analytical procedure LAP 004, NREL.

Eibaid, A., Bashari, M., Miao, M., Musa, A., Zhang, T., Jiang, B., 2014. Biosynthesis of 2-O-α-D-glucopyranosyl-L-Ascorbic Acid from Maltose by Cyclodextrin Glucanotransferase from Bacillus sp. SK 13.002. J. Food Nutr. Res. 2, 193–197. doi:10.12691/jfnr-2-4-10

England, G.R., Kelley, A., Mitchinson, C., 2010. Induction of gene expression using a high concentration sugar mixture. US Patent No. 7713725 B2 issued 11/05/2010.

Farinas, C.S., 2015. Developments in solid-state fermentation for the production of biomass-degrading enzymes for the bioenergy sector. Renew. Sustain. Energy Rev. 52, 179–188. doi:10.1016/J.RSER.2015.07.092.

Feng, X., Meng, X., Zhao, J., Miao, M., Shi, L., Zhang, S., Fang, J., 2015. Extraction and preparation of cellulose nanocrystals from dealginate kelp residue: structures and morphological characterization. Cellulose 22, 1763–1772. doi:10.1007/s10570-015-0617-z

Ferreira, S.M.P., Duarte, A.P., Queiroz, J.A., Domingues, F.C., 2009. Influence of buffer systems on *Trichoderma reesei* Rut C-30 morphology and cellulase production. Electronic Journal of Biotechnology 12. doi:10.2225/vol12-issue3-fulltext-6.

Galante, Y.M., De Conti, A., Monteverdi, R., 1998a. Application of *Trichoderma* enzymes in textile industry. In: Harman, G.F., Kubicek C.P., editors. *Trichoderma* & *Gliocladium* Enzymes, biological control and commercial applications. London: Taylor and Francis, 2, 327–42.

Galante, Y.M., De Conti, A., Monteverdi, R., 1998b. application of *Trichoderma* enzymes in the food and feed industeries. in: In: Harman, G.F., Kubicek C.P., editors. *Trichoderma & Gliocladium* Enzymes, biological control and commercial applications. London: Taylor and Francis,2. pp.311-325

Ghose, T.K., 1987. Measurement of cellulase activities. Pure Appl. Chem. 59, 257–268. doi:10.1351/pac198759020257.

Gonsalves, J.H., 2006. An Assessment if the Biofuels industry in India: A United Nations conference on trade and conference. UNCTAD/DIT/TED/2006/6.

Gourlay, K., Arantes, V., Saddler, J.N., 2012.Use of substructure-specific carbohydrate binding modules to track changes in cellulose accessibility and surface morphology during the amorphogenesis step of enzymatic hydrolysis. Biotechnol. Biofuels. 5 (1), p. 51.

Gourlay, K., Hu, J., Arantes, V., Andberg, M., Saloheimo, M., Penttilä, M., Saddler, J., 2013. Swollenin aids in the amorphogenesis step during the enzymatic hydrolysis of pretreated biomass. Bioresource Technology 142, 498–503. doi:10.1016/j.biortech.2013.05.053.

Graham, H., and Balnave, D., 1995. Dietary enzymes for increasing energy availability. In: Biotechnology in animal feeds and animal feeding. Ed Wallace,R.J., Chesson, V.CH., Weinheim 297-309.

Gubitz, G. M., Mansfield, S. D., Saddler, J.N., 1998. Effectiveness of two endoglucanases from *Gloeophyllum species* in deinking mixed office waste paper. Int Conf Biotechnol Pulp Pulp Ind (47th) C, 135–8.

Gusakov, A.V., 2014. Cellulases and hemicellulases in the 21st century race for cellulosic ethanol. Biofuels. 4, 567-9.

Hansen, G.H., Lübeck, M., Frisvad, J.C., Lübeck, P.S., Andersen, B., 2015. Production of cellulolytic enzymes from *ascomycetes*: Comparison of solid state and submerged fermentation. Process Biochem. 50, 1327–1341.

doi:10.1016/J.PROCBIO.2015.05.017.

Hayashida, S., Ohta, K., Kaiguo, M., 1988. Cellulases of *Humicola insolens* and Humicola grisea. Methods Enzymol. 160, 323–332. doi:10.1016/0076-6879(88)60134-0.

Heise, O.U., Unwin, J.P., Klungess, J.H., Fineran, W.G., Sykes, M., Abubakr., S. 1996. Industrila Scale up of enzyme -enhanced de-inking of nonimpact printed toners. Tappi J. 79(3):207-212.

Hendy, N., Wilke, C., Blanch, H., 1982. Enhanced cellulase production using Solka Floc in a fed-batch fermentation. Biotechnol. Lett. 4, 785–788. doi:10.1007/BF00131153.

Himmel, M. E., Ding, S., Johnson, D. K., Adney, W. S., Nimlos, M. R., Brady, J. W., Foust, T. D., 2007. Biomass recalcitrance: engineering plants and enzymes for biofuels production. Science 315, 804–7. doi:10.1126/science.1137016.

HöLker, U., HöFer, M., Lenz, J., 2004. Biotechnological advantages of laboratory-scale solid-state fermentation with fungi. Appl. Microbiol. Biotechnol. 64, 175–186. doi:10.1007/s00253-003-1504-3.

Hölker, U., Lenz, J., 2005. Solid-state fermentation — are there any biotechnological advantages? Curr. Opin. Microbiol. 8, 301–306. doi:10.1016/J.MIB.2005.04.006.

Hu, J., Arantes, V., Saddler, J.N., 2011. The enhancement of enzymatic hydrolysis of lignocellulosic substrates by the addition of accessory enzymes such as xylanase: is it an additive or synergistic effect? Biotechnol. Biofuels. 4, 36. DOI: 10.1186/1754-6834-4-36.

Igarashi, K., Koivula, A., Wada, M., Kimura, S., Penttilä, M., Samejima, M., 2009. High speed atomic force microscopy visualizes processive movement of *Trichoderma reesei* cellobiohydrolase I on crystalline cellulose. J. Biol. Chem. 284, 36186–90. doi:10.1074/jbc.M109.034611.

Ilmén, M., Saloheimo, A., Onnela, M.-L., Penttilä, M.E. 1997. Regulation of cellulase gene expression in the filamentous fungus *Trichoderma reesei*. Applied and Environmental Microbiology. 63, 1298-1306.

Inglis, P.W., and Tigano, M.S., 2006. Identification and taxonomy of some entomopathogenic *Paecilomyces spp*. (Ascomycota) isolates using rDNA-ITS Sequences. Genetics and Molecular Biology 29, 132–136. doi:10.1590/s1415-47572006000100025.

Jäger, G., Girfoglio, M., Dollo, F, Rinaldi, R., Bongard, H., Commandeur, U., Fischer, R., Spiess, A.C., Buchs, J., 2011. How recombinant swollenin from *Kluyveromyces lactis* affects cellulosic substrates and accelerates their hydrolysi. Biotechnol. Biofuels. 4 (33).

Jalak, J., Kurašin, M., Teugjas, H., Väljamäe, P., 2012. Endo-exo synergism in cellulose hydrolysis revisited. J. Biol. Chem. 287, 28802–15. doi:10.1074/jbc.M112.381624

Jeffries, T. W., Klungness, J. H., Marguerite, S., Cropsey, K. R., 1994. Comparison of enzyme enhanced with conventional deinking of xerographic and laser-printed paper. Tappi J. 77, 173–9.

Jeffries, T.W., Sykes, M.S., Cropsey, K.R., Klungnes, J.H., Abubakr, S., 1995. Enhancd removal of toners from office waste papers by micorbial cellulases. In: Proc. of 6th Intl. Conf. on Biotechnol. in Pulp and Paper Industry. 141-144.

Jorgensen, H., Eriksson, Borjesson, T.J., Tjerneld, F., Olsson, L., 2003. Purification and characterization of five cellulases and onexylanase from *Penicillium brasilianum* IBT20888. Enzyme Microb. Technol. 32, 7,pp.851–861,2003.

Jourdier, E., Ben-Chaabane, F., Poughon, L., Larroche, C., Monot, F., 2012. Simple kinetic model of cellulase production by *Trichoderma reesei* for productivity or yield maximization. Chem. Eng. Trans. 27, 313–318.

Jourdier, E., Cohen, C., Poughon, L., Larroche, C., Monot, F., Chaabane, F., 2013. Cellulase activity mapping of *Trichoderma reesei* cultivated in sugar mixtures under fed-batch conditions. Biotechnology for Biofuels 6, 79. doi:10.1186/1754-6834-6-79.

Juhasz, T., Szengyel, Z., Reczey, K., Viikari, L., 2005. Characterization of cellulases and hemicellulases produced by *Trichoderma reesei* on various carbon sources. Process Biochem. 40, 3519-3525.

Juhász, T.C.A., Szengyel, Z., Szijártó, N., Réczey, K., 2004. Effect of pH on Cellulase Production of *Trichoderma reesei* RUT C30. Appl. Biochem. Biotechnol. 113, 201–212. doi:10.1385/abab:113:1-3:201.

Jun, H., Bing, Y., Keying, Z., Xuemei, D., Daiwen, C., 2000. Strain improvement of *Trichoderma reesei* Rut C-30 for increased cellulase production. Indian J Microbiol 49:188–195.

Karnis, A.,1995. The role of latent and delatent mechanical pulp fines in sheet structure and pulp properties. Paperi Ja Puu-Paper and Timber. 77, 491–497.

Kenealy, W.R., Jeffries, T.W., 2003.Enzyme Processs for Pulp and Paper: A Review of Recent Developments. In: Goodell B, Nicolas, D.D., Schultz, T.P., (eds) Wood Deterioration and Preservation. ACS Symposium Series Vol. 845. pp 210-239.

Kilikian, B. V, Afonso, L.C., Souza, T.F.C., Ferreira, R.G., Pinheiro, I.R., 2014. Filamentous fungi and media for cellulase production in solid state cultures. Braz. J. Microbiol. 45, 279–86. doi:10.1590/S1517-83822014005000028.

Krishna, C., 2005. Solid-State Fermentation Systems—An Overview. Crit. Rev. Biotechnol. 25, 1–30. doi:10.1080/07388550590925383

Krishna, S. H., Rao, K. C., Babu, J.S., Reddy, D. S., 2000. Studies on the production and application of cellulase from *Trichoderma reesei* QM- 9414. Bioprocess Biosyst Eng. 22, 467–470.

Kubicek, C.P., 2012. Systems biological approaches towards understanding

cellulase production by *Trichoderma reesei*. J. Biotechnol. 163, 133–142. doi:10.1016/j.jbiotec.2012.05.020

Kubicek, C.P., Mikus, M., Schuster, A., Schmoll, M., Seiboth, B., 2009. Metabolic engineering strategies for the improvement of cellulase production by *Hypocrea jecorina*. Biotechnol. Biofuels 2, 19. doi:10.1186/1754-6834-2-19.

Kuhad, R.C., Gupta, R., Singh, A., 2011. Microbial Cellulases and Their Industrial Applications. Enzyme Research. doi:10.4061/2011/280696

Kumano, Y., Sakamoto, T., Egawa, M., Tanaka, M., Yamamoto, I., 1998. Enhancing Effect of 2-O-.ALPHA.-D-Glucopyranosyl-L-ascorbic Acid, a Stable Ascorbic Acid Derivative, on Collagen Synthesis. Biol. Pharm. Bull. 21, 662–666. doi:10.1248/bpb.21.662

Kung, L. Jr., Kreck, E.M., Tung, R.S., Hession, A., Sheperd, A.C., Cohen, M.A., Swain, H.E.,Leedle, J.A.. 1997.Effects of a live yeast culture and enzymes on in vitro ruminal fermentation and milk production of dairy cows. J Dairy Sci.80(9), 2045-51.

Kurasin, M., Valjamae, P., 2011. Processivity of Cellobiohydrolases Is Limited by the Substrate. J. Biol. Chem. 286, 169–177. doi:10.1074/jbc.M110.161059.

Lai, T.T., Pham, T.T.H., Adjallé, K., Montplaisir, D., Brouillette, F., Barnabé, S., 2017. Production of Trichoderma Reesei RUT C-30 Lignocellulolytic Enzymes Using Paper Sludge as Fermentation Substrate: An Approach for On-Site Manufacturing of Enzymes for Biorefineries. Waste and Biomass Valorization 8, 1081–1088. doi:10.1007/s12649-016-9686-y.

Leah, R., Kigel, J., Svendsen, I.b., Mundy, J., 1995. Biochemical and Molecular Characterization of a Barley Seed β-Glucosidase .J.Biol.Chem. 270, 15789-15797. doi: 10.1074/jbc.270.26.15789

Leah, R., Kigel, J., Svendsen, I., Mundy, J., 1995. Biochemical and molecular characterization of a barley seed beta-glucosidase. J. Biol. Chem. 270, 15789–97.

Li, C., Yang, Z., Zhang, R.H.C., Zhang, D., Chen, S., Ma, L., 2013. Effect of pH on cellulase production and morphology of *Trichoderma reesei* and the application in cellulosic material hydrolysis. J. Biotechnol. 168, 470–477. doi:10.1016/j.jbiotec.2013.10.003

Liming, X., Xueliang, S., 2004. High-yield cellulase production by *Trichoderma reesei* ZU-02 on corn cob residue. Bioresour. Technol. 91, 259–262. doi:10.1016/S0960-8524(03)00195-0

Lynd, L.R., Weimer, P.J., van Zyl, W.H., Pretorius, I.S., 2002. Microbial cellulose utilization: fundamentals and biotechnology. Microbiol. Mol. Biol. Rev. 66, 506–77, table of contents. doi:10.1128/mmbr.66.3.506-577.2002

Maeda, R.N., Barcelos, C.A., Anna, L.M.M.S., Pereira, N., 2013. Cellulase production by *Penicillium funiculosum* and its application in the hydrolysis of sugar cane bagasse for second generation ethanol production by fed batch operation. J. Biotechnol. 163, 38–44. doi:10.1016/J.JBIOTEC.2012.10.014

Mandels, M., Parrish, F.W., Reese, E.T., 1962. Sophorose as an inducer of cellulase in *Trichoderma viride*. J. Bacteriol. 83, 400–408.

Mandels, M., Weber, J., 1969. The production of cellulases, Adv. Chem. Ser. 95, 391–413.

Mathew, M. G., Sukumaran, R.K., Singhania, R.R., Pandey A.,2008 . Progress in research on fungal cellulases for lignocellulose degradation. Journal of scientific and industrial research 67, 898-907.

Mead, C.G., Finamore, F.J., 1969. The occurrence of ascorbic acid sulfate in the brine shrimp, Artemia salina. Biochem. 8, 2652–5.

Meng, Y., Wu, Q., Young, T.M., Huang, B., Wang, S., Li, Y., 2015. Analyzing three-dimensional structure and geometrical shape of individual cellulose nanocrystal from switchgrass. Polym. Compos. 38, 2368–2377. doi:10.1002/pc.23819

Miller, G.L., 1959. Use of Dinitrosalicylic Acid Reagent for Determination of Reducing Sugar. Analytical Chemistry. 31, 426–428. doi:10.1021/ac60147a030.

Mishra, M.M., 1980. Enzymatic hydrolysis of. cellulose: progress and problems. Ind. J.Microbiol. 20 (1), 87-94.

