β-glucosidases from *Aspergillus unguis* NII 08123: Molecular characterization, properties and applications

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DECLARATION

I hereby declare that the work presented in this thesis entitled "***β-glucosidases from Aspergillus unguis NII 08123: Molecular characterization, properties and applications***" is a bonafide record of the research carried out by Ms Rajasree K.P. (Reg No.3698), under my guidance and supervision, at the CSIR-National Institute for Interdisciplinary Science and Technology, Thiruvananthapuram, India. I declare that all suggestions made by the audience during Pre-synopsis seminar and recommended by the doctoral committee have been incorporated in this thesis. I also declare that this work or no part of this work had been submitted for the award of any degree, diploma, associateship or any other title or recognition.

Rajeev Kumar Sukumaran

Thiruvananthapuram
20 December 2013

DECLARATION

I hereby declare that the work presented in this thesis entitled "***β-glucosidases from Aspergillus unguis NII 08123: Molecular characterization, properties and applications***" is based on the original work done by me under the guidance of Dr Rajeev Kumar Sukumaran, Scientist, Biotechnology Division, CSIR-National Institute for Interdisciplinary Science and Technology, Thiruvananthapuram, 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.

Rajasree K.P.

Dedicated to my family and my teachers

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List of Publications

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2. Singhania RR, Sukumaran RK, **Rajasree KP**, Mathew A, LalithaDevi G, Pandey A. properties of majorβ- glucosidase BGL1 from Aspergillus niger NII 08121 expressed differentially in response to carbon sources. *Process Biochem*. 46(7), 2011, 1521-1524.

3. Aswathy US, Sukumaran RK, Lalitha Devi G, **Rajasree KP**, Singhania RR, Pandey A: Bio-ethanol from water hyacinth biomass: An evaluation of enzymatic saccharification strategy; *Bioresour. Technol.* 101 (2010) 925–930

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Book Chapters

1. Sukumaran RK, Gottumukkala LD, **Rajasree KP**, Alex D, Pandey A (2011), Butanol from Biomass:Revisiting ABE fermentation, In : Pandey A, Larroche C, Ricke SC, Dussap CG, Gnansounou E (eds) Biofuels: Alternaive Feedstocks and Conversion Processes, Academic Press, London, pp-571-586

Conference Papers/Presentations/Posters

1. Rajeev K Sukumaran, Abraham Mathew, **Rajasree KP**, Aravind Madhavan, Enzymes for Biomass Deconstruction, From moldy bran to tuning gene expression - The Centre for Biofuels Initiatives.Invited talk at the National Symposium on Emerging Trends in Biotechnology, organized by the Cochin University of Science and Technology, December 12-14, Kochi, India.

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5. Rajeev K Sukumaran, **KP Rajasree**, Reeta Rani Singhania, Abraham Mathew, Lalitha Devi G, KU Janu, K Satayanagalakshmi, KV Sajna, Noble Kurian, Vikram J Surender, Binod Parameswaran, Raveendran Sindhu & Ashok Pandey Technological challenges in the production and application of cellulases for lignocellulosic bioethanol production, International Symposium on Bioenergy, Indian Institute of Technology, Kharagpur, India, January 5-7, pp 70-71 (2010).

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Chapter 1. Introduction and Review of literature

1. 1. *β*- Glucosidases

β- Glucosidases (3.2.1.21) are ubiquitous enzymes which catalyze the hydrolysis of terminal non-reducing residues in β-glucosides. It acts on the β (1-4) bond linking two glucose units or glucose substituted molecules. Thus, under physiological conditions, β-glucosidase (BGL) catalyzes the hydrolysis of a β-glucosidic bond linking carbohydrate residues in aryl, amino or alkyl β-D-glucosides, cyanogenic glucosides, or short chain oligo saccharide (Bhatia et al. 2002a). Synthesis of glycosyl bonds, i.e. the reverse reaction can also be catalyzed under defined conditions by these enzymes and this is accomplished as either reverse hydrolysis or transglycosylation.

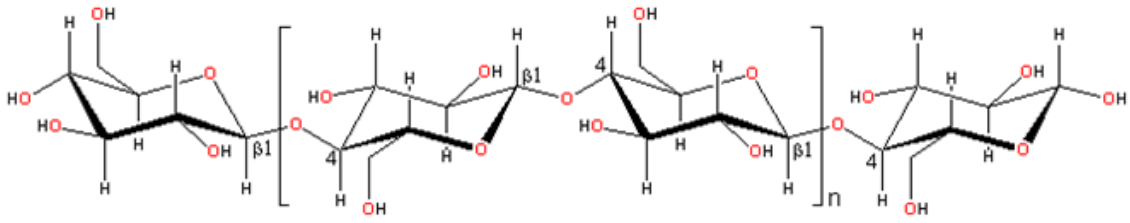
β- Glucosidases comprise a major group among glycosyl hydrolases which is now the focus of several researchers due to the importance of these enzymes in different physiological processes as well as due to their biotechnological applications (Czjzek et al. 2001). In plants BGLs are implicated in defence against pests, alkaloid metabolism, hydrolysis of cell wall derived oligosaccharides, phytohormone regulation and lignification (Seshadri et al. 2009). In enological yeasts, BGLs are responsible for liberation of terpenols, benzyl and phenyl ethyl alcohols from their respective glucoside precursors and thus accounts for the liberation of flavoring compounds (Rosi et al. 1994). In bacteria and fungi, BGLs are mainly part of the cellulolytic machinery and are responsible for hydrolysis of oligosaccharides and cellobiose resulting from hydrolysis of cellulose by the other synergistically acting cellulolytic enzymes. Here the role of BGL is pivotal in that it is the rate limiting enzyme in cellulose hydrolysis. In recent years there has been a lot of interest in β- glucosidases due to the importance of these enzymes in biomass hydrolysis for biofuel applications. As part of the cellulolytic systems sourced primarily from filamentous fungi or supplied externally to such enzyme preparations, BGLs have critical role in accomplishing complete hydrolysis of biomass to its component sugars.

1.2. Cellulase systems and the role of *β*- Glucosidases in cellulose hydrolysis

Plant cell wall contains mainly cellulose, hemicellulose and lignin (lignocellulose) with cellulose as the major component of the cell wall. It is also the most abundant biopolymer on earth which plays an important role in carbon cycle on earth. Lignocellulose thus makes an excellent renewable feedstock since the sugars derived from both cellulose and hemicellulose can be efficiently converted to biofuels like bioethanol or to other value added products.

Cellulose is a linear polymer of β-1,4 D-glucopyranose units (Figure 1.1) which exists in a number of crystalline and amorphous topologies. Despite its homogenous chemical composition, the insolubility and heterogeneity of native cellulose makes it a recalcitrant substrate for enzymatic hydrolysis. Microorganisms meet this challenge with the aid of a multi-enzyme system containing different classes of cellulases which when acting together can completely hydrolyze the native cellulose.

Figure 1.1 Cellulose structure showing β- 1, 4 linkage between glucose moieties

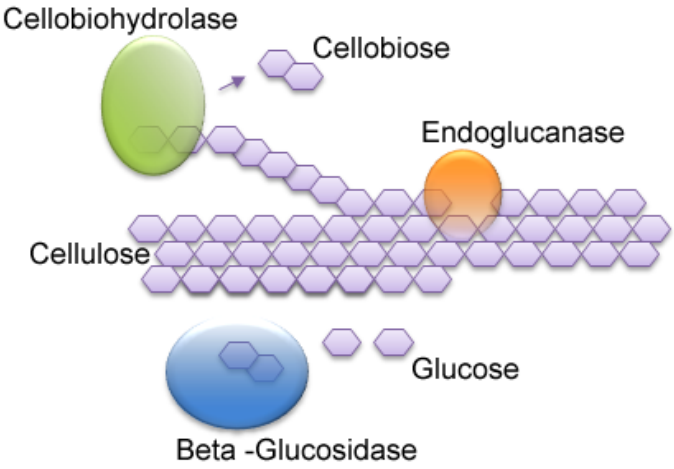


Cellulases are produced by several microorganisms and include different classes of enzymes. The β-1,4 -D glucan linkages in cellulose are degraded by these enzymes and the hydrolysis of native cellulose yields glucose as the main product and cellobiose and cello oligosaccharides as minor products. Synergistic effect of enzymes is widely observed in cellulose hydrolysis and three major classes of enzymes contribute to the hydrolysis of cellulose The three major enzyme classes that constitute the cellulase system are

a) Endoglucanases (ENG) – EC 3.2.1.4
b) Exoglucanases (EXG) – EC 3.2.1.91
c) β-glucosidases (BGL) – EC 3.2.1.21

Endoglucanase acts on the intramolecular β 1,4-glycosidic bonds of cellulose whereas exoglucanase hydrolyze from the reducing or non reducing ends of glucose chain and release cellobiose (Zhang et al. 2006). Thus, it progressively reduces the chain length of cellulose. Cellobiose is composed of glucose units linked by β-1, 4 bonds and β-glucosidase acts on this β-1, 4 linkages to release individual glucose molecules (Figure 1.2). β-glucosidase can also act on cellodextrins and the activity of BGLs decreases with increase in chain length of the substrate (Bisaria and Mishra 1989). Cellobiose acts as an inhibitor of endo and exoglucanase, which results in an overall reduction of reaction rates. Among the three enzymes, β-glucosidase is considered to be the rate limiting one because it catalyzes the reduction of cellobiose which in turn reduces the concentration of cellobiose. More over most of the BGLs are also inhibited by their product –glucose which leads to an accumulation of cellobiose which in turn results in both inhibition as well as repression of endo and exo glucanases (Barbagallo et al. 2004; Gottschalk et al. 2010; Passos et al. 2009).

Figure 1.2. Cellulase synergy in cellulose hydrolysis



Most of the fungal cellulase systems contain the endoglucanase, exoglucanase and β-glucosidase in different ratios. Majority of the cellulase producers can have high endoglucanase and exoglucanase content, but they are poor producers of β- glucosidase. While considering the list of cellulase producers, *Trichoderma reesei* is considered to be the undeniable leader among filamentous fungi. But wild-type *T. reesei* and its best extracellular

cellulase producer mutants (e.g.RUT-C30), produce only small amounts of β-glucosidase which are sensitive to the product. The enzyme plays an important role in regulation of celluloytic system of the fungus. In majority of the cellulolytic fungi including *T. reesei*, basal level expression of cellulase is implied in the generation of inducers. While the cellulolytic organism grows in vicinity of cellulosic substrate, it secretes the enzymes which will break down small quantities of cellulose to yield oligosaccharides. Basal level expression of β–glucosidase helps in the generation of inducers from the hydrolysis products by trans-glycosylation reactions. Once the inducer enters the cell, it triggers full-scale transcription of the cellulase gene mediated by activator proteins and activating elements. After the cellulose is degraded, a higher amount of glucose is liberated, which causes catabolite repression (Suto and Tomita 2001). Catabolite repression in *T. reesei* is mediated through a repressor protein called the "Cre I repressor protein (CreA in *Aspergilli*). At higher glucose concentrations, the Cre I protein binds to the promoters of cellulase genes causing the catabolite repression(Ilmen et al. 1996; Kubicek and Penttila 1998; Suto and Tomita 2001). Higher concentration of glucose also results in the feed back inhibition of BGL, all of these acting in synergy for an intricate control of the cellulolytic system.

These rate-limiting steps and precise control of cellulose hydrolysis-though advantageous to the cellulase producing microorganism, remain the major hurdles in the production of economically feasible cellulosic ethanol because of the difficulty to obtain enzyme cocktails that are not inhibited by high glucose concentrations (Dashtban et al. 2009).

1.3. Sources of β-glucosidases and their functional roles

β-glucosidases are widely distributed among bacteria, fungi, plants and animals. In plants, it is generally associated with defense mechanism, activation of plant hormones, lignin synthesis, cell wall degradation in endosperm during seed germination (Arthan et al. 2006; Czjzek et al. 2001; Dharmawardhana et al. 1995; Simos et al. 1994), β-glucan synthesis during cell wall development, pigment metabolism, fruit ripening (Khan and Akhtar 2010) etc. In animals, two different β-glucosidases have been identified – cystosolic and lysosomal β-glucosidases (Beutler et al. 2004). Cystosolic β-glucosidases are often found in liver,

kidney and intestine. They are thought to play a key role in detoxification of plant β-glucosides. They have broad specificity to sugars and have preference for hydrophobic aglycones (Hays et al. 1996; Hays et al. 1998). Lysosomal glucocerebrosidase cleaves glucosylceramide into ceramide and glucose. The enzyme is membrane associated and is seen in all types of tissues. Bacteria and fungi utilize β-glucosidase as a catabolic enzyme to break down complex cellooligosaccharides to obtain glucose. As part of the cellulase enzyme system, β-glucosidases are mainly produced by the filamentous fungi. Enzyme localization may be intracellular, extracellular or cell wall associated, but most of the studies have been done in extracellular enzymes due to its wide application especially in industries (Soewnsen 2010).

