Beta Glucosidase from *Aspergillus niger* NII 08121: Molecular Characterization and Applications in Bioethanol production

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Reeta Rani Singhania
Biotechnology Division

National Institute for Interdisciplinary
Science & Technology – CSIR
Thiruvananthapuram 695019, INDIA

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Reeta Rani Singhania

Reg. No. 3455

BIOTECHNOLOGY DIVISION
National Institute for Interdisciplinary Science & Technology –CSIR
Thiruvananthapuram 695019, INDIA
January 2011

Rajeev Kumar S, MSc PhD
Scientist, Biotechnology Division

National Institute for Interdisciplinary Science and Technology
Council of Scientific and Industrial Research, Government of India
Pappanamcode, Thiruvananthapuram 695019, Kerala, India
Phone : +91 471 2515368, Fax: +91 471 2491712
Email : rajeev.csir@gmail.com; rajeevs@niist.res.in

28 December 2010

DECLARATION

I hereby declare that the work presented in this thesis entitled " Beta Glucosidase from *Aspergillus niger* NII 08121- Molecular characterization and applications in bioethanol production" is based on the original work done by Ms Reeta Rani Singhania (Reg # 3455), under my guidance and supervision, at the National Institute for Interdisciplinary Science and Technology, CSIR, Trivandrum, India. I also declare that this work or no part of this work has been submitted for the award of any degree, diploma, associateship or any other title or recognition.

Rajeev Kumar S

Thiruvananthapuram
30 December 2010

DECLARATION

I hereby declare that the work presented in this thesis entitled "**β-Glucosidases from *Aspergillus niger* NII 08121- Molecular Characterization and Applications in Bioethanol Production"** is based on the original work done by me under the guidance of Dr Rajeev Kumar Sukumaran, Scientist, Biotechnology Division, National Institute for Interdisciplinary Science and Technology, CSIR, Trivandrum, India and the thesis or no part of it has been submitted elsewhere for the award of any Degree, Diploma, Associateship or any other Title or Recognition.

Reeta Rani Singhania

Dedicated to my parents and teachers

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CHAPTER 1

Introduction and Review of Literature

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1. Introduction

1.1. Enzymes for biomass-to-ethanol-conversion

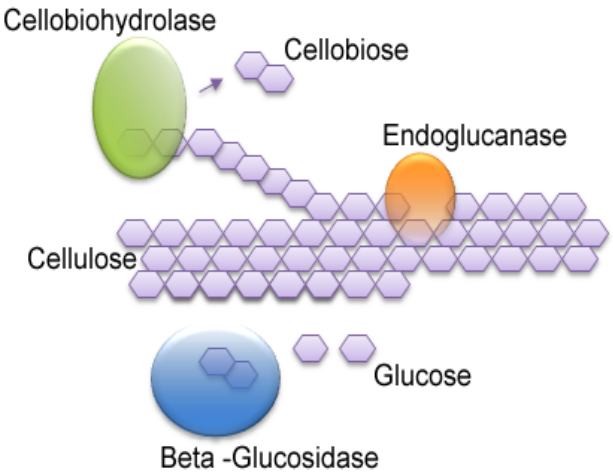
Bio-refinery concept of generating commodities replacing the conventional petrochemical route is now regarded as the future of industry and more and more research is now directed towards moving to carbohydrate based products. Bio-ethanol production from lignocellulosic biomass is emerging as one of the most important technologies for sustainable production of renewable transportation fuels. Ethanol has a higher octane rating than gasoline and produces fewer emissions, and is therefore widely recognized as a substitute and/or additive to gasoline (Wyman 1999). Due to these apparent advantages and also being a renewable alternative to existing transport fuels, there is now an increased interest in commercializing technologies for ethanol production from inexpensive biomass (Schell *et al*, 2004). Most of the fuel ethanol produced in the world is currently sourced from starchy biomass or sucrose (corn /beet starch, molasses or cane juice). These feedstock are also food or feed which leads to a direct competition with their use as food or feed. Therefore the technology for economical conversion non-food biomass/lignocellulosic biomass is actively sought worldwide and is expected to be realized in the in the coming years.

The production of ethanol from lignocellulosic biomass involves the different steps of pretreatment, hydrolysis (saccharification) and ethanol recovery (van Zessen *et al*, 2003). Hydrolysis of biomass is essential for generation of fermentable sugars which are then converted to ethanol by microbial action. Two methods, i.e. acid hydrolysis and enzymatic hydrolysis are primarily employed for biomass hydrolysis with varying efficiencies depending on treatment conditions, type of biomass and the properties of the hydrolytic agents. The former is a mature technology but with the disadvantages of generation of hazardous acidic waste and the technical difficulties in recovering sugar from the acid. The enzymatic method, however, is more efficient and proceeds under ambient conditions without generation of any toxic waste. The later method which is under rapid development has immense potentials for improvement in cost and efficiency (Mishima *et al*, 2006). Commercialization of ethanol production from lignocellulosic

biomass is hindered mainly by the prohibitive cost of the currently available cellulase preparations – the enzymes used for saccharification. Reduction in the cost of cellulases can be achieved only by concerted efforts which address several aspects of enzyme production from the raw material used for production to microbial strain improvement. Use of cheaper raw materials and cost effective fermentation strategies like solid-state fermentation can improve the economics of cellulase production. Reduction in cost of ‘‘bio-ethanol’’ may also be achieved by efficient technologies for saccharification which includes the use of better ‘‘enzyme cocktails’’ and conditions for hydrolysis.

Cellulases are multi enzyme complexes whose synthesis and action are intricately controlled by regulatory mechanisms in the organisms that produce these enzymes. Majority of commercial cellulases are currently produced from a species of fungus called *Trichoderma reesei*. The enzymatic hydrolysis of cellulose involves three types of cellulase activities (cellobiohydrolases, endoglucanases and β-glucosidases) working in synergy (Lynd *et al*, 2002). Endoglucanases (EC 3.2.1.4) randomly cleave the β-1,4 glycosidic linkages of cellulose; cellobiohydrolases (EC 3.2.1.91) attack cellulose chain ends to produce the cellobiose (a dimer of glucose linked by a β-1,4 glycosidic bond); and β-glucosidases (EC 3.2.1.21) that hydrolyze cellobiose into two molecules of glucose (Fig 1.1).

Fig 1.1 Enzymatic hydrolysis of cellulose schematic diagram showing cellulase synergy



Trichoderma reesei produces insufficient β-glucosidase (BGL) activity which results in cellobiose accumulation. Cellobiose inhibits the action of cellobiohydrolases and endoglucanase (Shewale 1982). Also the BGL from this fungus is subject to product

inhibition making the use of this cellulase complex less efficient. Blending of heterologus BGL which can work at elevated glucose concentrations can improve the saccharification efficiency of *T. reesei* cellulase complex (Krogh *et al,* 2004, Tu *et al,* 2006) and hence improve the economics of bioethanol production.

1.2. β-glucosidases

"*β*-glucosidases (β-D-glucoside glucohydrolase, EC 3.2.1.21) are well characterized, biologically important enzymes that catalyze the transfer of glycosyl group between oxygen nucleophiles. These transfer reaction results in the hydrolysis of β-glucosidic linkage present between carbohydrate residues in aryl-amino-, or alkyl-β-D-glucosides, cyanogenic-glucosides, short chain oligosaccharides and disaccharides under physiological conditions, whereas; under defined conditions, synthesis of glycosyl bond between different molecules can occur. It occurs by two modes reverse hydrolysis and transglycosylation. In reverse hydrolysis, modification of reaction conditions such as lowering of water activity (aw), trapping of product or high substrate concentration leads to a shift in the equilibrium of reaction toward synthesis. This reaction is under thermodynamic control. In transglycosylation approach, a preformed donor glycoside (e.g., a disaccharide or aryl-linked glucoside) is first hydrolyzed by the enzyme with the formation of an enzyme-glycosyl intermediate. This is then trapped by a nucleophile other than water (such as a monosaccharide, disaccharide, aryl-, amino-, or alkyl-alcohol or monoterpene alcohol) to yield a new elongated product. This reaction is under kinetic control" (Bhatia *et al,* 2002).

β-glucosidases are widely distributed in the living world and they play pivotal roles in many biological processes. The physiological roles associated with this enzyme are diverse and depend on the location of the enzyme and the biological system in which these occur. In cellulolytic microorganisms, β-glucosidase is involved in cellulase induction and cellulose hydrolysis (Bisaria & Mishra, 1989, Tomme *et al,* 1995). In plants, the enzyme is involved in β-glucan synthesis during cell wall development, pigment metabolism, fruit ripening, and defense mechanisms (Esen, 1993, Brozobohaty *et al,* 1993) whereas, in humans and other mammals, BGL is involved in the hydrolysis of glucosyl ceramides

(Libermann *et al,* 2007). Due to their wide and varied roles in nature, these versatile enzymes can be of use in several synthetic reactions as reviewed by Bhatia *et al,* (2002).

1.3. Microbial production of β-glucosidase

Microbial sources have been widely exploited for β-glucosidase production by both solid-state fermentation (SSF) and submerged fermentation. There are several reports available for β-glucosidase productions from filamentous fungi such as *Aspergillus niger* (Gunata & Vallier 1999), *A oryzae* (Riou *et al,* 1998), *Penicillium brasilianum* (Krogh *et al,* 2010) *P. decumbens* (Chen *et al,* 2010), *Phanerochaete chrysosporium* (Tsukada *et al,* 2006), *Paecilomyces* sp., (Yang *et al,* 2009) etc., though there are also various reports of β-glucosidase production from yeasts (majority of them from *Candida* sp.) and few bacteria. Submerged fermentation offers the advantage of controlled conditions such as aeration and pH whereas, solid-state fermentation provides a cheaper alternative production technology, as crude biomass can be employed as substrate for the production of the metabolites. SSF imitates natural habitat of these filamentous fungi and thus are better adapted and produces higher enzyme titers which can be directly employed for biomass hydrolysis (Reimbault, 1998, Pandey *et al,* 1999). High water activity is projected as a probable reason for high production of metabolites in SSF by microorganisms (Pandey *et al,* 1999).

1.4 Differential expression of β- glucosidase isoforms

Several filamentous fungi exhibit the property of expressing different isoforms of BGL depending on the culture conditions or carbon sources (Willick & Seligy 1985, Nazir *et al,* 2010). Various isoforms of endoglucanase and β-glucosidase are reported to be expressed in response to carbon sources in *Aspergillus terreus* (Nazir *et al,* 2010). The sequential induction of isoforms has been associated with the presence of distinct metabolites (Villas-Bôas *et al,* 2006, Panagiotou *et al,* 2005). As an accepted model, the induction of the cellulases is mediated either by low molecular weight soluble oligosaccharides that are released from complex substrates as a result of hydrolysis by constitutive enzymes or by the products (positional isomers) of transglycosylation reactions mediated by constituent β- glucosidase, xylanases, etc (Badhan *et al,* 2007).

These metabolites enter the cell and signal the presence of extracellular substrates and provide the stimulus for the accelerated synthesis of constituent enzymes of cellulase complex. However, this process is complex in view of the fact that many fungi and bacteria are known to express functionally multiple cellulases/hemicellulases in presence of different carbon sources. This multiplicity may be the result of genetic redundancy, differential mRNA processing or post translational modification such as glycosylation, autoaggregation or/and proteolytic digestion (Collins *et al,* 2005). However, the regulation of expression of these multiple isoforms is still not clear which necessitates further research regarding the sequential and differential expression of the isoforms. It must be emphasized that though the regulation of cellulases is apparently mediated through induction and repression as the two major mechanisms of controlling the expression of these enzymes, the existence of highly specialized and complex nature of regulating the expression of cellulases in diverse microorganisms has also been reported (Badhan *et al,* 2007a & b, Sánchez-Herrera *et al,* 2007). There may be relationship between the metabolites present in the culture extracts and the induction of different isoforms. The understanding about regulation would be important in designing culture conditions for overproducing desired kind of isoforms or secondary metabolites. The structure and nature of carbon source can also play an important role in differential induction of the enzyme system. Culturing under submerged or solid substrate fermentation also influences the expression of distinct isoforms.

Multiplicity of cellulases and hemicellulases is well known in the case of filamentous fungi (Willick & Seligy, 1985, Decker *et al,* 2000, Nazir *et al,* 2010) and probably this multiplicity is essential, considering the vast and diverse roles these enzymes play in fungal metabolism and survival. β-glucosidase multiplicity can be attributed to the presence of multiple genes or due to differential post transcriptional modifications (Collins *et al,* 2007, Iwashita *et al,* 1999). Differential expression of the various BGL proteins are reported in response to the carbon sources supplied in the medium or the conditions of culture (Willick & Seligy 1985, Nazir *et al,* 2010) and could be a probable adaptation of the fungi to respond to the changing immediate environments. This property however, could be exploited for selective expression of a desired isoform from a fungus by manipulating the culture conditions/carbon source carefully.

1.5 Classification of β-glucosidases

Beta-glucosidases are a heterogeneous group of hydrolytic enzymes and have been classified according to various criteria. There is no single well-defined method for the classification of these versatile enzymes. In general, two methods for their classification appear in the literature, on the basis of (1) substrate specificity, and (2) nucleotide sequence identity (NSI) (Henrissat and Bairoch, 1996).

Based on substrate specificity, these enzymes have been classified as (1) aryl β-glucosidases, which act on aryl-glucosides, (2) true cellobiases, which hydrolyze cellobiose to release glucose, and (3) broad substrate specificity enzymes, which act on a wide spectrum of substrates. Most of the β-glucosidases characterized so far are placed in the last category. The most accepted method of classification is by nucleotide sequence identity scheme, proposed by Henrissat and Bairoch (1996) based on sequence and folding similarities (hydrophobic cluster analysis, HCA) of these enzymes. HCA of a variety of such enzymes suggested that the α-helices and the β-strands were localized in similar positions in the folded conformation. Moreover, a number of highly conserved amino acids were also clustered near the active site. Such a classification is expected to reflect structural features, evolutionary relationships, and catalytic mechanism of these enzymes. Also, the identification of the nucleophile and the putative acid-base catalyst in one member of a family in effect identifies them in all members of the family. It is also expected that as the size of the family increases, the residues conserved in all members of the family usually will be important, structurally or catalytically. More sequence data and three-dimensional structure of enzymes belonging to these families are required to confirm this scheme. The sequence based classification is useful in characterizing the enzymes from the structural point of view but the substrate specificity with respect to the aglycone moiety still serves a primary, or, in some cases, the only lead in isolating and characterizing unknown or structurally undefined glucosidases. The β-glucosidases are mostly placed in either family 1 or family 3 of glycosyl hydrolases though these enzymes are also found in families 5, 9 and 30 of glycosyl hydrolases (Henrissat, 1991, Cantarel *et al*, 2009, Oppasiri *et al*, 2007) . Family 1 comprises nearly 62 β-glucosidases from archaebacteria, plants, mammals, and also includes 6-phosphoglycosidases and thioglucosidases. Most family 1 enzymes, also show significant β-galactosidase activity.

The family1 β-glucosidases are also classified as members of the 4/7 super family with a common eight-fold β/α barrel motif (Fig1.2).

Fig 1.2. Representative structure of 4/7 super family with the eight fold β/α barrel motif.

Thermoascus aurantiacus xylanase 10A (TAX) structure showing the eight fold β/α barrel motif (Lo Leggio *et al,* 2001)

Here, the active site is placed in a wide cavity defined along the axis of the barrel, with a putative acid/base catalyst located at the end of β-strand 4 and a catalytic nucleophile near the end of β-strand 7 (Kaper *et al*, 2000). The 4/7 super family also includes other enzymes like family 2 β-galactosidase, family 5 cellulases, family 10 xylanase, and family 17 barley glucanases (Jenkins *et al*, 1995).

"Family 3 of glycosyl hydrolases consists of nearly 44 β-glucosidases and hexosaminidases of bacterial, mold, and yeast origin. Structural data on representatives of GH3 are still scarce, since only three of their structures are known and only one of them has been thoroughly characterized—that of a β-D-glucan (exo1→3, 1→4) glucanase (Exo 1) from *Hordeum vulgare*, which catalyzes the hydrolysis of call-wall polysaccharides. The enzyme consists of N-terminal (α/β) 8 TIM barrel domain and a C-terminal domain of six stranded β sandwich. The non-homologous region, a helix-like strand of 16 amino acid residues, connects the two domains" (Bhatia *et al*, 2002). The catalytic center is located in the pocket at the interface of the two domains. Asp285 in the N-terminal domain acts as a catalytic nucleophile, while Glu491 in the C-terminal domain acts as a proton donor (Varghese *et al*, 1999).

1.6 Cloning of β-glucosidase genes

"The *β*-glucosidase genes from a large number of bacterial, mold, yeast, plant, and animal systems have been cloned and expressed in both *E.coli* and eukaryotic hosts such as *S. cerevisiae* and filamentous fungi. Cloning has been performed by two methods, either by (1) formation of a genomic DNA library followed by selection of the recombinant clones by screening for β-glucosidase production, or (2) starting with a cDNA library (or a genomic library), screening of recombinant clones by specific nucleotide probes designed from *a-priori* knowledge of the polypeptide sequence" (Bhatia *et al,* 2002). Though fungi are known to be good producers of the enzyme, reports on cloning of BGL from fungi are relatively low. This is mostly due to the complexities associated with the presence of introns in their genes and due to complexities associated with glycosylation. Nevertheless, several researchers have successfully cloned and expressed beta glucosidases from fungi including *Aspergilli* (Iwashita *et al,* 1999, Dan *et al*, 2000, Kim *et al*, 2007). Majority of the reports also mentions the existence of multiple genes and gene products that are differentially expressed. Fungal genes have been cloned and expressed mostly in eukaryotic expression systems like *Trichoderma reesei* (Barnet *et al,* 1991), *Aspergillus* sp (Takashima *et al,* 1999), *S. cerevisiae* (Dan *et al* ., 2000), and *Pichia pastoris* (Dan *et al*, 2000).

1.7 Importance of β-glucosidase in bioethanol production

The cellulolytic enzyme system secreted by the filamentous fungi *Trichoderma reesei* is the one mostly used in industrial applications. The hydrolysis step converting cellulose to glucose is recognized as the major limiting operation in the development of processes for production of biofuels from lignocellulosic raw materials because of the low efficiency of cellulases and their cost. Enzymatic hydrolysis of cellulose is a multistep complex process, the last step being a homogenous catalysis reaction involving the action of β-glucosidase on cellobiose (Lynd *et al,* 2002). Cellobiose is a strong inhibitor of both cellobiohydrolases and endocellulases, and the β-glucosidase action can reduce its effect. In addition, the produced glucose also inhibits cellulolysis, although to a lesser extent (Dekker, 1986). Glucose at high concentration can either block the active site for the substrate or prevent the hydrolyzed substrate from leaving (Krogh *et al,* 2010). The amount of β-glucosidase-1 (BGL1) generated by *T. reesei* hyper-producing strains represents a very low percentage of the total secreted proteins (Lynd *et al,* 2002, Herpoël-Gimbert *et al,* 2008). The less abundance of BGL even under conditions of cellulase induction and the product inhibition to which it is susceptible, limits the use of native cellulase preparations in lignocellulosic biomass hydrolysis for alcohol production. This limitation can be alleviated either by over expressing β-glucosidase in *T. reesei* or by adding extra β-glucosidase from other sources (Kumar *et al,* 2008, Xin *et al,* 1993). Supplementing the native *T. reesei* enzymatic cocktail with β-glucosidase from other fungi is often performed to avoid inhibition of cellobiose (Xiao *et al,* 2004).

Glucose tolerant BGL can circumvent the problem of feedback inhibition, and if available in an enzyme cocktail for biomass hydrolysis can improve the efficiency of hydrolysis by shifting the equilibrium towards a higher product concentration than otherwise achievable (Sukumaran *et al,* 2005). Few species of *Aspergilli* are known to produce glucose tolerant β-glucosidases and some of these enzymes have been cloned and characterized (Riou *et al,* 1998, Gunata & Vallier, 1999). It is expected that more of such glucose tolerant BGLs may be prevalent in nature especially in filamentous fungi. Isolation of such enzymes and knowledge about their properties, sequences and expression patterns can help in design of better enzyme cocktails for biomass hydrolysis as well as in targeted approaches for modifying the glucose tolerance of existing BGLs.

Commercially, the enzyme majors Genencor and Novozymes have launched a series of cocktails of cellulolytic enzymes for biomass hydrolysis, such as Accelerase® series of enzymes (Genecor, 2010) and the Cellic series of enzymes (Novozymes, 2010). The advanced enzyme preparations from both the companies contain beta glucosidase supplements indicating the importance of this enzyme in biomass hydrolysis.

1.8. Conclusions

The biochemical platform for fuel ethanol production from lignocellulosic biomass is now limited by the prohibitive cost of cellulases. Though the commercially exploited fungus *Trichoderma* produces a complete cellulolytic system, the rate limiting enzyme *β*-glucosidase is produced in very less quantities. Also this enzyme is slow acting. Supplementation of exogenous *β*-glucosidase to the *T. reesei* cellulase will enable improvement in efficiency of biomass hydrolysis and cost reduction of biomass-to ethanol conversion by reducing feedback inhibition and cellobiose mediated repression of cellulases. An *Aspergillus niger* strain isolated at NIIST was found to secrete very high titers of BGL which comprised of multiple isoforms of this enzyme. The enzyme preparation was also active at 250mM glucose concentration indicating its suitability as a supplement in the biomass hydrolyzing enzyme complex. It was therefore decided to study the production of this enzyme, its expression, multiplicity, and properties besides its evaluation along with *T. reesei* cellulase for biomass hydrolysis for ethanol production

1.9. Objectives and Scope of the Current Study

The scope of the present investigation was confined to the following objectives

1. Evaluation of carbon sources for BGL production by *A. niger* NII 08121
2. Production of BGL by *A. niger* NII 08121 under SmF and SSF
3. Optimization of BGL production by the fungus to improve yield
4. Studies on the differences in expression of BGL isoforms in response to carbon sources
5. Purification of the enzyme and characterization of its properties
6. Confirmation of BGL multiplicity by cloning of BGLs belonging to glycosyl hydrolase families 1, 3 and 5
7. Use of *A. niger* BGL in bioethanol production

    i. Production of *T. reesei* cellulase, and using *A. niger* BGL to create biomass hydrolyzing enzyme blends
    ii. Hydrolysis of alkali pretreated rice straw by enzyme cocktails
    iii. Optimization of enzyme cocktails for hydrolysis of alkali pretreated rice straw.
    iv. Production of ethanol from biomass hydrolysate

CHAPTER 2

Materials and Methods

Chapter 2
Materials and Methods

2.1. Microorganisms and preparation of inoculum

The fungal cultures *Aspergillus niger* NII 08121 and *Trichoderma reesei* RUT C30 were used in this study for the production of β-glucosidase (BGL) and cellulase respectively, and the yeast *Saccharomyces cerevisiae* NCIM 3288 was used for alcohol fermentation. *Aspergillus niger* was isolated from decaying wood at the Biotechnology division of NIIST and identified by the Microbial Type Culture Collection (MTCC), Chandigargh, India. *Trichoderma reesei* RUT-C30, was a kind gift from Prof George Scakacs, Technical University of Budapest, Hungary and *Saccharomyces cerevisiae* NCIM 3288 was procured from National Collection of Industrial Microorganisms (NCIM), Pune, India

Fungal cultures were inoculated on Potato Dextrose Agar slants and were incubated at 30^{o}C. The fully sporulated slants obtained after three days in case of *A. niger* and five days in case of *T. reesei* were either used immediately or stored at 4 $^{\circ}$C for short term preservation. Both cultures were also deposited in NII culture collection centre at NIIST. For preparing the spore inoculum, sterile distilled water containing 0.05% (w/v) Tween-80 was added to the slants and the spores were dislodged into it by gentle pipetting under aseptic conditions. The suspension was recovered by aspiration and transferred to sterile 15ml tubes. The suspension was appropriately diluted with sterile distilled water containing 0.05% Tween-80 to obtain the required spore count. Spore counts were done under a phase contrast microscope using a hemocytometer. One milliliter of this spore suspension was used to inoculate the medium. In case of submerged fermentation (SmF), the medium was inoculated with 10^{7} spores per 100ml.