Moon, R.J., Martini, A., Nairn, J., Simonsen, J., Youngblood, J., 2011. Cellulose nanomaterials review: structure, properties and nanocomposites. Chem. Soc. Rev. 40, 3941. doi:10.1039/c0cs00108b

Muto, N., Suga, S., Fujii, K., Goto, K., Yamamoto, I., 1990. Formation of a Stable Ascorbic Acid 2-Glucoside by Specific Transglucosylation with Rice Seed α -Glucosidase. Agric. Biol. Chem. 54, 1697–1703. doi:10.1080/00021369.1990.10870201

Myer, Y.P., Kumar, S., 1984. Ascorbate reduction of horse heart cytochrome c. A zero-energy reduction reaction. J. Biol. Chem. 259, 8144–50.

Nakkeeran, E., Gowthaman, M.K., Umesh-Kumar, S., Subramanian, R., 2012. Techno-economic analysis of processes for *Aspergillus carbonarius* polygalacturonase production. J. Biosci. Bioeng. 113, 634–640. doi:10.1016/J.JBIOSC.2011.12.021

Nieves, R.A., Ehrman, C.I., Adney, W.S., Elander, R.T., Himmel, M.E., 1998. Technical communication: Survey and analysis of commercial cellulase preparations suitable for biomass conversion to ethanol. World J. Microbiol. Biotechnol., 14, 301–304.

Nigam, P., Singh, D., 1996. Processing of agricultural wastes in solid state fermentation for cellulolytic enzyme production. J Sci Ind Res 55:457–467

Ong, L.G.A., Abd-Aziz, S., Noraini, S., Karim, M.I.A., Hassan, M.A., 2004. Enzyme Production and Profile by *Aspergillus niger* During Solid Substrate Fermentation Using Palm Kernel Cake as Substrate. Appl. Biochem. Biotechnol. 118, 073–080. doi:10.1385/ABAB:118:1-3:073

Ooijkaas, L.P., Weber, F.J., Buitelaar, R.M., Tramper, J., Rinzema, A., 2000. Defined media and inert supports: their potential as solid-state fermentation production systems. Trends Biotechnol. 18, 356–60.

Pajunen, E., 1986. Optimal use of b-glucanases in wort production. In: EBC-Symposium on world production, Monograph XI, Maffliers, France, pp. 137–48.

Pandey, A., 1992. Recent process developments in solid-state fermentation. Proc. Biochem. 27, 109–117. doi:10.1016/0032-9592(92)80017-w.

Pandey, A., 2003. Solid-state fermentation. Biochem. Eng. J. 13, 81–84. doi:10.1016/S1369-703X(02)00121-3.

Pandey, A., Biswas, S., Sukumaran, R. K., Kaushik,, N., 2009. Study on availability of Indian Biomass resources for exploitation : A report based on a nationwide survey, TIFAC, New Delhi.

Pandey, A., Selvakumar, P., Ashakumary, L., 1996. Performance of a column bioreactor for glucoamylase synthesis by *Aspergillus niger* in SSF. Proc. Biochem. 31, 43–46. doi:10.1016/0032-9592(94)00050-6.

Parekh, S., Vinci, V. A., Strobel, R. J., 2000. Improvement of microbial strains and fermentation processes. Appl. Microbiol. Biotechnol. 54, 287-301.

Payne, C.M., Knott, B.C., Mayes, H.B., Hansson, H., Himmel, M.E., Sandgren, M., Ståhlberg, J., Beckham, G.T., 2015. Fungal cellulases. Chem. Rev. 115(3):1308-1448.

Peciulyte, A., Anasontzis, G.E., Karlström, K., Larsson, P.T., Olsson, L., 2014. Morphology and enzyme production of *Trichoderma reesei* Rut C-30 are affected by the physical and structural characteristics of cellulosic substrates. Fung. Gen. Biol. 72, 64–72. doi:10.1016/j.fgb.2014.07.011.

Pere, J., Paavilainen, L., Siika-Aho, M., Cheng, Z., Viikari, L., 1996 Potential use of enzymes in drainage control of non- wood pulps. In: Proceedings of 3rd International Non-wood fibre pulping and paper making conference, Vol. 2. Beijing, pp. 421–30.

Pereira, B.M.P., Alvarez, T.M., da Silva Delabona, P., Dillon, A.J.P., Squina, F.M., da Cruz Pradella, J.G., 2013. Cellulase On-Site Production from Sugar Cane Bagasse Using *Penicillium echinulatum*. BioEnergy Res. 6, 1052–1062. doi:10.1007/s12155-013-9340-5

Pirota, R.D.P.B., Delabona, P.D., Farinas, C.S., 2014. Enzymatic Hydrolysis of Sugarcane Bagasse Using Enzyme Extract and Whole Solid-state Fermentation Medium of Two Newly Isolated Strains of *Aspergillus Oryzae*.

Chem. Eng. Trans. 38, 259-264.doi:10.3303/CET1438044.

Plackett, R.L., Burman, J.P., 1946. the design of optimum multifactorial experiments. Biometrika. 33, 305–325. doi:10.1093/biomet/33.4.305.

Prabavathy, V.R., Mathivanan, N., Sagadevan, E., Murugesan, K., Lalithakumari, D., 2006. Intra-strain protoplast fusion enhances carboxymethyl cellulase activity in *Trichoderma reesei*. Enzyme Microb.Technol. 38, 719–723.

Praestgaard, E., Elmerdahl, J., Murphy, L., Nymand, S., McFarland, K.C., Borch, K., Westh, P., 2011. A kinetic model for the burst phase of processive cellulases. FEBS J. 278, 1547–60. doi:10.1111/j.1742-4658.2011.08078.x

Prasad, D.Y., Heitmann, J.A., Joyce, T.W., 1993. Enzymatic de-inking of coloured offset newsprint Nord Pulp Pap Res J . 8, 284.

Qing, Y., Sabo, R., Zhu, J.Y., Agarwal, U., Cai, Z., Wu, Y., 2013. A comparative study of cellulose nanofibrils disintegrated via multiple processing approaches. Carbohydr. Poly.97,226–234. doi:10.1016/j.carbpol.2013.04.086

Raimbault, M., 1998. General and microbiological aspects of solid substrate fermentation. E.J.B. 1, 174–188. doi:10.2225/vol1-issue3-fulltext-9.

Rajasree, K.P., Mathew, G.M., Mathew, A., Pandey, A., Sukumaran, R.K., 2013. Highly glucose tolerant β-glucosidase from *Aspergillus unguis* NII08123 for enhanced hydrolysis of biomass. J. Ind.Microbiol.Biotechnol. 40, 967-975.

Rao, D. B., Ratnavathi, C. V., Sashidhar Reddy, C. h., and Rao, S. S., 2007. Potential alternate feedstock for bioethanol in India: Sweet sorghum, a bioenrgy crop. Agricultural Situation in India. 14 (6), 243-251.

Reese, E. T., and Mandels, M., 1984. Rolling with the times: production and applications of *Trichoderma reesei* cellulases. Annu Rep Ferm Proc. 7, 1-20.

Reese, E.T., Siu, R.G.H., Levinson, H.S., 1950. The biological degradation of soluble cellulose derivatives and its relationship to the mechanism of cellulose hydrolysis. J Bacteriol. 59, 485–497.

Rodriguez-Gomez, D., and Hobley, T.J., 2013. Is an organic nitrogen source needed for cellulase production by *Trichoderma reesei* Rut-C30? World J Microbiol Biotechnol. 29, 2157. https://doi.org/10.1007/s11274-013-1381-6.

Roslan, A.M., Yee, P.L., Shah, U.K.M., Aziz, S.A., Hassan, M.A., 2011. Production of Bioethanol from Rice Straw using Cellulase by Local *Aspergillus sp*. Int. J. Agric. Res. 6, 188–193. doi:10.3923/ijar.2011.188.193

Ryu, D. D. Y., Mandels, M., 1980. Cellulases: biosynthesis and applications. Enzyme Microb. Technol. 2, 91–102.

Saloheimo, M., Paloheimo, M., Hakola, S., Pere, J., Swanson, B., Nyyssonen, E., Bhatia, A., Ward, M., Penttila, M., 2002. Swollenin, a *Trichoderma reesei* protein with sequence similarity to the plant expansins, exhibits disruption activity on cellulosic materials. Eur J Biochem. 269, 4202–4211.

Schülein, M., 1988. Cellulases of Trichoderma reesei. Methods Enzymol. 160, 234–242. doi:10.1016/0076-6879(88)60125-X

Selig, M. J., Viamajala, S., Decker, S. R., Tucker, M. P., Himmel, M. E., Vinzant, T. B., 2007, Deposition of lignin droplets produced during dilute acid pretreatment of maize stems retards enzymatic hydrolysis of cellulose. Biotechnol Prog. 23(6), 1333-9.

Sheehan, J., Himmel, M., 1999. Enzymes, Energy, and the Environment: A Strategic Perspective on the U.S. Department of Energy's Research and Development Activities for Bioethanol. Biotechnol. Prog. 15, 817–827. doi:10.1021/bp990110d

Shibuya, H., Magara, K., Nojiri, M., 1981. Cellulase production by *Trichoderma reesei* in fed-batch cultivation on soda-anthraquinone pulp of the Japanese cedar. FFPRI. 14(1), 29-35.

Shin, C.S., Lee, J.P., Lee, J.S., Park, S.C., 2000. Enzyme Production of *Trichoderma reesei* RUT C-30 on various lignocellulosic substrates. Appl. Biochem. Biotechnol. 84-86, 237-245.

Shrinath, A., Szewczak, J.T., Bowen, I.J., 1991. A review of inkremoval techniques in current deinking technology. Tappi J. 74, 85–93.

Singhania, R.R., Saini, R., Adsul, M., Saini, J.K., Mathur, M., Tuli, D.K., 2015. An integrative process for bio-ethanol production employing SSF produced cellulase without extraction. Biochem. Eng. J. 102, 45-48.

Singhania, R.R., Sukumaran, R.K., Pandey, A., 2007. Improved cellulase production by *Trichoderma reesei* RUT C30 under SSF through process optimization. Appl. Biochem. Biotechnol. 142, 60–70. doi:10.1007/s12010-007-0019-2.

Singhania, R.R., Sukumaran, R.K., Patel, A.K., Larroche, C., Pandey, A., 2010. Advancement and comparative profiles in the production technologies using solid-state and submerged fermentation for microbial cellulases. Enzyme Microb. Technol. 46, 541–549. doi:10.1016/j.enzmictec.2010.03.010.

Sluiter, A., Hames, B., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D., 2008a. Determination of Sugar, byproduct, and Degradation Products in Liquid Fraction Process Sample: Laboratory Analytical Procedure (LAP).

Sluiter, A., Hames, B., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D., Crocker, D., 2008b. Determination of Structural Carbohydrates and Lignin in Biomass: Laboratory Analytical Procedure (LAP) (Revised July 2011).

Smits, J.P., Rinzema, A., Tramper, J., Sonsbeek, H.M.V., Knol, W., 1996. Solid-state fermentation of wheat bran by *Trichoderma reesei* QM9414: substrate composition changes, C balance, enzyme production, growth and kinetics. Appl. Microbiol. Biotechnol. 46, 489–496. doi:10.1007/s002530050849.

Sternberg, D., Mandels, G.R., 1979. Induction of cellulolytic enzymes in *Trichoderma reesei* by sophorose. J. Bacteriol. 139, 761–9.

Sukumaran, R.K., Singhania, R.R., Mathew, G.M., Pandey, A., 2009. Cellulase production using biomass feed stock and its application in lignocellulose saccharification for bio-ethanol production. Renew. Energy 34, 421–424. doi:10.1016/j.renene.2008.05.008

Sukumaran, R.K., Singhania, R.R., Pandey, A., 2005. Microbial cellulases - Production, applications and challenges. J. Sci. Ind. Res. (India). 64, 832–844.

Syverud, K., Chinga-Carrasco, G., Toledo, J., Toledo, P.G., 2011. A comparative study of Eucalyptus and Pinus radiata pulp fibres as raw materials for production of cellulose nanofibrils. Carbohydr. Polym. 84, 1033–1038. doi:10.1016/J.CARBPOL.2010.12.066

Teeri, T.T., Koivula, A., Linder, M., Wohlfahrt, G., Divne, C., Jones, T.A., 1998. Trichoderma reesei cellobiohydrolases: Why so efficient on crystalline cellulose? Biochem. Soc. Trans. 26, 173–177. doi:10.1042/bst0260173

Teleman, A., Harjunpaa, V., Tenkanen, M., Buchert, J., Hausalo, T., Drakenberg, T., Vuorinen, T., 1995. Characterisation of 4-deoxy-ß-L-threo-hex-4-enopyranosyluronic acid attached to xylan in pine Kraft pulp and pulping liquor by 1H and 13C-NMR spectroscopy. Carbohydr Res 272, 55–71.

Templeton, D., Ehrman, T., 1995. Determination of acid-insoluble lignin in biomass. In: Laboratory analytical procedure-LAP 003, NREL.

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

Tengerdy, RP., Szakacs, G.,2003. Bioconversion of lignocellulose in solid substrate fermentation. Biochem. Eng. J 13, 169–179.

Teugjas, H., Väljamäe, P., 2013. Selecting β-glucosidases to support cellulases in cellulose saccharification. Biotechnol. Biofuels 6, 105. doi:10.1186/1754-6834-6-105

Thomas, W.J., 1994. Comparison of enzyme-enhanced with conventional deinking of xerographic and laser-printed paper. Tappi J. 77, 173–179.

Toyada-ono, Y., Maeda, M., Nakao, M., Yoshimura, M., Sugiura-tomimori, N., Fukami, H., Nishioka, H., Miyashita, Y., Kojo, S., 2005. A novel vitamin C analog, 2-O-(β-D-Glucopyranosyl)ascorbic acid: examination of enzymatic synthesis and biological activity. J. Biosci. Bioeng. 99, 361–365. doi:10.1263/jbb.99.361

Uhlig, H.,1998. Industrial enzymes and their applications, New York: John Wiley & Sons, Inc., pp. 435.

Vaaje-Kolstad, G., Westereng, B., Horn, S.J., Liu, Z., Zhai, H., Sørlie, M., Eijsink, V.G.H., 2010. An Oxidative Enzyme Boosting the Enzymatic Conversion of Recalcitrant Polysaccharides. Science 330, 219–222. doi:10.1126/science.1192231.

Vaheri, M., Leisola, M., Kauppinen, V., 1979. Transglycosylation products of cellulase system of *Trichoderma reesei.* Biotechnol. Lett. 1, 41–46. doi:10.1007/BF01395789

Viniegra-González, G., Favela-Torres, E., 2005. Why Solid-State Fermentation Seems to be Resistant to Catabolite Repression?. 2005 Food Technology and Biotechnology. 44,3.

Viniegra-González, G., Favela-Torres, E., Aguilar, C.N., Rómero-Gomez, S. de J., Díaz-Godínez, G., Augur, C., 2003. Advantages of fungal enzyme production in solid state over liquid fermentation systems. Biochem. Eng. J. 13, 157–167. doi:10.1016/S1369-703X(02)00128-6

Vyas, S., and Lachke, A., 2003. Biodeinking of mixed office waste paper by alkaline active cellulases from alkalotolerant Fusarium sp. Enzyme Microb. Technol. 33, 236–245.