Table 1.1 Bacterial β-glucosidases

Organism	**Amino acid**	**Glycosyl Hydrolase Family**	**Citation**
Aeromonas salmonicida	793	GH3	(Reith et al. 2008)
Arthrobacter chlorophenolicus	832	GH3	(Lucas et al. 2009)
Borrelia recurrentis	551	GH3	(Lescot et al. 2008)
Clostridium botulinum	481	GH1	(Smith et al. 2007)
Clostridium perfringens	459	GH1	(Shimizu et al. 2002)
Enterococcus faecium	471	GH1	(Earl et al. 2013)
Geobacillus sp.	455	GH1	(van et al. 2013)
Kitasatospora setae (Streptomyces setae)	447	GH1	(Ichikawa et al. 2010)
Lactobacillus casei	731	GH3	(Broadbent et al. 2012)
Lactococcus lactis subsp. cremoris	475	GH1	(Bolotin et al. 2011)
Listeria monocytogenes str.	483	GH1	(Briers et al. 2011)
Prevotella denticola	806	GH3	(Durkin et al. 2011)
Pseudomonas fluorescens	763	GH3	(Loper et al. 2012)
Pseudoxanthomonas spadix	728	GH3	(Lee et al. 2012)
Salmonella enterica subsp. enterica serovar Adelaide str.	765	GH3	(den Bakker et al. 2011)
Streptococcus pneumoniae	469	GH1	(Donati et al. 2012)
Streptomyces coelicolor	448	GH1	(Bentley et al. 2002)
Streptomyces rochei (Streptomyces parvullus)	400	GH1	(Mastromei et al. 1993)
Yersinia pestis	793	GH3	(Doggett et al. 2011)
Zunongwangia profunda	796	GH3	(Qin et al. 2010)

In bacteria, BGLs are produced as part of the cellulolytic complex (e.g. Cellulosomes of *Cellulomonas* and *Clostridia*) and in some cases they are even implicated in pathogenesis. Higher BGL activity has been associated with higher virulence in *Agrobacterium tumefaciens* and in some cases inducers of virulence genes are synthesized by bacterial β-glucosidases (Castle et al. 1992). Table 1.1 represents some of the bacterial sources of β-glucosidase reported.

Even though there are several microbes capable of producing β-glucosidase, the hydrolytic activity of fungal originated β-glucosidase is exploited in several biotechnological processes to increase the rate and extent of saccharification of several cellulosic materials by hydrolyzing the cellobiose which inhibits cellulases (García Kirchner et al. 2005). Among the fungi, species of *Aspergillus* is known to produce high titers of the enzyme, while *Trichoderma* produces only very less quantities of the enzyme. Species of *Penicillium* and yeasts like *Candida* are also capable of producing β- glucosidases. Nevertheless, *Aspergilli are* considered to be the best producers of BGL and are commercially exploited for production of the enzyme. A list of fungi reported to produce BGL is given in Table 1.2

Table 1.2 β-glucosidase production by filamentous fungi

Organism	**Amino acid**	**Glycosyl Hydrolase Family**	**Reference**
Acremonium cellulolyticus	142	GH3	(Hideno et al. 2011)
Ajellomyces capsulata (Histoplasma capsulatum)	236	GH1	(Champion et al. 2009)
Ajellomyces dermatitidis (Blastomyces dermatitidis)	419	GH 5	(Cuomo et al. 2010)
Amanita thiersii	116	GH3	(Wolfe and Pringle 2011)
Amanita altipes	114	GH3	(Wolfe and Pringle 2011)
Arthroderma gypseum (Microsporum gypseum)	864	GH3	(Martinez et al. 2012)
Arthroderma otae (Microsporum canis)	1,231	GH5	(Martinez et al. 2012)
Aspergillus aculeatus	860	GH 3	(Zou et al. 2011)
Aspergillus avenaceus	858	GH3	(Pepin et al. 2005)
Aspergillus awamori (Black koji mold)	151	GH3	(Beena et al. 2009)
Aspergillus clavatus	867	GH3	(Fedorova et al. 2008)
Aspergillus flavus	861	GH3	(Payne et al. 2007)
Aspergillus kawachii (White koji mold,Aspergillus awamori var. kawachi)	846	GH1	(Futagami et al. 2011)

Aspergillus niger	860	GH3	(Pan et al. 2012)
Aspergillus oryzae (Yellow koji mold)	532	GH3	(Zhao et al. 2012)
Aspergillus terreus	861	GH3	(Sidik et al. 2004)
Aspergillus wentii	63	GH3	(Bause and Legler 1980)
Beauveria bassiana (White muscardine disease fungus,Tritirachium shiotae)	792	GH3	(Xiao et al. 2012)
Blumeria graminis f. sp. hordei (Barley powdery mildew,Oidium monilioides)	358	GH16	(Spanu et al. 2010)
Botryosphaeria parva (Grapevine canker fungus,Neofusicoccum parvum)	480	GH3	(Blanco-Ulate et al. 2013a)
Candida albicans (Yeast)	438	GH81	(Jones et al. 2004)
Candida dubliniensis (Yeast)	815	GH5	(Jackson et al. 2009)
Candida maltosa (Yeast)	814	GH3	(Yu et al. 2013)
Candida oleophila (Yeast)	425	GH5	(Segal et al. 2002)
Candida tropicalis (Yeast)	471	GH3	(Butler et al. 2009)
Candida wickerhamii	609	GH1	(Skory and Freer 1995)
Chaetomium thermophilum	589	GH3	(Xu et al. 2009)
Chrysosporium lucknowense	1,106	GH5	(Schardl et al. 2013)
Claviceps purpurea (Ergot fungus,Sphacelia segetum)	778	GH3	(Schardl et al. 2013)
Coccidioides immitis (Valley fever fungus)	901	GH5	(Sharpton et al. 2009)
Colletotrichum gloeosporioides	438	GH3	(Gan et al. 2013)
Coprinopsis cinerea (Inky cap fungus,Hormographiella aspergillata)	775	GH17	(Stajich et al. 2010)
Cordyceps militaris (Caterpillar fungus)	760	GH3	(Zheng et al. 2011)
Cryptococcus neoformans var. grubii (Filobasidiella neoformans var. grubii)	822	GH3	(Birren et al. 2012)
Dacryopinax sp. (Brown rot fungus)	852	GH3	(Curtin et al. 2012)
Dekkera bruxellensis	841	GH3	(Floudas et al. 2012)
Emericella nidulans (Aspergillus nidulans)	863	GH3	(Galagan et al. 2005)
Hypocrea rufa (Trichoderma viride)	450	GH3	(Liu et al. 2009)
Kluyveromyces marxianus (Yeast) (Candida kefyr)	845	GH3	(Yoshida et al. 2009)
Komagataella pastoris (Yeast, Pichia pastoris)	839	GH5	(Xu et al. 2006)
Kuraishia capsulata	763	GH16	(Martin et al. 2008)
Lodderomyces elongisporus (Yeast,Saccharomyces elongisporus)	228	GH5	(Jackson et al. 2009)
Magnaporthe oryzae (Rice blast fungus,Pyricularia oryzae)	873	GH17	(Dean et al. 2005)

Mycosphaerella graminicola (Speckled leaf blotch fungus,Septoria tritici)	619	GH3	(Goodwin et al. 2011)
Neosartorya fischeri (Aspergillus fischerianus)	873	GH3	(Fedorova et al. 2008)
Neosartorya fumigata (Aspergillus fumigatus)	863	GH55	(Pain et al. 2004)
Neurospora tetrasperma	894	GH3	(Ellison et al. 2011)
Ophiostoma piceae	877	GH3	(Haridas et al. 2013)
Paecilomyces sp.	479	GH3	(Yang et al. 2013)
Paracoccidioides brasiliensis	874	GH17	(McEwen et al. 2008)
Penicillium brasilianum	878	GH3	(Krogh et al. 2010)
Penicillium funiculosum (Fruitlet core rot fungus)	490	GH3	(Ramani et al. 2012)
Phaeosphaeria avenaria f. sp. avenaria	874	GH3	(Liu et al. 2013)
Phaeosphaeria nodorum (Glume blotch fungus,Septoria nodorum)	874	GH3	(Ueng et al. 2004)
Phanerochaete chrysosporium (White-rot fungus,Sporotrichum pruinosum)	462	GH1	(Tsukada et al. 2006)
Pichia angusta (Yeast, Hansenula polymorpha)	1,702	GH3	(Ravin et al. 2011)
Piriformospora indica	800	GH3	(Zuccaro et al. 2011)
Postia placenta (Brown rot fungus,Poria monticola)	847	GH3	(Martinez et al. 2009)
Pseudozyma hubeiensis (Yeast)	177	GH3	(Konishi et al. 2013)
Pyrenophora tritici-repentis (Wheat tan spot fungus, Drechslera tritici-repentis)	719	GH5	(Birren BW et al. 2007)
Rhizomucor miehei	717	GH1	(Tang et al. 2010)
Rhodotorula glutinis (Yeast)	873	GH5	(Langkjaer et al. 2003)
Saccharomyces cerevisiae (Baker's yeast)	71	GH3	(Raynal et al. 1987)
Saccharomycopsis fibuligera (Yeast)	876	GH3	(Machida et al. 1988)
Scheffersomyces stipitis (Yeast, Pichia stipitis)	738	GH17	(Jeffries et al. 2007)
Schizophyllum commune (Split gill fungus)	192	GH3	(Moranelli et al. 1986)
Spathaspora passalidarum	835	GH3	(Wohlbach et al. 2011)
Sporisorium reilianum (Maize head smut fungus)	801	GH1	(Schirawski et al. 2010)
Stachybotrys microspora	422	GH1	(Abdeljalil and Gargouri 2012)
Talaromyces emersonii	857	GH1	(Murray et al. 2002)
Talaromyces stipitatus (Penicillium stipitatum)	618	GH3	(Fedorova et al. 2007)
Taphrina deformans (Peach leaf curl fungus, Lalaria deformans)	884	GH3	(Cisse et al. 2013)
Thanatephorus cucumeris (Black scurf of potato, Rhizoctonia solani)	845	GH3	(Rivard et al. 2006)

Thermoascus aurantiacus	861	GH3	(Hong et al. 2007)
Togninia minima (Esca disease fungus, Phaeoacremonium aleophilum)	683	GH3	(Blanco-Ulate et al. 2013b)
Trichoderma harzianum (Hypocrea lixii)	447	GH1	(Huang and Peng 2011)
Trichophyton equinum (Horse ringworm fungus)	920	GH3	(Martinez et al. 2012)
Trichophyton verrucosum	881	GH3	(Burmester et al. 2011)
Uncinocarpus reesii	863	GH3	(Sharpton et al. 2009)
Ustilago esculenta	819	GH3	(Nakajima et al. 2011)
Verticillium albo-atrum (Verticillium wilt)	685	GH3	(Ma et al. 2008)

1.4. *Aspergilli* as a major source of β-glucosidases

Species of *Aspergillus* have a significant role in the decomposition of plant materials as in composting and it is ubiquitous in nature.The growth of Aspergilli require 1-3 weeks and it is a filamentous ascomycetous fungi. Its growth is mainly on carbon source, but it can survive even in nutrient depleted environments. For achieving commercially competent enzyme yield, strain selection, nature of substrate and environmental conditions are important. Most of the commercial enzymes produced by fungi come from the *Aspergillus* sps due to its ease of growth and resistance to several inhibitory agents. There has been several reports on the producton of BGL from *Aspergilli* (Juhasz et al. 2003; Liu et al. 2012; Wang et al. 2012)and studies on their structure and function (Lima et al. 2013). *Aspergillus niger* is considered to be one of the most potent producers of β-glucosidase and the most successful commercial BGLs are now sourced from this fungus (Vaithanomsat et al. 2011). Most of the commercially available BGLs (eg Novozyme 188 from *Aspergillus niger*) are used as components of cellulase preparations for biomass hydrolysis along with *Trichoderma reesei* cellulases (eg Celluclast and Novozyme 188 from Novozymes used together for biomass hydrolysis).

1.5. Multiplicity of β-glucosidase in filamentous fungi

Isoforms are highly related gene products but with different amino acid sequence that performs essentially the same biological function. Isoforms are either the products of one gene or of multiple genes that evolved from a single ancestor gene. According to Tomaiuolo et al (2008) isoforms of the enzyme can increase the phenotypic system robustness. They

may catalyze the same catalytic reaction but still have different affinity towards the substrate. In order to study the substrate specificity and catalytic efficiency, purification of the isoforms are important. The multiplicity of β-glucosidase is common among fungi. Maximum of seven isoforms were reported in *Trichoderma reesei* (Takashima et al. 1999); six isoforms in *Humicola grisea* (Sonia et al. 2008); five isoforms in *Phanerochaete chrysosporium*(Elena et al. 1995); four isoforms in *Penicillium funiculosum* (Ramani et al. 2012), *Aspergillus tubingenesis* (Decker et al. 2001), *Aspergillus terreus* (Nazir et al. 2009); three isoforms in *Aspergillus kawachii* (Iwashita et al. 1998); *Candida wickerhamii* (Freer 1993) and two isoforms among *Pichia etchellsii* (Wallecha and Mishra 2003); *Penicillium decumbens* (Mamma et al. 2004); *Aspergillus oryzae* (Gunata and Vallier 1999); and *Aspergillus nidulans* (Kwon et al. 1992). Some of the properties of these isoforms are listed in Table 1.3. It is considered that different isoforms of β-glucosidase cooperate to enhance the hydrolysis of cellobiose to glucose. Literature cites that the expression levels of different isoforms may vary based on different factors including the carbon source it is grown on (Singhania et al. 2011), period of incubation, culture condition etc. and based on their expression levels isoforms can be often called major and minor isoforms. Several studies have been performed on highly expressing or the major isoforms whereas, the minor isoforms are difficult to purify due to their low level expression resulting in very limited studies on them. In *Aspergillus terreus*, rice straw acts as a major inducer when the culture was grown under Solid State Fermentation whereas additional supplementation of glucose, fructose, and mannitol to the media containing corn cob weakly represses the expression of β-glucosidase (Nazir et al. 2009). In *Candida wickerhamii*, under aerobic condition high concentration of glucose represses extra-cytoplasmic cell associated β-glucosidase expression (Freer 1993). According to Iwashita et al (1999), the culture conditions also determine the secretion of the isoforms in *Aspergillus kawachii*. In solid state cultivation, most of the BGL was secreted in to the medium whereas, in the case of liquid cultivation, BGL was bound to the mycelia or intracellular isoforms play major role in the overall activity of the β-glucosidase enzymes. All the three BGL isoforms (two extracellular and one intracellular) in *Aspergillis kawachii* are encoded by the same gene (bglA) but it is not clear whether they are translated from the same mRNA, transcribed from bglA gene (Iwashita et al. 1999). Thus more studies are required to understand the factors affecting the differential expression of isoforms.