S. cerevisae was grown in YEP broth (Himedia, India) for 12h with 180 rpm agitation on rotary shaker and the culture was used at 10% v/v as inoculum for alcohol fermentation.

2.2 Medium preparation

2.2.1 *Solid State Fermentation (SSF)*

Wheat bran (WB) was used as substrate for SSF. WB purchased locally from a flour mill was dried overnight at 60 °C in a hot air oven to remove moisture. Five grams of the substrate was weighed into 250 ml Erlenmeyer flasks and was moistened with a specific amount of mineral salt medium. Distilled water was added in addition to the medium to attain the appropriate initial moisture content wherever applicable. The basal mineral salts solution used for the experiment for β-glucosidase production had following composition in g/l: Urea - 0.3, $(NH_4)_2SO_4$ - 1.4, KH_2PO_4 - 0.4, $MgSO_4.7H_2O$ - 0.3, Peptone - 0.75, Yeast extract - 0.25, $FeSO_4.7H_2O$ - 0.05, $MnSO_4.7H_2O$ - 0.01, $ZnSO_4.7H_2O$ - 0.01, $CoCl_2$ - 0.01 (Mandels & Weber, 1969). The basal medium used for cellulase production by *T. reesei* had the following composition in g/l- KH_2PO_4 - 0.5%, NH_4NO_3 - 0.5%, $MgSO_4.7H_2O$ - 0.1%, Peptone - 0.1%, NaCl - 0.1% and $CaCl_2$ - 0.05%. Trace elements: $FeSO_4.7H_2O$ - 0.005%, $MnSO_4.7H_2O$ - 0.001%, $ZnSO_4.7H_2O$ - 0.001% and $CoCl_2$ - 0.0002%. The pH of the media was adjusted with 1N HCl or 1N NaOH wherever required. The moistened bran was mixed well and was sterilized by autoclaving at 121 °C for 15 min at 15lbs pressure.

2.2.2 *Submerged Fermentation (SmF)*

Mandel and Weber medium added with 1% of an additional carbon source were used for the production of β-glucosidase. Hundred milliliters of medium was taken in 500ml Erlenmeyer flasks and sterilized by autoclaving at 121 °C, 15lbs pressure for 15 min.

2.3 Enzyme Production and Extraction

2.3.1. *Solid State Fermentation*

Medium prepared for SSF was inoculated with 1ml of either *A. niger* or *T. reesei* spores suspension containing the desired number of spores. The contents were mixed thoroughly and were incubated under controlled conditions of temperature and humidity. Incubation was

continued for the duration indicated in the experimental designs and at the end of incubation period, enzyme was recovered by extraction with 100ml of 0.05M citrate buffer (pH 4.8). The buffer was added to each flask and the flasks were kept on a rotary shaker for 1h at 200 rpm, after which the entire slurry was recovered and was filtered using glass wool. The filtered solution containing enzyme was centrifuged at 6000 rpm for 10min at 4 °C to remove debris and was filtered again using 1.6µm glass microfiber filters (Whatman® GF/A). This filtrate was used as the crude enzyme preparation.

2.3.2. *Submerged fermentation*

Mandel and Weber medium inoculated with the specified amount of spores were incubated for 96 hours at 30°C on an incubated shaker at 180 rpm agitation. At the end of fermentation, biomass was separated by centrifugation at 6000 rpm for 10 min at 4 °C, followed by filtration using 1.6µm glass microfiber filters (Whatman® GF/A) and the supernatant was used as the crude enzyme preparation.

2.4 Analytical Methods

2.4.1 Enzyme assays:

2.4.1.1. β-glucosidase (BGL) assay:

β-glucosidase assay was performed using p-nitrophenyl β-D glucopyranoside (pNPG) (Sigma-Aldrich, India) as substrate as specified in Ghose & Bisaria (1987). Appropriately diluted enzyme sample of 0.5ml was incubated with 0.5ml of 10mM pNPG in citrate buffer (0.05M, pH 4.8) and 1ml of citrate buffer (0.05M, pH 4.8) at 40 °C for 15 min. The reaction was terminated by adding 2ml of 0.2M Na_2CO_3 solution. Appropriate blanks devoid of enzyme or substrate were also run in parallel to the enzyme assay. The color developed due to liberation of p-Nitrophenol (pNP) was read in a UV-Visible spectrophotometer (Shimadzu, Japan) and the amount of pNP liberated was calculated by comparing the reading corrected for blanks against a standard curve generated using varying concentrations of pNP. One unit of BGL activity was defined as the amount of enzyme needed to liberate 1µM of p-nitrophenol (pNP) per minute under the standard assay conditions and was expressed in units

per gram dry substrate (U/gDS), in the case of SSF or as units per milliliter (U/ml) in the case of submerged fermentation. For measurement of glucose tolerance, glucose was added in the assay mixture to a final concentration of 0.25M and assays were performed exactly as above. Glucose tolerance was expressed as percentage of activity compared to assay performed without glucose and was expressed as % Activity Retention.

2.4.1.2 Cellulase 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 x 6 cm (~50mg) was placed into each assay tube. The filter paper strips were saturated with 0.5 ml of Na-citrate buffer (0.05M, pH 4.8) and were equilibrated for 10 min at 50 °C in a water bath. Half milliliter 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, each tube was removed from the water bath and the reaction was stopped by addition of 3ml of DNS reagent. The tubes were incubated for 5 min in a boiling water bath for color development and were cooled rapidly by transferring into a cold water bath. The reaction mixture was diluted appropriately and was measured against a reagent blank at 540nm in a UV-VIS spectrophotometer (Shimadzu, Japan). 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 2mg of glucose under the above assay conditions and was expressed as Filter Paper Units (FPUs)

2.4.1.2 Endo Glucanase (CMCase) Activity

Endoglucanase activity was determined as outlined above for filter paper assay but using Carboxy Methyl Cellulose as substrate (0.5ml of a 2% Na-CMC solution in citrate buffer [0.05m, pH4.8]) instead of filter paper (Ghosh, 1987). 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. CMCase activity was

calculated following the concept that 0.185 U of enzyme will liberate 0.5 mg of glucose under the assay conditions and was expressed as U/ml.

2.4.2. *Protein Assay*

Protein assays were done using the Folin-Ciocalteau reagent according to Lowry's Method (Lowry *et al*, 1951) and were expressed as mg/ml.

2.4.3. *Reducing sugar Estimation*

Estimation of total reducing sugar in the enzymatic hydrolysate of biomass was done by DNS method (Miller, 1959) and was expressed as mg/ml and/or mg/g biomass.

2.4.4. *Estimation of Ethanol*

Ethanol estimation was done by gas chromatography as outlined in NREL Laboratory Analytical protocol # 011 (Templeton, 1994). One milliliter fermented broth was centrifuged at 12000 rpm for 5 minutes at 4 °C and the supernatant was filtered through a 0.45μm PES membrane (Pall, USA) before injecting into the GC. Ethanol was detected using an FID detector kept at 250 °C. Other conditions of operation were mobile phase – N_2 (30ml/min), Column temperature – 150 °C, Injector temperature -175 °C and injection volume 1μl. Ethanol was detected by its elution time compared against a standard sample of pure ethanol and the concentrations were calculated based on the peak areas of known concentrations of ethanol. Ethanol concentrations were expressed as % v/v.

2.5. Electrophoresis and Zymogram Analyses

Standard protocol for SDS and Native PAGE were employed to prepare gels with 10% strength and were used throughout the study. Samples were concentrated using a vacuum concentrator (Eppendorf, Germany) before loading on to the gels. Protein was estimated by Lowry's method and samples were normalized to contain equal protein concentration before loading the gel in duplicates. Gels were loaded as two halves with each half containing the same samples exactly in the same order and concentration. After completion of the electrophoresis, the gels were washed once in distilled water and were divided into two parts

each corresponding to a half containing all the samples as the other one. One of the halves was incubated with 10mM MUG solution in citrate buffer (0.05M, pH 4.8) for 10 min at room temperature (28+/- 2 °C. The second half was treated similarly but with a substrate solution containing 250 mM of glucose to determine the BGL activity inhibition. BGL activity was visualized as blue –green fluorescence under long wavelength UV trans-illumination. Both halves were photographed simultaneously using an imaging system (Syngene-GBox, UK), to avoid differences in lighting and exposure. Differences in fluorescence intensities of bands were measured by pixel density analyses of the photographs using the software Scion Image ® (Scion Corp, USA). Glucose tolerance of BGL bands were expressed in terms of activity retention which was calculated as the % of fluorescence intensity remaining in the BGL activity band in the gel incubated in presence of glucose to that in the gel incubated in MUG without glucose.

2.6. Biomass (rice straw) pretreatment

Rice straw (RS) was procured locally. The biomass feed stock was brought to the lab and further dried overnight at 70 °C in a hot air oven to remove residual moisture. Feed stock was milled in a Knife mill to reduce the size prior to pretreatment. Milled feedstock with a particle size range 100- 2000 µm was pretreated with dilute alkali. Briefly, the sample was reacted with 0.1N NaOH for 1h at 121 °C in an autoclave. After cooling, the slurry was dewatered by filtration using a 140 mesh nylon sieve and washed several times in tap water to neutralize the pH followed by a final rinse in distilled water. The pretreated rice straw was air dried at room temperature to remove moisture by spreading on paper sheets. The pretreated feed stock was either used immediately for hydrolysis experiments or stored in airtight containers at 4 °C until used.

2.7. Biomass saccharification

Enzymatic saccharification of biomass was done by incubating 1g of pretreated biomass (rice straw) with the *T. reesei* crude cellulase alone or with various enzyme cocktails containing

different amounts of *A. niger* BGL along with the *T. reesei* cellulase preparation. The saccharification studies were conducted at 45 °C, in 100 ml stoppered Erlenmeyer flasks in a total volume of 50 ml made up with 50 mM citrate buffer (pH 4.8). The flasks were agitated at 100 rpm in a water bath shaker. Hydrolysis efficiencies were measured as the amount of total reducing sugars liberated from biomass according to the reducing sugar assay (Section 2.4.3).

2.8. Ethanol production

Ethanol production was studied using the enzymatic hydrolysate of rice straw as the substrate for alcohol fermentation. The rice straw hydrolysate generated by enzymatic saccharification (section 2.8) was clarified by centrifugation at 8000 rpm for 15 min and was concentrated by evaporation (50 °C) to reducing sugar content of either 6% or 12% w/v. Ten milliliters of the hydrolysate was sterilized by filtration through a 0.22µm syringe filter and was inoculated with 10% v/v of a 12h old seed culture of *S. cerevisiae*. Incubation was carried out in stoppered 15ml glass vials at room temperature (28±2°C) without agitation. Samples (1ml) were withdrawn at 24h intervals. The samples were centrifuged at 13000 rpm for 10 min at 4 °C. The supernatant was filtered using 0.45µ syringe filter and the ethanol content was analyzed by gas chromatography (Section 2.4.4).

CHAPTER 3

Fermentative Production of β-Glucosidase

Chapter 3
Fermentative production of β-glucosidase

3. Introduction

Commercial production of *β*-glucosidase is often achieved by use of species of *Aspergilli*. *Aspergilli* are known to produce higher titers of the enzyme. Nevertheless, reports on large scale production of BGL are limited. Relatively pure forms of cellulose and native as well as pretreated biomass have been used successfully as carbon sources for production of the enzyme under both submerged (SmF) and solid state fermentations (SSF). While submerged fermentation is the most common strategy employed for commercial production of microbial enzymes due to its inherent advantages of better sterility, heat and mass transfer, easiness in process monitoring and automation etc, SSF is popular in the case of fungal fermentations for high volume low value enzymes like amylases, cellulases etc. This is because SSF has better productivity, low capital investment, low energy requirement, lesser waste water output, higher product concentration, and lack of foam build up (Reimbault, 1998). However, knowledge on process automation is limited, and there is intense heat generation in SSF systems.

Process optimizations are essential in improving the productivity and to understand the effect of parameters on the fermentation. In the conventional method for the optimization of enzyme production, the “one variable at a time” approach is used, which involves changing one parameter at a time while keeping the other entire parameters constant (Greasham & Inamine, 1986, Chen, 1994). The optimized concentration of the previous experiment is then incorporated in the next experiment. The same procedure is followed for all the parameters to complete the optimization (Young *et al*, 1985). But this process is cost, labor and time intensive, and also does not consider the interaction between variables. An alternative and more efficient approach is the use of statistical methods. Several statistical methods ranging from two factorial to multi-factorial designs are available (Monoghan & Koupal, 1989). Placket and Burman designs (Plackett & Burman 1946) are fractional factorial designs used when one needs to screen a large number of factors to identify those that may be important

(i.e., those that are related to the dependent variable of interest), In such situations a design that allows one to test the largest number of factor main effects with the least number of observations is desired. To enable this, the Placket and Burman design has the interaction effects of variables confounded with new main effects. Because the added factors are created by equating (aliasing), the "new" factors with the interactions of a full factorial design, these designs always will have 2^k runs e.g., 4, 8, 16, 32, and so on. Full factorial design is fractionalized in a different manner, to yield saturated designs where the number of runs is a multiple of 4, rather than a power of 2.

In an experimental procedure for studying the effects of process parameters (independent variables) under question, the selection of high (1) and low (-1) values of the variable is very critical (Greasham & Inamine, 1986). The difference between the levels of each variable must be large enough to ensure that the optimum response will be included. After performing the experiments, the responses obtained are analyzed statistically to determine the effect of that variable on the response, experimental errors and the significance of the influence of each variable on the response (Nelson, 1982). The effect of a variable is the difference between the average response of that variable at higher and lower levels. Probability tests are run to determine the level of significance of the effects of each variable. The design of experiments and analyses of responses are now routinely done using software made for the purpose eg – Statistical (Statsoft Inc, USA), Design Expert (Stat-Ease, USA) etc.

A filamentous fungus capable of producing moderately glucose tolerant beta glucosidase was isolated at NIIST and was identified as *Aspergillus niger* (MTCC 7956 /NII 08121). The objective of present study was to determine the effect of carbon sources in production of beta glucosidases by this fungus under SmF, and also to identify and optimize the production of BGL under SSF using a fractional factorial (Plackett & Burman) experiment design.

3.1 Materials and Methods

3.1.1 *Screening of Carbon sources for BGL production*

The effect of carbon sources on BGL production by *A. niger* NII 08121 and the level of glucose tolerance of the enzyme secreted by it was studied under SmF by incorporating the carbon sources in enzyme production medium. Mandel and Weber medium with composition as given under section 2.2.1 was used with supplementation of one the carbon sources -Wheat bran, Rice straw, Glucose, Lactose and Cellulose at 1 % (w/v) level. Enzyme production and extraction was carried out as outlined under section 2.3.2. Enzyme assays were conducted to determine the activity and glucose tolerance of each sample as indicated in section 2.4.1.1.

3.1.2 *Bioreactor studies*

Bioreactor studies for BGL production were done using a parallel fermentation system with six 1L vessels (Infors HT, Switzerland). Three reactors were operated in parallel for each BGL production experiment, each containing 350 ml of medium. The DO level was set at 60% and rpm was set in the range 100- 600/min to be operated in cascading mode (Fig 3.1). The culture was aerated at 0.5 vvm level using compressed air passed through a sterile 0.22µm filter. 1N HCl and 1N NaOH was used for maintaining the pH 4.8 and heat sterilized silicone oil was used for foam control. The reactors were inoculated with 2 x 10^5 spores/ml. Operating temperature was maintained at 30°C. After 96h of cultivation, the fermentation broth was recovered and biomass was separated by centrifugation at 8000 rpm for 15 min, followed by filtration using a 1.6 µm pore glass microfiber filter (Whatman ® GF/A). Control experiments were run in shake flasks under similar conditions but with 180 rpm agitation and without control for pH and DO.

Fig 3.1: Reactor setup for SmF production of BGL using *A. niger*

3.1.3. *Optimization of the SSF production of BGL by* A. niger

Solid State Fermentation production of BGL was done as outlined under section 2.3.1. A Plackett and Burman (Plackett & Burman, 1946) design was employed to determine the effect of individual parameters affecting BGL production by the fungus under SSF. The composition of mineral salt solution used for wetting the substrate and the important physical parameters affecting enzyme production were screened in a design with 7 variables at two levels in a total of 8 experimental runs (Table 3.1).

Table 3.1: Plackett & Burman design matrix for the optimization of variables influencing BGL production

Std Order	X_1	X_2	X_3	X_4	X_5	X_6	X_7
1	-1	-1	-1	1	1	1	-1
2	1	-1	-1	-1	-1	1	1
3	-1	1	-1	-1	1	-1	1
4	1	1	-1	1	-1	-1	-1
5	-1	-1	1	1	-1	-1	1
6	1	-1	1	-1	1	-1	-1
7	-1	1	1	-1	-1	1	-1
8	1	1	1	1	1	1	1

The parameters tested were: Incubation time, Peptone concentration, Initial moisture content of the medium, Concentrations of Urea, Lactose, Tween 80 and Incubation temperature. The variables were tested at two levels: a higher level designated as +1 and a lower level designated as -1. The actual and coded values tested for each parameter are given in Table 3.2.

Table 3.2: Actual levels of variables tested with the factorial design and their effects on BGL production

Code	Parameter name	Low level (-1)	High Level (+1)
X_1	Incubation time (h)	96	144
X_2	Peptone (g/L)	1	3
X_3	Initial moisture (%)	50	70
X_4	Urea (g/L)	0	2
X_5	Lactose (g/L)	0	1
X_6	Tween 80 (g/L)	1	3
X_7	Temp of Incubation (^oC)	27	32

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 BGL production was calculated by the following equation (Eqn.1)

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

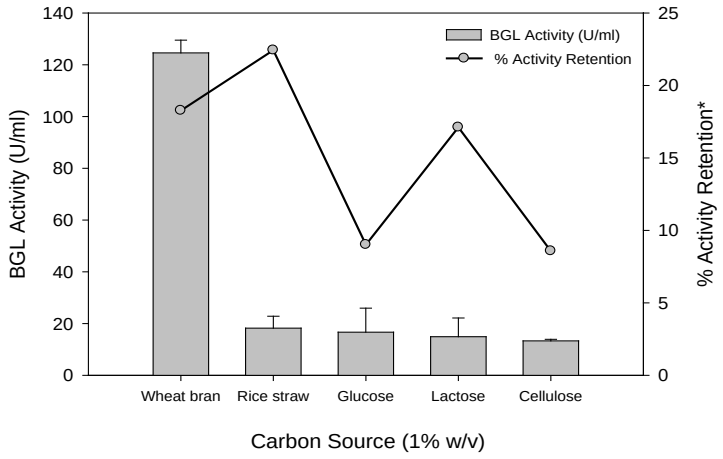
Where ε is the effect of parameter under study and "μ_+" and "μ_-"are responses (BGL activity) of trials at which the parameter was at its higher and lower levels respectively and "n" is the total number of trials. Analysis of variance (ANOVA) was performed on the data to determine the significance of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

3.2 Results and discussion

3.2.1. *Screening of Carbon sources for BGL production by* A. niger

Among the different carbon sources (wheat bran, rice straw, glucose, lactose and cellulose) tested in SmF, maximal BGL activity was obtained with Wheat Bran (128 U/ml) followed by Rice straw, Glucose, Lactose and Cellulose (Figure 3.2). Similar levels of BGL production was observed when the carbon sources other than Wheat Bran (WB) were used.

Fig 3.2: Effect of carbon sources on BGL production by *A. niger*



[* % Activity retention is the amount of activity retained by the enzyme while assayed in presence of 0.25M glucose in comparison to a control experiment where the assay in conducted without glucose]

Wheat bran is a crude substrate that contains proteins, cellulose, starch and minerals and these nutrients can apparently promote enhanced growth and consequently enzyme production. High productivity in crude biomass could also be due to the dual role of biomass as a nutrient source and a support matrix for fungal adherence. *A. niger* mycelia had grown as spherical beads in the shake flaks culture and the size of the beads were also different with the different carbon sources studied. With the simpler sugars and pure cellulose the mycelia beads were comparatively larger than those in wheat bran or rice straw medium. The latter two carbon sources are particulate in nature which would result in increased turbulence while mixing.

This in turn aids in better aeration as well as breakage of large pellets. Smaller pellet size in improved mass transfer due to an increased surface.

The comparative lack of BGL production in the presence of glucose may be because; glucose is a catabolite repressor of many cellulase genes or due to the inhibitory effect of glucose on BGL activity (Gulati & Mahadevan, 2000). The growth of fungi on mineral salt medium containing glucose generally represses cellulase activity and only after depletion of glucose, the fungi resume production of the enzyme (Jørgensen *et al*, 2004).

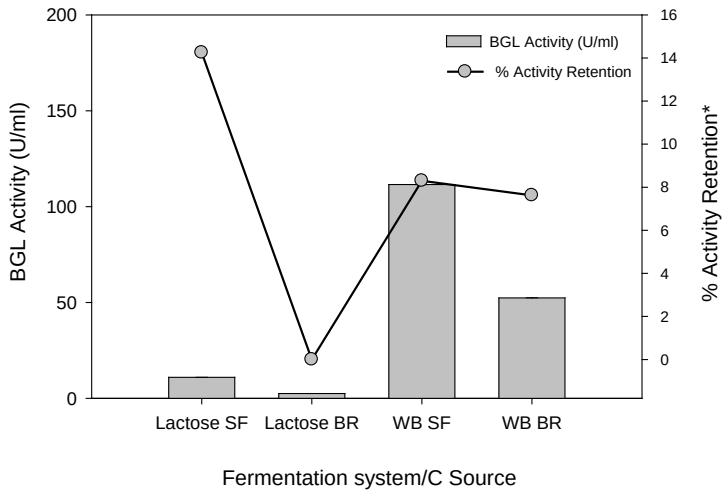
Differences were also apparent in the glucose tolerance of the enzyme recovered from cultures grown in the different carbon sources. The difference in activity retention in presence of glucose for the enzymes prepared in different media could be due to the differences in expression of multiple BGL proteins since it is known that multiple isoforms of BGL is present in *Aspergilli* and a few of them are reported to be glucose tolerant (Gunata & Vallier, 1999, Decker *et al*, 2000). The properties of substrate can determine the type and level of beta glucosidase expression (Roy *et al*, 1988, Perez-Pons *et al*, 1995). In a different context, the expression of BGL proteins were studied (Chapter 4) which confirmed the presence of multiple isoforms of the enzyme in *A. niger* NII 08121 that were expressed differently in response to the carbon source in the medium. It was also demonstrated that at least one of the isoforms is glucose tolerant. The possibility of driving the expression of an isoform of the enzyme to larger extent by modulating the carbon source opens up great possibilities in enzyme production for biomass hydrolysis since glucose tolerance is a highly desired feature for BGLs to be used in biomass saccharification.