Wang, Q., Zhao, X., Zhu, J.Y., 2014. Kinetics of Strong Acid Hydrolysis of a Bleached Kraft Pulp for Producing Cellulose Nanocrystals (CNCs). Ind. Eng. Chem. Res. 53, 11007–11014. doi:10.1021/ie501672m

Wang, Q.Q., Zhu, J.Y., Reiner, R.S., Verrill, S.P., Baxa, U., McNeil, S.E., 2012. Approaching zero cellulose loss in cellulose nanocrystal (CNC) production: recovery and characterization of cellulosic solid residues (CSR) and CNC. Cellulose 19, 2033–2047. doi:10.1007/s10570-012-9765-6

Wen, Z., and Liao, W., Chen Sh., 2005. Production of cellulase by *Trichoderma reesei* from dairy manure. Bioresour. Technol 96, 491-499.

Wen, Z., Liao, W., Chen, S., 2005. Production of cellulase by *Trichoderma reesei* from dairy manure. Bioresour. Technol. 96, 491–499. doi:10.1016/j.biortech.2004.05.021

White, T., Bruns, T., Lee, S., Taylor, J., 1990. Amplification And Direct Sequencing Of Fungal Ribosomal RNA Genes For Phylogenetics. PCR Protocols. Edited by GD Innis MA, Sninsky JJ, White TJ: Academic Press, Inc, New York. 315–322. doi:10.1016/b978-0-12-372180-8.50042-1.

Yarbrough, J.M., Zhang, R., Mittal, A., Vander Wall, T., Bomble, Y.J., Decker, S.R., Himmel, M.E., Ciesielski, P.N., 2017. Multifunctional Cellulolytic Enzymes Outperform Processive Fungal Cellulases for Coproduction of Nanocellulose and Biofuels. ACS Nano. 11, 3101–3109. doi:10.1021/acsnano.7b00086

Yoon, L.W., Ang, T.N., Ngoh, G.C., Chua, A.S.M., 2014. Fungal solid-state fermentation and various methods of enhancement in cellulase production. Biomass and Bioenergy. 67, 319–338. doi:10.1016/J.BIOMBIOE.2014.05.013

Zhang, X.H., Zhang, Y.H.P., 2013. Cellulases: Characteriztics, sources, Production, and Applications. In: Yang, S.T.,El-Enshasy ,H.A., Thongchul, N., (Eds).Bioprocessing Technologies in Biorefi nery for Sustainable Production of Fuels, Chemicals, and Polymers, Ist Ed. John Wiley & Sons, Inc.New York, USA. pp 131-146.

Zhang, Z., Li, J., Liu, L., Sun, J., Hua, Z., Du, G., Chen, J., 2011. Enzymatic transformation of 2-O-α-D-glucopyranosyl-L-ascorbic acid by α-cyclodextrin glucanotransferase from recombinant *Escherichia coli*. Biotechnol. Bioprocess Eng. 16, 107–113. doi:10.1007/s12257-010-0161-5

Zhou, Q., Xu, J., Kou, Y., Lv, X., Zhang, X., Zhao, G., Zhang, W., Chen, G., Liu, W., 2012. Differential involvement of ß-glucosidases from *Hypocrea jecorina* in rapid induction of cellulase genes by cellulose and cellobiose. Eukaryot. Cell 11, 1371–1381. doi:10.1128/EC.00170-12.

Zhu, H., Luo, W., Ciesielski, P.N., Fang, Z., Zhu, J.Y., Henriksson, G., Himmel, M.E., Hu, L., 2016. Wood-Derived Materials for Green Electronics, Biological Devices, and Energy Applications. Chem. Rev. 116, 9305–9374. doi:10.1021/acs.chemrev.6b00225.

Zhuang, J., Marchant, M., Nokes, S., Strobel, H., 2007. Economic analysis of cellulase production methods for bio-ethanol. Appl. Eng. Agric. 23, 679–687.

Zimmermann, T., Bordeanu, N., Strub, E., 2010. Properties of nanofibrillated cellulose from different raw materials and its reinforcement potential. Carbohydr. Polym. 79, 1086–1093. doi:10.1016/J.CARBPOL.2009.10.045.

APPENDIX 1

List of abbreviations

AA	Ascorbic Acid
AA2G	Ascorbic Acid 2Glusoide
ANOVA	Analysis of Variance
APBCS	alkali pretreated and bleached cotton stalk
BBD	Box Behnken Design
CBM	Cellulose Binding Module
CCD	Central Composite Design
CMC	Carboxy Methyl Cellulose
CMCase	Carboxy Methyl Cellulase
CNF	Cellulose nano fiber
CSL	Corn Steep Liquor
DAHP	Di ammonium hydrogen phosphate
DLS	Dynamic Light Scattering
DNS	3'5'Dinitrosalicylic acid
EG	Endoglucanase
FPU	Filter Paper Units
HMF	Hydroxy Methyl Furfural
HPLC	High Performance Liquid Chromatography
IU/ml	International Units per milliliter
NC	Nanocellulose
PDA	Potato Dextrose Agar
RPM	Rotations per minute
SmF/SMF	submerged fermentation
SSF	solid state fermentation
WB	Wheat Bran

DE GRUYTER

Biologia **71**/3: 265—271, 2016
Section Cellular and Molecular Biology
DOI: 10.1515/biolog-2016-0046

Production of endoglucanase from *Trichoderma reesei* RUT C30 and its application in deinking of printed office waste paper

Ayman Salih Omer Idris[1,2], Ashok Pandey[1] & Rajeev Kumar Sukumaran[1]*

[1] *Centre for Biofuels, Biotechnology Division, CSIR – National Institute for Interdisciplinary Science and Technology, Industrial Estate P.O., Thiruvananthapuram – 695019, India; e-mail: rajeevs@niist.res.in*
[2] *Academy of Scientific and Innovative Research, New Delhi – 110025, India*

Abstract: Media components were optimized using two-step statistical design of experiments for enhancing endoglucanase/carboxymethyl cellulase (CMCase) production by *Trichoderma reesei* RUT C30. A Placket-Burman design identified cellulose concentration and pH as the most significant variables, which influenced the CMCase activity. Central composite design was employed to optimize these selected parameters. The optimal activity was obtained at cellulose concentration 19.7 g/L and pH of 7.2. Under the optimized conditions, CMCase activity was 83.63 ± 1.86 IU/mL and filter paper activity was 2.58 ± 0.2 filter paper units per mL. Enzyme productivity was higher compared to previous reports. The enzyme produced from *T. reesei* was concentrated and was evaluated for deinking of printed paper, which demonstrated the suitability of the enzyme for this application.

Key words: cellulase; *Trichoderma*; endoglucanase; deinking; paper and pulp; response surface.

Abbreviations: ANOVA, analysis of variance; CCD, central composite design; CMC, carboxymethyl cellulose; CMCase, carboxymethyl cellulase; CSL, corn steep liquor; DAHP, diammonium hydrogenphosphate; DNS, 3'5'-dinitrosalicylic acid; IU, international unit.

Introduction

The hypercellulolytic fungus *Trichoderma reesei* RUT C30, the anamorph of the pantropical ascomycete *Hypocrea jecorina*, all of the strains that are currently used on a commercial scale have been ultimately derived from one single isolate, which was collected on the Solomon Islands during World War II (Reese & Mandels 1984; El-Gogary et al. 1990). *T. reesei* has been improved throughout the years by random mutagenesis, which has resulted in substantially improved enzyme productivity of this fungus (Mandels et al. 1971; Montenecourt & Eveleigh 1979; Cherry & Fidantsef 2003). *T. reesei* RUT C-30 is the parent strain of many commercially strains used today and is noted for its partial catabolite de-repression (Montenecourt & Eveleigh 1979).

Cellulase comprises three major categories of enzymes: (i) endoglucanases, whose major activity involves the cleavage of β-glycosidic bonds in the cellulose chain to liberate reducing and non-reducing ends in the chain; (ii) cellobiohydrolases, whose major activity involves the cleavage of cellobiose residues consecutively from the ends of the cellulose chains; and (iii) β-glucosidases, which cleave cellobiose units to liberate glucose. The members of this system act synergistically and are necessary for the efficient hydrolysis of cellulose to soluble oligosaccharides (Kubicek et al. 2009).

T. reesei genome contains five endoglucanases (Saloheimo et al. 1988, 1997; Okada et al. 1998), and about 20–36% of its cellulase production is accounted for by endoglucanase, next to cellobiohydrolases (Zaldivar et al. 2001). It has been demonstrated that *T. reesei* can secrete up to 40 g/L of cellulases (Durand et al. 1998), which make it an excellent organism for industrial scale production of these enzymes. Cellulases are used in several industrial applications, such as textile and laundry, food and feed, baking, biomass to alcohol and in the paper and pulp industry (de Castro et al. 2010).

Recycled paper is an important raw material for the paper and pulp industry. Utilization of these secondary fibres is important from the environmental point of view to reduce deforestation and the use of recycled paper has assumed significant proportions all over the world. Deinking of printed paper is an important step in the recycling process for white grade papers. In the traditional deinking process, large quantities of chemicals are used (Bajpai 2014), which makes the method expensive, environmentally damaging, and also increases the release of contaminants. Enzymes could reduce the demand of chemicals and would also lower the process

* Corresponding author

Table 1. Placket-Burman design matrix showing CMCase production by *T. reesei*.

Run	WB (g/L)	Cellulose (g/L)	$FeSO_4$ (mg/L)	Inoculum conc. (spore count)	DAHP (g/L)	Yeast extract (g/L)	Urea (g/L)	pH	$CoCl_2$	Tween 80 (mg/L)	Lactose (g/L)	CMCase (g/L)	(IU/mL)
1	15	10	5.0	5E+07	2.1	2	0.3	7	20	0.5	1	75.5	
2	25	10	5.0	1E+07	1.4	4	0.1	7	20	0.5	2	67.1	
3	15	10	7.5	1E+07	2.1	4	0.3	5	2	0.5	2	57.9	
4	15	5	5.0	5E+07	1.4	4	0.3	5	20	1.0	2	35.4	
5	15	5	5.0	1E+07	1.4	2	0.1	5	2	0.5	1	32.5	
6	25	5	7.5	5E+07	1.4	4	0.3	7	2	0.5	1	32.5	
7	15	5	7.5	1E+07	2.1	4	0.1	7	20	1.0	1	35.0	
8	25	5	7.5	5E+07	2.1	2	0.1	5	20	0.5	2	33.8	
9	15	10	7.5	5E+07	1.4	2	0.1	7	2	1.0	2	63.8	
10	25	10	5.0	5E+07	2.1	4	0.1	5	2	1.0	1	50.2	
11	25	10	7.5	1E+07	1.4	2	0.3	5	20	1.0	1	43.9	
12	25	5	5.0	1E+07	2.1	2	0.3	7	2	1.0	2	43.6	

costs and the environmental impact (Ibarra et al. 2012; Virk et al. 2013).

The aim of this study was therefore to improve the endoglucanase production by *T. reesei* RUT C-30, while trying to reduce the production costs by using economically attractive natural media ingredients, like wheat bran and cellulose as main carbon sources, and the evaluation of the so produced enzyme for deinking of waste office paper.

Material and methods

Microorganism

T. reesei RUT C-30 used in the present study was kind gift from Prof. George Szakacs of the Technical University of Budapest, Hungary. The fungus was maintained as spore suspension in distilled water for long term storage and on potato dextrose agar at 4°C for working stocks. For inoculum preparation, 2-5 mL of sterile distilled water containing 0.05% Tween 80 was aseptically added into fully sporulated slants (7-day old) and was pipetted up and down gently to dislodge the spores into the liquid. The spore suspension thus obtained was aspirated into sterile vials, counted and was adjusted to desired number using sterile 0.05% Tween 80 solution.

Medium

Mandels & Weber (1969) medium was used, supplemented with different carbon sources for carboxymethyl cellulase (CMCase) production. The medium contained in g/L: $(NH_4)_2SO_4$ – 1.4, K_2HPO_4 – 2, urea - 0.3, $CaCl_2.2H_2O$ – 0.3, $MgSO_4.7H_2O$ – 0.3, peptone – 0.75, yeast extract – 0.25, Tween 80 – 1, NaCl – 0.5, $FeSO_4.7H_2O$ – 0.005, $MnSO_4.7H_2O$ – 0.0016, $ZnSO_4.7H_2O$ – 0.0014, and $CoCl_2.6H_2O$ – 0.002.

Enzyme production

To optimize the endoglucanase production, the experiments were carried out in 250 mL Erlenmeyer flasks with 50 mL of fermentation medium containing different carbon and nitrogen sources. The flasks were inoculated with 1.0 mL of a spore suspension containing approximately 1×10^7 spores/mL. The flasks were kept in incubator shaker at 30 ± 2°C and 200 rpm for the required time (as per the experiment design). At the end of fermentation, the culture was filtered through a nylon sieve and then centrifuged at 10,000 rpm and 4°C for 10 min to remove debris and the supernatant was used as crude enzyme preparation.

Determination of enzyme activity

The CMCase (endo-1,4-β-D-glucanase; EC 3.2.1.4) activity was assayed in a total reaction volume of 1.0 mL containing 0.5 mL of diluted enzyme and 0.5 mL of 2.0% (w/v) carboxymethyl cellulose (CMC) solution in citrate buffer (50 mM, pH 4.8). This reaction mixture was incubated at 50°C for 30 min. Dinitrosalicylic acid (DNS) reagent (3.0 mL) was added to the reaction mixture and boiled in a vigorously boiling water bath for 5 min. Reaction mixture was diluted 10-times by adding distilled water and the absorbance was measured at 540 nm. Appropriate blanks were also included, which lacked either enzyme or substrate. Standards prepared with varying glucose concentrations were run in parallel to calculate the glucose release. The CMCase activity was calculated and defined as outlined by Ghose (1987), and was expressed as international unit (IU) per mL.

Optimization of endoglucanase production

Initially, different carbon sources and nitrogen sources were screened in a one factor at a time approach and these were incorporated in the medium for further optimization using a two stage, statistical experimental design strategy. In the first stage a fractional factorial design was used to screen 11 process variables to identify the most significant ones affecting the CMCase production and in the second stage a response surface design was used to optimize the levels of these selected variables.

Plackett-Burman design. The experimental matrix for the fractional factorial screening design was a Plackett & Burman (1946) design, where 11 variables were evaluated at 2 levels. The actual factors tested and their levels are given in Table 1. Experimental runs were performed according to the design and the response (enzyme activity) was recorded. A factorial model was fitted for the main effects using Design Expert® software (Statease Corp., USA). The effects of individual parameters on CMCase production was calculated by the following Equation (1):

$$\varepsilon = \left(\sum \mu_+ - \sum \mu_-\right)/n \quad (1)$$

where ε is the effect of parameter under study and μ_+ and μ_- are responses (CMCase activity) of trials, at which the parameter was at its higher and lower levels, respectively; the "n" is the total number of trials. Analysis of variance (ANOVA) was performed on the data to determine the significance of fitted model and to test the significance

Table 2. CCD matrix for optimization of CMCase production.

Run	Cellulose (g/L)	pH	CMCase activity (IU/mL)
1	30	8	49.93
2	10	8	57.36
3	34.1	7	66.21
4	20	7	83.95
5	10	6	57.78
6	20	7	77.75
7	20	8.4	56.49
8	20	7	82.41
9	20	5.6	27.74
10	20	7	79.52
11	30	6	21.37
12	5.9	7	48.53
13	20	7	80.73

of the effect of individual parameters on CMCase production. The most significant parameters affecting the CMCase production were identified.