Table 1.3. Multiplicity of β-glucosidase in filamentous fungi

Organism	**Localization**	**Mol. Wt (Native)**	**Mol. Wt (SDS)**	**Temp. Optimum**	**pH Optimum**	***Km* value**	***Ki* for Glucose**	**References**
Aspergillus sp.	Ex	44 kDa	-	60 °C	4	-	-	(Elyas et al., 2010)
	Ex	30 kDa		60 °C	3	-	-	
Pichia etchellsii	Ce	186 kDa	-	50 °C	6	0.33 mM pNPG	12 mM	(Wallecha and Mishra, 2003)
	Ce	340 kDa		50 °C	6	0.33 mM pNPG		
			-			24.0 mM Cellobiose	25 mM	
Penicillium decumbens	In	50 kDa	28 kDa (dimer)	65 °C	7	0.007 mM pNPG	0.24 mM	(Mamma et al., 2004)
	In	460 kDa	115 kDa (tetramer)	75 °C	7	0.013 mM pNPG	0.29 mM	
Aspergillus tubingensis	Ex	-	131 kDa	65 °C	4.6	0.76 mM pNPG	5.8 mM	(Decker et al., 2001)
	Ex	-	126 kDa	65 °C	4	0.35 mM pNPG	1.3 mM	
	Ex	-	54 kDa	60 °C	5	3.2 mM pNPG	470 mM	
	Ex	-	54 kDa	60 °C	5	6.2 mM pNPG	600 mM	
Aspergillus terreus	Ex	-	29 kDa	60 °C	2.0-8.0	14.2 mM pNPG	-	(Nazir et al., 2009)
	Ex	-	43 kDa	60 °C	5	4.37 mM pNPG		
	Ex	-	98 kDa	70 °C	5	11.1 mM pNPG		
Aspergillus oryzae	Ex	>130 kDa	-	-	-	-	3.5 mM	(Günata and Vallier, 1999)
	Ex	30 kDa	-	-	4.5-6.0	6.4 mM pNPG	953 mM	
Ceriporiopsis subvermispora	Ex	-	110 kDa	60 °C	3.5	3.29 mM pNPG 2.63 mM cellobiose	-	(Magalhães et al., 2006)
	Ex	-	53 kDa	-	-	-	-	

1.6. Glucose tolerant β-glucosidases

The product as well as the substrate inhibition is a common characteristic of β-glucosidases and it is an important constraint for industrial use of this enzyme especially for biomass hydrolysis. Most microbial β-glucosidases that catalyze the hydrolysis of cellobiose are very sensitive to glucose inhibition, which limits their activity (Gueguen et al. 1995; Saha and Bothast 1996). Furthermore, the enzyme is also inhibited by its own substrate, cellobiose (Yan and Lin 1997). In this respect, the availability of β-glucosidase insensitive to glucose (glucose tolerant β-glucosidase) and cellobiose inhibition will have a significant impact on the enzymatic conversion of cellulosic biomass to glucose. Aspergilli are known to produce high titers of β-glucosidase and some species of these fungi are reported to produce glucose tolerant β –glucosidases. Glucose competitively inhibit the β-glucosidase activity in *Sporotrichum thermophile*, a *Monilia* sp., *Fusarium oxysporum*, *Neocallimastrix frontalis*, *Botrytis cinerea*, and *Streptomyces* sp. with a *K*i range between 0.6 mM – 8mM for glucose (Bhat et al. 1993; Christakopoulos et al. 1994; Dekker 1981; Gueguen et al. 1995; Li and Calza 1991; Perez-Pons et al. 1994). Maximum glucose tolerance was reported from *Candida peltata* β-glucosidase with a *K*i of 1.4M and further increase in glucose concentration competitively inhibited the enzyme activity (Saha and Bothast 1996). Moreover the enzyme was also highly tolerant to the substrate cellobiose (15% cellobiose). In *Trichoderma viridae* the inhibition was mixed type with competitive character predominating (Montero and Romeu 1992). Highly Glucose tolerant β-glucosidases already reported from fungi are summarized in Table 1.4.

Table 1.4. Highly glucose tolerant β-glucosidases from fungi

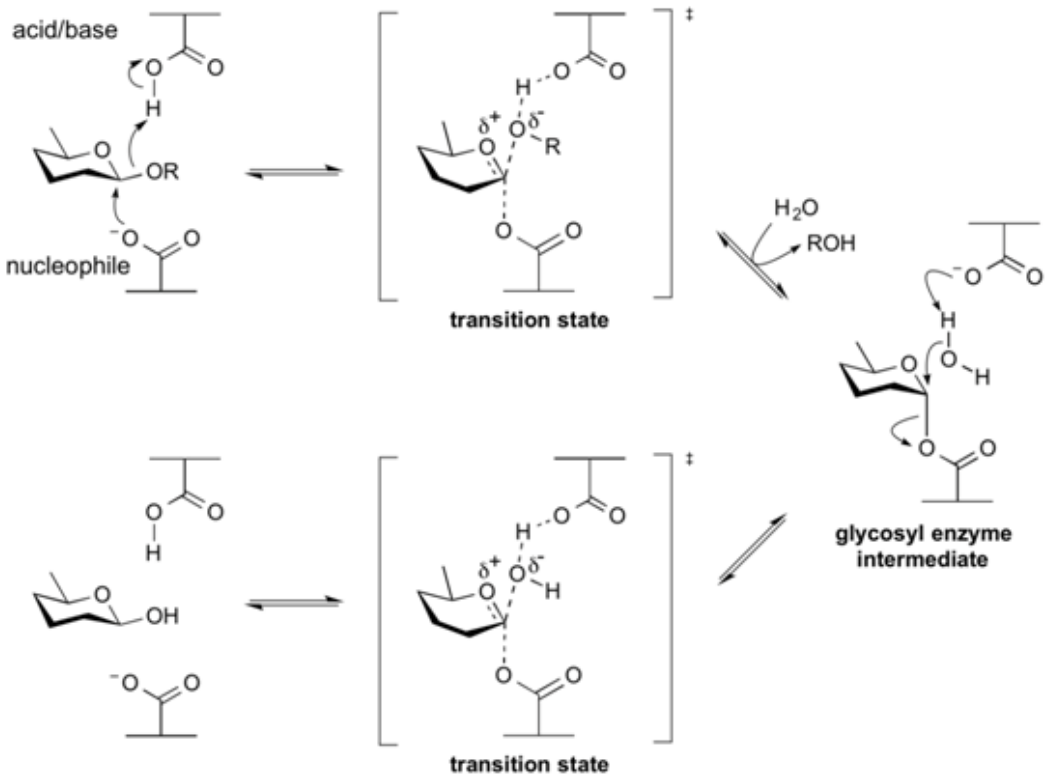
Organism	**Glucose Tolerance (*Ki* in mM Glucose)**	**Reference**
Pyrococcus furiosus	300	(Kengen et al. 1993)
Aspergillus tubingenesis	470 & 600	(Decker et al. 2001)
Aspergillus oryzae	953	(Gunata and Vallier 1999)
Aspergillus oryzae	1360	(Riou et al. 1998)
Candida peltata	1400	(Saha and Bothast 1996)
Aspergillus niger	543	(Yan and Lin 1997)
Byssochlamus fulva	800	(Abraham et al. 2012)

1.7. Mechanism of enzyme catalysis

1.7.1. Hydrolysis

β-Glucosidases are hydrolytic enzymes catalyzing the hydrolysis of β-glucosidic linkages. Enzymatic hydrolysis occurs via two major mechanisms 1) Single displacement mechanism resulting in Inversion 2) Double displacement mechanism resulting in Retention. Enzymatic hydrolysis of the glycosidic bonds takes place via acid catalysis that requires 1) Proton donor 2) a nucleophile/base. An acid/base catalytic group exists for the protonation of the glycosidic group to release 'aglycon'. A nucleophile catalyst stabilizes an oxocarbenium-ion-like transition state, which collapses into a covalent glycosyl-enzyme intermediate (Davies and Henrissat 1995). According to Koshland, β-glucosidase reaction proceed with double displacement mechanism resulting in retention. In retention, the acidic group (Aspartic /glutamic acid) in the active site donates a proton to glycosidic oxygen and a nucleophilic group facilitates the bond breaking by attacking the C1 of glucose. In retention mechanism, the position of proton donor is identical (ie.within the hydrogen bonding distance of the glycosidic oxygen) to the inversion mechanism. But the nucleophile catalytic base is in close vicinity (~5.5A°) of the sugar anomeric carbon as opposed to inversion where the distance is more (~10A°).Classical Koshland retaining mechanism is given below. Structurally, the active site topologies fall into three main classes1) Pocket or Crater 2) Cleft or groove and 3) Tunnel. β-glucosidases having a pocket or crater topology at the active site is suitable for substrates with large number of chain ends. But these enzymes are not suitable for fibrous substrates like native cellulose which almost has no chain ends (Davies and Henrissat 1995).

Figure 1.3. Classical Koshland double displacement mechanism for β-glucodidase action (Koshland 1953)



The retaining mechanism, in which the glycosidic oxygen is protonated by the acid catalyst (AH) and nucleophilic assistance to aglycon departure is provided by the base B-. The resulting glycosyl enzyme is hydrolyzed by a water molecule and this second nucleophilic substitution at the anomeric carbon generates a product with the same stereochemistry as the substrate)

1.7.2. Biosynthesis

The enzymatic synthesis of glycosides has certain advantages over chemical synthesis; since it is carried out under mild conditions in one stereoselective step. β-glucosidase mediated biosynthesis of glycoconjugates occur either by 1) reverse hydrolysis or by 2) transglycosylation. The two-step mechanism employed by retaining enzymes such as β-glucosidases allows these enzymes to transglycosylate.

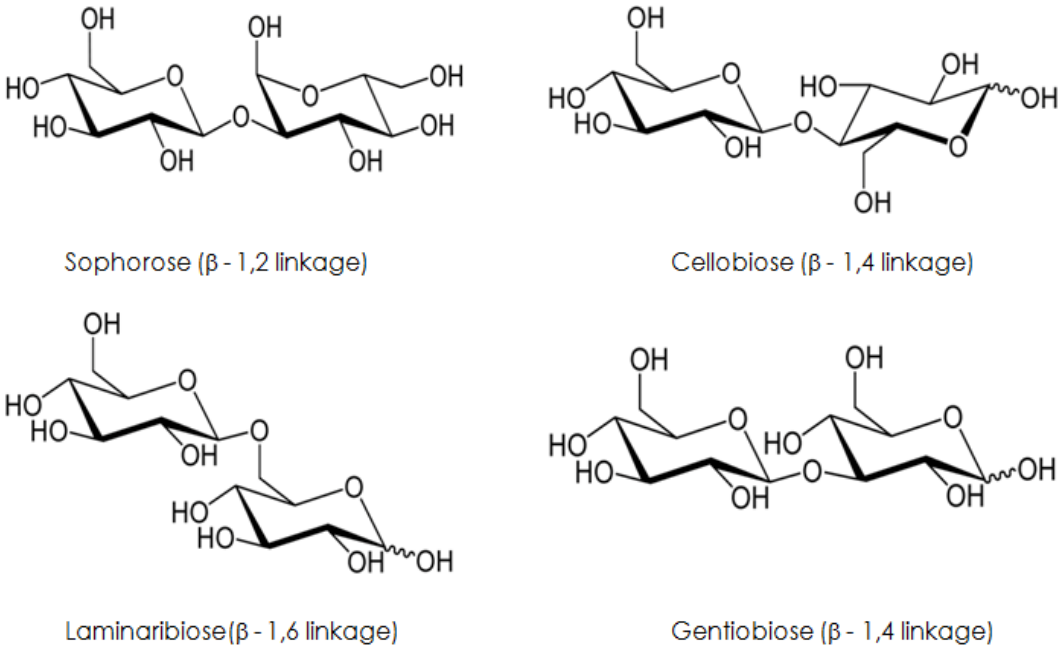
1.7.2.1. Reverse hydrolysis

In reverse hydrolysis, the substrate has an H in place of R. The enzyme-glycosyl intermediate is intercepted by R′OH where R′ is another sugar, yielding a disaccharide product. The reaction is under thermodynamic control. In the transglycosylation method, the substrate has R in place of H and is an "activated" donor. The enzyme-glycosyl intermediate may be trapped by a nucleophile other than water, viz., aryl or alkyl alcohol (as R′OH) to yield a new glycoside. Here, the efficiency of the formation of the product is determined by competition between water and the acceptor R′OH for the enzyme-glycosyl intermediate (Bhatia et al. 2002a)

1.7.2.2. Transglycosylation.

Transglycosylation is a kinetically controlled reaction. It also occurs through a double displacement mechanism of retention. First step is the enzyme substrate complex formation which is followed by cleavage of the glycosidic linkage via protonation of the leaving aglycon moiety. When the substrate molecule in a hydrolysis reaction starts as an acceptor, substrate transglycosylation occurs and this leads to the slowdown of hydrolysis. Even though glycosyl transferases are highly efficient in synthesis of glycosidic linkages, their applications to industrial process is limited because of the expensive nucleotide precursors, narrow substrate specificities and low enzyme availability. In contrast, β-glucosidases are attractive for industrial applications since they are more abundant, commercially available and exhibit a relatively broad acceptor-substrate specificity with simple substrates (Kren and Thiem 1997). The major oligosaccharides synthesized by β-glucosidase through transglycosylation by using glucose substrate are sophorose (β-1,2 linkage), laminaribiose (β- 1,3 linkage), cellobiose (β-1,4 linkage), and gentiobiose (β-1,6 linkage).