3.2.2. *β- glucosidase production under SmF in Bioreactor*

A. niger is known to produce higher levels of metabolites under controlled conditions of pH and aeration (Liu *et al*, 2003, Znad *et al*, 2004). So BGL production was tried either wheat bran or lactose as substrate in a bioreactor (Infors, Switzerland, See 3.2.1). Fermentations were carried out for 96h and the BGL activities obtained were compared with that obtained in shake flask cultures run in parallel, under similar conditions. BGL activities obtained under unregulated conditions (Shake flasks) when compared with those where pH

and DO levels were regulated, were higher. Also the enzyme produced by shake flask cultivation had better glucose tolerance (Fig 3.3). One of the reasons for this observation is the mechanical breakage of fungal mycelia by the impellers which resulted in a comparatively lower biomass and hence lower enzyme yield. Though the initial growth of mycelia were faster in the bioreactor, this also resulted in a faster depletion of DO causing the reactor to cascade to a higher rpm which had proved deleterious of the fungus. So it would be safe to assume that *A. niger* cultivation at larger scales in STR's needs to be approached with caution and extreme care needs to be taken in maintaining the correct mixing levels and DO. Alternatively, other reactor configurations like membrane reactors (Chang *et al*, 1990), airlift reactors (Park *et al*, 1994) or tubular reactors (Papagianni *et al*, 2003) may be employed.

Fig 3.3: Comparison of BGL production and glucose tolerance in shake flasks and bioreactor



[* % Activity retention is the amount of activity retained by the enzyme while assayed in presence of 0.25M glucose in comparison to a control experiment where the assay in conducted without glucose]

BGL production by *A. niger* was less when lactose was used as carbon source which was surprising since lactose is a known inducer of cellulases and is employed in production of cellulases (Sehnem *et al*, 2006, Fang *et al*, 2008). Morikawa *et al* (1995), had proposed a mechanism of cellulase induction in *T. reesei* by lactose where they had proposed that lactose alone is not an effective inducer and it can promote cellulase production only when it is taken inside the cell. The role of a putative lactose permease which is inducible by other sugars like

sophorose was also proposed. Assuming that a similar mechanism is operational in *A. niger*, wheat bran could have provided enough carbon source that the induction of the permease was probably not necessary which could explain the reduced BGL production.

3.2.3. *Optimization of the SSF production of BGL by* A. niger *NII 08121*

To determine the important parameters which significantly affect BGL production by *A. niger* NII 08121 on wheat bran under SSF, a Plackett–Burman design was used. Seven variables were screened in 8 experimental runs. The results of the runs based on the Plackett and Burman design showed a variation in BGL production ranging from 22100 U/gds to 29560 U/gds (Table 3.3). This variation shows the importance of optimizing these parameters for improving BGL production by *A. niger* on wheat bran

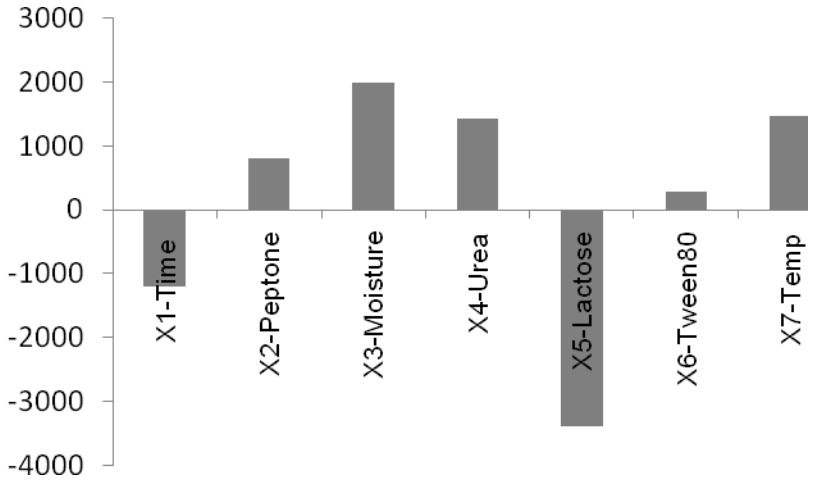
Table 3.3: BGL yields for experimental runs performed according to the Placket and Burman design matrix

Std Order	X_1 Time (h)	X_2 Peptone (g/L)	X_3 Moisture (%)	X_4 Urea (g/L)	X_5 Lactose (g/L)	X_6 Tween 80 (g/L)	X_7 Temp (oC)	Y BGL activity (U/gDS)
1	96	1	70	2.0	0.0	1	32	29560
2	144	3	50	2.0	0.0	1	27	25720
3	144	1	50	0.0	0.0	3	32	25240
4	96	3	70	0.0	0.0	3	27	27760
5	96	3	50	0.0	1	1	32	23580
6	144	3	70	2.0	1	3	32	26060
7	144	1	70	0.0	1	1	27	22100
8	96	1	50	2.0	1	3	27	23020

The average effect of the variables at their assigned levels on BGL production was calculated. The difference between average response of each factor at level +1 and -1 indicates the relative influence of their effect. The magnitude of the value indicates the relative extent of their influence while the direction (the sign of the value either + or -) indicates whether the

change from level -1 to +1 resulted in an increase or decrease in the response. The results obtained for the calculation of effects is given in Figure 3.4

Figure 3.4: Estimated effect of process parameters on BGL production by *A. niger*



The parameters with largest effects were Lactose concentration, initial moisture content and incubation temperature. Lactose influenced BGL production negatively while the other two has a positive effect.

Analysis of the BGL production data was further performed using the Design Expert ® software. The responses obtained for the experimental runs were analyzed by Analysis Of Variance (ANOVA) to test the fitness of the model and to determine the parameters with significant effects. The ANOVA test for 7 process variables indicated that BGL production by *A. niger* NII 08121 under SSF on wheat bran can be described by the factorial model considered (Table 3.4)

Table 3.4: Analysis of Variance (ANOVA) for the factorial model

Source	Sum of Squares	DF	Mean Square	F value	p-value (Prob >F)
Model	3.07×10^7	2	1.53×10^7	6.08	0.0458
X2-Moisture	7.84×10^6	1	7.84×10^6	3.11	0.1382
X5-Lactose	2.28×10^7	1	2.28×10^7	9.06	0.0298
Residual	1.26×10^7	5	2.52×10^7		
Cor Total	4.33×10^7	7			

The results of the ANOVA indicated that the model is significant and can be used to define the influence of parameters on BGL production by *A. niger* under SSF on wheat bran. Lactose (X_5) was the only parameter to have significant influence on BGL production with a p value of 0.0298.

The variables X_2 (initial moisture of the medium), X_5 (Lactose concentration), were included in the model. Coefficients of the model terms were calculated using design expert software and is given in Table 3.5.

Table 3.5: Coefficients of variables included in the model

Model Term	Co-efficient estimate
β_0	25380
X_2	990
X_5	1690

β_0 is the overall interaction coefficient

The equation representing the model was derived as

$Y = 25380 + 990X_2 - 1690X_5$

Where

Y = BGL yield

X_2 = Initial moisture content

X_5 = Lactose concentration in the medium

Lactose was the only significant model term and this was having negative impact on β-glucosidase production. Wheat bran itself is a rich source of nutrients and can support the growth and nutrition of microorganisms. The effect of other parameters could be influenced significantly or even masked by the dominating influence of wheat bran. Nevertheless, the production level was increased from 22100 U/gds to 29560U/gds which is considerably high. The combinations of process variables that would give high BGL yields were derived by solving the model equation using the numerical optimization function in Design Expert®. Three combinations identified by the software are given in Table 3.6

Table 3.6: Optimal combinations of variables predicted for improved BGL yield and expected enzyme yields

Sl #	Time (h)	Peptone (g/L)	Moisture (%)	Urea (g/L)	Lactose (g/L)	Tween80 (g/L)	Temp (^{o}C)	Expected BGL yield
1	120	0.20	70.00	0.10	0.00	0.20	30	28060
2	120	0.20	70.00	0.10	0.00	0.20	30	27974
3	120	0.20	69.04	0.10	0.00	0.20	30	27964

3.3. Conclusions

SSF and SmF both offer several advantages over each other and each of these fermentation techniques also have their own limitations. The studies done on screening of carbon sources identified that wheat bran can serve as a cheap and effective substrate for BGL production by *A. niger* NII 08121. For bio-fuel applications, BGL produced by SSF would be more appropriate as production titers are very high compared to SmF. Also the crude enzyme may be utilized without any purification for hydrolysis along with other cellulases for enzyme cocktails. Wheat bran also supports good activity retention of BGL in presence of glucose (~19 % in 0.25M glucose).

CHAPTER 4

Differential expression of β-glucosidase by *Aspergillus niger* NII-08121 in response to carbon sources

Chapter 4

Differential expression of β-glucosidase by *Aspergillus niger* NII-08121 in response to carbon sources

4. Introduction

β-glucosidases are enzymes found universally across all domains of living organisms where they play a variety of functions (Ketudat Cairns & Esen, 2010). They are important in plant microbe interactions and in the breakdown of plant cell wall. These enzymes have been implicated primarily in biomass to glucose conversions or in breaking of plant cell wall to establish parasitic or symbiotic relationships (Gilbert *et al*, 2008). β-glucosidases play multiple roles in filamentous fungi, where they are involved in fungal cell wall metabolism (Lymar *et al*, 1995), modification of the biological activities of self produced or exogenous glycosides (Quiros *et al*, 1998, Xue *et al*, 1998), generation of the inducers of cellulase genes by trans-glycosylation (Fowler *et al*, 1992, Mach *et al*, 1995), in resistance against plant saponins (Bowyer *et al*, 1995, Osbourn *et al* 1995) etc. Multiplicity of cellulases and hemicellulases is well known in the case of filamentous fungi (Willick & Seligy, 1985, Dekker *et al*, 2000, Nazir *et al*, 2010) and probably this multiplicity is essential, considering the vast and diverse roles these enzymes play in fungal metabolism and survival. β-glucosidase multiplicity can be attributed to the presence of multiple genes or due to differential post transcriptional modifications (Collins *et al*, 2007, Iwashita *et al*, 1999). Differential expression of the various BGL proteins are reported in response to the carbon sources supplied in the medium or the conditions of culture (Willick & Seligy 1985, Nazir *et al*, 2010) and could be a probable adaptation of the fungi to respond to the changing immediate environments. This property however, could be exploited for selective expression of a desired isoform from a fungus by manipulating the culture conditions/carbon source carefully.

A query of the *A. niger* genome sequence (*Aspergillus niger v3, JGI, 2010*) returned several genes listed as β-glucosidase implicating the abundance and multiplicity of the enzyme in this fungus. Also the strain under study - *Aspergillus niger* NII 08121 produced high titers of β-glucosidase and exhibited differences in BGL expression and glucose sensitivity in response to different carbon sources supplemented in the medium. It was therefore speculated that there could be differences in expression of the various BGL

proteins/isoforms. *A. niger* NII 08121 was grown under submerged fermentation, supplementing different crude and pure carbon sources and the culture filtrates were analyzed for the presence of different isoforms, their level of expression and glucose sensitivity.

4.1. Materials and Methods

4.1.1. *Production of BGL using different carbon sources under SmF*

Studies were carried out under submerged fermentation using the basal mineral salt medium (Mandels & Weber, 1969) supplemented with one of the different carbon sources – ie -wheat bran, rice straw, glucose, lactose or cellulose at 1% w/v concentration. Erlenmeyer flasks (500ml) containing 100 ml of the medium with appropriate carbon source was prepared and sterilized as outlined under section 2.2.2. Each flask was inoculated with 1ml of a spore suspension containing 10^7 spores (Section 2.1) and was incubated for 96h at 30 °C in a refrigerated incubator. Enzyme preparation was done according to section 2.3.2.

The crude enzyme samples were concentrated 10 fold by vacuum evaporation before being analyzed for the presence of BGL isoforms. β-glucosidase assay was done for the concentrated samples according to the method outlined under section 2.4.1.1. One unit of BGL activity was defined as amount of 4-nitrophenol (in µM) released per ml of enzyme per minute under the standard assay conditions.

4.1.2. *Detection of BGL protein isoforms by PAGE and Activity staining*

BGL isoforms produced by the fungus in media supplemented with the different carbon sources were visualized on poly acrylamide gels by activity staining. The crude enzyme preparation was concentrated by a centrifugal concentrator and zymogram analyses were performed as described in section 2.5. Native PAGE (10%) was run with the same amount of protein from each sample loaded on to the wells. Sample loadings were duplicated in the same order in the same gel so as to stain one set (wells 1-4) in absence of glucose and the other set (wells 5-8) in presence of 250 mM glucose. Activity

staining on electrophoresis gels was done by incubating the gels with 10mM Methyl Umbelliferyl β-D-Glucopyranoside (MUG) solution in 0.05 M, pH 4.8 citrate buffer. While staining, all the parameters and concentration of substrate were kept constant for both the sets with the only difference being the presence of 250 mM glucose in one. Activity bands were visualized by illumination with long wavelength UV trans-illumination and pictures were taken using an imaging system (Syngene-GBox, UK). Both the sets of gel were pictured simultaneously to ensure even conditions of illumination and exposure. Differences in fluorescence intensities of bands were measured by pixel density analyses of the photographs using the software Scion Image ® (Scion Corp, USA). Glucose tolerance of BGL bands were expressed in terms of activity retention which was calculated as the % of fluorescence intensity remaining in the BGL activity band in the gel incubated in presence of glucose to that in the gel incubated in MUG without glucose.

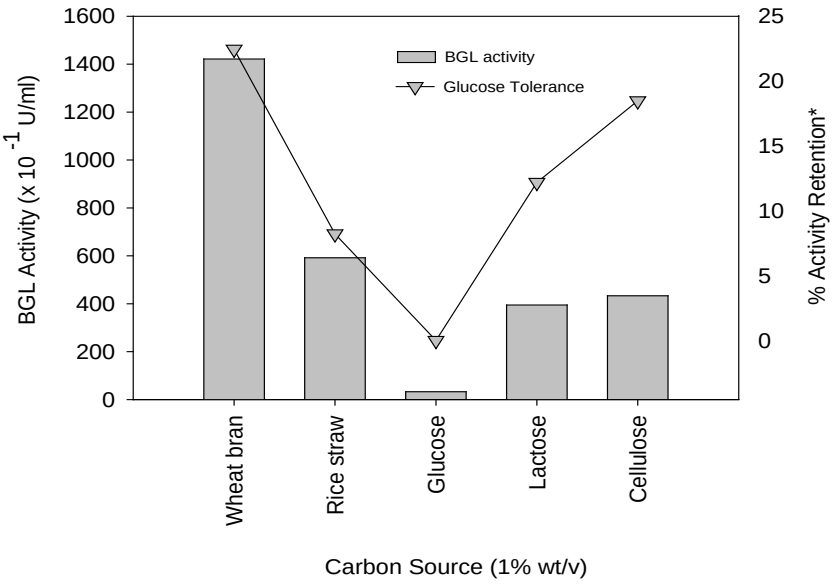
4.2. Results and discussion

4.2.1. *Production of BGL by* A. niger *in media containing different carbon sources*

Enzyme assays done on the concentrated enzyme samples showed that wheat bran supported the maximum production of β-glucosidase (1400U/ml for 10X sample) followed by rice straw, cellulose, lactose and glucose respectively (Figure 4.1). Glucose did not support BGL production, apparently due to carbon catabolite repression which is effective in this fungus. Cellulase genes are normally expressed at a low basal level, and the induction or repression of the cellulose degrading enzymes in several fungi are tightly controlled with the production of cellulolytic enzymes induced only in presence of the substrate, and repressed when easily utilizable sugars like glucose are available (Suto & Tomita, 2001). The growth of fungal species on mineral medium containing glucose generally represses enzyme production, and only after glucose depletion the fungi begins the production of BGL (Jorgensen *et al*, 2004). Wheat bran supported maximum production of BGL. Wheat bran is a rich source of nutrients, containing proteins, cellulose, starch and minerals which might help in a better growth as well as enzyme production. Rice straw which is another crude biomass resource had supported the next higher production of the enzyme. However with rice straw as carbon source, the glucose tolerance of the enzyme preparation was comparatively lower. High productivity in crude

biomass could be due to the dual role of biomass as it provides nutrients as well as support for the fungal adherence (Pandey *et al*, 1999). The fact that the glucose tolerance of enzyme preparations produced using different carbon sources are widely different, provides further evidence to the fact that there is a differential induction of BGL isoforms in the fungus. The differences in percentile activity retention in presence of glucose might be due to the differences in the amount of glucose tolerant isoform in the preparation which in turn is due to the differences in expression of the protein. This speculation was tested by monitoring the level of expression of the different isoforms using zymogram analysis.

Figure 4.1: BGL production and its activity retention in response to different carbon sources



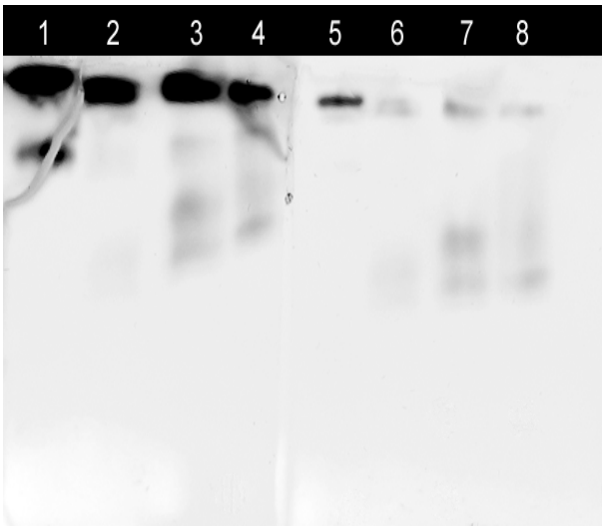
[* % Activity retention is the amount of activity retained by the enzyme while assayed in presence of 0.25M glucose in comparison to a control experiment where the assay in conducted without glucose]

4.2.2. *Differential expression of BGL protein in response to carbon sources*

Zymogram analysis showed the presence of 4 different bands showing BGL activity in enzyme samples produced using lactose and cellulose which are known inducers of cellulases in fungi (Fig 4.2, Lanes 3 & 4). Three of the low molecular weight bands were visible only in these two samples whereas the high molecular weight band was visible in samples produced on all different carbon sources. Also this was the BGL isoform which was highly expressed, as evident from the high band intensity (Fig 4.3 &

Table 4.1). Activity staining done in presence of 250mM glucose showed that the two low molecular weight isoforms retained their activity in presence of glucose. These BGL bands in case of lactose induced sample, showed activity retention of 95% and 83% respectively on the basis of pixel density analysis (Table 4.1)

Figure 4.2. Differential induction of BGL in response to different carbon sources

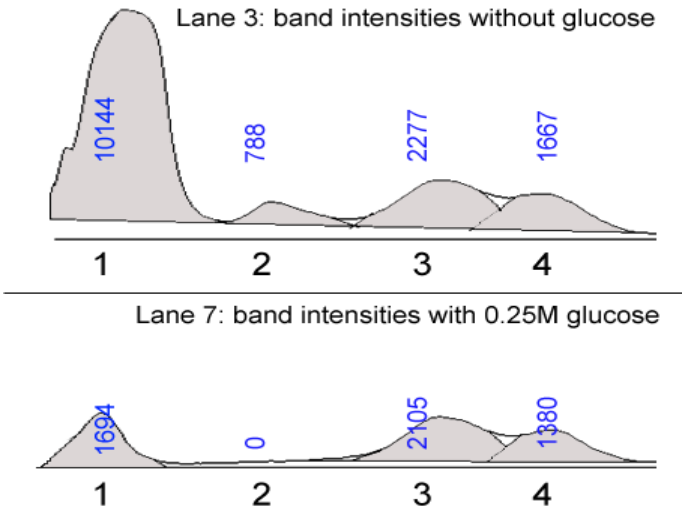


Lane Information

1&5	Sample produced in WB as C source
2&6	Sample produced in RS as C source
3&7	Sample produced in Lactose as C source
4&8	Sample produced in Cellulose as C source
1-4	Staining done without glucose
5-8	Staining done in presence of 0.25M glucose

[* Zymogram of BGL was prepared as described under section 2.5. The image was inverted for better clarity and to aid in pixel density analysis]

Figure 4.3: Pixel density analysis of the activity bands obtained by MUG staining of BGL produced using lactose as carbon source



Pixel density analysis of the enzyme sample produced using lactose medium in presence (Lane7, Fig 4.2) and absence (Lane 3, Fig 4.2) of glucose. Area under each peak corresponds to the pixel densities (intensity of bands) and the values are indicated on the respective peaks.

Table 4.1. Glucose tolerance of BGL isoforms produced in lactose medium

V	Lane No. / Band No. >	1	2	3	4
3	(Glucose-)	10144	788	2217	1667
7	(0.25M Glucose)	1694	0	2105	1380
Glucose Tolerance (% Activity retention)		16.7	0	94.9	82.8

It may thus be assumed that the lower two bands correspond to glucose tolerant BGLs and the expression of these are induced more by lactose compared to other carbon sources used in the medium. Lactose is known to induce BGL production in some fungi (Abdel Fattah *et al*, 1997). It supports the glucose tolerant BGL production and it is utilized in the commercial production of the enzyme owing to economic considerations (Sukumaran *et al*, 2005). The mechanism of lactose induction of BGL however, is not fully understood (Fekete *et al*, 2008).

It is generally believed that oligosaccharides play an important role in regulating the synthesis of wood-degrading enzymes which includes BGL (Hrmova *et al*, 1991, Schmoll & Kubicek, 2003) and oligosaccharides has been proved to be converted to inducer (such as sophorose and gentiobiose) by transglyosylation (Suto & Tomita, 2001, Kubicek, 1987). As an accepted model, the induction of the cellulases is mediated either by low molecular weight soluble oligosaccharides that are released from complex substrates as a result of hydrolysis by constitutive enzymes or by the products (positional isomers) of transglycosylation reactions mediated by constituent β-glucosidase, xylanases, etc. (Badhan *et al*, 2007a). Also, the effect of the growth environment manifests itself markedly in the type of carbohydrate metabolism of heterotrophic microorganisms. Nature and quantity of the excreted metabolites is dependent on the nature of the growth limitations and carbon source (Neijssel, 1979).

A. niger, thus produced four different BGL proteins when induced with different carbon sources of which the high molecular weight activity band was the most highly expressed β-glucosidase. This β-glucosidase designated as BGL1 was the major BGL protein which was responsible for a very significant percentage of the total BGL activity of all samples. Sequential induction of isoforms have been associated with the presence of

distinct metabolites which has direct or indirect bearing on biomass degradation (Villas-Bôas *et al*, 2006, Panagiotou *et al*, 2005). The changed profiles of the metabolites during culture might be important in differential expression of enzymes and vice versa. The different BGL isoforms induced differentially may be assumed to belong to different glycosyl hydrolase families especially glycosyl hydrolase families 3, 5 and 1 since most of the biomass active glycosyl hydrolases are grouped in these families. Chapter 5 deals with the studies which confirm the presence of β-glucosidase belonging to above mentioned GH families.

4.3 Conclusions

Aspergillus niger NII 08121 exhibited differences in expression of β-glucosidase (BGL) in response to different carbon sources. Among four isoforms of BGL expressed using lactose as carbon source, two isoforms were found to retain of 92% and 82% activity respectively in presence of glucose indicating that they are at least moderately glucose tolerant. The study provides conclusive evidence to the fact that multiple BGLs are expressed in *A. niger* differentially in response to the carbon source provided in the culture medium. Carbon source and other components play an important role to direct the metabolic pathway of *A. niger* towards production of a desired metabolite. Careful leveraging of the medium components could be useful for enhanced production of specific BGL isoforms in the fungus.