Central composite design (CDD). The significant parameters identified by the Placket and Burman design were optimized using a response surface methodology. Specifically, a central composite design (CCD) was used for this study, where the effect of the significant variables was studied at 5 different levels (–2, –1, 0, +1, +2) (del Castillo 2007). The design matrix with 13 experimental runs in a single block, where the midpoint was replicated 5 times, is shown in Table 2. The behaviour of the system was modelled by a second order polynomial equation. The model equation used for the analysis is the following Equation (2):

$$Y = \beta_0 + \sum_{i=1}^{n} \beta_i X_i + \sum_{i=1}^{n} \beta_{ii} X_i^2 + \sum_{i=1}^{n} \sum_{j=1}^{n} \beta_{ij} X_i X_j + \varepsilon \quad (2)$$

where Y is the predicted response, β_0 is the offset term, β_i is the linear effect, β_{ii} is the squared effect, β_{ij} is the interaction effect, X_i and X_j are coded terms for independent variables under study and ε is the error factor. Regression analysis and estimation of the coefficients were performed and the three-dimensional response surfaces were generated using Design Expert® software (Statease Corp., USA). The ideal levels and combinations of parameters were identified by optimization functions in the Design Expert software, and experiments were run at these levels for validation of the model.

Deinking laser-printed paper using the endoglucanase

Modified method of Vyas & Lachke (2003) was employed for deinking studies using printed A4-size pages from a laser printer. The papers were printed with black ink, covering 100% printable area on the both sides of the paper. The sheets were cut into 1 × 6 cm strips (50 mg) and were incubated in citrate buffer (0.05 M, pH 4.8) with crude enzyme from *T. reesei* at 50 °C for 2 h. A control with heat-denatured enzyme was also performed in parallel. The whole experiment was performed in triplicates. The deinking was measured by recording the absorbance of released colour in filtrate at 596 nm. Microscopic images (100 × magnification) of the treated and control paper strips were taken using a stereo microscope.

Results and discussion

Selection of carbon and nitrogen sources for CMCase production

The enzyme production by the *T. reesei* RUT C-30 was evaluated using the carbon sources – cellulose, microcrystalline cellulose, wheat bran, wheat straw, and sugar cane trash at 1.0% (w/v) level. Apparently, cellulose and microcrystalline cellulose could induce endoglucanase synthesis to the maximal levels (21.1 and 25.6 IU/mL, respectively). Wheat bran (10.5 IU/mL) was also a good raw material for endoglucanase production compared to wheat straw (5.9 IU/mL), and sugar cane trash (0.21 IU/mL). However, no growth was detected with rice straw as the carbon source. While pure celluloses supported maximum enzyme yields, wheat bran could support nearly half of the enzyme yields obtained on using pure celluloses. Since wheat bran is a cheap carbon source it was selected as the carbon source in production medium for further optimization. Studies performed with different concentrations of wheat bran indicated that the maximum enzyme yield of 28.3 IU/mL was obtained at 2 % concentration.

It has been demonstrated in *T. reesei* that the nitrogen demand for cellulase production can be fulfilled by the use of inorganic nitrogen sources. Ilmen et al. (1979) showed that the peptone does not exert an inducing effect on cellulase and it just increases the growth of *T. reesei* strain QM9414, subsequently elevating cellulase levels. However, it is a common practice to add peptone or some other organic nitrogen source to enzyme production media to improve cellulase yields (Krishna et al. 2000; Jun et al. 2009). Here, supplementation of diammonium hydrogenphosphate (DAHP) either alone as inorganic nitrogen source or in combination with corn steep liquor (CSL), peptone or yeast extract each at 1 or 2 g/L concentrations were tried as nitrogen sources. Results shown in Figure 1 indicated that DAHP alone was not as effective as the nitrogen source compared to DAHP supplemented with CSL, peptone or yeast extract. Maximal enzyme yields were obtained on using DAHP supplemented with 2 g/L yeast extract. Complex organic nitrogen sources, like peptone or yeast extract can also supplement essential nutrients, vitamins, cofactors, amino acids, etc., and this could be the reason for enhanced growth and enzyme production with their supplementation.

Since cellulose as carbon source seemed to be a dominating factor for enzyme production, it was decided to include minimal amount of cellulose along with wheat bran and to optimize the medium components and production parameters using statistical experiment designs. To determine the best sampling time, enzyme production studies were conducted in a medium containing 1.5% wheat bran, 0.5% cellulose, 0.2% yeast extract and 10 mM DAHP incubated for 96, 120 and 144 h. CMCase yields obtained for 96, 120 and 144 h were 56, 68 and 66 IU/mL, respectively. Since 120 h incubation gave maximal enzyme yield, the incubation

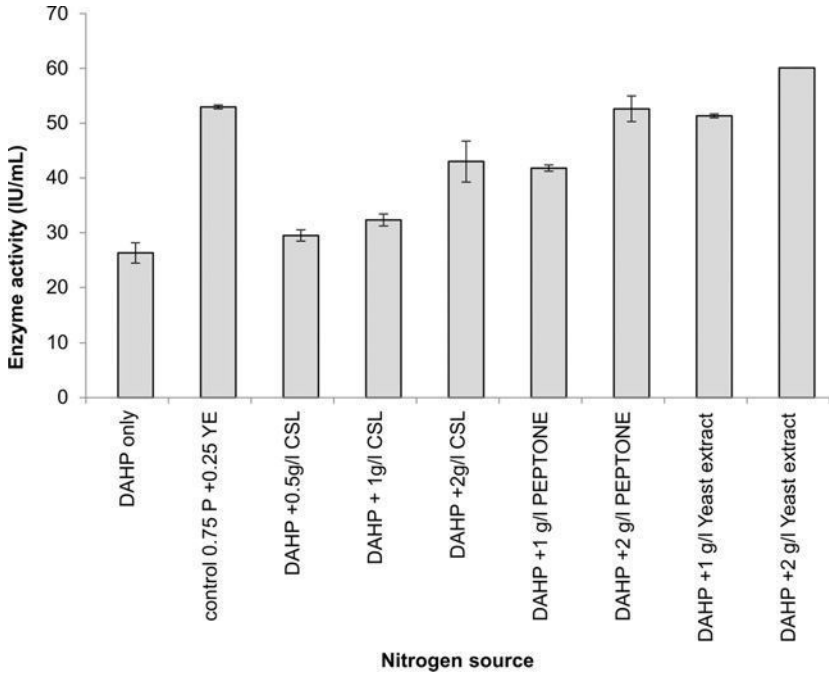


Fig. 1. Effect of different organic nitrogen sources supplemented to DAHP on CMCase production. DAHP, diammonium hydrogen phosphate; CSL, corn steep liquor; P, peptone; YE, yeast extract.

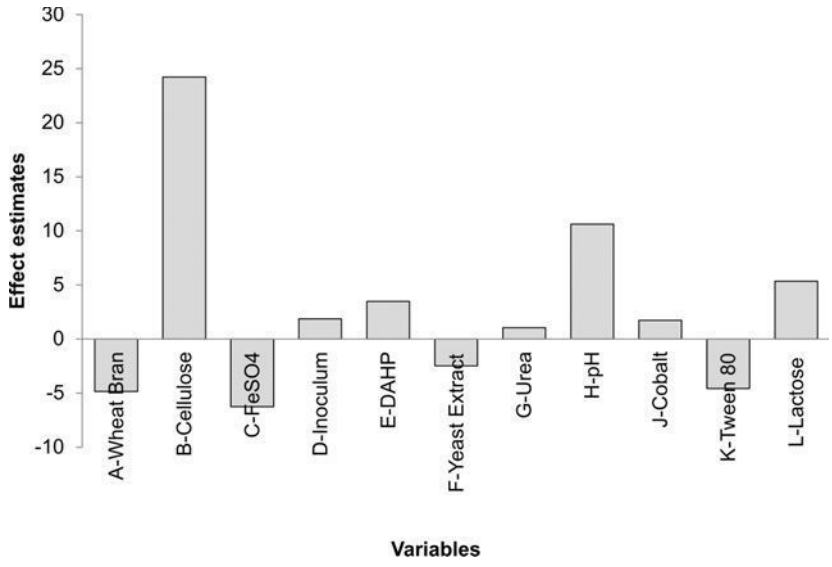


Fig. 2. Pareto chart showing effect estimates of different parameters on CMCase production. DAHP, diammonium hydrogen phosphate.

time was fixed as 120 h for the factorial screening experiments.

Optimization of CMCase production

Screening of parameters affecting CMCase production using Placket and Burman design. The Placket and Burman experiments showed a wide variation in CMCase production ranging from 32.5 to 75.5 IU/mL (Table 2), indicating the importance of optimizing the production parameters for enhancing the enzyme production. The effects of various parameters on CMCase production was estimated using the Equation (1) and the Pareto chart showing the effect estimates are presented in Figure 2. The magnitude and direction of effects give the importance of the variables. Negative values for effect estimate indicate that increase in level of the concerned parameter will result in a decrease in yield. The factors with the highest effects were cellulose concentration and pH of the medium. Interestingly, in presence of cellulose, an increase in wheat bran concentration was shown to influence the CMCase production negatively. The ANOVA of the results also confirmed the importance of cellulose concentration and pH with p values of 0.002 and 0.024, respectively. Cellulose is a strong inducer of cellulases and, apparently, the dominant influence of cellulose do not come as a surprise. However, the positive influence of increased pH is indeed interesting, since in several cases the optimal pH

of cellulase production and activity have been reported in the acidic range. It has been shown that depending on the nature of the carbon source used to induce the cellulase production in *Trichoderma* strains, the optimal pH for maximum cellulose yield might be different. Ryu & Mandels (1980) had reported that a pH range of 3.0–4.0 was optimal for pure cellulose carbon source, but a higher initial pH was recommended for lignocelluloses. While the pH of production medium drops from the typical 4.8 to as low as 2.5 after 2 days due to acid formation, it was also shown that the decrease of medium pH from 3.0 to 2.4 resulted in a loss of 50% of filter paper activity (Sternberg, 1976). Since cellulose concentration and medium pH were the most significant parameters that were identified by Placket and Burman design, their levels were further optimized using a response surface CCD.

Optimization of the significant parameters using CCD. Two critical parameters that were found to have the maximum effect on the CMCase production were taken up for further studies to optimize their levels using response surface methodology. The levels of wheat bran, DAHP, yeast extract, lactose, etc., were fixed at the level that gave the maximum production of CMCase (the run No. 1 in Table 1) and the levels of the significant parameters identified by the factorial design, i.e. the cellulose concentration and pH were optimized using the CCD. The CCD and the experimental and predicted responses obtained for the CMCase production by *T. reesei* are shown in Table 2. The data were analyzed by multiple regression analysis and the following second order polynomial Equation (3) was derived to represent the CMCase production as a function of the independent variables tested:

$$Y = 80.87 - 2.35X_1 + 8.60X_2 + 7.24X_1X_2 - \\ - 12.53X_{12} - 20.16X_{22} \qquad (3)$$

where Y is the predicted response (the CMCase yield), and X_1 and X_2 are coded values of cellulose concentration and pH, respectively.

Testing of the model was performed by the Fisher's statistical test for the ANOVA using Design Expert software. The ANOVA of the quadratic regression model suggested that the model was significant with a computed F value of 9.34 and a p value of 0.005. The regression coefficient (R^2) was 0.870, which indicated

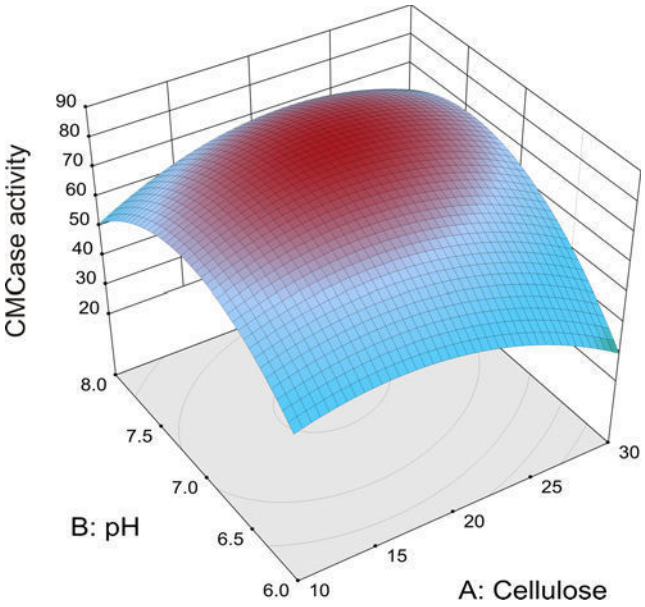


Fig. 3. Response surface graph showing interaction of cellulose concentration and pH on CMCase production by *T. reesei*.

that 87.0% experimental data fitted with expected data in model. The p values for individual model terms indicated that pH and the quadratic effects of both cellulose concentration and pH were significant (p values of 0.040, 0.011 and 0.001, respectively). The interaction between cellulose concentration and pH was not significant.

Nevertheless, response surface curve was plotted to understand the level of interaction of variables and for identifying the optimal levels of each parameter for attaining maximal CMCase yield. Figure 3 represents the response surface for the interaction of cellulose concentration and pH. It was observed that the enzyme production increased with increase in cellulose concentration from 10 g/L to about 20 g/L and thereafter decreased at all the pH tried. Similarly, pH effect was also independent and the CMCase activity increased with increase in pH till about 7.0 and then decreased with further increase of pH. Numerical optimization function in Design Expert software was used to optimize the levels of these parameters and the optimized conditions were a cellulose concentration of 19.7g/l and a pH of 7.2. Under these conditions, the predicted CM-

Table 3. Comparison of CMCase production and productivities of *Trichoderma reesei* with others studies.

T. reesei strain[a]	Production[b]	Formation rate[c]	Days	Condition	Reference
RUT C30	16.00	94.11	7	Pre-treated corn stover	Juhasz et al. 2005
PTr2 isolate	120	833.33	6	5g/L CMC	Prabavathy et al. 2006
RUT C30	18.2	252.77	3	BM (1%) and WB (1%)	Jun et al. 2009
NU-6	54.2	752.77	3	BM+YC (1%) and lactose (1%)	Jun et al. 2009
RUT C30	12.22	84.86	6	Dairy manure with minimum salt media	Wen et al. 2005
RUT C30	150	446.42	14	6% (w/v) roll-milled cotton in a 10 L fermenter	Ryu & Mandels 1980
RUT C30	81.64	680.33	5	1.9% cellulose + 1.5% cheat bran, pH 7.2	This study

[a] The strain NU-6, random mutagenesis by using UV. [b] IU/mL. [c] IU/L per hour.