Figure1.4. Disaccharides generated by transglycosylation using β-glucosidase



β-glucosidases from *Aspergillus* as well as *Trichoderma* sp exhibit transglycosylation resulting in the formation of β-1,3 as well as β-1,6 glycosidic linkages. *Pitchia etchellsii* β-glucosidase produces disaccharides in the following order sophorose > gentiobiose > cellobiose (Bhatia et al. 2002b). Eventhough the products are same, the ratio of formation of the disaccharides varies among the sps. In *Trichoderma pseudokoningii,* the major transglycosylation products are gentiobiose along with small amounts of sophorose, laminaribiose and cellobiose, when cellulose and glucose was used as the substrate (Dong et al. 1996). β-glucosidase from *Aspergillus niger*, *Corynascus* sp. and *Penicillium verruculosum* also synthesized gentiobiose as the major tranglycosylation product where as in *Trichoderma reesei* sample, laminaribiose was the main product (Semenova et al. 2009). So the enzymatic synthesis of these disaccharides from glucose is a simple and inexpensive way compared to the chemical synthesis.

1.8. Classification of β-glucosidases

Initial classification of β-glucosidase was based on the substrate specificities and they were mainly classified as 1) aryl β-glucosidase 2) true cellobiases and 3) broad substrate specificity enzymes. Another classification was proposed by Henrissat in 1991 based on the structure and folding similarities of glycosyl hydrolase and this is the widely accepted method for classification (Henrissat 1991). Glycoside hydrolase consists of more than hundred families and the classification is mainly based on amino acid sequence similarities. This nomenclature system for glycosyl hydrolase is continuously updated at *http://www.cazy.org/Glycoside-Hydrolases.html.* About 136 families have been listed in the CAZY website (Carbohydrate Active Enzyme Website). The bacterial and fungal β-glucosidases reported in the literature are primarily included in three main families of glycosyl hydrolases ie.-GH1, GH3 and GH5. Eventhough in limited numbers, few more fungal β-glucosidases have been reported in glycosyl hydrolase families other than these three. In *Blumeria graminis* f. sp. *hordei* (Barley powdery mildew) and in *Kuraishia capsulate* the β-glucosidase belongs to GH16 (Martin et al. 2008; Spanu et al. 2010). β-glucosidase from *Coprinopsis cinerea* (Inky cap fungus), *Magnaporthe oryzae* , *Paracoccidioides brasiliensis* , *Scheffersomyces stipitis* (*Pichia stipitis*) shows close similarities to Glycosyl hydrolase Family 17 (GH17) (Dean et al. 2005; Jeffries et al. 2007; McEwen et al. 2008; Stajich et al. 2010).

1.8.1. Glycosyl hydrolase Family 1 (GH1)

Enzymes from Glycosyl Hydrolase F1 are the highly characterized among the Glycosyl hydrolases. Henrissat and Bairoch (1996) put forward the updated version of the existing classification and the electronic version was also announced. In order to improve the sequence comparison strategies, glycosyl hydrolases are grouped in to 'clans'. A 'clan' is a group of families that are thought to have a common ancestry and are recognized by significant similarities in tertiary structure together with conservation of the catalytic residues and catalytic mechanism.

Glycosyl hydrolase Family1 and 5 are grouped in to the same clan GH-A. The mechanism of catalysis involves the *retaining* mechanism and mostly all of them have a catalytic

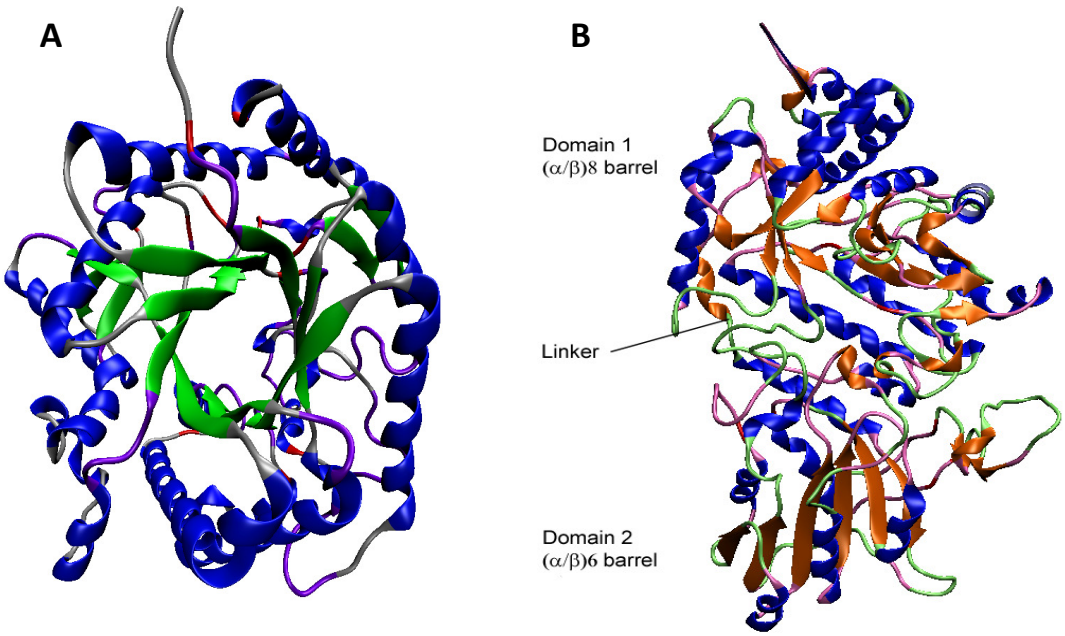
proton donor/base and a catalytic nucleophile. The conserved glutamate residue in **NEP** motif has been identified as the catalytic proton donor and the glutamate in **ENG** motif was identified as the catalytic nucloephile in the active site region in Glycosyl Hydrolase F1(Bhatia et al. 2002a). Family GH1 enzymes are found in several different groups of organisms but are more common in bacteria and plants. The β-glucosidases belonging to families GH-1 are grouped under Clan GH-A where they have a similar $(\beta/\alpha)_8$-barrel domains that contain their active site (Figure 1.5A). Their catalytic site contains two active site carboxylic acid residues in the β strand at a site 4 and 7 (4/7 superfamily) which are conserved in GH1 Family (Cairns and Esen 2010). In GH F1 β-glucosidases, glutamic acid acts as acid/base catalyst and catalytic nucleophile.

1.8.2. Glycosyl Hydrolase Family 3 (GH3)

The enzymatic reactions of Glycosyl hydrolase Family 1 and 3 are similar in nature but the detailed studies are less reported in Family 3 as compared to Family 1. The first detailed report on the complete resolution of β-glucosidase from GHF3 was of the β-glucosidase from Barley (Varghese et al. 1999). Based on this structure, Aspartate (ASP-285) is the nucleophile in GHF3 instead of conserved glutamate residue in GHF1 and glutamate residue (GLU-491) is the acid/base catalyst in GHF3. In Family 3 Glycosyl hydrolases, the major two motifs are SDW at the active site and KHF motif is the proposed proton donor. In Family 3, β- glucosidase E/D is the active site nucleophile where H/E is the putative proton donor (Bhatia et al. 2002a; Iwashita et al. 1999).

Enzymes from GH F3 generally has two distinct domains connected by a helix strand made of 16 residues.The first domain is an $(\alpha/\beta)_8$ TIM barrel domain and second domain is a six stranded β sandwich which contains 5 parellel β strands and one antiparellel β strand with three α-helices on either side of it (Figure 1.5.B). Glucose moiety is believed to be the interface of the two domains (Varghese et al. 1999)

Figure 1.5. Representative structure of GH1 and GH3 β-glucosidases



GH1 β-glucosidase from *Phanerochaete chrysosporium* (PDB file 2E3Z)

GH3 β-glucosidase from *Hordeum vulgare* (PDB file 1EX1)

1.8.3. Glycosyl hydrolase Family 5 (GH5)

Glycosyl hydrolase family 5 enzymes are also classified under Clan A, similar to GHF1 and contains the enzymes with a wide range of catalytic activities including endoglucanse and exoglucanase. There are 22 known three dimensional structures for GHF5. The enzymes have similar $(\alpha/\beta)_8$ barrel structures, the difference being at the active site cleft which varies from long grooves to an active site pocket (Opassiri et al. 2007). Similar to GH F1 β-glucosidases these enzymes also have glutamic acid as the acid base catalyst and the catalytic nucleophile.

1.9. Biotechnological Applications of β-glucosidase

The hydrolytic activity of fungal originated β-glucosidase is exploited in several biotechnological processes:

- For increasing the rate and extent of saccharification of cellulosic material by hydrolyzing the cellobiose which is an inhibitor of cellulases (Woodward and Wiseman 1982)
- For flavor enhancement in fruit juices and derived beverages through the liberation of flavor compounds from glucosidic precursors (Günata et al. 1993)
- For de-bittering of citrus juices through the hydrolysis of prunin (Roitner et al. 1984)
- For detoxification of cassava through the hydrolysis of cyanogenic glycosides (Birk et al. 1996)
- Release of aromatic compounds in the flavor industry (Gueguen et al. 1997)
- Synthesis of useful β-glucosides (Millqvist-Fureby et al. 1998)

One of the most important applications of β-glucosidase is in the saccharification of biomass for biofuel production

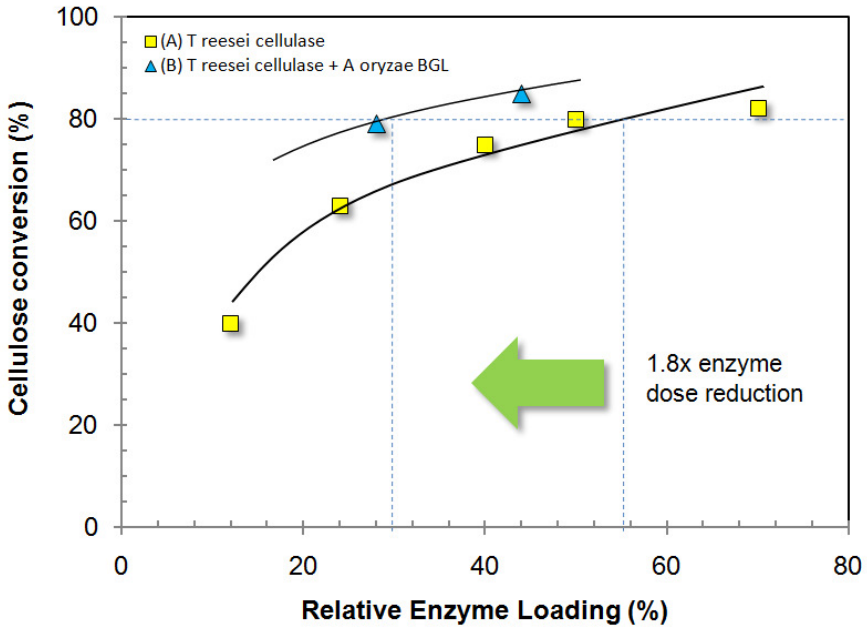
1.9.1. β-glucosidases for biomass hydrolysis

Enhancement of the biofuel production using enzymatic saccharification requires additional supplementation of β-glucosidase in the current commercial cellulase preparations as most of the cellulase producers lack the ability to produce sufficient amount of β-glucosidase. In order to improve the saccharification efficiency of the enzymes, commercial cellulase products are made as enzyme cocktails, initially prepared as seperate fermentation products and later combined in different ratios for getting the most appropriate enzyme ratios to obtain high sugar yields from biomass. *Trichoderma reesei* and *Aspergillus niger* are the most common sources currently employed for cellulase and β-glucosidase production respectively at the industrial level. However, most of the commercial β-glucosidases are also susceptible to product as well as substrate inhibition which limits the overall efficiency of the process. This limitation is largely addressed now by adding very high concentrations of these enzymes in the cocktail. β-

glucosidases insensitive to glucose and cellobiose inhibition can have a significant impact on enzymatic conversion of biomass to sugars (Saha and Bothast 1996). Supplementation of such glucose tolerant β-glucosidases can be considered as an alternate strategy to overcome the limitations of the current enzyme cocktails for biomass hydrolysis.