CHAPTER 5

Purification and characterization of the major BGL (BGL1) from *Aspergillus niger* NII 08121

Chapter 5
Purification and characterization of the major BGL (BGL1) from *Aspergillus niger* NII 08121

5. Introduction

Purification of enzymes is essential for detailed studies on their properties. Also it is essential when a pure enzyme is needed for a given target application. In general, the method of purification involves concentration of the enzyme sample followed by separation of different proteins which is done by chromatographic techniques. Several researchers have successfully purified beta-glucosidases from *Aspergilli* and have established their characteristics. Notably Gunata & Vallier (1999) had reported the production of glucose tolerant beta glucosidases from *Aspergillus niger* and *Aspergillus oryzae.* They had reported the purification of two BGLs from *A. oryzae* and the major protein was strongly inhibited by glucose. The minor protein was glucose tolerant with a *Ki* of 0.95M but was expressed only in minor quantities. The molecular mass determined for the minor BGL was 30KDa, whereas this was 80KDa for the major BGL. While Gunata & Vallier (1999) employed ultra-filtration followed by gel permeation chromatography, Riou *et al* (1998) had used ammonium sulfate fractionation, followed by gel permeation and ion exchange chromatography to purify BGLs from *Aspergillus niger*. Other methods including affinity chromatography (Watanabe *et al*, 1992) and aqueous two phase partitioning (Johansson & Reczey, 1998), have also been used for purification of BGLs from *Aspergilli*. It is now an established fact *Aspergillus* produces different BGL and at least two BGLs are secreted by the fungus one which is highly expressed but with lower glucose tolerance and the second which is glucose tolerant but produced in lower quantities. It is also observed that the major BGL has a higher molecular weight while the glucose tolerant enzyme has a low molecular weight (Woodward & Wiseman, 1982, Kwon *et al*, 1992, Gunata *et al*, 1993, Riou *et al*, 1998). Characterization of the glucose inhibition constant (*Ki*), and the temperature and pH optima of BGLs are important in view of assessing their suitability in biomass hydrolysis. A BGL that is better tolerant to glucose and capable of acting at elevated temperatures is desirable in biomass

hydrolysis (Saha & Bothast, 1996, Pandey & Mishra, 1997, Ghosh & Ghose, 2003). In the present study the major BGL from *A. niger* NII 08121 was purified to homogeneity using a combination of steps including acetone precipitation, cation exchange chromatography and electro-elution.

5.1. Materials and Methods

5.1.1 *Enzyme purification*

5.1.1.1 *Acetone fractionation of crude enzyme preparation*

Enzyme production was carried out using solid state fermentation using the optimized conditions as described in Chapter 3(Section 3.2.3). Extraction of the enzyme was performed as outlined under section 2.3.1. Partial purification of the enzyme was carried out using acetone fractionation with increasing volume of solvent. Approximately 500ml of the crude enzyme preparation was used for acetone fractionation. Chilled acetone was added to pre-chilled crude enzyme extract at a ratio of 1: 0.5 and the mixture was kept for 4h at -20 °C, followed by centrifugation at 8000 rpm for 15 min at 4 °C. The supernatant was recovered and acetone was added to further increase the ratio to 1:1. These steps were continued to obtain ratios of 1:2 and 1:3 for fractional precipitation. The pellet from each step was allowed to dry at room temperature to remove residual acetone. The pellets were then resuspended in 12.5 ml of 0.05M Citrate Buffer (pH 4.8), and were used for further studies.

5.1.1.2 *Isoelectric focusing*

Liquid phase isoelectric focusing (IEF) was performed using a Biorad Rotofor® unit (Biorad, USA) according to manufacturer's protocols for determination of the isoelectric point (pI) of BGL and for separating the enzymes/proteins based on their pI. Ampholytes ranging from pH 3.0 to 12.0 were employed for the separation. Samples withdrawn from different regions of the Rotofor separation cell using a syringe and were dialyzed against 0.05M Citrate buffer (pH 4.8). The samples were then concentrated by vacuum evaporation

using a vacuum centrifugation unit. The dialyzed samples and concentrated (to original volume) were analyzed for enzyme activity and were used for further studies.

5.1.1.3 *Chromatography*

Crude BGL preparation was purified by ion exchange chromatography on a cation exchange column (Macro-Prep High S matrix, Biorad, USA). Experiments were performed on a Biorad LP system. Sample in citrate buffer (0.05M, pH 3.0) was loaded by running it at 0.5 ml/min flow rate through the column and washed by running three column volumes of the loading buffer (0.05M Citrate buffer, pH 3.0) through the column at 0.5ml/min. Elution was performed using a continuous gradient of NaCl from 0.0-1M and 2 ml fractions were collected. Fractions giving the maximum activity were pooled together, dialyzed against 10 mM citrate buffer (pH 4.8) and concentrated by vacuum evaporation where applicable and were used for further studies.

5.1.1.4 *Electro-Elution*

Since multiple BGL isoforms could be detected in the crude preparation which eluted together in ion-exchange chromatography, electro-elution was performed to separate the BGL activities. Crude BGL samples concentrated using vacuum evaporation was run on 10% Native PAGE and the BGL activity bands were visualized by activity staining as outlined under section 2.5. The fluorescent bands were cut from the gel using a surgical blade, chopped into small pieces and were transferred into dialysis bags containing citrate buffer (0.05M, pH 4.8). Bags so prepared were kept horizontally on a submarine electrophoresis unit with the citrate buffer (0.05M, pH 4.8) and was operated at 20V for 3h. After 3 hours it was inverted and again run for another 5 minutes to detach the protein from the dialysis bag and then the buffer from the dialysis bag was extracted. This extract was used for further characterization of the BGL.

5.1.2. *Characterization of the major beta-glucosidase (BGL1) from* A. niger *NII 08121*

Purified major BGL of *A. niger* was characterized for its molecular weight by SDS PAGE. The optimal temperature and pH of the enzyme and its temperature stability was determined.

5.1.2.1. *Determination of the molecular weight of major BGL from* A. niger

The major BGL isoform purified by electro-elution was run on SDS PAGE (Laemmli, 1970) along with standard protein markers (Page Ruler ^{TM} Prestained Protein ladder, Fermentas, USA). BGL activity band was visualized by MUG staining and the Zymogram was photographed as outlined in section 2.5. The gel was then washed twice in distilled water and was then silver stained according to a modified rapid staining protocol (Merril *et al*, 1981). The silver stained gel was also photographed similar to the Zymogram. The position of major BGL isoform was confirmed by overlapping the photographs of Zymogram and the silver stained gel and the molecular weights were determined by comparison with the standard protein markers of known molecular weights.

5.1.2.2. *Determination of the optimal temperature of activity for the* A. niger *major BGL*

The purified major BGL protein was assayed at different temperatures (33*, 40, 50, 60 and 80 °C) using the pNPG assay as outlined under section 2.4.1.1. Optimal temperature was defined as the temperature at which maximum pNPG hydrolysis occurred, detected by increase in activity. [* Room temperature while this study was conducted]

5.1.2.3. *Determination of the optimal pH of activity for the* A. niger *major BGL*

Optimal pH for the activity of the major BGL protein was determined by assaying the enzyme at different pH. The differences in pH were attained by use of 0.05M concentration of different buffers as given below (pH 3, 4, 4.8 and 6 – Citrate buffer, pH 7 and 8 – Na-

Phosphate buffer, pH 9 – Tris-HCl buffer). Optimal pH was defined as the pH at which maximal enzyme activity was obtained in the assay.

5.1.2.4. *Temperature stability of the major BGL of* A. niger

Temperature stability of purified BGL was studied at 50 °C which is the temperature at which cellulases are used for biomass hydrolysis. The enzyme preparation was incubated in a water bath maintained at 50 °C and samples withdrawn at different intervals were assayed (according to section 2.4.1.1). Activity retention was calculated for each time interval and plotted against incubation time. Half life of enzyme was defined as the time required for 50% activity loss under conditions of assay.

5.1.2.5. *Glucose inhibition kinetics of the* A. niger *major BGL*

The glucose inhibition constant (*Ki*) of the major BGL was determined graphically using a Dixon Plot (Dixon, 1953). BGL assays were performed (section 2.4.1.1) with different concentrations of inhibitor/glucose (50mM -500mM) for multiple substrate/pNPG concentrations (2mM -20mM). The initial reaction velocities [1/*V*] were plotted against different inhibitor [I] concentrations at a given substrate concentration [S] for each substrate concentration. The value of [I] at which the different lines intersect which corresponds to – K*i* was determined and the absolute value of this was taken as the K*i* for glucose inhibition of the major BGL.

5.2. Results and discussion

5.2.1 *Acetone fractionation of BGL*

Maximum activity as well as glucose tolerance was exhibited by the fraction obtained using 1:2 volume acetone precipitation (Figure 5.1). No activity was obtained in 1: 0.5 acetone fraction and this fraction was not used for zymogram analysis. The zymogram analysis of the different fractions showed a prominent high molecular weight band and a low

molecular weight band. The sample from 1:1 acetone precipitate showed only the high molecular weight band while the sample from 1:3 acetone precipitate showed only the low molecular weight band. Both the high molecular weight band and the low molecular weight band were visible in 1:2 acetone precipitate (Figure 5.2A). The differences in glucose tolerance of the two bands were also evident from the zymogram and only the low molecular weight band showed activity in presence of 0.25M glucose. Besides, silver staining of the gels showed the presence of only one low molecular weight band in 1:3 acetone fractions indicating an effective separation of BGL activities (Figure 5.2B). The results indicate that fractional acetone precipitation could be an effective strategy for preliminary separation of the BGL isoforms.

Figure 5.1. Fractional precipitation of BGL using Acetone

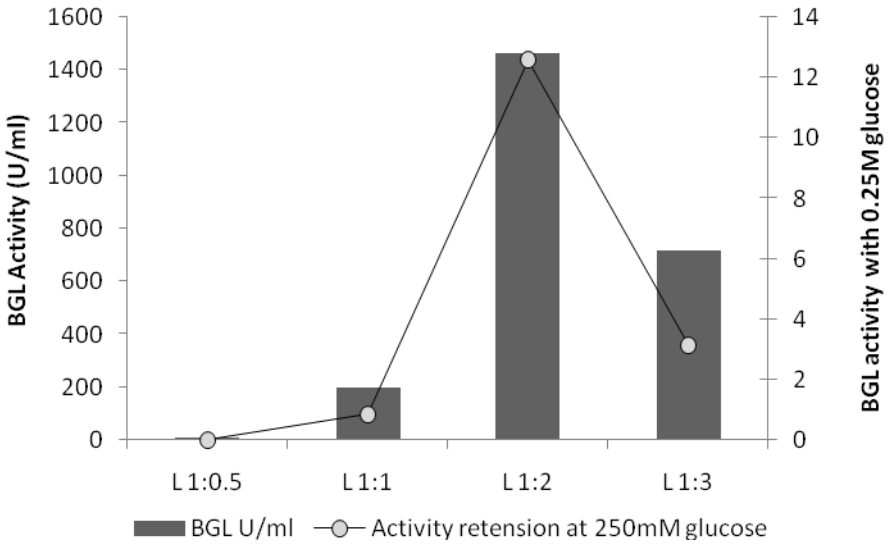
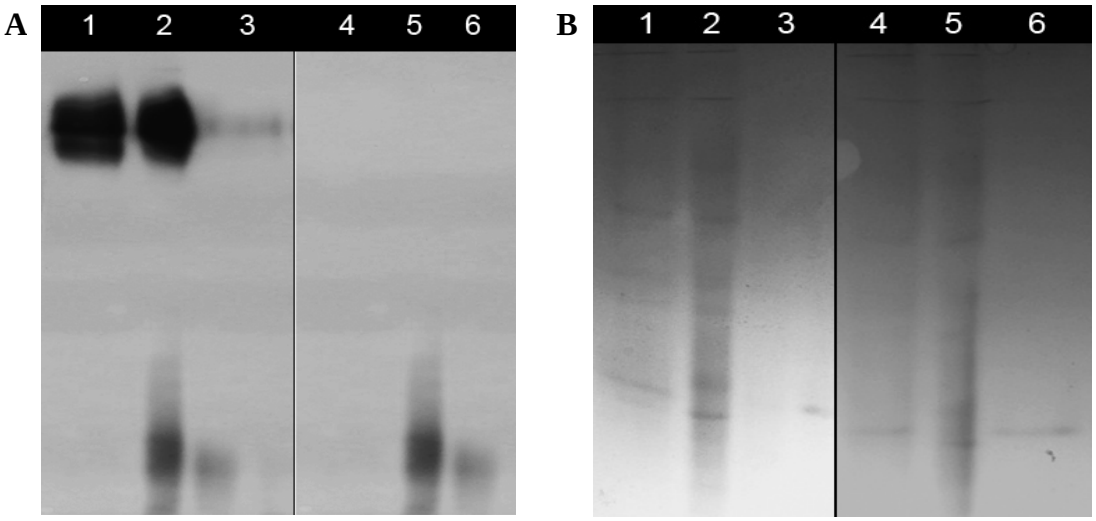


Figure 5.2. Activity staining (A) and Native PAGE (B) analysis of acetone fractions of BGL



MUG staining of acetone fractions

1 - 1:1 acetone precipitate
2 - 1:2 acetone precipitate
3 - 1:3 acetone precipitate

Silver staining of acetone fractions

4 - 1:1 acetone precipitate (0.25M Glucose)
5 - 1:2 acetone precipitate (0.25M Glucose)
6 - 1:3 acetone precipitate (0.25M Glucose)

[* Fig 5.2 A: Zymogram of BGL was prepared as described under section 2.5. The image was inverted for better clarity. The gels used for activity staining was washed and were used for silver staining (Fig 5.2B)]

Fractional acetone precipitation has been employed to separate proteins by several authors and even very early reports on the technique are available in the literature (Petersen & Sheridan, 1978, Bradley & Whitaker, 1986). The solubility of protein depends on, among other things, the di-electric constant of the solution. Acetone added to aqueous enzyme preparation can reduce the dielectric contestant and thereby help in precipitating the proteins (Bell *et al*, 1983). Acetone has the advantage that it is relatively inexpensive and is available in a pure form with few contaminants that may inhibit or poison the enzyme. It can also be recovered and recycled. Molecular weight of the protein is an important determinant of the amount of acetone required for precipitation and in general, low molecular weight proteins are precipitated in a higher ratio of acetone (Simpson, 2004).

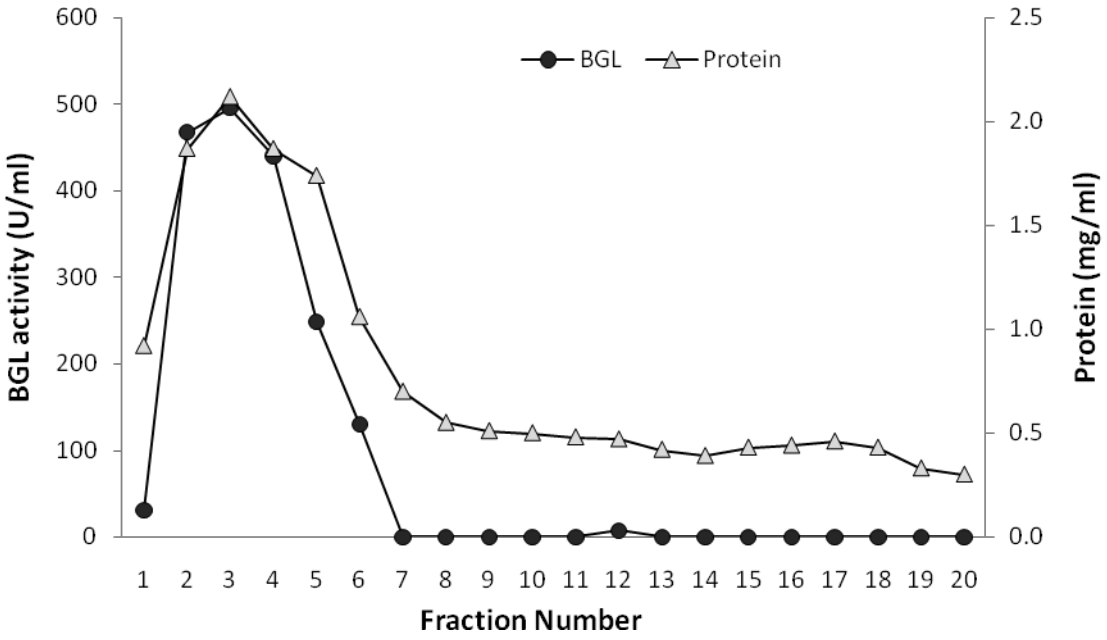
5.2.2. Purification of the major BGL (BGL1) of A. niger *NII 08121*

5.2.2.1. Determination of the isoelectric point (pI) of BGL from 1:1 acetone fraction

Purification of the major BGL from *A. niger* NII 08121 was done using the enzyme samples produced under SSF and concentrated by acetone fractionation as mentioned earlier (Section 5.1.1.2). The samples generated using 1:1 and 1:3 ratio acetone fractionations were used for purification studies since at these ratios, it was observed that the major and minor BGL activities with different molecular weights are getting separated. Purification was effected through sequential steps of ion exchange chromatography and electro-elution. The isoelectric point of BGL was determined for selection of the appropriate ion exchange resin.

Liquid phase isoelectric focusing done on the enzyme sample from 1: 1 acetone fraction using Rotophore®, resulted in the BGL activity getting focused in the pH 4.5-5.0 region of the focusing column which corresponds to fractions 2-5 (Figure 5.3).

Figure 5.3: Determination of the *pI* of BGL using liquid phase isoelectric focusing



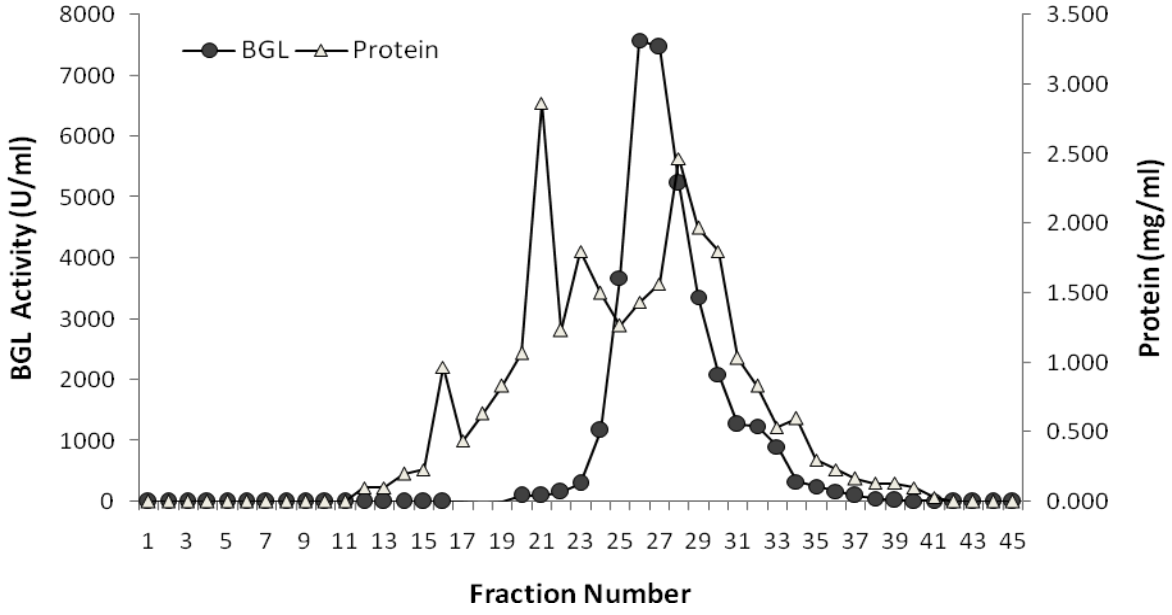
It was therefore presumed that the isoelectric point (*pI*) of the BGL in 1: 1 acetone fraction is close to this value (pH 4.5-5). Several authors have listed the pI of high molecular weight

BGL proteins from *Aspergilli* in this range (Rodinova *et al*, 1987, Galas & Romanovska, 1997, Langston *et al*, 2006). Cation exchange chromatography was therefore selected for further purification the enzyme.

5.2.2.2. Chromatographic separation of BGL activities from acetone precipitated enzymes

On the basis of information generated on the *pI* of BGL, 1:1 and 1:3 acetone precipitated fractions from the enzyme preparation was loaded separately on a cation exchange column and elution was carried out using continuous salt gradient as outlined under section 5.1.1.3. The best conditions of binding occurred at a pH of 3.0, where most of the proteins occur as cation since this pH is below the *pI* of the major BGL. However, no activity was recovered in 1:3 acetone fractions and hence this fraction was not studied further. The 1:1 acetone fraction when run on cation exchange column resolved into a single activity peak though there were multiple protein peaks (Figure 5.4). There were 5 major protein peaks spanning fractions 15-17, 20-22, 22-25, 27-31, and 33-35 respectively of which the BGL activity corresponded to part of the peak spanning fractions 27-31. The activity peak spanned fractions 24 -30. These fractions were pooled, and used for further studies.

Figure 5.4. Chromatographic purification of major BGL from *A. niger*



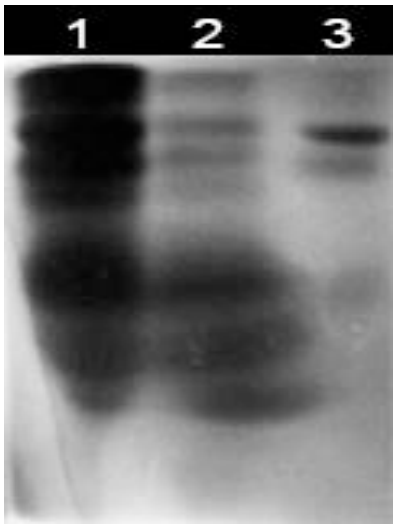
Zymogram analysis of the acetone precipitates and the pooled BGL fractions from ion exchange chromatography indicated the presence of only the high molecular weight band in both 1:1 and 1:3 ratio acetone precipitated samples and the pooled ion exchange fractions (Figure 5.5A). Native PAGE followed by silver staining of the pooled BGL fractions from ion exchange chromatography however showed two high molecular weight protein bands that are close together (Figure 5.5B). The lower band was less intense indicating lesser expression. There was also a faint low molecular band in the pooled ion exchange sample indicating that the separation was not effective. It was therefore decided to separate the activity band using electro elution after separating the proteins by gel electrophoresis.