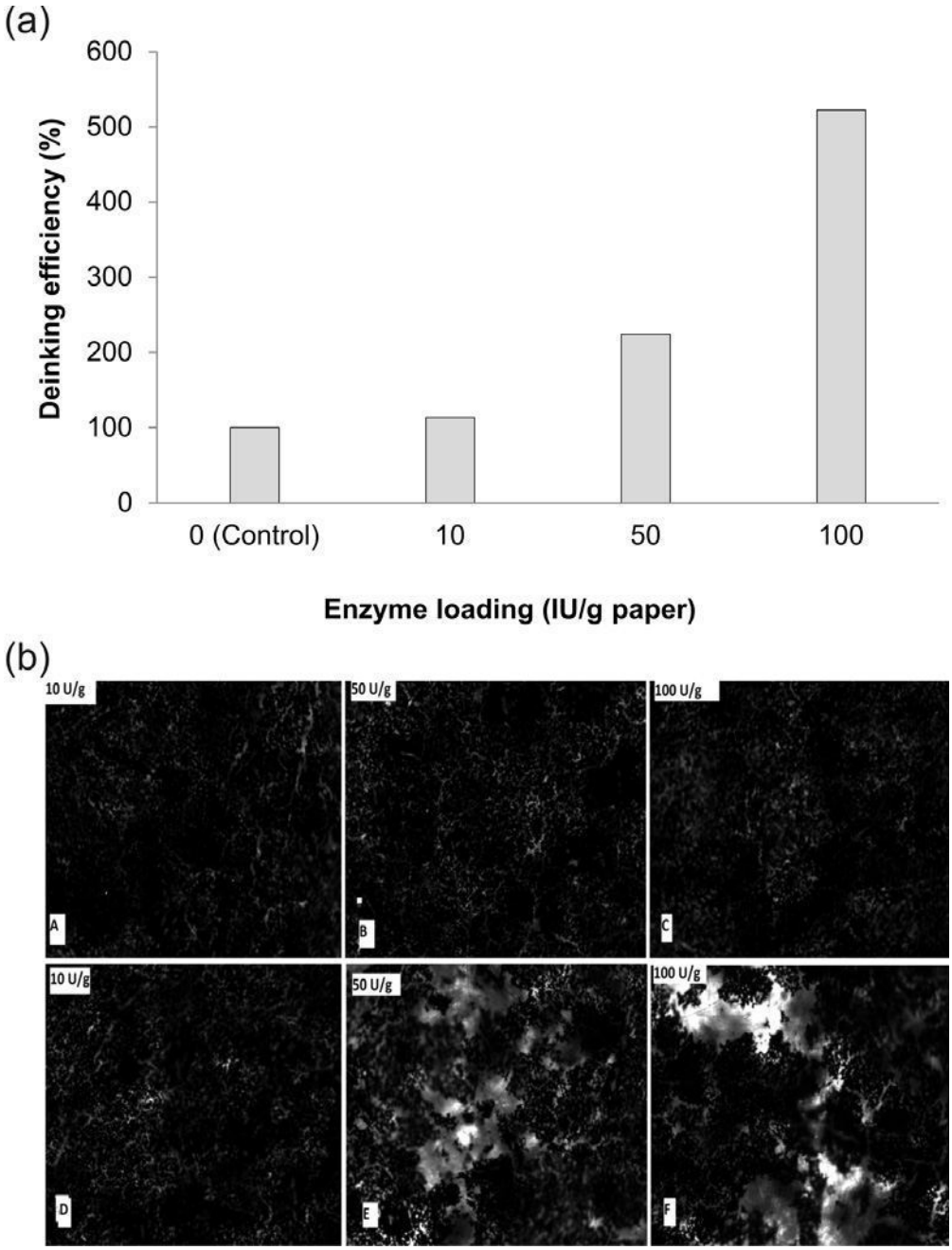


Fig. 4. Deinking performance of *T. reesei* CMCase on printed office waste paper. (a) Dosage dependent enhancement of deinking efficiency. (b) Microscopic images showing deinking of printed paper using *T. reesei* CMCase. A, B, C: treatment controls 10, 50 and 100 μg/g of heat-denatured enzyme, respectively. D, E, F: deinking of paper using 10, 50 and 100 μg/g of CMCase, respectively.

Case yield was 82 IU/mL. Replicated experiments performed under these optimized conditions gave an enzyme yield of 83.63 ± 1.86 IU/mL validating the optimization. A comparison of the productivities of CMCase from *T. reesei* under different conditions showed that the productivities obtained in the current study is one of the highest reported (Table 3).

Deinking of laser-printed paper

Deinking experiments were performed under three different enzyme loadings and the samples were compared for optical density as well as microscopic images in order to evaluate the efficacy of the *T. reesei* CMCase preparation on deinking. The deinking process resulted in increase in ink removal as compared to control samples, which were treated with denatured enzyme (Fig. 4a). Microscopic images confirmed the result. Clearly, the increase in ink removal was increased with increase in enzyme loading (Fig. 4b). Several studies have found that during enzymatic deinking, the cellulases act preferentially on the fines and microfibrils protruding out from the surfaces. This enzymatic action increases the freeness of the pulp (Jeffries et al. 1994; Gubitz et al. 1998).

Conclusions

By using single parameter optimizations the combination of wheat bran and cellulose was selected for en-

hanced endoglucanase production using *T. reesei* RUT C30. Supplementation of 2 g/L of yeast extract and 10 mM DAHP instead of ammonium sulphate, peptone and yeast extract as nitrogen sources resulted in an improved CMCase yield.

Placket-Burman design identified that cellulose concentration and pH were the most significant variables that significantly enhanced the CMCase activity. Optimization of these parameters using a central composite design led to a final CMCase yield of 83.63 ± 1.86 IU/mL and filter paper units of 2.58 ± 0.2 IU/mL. While the enzyme activity is not the highest, productivity was higher compared to several previous reports. The crude enzyme was demonstrated to be useful in deinking of waste office paper.

Acknowledgements

ASOI gratefully acknowledges the financial assistance provided by Department of Biotechnology (DBT), Government of India, and The World Academy of Sciences for the Advancement of Science in developing countries (TWAS) in the form of fellowship for supporting his PhD program, of which this study forms a part.

References

Bajpai P. 2014. Recycling and Deinking of Recovered Paper. Elsevier, Amsterdam.

Cherry J.R. & Fidantsef A.L. 2003. Directed evolution of industrial enzymes: an update. Curr. Opin. Biotechnol. **14:** 438–443.

de Castro A.M., Ferreira M.C., da Cruz J.C., Rodrigues P.K., Carvalho D.F., Leite S. & Pereira J.N. 2010. High-yield endoglucanase production by *Trichoderma harzianum* IOC-3844 cultivated in pretreated sugarcane mill byproduct. Enzyme Res. **2010:** 854526.

del Castillo E. 2007. Process Optimization: A Statistical Approach. Springer Science, New York.

Durand H., Baron M., Calmels T. & Tiraby G. 1998. Classical and molecular genetics applied to Trichoderma reesei for the selection of improved cellulolytic industrial strains, pp. 135–151. In: Aubert J.P., Beguin P. & Millet J. (eds) Biochemistry and Genetics of Cellulose Degradation, FEMS Symposium No. 43. Academic Press, London.

El-Gogary S., Leite A., Crivellaro O., El-Dorry H. & Eveleigh D.E. 1990. *Trichoderma reesei* cellulose – from mutants to induction, pp. 200–211. In: Kubicek C.P., Eveleigh D.E., Esterbauer H., Steiner W., Kubicek-Pranz E.M (eds) *Trichoderma reesei* Cellulases. Royal Society of Chemistry, Cambridge.

Ghose T.K. 1987. Measurement of cellulase activities. Pure Appl. Chem. **59:** 257–268.

Gubitz G.M., Mansfield S.D. & Saddler J.N. 1998. Effectiveness of two endoglucanases from *Gloeophyllum* species in deinking mixed office waste paper, pp. C135–C138. In: Proceedings of The 47th International Conference on Biotechnology of the Pulp and Paper Industry, Montreal.

Ibarra D., Concepción Monte M., Blanco A., Martínez A.T. & Martínez M.J. 2012. Enzymatic deinking of secondary fibers: cellulases/hemicellulases versus laccase-mediator system. J. Ind. Microbiol. Biotechnol. **39:** 1–9.

Ilmen M., Saloheimo A., Onnela M.L. & Penttilä M.E. 1997. Regulation of cellulase gene expression in the filamentous fungus *Trichoderma reesei*. Appl. Environ. Microbiol. **63:** 1298–306.

Jeffries T.W., Klungness J.H., Marguerite S. & Cropsey K.R. 1994. Comparison of enzyme enhanced with conventional deinking of xerographic and laser-printed paper. Tappi J. **77:** 173–179.

Juhasz T., Szengyel Z., Reczey K. & Viikari L. 2005. Characterization of cellulases and hemicellulases produced by *Trichoderma reesei* on various carbon sources. Process Biochem. **40:** 3519–3525.

Jun H., Bing Y., Keying Z., Xuemei D. & Daiwen C. 2009. Strain improvement of *Trichoderma reesei* Rut C-30 for increased cellulase production. Indian J. Microbiol. **49:** 188–195.

Krishna S.H., Rao K.C., Babu J.S. & Reddy D.S. 2000. Studies on the production and application of cellulase from *Trichoderma reesei* QM- 9414. Bioprocess Biosyst. Eng. **22:** 467–470.

Kubicek C.P., Mikus M., Schuster A., Schmoll M. & Seiboth B. 2009. Metabolic engineering strategies for the improvement of cellulase production by *Hypocrea jecorina*. Biotechnol. Biofuels **2:** 19.

Mandels M. & Weber J. 1969. The production of cellulases. Adv. Chem. **95:** 391–413.

Mandels M., Weber J. & Parizek R. 1971.Enhanced cellulase production by a mutant of *Trichoderma viride*. Appl. Microbiol. **21:** 152–154.

Montenecourt B.S. & Eveleigh D.E. 1979. Selective screening methods for the isolation of high yielding cellulase mutants of *Trichoderma reesei*. Adv. Chem. **181:** 289–301.

Okada H., Tada K., Sekiya T., Yokoyama K., Takahashi A., Tohda H., Kumagai H. & Morikawa Y. 1998. Molecular characterization and heterologous expression of the gene encoding a low-molecular mass endoglucanase from *Trichoderma reesei* QM9414. Appl. Environ. Microbiol. **64:** 555–563.

Plackett R.L. & Burman J.P. 1946. The design of optimum multifactorial experiments. Biometrika **37:** 305–325.

Prabavathy V.R., Mathivanan N., Sagadevan E., Murugesan K. & Lalithakumari D. 2006. Intra-strain protoplast fusion enhances carboxymethyl cellulase activity in *Trichoderma reesei*. Enzyme Microb. Technol. **38:** 719–723.

Reese E.T. & Mandels M. 1984. Rolling with the times: production and applications of *Trichoderma reesei* cellulases. Annu. Rep. Ferm. Proc. **7:** 1–20.

Ryu D.D.Y. & Mandels M. 1980. Cellulases: biosynthesis and applications. Enzyme Microb. Technol. **2:** 91–102.

Saloheimo M., Lehtovaara P., Penttila M., Teeri T.T., Stahlberg J., Johansson G., Pettersson G., Claeyssens M., Tomme P. & Knowles J.K. 1988. EGIII, a new endoglucanase from *Trichoderma reesei*: the characterization of both gene and enzyme. Gene **63:** 11–22.

Saloheimo M., Nakari-Setala T., Tenkanen M. & Penttila M. 1997. cDNA cloning of a *Trichoderma reesei* cellulase and demonstration of endoglucanase activity by expression in yeast. Eur. J. Biochem. **249:** 584–591.

Sternberg D. 1976. Production of cellulase by *Trichoderma*. Biotechnology and Bioengineering Symp. **6:** 35–53.

Virk A.P., Puri M., Gupta V., Capalash N. & Sharma P. 2013. Combined enzymatic and physical deinking methodology for efficient eco-friendly recycling of old newsprint. PLoS One **8:** e72346.

Vyas S. & Lachke A. 2003. Biodeinking of mixed office waste paper by alkaline active cellulases from alkalotolerant *Fusarium* sp. Enzyme Microb. Technol. **33:** 236–245.

Wen Z., Liao W., & Chen S. 2005. Production of cellulase by *Trichoderma reesei* from dairy manure. Bioresour. Technol. **96:** 491–499.

Zaldivar M., Velásquez J. C., Contreras I. & María Pérez L. 2001. *Trichoderma aureoviride* 7-121, a mutant with enhanced production of lytic enzymes: its potential use in waste cellulose degradation and/or biocontrol. Electronic J. Biotechnol. **4:** a07.

Received December 31, 2015
Accepted March 19, 2016

Bioresource Technology 242 (2017) 265–271

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Cellulase production through solid-state tray fermentation, and its use for bioethanol from sorghum stover

Ayman Salih Omer Idris [a,b], Ashok Pandey [c], S.S. Rao [d], Rajeev K. Sukumaran [a,b,*]

[a] Academy of Scientific and Innovative Research (AcSIR), CSIR-NIIST Campus, Thiruvananthapuram, India
[b] Centre for Biofuels, Microbial Processes and Technology Division, CSIR-National Institute for Interdisciplinary Science and Technology, Industrial Estate P.O., Thiruvananthapuram 695019, India
[c] Centre for Innovative and Applied Bioprocessing, Mohali 160 071, Punjab, India
[d] Indian Institute of Millets Research, Hyderabad, India

HIGHLIGHTS

- *Trichoderma reesei* produces high level of endoglucanase when cultivated under SSF.
- Process optimizations enhance the cellulase production 3.2-fold in SSF using *T. reesei*.
- Alkali treated sorghum stover is hydrolyzed effectively by *in house* cellulase.
- Addition of *A. niger* BGL improves biomass hydrolysis by 174%.
- 80% efficiency in ethanol fermentation achieved with sorghum stover hydrolysates.

ARTICLE INFO

Article history:
Received 31 January 2017
Received in revised form 14 March 2017
Accepted 17 March 2017
Available online 20 March 2017

Keywords:
Trichoderma
Solid-state fermentation
Cellulase
Beta glucosidase
Bioethanol
Sorghum

ABSTRACT

The production of cellulase by *Trichoderma reesei* RUT C-30 under solid-state fermentation (SSF) on wheat bran and cellulose was optimized employing a two stage statistical design of experiments. Optimization of process parameters resulted in a 3.2-fold increase in CMCase production to 959.53 IU/gDS. The process was evaluated at pilot scale in tray fermenters and yielded 457 IU/gDS using the lab conditions and indicating possibility for further improvement. The cellulase could effectively hydrolyze alkali pretreated sorghum stover and addition of *Aspergillus niger* β-glucosidase improved the hydrolytic efficiency 174%, indicating the potential to use this blend for effective saccharification of sorghum stover biomass. The enzymatic hydrolysate of sorghum stover was fermented to ethanol with ~80% efficiency.

1. Introduction

The enzymatic saccharification of plant biomass is the most efficient method for lignocellulose breakdown to sugars, though the cost of this operation is still one of the major bottlenecks in commercialization of second generation alcohol. Cellulase cost reduction remains the top agenda of establishments involved in bioethanol research, and several methods including onsite production, use of cheap raw materials for production of the enzyme and use of enzyme in crude concentrated form without purification are being investigated to further reduce the cost of biomass hydrolyzing enzymes – primarily cellulases. Cellulases are a group of enzymes including endo and exoglucanases and beta glucosidases that act synergistically and are necessary for the efficient hydrolysis of cellulose to soluble oligosaccharides (Beckham et al., 2010; Kubicek et al., 2009).