Olsson et al (2007) had addressed the beneficial effects of β-glucosidase in biomass hydrolyzing enzymes by peforming the hydrolysis of pretreated corn stover (PCS) with *Trichodrema reesei* derived cellulases with and without supplementation of a β-glucosidase from *Aspergillus oryzae.* With addition of β-glucosidase (without product inhibition) at high solids loadings (13.5% w/w in this example), the amount of total enzyme protein required to hydrolyze 80% of the cellulose to glucose was reduced by nearly two fold (Figure 1.6). At this solid loading, the beneficial effect of β-glucosidase addition was saturated when it reached ~ 5% of the total enzyme protein, but it was concluded that higher solids would require higher β-glucosidase levels or a more active β-glucosidase enzyme.

Figure 1.6. Reducion in enzyme requirement for biomass hydrolysis with β-glucosidase supplementation (Adapted from Olssson et al, 2007)



Improvement of biomass-hydrolyzing cellulases by increasing levels of β-glucosidase (BGL) activity. Comparison of *T. reesei* cellulase preparations, with (*B*) and without (*A*) supplementation of purified *A. oryzae* BGL, in the hydrolysis of cellulose present in acid pretreated corn stover demonstrates a significant benefit in reducing the amount of enzyme required. Addition of small amounts of BG, present as a few percent of total protein,allowed hydrolysis of 80% of the cellulose to glucose with an enzyme protein dosage 1.8-fold lower that the unsupplemented cellulase (Olsson et al. 2007)

1.10. Objectives of the Current study

Biofuel from biomass is a potential area of growth throughout the world, since it has an important role to play in energy sector. Developing suitable enzyme cocktails for biomass hydrolysis required for the complete conversion of cellulose to glucose is a major challenge in any biomass to biofuels program. There are already few enzyme cocktails tailored for biomass hydrolysis developed by the world's leading enzyme manufacturers, but the economic feasibility as well as universal availability is still a prevailing question with the ambiquities surrounding these products. Current enzyme cocktails for biomass hydrolysis do use β-glucosidase supplementation, but these enzymes are susceptible to product as well as substrate inhibition which limit the efficiency of conversion of cellulose to glucose. This necessitates the β-glucosidase supplementation to be raised to very high levels so as to combat product and substrate inhibitions. Another important hurdle in the enzyme process is the high cost of the enzyme. Current study was undertaken mainly to address these two issues. The main aim of the study was to explore and develop an enzyme capable of acting in presence of high amounts of glucose and evalualte its application in the enzymatic saccharification of biomass which further leads to biofuel production. The objectives of the study are listed below,

1. Isolation and identification of a potent fungus producing glucose tolerant β-glucosidase
2. Developing a process for production of β-glucosidase using the fungus
3. Purification and characterization of the glucose tolerant β-glucosidase protein from the fungus.
4. Purification and characterization of β-glucosidase isoforms if present from this fungus
5. Partial gene sequencing of β-glucosidases from the genomic DNA of the fungus.
6. Bioinformatics analyses of the β-glucosidase gene sequence(s) and translated protein sequence of the enzyme to understand more about the properties and features of the enzyme at molecular level
7. Analysis of β-glucosidase protein sequence for structural features and homology modeling
8. Application of β-glucosidase in enzymatic hydrolysis of biomass.

Chapter 2. Materials and Methods

2.1. Microorganisms and culture conditions

The microorganism used for the current study was a filamentous fungus previously isolated from soil samples at the Biotechnology division of CSIR – National Institute for Interdisciplinary Science and Technology (NIIST), Thiruvananthapuram, India. The fungus code named BTCF58 was producing β-glucosidase active at 0.5M glucose concentration and hence was selected for the study. The isolate was identified as *Aspergillus unguis* and was deposited in the NII culture collection centre CSIR-NIIST with an accession No. NII08123. *Penicillium janthinellum* was used for cellulase production and this strain was a kind gift from Dr. DV Gokhale, former head, National Collection of Industrial Microorganisms (NCIM), National Chemical Laboratory, Pune India.

Potato Dextrose Agar (PDA) slants was used for maintenance of the cultures. The cultures were streaked on PDA slants and were incubated at 30 °C for 5 days. After complete sporulation, the slants were either used directly or stored at 4 °C. Sub-culturing was done every month onto fresh PDA slants.

Escherichia coli strains DH5α, BL21(DE3) and JM109 used for transformation studies were grown on Luria Bertani Agar at 37 °C and were stored at 4 °C for short term preservation. The cultures were preserved as 50% glycerol stocks at -80 °C for long term storage. The bacterial strains and vectors used in the present study are listed in Table 2.1

Table 2.1. List of Bacterial strains and vectors used in the present study

Bacterial strains (*E.coli*)	Characteristics	Source or Reference
DH5α	*fhuA2 lac(del)U169 phoA glnV44 Φ80' lacZ(del)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17*	Fermentas
JM109	*end*A1, *rec*A1, *gyr*A96, *thi*, *hsd*R17 (r_k^-, m_k^+), *rel*A1, *sup*E44, Δ(lac-proAB)	Fermentas
BL21(DE3)	*E. coli B dcm ompT hsdS* ($r_B^- m_B^-$) *gal*	New England Biolabs

Vectors		
pTZ57R/T	Blue-White Screening, M13/pUCprimer sites, T7 promoter	Fermentas
pJET1.2/blunt	Contains lethal gene which get disrupted by ligation of DNA inserts	Fermentas

2.2. Medium for Enzyme production

Medium used for production of β-glucosidase was the basal mineral salt solution according to Mandel and Weber (1969) with the following composition

Table 2.2. Composition of Mandel and Weber medium

Components	g/l	Trace elements	g/l
Urea	1.3	$FeSO_4.7H_2O$	0.05
$(NH_4)_2SO_4$	1.4	$MnSO_4.7H_2O$	0.016
KH_2PO_4	2	$ZnSO_4.7H_2O$	0.034
$CaCl_2.2H_2O$	0.3	$CoCl_2.6H_2O$	0.02
$MgSO_4.7H_2O$	0.3		
Peptone	0.75		
Yeast extract	0.25		

The pH of the medium was adjusted with 1N HCl or 1N NaOH wherever required.

2.3. Preparation of spore inoculum

Spore suspensions were prepared in sterile saline containing 0.05 % Tween 80. Five milliliters of the sterile saline was added to fully grown slants and spores were dislodged in to it by gentle pipetting using a 1.0 ml micro pipette under aseptic conditions. The suspension was recovered by aspiration and transferred to a sterile 15 ml glass vial. Spores were counted under a phase contrast microscope using a hemocytometer to obtain the spore count. The suspension was appropriately diluted with sterile saline to obtain the required spore count (10^6 spores/ml unless otherwise specified) and was used as inoculum.

2.4. Enzyme Production

2.4.1. Submerged Fermentation

Submerged Fermentation (SmF) for enzyme production was carried out in 500 ml Erlenmeyer flasks having 100 ml of Mandels and Weber medium (1969) containing the desired concentration of carbon source and inducer (eg. wheat bran & lactose). The contents of the flask were mixed thoroughly after the addition of the basal medium and substrate and the flasks were sterilized by autoclaving for 15 minutes at 121 $^{\circ}$C and 15 lbs pressure. After cooling, the flasks were inoculated with 1 ml of a spore inoculum containing 1 x 10^6 spores/ml (unless otherwise specified). The flasks were incubated at 30 ± 2 °C and 180 rpm agitation for the specified time interval. The culture broth after fermentation was centrifuged at 8000 rpm for 20 min at 4 ^{o}C and the supernatant was used as the crude enzyme preparation.

The effect of additional carbon source/inducer was studied by supplementing these in the enzyme production medium. The basal enzyme production medium (EPM) was supplemented with 1% of any one of the carbon sources/inducers. Fermentation was carried out as above and enzyme assays were conducted on the culture supernatants to determine BGL activity. Lactose was used as the carbon source during production of enzyme for the purification studies.

2.4.2. Solid State Fermentation

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 constant moisture. Five gram (dry weight) of the substrate was weighed into 250 ml Erlenmeyer flasks and was moistened with a 6 ml of double strength Mandels and Weber (1969) basal mineral salt medium. The pH of the medium was adjusted with 1N HCl or 1N NaOH to be between 4.5-5.0. Distilled water was added in addition to the medium to attain 60 % initial moisture content (including the water added along with spore suspension). The moistened bran was mixed well and was sterilized by autoclaving at 121 °C for 15 min at 15 lbs pressure. Each flask was inoculated with 1ml of a spore inoculum containing 1 x 10^6 spores/ml prepared as above (section 2.3).

The contents were mixed thoroughly and were incubated under controlled conditions of temperature and relative humidity. Incubation was continued for 96-120h and at the end of incubation period enzyme was recovered by extraction with 50 ml of 0.05 M citrate buffer (pH 4.8) containing 0.05 % Tween-80. The buffer was added to each flask and the flasks were kept on a rotary shaker for 30 min at 150 rpm, after which the entire slurry was recovered and was filtered using nylon mesh. Filtered solution, containing the enzyme was centrifuged at 8000 rpm for 20 min at 4 °C and the supernatant was used as the crude enzyme preparation.

2.4.3. Enzyme Concentration

For obtaining a concentrated enzyme (for purification as well as for application studies), 3 volume of chilled acetone (-20 °C) was slowly added to the pre-cooled extract (~4-8 °C) with constant stirring. The mixture was kept for 12 h at 4 °C, followed by centrifugation at 8000 rpm for 15 min at 4 °C. The pellet was allowed to air dry at room temperature (28 ± 2 °C) to remove residual acetone. The pellets were then dissolved in minimal volume of 0.05 M citrate buffer (pH 4.8), and were used for further studies

2.5. Analytical methods

2.5.1. β-glucosidase assay

BGL activity was determined according to Ghose (1987) using p-nitro phenyl β-D gluco-pyranoside (pNPG) as substrate. Appropriately diluted enzyme sample of 25µl was incubated with 25µl of 10mM pNPG (prepared in 0.05M, pH 4.8 Citrate buffer) and 50µl of Citrate buffer (0.05M, pH 4.8) at 50 °C for 15 min. The reaction was terminated by adding 100µl of 0.2M Na_2CO_3 solution. Blanks without enzyme or substrate were also run in parallel. The color developed was read in a microplate reader (Tecan Infinite 200 Pro, Switzerland). A standard curve generated using varying concentrations of pNP was then used to calculate the amount of pNP liberated. One unit of BGL activity was defined as the amount of enzyme liberating 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 international units per milliliter (IU/ml) in the case of submerged fermentation.

2.5.2. Assay of the glucose tolerance of β-glucosidase

Glucose tolerance of the enzyme was determined wherever mentioned by incorporating the appropriate concentration of glucose in the assay mixture. Assays were done as above (section 2.5.1) with glucose added to the buffer. Glucose tolerance was expressed as percentage of activity compared to assay performed without glucose and was expressed as % activity retention.

2.5.3. 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 (~50 mg) was placed into each assay tube. The filter paper strips were saturated with 0.5 ml of citrate buffer (0.05 M, pH 4.8). Half milliliter of an appropriately diluted enzyme was added to the tube and incubated at 50 °C for 60 min. 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 3.0 ml 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 540 nm 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 with known concentrations of glucose. Filter Paper Activity (FPA) was calculated following the concept that 0.37 FPU of enzyme will liberate 2 mg of glucose under the above assay conditions and was expressed as Filter Paper Units (FPUs).

2.5.4. Sugar estimation

Reducing sugar concentration was estimated using 3'5-Dinitro Salicylic Acid (DNS) reagent following the method of Miller (1959). Estimation of sugars was also performed using HPLC wherever indicated following the NREL method (Ruiz and Ehrman 1996). BioRad Aminex HP-87H HPLC column was used for the analysis and a Refractive Index detector was used for detection. This analysis was performed at 55 $^{\circ}$C column temperature and 40 $^{\circ}$C detector cell

temperature. The flow rate was 0.6 ml/min using 0.01 N H_2SO_4 as mobile phase. Glucose, Xylose and Cellobiose standards used were HPLC grade from Sigma Aldrich India.

2.5.5. Protein Assay

Protein assay was done using the Bradford's reagent according to the method of Bradford (1976) and the protein concentration was expressed as mg/ml.