Figure 5.5. Zymogram and Electrophoretic analysis of acetone precipitates and pooled chromatography fractions

A Zymogram

Lane 1 1:1 Acetone Precipitate
Lane 2 1:3 Acetone Precipitate
Lane 3 1:1 Acetone precipitate after ion exchange
Lane 4 1:3 Acetone precipitate after ion exchange

B Silver Staining of Native PAGE



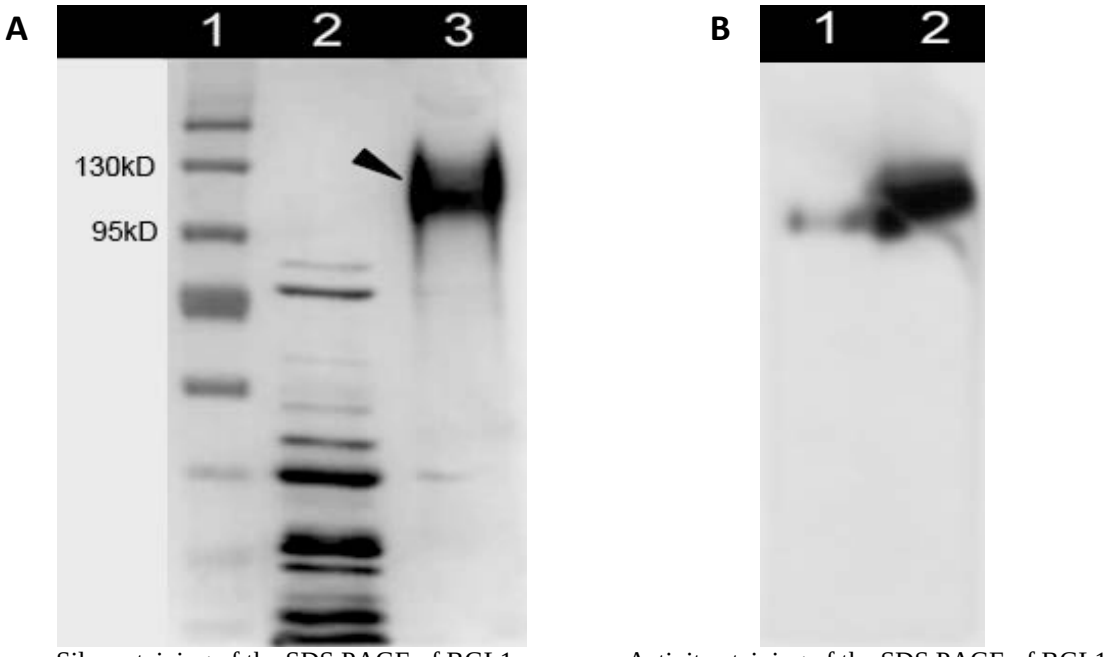
Lane 1 1:1 Acetone Precipitate
Lane 2 1:3 Acetone Precipitate
Lane 3 1:1 Acetone precipitate after ion exchange

5.2.2.3. Purification of major BGL using Electro-elution

The major BGL activity as indicated by the high molecular weight band in the activity staining and protein staining (Figure 5.5A &B, Lane 3) was purified to homogeneity by electrophoresis followed

by electro-elution of the band from the gel as outlined in section 5.1.1.4. Figure 5.6A shows the silver stained SDS PAGE of BGL1 where 1st lane corresponds to the protein marker and 3rd lane corresponds to the electro-eluted major band (BGL1). The major BGL band (BGL1) had a molecular weight of ~120 kDa as obtained by comparison with known molecular weight standards (Page Ruler™, protein ladder-Fermentas). The confirmation of the BGL activity of this band was done by activity staining using MUG as substrate (Section 2.5). Fluorescence of the electro-eluted band was detected by the MUG assay in the same position as the 120KDa major BGL protein in the silver staining (Figure 5.6B), which indicates the identity of this band as the major BGL (BGL1) of *Aspergillus niger* NII 08121. Several authors have reported the molecular weight of major BGL protein from *Aspergilli in* this range (Riou *et al*, 1998, Gunata & Vallier, 1999). The fact that both the Zymogram analysis and silver stain of the SDS PAGE of electro-eluted BGL shows only a single band indicates that probably the major BGL is a monomeric protein.

Figure 5.6: SDS PAGE and activity staining of the purified major BGL



Silver staining of the SDS PAGE of BGL1

Lane 1 Molecular weight marker (Page Ruler –SM0671, Fermentas)
Lane 2 Sample for part of this study
Lane 3 Electro eluted major BGL

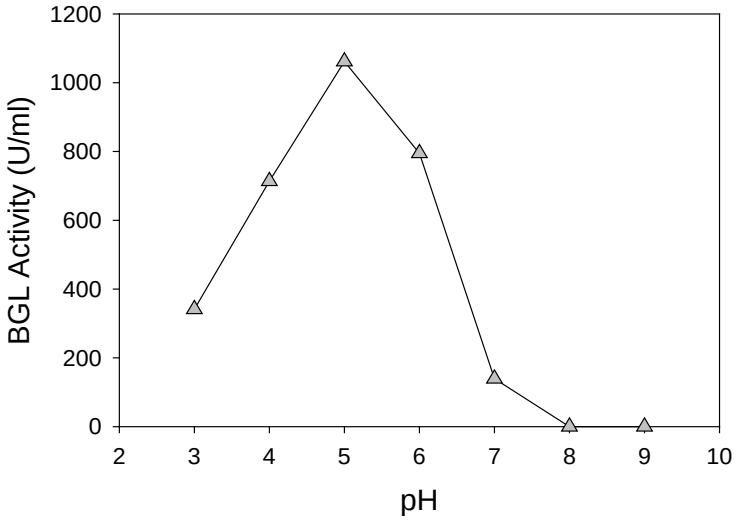
Activity staining of the SDS PAGE of BGL1

Lane 1 Electro eluted major BGL (low concentration)
Lane 2 Electro eluted major BGL (high concentration)

5.2.3. Characterization of the major BGL (BGL1) from A. niger *NII 08121*

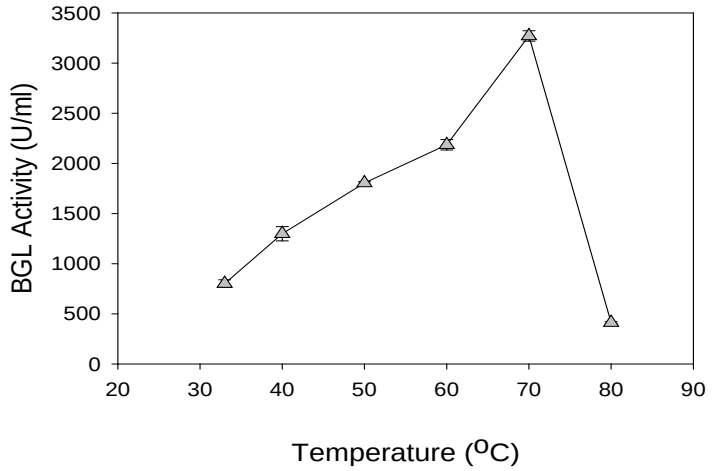
Electro-eluted 120kDa major BGL protein was characterized for its thermo tolerance, pH range and thermo stability as outlined in the materials and methods section. The glucose inhibition constant (*Ki*) which is a determinant of the glucose tolerance was also determined. Data shown in figure 5.7 indicated that the BGL1 has an acidic range of pH optima and the enzyme was most active at a pH of 5.0. There was a drastic decrease in activity beyond pH 7.0.

Figure 5.7. Determination of the Optimal pH for BGL1 activity



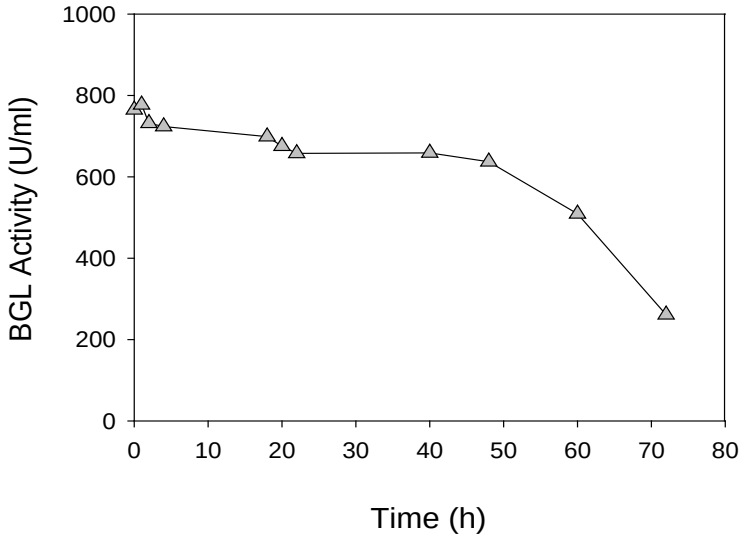
BGL assays done at various temperatures (33° C, 40°C, 50°C, 60°C, 70°C and 80°C) showed that the BGL activity increased with an increase in temperature till 70°C and there was a drop at 80°C (Figure 5.8). Maximum activity was observed at 70°C. BGLs active at higher temepartures are very advantageous for biomass saccharification since they can mainatin a rapid reaction rate and also help in preventing contamination by allowing the reaction to proceed at a higher temperatures. In this respect BGL1 from *A. niger* NII 08121, can be a potent supplement to the cellulases for creating better enzyme cocktails that can also act at elevated process temperatures

Figure 5.8. Determination of the Optimal Temperature for BGL1 activity



Since long term stability of BGL at elevated temperatures is critical in it's use for biomass hydrolysis, this property was tested by extended incubation at 50° C which is the most commonly employed temperature for biomass hydrolysis (Sukumaran *et al*, 2005, Rivera e al, 2010). BGL activity was estimated till 72 h and BGL1 was active at 50° C till 72 h. It retained about 90% activity till 48 h and almost half of the activity was still there at 72 h (Figure 5.9). The standard retention time used for biomass hydrolysis are often less than 48h.

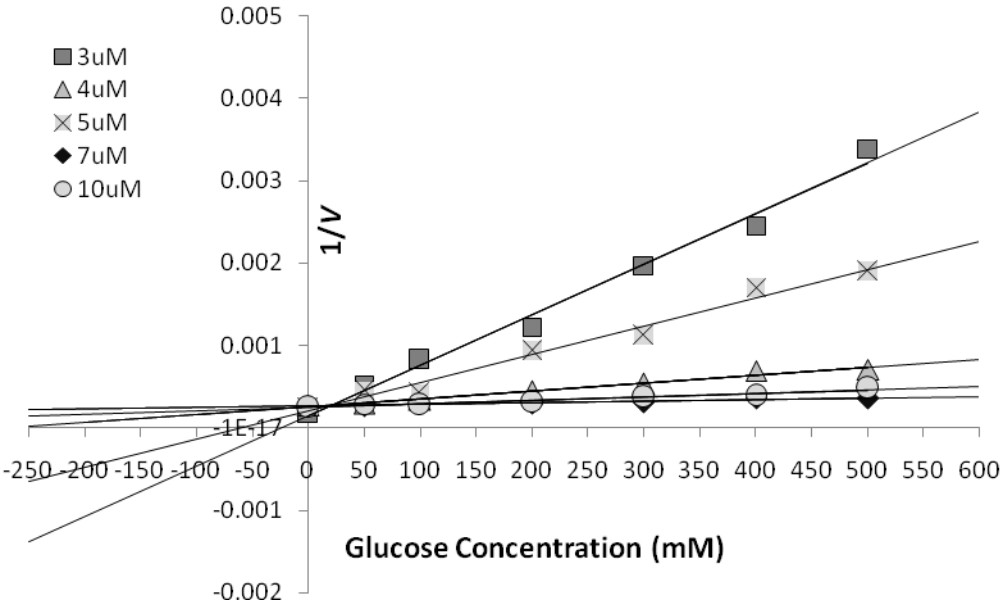
Figure 5.9. Temperature stability of BGL1 at 50 °C



5.2.3.1. Determination of the Glucose inhibition constant (Ki) of BGL1

Sensitivity / tolerance to glucose can be a major parameter which determines the suitability of a β-glucosidase for biomass hydrolysis. Major BGL protein of *Aspergilli* are reported to be sensitive to glucose though it is highly active and is expressed in very high titers compared to the glucose tolerant isoform (Riou *et al*, 1998, Gunata & Vallier, 1999). BGL1 from *A. niger* NII 08121 was also expressed in higher quantities compared to the other isoforms and the studies done on glucose inhibition kinetics of the major BGL revealed that it has a *Ki* of 20mM as estimated using a Dixon plot (Figure 5.10). The data also indicated competitive inhibition of the enzyme by glucose.

Figure 5.10. Dixon plot for determination of glucose inhibition constant (*Ki*) of BGL1



5.3. Conclusions

A. niger NII 08121 produced different BGL isoforms when grown on different carbon sources. Fractional acetone precipitation could separate out a high molecular weight major BGL (BGL1) and a low molecular weight and glucose tolerant minor BGL. The major beta glucosidase (BGL1) upon purification and characterization showed interesting properties

including an optimum temperature of 70 °C, optimum pH of 5.0 and extended stability at 50 ^{o}C for more than 48h. However, it was inhibited at 20mM glucose concentration. The characterization of other BGL activities from *A. niger* would be interesting, especially in light of the glucose tolerance exhibited by the low molecular weight BGLs observed in this study. The major BGL from *A. niger* NII 08121 is a potent candidate for use in biomass hydrolysis since normal enzymatic hydrolysis of biomass is performed at 50 °C over 24-72h and loss of enzyme activity is a major limitation in such processes. Enhanced level of production of BGL1 by the fungus and the improved temperature tolerance and stability of the enzyme are highly advantageous for biomass hydrolysis.

CHAPTER 6

Multiplicity of *Aspergillus niger* BGL: Confirmation by partial gene cloning of BGLs belonging to three glycosyl hydrolase families

Chapter 6

Multiplicity of *A. niger* BGL: Confirmation by partial gene cloning of BGLs belonging to three glycosyl hydrolase families

6. Introduction

β-glucosidases are found in all living organisms where they play multiple essential roles (Ketudat Cairns & Esen, 2010). In microorganisms, the function of BGL has been often studied in relation to biomass degradation (Gilbert *et al*, 2008). However, to state that BGLs are mostly involved in biomass degradation would be an underestimation of the roles of these versatile enzymes since several other roles are already known for these enzymes in microbes (Osbourn *et al*, 1995, Hung *et al*, 2001). BGLs have been classified into glycosyl hydrolase (GH) GH1, GH3, GH5, GH9 and GH30 based on their amino acid sequences (Henrissat, 1991, Cantarel *et al*, 2009, Oppasiri *et al*, 2007) families. BGLs belonging to different GH families are present and often expressed in response to inducers of cellulose metabolism in fungi. A search of the *Aspergillus niger* genome (*Aspergillus nigerV3*, JGI, 2010) returns several different genes and ESTs which indicates the multiplicity of these enzymes in this fungus.

Several researchers have successfully cloned and expressed beta glucosidases from *Aspergilli* (Iwashita *et al*, 1999, Dan *et al*, 2000, Kim *et al*, 2007) and majority of the reports also mentions the existence of multiple genes and gene products that are differentially expressed. The studies done on differential expression of BGL proteins in *A. niger* NII 08121 and the purification of the BGL proteins (Chapter 4 and 5) indicated the existence of at least 4 different secreted BGL proteins in this fungus while grown on biomass. To confirm the existence of different BGL genes which could be responsible for producing these proteins, it was proposed to look at the existence of multiple genes by PCR amplification and cloning of the partial gene sequences. Since BGLs belong to different GH families, degenerate PCR probes specific for amplification of GH1, GH3 and GH5 family beta glucosidases were designed and the PCR products were cloned and sequenced. The sequences were analyzed by

BLAST (Atschul *et al*, 1990) to prove the existence of BGL genes belonging to different GH families in *A. niger* NII 08121.

6.1. Materials and Methods

All of the DNA manipulation/molecular biology techniques were performed according to standard procedures as described by Sambrook *et al* (2001), unless otherwise specified.

6.1.1. Molecular cloning of the partial gene sequences of *A. niger* β-glucosidases

6.1.1.1. *Chromosomal DNA Isolation from* A. niger

A. niger was cultivated in potato dextrose broth (PDB) by inoculating 50 ml sterile PDB taken in 250ml Erlenmeyer flasks, with 10^6 spores and incubating at room temperature (28±2 °C) for 48h when the mycelia had grown as 1-5mm diameter balls. Five hundred milligram wet weight of the mycelium were frozen in liquid nitrogen and were ground with glass powder using a mortar and pestle to a fine paste. It was resuspended into 10ml of CTAB buffer (1% Cetyl Trimethyl Ammonium Bromide (CTAB), 0.7M NaCl, 10mM EDTA, 1% β-mercaptoethanol and 0.3mg/ml Proteinase K in 0.1M Tris buffer pH 7.5). The mycelial suspension was incubated at 65 °C for 30 min and after cooling an equal volume of 24:1 chloroform: iso-amyl alcohol was added. The tubes were inverted gently to mix the sample and the aqueous phase was recovered by centrifugation at 12000 rpm for 10min. Chromosomal DNA was precipitated by adding 1 volume of chilled iso-propanol and recovered by centrifugation at 12000 rpm for 10 min. The DNA precipitate was washed twice with 70 % ethanol, allowed to air dry and was resuspended in 10mM Tris-EDTA buffer (pH 8.0).

6.1.1.2. *Primer designing*

Protein sequences of β-glucosidases belonging to families 1, 3 and 5 of glycosyl hydrolases were collected from NCBI Proteins (http://www.ncbi.nlm.nih.gov/protein). Multiple sequence alignment was done by using a local copy of ClustalW (Larkin *et al*, 2007) and consensus

BLOCKS were generated using Blocks Multiple Sequence Alignment Processor web server (Henikoff *et al*, 2000). The blocks data was submitted to **CO**nsensus **DE**generate **H**ybrid **O**ligonucleotide **P**rimers (CODEHOP) web server (Rose *et al*, 1998) to design degenerate primers. As it was evident from the current study that *A. niger* produced several isoforms, BGLs belonging to more than one family of glycosyl hydrolase (GH) were expected to be produced by the fungus. So, degenerate primers for three major GH families ie – family 1 (GH1), family 3 (GH3) and family 5 (GH5) were designed and were tried for PCR amplification of partial BGL genes from the chromosomal DNA of the fungus. Specific primers were designed wherever sequence information was available using either of the software – PerlPrimer (Marshall, 2004) or FastPCR (Kalendar *et al*, 2009). The degenerate primers designed for amplification of GH1, GH3 and GH5 family BGLs are listed in Table 6.1, and the specific primers designed for amplification of a short region of GH3 family BGL of *A. niger* is given in Table 6.2.

Table 6.1. List of Degenerate Primers designed for PCR amplification of GH1, GH3 and GH5 family β- glucosidases

SL #	SEQ CODE	SEQUENCE
		FAMILY 1 GLYCOSYL HYDROLASE
1	BGL1_AF1	5'-GCA ACG GCG GCT TAT CAR RTN GAR GG -3'
2	BGL1_AF2	5'-TGG TAA AGG ACA ATC TAT CTG GGA YAC NTT -3'
3	BGL1_DR1	5'-TGT AAC ACC ATG GCT CGT TGA RNG TNA YCC A -3'
4	BGL1_DR2	5'-CGT GAT GCC CGA ATA TAG TGT AAC ACY MNG GYT CRT T -3'
5	BGL1_FF1	5′-CAC TCT ACA CGG TCA CTG GTA TGA RCC NTG GRA -3'
6	BGL1_FR1	5'-TGT TGA TCC GCC GCG TYC CAN GGY TC -3'
7	BGL1_JR1	5'-CTC CCT TAC ACG TGC AGC CRT TYT CNG T -3'
8	BGL1_IR1	5'-CGG TTC CAT ACC CAA TTC AGG WRY TTN CKR AA -3'

FAMILY 5 GLYCOSYL HYDROLASE		
9	BGL5_BF1	5'- TGG GTG GCT GGT GCT AGA RCC NTG GHT -3'
10	BGL5_BF2	5'- TGC GGG GCG TTA ATC TAG GNG GNT GGY T-3'
11	BGL5_DR1	5'- GGT ACT CCA GTT GTC CGT CGA CRT ANG GNT C-3'
12	BGL5_DR2	5'- CGA TCA TTA CCT TCA GAC CAG CAK CNC BNG CCC A-3'
13	BGL5_ER1	5'- GCC CTT TCG CCC AGA ATT RTC RAA NCC-3'
14	BGL5_FF1	5'- ACG GCG ATC GAG GCC NTN AAY GAR CC-3'
15	BGL5_FF2	5'- GGA CAC TAC CGT ATT CTT CCA TGA YGG NTT YNT-3'
16	BGL5_HR1	5'- ATC GGT CAT TGC CCC GSW CCA YTC NCC-3'
17	BGL5_IR1	5'- CCC AGG CGT CTA GCT GTG CYT CDA TRA A-3'
18	BGL5_JR1	5'- CCC CAT TTT CTA GTA GAT CTT GCA TRT CCC ANC C-3'
FAMILY 3 GLYCOSYL HYDROLASE		
19	BGL3a_DF1	5'-TGC GGT ATA AAC GTA GGG GCN ACN TKB GA -3'
20	BGL3a_EF1	5'-TGC GCA AAG CAT TAT ATT TGC WAY GAN MWR GA -3'
21	BGL3a_GR1	5'-GGA ATG GTG TGC GAA CCA RTC NSW CAT -3'
22	BGL3a_GR2	5'-ACC GGA ATG GTG TGC GAN CCA RTC NSW CA -3'
23	BGL3_GF1	5′-CGG TTT CCA AGG CTT CGT AAT GWS NGA YTG G-3'
24	BGL3_GF2	5'-CCA ACG CTG GGC TGG AYA TGW SNA TG -3′
25	BGL3_KR1	5'-GCT TCG TCC GGC GTA ACA ARR TAN GGR WA -3'
26	BGL3_KR2	5'-CAA AAT AGG GAA AGT TAG CAG TTC CAS WNC CCC ANC C -3'

Table 6.2. List of Specific primers designed for PCR amplification of a short stretch of GH3 family β- glucosidase of *A. niger*

SL #	SEQ CODE	SEQUENCE
	***A. niger* GH family 3 primers**	
1	AN_1F	AGG TCC TTG TGG GGA CCA GAG
2	AN_2F	AGA GTT GAC CCG CGC AGT GTT G
3	AN_1R	GTT AGC AGT TCC AGA GCC CCA
4	AN_2R	GGC CAG AGT ACC CTG GTT GCA
5	AN_4R	CCC GAT AGG GAC CAC CGG ATG

6.1.1.3. *PCR Amplification of partial gene sequences of BGL*

Amplification of partial gene sequences of beta glucosidases belonging to glycosyl hydrolase families 1, 3 and 5 were performed by polymerase chain reaction using different sets of the degenerate primers designed for each family. PCR reactions contained 0.5 unit of Fermentas (USA) *Taq* DNA polymerase, 1X buffer, 200µM of each deoxynucleotide triphosphate, 2µM $MgSO_4$ and 0.5µM forward and reverse primers. Reaction conditions for PCR amplification were an initial 95 °C for 4 min, followed by 25 cycles of 95 °C for 30 sec, 50 °C for 1 min and 70 °C for 1 min; and a final extension step at 72 °C for 5 min. An Eppendorff 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.

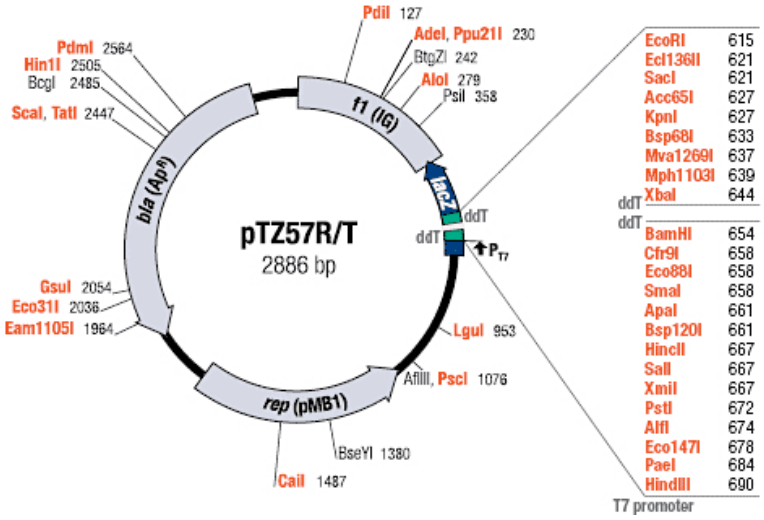
6.1.1.4. *Cloning of PCR products and sequencing of inserts*

PCR fragments obtained using degenerate primers were gel purified using GeneJET ™ PCR purification kit (Fermentas, USA) and were then cloned into the T/A cloning vector pTZ57R/T from Fermentas (Figure 6.1). Cloning of the PCR amplicons were performed using "InsT/Aclone™ PCR product cloning kit (Fermentas, USA) according to the manufacturer's protocols. In brief, the following components were added into the 1.5ml micro centrifuge tube to make up the final volume to 10µl (Table 6.3).