While cellulases are produced by various microorganisms, commercial sources of the enzyme are mostly the filamentous fungi *Trichoderma reesei* and *Aspergillus species*, mainly due to their high cellulase productivity, safe use in industry and the availability of their whole genome sequences for genetic interventions (Le Crom et al., 2009; Martinez et al., 2008). Both submerged fermentation and solid-state fermentation (SSF) have been used for cellulase production (Singhania et al., 2010). While each of these strategies has their own advantages and disadvantages, SSF is con-

* Corresponding author at: Centre for Biofuels, Microbial Processes and Technology Division, CSIR-National Institute for Interdisciplinary Science and Technology, Industrial Estate P.O., Thiruvananthapuram 695019, India.
E-mail address: rajeevs@niist.res.in (R.K. Sukumaran).

http://dx.doi.org/10.1016/j.biortech.2017.03.092
0960-8524/

sidered as less capital intensive, easy to operate and does not require skilled manpower. Another major advantage is the ability to use cheap insoluble lignocellulosic feedstock as substrates/cheap carbon sources for enzyme production (Pandey, 2003).

Sweet sorghum is an ideal food cum bioenergy crop that can produce grain, stalk sugar, forage as well as large amounts of biomass in the order of 10–20 dry tons/ha/3.5 months (Gonsalves, 2006; Rao et al., 2007). Since it require less water compared to sugarcane and since the crop duration is less, cultivation of sorghum offers an interesting opportunity for better utilization of available arable land. With an annual surplus availability of ~1.6 million metric tons (MMT), sorghum biomass in India is a formidable feedstock resource for second generation ethanol and very less work has been done on this feedstock towards its conversion to ethanol (Pandey et al., 2009). Also there is very less information on the enzyme cocktails suitable for optimal sorghum biomass hydrolysis. Hence, the present investigation was undertaken for *in-situ* production of cellulases using *Trichoderma reesei* utilizing cheap raw material under solid-state fermentation and the enzyme was used for hydrolysis of pretreated sorghum stover to generate fermentable sugars and their fermentation to produce bioethanol.

2. Materials and methods

2.1. Microorganism and inoculum preparation

Trichoderma reesei RUT C-30 used for the present study was a kind gift from Prof George Szakach, Technical University of Budapest, Hungary. The fungus was maintained on Potato Dextrose Agar (PDA) at 4 °C for working stocks. For inoculum preparation, 2–5 ml of sterile saline containing 0.05% Tween 80 was aseptically added into fully sporulated slants (7 day old culture) and was gently pipetted up and down to dislodge the spores into the liquid. The spore suspension thus obtained was aspirated into sterile vials, counted under microscope using a haemocytometer and was adjusted to desired count using sterile saline containing 0.05% Tween 80. The spore suspension with the desired spore count was used as inoculum.

2.2. Enzyme production

Different agricultural waste residues were evaluated initially as substrate in the present study, and wheat bran was selected for production studies with supplementation of the added inducer-cellulose. Total of 5 g dry weight of the substrate of choice or wheat bran plus cellulose was weighed into 250-ml Erlenmeyer flasks and was moistened with mineral salt medium (Mandels and Weber, 1969) to obtain the appropriate initial moisture content. The flasks were then sterilized by autoclaving. Medium used for the experiment had following composition in g/l $(NH_4)_2$ SO_4-1.4, K_2HPO_4-2, Urea-0.3, $CaCl_2{\cdot}2H_2O$-0.3, $MgSO_4{\cdot}7H_2O$-0.3 g, Peptone - 0.75, Yeast Extract-0.25, Tween 80-1.0, $FeSO_4{\cdot}7H_2O$-0.005, $MnSO_4{\cdot}7H_2O$-0.0016, $ZnSO_4{\cdot}7H_2O$-0.0014, $CoCl_2{\cdot}6H_2O$-0.002.The pH of the medium was adjusted to 4.8 with 1 N HCl or 1 N NaOH wherever required. The flasks were inoculated with 1.0 ml of the spore suspension containing approximately 1×10^7 spores/ml or the appropriate volume as per the experiment design. The flasks were incubated at 30 ± 2 °C for the required duration as per the experiment design. Enzyme extraction was performed using 50 mM citrate buffer (pH 4.8). Fifty milliliter of the buffer was added to each flask containing 5 g of moldy bran and was incubated with shaking (200 rpm, 30 °C, 60 min). The slurry was filtered using a nylon mesh to remove debris and the filtrate was centrifuged at 7000 rpm for 10 min at 4 °C to remove fine suspended particles. The supernatant was taken as the crude enzyme, and the enzyme activity of the enzyme was determined.

2.3. Enzyme assays

2.3.1. Carboxymethyl cellulase (CMCase) activity

Endoglucanase (CMCase, endo-1,4-β-D-glucanase; EC 3.2.1.4) activity was assayed as per the IUPAC guidelines (Ghose, 1987) in a total reaction volume of 1.0 ml containing 0.5 ml of diluted enzyme and 0.5 ml of 2.0% (w/v) carboxymethyl cellulose (CMC) solution in citrate buffer (50 mM, pH 4.8). This reaction mixture was incubated at 50 °C for 30 min. Dinitrosalicylic acid (DNS) reagent (3.0 ml) was added to the reaction mixture and boiled for 5 min. Reaction mixture was diluted by adding distilled water and the absorbance was measured at 540 nm. Appropriate blanks were also included which lacked either enzyme or substrate. Standards prepared with varying glucose concentrations were run in parallel to calculate the glucose release. CMCase activity was calculated and defined as outlined by Ghose (1987), and was expressed as international units per gram dry substrate (IU/gDS).

2.3.2. Filter paper activity assay

Total cellulase activity was measured using the filter paper assay according to IUPAC (Ghose, 1987). A rolled Whatman #1 filter paper strip of dimension 1.0 × 6 cm (~50 mg) was used as substrate. The filter paper strips were saturated with 0.5 ml of Na-citrate buffer (0.05 M, pH 4.8) and 0.5 of an appropriately diluted (in Na-citrate buffer −0.05 M, pH 4.8) enzyme was added to the tube and incubated at 50 °C for 60 min. Appropriate controls were also run along with the test. After incubation, 3.0 ml of DNS reagent was added and boiled for 5 min after which it was diluted appropriately and absorbance was measured at 540 nm. Glucose released by different enzyme dilutions was obtained from a similarly processed standard curve. Filter Paper Activity (FPA) was calculated following the concept that 0.37 FPU of enzyme will liberate 2 mg of glucose under the above assay conditions and was expressed as Filter Paper Units (FPUs) per gram dry substrate (FPUs/gDS).

2.4. Optimization of cellulase production

Different carbon sources and others parameters were screened in a one factor at a time approach and these were incorporated in the medium for further optimization using a two stage statistical experimental design strategy. In the first stage, a fractional factorial design was used to screen 10 process variables to identify the most significant ones affecting cellulase (measured as CMCase activity) production and in the second stage, a response surface Box Behnken design (Box and Behnken, 1960) was used to optimize the levels of these selected variables.

2.4.1. Screening of variables affecting production

The screening of important variables affecting cellulase production was performed using a Plackett and Burman design (Plackett and Burman, 1946), where 11 variables were evaluated at 2 levels (Table 1). Experiments were performed as per the design and the response (CMCase activity) was recorded. A factorial model was fitted for the main effects using the software Design Expert® (Stat-ease, USA). The effects of experimental variables on CMCAse production was computed using the following equation (Eq. (1))

$$\varepsilon = \left(\sum \mu_{+} - \sum \mu_{-}\right)/n \quad (1)$$

where ε is the effect of parameter under study and "μ_+" and "μ_-"are responses (CMCase activity) of trials at the higher and lower levels of each variable respectively and "n" is the total number of trials. Analysis of variance (ANOVA) was performed on the data to determine the significance of fitted model and to test the significance of the effect of individual parameters on CMCase production. Variables

Table 1
Placket and Burman Design matrix for screening of process variables.

Run #	Cellulose (g/g)	Peptone (g/L)	Yeast Extract (g/L)	Moisture (%)	Amm Sulfate (g/L)	Lactose (g/L)	Tween 80 (g/L)	Inoculum size (spores/g)	Time (Days)	pH	Dummy	Enzyme Activity (IU/gDS)
1	0.80	3	5	65	2	20	0.25	2.00E+06	7	5.5	−1	260.59
2	0.2	3	7.5	55	2	20	1	2.00E+06	7	4.8	1	360.95
3	0.80	0	7.5	65	1	20	1	1.00E+07	7	4.8	−1	293.69
5	0.20	0	7.5	55	2	20	0.25	1.00E+07	9	5.5	−1	434.69
6	0.20	0	5	65	1	20	1	2.00E+06	9	5.5	1	237.67
4	0.20	3	5	65	2	0	1	1.00E+07	9	4.8	−1	274.53
7	0.80	0	5	55	2	0	1	1.00E+07	7	5.5	1	502.13
8	0.80	3	5	55	1	20	0.25	1.00E+07	9	4.8	1	547.28
9	0.80	3	7.5	55	1	0	1	2.00E+06	9	5.5	−1	531.88
10	0.20	3	7.5	65	1	0	0.25	1.00E+07	7	5.5	1	252.94
11	0.8	0	7.5	65	2	0	0.25	2.00E+06	9	4.8	1	241.03
12	0.20	0	5	55	1	0	0.25	2.00E+06	7	4.8	−1	310.55

affecting the production of CMCase most significantly were production.

2.4.2. Box Behnken design (BBD)

The levels of the variables identified as significant by the initial screening test were optimized for enhancing the endoglucanase yield using a response surface Box– Behnken experiment design (Box and Behnken, 1960). The variables selected for optimization, i.e., initial moisture, cellulose concentration, and inoculum size, were coded as X_1, X_2, and X_3, respectively (Table 2). A second-order polynomial (Eq. (2)) was used to represent the response as a function of the tested variables

$$Y = \beta_0 + \sum_{i=1}^{n} \beta_i X_i + \sum_{i=1}^{n} \beta_{ii} X_i^2 + \sum_{i=1}^{n} \beta_{ij} Xi X_j \quad (2)$$

where Y is the measured response (CMCase yield), β, β_i, and β_{ii} are the regression coefficients, and X_1–X_3 are the factors under study. Regression analysis and estimation of the coefficients were performed using Design Expert® software (Stat Ease, USA). The contributions of individual parameters and their quadratic and interaction effects on cellulase production were determined.

2.5. Enzyme evaluation for hydrolysis of sorghum stover and bioethanol production

2.5.1. Raw material, pre-processing and composition analyses

Sorghum stover (SS) samples were kindly provided by the Indian Institute of Millets Research, Hyderabad. The samples were air-dried and were milled in knife mill to a particle size less than 2 mm and were stored in air tight containers until used. Moisture content was estimated prior to use using an infrared moisture analysis balance (AND, Japan). Milled SS biomass was pretreated by dilute acid or alkali using previously optimized conditions and used a 10% biomass loading in either case. In the case of acid pretreatment, 1.5% H_2SO_4 was used for 60 min at 121 °C in an autoclave and in the case of alkali, 2% NaOH was used for 60 min at 121 °C in an autoclave. After cooling, the biomass was washed with tap water until the pH became neutral. The wet biomass after solid liquid separation using a nylon mesh was air dried and used for enzymatic saccharification experiments. Compositional analyses of the untreated and pretreated sorghum stover was carried out based on the NREL protocol (Sluiter et al., 2008a) and Scanning electron microscopy of the samples were performed as described previously (Kuttiraja et al., 2013).

Table 2
Box Behnken Design matrix for optimization of CMCase production by *T. reesei*.

Run No	X_1 Cellulose (g/5 g)	X_2 Inoculum Density (Spores/g)	X_3 Moisture (%)	CMCase (IU/gDS)
1	0.8	3.00E+06	55.0	733.06
2	1.0	3.00E+06	57.5	940.95
3	1.0	3.00E+06	57.5	954.71
4	1.0	3.00E+06	57.5	956.02
5	1.2	3.00E+06	60.0	774.00
6	1.2	5.00E+06	57.5	830.16
7	1.2	1.00E+06	57.5	778.29
8	1.2	3.00E+06	55.0	774.00
9	1.0	3.00E+06	57.5	959.53
10	0.8	1.00E+06	57.5	755.23
11	1.0	5.00E+06	55.0	878.29
12	0.8	3.00E+06	60.0	723.13
13	1.0	5.00E+06	60.0	756.62
14	1.0	1.00E+06	55.0	857.18
15	0.8	5.00E+06	57.5	795.42
16	1.0	1.00E+06	60.0	794.73
17	1.0	3.00E+06	57.5	827.38

2.5.2. Enzymatic hydrolysis and ethanol fermentation

Enzymatic saccharification of biomass was performed at 10% biomass loading by incubating 2 g of pretreated biomass with the crude *T. reesei* cellulase produced under the optimal conditions derived from the experiments above. Cellulase was used at a concentration of 20FPUs/g with or without β-glucosidase (BGL supplementation at a concentration of 50 IU/g or 100 IU/g biomass. The BGL used was from *Aspergillus niger,* produced in the lab as described earlier (Singhania et al., 2011). Hydrolysis was performed in 150 ml screw capped flasks in a reaction volume of 20 ml at 50 °C, and with 100 rpm agitation in a water bath shaker to a maximum duration of 48 h. After hydrolysis, the unhydrolyzed debris was separated by filtration and concentrated by vacuum evaporation. The concentrated filtrate was filter sterilized and was inoculated at 10% v/v using a 12 h old seed culture of *Saccharomyces cerevisiae* cultivated in YPD broth (Himedia, India). Incubation was carried out for 24 h in stopper flasks at room temperature under static condition. Samples (1.0 ml) were withdrawn at 0, 12, and 24 h; were centrifuged for 10 min at 4 °C and supernatant was filtered and analyzed for ethanol by gas chromatography as described previously (Kuttiraja et al., 2013). Total reducing sugars were estimated in hydrolysates by DNS method (Miller, 1959) and glucose estimations were performed by HPLC following NREL protocols (Sluiter et al., 2008b). Sugar concentrations were expressed as mg/g of biomass. Hydrolysis efficiencies were calculated according to the following formula

$$\in = \left(\frac{\textit{concentration of sugar released}}{(\textit{cellulose conc.} \times 1.11) + (\textit{hemicellulose conc.} \times 1.12)} \right) \times 100 \quad (3)$$

where "∈" is the hydrolysis efficiency in percentage and the concentration of sugar released is the total sugar release in the hydrolysate

measured in mg/g of biomass. Cellulose and hemicellulose concentrations are expressed in g/g biomass and 1.11 and 1.12 are the standard conversion factors for calculating the amount of monomeric sugars released on hydrolysis of the respective sugar polymers.

3. Results and discussion

3.1. Selection of carbon sources for CMCase production

Cellulase production by *Trichoderma reesei* RUT C-30 under solid-state fermentation was evaluated using agricultural residues (rice straw (RS), sorghum straw (SS), sugar cane trash (SCT), wheat straw (WS), and wheat bran (WB)) as carbon sources. Among these crude carbon sources, wheat bran could induce the maximum cellulase (CMCase) activity of 298.35 IU/gDS. While the lignocellulosic substrates did induce cellulase activity, the enzyme yields were considerably lower compared to wheat bran as the substrate. Among these substrates, WS supported maximum yield of 80.65 IU/gDS whereas the enzyme yields for RS, SS and SCT were 58.2, 55.75 and 69.7 respectively. Wheat bran is a cheap carbon source routinely used as substrate for SSF and is rich in several nutrients, in addition carbon, and this could be the reason for its better performance since it can enhance cell growth and metabolism. It was selected as the carbon source for all further experiments.