2.6. Electrophoresis and Zymogram Analysis

2.6.1. Poly Acrylamide Gel Electrophoresis (PAGE)

The molecular weight as well as the homogeneity of the protein was determined by Sodium Dedecyl PAGE (SDS PAGE) as well as Native PAGE using the method of Laemmli (1970). Gels with 12% strength of acrylamide were used for the electrophoresis. Protein in the samples was estimated by Bradford's method. 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 10 mM MUG solution in citrate buffer (0.05 M, pH 4.8) for 10 min at room temperature (28 °C ± 2 °C). The second half was treated similarly but with a substrate solution containing 0.5 M or 1.0 M 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. Band intensities were measured using ImageJ software (Schneider et al. 2012). The fluorescence intensities of the bands in presence and absence of glucose was compared to calculate the percentage activity retention as follows

$$\% \text{ Activity Retention (Glucose Tolerance)} = T_g = (I_g/I_0) \times 100$$

where I_g and I_0 are the fluorescence intensities in presence and absence of glucose, respectively, for each band.

2.6.2. Agarose Gel Electrophoresis

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

2.7. Chromosomal DNA isolation from the fungus

Fungal spores (10^6 spores) were inoculated in Mandels and Weber (1969) medium with 1% glucose as carbon source in 250 ml Erlenmeyer flasks and were incubated at room temperature (28 ± 2 °C) for 48 h. One gram wet-weight of the mycelium was frozen in liquid nitrogen and was ground to a fine powder. It was suspended in 10 ml of lysis buffer (250 mM NaCl, 25 mM EDTA, 0.5 % w/v CTAB and 200 mM Tris–HCl, pH 8.5). The suspension was incubated at 60 °C for 30min with occasional gentle mixing. After centrifugation at 13,000 rpm for 15 min (4 ºC), the supernatant was transferred to a new tube and polysaccharides and proteins were precipitated by adding equal volume of Phenol: Chloroform: Isoamyl Alchohol (25:24:1). Clear aqueous fraction was transferred with wide bore pipette into a clean nuclease free centrifuge tube. DNA was precipitated by adding 2/3 volumes of chilled iso-propanol to aqueous fraction. The solution was gently mixed by inversion, placed at -20 ºC for 20 minutes and DNA was recovered by centrifugation at 13000 rpm for 15 min (4 ºC). The DNA precipitate was washed twice with 70 % ethanol and was allowed to air dry after which it was resuspended in 10 mM Tris-EDTA buffer (pH 8.0). RNAase was added to a final concentration of 10 µg/ml at 37 °C (Doyle and Doyle 1987). The DNA was purified again using phenol: chloroform: isoamyl alchohol precipitation method. After a final wash with ethanol the DNA was allowed to air dry and was resuspended in 10 mM Tris-EDTA buffer (pH 8.0). For long term storage DNA was kept at -20 °C.

2.8. Plasmid DNA isolation

Plasmid DNA was isolated based on the alkaline lysis method (Bimboim and Doly 1979). For DNA sequencing the plasmid DNA was isolated using Qiagen Mini Prep Plasmid isolation kit following the manufacturer's instructions.

Chapter 3. Identification of the fungal isolate producing glucose tolerant β-glucosidase and differential expression of the enzyme in response to carbon sources

3.1. Introduction

β-Glucosidases (EC 3.2.1.21; β-D-glucoside glucohydrolase) are key enzymes in cellulose hydrolysis, being the rate-limiting enzyme that is regulated by feedback inhibition from its own product-glucose. These enzymes are therefore of considerable interest as constituents of cellulose-degrading systems to be used for biomass conversion applications (Sørensen et al. 2011). *Aspergilli* are known to be potent sources of β-glucosidase (BGL) and the most common industrial source of BGL is *Aspergillus niger* (Chauve et al. 2010). Inhibition by the product and substrate limits its use, especially in the context of biomass hydrolysis. β-glucosidase insensitive to glucose and cellobiose will significantly improve enzymatic conversion of cellulosic biomass to glucose for the subsequent production of fuel ethanol (Saha and Bothast 1996). Glucose-tolerant β-glucosidases (GT-BGLs) have been reported from a few filamentous fungi, and differences in their properties were studied including molecular mass, isoelectric points, pH optima, and glucose inhibition constants (Decker et al. 2001; Gunata and Vallier 1999; Job et al. 2010; Saha and Bothast 1996). From the limited number of reports about GT-BGLs, it may be speculated that such enzymes are rather uncommon or understudied. However, the impact of the addition of GT-BGLs in the cellulase cocktails for biomass hydrolysis can be significant, and improvements in the yield and concentration of sugars may be achieved by using such blends. Recently, the search for β-glucosidases that are insensitive to glucose has increased significance, for these enzymes can improve various industrial processes, especially biomass hydrolysis. 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 β-glucosidases.

As filamentous fungi are the major decomposers of plant material, they are the potent source of cellulases and hence β-glucosidases. Also since these enzymes have multitudes of functions, obtaining enzymes with different kinetic properties from fungi may not be a difficult task. Due to the wide range of distribution of filamentous fungi, each with several strains adapted

to specific environments, it is expected that screening of filamentous fungi from different ecological niches would definitely provide isolates capable of producing glucose tolerant BGL(s). Identification of such fungi and developing process(s) for production of the enzyme are also important. For enzyme production using microbes, all the nutritional requirements have to be met and need to be optimized for maximum yield of the product of interest. Two processes widely employed in microbial production include submerged fermentation (SmF) and solid state fermentation (SSF). Industrial production of enzymes generally employs submerged fermentation owing to better automation, reproducibility, ease of handling and easier purification strategy. SSF is defined as the cultivation of microbes on moist solid supports which may be inert carriers or insoluble substrates. The fermentation takes place in the absence or near absence of free water (Hölker et al. 2004; Pandey et al. 2000). Solid state fermentation is sometimes preferred for fungal fermentations as conditions similar to natural habitat may be provided. The advantages of SSF include - high productivity, concentrated products, lesser catabolite repression, higher product stability, ability to cultivate microbes in water insoluble substrates, mixed cultivation of fungi and lower demand of sterility due to low water activity (Hölker et al. 2004).

An important aspect in the large scale production of enzymes is the optimization of process parameters. Currently, most of the fermentation optimizations are performed through statistical design of experiments (DOE) and quite often a fractional factorial design is performed when a large number of parameters need to be screened/optimized. 2^k factorial designs are a popular choice of screening experiments which measure first order effects of k factors as well as the interactions between those factors. Each factor is represented only at two levels – a high and a low level. The restriction to measuring a linear effect of each factor is often reasonable and provides an efficient design for identifying the significant factors from a large collection of candidates. These designs require 2^k runs (Montgomery 2001)

Studies conducted previously at the Centre for Biofuels, CSIR-NIIST on prospecting of fungal strains producing glucose tolerant β-glucosidase had resulted in the isolation of a filamentous fungal isolate code named BTCF58. The culture was found to secrete β-glucosidase which in the crude form was active at 0.5M glucose concentration. The current study was therefore undertaken to identify this fungus and produce β-glucosidase using it, to characterize the glucose tolerant BGL enzyme, and evaluate its application in biomass hydrolysis.

3.2. Materials and methods

3.2.1. Identification of fungal strain

Fungal isolate BTCF58 which produced glucose tolerant β-glucosidase was identified by morphological features and the identification was confirmed by molecular analyses.

3.2.1.1. Morphological identification of the fungal strain and β-glucosidase production

Vegetative and reproductive features of fungal strain were studied by microscopic observations under a Phase Contrast Microscope (Leica DM 2500). Scanning Electron Microscopy (SEM) was also performed for detailed observation of morphological features at higher resolution. For SEM analysis, the fungal mycelia and conidia were coated with gold palladium using a JEOL JFC-1200 fine coater and their structures were observed using scanning electron microscope (SEM, JEOL Model JSM -5600, Japan) at 15kV and 500-5000x magnification.

Production of β-glucosidase by the fungal isolate was tested on plates using a fluorescence assay which employs 4-Methyl Umbelliferyl -β-D-Glucopyranoside (MUG) as substrate for β-glucosidase in the plates. MUG on hydrolysis by BGL liberates methylumbelliferone, which fluoresces under UV light. The fungal isolate was streaked on agar plates and were incubated for 72h to allow growth of the cultures. After this, 10 ml of a soft agar (0.5 %) containing 10 µg/ml MUG was layered over the plates containing the fungal colonies and were incubated at 30 ± 2 ºC for 1h followed by visualization under long wavelength (340nm).

3.2.1.2. Molecular identification of the fungal strain

Molecular identification was carried out by sequencing and BLAST analysis of amplified regions of 18S rRNA gene and further confirmation was done using ITS region amplification and sequence analysis.

3.2.1.2.1. Chromosomal DNA isolation from fungal strains

Chromosomal DNA from fungal strains was isolated as per the protocol stated in section 2.7. The DNA precipitate was washed twice with 70 % ethanol, allowed to air dry and was resuspended in 10 mM Tris-EDTA buffer (pH 8.0).

3.2.1.2.2. PCR Amplification and analysis of 18S rRNA gene

A portion of the 18S rRNA gene was amplified from the genomic DNA by polymerase chain reaction (PCR) using the universal primers NS1, NS4, NS3, NS8(White et al. 1990). PCR reactions contained 0.5 units of *Taq* DNA polymerase, 1x *Taq* buffer, 200 µM of each dNTPs, 2.0 µM $MgSO_4$ (All from Fermentas, USA), 0.2 µg genomic DNA, and 0.5 µM forward and reverse primers. Reaction conditions for PCR amplification were an initial 94 °C for 3 min, followed by 30 cycles of 94 °C for 30 sec, 55 °C for 30 sec and 72 °C for 1 min; and a final extension step at 72 °C for 10 min. An Eppendorf ® 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. Nucleotide sequences of the PCR amplicons were determined by dye terminator sequencing (outsourced to Scigenom, Kochi, India). Identity of the sequence assembly was established by BLAST analysis (Altschul et al. 1990). Later a homology search was performed and based on the results; a Phylogenetic tree was constructed using the neighbor joining method implemented in PHYLIP (Felsenstein 1989)

3.2.1.2.3. ITS amplification and analysis

PCR amplification of Internal Transcribed Spacer region (ITS) of rDNA was carried out using universal eukaryotic ITS primers. The primers used for ITS amplification is given in Table 3.1. The forward primer binds to 3′ end of 18S rDNA and the reverse primer binds to 5′ end of 28S rDNA (Inglis and Tigano 2006). Conditions provided for PCR amplification were similar, and sequence analysis was performed using BLAST as mentioned above (section 3.2.1.2.2).

Figure 3.1. Diagrammatic representation of the ITS region in Eukaryotes

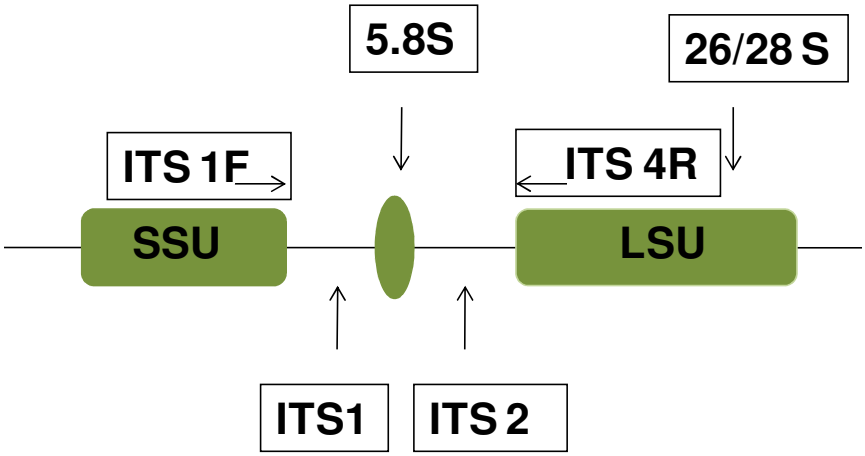


Table 3.1: Primers used for amplification of ITS region

Primer name	Primer Sequence(5`-3`)	Reference
ITS1F	TCCGTAGGTGAACCTGCGG	(White et al. 1990)
ITS4R	TCCTCCGCTTATTGATATGC	(White et al. 1990)

3.2.2. Differential induction of BGL in response to carbon source

The carbon sources - cellulose, xylose, lactose, glucose, maltose, and sucrose - were supplied in the medium at 1 % w/v level and fermentation was carried out as outlined under section 2.4.1. Culture filtrates were assayed for BGL activity as outlined under section 2.5.1, and the samples were concentrated using vacuum centrifugation (Eppendorf, Germany). Secretion of BGL isoforms by the fungus in response to the carbon source was studied by analyzing the zymogram generated by performing Native PAGE followed by activity staining of the gels (Section 2.6.1). Glucose tolerance of the enzyme was represented as percentage activity retention of the bands in presence of glucose compared to that of native bands as outlined under section 2.6.1.

3.2.3. Optimization of BGL production

For enzyme production, the culture was grown on Mandels & Weber medium (1969) with the base composition as outlined under section 2.2. Submerged fermentation for β-glucosidase production was performed as mentioned in section 2.4.1. A 2^k design (Montgomery 2001) was used to determine the relative significance of 7 factors considered to be important for production of enzyme by the fungus. Each variable was investigated at two levels, ie. a high level (+) and low level (-). The complete experimental design matrix is given in Table 3.2. The software Design Expert ® (version 8.0.1., State Ease Inc, Minneapolis, USA) was used for experiment design and data analyses. Each run was carried out in triplicate and the average values of the responses were taken for analyses.