Table 6.3. Ligation reaction for cloning PCR products

Plasmid vector pTZ57R/T (0.05 µg)	1µl
Purified PCR fragment (0.15 µg)	3µl
10x ligation buffer	2.5µl
T4 DNA Ligase, 5U	1µl

Fig 6.1. Restriction map of T/A cloning vector pTZ57R/T



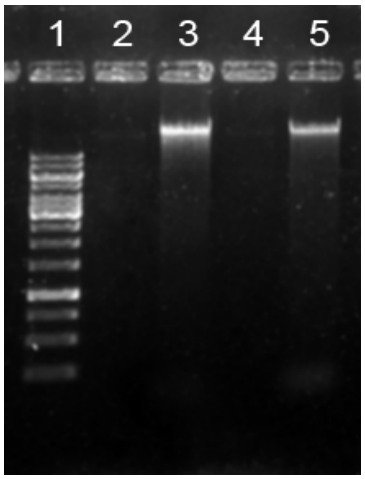
The ligation reaction was done at 16 °C in a refrigerated water bath for 8h. Five micro liters of the ligation mix was used for transformation. Competent cells (*E. coli* strains of XL1Blue/JM107) were prepared and transformation was performed using "TransformAid^{TM} Bacterial Transformation kit (Fermentas, USA) according to manufacturer's protocols. LB plates with XGal (40µg/ml), IPTG (0.1mM) and Ampicillin (100µg/ml) were plated with transformed *E. coli* and were incubated for 12-16h at 37 °C. Colonies that appeared in the plates were selected based on the blue white screening. White colonies were expected to harbor the plasmid with insert and were inoculated into LB medium with Ampicillin for 12-16h. Plasmids were isolated from the bacterial cells by "GeneJET^{TM} Plasmid Miniprep Kit (Fermentas, USA). Presence and size of inserts were determined by restriction digestion of the purified plasmids (According to enzyme manufacturer's protocols), and also by PCR amplification of the desired fragment using the same set of primers as was used for generating the amplicons. Sequencing of the inserts was outsourced and purified plasmids were sent to either Scigenome (Kochi, India) or Axygen (New Delhi, India) for sequencing using M13 forward and reverse primers which are complementary to the flanking regions of pTZ57R/T multiple cloning site. Sequence reads received from the companies were analyzed by BLAST (Altschul *et al*, 1997).

6.2. Results and Discussion

6.2.1. *Chromosomal DNA isolation*

DNA preparation from *A. niger* was successful and the CTAB method yielded high molecular weight chromosomal DNA (Figure 6.2). The DNA isolated was suspended in 10mM Tris EDTA buffer (pH 8.00) and was used as template for PCR reactions. Long term storage of DNA was done at -80 ºC.

Figure 6.2: Chromosomal DNA isolation from *A. niger*



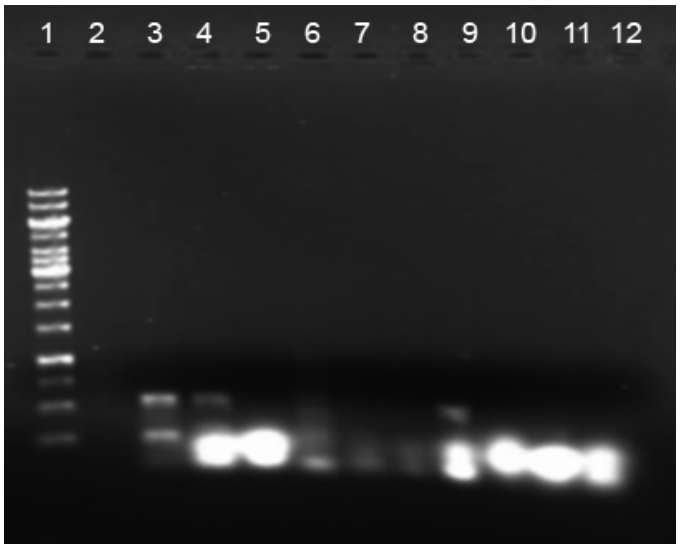
Lane Information

1 -Generuler® -1kb ladder (Fermentas)
3 -Chromosomal DNA from *T. reesei*
5 -Chromosomal DNA from *A. niger*

6.2.2. *PCR amplification of BGL genes*

PCR Amplification of BGL genes belonging to the glycosyl hydrolase families GH1, GH3 and GH5 were performed using combination of degenerate primers designed to amplify the BGLs from these families. The different sets of primers were designed to amplify different regions of the gene. Figures 6.3, 6.4 and 6.5 shows the amplicons obtained with primer pairs corresponding to GH1, GH3 and GH5 respectively.

Figure 6.3: PCR amplification of GH1 family BGL from *A. niger* genomic DNA

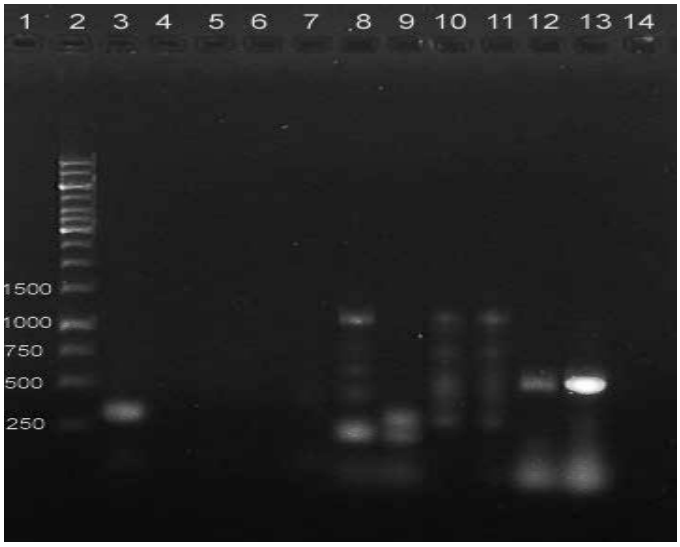


Lane Information

1	GeneRuler ® 1kb ladder(Fermentas)
3	Primer pair AF1 –DR1
4	Primer pair AF1 –DR2
5	Primer pair AF2 –DR1
6	Primer pair AF2 –DR2
7	Primer pair FF1 –JR1
8	Primer pair FF1 –IR1
9	Primer pair AF1 –FR1
10	Primer pair AF2 –FR1
11	Primer pair AF1 –JR1
12	Primer pair AF1 –IR1

Most of the primer pairs successfully amplified an ~200 bp region while certain combinations of primers (AF1 –DR1, AF1 –DR2, AF2 –DR2, AF1 –FR1) produced 2 amplicons , one ~200bp and the other one >500bp. Amplicons obtained using primer pair *BGL1*_AF1 – *BGL1*_DR1 and *BGL1*_AF1 – *BGL1*_DR2 was sharp and clear indicating better products and it was decided to clone fragments from these two combinations separately after purification of the fragments from gel.

Figure 6.4. PCR amplification of GH3 family BGL from *A. niger*

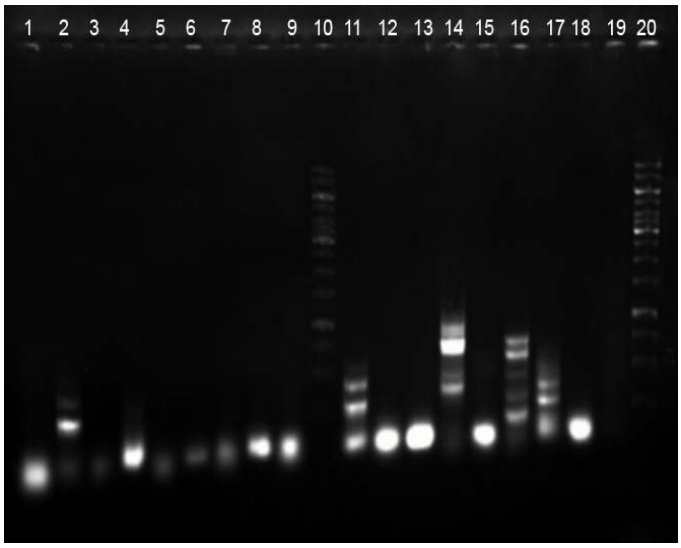


Lane Information

2	GeneRuler ® 1kb ladder(Fermentas)
3	Primer pair EF1 -GR1
8	Primer pair DF1 -KR2
9	Primer pair EF1 -GR2
10	Primer pair DF1 -KR2
11	Primer pair EF2 -KR2
12	Primer pair GF1 -KR2
13	Primer pair GF2 -KR2

Most of the primer pairs in the case of GH3 family amplified multiple sequences as evident from the distribution of amplicons in lanes 8, 9, 10 and 11 in Figure 6.4. Primer pair *BGL3*_EF1- *BGL3*_GR2 produced an ~ 300bp amplicon while primer pairs *BGL3*_GF1- *BGL3*_KR2 and *BGL3*_GF2- *BGL3*_KR2 produced 2 amplicons each of size ~ 700 bp and ~100bp respectively. It was decided to clone the high molecular weight amplicon from *BGL3*_GF2- *BGL3*_KR2 combination primers.

Figure 6.5. PCR amplification of GH5 family BGL from *A. niger*



Lane Information

1	Primer pair BF1 -DR1
2	Primer pair BF2 -DR1
4	Primer pair BF2 -DR2
8	Primer pair BF2 -HR1
10	GeneRuler ® 1kb ladder(Fermentas)
11	Primer pair BF2 -IR1
12	Primer pair BF1 -JR1
13	Primer pair BF2 -JR1
14	Primer pair FF1 -HR1
16	Primer pair FF1 -JR1
17	Primer pair FF2 -HR1
20	GeneRuler ® 1kb ladder(Fermentas)

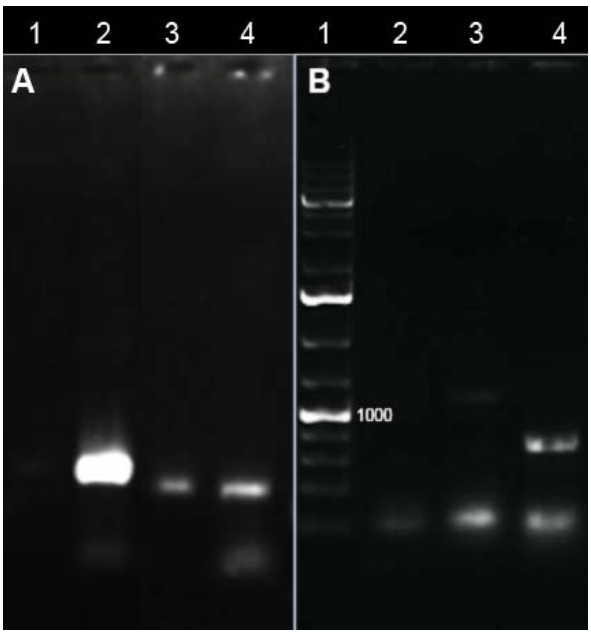
Use of primer pairs *BGL5*_BF2-*BGL5*_DR1 and *BGL5*_FF2-*BGL5*_HR1 produced two amplicons each, with a molecular weight of ~600 bp for the upper band. Other amplicons were either small (~100bp) or with noise (several bands for same set of primers). It was therefore decided to clone the upper band from these primer combinations.

6.2.3. *Cloning, Sequencing and BLAST analysis of the partial BGL genes of families GH1, GH3 and GH5*

6.2.3.1. Cloning of partial genes of GH1, GH3 and GH5 BGLs

Amplicons of the BGLs belonging to GH1, GH3 and GH5 as selected above were gel purified and cloned as outlined under section 6.1.1.4. Plasmids were isolated from transformants and was purified using a GeneJet ™ Plasmid Miniprep kit from Fermentas. The isolated plasmids were used as templates for PCR amplification of the gene using the same set of degenerate primers used for amplifying them from the genomic DNA. The presence of amplicons with identical size from genomic DNA and plasmid confirmed successful cloning of the partial BGL genes. Figures 6.6 – 6.7 provides the confirmation of partial gene cloning of BGLS belonging to families GH1, GH3 and GH5.

Figure 6.6A &B: Confirmation of GH1and GH5 family BGL partial gene cloning



Lane Information

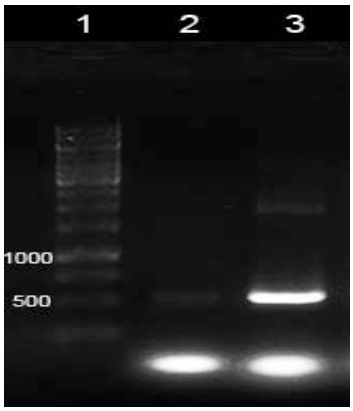
A: Amplification of GH1-BGL and GH5-BGL partial genes from genomic DNA and recombinant plasmids

1 GH1-BGL gene amplification using genomic DNA as template (Primers AF1 & DR1)
2 GH1-BGL gene amplification using plasmid DNA as template (Primers AF1 & DR1)
3 GH5-BGL gene amplification using genomic DNA as template (Primers BF2 & DR1)
4 GH5-BGL gene amplification using plasmid DNA as template (Primers BF2 & DR1)

B: Size determination of GH1-BGL and GH5-BGL partial genes amplified from plasmids

1 GeneRuler ® 1kb ladder(Fermentas)
2 GH1-BGL gene amplicon (AF1 & DR1) (No amplification)
3 GH1-BGL gene amplicon (AF1 & DR2) (~1200bp)
4 GH5-BGL gene amplicon (BF2 & DR1) (~700bp)

Figure 6.7: Confirmation of GH3 family BGL partial gene cloning



Lane Information

1 GeneRuler ® 1kb ladder(Fermentas)

2 GH3-BGL amplicon (GF2-KR2) Amplified from genomic DNA

3 GH3-BGL amplicon (GF2-KR2) Amplified from plasmid DNA (~500bp major band and >1000bp minor band)

6.2.3.2. Sequencing and BLAST analysis of partial genes of GH1, GH3 and GH5 BGLs

Cloned partial genes of GH1, GH3 and GH5 family BGLs were sequenced using di-deoxy chain termination method using M13 forward and reverse primers since the multiple cloning site of pTZ57R/T vector contain flanking sequences of M13 forward and reverse primers to facilitate sequencing. The sequences reads of appropriate quality obtained for each gene was compared against non redundant nucleotide sequence collection at Genbank using the web interface of NCBI-BLAST. Results are given below

6.2.3.2.1. Sequence of GH1-BGL insert and BLAST results

Sequencing of the GH1 –BGL insert returned a 454bp fragment as given below

>GH1_BGL
GCGACGGTCTCGCGATGCATCTAGATTCGTGATGCCCGAATATAGTGTAACACTACGGTTCGTTGAATGTAATCCAATG
CTTGACTTTCGAGCCCAGGGCCTGGAACATGACTCGAGCGTAGTGTGCGAAATCCGCTACGAATTCCTCCTTGTTCAGG
AGTCCACCGTAGCGCTTGTCGAGTTCATCGGGCAGATCCCAATGGAAGAGTGTCACCAGGGGTGTGATCCCCGCTGCTA
GGAGGTCGTCGACAAATTTGACGTAGTGTTGGATTCCCTTTTCGTTGATGGGGTCGTTGCGGCCTCCCAGGGGGATGAT
GCGTGACCTGAAGGATGGGTCAGAATGATGTTCGGGGTGTGATTGAATTGAATGTTCGTCTTGGTATGGTATGGTATTA
AACTCACCATGAGATTGAAAAGCGGTAGGCTTGGGCACCGCATTCTTTTAACAAGGCGA

BLAST analysis indicated that the sequence has 87 % identity with 77% coverage to *Aspergillus niger* CBS 513.88 hypothetical protein An03g03740 which has the domain signatures of glycosyl hydrolase family 1 and is thought to be a putative GH1 beta glucosidase. Also the sequence had close similarity to the following

Aspergillus clavatus NRRL 1 beta-glucosidase, putative (ACLA_020660), (XM_001268784.1),

Neosartorya fischeri NRRL 181 beta-glucosidase, putative (NFIA_010690) (XM_001264284.1),

Aspergillus fumigatus Af293 beta-glucosidase (XM_747747.1),

Aspergillus flavus NRRL3357 beta-glucosidase, putative, mRNA (XM_002374789.1),

Talaromyces emersonii beta-glucosidase 1 (bg1) gene, complete cds (AY081764.2)

The results indicated the existence of GH1- family BGL in *A. niger* NII 08121.

6.2.3.2.2. *Sequence of GH3-BGL insert and BLAST results*

GH3 –BGL insert was a 1169bp fragment as given below

```
> GH3_BGL
GGCGTAGCATCGCGTGTGCCACTCGGGCGGACGGAAGGTCCTTGTGGGGACCAGAGTTGACCCGCGCAGTGTTGA
ACACCTCTGTCCCGATGGACCGGTTGAACGACATGGTGACCCGCATTGTGGCCTCCTGGTACCATCTTGGCCAGG
ACTTGTGGCCTCGGCCTGCTCCGGACGGTGATGGCGGGCCTACCTTTTCCTCGTGGACCAACGATGAGGTCGGAT
GGCTGCACCAAGGGTCTCCGGATGACGAGGCCTACGGTATCGTGAACCGGTTCATTAATGCCCAGGGCGAAGGCG
AGAATGCACACTCCATCATTGCTCGCAAGGTGGCGGCCGAAGGCATCGTGATGGTGAAGAATGACCAGAACACAC
TGCCATTGTCCCGCCGGCCAGTGAGCCCATCCGGTGGTCCCTATCGGGTGGGCGTGTATGGCGACGACGCCGGAC
CGGCTGGAGGACCCAACGCCTGCCCAGACCGGGGCTGCAACCAGGGTACTCTGGCCATGGGCTGGGGCTCTGGAA
CTGCTAACTTTCCCTATTTTGAATCGGATCCCGGGCCCGTCGACTGCAGAGGCCTGCATGCAAGCTTTCCCTATA
GTGAGTCGTATTAGAGCTTGGCGTAATCATGGTCATAGCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTC
CACACAACATACGAGCCGGAAGCATAAAGTGTAAAGCCTGGGGTGCCTAATGAGTGAGCTAACTCACATTAATTG
CGTTGCGCTCACTGCCCGCTTTCCAGTCAGGAAACCTGTCGTGCCAGCTGCATTAATGAATCGGCCAACGCGCGG
GGAGAGGCGGTTTGCGTATTGGGCGCTCTTCCGCTTCCTCGCTCACTGACTCGCTGCGCTCGGTCGTTCGGCTGC
AGCGAGCGGTATCAGCTCACTCAAAGGCGGTATACGATATCCACAGATCAGGGATACGCAGAAAGAACATGTGAG
CAAAGTCAGCAAATGCAGATCGTAAAAGCCGCGTTGCTGGCGTTTTCATAGCTCCGCCCCCTTGACTGAGCATAC
ACATTCGAACGCCTCAGTCGAAGTGGCGAACCCGACGACTATGAGAATACAGGCGTTACCCCTGGGAGTTCCTCT
TGGGCGCCTTTCCTGTTTCGAACCACTGTGCCGCGTTATCCTGA
```

The GH3_BGL insert sequence had 91% identity over a 44% region with the hypothetical protein An15g01890 of *Aspergillus niger CBS 513.88*. This protein sequence has both glycosyl hydrolase N terminal and C terminal domain signatures and is listed as a putative GH family 3 beta glucosidase. Results indicate the presence of GH3-BGL in *A. niger* NII 08121.

6.2.3.2.2. *Sequence of GH5-BGL insert and BLAST results*

```
>GH5_BGL
ACTACCGGTCTCGCGATGCATCTAGATTACGGCGATCGAGGCCCTGAACGAACCCAACATTCCTGGTGGCGTAAA
CGAGGGTGACCTCAAGAACTACTATTACGGTGCTTTGGCCGATGTCCAGCGTCTCAACCCTTCTACTACTTTGTT
CATGTCGGACGGCTTTCAGCCTGTAGAGTCCTGGAACGGCTTTATGCAAGGTAGCAACGTTGTCATGGATACGCA
TCACTATCAGGTTTTTGACACTGGCTTGCTTTCTATGAGCATCGATGATCATGTCAAAACGGCCTGTTCTCTCGC
CACACAGCATACGATGCAATCAGATAAGCCCGTCGTCGTGGGCGAATGGTCCGGGGCAATGACCGATAATCGGAT
CCCGGGCCCGTCGACTGCAGAGGCCTGCATGCAAGCTTTCCCTATAGTGAGTCGTATTAGAGCTTGGCGTAATCA
TGGTCATAGCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCCACACAACATACGAGCCGGAAGCATAAAG
TGTAAAGCCTGGGGTGCCTAATGAGTGAGCTAACTCACATTAATTGCGTTGCGCTCACTGCCCGCTTTCCAGTCG
GGAAACCTGTCGTGCCAGCTGCATTAATGAATCGGGCCAACGCGCGGGGAGAGGCGGTTT
```

The sequence of GH5-BGL insert (660bp) matched *Aspergillus niger* CBS 513.88 hypothetical protein (An18g04100) partial mRNA. The hypothetical protein showed strong similarity to the *Aspergillus oryzae* beta-glucosidase gene from patent WO9955884 (Riou & Gunata, 1999) and has the pfam domain signature PF00150 which is specific for glycosyl hydrolase family 5. Similarity to this hypothetical protein which is putative GH family 5 beta glucosidase indicates that the G5-BGL gene is also present in *A. niger* NII 08121.

6.3. Conclusions

Multiplicity of BGLs in filamentous fungi is a known fact and the presence of multiple BGLs can be due to the presence of different BGL genes or due to the differential processing of transcripts. Existence of different BGL activities in organisms is thought to be essential due to the diverse roles of these enzymes. *A. niger* NII 08121 was found to secrete at least four different BGL proteins as determined by electrophoretic separation and activity staining. However, the results from the present study show that the organism has genes for BGLs belonging to three major glycosyl hydrolase families ie GH1, GH3 and GH5. While most of the reported BGLs belong to families GH1 and GH3, there are also reports on the existence of GH5 BGLs in filamentous fungi. Partial gene sequences obtained from the study can be used to probe a cDNA library or even the chromosomal DNA for isolation of full length sequences which will be useful in characterizing the beta glucosidase activities in the fungus.