3.2. Optimization of cellulase production

Screening experiments performed following the Placket and Burman design indicated considerable variation in CMCase production ranging from 237.67 IU/gds to 547.28 IU/gds (Table 1). The levels of influence of tested variables on CMCase production was computed using Eq. (1). The factors with highest effects were initial moisture content (negative), cellulose concentration (positive), and inoculum size (positive). Cellulases are inducible enzymes and production of the enzyme by *T. reesei* depends on the induction mediated by the lignocellulosic substrate under natural conditions. Pure cellulose is a strong inducer of cellulases and apparently a dominant factor of cellulase production (Bischof et al., 2013). Cellulose was supplemented in the production medium to have strong induction of the enzymes and the results indicated that this approach was successful. Increase in moisture content had a negative influence on cellulase production, and the results confirmed to our previous observations that *Trichoderma reesei* produce more cellulase at low moisture contents under SSF (Singhania et al., 2007). *T. reesei* is a highly aerobic culture higher moisture content is reported to result in oxygen limitation through the filling of voids in the substrate with water (Vitcosque et al., 2012). Increase in inoculum concentration within tested range resulted in enhanced activities which might be attributed to higher growth rates achieved initially. The levels of initial moisture content, cellulose concentration and inoculum size was identified as most significant from the screening study (Plackett & Burman Design) and were further optimized following a Box Behnken design (BBD).

The maximum response (959.53 IU/gDS) was obtained in run 9, and in general, runs with middle levels of parameters gave higher enzyme production compared to other combinations (Table 2). Regression coefficients for the design model were determined by multiple regression analysis using the software Design Expert® v8.0 and cellulase production as represented as a function of the independent variables tested through a second-order polynomial equation (Eq. (4))

$$Y = 927.718 + 18.70X_1 + 9.38X_2 - 24.25X_3 - 104.3X_1^2 - 33.64X_2^2 - 72.37X_3^2 + 2.92X_1X_2 + 2.48X_1X_3 - 14.80X_2X_3 \quad (4)$$

where, Y is the predicted response, and X_1, X_2, and X_3 are coded values of cellulose content, inoculation size and moisture content, respectively. The quadratic regression model adopted was suggested significant by the Analysis of Variance (ANOVA) which indicated computed F value of 3.7821and a P value lower than 0.05 (Table 3). X_1^2, X_3^2 were found to be significant model terms based on their low p-values (<0.05). Response surface curves were plotted to view the interaction effects of variables and for identifying the optimal levels of each parameter for obtaining maximum yield (Fig. 1). Increase in cellulose concentration had a positive impact on cellulase production up to a concentration of about 1.0–1.1 g/5 g (20–22% w/w) of total substrate beyond which it decreased (Fig. 1A). Probably this concentration may indicate the threshold maximum attainable with the given biomass growth under the tested SSF conditions. Increase in cellulose concomitantly decreases the wheat bran content in total substrate and this could affect the amount of fungal biomass achieved, since wheat bran can be used more easily by the organism. Similar to cellulose concentration, inoculum density also had a positive effect till $\sim 3.5 \times 10^6$ spores/g concentration beyond which it diminished gradually. Decrease in enzyme yield at higher spore density may be attributed to the increased growth and biomass accumulation where majority of the resources are used for growth and not for cellulase production. Analyses of the response curve for moisture against inoculum density showed the same trend for inoculum concentration with the optimal concentration remaining between 3.0×10^6 and 4×10^6 spores/g dry substrate. The ideal moisture content was found to be between 55% and 57% (Fig. 1B). The parameters cellulose concentration and moisture content acted totally independently as indicated by the data in Fig. 1C. The optimal cellulose concentration was between 1.0 and 1.1 g/5 g and the moisture content between 55% and 57%. Incidentally, the maximum activity of ~950 IU/gDS was obtained at near middle levels of each of the parameters indicating that the choice of levels have been very appropriate to arrive at the optimal concentration. Many factors such as moisture content, cellulose content, inoculum size, oxygen transfer and concentrations of nutrients are known to influence growth and enzyme production under SSF, and the selection of optimum level for these parameters can lead to increase in enzyme production under SSF. *Trichoderma reesei* produced 298.35 IU/gds SSF on wheat bran in the basal medium and under the optimal combination of parameters the enzyme titers had gone up 3.2-fold higher to 959.53 IU/gDS. This indicated that SSF may be used for cellulase production from plant biomass. In this study, initial moisture content had the largest influence on production of CMCase. Different moisture ranges (37.56–80%) have been used for cultivation of *T. reesei* for production of the enzyme (Kilikian et al., 2014; Singhania et al., 2007). In the current study 57% was found to be optimal for enzyme production. Under the optimal conditions of moisture content, cellulose concentration and inoculum size, the ideal incubation time was found to be 9.0 days. Filter paper activity was determined for the enzyme produced under optimal conditions and it was found to be 22.37 FPUs/gDS.

3.3. Tray fermentation for scaled up cellulase production

Solid-state fermentation on production scales are usually performed in trays and inside climate controlled chambers called 'Koji rooms'. Initial evaluation of the larger scale production of enzymes through SSF using the *T. reesei* culture was attempted in 50 g capacity trays in the Pilot scale SSF facility (Koji Room) of CSIR NIIST with relative humidity and temperature maintained at 80% and 30 ± 2 °C respectively. Fifty grams of total dry substrate containing 20% cellulose was mixed well and moistened with the production medium according to the conditions optimized in flask cultivation. Inoculation was carried out with spore inoculum

Table 3
ANOVA for Response Surface Quadratic Model.

Source	Sum of Squares	DF	Mean of Square	F Value	p-value Prob > F
Model	88543.35	9	9838.15	3.78	0.0466
A-Cellulose	2797.89	1	2797.89	1.08	0.3342
B-Inoculum density	704.25	1	704.25	0.27	0.6189
C-Moisture	4706.93	1	4706.93	1.81	0.2205
AB	34.11	1	34.11	0.01	0.9121
AC	24.65	1	24.65	0.01	0.9252
BC	876.75	1	876.75	0.34	0.5797
A^2	45804.39	1	45804.39	17.61	0.0041
B^2	4765.62	1	4765.62	1.83	0.2180
C^2	22052.43	1	22052.43	8.48	0.0226

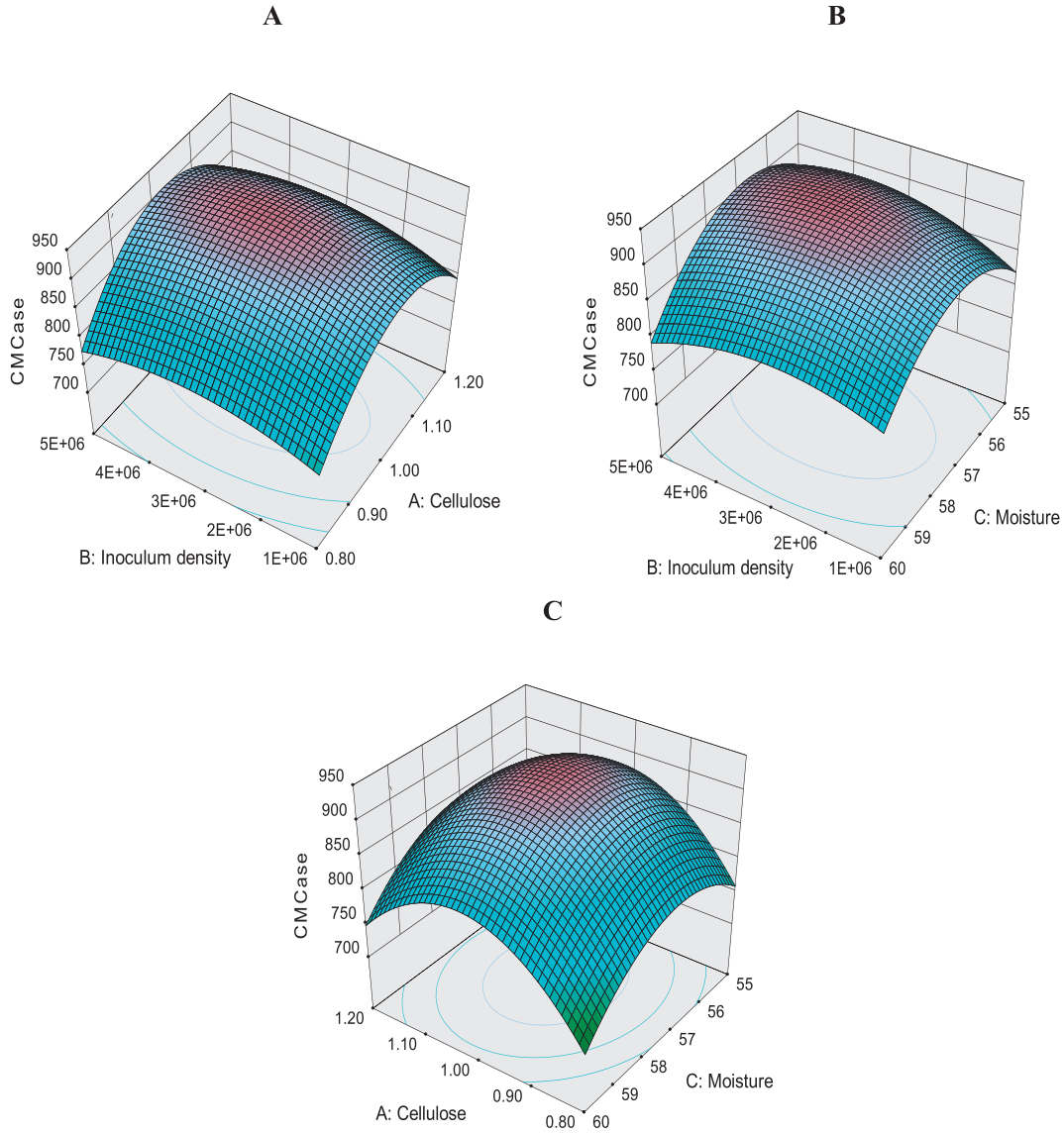


Fig. 1. Response surface plots showing the interaction effects of process variables on production of cellulase by *T. reesei*. A) Interaction of inoculum density and cellulose concentration B) Interaction between inoculum density and moisture content C) Interaction between cellulose and moisture content.

prepared with the optimized concentration and fermentation was carried out till 14 days when mycelial growth was visible and sporulation set in; the conditions when maximum cellulase production was observed in flask fermentation. Enzyme extraction and assay indicated that the yield as almost half (457.27 ± 24.7 IUs/gDS) of what was obtained in flasks, though there was no contamination. It was observed that the trays were warm due to metabolic heat and the mycelial growth was lesser in the centre of trays. It is speculated that the heat and oxygen transfer could be limiting and more studies and optimization in trays have to be performed to obtain the same levels of production as obtained in flasks. It has been demonstrated that there could be very significant temperature gradients with increase in substrate packing height, properties of the substrate, moisture of the atmosphere in

which the trays are incubated etc (Chen, 2013). It is therefore required to perform detailed studies on energy and water balances to determine the appropriate scale up criteria (Figueroa-Montero et al., 2011). Nevertheless, the results seems promising, since the production is still higher than base level and even after performing the experiments in a semi sterile atmosphere, there was no contamination and about 450 IUs/gDS of enzyme was obtained from the system, indicating scope for large scale production of the enzyme using SSF.

3.4. Hydrolysis of sorghum stover using in-situ produced T. Reesei cellulase and fermentation of the hydrolysate to ethanol

Chemical composition of biomass varies with respect to several parameters and depending on the method of pretreatment. Also the composition of the biomass is essential to estimate the efficiencies of hydrolysis and for calculation of the potential yield of bioethanol. The composition of biomass was analyzed experimentally for the native sample as well as for dilute acid and alkali pretreated sorghum stover. As expected, dilute acid pretreatment removed more of hemicellulose by its break down while alkali pretreatment removed more of lignin (Table 4). While the improvement in cellulose content was similar with all the different pretreatments (From 40% in Native to ~50% in the pretreated samples), the major differences were in the removal of lignin and hemicellulose. While acid pretreatment actually resulted in an increased final percentage of lignin, there was a considerable hemicellulose removal with this treatment. On the contrary, alkali removed more of lignin and the final lignin percentage was only 7.36%.

The efficiency of the SSF produced *T. reesei* cellulase for hydrolysis of pretreated sorghum stover was evaluated with and without supplementation of *in-house* produced *Aspergillus niger* BGL at 50 or 100 IU/g biomass concentration. Cellulase was used at 20FPUs/g biomass in all the hydrolysis experiments. Fig. 2A & B indicate the sugar yields obtained for hydrolysis employing the *T. reesei* cellulase for acid and alkali pretreated sorghum stover respectively. Hydrolytic efficiencies were lower for acid pretreated biomass compared to alkali pretreated in all the enzyme combinations attempted. The conversion efficiency was only about 25% of theoretical maximum in the case of native unblended *T. reesei* cellulase for acid pretreated SS. With addition of either 50 or 100 IUs of BGL per gram biomass, the hydrolytic efficiency improved to 30% of theoretical maximum. The situation was totally different in the case of alkali pretreated biomass. The native *T. reesei* cellulase had an efficiency of 47% whereas with supplementation of 50 or 100 IUs/g BGL to the cellulase, the efficiencies improved significantly to almost 60% and 82% respectively indicating excellent synergy between the enzymes. Apparently, the type of pretreatment had influenced the hydrolysis efficiencies significantly, since the acid pretreated material was hydrolyzed poorly. This is speculated to be due to the poor removal of lignin in dilute acid pretreatment and its re-deposition on the biomass preventing enzyme access a phenomenon which is already reported (Selig et al., 2007). With a better pretreated sample, the hydrolysis efficiencies were superior and with BGL supplementation it improved to 82%. *T. reesei* is known to produce very less of BGL enzymes and BGL blending

Table 4
Composition analysis of native and pretreated sorghum stover.

	Cellulose	Hemicellulose	Lignin
Native	39.58	20.15	21.72
1.5% Acid	50.4	9.20	25.85
2% NaOH	57.16	27.16	7.36

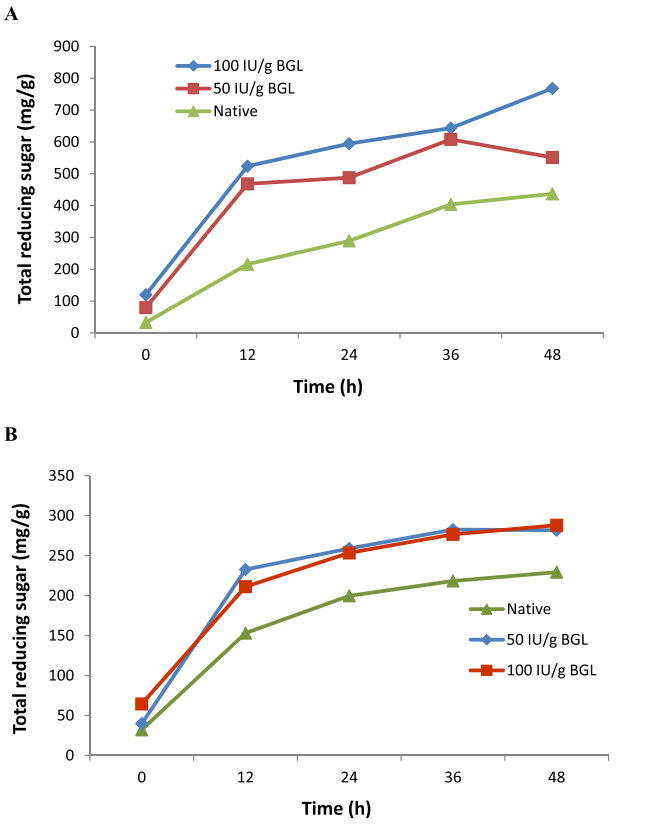


Fig. 2. Hydrolysis of sorghum stover biomass by native and BGL blended *T. reesei* cellulase. A) Enzymatic hydrolysis of acid pretreated SS B) Enzymatic hydrolysis of pretreated SS.