Table 3.2. 2^k design matrix for optimization of β-glucosidase production under SmF

Std	Wheat Bran (g/L)	Ammonium Sulfate (g/L)	Peptone (g/L)	pH	Lactose (% w/v)	Tween 80 (% w/v)	Inoculum (spore count)
1	2	0.5	0.10	7.5	1.0	0.10	1.0E+05
2	10	0.5	0.10	4.5	0.0	0.10	1.0E+07
3	2	2.0	0.10	4.5	1.0	0.05	1.0E+07
4	10	2.0	0.10	7.5	0.0	0.05	1.0E+05
5	2	0.5	1.00	7.5	0.0	0.05	1.0E+07
6	10	0.5	1.00	4.5	1.0	0.05	1.0E+05
7	2	2.0	1.00	4.5	0.0	0.10	1.0E+05
8	10	2.0	1.00	7.5	1.0	0.10	1.0E+07

The effects of individual parameters on BGL production and glucose tolerance was calculated by the equation $\varepsilon = (\Sigma\mu_+ - \Sigma\mu_-)/n$ 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 to determine the significance of fitted model and individual parameters. The most significant parameters affecting BGL production were identified. Optimization of enzyme production was performed using the numerical optimization function in Design Expert ® and three randomly selected solutions were validated by performing the experiment under predicted conditions and comparing the experimental yield against the predicted ones.

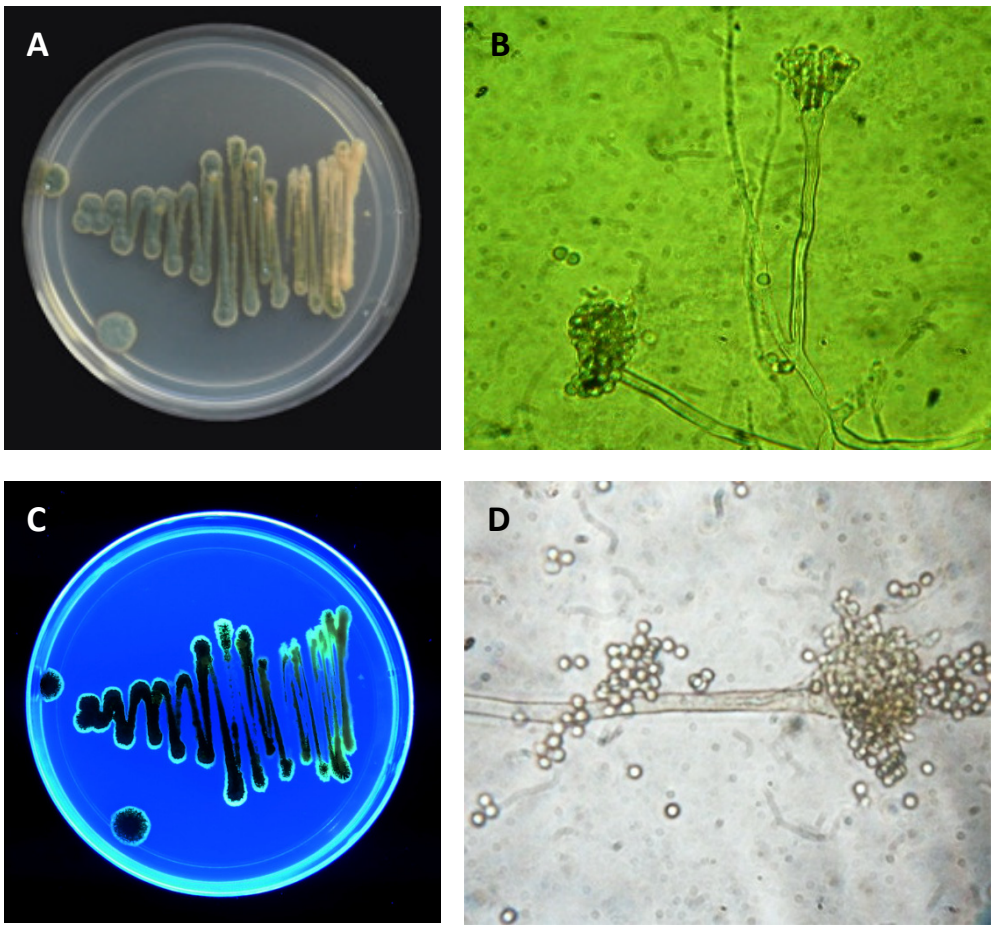
3.3. Results and Discussion

3.3.1. Identification of the fungus

3.3.1.1. Morphological identification

Fungal isolate BTCF58 formed dark green colonies on PDA, and the microscopic features resembled that of *Aspergillus* sp. Morphological examination of the fungus under microscope revealed long roughened and thick walled spicular hyphae, hemispherical vesicles, biseriate phialides supported by metulae, and globose conidia dull green in color (Figure 3.2A-D & Figure 3.3 A-D) the features characteristic of *Aspergillus unguis* (Thom and Raper 1939).

Figure 3.2. *Aspergillus unguis* strain morphology and detection of β-glucosidase production



A) Colony on PDA agar.
B) Conidiophore showing thick wall and biseriate phialides
C) Screening of β-glucosidase production using MUG soft agar method.
D) Conidia visualized under phase contrast

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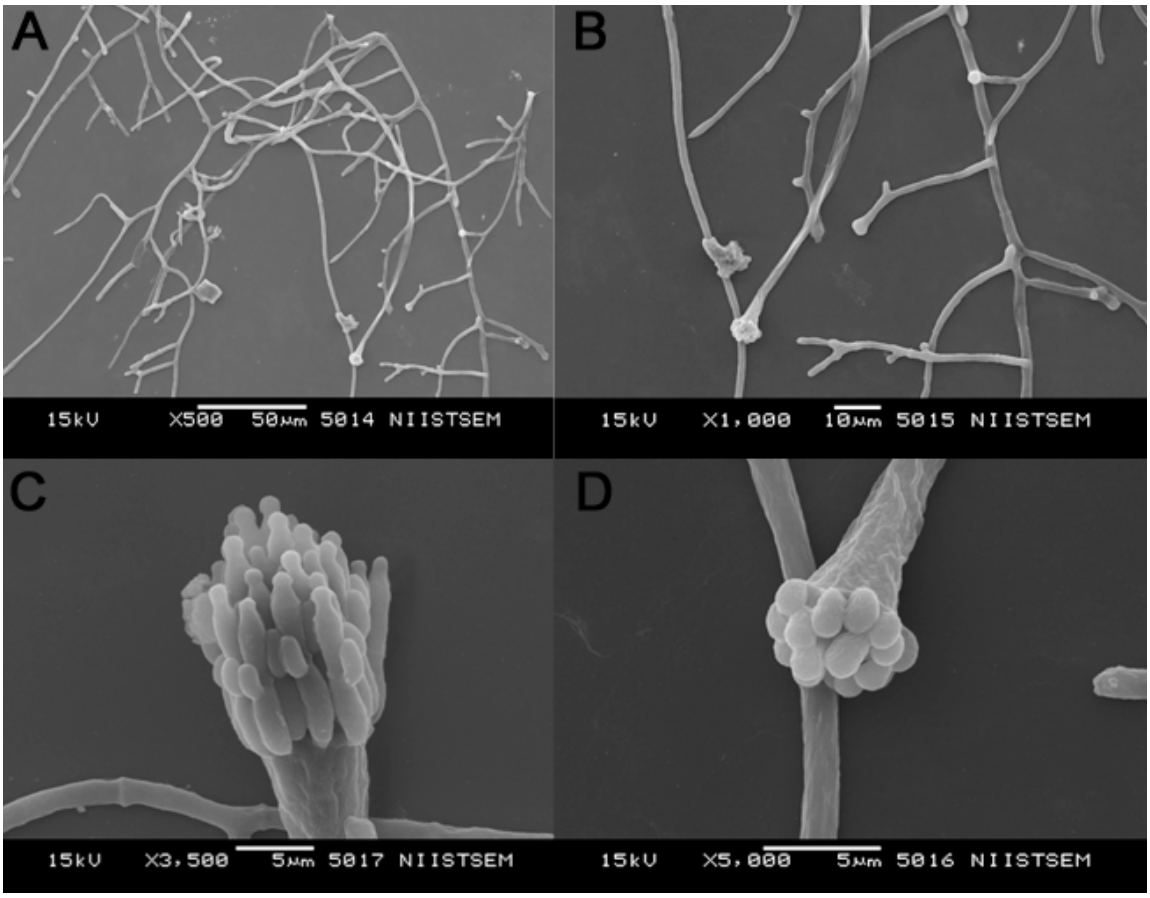
Figure 3.3. *Aspergillus unguis* strain morphology by Scanning Electron Microscopy



A & B-Sterile setae like hyphae, C- conidiophore showing biseriate phialides with metulae, D –Conidiophore showing globose conidia

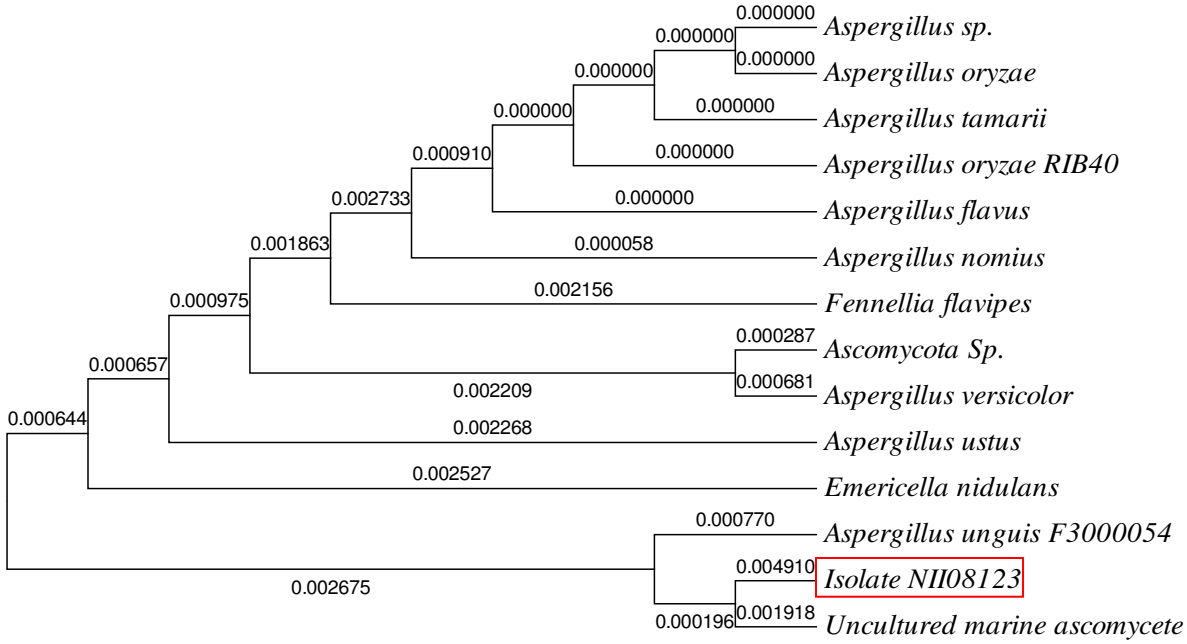
3.3.1.2. Molecular identification using 18S rRNA sequence analysis

PCR amplification of the 18S rRNA region yielded a 1678 bp sequence which was submitted to Genbank with the accession JQ726491 (gi|387966750). The BLAST analyses of the partial DNA sequence showed 99 % similarity with 18S rRNA sequence of *Aspergillus unguis* and progressively lesser similarity with those from other *Aspergilli* confirming that the isolate is a strain of *Aspergillus unguis*. The isolate was deposited in the NII culture collection (CSIR-NIIST) with accession NII 08123. Pair wise alignments of the sequence with closest matches in the sequence depositories identified by BLASTN (http://www.ncbi.nlm.nih.gov/BLAST/) searches were performed, and the phylogenetic relationship were derived using neighbourhood joining methods and was represented as a phylogenetic tree (Figure 3.4.).