CHAPTER 7

Studies on the application of *Aspergillus niger* β-glucosidase for bioethanol production

Chapter 7

Studies on the application of *A. niger* β-glucosidase for bioethanol production

7. Introduction

One of the major applications projected for beta glucosidases is as a component in the cellulase cocktail for biomass hydrolysis. Enzymatic conversion of biomass is the most efficient way of breaking down the pretreated lignocellulosic feedstock to their component sugars which can then be used for producing bioethanol. However, no single enzyme is capable of efficient deconstruction of a wide variety of highly complex pretreated substrates (Decker & Teter, 2008, Meyer *et al*, 2009). Therefore the properties desired in enzyme are also wide and varied and often dependent on the type feedstock and the method of pretreatment. Cellulase preparations from commercially used enzyme producer *T. reesei* RUT C 30, though capable of biomass hydrolysis contains very less quantities of beta glucosidase which necessitates the addition of exogenous BGL to improve the efficiency of saccharification. BGL from *A. niger* have been used by several researchers for creating enzyme blends suited for hydrolysis of different feedstock (Sternberg *et al*, 1977, Mohagheghi *et al*, 1992, Xiao *et al*, 2004). *A. niger* NII 08121 was found to produce very high titers of beta glucosidase which could be a potent supplementation in enzyme cocktails for biomass hydrolysis. It was therefore decided to test the effectiveness of the BGL preparation from the fungus as component in the enzyme cocktail for biomass hydrolysis. Since cellulase preparation with only *T. reesei* enzyme was not available, it was decided to produce the *T. reesei* cellulase and use this in blends with *A. niger* BGL. The combination of enzymes and the hydrolysis conditions were optimized for best sugar yields. Alkali pretreated rice straw was used as a model substrate. The biomass hydrolysate generated by enzymatic saccharification with enzyme cocktail was also used for alcohol production by inoculating this with *Saccharomyces cerevisiae.*

7.1 Materials and Methods

7.1.1. *Organisms and culture conditions*

Trichoderma reesei RUT-C30, was a kind gift from Prof George Scakacs, Technical University of Budapest, Hungary and *Saccharomyces cerevisiae* NCIM 3288 was procured from National Collection of Industrial Microorganisms (NCIM), Pune, India. Media and Inoculum preparation, Solid state fermentation, enzyme extraction and concentration and assays were done as detailed under appropriate sections in the Materials and Methods (Chapter 2).

7.1.2. *Cellulase production using Trichoderma reesei RUT C30.*

Production of cellulase by Solid State Fermentation (SSF) using wheat bran as carbon source was done as outlined in sections 2.2.1 and 2.3.1.

7.1.2.1 Optimization of cellulase production by T.reesei RUT C30 under Solid State Fermentation

Optimization of parameters for cellulase production was performed in two stages. Initially 11 variables were screened using a fractional factorial design to identify the parameters which significantly influenced enzyme production and in the second stage the levels of these parameters were optimized using a response surface design.

7.1.2.1 A. Screening of parameters affecting cellulase production by fractional factorial design

A Plackett and Burman (1946) design was employed to determine the effect of individual parameters affecting cellulase production by the fungus under SSF. The composition of mineral salt solution used for wetting the substrate and the important physical parameters affecting enzyme production were screened in a design with 11 variables at two levels in a total of 12 experimental runs (Table 7.1). The variables were tested at two levels

+1 and -1. The actual and coded values tested for each parameter are given in Table 7.2. 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 cellulase production was calculated by the following equation (Eqn.1)

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

Where ε is the effect of parameter under study and "μ_{+}" and "μ_{-}"are responses (cellulase activities) 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 cellulase production. The most significant parameters affecting cellulase production were identified.

Table 7.1 Plackett & Burman design matrix for the screening of variables influencing cellulase production

Std order	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	X_9	X_{10}	X_{11}
1	1	-1	1	-1	-1	-1	1	1	1	-1	1
2	1	1	-1	1	-1	-1	-1	1	1	1	-1
3	-1	1	1	-1	1	-1	-1	-1	1	1	1
4	1	-1	1	1	-1	1	-1	-1	-1	1	1
5	1	1	-1	1	1	-1	1	-1	-1	-1	1
6	1	1	1	-1	1	1	-1	1	-1	-1	-1
7	-1	1	1	1	-1	1	1	-1	1	-1	-1
8	-1	-1	1	1	1	-1	1	1	-1	1	-1
9	-1	-1	-1	1	1	1	-1	1	1	-1	1
10	1	-1	-1	-1	1	1	1	-1	1	1	-1
11	-1	1	-1	-1	-1	1	1	1	-1	1	1
12	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1

Table 7.2 Actual levels of variables tested with the factorial design and their effects on cellulase production

Code	Parameter name	Low level (-1)	High Level (+1)
X_1	Moisture (%)	40	60
X_2	Particle size (μM)	300-500	500-1000
X_3	pH	4	7
X_4	Temp of Incubation (^oC)	27	32
X_5	Inoculum size (spores/ml)	10^5	10^8
X_6	Inoculum age (days)	5	7
X_7	NH_4NO_3 (g/L)	2.5	7.5
X_8	Peptone (g/L)	1	3
X_9	Cellobiose (g/L)	0.001	0.01
X_{10}	Tween 80 (g/L)	0.1	0.5
X_{11}	Incubation time (h)	96	144

7.1.2.1. B *Optimization of significant parameters for improving cellulase production*

The significant parameters identified by the Placket and Burman design were optimized using a Response Surface Central Composite rotary design (CCD) (Box & Wilson, 1951). The effect of the significant variables was studied at five different levels (Table 7.3). The design matrix with 14 experimental runs in two blocks where the midpoint is replicated 6 times is shown in Table 7.4. The screened variables: initial moisture content of the medium and the incubation temperature were coded as X_1 and X_2 respectively. The behavior of the system was modeled by a second order polynomial equation. The model equation used for the analysis is given below (Eqn. 2)

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

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. For two variable systems the model equation is given below (Eqn. 3)

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{12} X_1 X_2$$

(3)

Regression analysis and estimation of the coefficients were performed using Design Expert®. Pareto chart of the effect estimates, the three dimensional response surfaces and contour plots were generated using Statistica software (Statsoft Inc. USA) or Design Expert. The ideal levels and combinations of parameters were identified by optimization functions in the software and experiments were run at these levels for validation of the model.

Table 7.3 Actual and coded levels of variables tested with the central composite design

Code	Parameter name	Levels of parameters				
		-α (–1.414)	-1	0	+1	+α (+1.414)
X_1	Moisture (%)	33.96	35	37.5	40	41.04
X_2	Temp of Incubation (^oC)	28.2	29	31	33	33.8

α = Axial /Star point (s) are levels larger than the chosen range of parameters

Table 7.4 Central Composite Design matrix for optimization of parameters identified by the fractional factorial design

Std order	Block	Moisture (%)		Temperature (^oC)	
		Actual	Coded	Actual	Coded
1	1	35	-1	29	-1
2	1	40	1	29	-1
3	1	35	-1	33	1
4	1	40	1	33	1
5	1	37.5	0	31	0
6	1	37.5	0	31	0
7	1	37.5	0	31	0
8	2	33.96	-1.41	31	0
9	2	41.04	1.41	31	0
10	2	37.5	0	28.17	-1.41
11	2	37.5	0	33.83	1.41
12	2	37.5	0	31	0

13	2	37.5	0	31	0
14	2	37.5	0	31	0

7.1.3. *Optimization of Enzyme cocktail for hydrolysis of alkali pretreated rice straw*

Blends of *T. reesei* cellulase and *A. niger* BGL were prepared with different ratios to make enzyme cocktails for biomass hydrolysis. The performance of these cocktails was assessed by using them for hydrolysis of alkali pretreated rice straw which was prepared according to section 2.6. The carbohydrate and lignin content of pretreated biomass was analyzed by NREL protocols (Sluiter *et al*, 2008). Theoretical maximum of total sugar yield was calculated as C × 1.11 + H × 1.136 where C is the cellulose content and H is the hemicellulose content in g/g biomass and were expressed in percentage values. The factors used in either case represent the increase in total weight due to addition of water molecule upon breakage of the sugar bonds. Saccharification of biomass was performed as outlined under section 2.7. Efficiency of saccharification was gauged as the yield of reducing sugars from the pretreated biomass. Total reducing sugars in the hydrolysate was measured by DNS method (Miller, 1959) and was expressed in either g/g or mg/g (grams/milligrams of reducing sugar liberated/gram pretreated biomass)

Optimization of enzyme cocktails was done using a response surface Box Behnken design. The design matrix with 15 experimental runs in a single block is shown in Table 7.5. The screened variables: biomass loading, enzyme loading, surfactant concentration and residence time were coded as X_1, X_2 and X_3 respectively.

Table 7.5: Box –Behnken design matrix for optimization of saccharification

Std Order	Block	*T. reesei* cellulase (FPUs/g biomass) X_1	*A. niger* BGL (U/g biomass) X_2	Time (h) X_3
1	1	5.00	0.00	36.00
2	1	10.00	0.00	36.00
3	1	5.00	866	36.00
4	1	10.00	866	36.00
5	1	5.00	433	24.00
6	1	10.00	433	24.00
7	1	5.00	433	48.00
8	1	10.00	433	48.00
9	1	7.50	0.00	24.00
10	1	7.50	866	24.00
11	1	7.50	0.00	48.00
12	1	7.50	866	48.00
13	1	7.50	433	36.00
14	1	7.50	433	36.00
15	1	7.50	433	36.00

The behavior of the system was modeled by a second order polynomial equation. The model equation used for the analysis is given in equation 2 (Section 7.1.2.1B)

For three variable systems, the model equation was as given below (Eqn. 4)

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X3^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 \quad (4)$$

Regression analysis and estimation of the coefficients were performed using the DOE software Design Expert ® (Statease Corp, USA). Three dimensional response surfaces were generated using the software. The optimal levels and combinations of parameters were identified by optimization functions in the software.

7.1.4. *Ethanol production using the enzymatic hydrolysate of rice straw.*

The suitability of the rice straw hydrolysate generated using optimized enzyme cocktail was tested by using it as substrate for ethanol production. Ethanol production and assay were performed according to sections 2.8 and 2.4.4 respectively.

7.2. Results and Discussion

7.2.1. *Optimization of parameters for improving cellulase production from* T. reesei *RUT C30.*

Trichoderma reesei RUT C30 produced 0.605 U/gds of cellulase activity in the un-optimized basal medium (Mandels & Weber, 1969). Screening of most important variables and their optimization was attempted to improve the yield of cellulase under SSF on wheat bran substrate.

7.2.1.1. Screening of parameters affecting cellulase production

Eleven variables were screened for determining the parameters with most significant effects on cellulase production. These parameters included environmental variables and media components as given in Table 7.2. Placket and Burman experiments showed a wide range of difference in the cellulase yield from *T. reesei*. The minimal and maximal activities obtained were 0.17 U/gds and 2.97 U/gds respectively (Table 7.6).

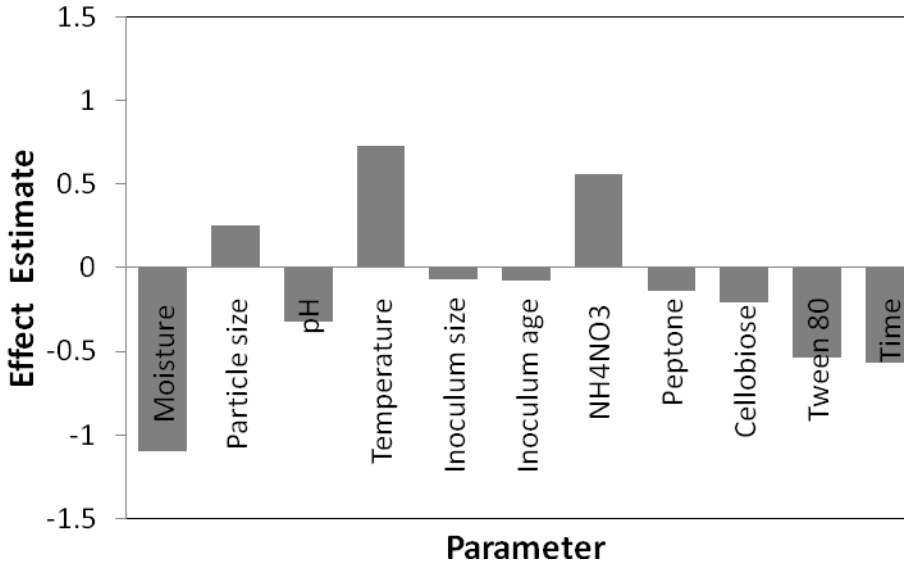
Table 7.6. Plackett & Burman design matrix for the screening of variables influencing cellulase production with the observed responses

Std order	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	X_9	X_{10}	X_{11}	Cellulase Activity (U/gDS)
1	1	-1	1	-1	-1	-1	1	1	1	-1	1	0.26
2	1	1	-1	1	-1	-1	-1	1	1	1	-1	1.03
3	-1	1	1	-1	1	-1	-1	-1	1	1	1	0.59
4	1	-1	1	1	-1	1	-1	-1	-1	1	1	0.17
5	1	1	-1	1	1	-1	1	-1	-1	-1	1	1.85
6	1	1	1	-1	1	1	-1	1	-1	-1	-1	0.58
7	-1	1	1	1	-1	1	1	-1	1	-1	-1	2.97
8	-1	-1	1	1	1	-1	1	1	-1	1	-1	2.27
9	-1	-1	-1	1	1	1	-1	1	1	-1	1	1.70
10	1	-1	-1	-1	1	1	1	-1	1	1	-1	0.60

11	-1	1	-1	-1	-1	1	1	1	-1	1	1	1.52
12	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	2.04

The main effects of parameters on cellulase production were calculated as ε values as given above (7.1.2.1A). The parameters with statistically significant effects were identified using the Fisher's test for analysis of variance (ANOVA). Initial moisture content (X_1) and incubation temperature (X_7) had a confidence level higher than 95 % (P >F = 0.05 or lesser). The ε-values (effect estimates of individual parameters on cellulase production) are represented in Figure 7.1.

Figure 7.1. Influence of process parameters on cellulase production by *T. reesei* under SSF on wheat bran



It can be deduced that the cellulase production increased with increase in incubation temperature and with a decrease in initial moisture content of the medium. Based on these results, initial moisture content and incubation temperature were selected for further optimization to improve cellulase production.

7.2.1.2. Optimization of the levels of significant parameters identified by Placket & Burman experiments

The two variables which showed a confidence level above 95% in the screening experiment were selected and were optimized further using a central composite design (CCD). Based on the results of Plackett & Burman experiment, the levels of media components were set at their middle levels in the CCD with the following exceptions. The inducer cellobiose was eliminated from the medium since it had an insignificant and negative effect. Particle size had a positive effect and considering its role in proper aeration, the higher level was taken. The level of Tween 80 was fixed at its lower level and incubation was performed till 96h since the effects of surfactant and incubation time were negative. Table 7.7 shows the central composite experiment design and the experimental and predicted responses obtained for cellulase production by *T. reesei.*

Table 7.7. Responses obtained for the experimental runs for optimizing cellulase production using CCD design matrix with the corresponding predicted responses

Std order	Block	Moisture (%)	Temperature (^{o}C)	Cellulase Activity (U/gds)	
				Observed	Predicted
1	1	35	29	3.12	3.19
2	1	40	29	3.1	3.24
3	1	35	33	2.9	2.75
4	1	40	33	2.8	2.72
5	1	37.5	31	3.6	3.53
6	1	37.5	31	3.5	3.53
7	1	37.5	31	3.48	3.53
8	2	33.96	31	2.9	2.95
9	2	41.04	31	3	2.96
10	2	37.5	28.17	3.26	3.11
11	2	37.5	33.83	2.27	2.43
12	2	37.5	31	3.78	3.42
13	2	37.5	31	3.26	3.42
14	2	37.5	31	3.24	3.42

The data was analyzed by multiple regression analysis and a second order polynomial equation (Eqn.5) was derived to represent the cellulase production as a function of the independent variables tested.

$$Y = 3.48 + 0.0026X_1 - 0.24X_2 - 0.23X_1^2 - 0.32X_2^2 - 0.02X_1X_2 \quad (5)$$

Where Y = predicted response (cellulase yield), X_1, X_2 are coded values of initial moisture content and incubation temperature respectively.

Testing of the model was performed by the Fisher's statistical test for the analysis of variance (ANOVA) using Design Expert software and the results are shown in Table 7.8. ANOVA of the quadratic regression model suggests that the model is significant with a computed F value of 7.26 and a p value lower than 0.05. The value of multiple correlation coefficient (R) was 0.9156. The closer the value of R to 1, the better is the correlation between the observed and predicted values and the R value obtained indicated a better correlation. A lower value for the coefficient of variation suggests higher reliability of the experiment and in this case the obtained CV value of 6.57 % demonstrated a greater reliability of the trials. Table 7.8 also gives the P values of each of the parameters and their quadratic and interaction terms. The significant influences on cellulase production were incubation temperature, quadratic effects of initial moisture content and incubation temperature. The significance of individual variables can be evaluated from their P values, the more significant terms having a lower P value. The values of P>F less than 0.05 indicated that the model terms are significant and this case X_2, X_1^2 and X_2^2 were found to be significant model terms. There was no significant interaction between the parameters.

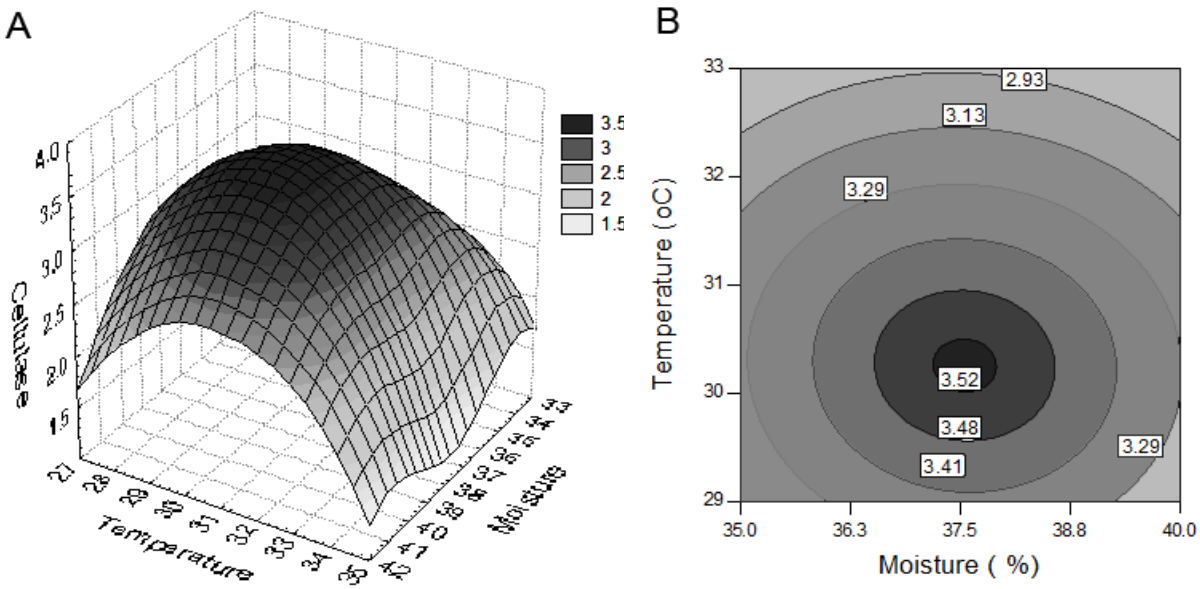
Table 7.8 Analysis of variance for the selected quadratic model *

Source	Sum of Squares	DF	Mean Square	F Value	Prob > F
Model	1.564	5	0.3128	7.26	0.0108
X_1	0.000	1	0.0001	0.00	0.9719
X_2	0.461	1	0.4608	10.70	0.0137
X_1^2	0.400	1	0.3999	9.28	0.0187
X_2^2	0.781	1	0.7810	18.13	0.0038
X_1X_2	0.002	1	0.0016	0.04	0.8526
Residual	0.302	7	0.0431		
Lack of Fit	0.106	3	0.0353	0.72	0.5898
Pure Error	0.196	4	0.0489		
Corrected Total	1.910	13			

* Coefficient of variation (CV) = 6.57 %, coefficient of determination (R^2) =0.8384
correlation coefficient (R) = 0.9156, and adjusted R^2 = 0.7229.

Response surface curve was plotted to understand the interaction effects of variables and for identifying the optimal levels of each parameter for attaining maximal cellulase yield. Figure 7.2 A & B represent the response surface and the contour plot obtained for the effects of incubation temperature and initial moisture content on cellulase yield respectively. The shapes of contour plots indicate the nature and extent of the interactions. Prominent interactions are shown by elliptical plots whereas less prominent or negligible or less prominent interactions are shown by circular contour maps. It is clearly observed from the response surface and contour plot that there is no significant interaction between the tested variables. Regardless of the incubation temperature, the maximal cellulase yield was obtained at an initial moisture content between 37 & 38 % and variations in initial moisture content did not affect the temperature optima between 30 -31 ^{o}C, confirming the lack of interaction between these parameters. The optimal temperature and initial moisture content was within these ranges where the maximal activity of 3.52 U/gds was predicted by the model.

Figure 7.2 Three dimensional response surface (A) and the corresponding contour plot (B) for cellulase production in relation to initial moisture content and incubation temperature



Moisture content of the medium in SSF systems is an important parameter which affects the productivity (Pandey *et al*, 1999). The moisture levels in SSF processes, which vary between 30 and 85 %, have a marked effect on growth kinetics (Oriol *et al*, 1988). The optimal moisture content for growth of microorganisms differ with respect to the substrate since the water holding capacity of substrates are different which have significant effects on water activity- A_w (Reimbault M, 1998). Wheat bran has a higher water holding capacity compared to the lignocellulosic substrates like sugar cane bagasse and this would account for the lower value of initial moisture content for optimal yield of the enzyme. Effect of incubation temperature is rather an organism dependent parameter. Though a large number experiments for cellulase production has used an incubation temperature of 28 ^{o}C (Nakari-Setala & Pentilla, 1995, Ilmen *et al*, 1997) the fungus had been cultivated largely at 30 ^{o}C for production of the enzyme (Reczey *et al*, 1996, Gutierrez-Correa *et al*, 1999). In the current study temperature was found to be the parameter with largest significant effect on cellulase production. The regression equation (Eqn. 5) was solved using Design expert and the optimal values for obtaining the highest yield was determined to be an initial moisture content of 37.56 % and an incubation temperature of 30 ^{o}C. At this combination the maximum predicted yield was 3.52 U/gds.

7.2.1.3. Validation of the model

The adequacy of the model equation (Eqn.5) was validated by performing a total of three verification experiments within the experimental range as given in Table 7.9. The data of the validation runs were also statistically analyzed to find the correlation between observed and predicted values. The correlation coefficient (R) between experimental and predicted values was found to be 0.9703 indicating that the group of experimental values is in good agreement with those of the predicted demonstrating the accuracy of the model.

Table 7.9. Model validation experiments

Trial No.	Moisture (%)	Temperature (^{o}C)	Cellulase Activity (U/gds)	
			Observed	Predicted
1	36.0	30	3.58	3.49
2	37.0	30	3.8	3.58
3	37.5	30	3.78	3.59

Cellulase was produced under optimal conditions for further experiments on biomass hydrolysis.

7.2.2. *Preparation of Enzyme Cocktails and biomass hydrolysis*

T. reesei cellulase and *A. niger* BGL were produced under the optimized conditions for the preparation of enzyme cocktails. The enzymes were concentrated by acetone precipitation (Enzyme: Acetone = 1:3) and the precipitates were resuspended in citrate buffer (pH 4.8, 0.05M). The cellulase and BGL activities for each preparation were; *T. reesei* enzyme – Cellulase- 8.98 FPUs/ml, BGL – 710 U/ml; *A. niger* enzyme – Cellulase -0.39 FPUs/ml, BGL – 9390 U/ml. Two different combinations of cellulase and BGL loading and incubation times were tried for biomass saccharification using alkali pretreated rice straw as a model substrate (Table 7.10). From the results presented in Table 7.10, it can be seen that method B which used a BGL loading of 542 U along with 5 FPU cellulase per gram of biomass; and an incubation time of 48 h performed much better than method A where there is no supplementation of BGL. The results show that with the addition of BGL, the saccharification efficiency has gone up 31 % (1.37 g/100ml to 2g/100ml). Alkali pretreated Rice Straw had a composition of 60.41 % cellulose, 29.51% hemicellulose and 13.05% lignin

making the theoretical maximum of reducing sugar possible 1.006 g/g biomass (0.6706 g and 0.3352g of sugars from cellulose and hemicellulose fractions respectively). The maximum efficiency of hydrolysis attained therefore was 39.73 %.