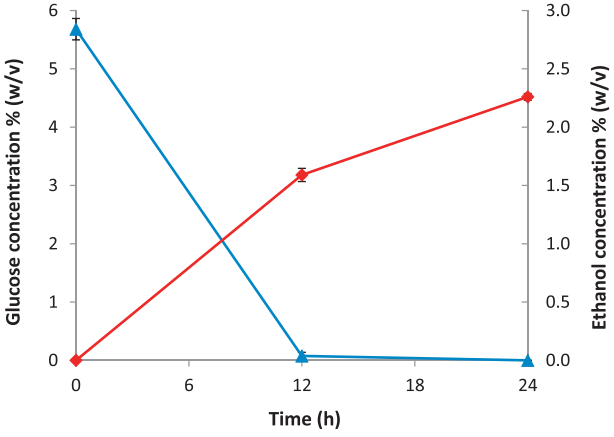


Fig. 3. Bioethanol production using sorghum stover hydrolysate.

is known to improve its hydrolytic potential (Chir et al., 2011; Hu et al., 2011). Apparently, with BGL blending the SSF produced *T. reesei* cellulase is an effective way for improving its hydrolytic efficiency. With the results, it becomes clear that the strategy works well with sorghum biomass as well.

Ethanol production from the enzymatic hydrolysate of alkali pretreated sorghum stover was studied using a lab strain of *Saccharomyces cerevisiae*. The hydrolysate generated using *T. reesei* cellulase supplemented with 100 IU/g of *A niger* BGL contained 56.67 g/L of glucose after concentration. Almost entire glucose was consumed in about 12 h duration and the maximal ethanol yield of 22.2 g/L was obtained in 24 h (Fig. 3) which corresponded to

76.8% conversion efficiency. This proved that the hydrolysate is efficiently fermented by a standard yeast strain.

The current work has addressed onsite production of cellulase and its use for hydrolysis of sorghum stover biomass which is one of the important but under exploited feed stock resources in India. Fermentation process for cellulase production has been demonstrated at pilot scale and the enzyme was successfully used for biomass hydrolysis. Onsite enzyme production reduces the enzyme cost due to the savings on logistics, storage and the stabilization of enzyme and this in turn is expected to reduce the cost of bioethanol from biomass.

4. Conclusions

SSF was used for cellulase production employing *T. reesei* and by optimizing process variables a 3.2-fold increased yield was obtained. The process was demonstrated at pilot scale in tray fermenter system. Supplementation of *A. niger* BGL to the cellulase improved its hydrolytic efficiency 174% indicating synergy between these enzymes. Sorghum stover hydrolysate was efficiently fermented to ethanol. Results indicate the potential of using on-site enzyme production using SSF, and its use for sorghum stover hydrolysis and ethanol production.

Acknowledgements

ASOI gratefully acknowledge the fellowship provided by Department of Biotechnology (DBT) Government of India, and The World Academy of Sciences for the Advancement of Science in developing countries (TWAS) for his PhD work. Authors would like to acknowledge financial support from Technology Information, Forecasting and Assessment Council (TIFAC) and Department of Science and Technology Government of India, for Centre for Biofuels through projects TIFAC/CBF-II/14 and DST/INT/AUS/GCP-5/13(G).

References

Beckham, G.T., Bomble, Y.J., Matthews, J.F., Taylor, C.B., Resch, M.G., Yarbrough, J.M., Decker, S.R., Bu, L., Zhao, X., McCabe, C., Wohlert, J., Bergenstrahle, M., Brady, J. W., Adney, W.S., Himmel, M.E., Crowley, M.F., 2010. The O-Glycosylated linker from the *Trichoderma reesei* family 7 cellulase is a flexible, disordered protein. Biophys. J. 99, 3773–3781. http://dx.doi.org/10.1016/j.bpj.2010.10.032.

Bischof, R., Fourtis, L., Limbeck, A., Gamauf, C., Seiboth, B., Kubicek, C.P., 2013. Comparative analysis of the *Trichoderma reesei* transcriptome during growth on the cellulase inducing substrates wheat straw and lactose. Biotechnol. Biofuels 6, 127. http://dx.doi.org/10.1186/1754-6834-6-127.

Box, G.E.P., Behnken, D.W., 1960. Some new three level designs for the study of quantitative variable. Technometrics 2, 455–475.

Chen, H. 2013. Modern Solid State Fermentation, Theory and Practice. Springer, Heidelberg.

Chir, J.L., Wan, C.F., Chou, C.H., Wu, A.T., 2011. Hydrolysis of cellulose in synergistic mixtures of β-glucosidase and endo/exocellulase Cel9A from *Thermobifida fusca*. Biotechnol. Lett. 33, 777–782. http://dx.doi.org/10.1007/s10529-010-0500-9.

Figueroa-Montero, A., Esparza-Isunza, T., Saucedo-Castaneda, G., Huerta-Ochoa, S., Gutierrez-Rojas, M., Favela-Torres, E., 2011. Improvement of heat removal in solid-state fermentation tray bioreactors by forced air convection. J. Chem. Technol. Biotechnol. 86, 1321–1331.

Ghose, T.K., 1987. Measurement of cellulase activities. Pure Appl. Chem. 59, 257–268. http://dx.doi.org/10.1351/pac198759020257.

Gonsalves, J.H., 2006. An Assessment if the Biofuels industry in India: A United Nations conference on trade and conference. UNCTAD/DIT/TED/2006/6.

Hu, J., Arantes, V., Saddler, J.N., 2011. The enhancement of enzymatic hydrolysis of lignocellulosic substrates by the addition of accessory enzymes such as xylanase: is it an additive or synergistic effect? Biotechnol. Biofuels 4, 36. http://dx.doi.org/10.1186/1754-6834-4-36.

Kilikian, B.V., Afonso, L.C., Souza, T.F.C., Ferreira, R.G., Pinheiro, I.R., 2014. Filamentous fungi and media for cellulase production in solid-state cultures. Braz. J. Microbiol. 45, 279–286. http://dx.doi.org/10.1590/S1517-83822014005000028.

Kubicek, C.P., Mikus, M., Schuster, A., Schmoll, M., Seiboth, B., 2009. Metabolic engineering strategies for the improvement of cellulase production by *Hypocrea jecorina*. Biotechnol. Biofuels 2, 19. http://dx.doi.org/10.1186/1754-6834-2-19.

Kuttiraja, M., Sindhu, R., Preeti, E.V., Sandhya, S.V., Binod, P., Vani, S., Pandey, A., Sukumaran, R.K., 2013. Bioethanol production from bamboo (*Dendrocalamus* sp) process waste. Biomass Bioenergy 59, 142–150.

Le Crom, S., Schackwitz, W., Pennacchio, L., Magnuson, J.K., Culley, D.E., Collett, J.R., Martin, J., Druzhinina, I.S., Mathis, H., Monot, F., Seiboth, B., Cherry, B., Rey, M., Berka, R., Kubicek, C.P., Baker, S.E., Margeot, A., 2009. Tracking the roots of cellulase hyperproduction by the fungus *Trichoderma reesei* using massively parallel DNA sequencing. Proc. Natl. Acad. Sci. U.S.A. 106, 16151–16156. http://dx.doi.org/10.1073/pnas.0905848106.

Mandels, M., Weber, J., 1969. The production of cellulases. Adv. Chem. Ser. 95, 391–413.

Martinez, D., Berka, R.M., Henrissat, B., Saloheimo, M., Arvas, M., Baker, S.E., Chapman, J., Chertkov, O., Coutinho, P.M., Cullen, D., Danchin, E.G.J., Grigoriev, I. V., Harris, P., Jackson, M., Kubicek, C.P., Han, C.S., Ho, I., Larrondo, L.F., de Leon, A. L., Magnuson, J.K., Merino, S., Misra, M., Nelson, B., Putnam, N., Robbertse, B., Salamov, A.A., Schmoll, M., Terry, A., Thayer, N., Westerholm-Parvinen, A., Schoch, C.L., Yao, J., Barabote, R., Barbote, R., Nelson, M.A., Detter, C., Bruce, D., Kuske, C.R., Xie, G., Richardson, P., Rokhsar, D.S., Lucas, S.M., Rubin, E.M., Dunn-Coleman, N., Ward, M., Brettin, T.S., 2008. Genome sequencing and analysis of the biomass-degrading fungus *Trichoderma reesei* (syn. *Hypocrea jecorina*). Nat. Biotechnol. 26, 553–560. http://dx.doi.org/10.1038/nbt1403.

Miller, G.L., 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal. Chem. 31, 426–428. http://dx.doi.org/10.1021/ac60147a030.

Pandey, A., 2003. Solid-state fermentation. Biochem. Eng. J. 13, 81–84. http://dx.doi.org/10.1016/S1369-703X(02)00121-3.

Pandey, A., Biswas, S., Sukumaran, R.K., Kaushik, N., 2009. Study on Availability of Indian Biomass Resources for Exploitation: A Report Based on a Nationwide Survey. TIFAC, New Delhi.

Plackett, R.L., Burman, J.P., 1946. The design of optimum multifactorial experiments. Biometrika 33, 305–325. http://dx.doi.org/10.1093/biomet/33.4.305.

Rao, D.B., Ratnavathi, C.V., Sashidhar, R.C., Rao, S.S., 2007. Potential alternate feedstock for bioethanol in India: Sweet sorghum, a bioenergy crop. Agric. Situat. India 14, 243–251.

Selig, M.J., Viamajala, S., Decker, S.R., Tucker, M.P., Himmel, M.E., Vinzant, T.B., 2007. Deposition of lignin droplets produced during dilute acid pretreatment of maize stems retards enzymatic hydrolysis of cellulose. Biotechnol. Prog. 23, 1333–1339. http://dx.doi.org/10.1021/bp0702018.

Singhania, R.R., Sukumaran, R.K., Pandey, A., 2007. Improved cellulase production by *Trichoderma reesei* RUT C30 under SSF through process optimization. Appl. Biochem. Biotechnol. 142, 60–70. http://dx.doi.org/10.1007/s12010-007-0019-2.

Singhania, R.R., Sukumaran, R.K., Patel, A.K., Larroche, C., Pandey, A., 2010. Advancement and comparative profiles in the production technologies using solid-state and submerged fermentation for microbial cellulases. Enzyme Microb. Technol. 46, 541–549. http://dx.doi.org/10.1016/j.enzmictec.2010.03.010.

Singhania, R.R., Sukumaran, R.K., Rajasree, K.P., Mathew, A., Gottumukkala, L., Pandey, A., n.d. Properties of a major β-glucosidase-BGL1 from Aspergillus niger NII-08121 expressed differentially in response to carbon sources. Process Biochem. 46, 1521–1524.

Sluiter, A., Hames, B., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D., Crocker, D., 2008a. Determination of Structural Carbohydrates and Lignin in Biomass: Laboratory Analytical Procedure (LAP) (Revised July 2011).

Sluiter, A., Hames, B., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D., 2008b. Determination of Sugar, byproduct, and Degradation Products in Liquid Fraction Process Sample: Laboratory Analytical Procedure (LAP); Issue Date 12/08/2006.

Vitcosque, G.L., Fonseca, R.F., Rodríguez-Zúñiga, U.F., Neto, V.B., Couri, S., Farinas, C. S., 2012. Production of biomass-degrading multienzyme complexes under solid-state fermentation of soybean meal using a bioreactor. Enzyme Res. 2012,, 9. http://dx.doi.org/10.1155/2012/248983 248983.

Title: Applications of computational science for understanding enzymatic deconstruction of cellulose

Author: Gregg T Beckham,Yannick J Bomble,Edward A Bayer,Michael E Himmel,Michael F Crowley

Publication: Current Opinion in Biotechnology

Publisher: Elsevier

Date: April 2011

Order Completed

Thank you for your order.

This Agreement between Rajeev Sukumaran ("You") and Elsevier ("Elsevier") consists of your license details and the terms and conditions provided by Elsevier and Copyright Clearance Center.

Your confirmation email will contain your order number for future reference.

printable details

License Number	4275300602594
License date	Jan 24, 2018
Licensed Content Publisher	Elsevier
Licensed Content Publication	Current Opinion in Biotechnology
Licensed Content Title	Applications of computational science for understanding enzymatic deconstruction of cellulose
Licensed Content Author	Gregg T Beckham,Yannick J Bomble,Edward A Bayer,Michael E Himmel,Michael F Crowley
Licensed Content Date	Apr 1, 2011
Licensed Content Volume	22
Licensed Content Issue	2
Licensed Content Pages	8
Type of Use	reuse in a thesis/dissertation
Portion	figures/tables/illustrations
Number of figures/tables/illustrations	1
Format	both print and electronic
Are you the author of this Elsevier article?	No
Will you be translating?	No
Original figure numbers	Figure 2
Title of your thesis/dissertation	Fungal Cellulase Production and Applications
Expected completion date	Jan 2018
Estimated size (number of pages)	120
Requestor Location	Rajeev Sukumaran Centre for Biofuels, CSIR-NIIST Industrial Estate P.O. Trivandrum, 695019 India Attn: Rajeev K Sukumaran
Customer VAT ID	INBCRPS0195Q
Total	0.00 USD

ORDER MORE | CLOSE WINDOW

SPRINGER NATURE

Title: Enzymes for Bioenergy
Author: Rajeev K. Sukumaran, Amith Abraham, Anil K. Mathew
Publication: Springer eBook
Publisher: Springer Nature
Date: Jan 1, 2017

Order Completed

Thank you for your order.

This Agreement between Rajeev Sukumaran ("You") and Springer Nature ("Springer Nature") consists of your license details and the terms and conditions provided by Springer Nature and Copyright Clearance Center.

Your confirmation email will contain your order number for future reference.

printable details

License Number	4275301452372
License date	Jan 24, 2018
Licensed Content Publisher	Springer Nature
Licensed Content Publication	Springer eBook
Licensed Content Title	Enzymes for Bioenergy
Licensed Content Author	Rajeev K. Sukumaran, Amith Abraham, Anil K. Mathew
Licensed Content Date	Jan 1, 2017
Type of Use	Thesis/Dissertation
Requestor type	academic/university or research institute
Format	print and electronic
Portion	full article/chapter
Will you be translating?	no
Circulation/distribution	<501
Author of this Springer Nature content	yes
Title	Fungal Cellulase Production and Applications
Instructor name	n/a
Institution name	n/a
Expected presentation date	Jan 2018
Portions	Portions from Sections 2, 3, 4, 5
Requestor Location	Rajeev Sukumaran Centre for Biofuels, CSIR-NIIST Industrial Estate P.O. Trivandrum, 695019 India Attn: Rajeev K Sukumaran
Customer VAT ID	INBCRPS0195Q
Billing Type	Invoice
Billing address	Rajeev K Sukumaran Centre for Biofuels, CSIR-NIIST Industrial Estate P.O. Trivandrum, India 695019 Attn: Rajeev K Sukumaran
Total	0.00 USD

ORDER MORE | CLOSE WINDOW