Table 7.10: Effect of BGL supplementation on saccharification of Rice straw

Method	Cellulase (FPU/g)	Additional BGL (U/g)	Time (h)	Reducing Sugar Yield (mg/g)
	5.00	0.00	24.00	202.90
A	5.00	0.00	48.00	273.10
	5.00	542	24.00	315.44
B	5.00	542	48.00	399.63

Most of the commercial cellulases available are produced from *T. reesei* and *Aspergillus niger* but *T. reesei* lack sufficient amount of β-glucosidase to perform a proper and complete hydrolysis (Gusakov *et al*, 2007). Thus, the cellobiose accumulated due to an incomplete conversion caused by the limiting amounts of β-glucosidase inhibits exo- and endo-glucanases (Sukumaran *et al*, 2005). *β* -Glucosidases are also subject to product inhibition by the glucose, beyond certain levels that vary between the different preparations and sources of the enzyme. One way to solve this discrepancy is to add a glucose tolerant *β*-glucosidase to the reaction mixture containing other cellulase components and to employ this cocktail for biomass hydrolysis which would increase the efficiency of hydrolysis (Sukumaran *et al*, 2005). The result indicates the importance of supplementing BGL and also indicates that with further optimizations in the enzyme cocktail and other parameters, an even higher efficiency may be achieved. Alkali treatment tends to preserve the hemicellulose polymer and remove lignin (Grey *et al*, 2006). Rice straw contains up to 25-30% lignin and the removal of lignin facilitates the loosening of cellulose fibers making them accessible to the cellulase. Cellulases need to penetrate the polymer to access and hydrolyze it. Cellulases have specific domains for binding their substrate so that the enzyme sits on the polymer and effects a slow degradation (reviewed in Lynd *et al*, 2002) which probably could be the reason for more efficient hydrolysis at an increased incubation time.

7.2.3. *Optimization of enzyme cocktails for biomass saccharification*

Reducing sugar yields from the different runs ranged between 373 mg/g to 535mg/g which represented conversion efficiencies of 37.1 % and 53.2 % of the theoretical maximum (1006 mg/g) respectively. The responses obtained for each experimental run performed according to the Box Behnken design as represented in Table 7.11, were analyzed by multiple regression analysis and a second order polynomial equation (Eqn.6) was derived to represent the cellulase production as a function of the independent variables tested. Maximal reducing sugar yield obtained was 53.2 % of the theoretical maximum and 1.34 fold (34%) improvement over method B (Section 7.2.2) above.

Table 7.11. Responses obtained for the experimental runs for optimizing biomass hydrolysis using Box-Behnken design matrix

Std	Cellulase (FPUs/g) X_1	Additional BGL (U/g) X_2	Time (h) X_3	Reducing sugar (mg) Y
1	25	542	36	392
2	50	542	36	472
3	25	1626	36	436
4	50	1626	36	494
5	25	1084	24	373
6	50	1084	24	493
7	25	1084	48	445
8	50	1084	48	528
9	37.5	542	24	477
10	37.5	1626	24	482
11	37.5	542	48	516
12	37.5	1626	48	535
13	37.5	1084	36	447
14	37.5	1084	36	466
15	37.5	1084	36	456
16	37.5	1084	36	461
17	37.5	1084	36	456

$$Y = 457.2 + 42.62X_1 + 11.25X_2 + 24.88X_3 - 25.72X_1^2 + 17.03X_2^2 + 28.27X3^2 - 5.5X_1X_2 - 9.25X_1X_3 + 3.5X_2X_3 \quad (6)$$

Where Y = predicted response (reducing sugar yield), X_1, X_2 and X_3 are coded values of cellulase loading, BGL loading and residence time respectively. Testing of the model was performed by the Fisher's statistical test for the analysis of variance (ANOVA) using Design Expert software and the results are presented in Table 7.12.

Table 7.12. Analysis of variance for the selected quadratic model

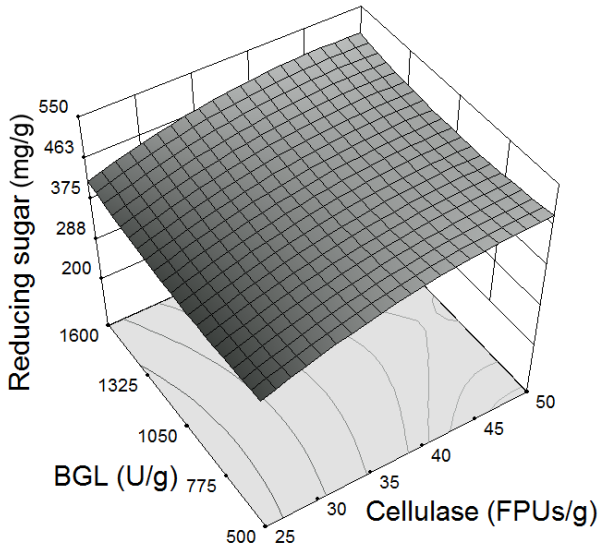
Source	Sum of Squares	DF	Mean Square	F Value	p-Value (Prob > F)
Model	28105	9	3123	22.41	0.0002
A-Cellulase	14535	1	14535	104.30	< 0.0001
B-BGL	1013	1	1013	7.27	0.0308
C-Time	4950	1	4950	35.52	0.0006
AB	121	1	121	0.87	0.3825
AC	342	1	342	2.46	0.1611
BC	49	1	49	0.35	0.5719
A^2	2786	1	2786	19.99	0.0029
B^2	1220	1	1220	8.76	0.0211
C^2	3366	1	3366	24.15	0.0017
Residual	976	7	139		
Lack of Fit	777	3	259	5.21	0.0724
Pure Error	199	4	50		
Cor Total	29080	16			

ANOVA of the quadratic regression model suggested that the model is significant with a computed F value of 22.41 and a p-value lower than 0.05 (0.0002). P values of each of the parameters and their quadratic and interaction terms were also determined by the ANOVA test. The significance of individual variables can be evaluated from their P values, the more significant terms having a lower P value. The values of p-value less than 0.05 indicates that the model terms are significant and this case X_1, X_2, X_3, X_1^2, X_2^2, X_3^2 (Cellulase loading, BGL loading, residence time and the quadratic effect of these parameters) were found to be significant model terms. There were no statistically significant interactions as could be gauged from the p-values. Nevertheless, response surface curves were plotted to understand the interaction effects of variables and for identifying the optimal levels of each parameter for attaining maximal reducing sugar yield.

Beta glucosidase loading was found to have a significant effect on the efficiency of saccharification and the reducing sugar yield was comparatively lower in all trials conducted without adding BGL. Cellulase loading and residence time also influenced reducing sugar

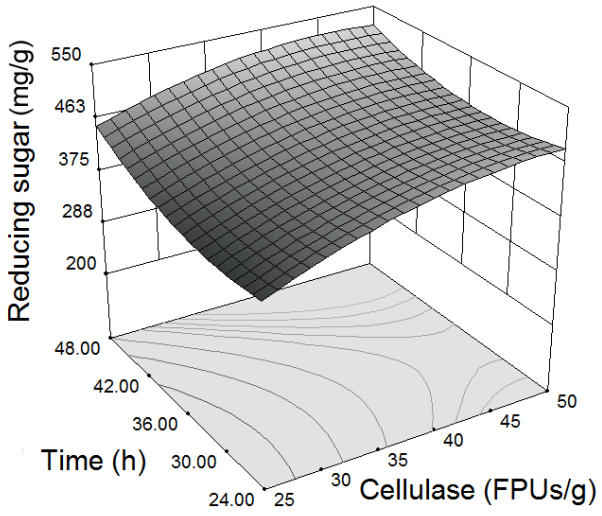
yield significantly. The longer residence time of 48h was better for saccharification. The reason could be that cellulases need to penetrate the polymer to access and hydrolyze it, unlike many common enzymes which take in their substrates to the active site pockets. Cellulases have specific domains for binding their substrate so that the enzyme sits on the polymer and effects a slow degradation (Lynd *et al*, 2002). At the levels added, the effect of FPAse concentration however was the highest, indicating that the initial steps in cellulose hydrolysis are critical. BGL activity of *T. reesei* cellulase can itself drive a saccharification reaction but the addition of exogenous BGL improves the efficiency and reducing sugar yield. Quadratic effects of all the 3 parameters were also significant. Response surface plots were generated for the effect of different parameters (Figure 7.3-7.5).

Figure 7.3, Combined effect of *T. reesei* cellulase and *A. niger* BGL on biomass saccharification



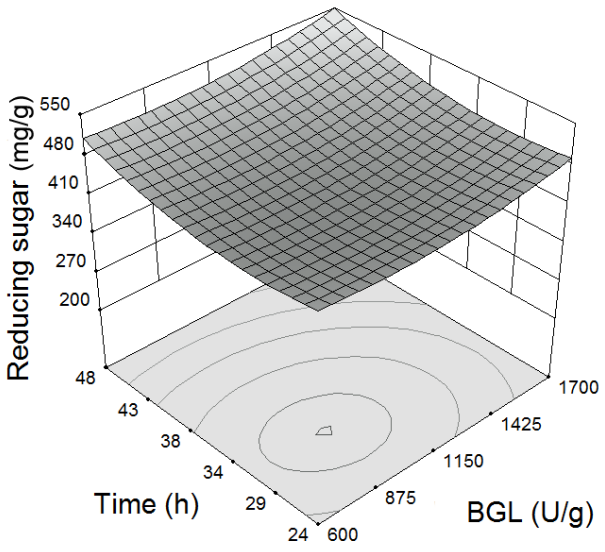
Maximal biomass saccharification was obtained with a cellulase loading of 50FPUs/g and an additional BGL loading of 1626 U/ml both of which were the higher extremes tested.

Figure 7.4, Effect of cellulase loading and residence time on sugar yield



Both at higher and lower cellulase loadings, increase in residence time resulted in a better yield of sugars. Highest reducing sugar yield was observed at 50FPUs/g cellulase loading and with a residence time of 48h.

Figure 7.5. Effect of BGL loading and residence time on sugar yield



Similar to the effect of cellulase, with both lower and higher BGL loadings, there was an increase in sugar yield when the residence time was increased. Highest yield of sugar was obtained with the highest BGL loading and residence time. Though the interaction effects were insignificant, response surface analyses indicated that a higher cellulase loading should improve the sugar yield and with increase in cellulase loading, a corresponding increase in BGL concentration was essential to maintain the hydrolysis.

The optimal combination of variables for obtaining higher saccharification efficiency was predicted using the numerical optimization function in Design Expert. The top 5 solutions are given in Table 7.13.

Table 7.13. Optimized combination of parameters predicted for increasing saccharification efficiency

Sl #	Cellulase (FPUs/g)	BGL (U/g)	Time (h)	Predicted Reducing sugar yield (mg/g)
1	37	1582	48	536
2	46	1511	47	536
3	49	1596	48	542
4	44	1526	48	540
5	38	1626	48	542

The experiment proved the indispensable role of BGL in saccharification and the improvement in efficiency which can be achieved with addition of even a moderately glucose tolerant beta-glucosidase (Since the crude BGL preparation from *A. niger* NII 08121 has a *Ki* of ~200mM). The obtained efficiency of ~ 53 % of theoretical maximum is not very high. Nevertheless it was achieved with a highly recalcitrant biomass with the minimal pretreatment. Improvement in pretreatment strategies and also further optimization of enzyme cocktails and saccharification conditions can definitely improve the saccharification efficiencies.

7.2.4. *Ethanol production from rice straw hydrolysate*

Enzymatic hydrolysate of rice straw was concentrated by evaporation to a reducing sugar concentration of 60g/L or 120g/L and was used for the fermentation experiments. The results of the fermentation of alkali pretreated and enzymatically saccharified rice straw are given in Table 7.14. The maximum ethanol concentration (25.56g/L) was obtained after 24h using hydrolysate with 12% reducing sugar. With the hydrolysate having initial reducing sugar concentration of 6%, the maximum ethanol concentration obtained was 12.34 g/L in 24h. The ethanol yield obtained was 0.093g and 0.096g per gram of dry substrate (gDS) for initial sugar concentrations of 6% and 12% respectively.

The results indicated that concentration of hydrolysate is needed to obtain an ethanol yield which is needed practically for a distillation step (>5% v/v). However, the efficiency attained for sugar to ethanol conversion was only ~40% which could be due to the presence of inhibitors of yeast growth in the hydrolysate. Concentration of the hydrolysate results in concentration of the inhibitors present in it. This might prevent yeast growth and hence the production of ethanol which could be the reason for a lower efficiency in alcohol production. These points to the necessity of a detoxification step before the hydrolysate is used for fermentation to produce alcohol.

Table 7.14: Ethanol production from rice straw hydrolysate by *Saccharomyces cerevisiae*

Reducing sugar (RS) concentration. (g/L)	Duration of incubation (h)	Ethanol Concentration (g/L)	Ethanol Yield (g/gDS)	Efficiency (RS to ethanol) %
60	24	12.34	0.093	40.33
120	24	25.56	0.096	41.76

7.3. Conclusions

The study demonstrates the feasibility of using crude *T. reesei* RUT C30 cellulase and *A. niger* NII 08121 *β*-glucosidase in combination for hydrolysis of biomass residues (at least

pretreated rice straw), and shows the effectiveness of enzyme cocktails over preparations from single sources. Optimization of enzyme cocktail was performed using pretreated rice straw as feedstock and hence would be more effective for the saccharification of this biomass type. However, the study brings about the potential of optimized enzyme cocktails for biomass saccharification in general. The experiment proves the indispensable role of BGL in saccharification and the improvement in efficiency which can be achieved with addition of even a moderately glucose tolerant beta-glucosidase. Enzymatically hydrolyzed biomass was successfully fermented to alcohol. Further optimization of the cocktails to suit particular biomass type and the design of efficient fermentation strategies can tremendously improve the biomass-to-ethanol process.

The BGL preparation from *A. niger* NII 08121 was successfully demonstrated as a potent enzyme for efficient biomass hydrolysis. The enzyme preparation can be used in the formulation of highly efficient enzyme cocktails. Since the production of *A. niger* BGL is easy, cost and time efficient when produced using solid state fermentation, an since the titers of enzyme is high, the organism can be considered for industrial production of BGL for biomass hydrolyzing enzyme cocktail preparations.

CHAPTER 8

Summary & Conclusions

Chapter 8
Summary and Conclusions

8.1. Summary

Beta- Glucosidases (BGLs) are enzymes that hydrolyze aryl beta-glucosides and/or cellobiose and cello-oligosaccharides. In cellulolytic fungi, BGL completes the final step in cellulose hydrolysis by converting cellobiose to glucose. In several fungi, especially *Trichoderma reesei* the best known cellulase producer, the enzyme is produced in lower quantities compared to the other two classes of cellulases, and it is slow acting, making BGL the rate limiting component in cellulose hydrolysis. The less abundance of BGL even under conditions of cellulase induction and the product inhibition to which it is susceptible, limits the use of native cellulase preparations in lignocellulosic biomass hydrolysis for alcohol production.

Glucose tolerant BGL can circumvent the problem of feedback inhibition, and if available in an enzyme cocktail for biomass hydrolysis can improve the efficiency of hydrolysis by shifting the equilibrium towards a higher product concentration than otherwise achievable. Few species of *Aspergilli* are known to produce glucose tolerant β-glucosidases and some of these enzymes have been cloned and characterized. It is expected that more of such glucose tolerant BGLs may be prevalent in nature especially in filamentous fungi. Isolation of such enzymes and knowledge about their properties, sequences and expression patterns can help in design of better enzyme cocktails for biomass hydrolysis as well as in targeted approaches for modifying the glucose tolerance of existing BGLs.

An *Aspergillus* strain was isolated at NIIST from decaying wood which produced BGL that was active at 0.5M Glucose concentration. The fungus was identified as *Aspergillus niger* and was deposited in NII culture collection with accession number NII 08121. Multiple BGL isoforms could be detected in this fungus by Native PAGE followed by activity staining using a fluorescent substrate for BGL. The isoforms were differentially induced in response to carbon sources and four isomers were detected, two of which showed glucose tolerance at 250mM glucose. Degenerate primers designed for BGL gene belonging to families 1, 3 and 5 of glycosyl hydrolases could amplify stretches from the genomic DNA. The amplicons on

sequencing confirmed homology to BGLs belonging to respective families indicating presence of BGLs belonging to all three families glycosyl hydrolases in the fungus. The major BGL which was the highly expressed isoform in native strain was purified and characterized. It was 120kDa in size and was most active at pH 5.0 and stable at 50°C till 72 hours. It retained more than 90% of the activity up to 48h which indicated the suitability for biomass hydrolysis.

Conditions for BGL production were optimized in Solid State Fermentation (SSF) employing *A. niger* which resulted in improved enzyme yield. The enzyme produced under SSF retained 19 % activity in presence of 0.25M glucose. The major beta glucosidase (BGL1) of this fungus showed interesting properties including an optimum temperature of 70 °C, optimum pH of 5.0 and extended stability at 50 ^{o}C for more than 48h. To test the efficacy of *A. niger* BGL in biomass saccharification and ethanol production, enzyme blends were created using *T. reesei* cellulase. Cellulase production from *T. reesei* under solid state fermentation was optimized and the enzyme was produced using this fungus under optimized conditions for saccharification trials. *T. reesei* cellulase and the BGL from *A. niger* were used to create blends for biomass hydrolysis. Optimized enzyme cocktails were derived for efficient hydrolysis of pretreated rice straw, and the hydrolysate was used to demonstrate ethanol production by alcohol fermentation employing *Saccharomyces cerevisiae*.

The study has helped to reveal the property of differential induction of BGL isoforms in *Aspergillus niger* which can be used to manipulate culture conditions to produce the desired isoform in excess. Also, a laboratory process for producing BGL enzyme in high titers was derived. Major BGL isoform was purified and characterized and partial gene sequences of BGLs belonging to families 1, 3 and 5 of glycosyl hydrolases were obtained. This information would be very useful in isolating the full length cDNAs of these genes for further studies and possible over expression in suitable vectors.

8.2 Conclusions

Aspergillus niger NII 08121 isolated at NIIST was revealed to be a potent source of β-glucosidase for biomass conversion. The fungus produced very high titers of the enzyme and

also exhibited moderate glucose tolerant –properties that are useful in biomass hydrolysis. The study provided also conclusive evidence to the fact that multiple BGLs are expressed in *A. niger* differentially in response to the carbon source provided in the culture medium. Carbon source and other components play an important role to direct the metabolic pathway of *A. niger* towards production of a desired metabolite. Careful leveraging of the medium components could be useful for enhanced production of specific BGL isoforms in the fungus.

The major BGL from *A. niger* NII 08121 can be considered as a potent candidate for use in biomass hydrolysis due to its enhanced thermal stability and high activity under hydrolysis conditions.

Molecular cloning of the partial gene sequences confirmed the presence of BGL belonging to different glycosyl hydrolases families which points to the multiple roles these enzymes play in the organism. It would be interesting to look at the physiological roles of these different BGL isoforms and the information on partial gene sequences would greatly aid in undertaking such studies.

The study also demonstrated the feasibility of using crude *T. reesei* RUT C30 cellulase and *A. niger* NII 08121 β-glucosidase in combination for hydrolysis of biomass residues and showed the effectiveness of enzyme cocktails over preparations from single sources. The study therefore brings about the potential of optimized enzyme cocktails for biomass saccharification in general. It has also proved the indispensable role of BGL in saccharification and the improvement in efficiency which can be achieved with addition of even a moderately glucose tolerant beta-glucosidase.

The BGL preparation from *A. niger* NII 08121 was successfully demonstrated as a potent enzyme for efficient biomass hydrolysis and the characteristic of the major BGL was established. *A. niger* NII 08121 is a fast grower and producer of BGL in high titers and therefore could be considered for industrial production of BGL for biomass hydrolyzing enzyme cocktail preparations.

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APPENDICES

APPENDIX 1
LIST OF ABBREVIATIONS

#	Number
µ	Micron/ micrometer
µM	micromol
ANOVA	Analysis of Variance
BGL	β- glucosidase
BLAST	Basic Local Alignment Search Tool
CBH	Cellobiohydrolase
CCD	Central Composite Design
cDNA	Complementary DNA
cm	centimeter
CMC	Carboxy Methyl Cellulose
CMCase	Carboxy Methyl Cellulase
CODEHOP	COnsensus DEgenerate Hybrid Oligonucleotide Primers
CSIR	Council of Scientific and Industrial Research
CTAB	Cetyl Trimethyl Ammonium Bromide
DNA	Deoxy Ribonucleic Acid
DNS	3, 5' Dinitro Salicylic Acid
DO	Dissolved Oxygen
DOE	Design of Experiments
EDTA	Ethylene Diamine Tetra Acetic Acid
EG	Endogluanase
FID	Flame Ionization Detector
FPAase	Filter Paper Hydrolyzing Activity
FPU	Filter paper units
g	Grams
GC	Gas Chromatography, Gas Chromatogram
gDS	Grams Dry Substrate
GH	Glycosyl Hydrolase

GH1	Glycosyl Hydrolase Family1
GH3	Glycosyl Hydrolase Family3
GH5	Glycosyl Hydrolase Family5
h	Hour
HCA	Hydrophobic Cluster Analysis
IEF	Isoelectric Focussing
IPTG	Isopropyl β-D-1-thiogalactopyranoside
IU	International Units
IUPAC	International Union for Pure and Applied Chemistry.
kDa	kilo Daltons
Ki	Inhibition constant
L	Liter
LB Broth	Luria Bertani Broth
lbs	Pounds Inch $^{-2}$
M	Molar
mg	Milligram
min	Minute
ml	Milliliter
mM	Millimolar
MTCC	Microbial Type Culture Collection
MUG	Methyl Umbelliferyl β-D glucopyranoside
NCIM	National Collection of Industrial Microorganisms
NII	NIIST Culture Collection ID
NIIST	National Institute for Interdisciplinary Science and Technology
NREL	National Renewable Energy Laboratory , USA
PAGE	PolyAcryl Amide Gel Electrophoresis
PCR	Polymerase chain reaction
PDB	Potato Dextrose Broth
PES	Poly Ether Sulphone
pI	Isoelectric Point

pNP	para Nitrophenol
pNPG	para –Nitrophenyl β-D glucopyranoside
RNA	Ribonucleic Acid
rpm	Rotations/minute
RS	Rice Straw
RSM	Response Surface Method/Methodology
SCB	Sugar Cane Bagasse
SDS	Sodium Dodecyl Sulfate
SmF	Submerged Fermentation
SSF	Solid State Fermentation
U	Units
U/gDS	Units per gram dry substrate
U/ml	Units/milliliter
UV	Ultra Violet
v/v	Volume /Volume
vvm	Volume/volume/minute
w/v	Weight/Volume
WB	Wheat Bran
X Gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
YEP broth	Yeast Extract Peptone Broth

APPENDIX 2

LIST OF TABLES

APPENDIX 3

LIST OF FIGURES