>gi|387966750|gb|JQ726491.1| Aspergillus unguis isolate NII08123 18S ribosomal RNA gene, partial sequence

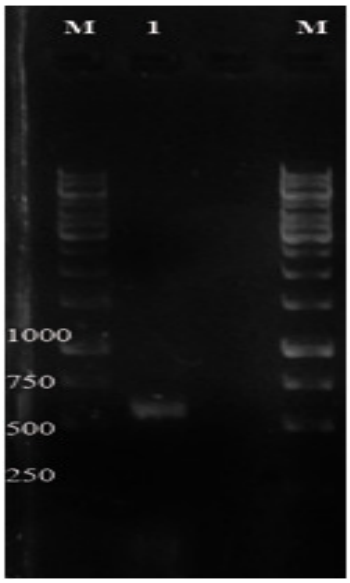
CTTTATACCGTGAAACTGCGAATGGCTCATTAAATCAGTTATCGTTTATTTGATAGTACCTTACTACATGGATACCT
GTGGTAATTCTAGAGCTAATACATGCTAAAAACCCCGACTTCGGGAGGGGTGTATTTATTAGATAAAAAACCAATGC
CCCTCGGGGCTCCTTGGTGATTCATAATAACTTAACGAATCGCATGGCCTTGCGCCGGCGATGGTTCATTCAAATTT
CTGCCCTATCAACTTTCGATGGTAGGATAGTGGCCTACCATGGTGGCAACGGGTAACGGGGAATTAGGGTTCGATTC
CGGAGAGGGAGCCTGAGAAACGGCTACCACATCCAAGGAAGGCAGCAGGCGCGCAAATTACCCAATCCCGACACGGG
GAGGTAGTGACAATAAATACTGATACGGGGCTCTTTTGGGTCTCGTAATTGGAATGAGAACAATCTAAATCCCTTAA
CGAGGAACAATTGGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCCAATAGCGTATATTAAAGTTGTT
GCAGTTAAAAAGCTCGTAGTTGAACCTTGGGTTCGGCTGGCCGGTCCGCCTCACCGCGAGTACTGGTCCGGCTGGAC
CTTTCCTTCTGGGGAACCTCATGGCCTTCACTGGCTGTGGGGGGAACCAGGACTTTTACTGTGAAAAAATTAGAGTG
TTCAAAGCAGGCCTTTGCTCGGATACATTAGCATGGAAATAATAGAATAGGACGTGCGGGTTCTATTTTGTTGGTTT
CTAGGACCGCCGTAATGATTAATAGGGATAGTCGGGGGCGTCAGTATTCAGCTGTCAGAGGTGAAATTCTTGGATTT
GCTGAAGACTAACTACTGCGAAAGCATTCGCCAAGGATGTTTTCATTAATCAGGGAACGAAAGTTAGGGGATCGAAG
ACGATTAGATACCGTCGTTGTCTTAACCATAAACTATGCCGACTAGGGATCGGGCGGCGTTTCTATTGCCGAACTAG
GGATCGGGCGGCGGTTTCTTATTATGACCCGCTCGGCACCTTAACGGAGAAAATCAAAGTTTTTGGGTTCTGGGGGG
GAGGTATGGTCGCAAGGCTGAAAACTTAAAGAAAATTGACGGAAGGGCACCACAAGGCGTGGAGCCTGCGGCTTTAA
TTTGACTCAAACACGGGGAAACTCACCAGGTCCAGACAAAATAAGGATTGACAGATTGAGAGCTCTTTCTTGATCTT
TTGGATGGTGGTGCATGGCCGTTCTTAGTTGGTGGAGTGATTTGTCTGCTTAATTGCGATAACGAACGAGACCTCGG
CCCTTAAATAGCCCGGTCCGCGTCCGCGGGCCGCTGGCTTCTTAGGGGGACTATCGGCTCAAGCCGATGGAAGTGCG
CGGCAATAACAGGTCTGTGATGCCCTTAGATGTTCTGGGCCGCACGCGCGCTACACTGACAGGGCCAGCGAGTACAT
CACCTTGGCCGAGAGGCCCGGGTAATCTTGTTAAACCCTGTCGTGCTGGGGATAGAGCATTGCAATTATTGCTCTTC
AACGAGGAATGCCTAGTAGGCACGAGTCATCAGCTCGTGCCGATTACGTCCCTGCCCTTTGTACACACCGCCCGTCG
CTACTACCGATTGAATGGCTCGGTGAGGCCTTCGGACTGGAAACAGGAGGGTTGGCAACGA

Figure 3.4. Phylogenetic tree of *Aspergillus unguis* NII 08123 (BTCF58) showing relationship of the fungus to other Aspergilli



For further confirmation, ITS region of the isolate was amplified using the universal primers ITS 1F and ITS 4R when a ~500bp amplicon was obtained (Figure 3.5). The amplicon was purified from gel and was sequenced. BLAST analysis performed with the sequence indicated that the isolate shared 99 % identity with *Aspergillus unguis* isolate BP321 internal transcribed spacer, partial sequence (Table 3.3)

Figure 3.5. PCR amplification of the ITS region of *Aspergillus unguis* NII 08123



Lane information

Lane M GeneRuler ® 1kb ladder (Fermentas)

Lane 1 Primer pair_ ITS 1F and ITS 4R

Partial sequence of the ITS region from *Aspergillus unguis* NII 08123

>A_unguis ITS
CCTCCCACCCTTGAATACTAAACACTGTTGCTTCGGCGGGGAGCCCCTTCCGGGGGGCAAGCCGCCGGGGACCACTG
AACTTCATGCCTGAGAGTGATGCAGTCTGAGTCTGAATTATAAATCAGTCAAAACTTTCAACAATGGATCTCTTGGT
TCCGGCATCGATGAAGAACGCAGCGAACTGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAGTCTTTG
AACGCACATTGCGCCCCCTGGCATTCCGGGGGGCATGCCTGTCCGAGCGTCATTGCTGCCCTTCAAGCCCGGCTTGT
GTGTTGGGTCGTCGTCCCCCCCCGGGGGACGGGCCCGAAAGGCAGCGGCGGCACCGTGTCCGGTCCTCGAGCGTATGG
GGCTTTGTCACCCGCTCGATTAGGGCCGGCCGGGCGCCAGCCGGCGTCATCAATCTATTTTACCAGGTTGACCTCGG
ATCAGGTAGGGATACCCGCTGAACTTAAGCATATCAAAGGCCGGAGGAAATTACCGAGTGCGGGCTGCCTCCGGGCG
CCCAACT

Table 3.3. Sequences with significant similarity to *Aspergillus unguis* NII 08123 ITS region identified by BLAST analysis

Description	Max score	Total score	Query coverage	E value	Max identity	Accession
Aspergillus unguis isolate BP321 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	935	935	93%	0.0	99%	JF731256.1
Aspergillus unguis isolate N2 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	933	933	93%	0.0	99%	JN974764.1
Aspergillus unguis isolate NRRL 6328 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	929	993	99%	0.0	99%	EF652509.1
Aspergillus unguis isolate NRRL 216 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	929	993	99%	0.0	99%	EF652436.1
Aspergillus unguis isolate NRRL 216 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	928	928	93%	0.0	99%	EF067337.1
Aspergillus unguis genes for ITS1, 5.8S rRNA and ITS2, partial and complete sequence, strain: IFM 42017	928	998	99%	0.0	99%	AB248983.1

The systematic position of β-glucosidase producing fungal isolate was derived as below,

3.3.1.3. Systematic position of the strain

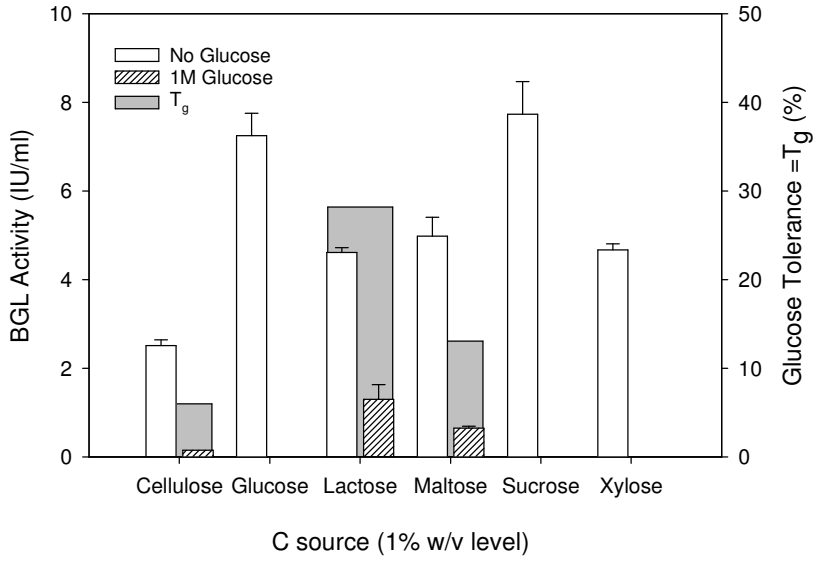
Super kingdom	-	Eukaryota
Kingdom	-	Fungi
Sub kingdom	-	Dikarya
Phylum	-	Ascomycota
Sub phylum	-	Pezizomycotina
Class	-	Euratiomycetes
Sub class	-	Eurotiomycetidae
Order	-	Eurotiales
Family	-	*Aspergillaceae*
Genus	-	*Aspergillus*
Species	-	*unguis*

3.3.2. Differential expression of BGL in response to C-sources and the existence of multiple BGL isoforms

Since during the fermentation for BGL production by *A. unguis NII 08123*, there was a large amount of fluctuation in the glucose tolerance of BGL, it was speculated that there could be differences in the expression of enzyme depending on the immediate environment, especially the type of C source. To evaluate this, the isolate was cultured in the same basal medium under identical conditions but with different C sources, and the BGL activity and glucose tolerance was monitored. Effect of carbon source/inducer on β-glucosidase production by *A. unguis* and the expression of different isoforms are shown in Figure 3.6 and 3.7. Maximum glucose tolerance (32.75 % activity retained) was observed with lactose as C source, and the glucose tolerance and levels of BGL yield varied with C-sources (Figure 3.6).

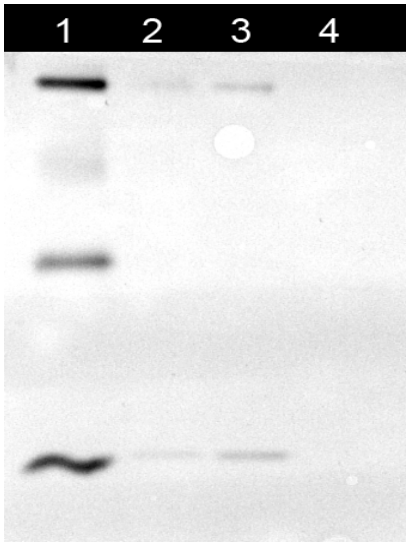
Multiplicity of BGLs has been reported earlier by several authors (Brumbauer et al. 2000; Decker et al. 2001; Gunata and Vallier 1999; Riou et al. 1998; Singhania et al. 2011). Difference in expression of the BGL isoforms could be the reason for the differences in glucose tolerance of the crude enzyme preparation, since the actual amount of the glucose tolerant isoform could vary according to the carbon source. Existence of multiple isoforms of BGL in *A. unguis* was studied by growing the fungus in different C-sources and performing zymogram analysis. Since it was noted that the crude carbon source-wheat bran induced most of the BGL isoforms in *A. unguis*, BGL production was also conducted in media supplemented 1% w/v of either wheat bran or rice straw.

Figure. 3.6. BGL production in response to carbon sources in the medium



BGL activity in media supplemented with different C sources (Open bars). Hatched bars represent the BGL activity when assayed in the presence of 1.0 M glucose and % activity retention/glucose tolerance (Tg) is represented by filled bars in the background

Figure. 3.7. Differential expression of BGL in response to carbon sources in the medium

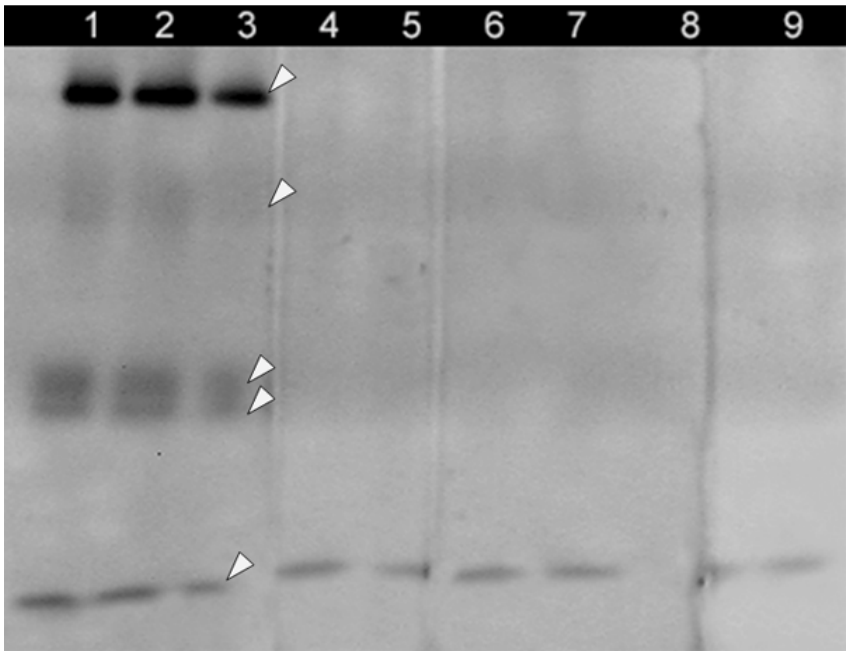


Differential expression of BGL in response to carbon source. Enzyme samples were subjected to Native PAGE and incubated with MUG for detection of activity. Lane information: carbon source followed by T_g in parenthesis: lane 1 wheat bran (23 %), lane 2 rice straw (1.9 %), lane 3 cellulose (20.2 %), lane 4 glucose (0.0 %). Image inverted for clarity

Results shown in figures 3.7 and 3.8 indicated the existence of multiple isoforms (5 detected here) of the enzyme. While wheat bran induced the production of 5 BGL isoforms (Figure 3.8, Lanes 1-3), rice straw and cellulose could induce only the high molecular weight and low molecular weight BGL isoforms. There was also a direct correlation between the signal intensity of the low molecular weight band and the glucose tolerance (as detected by pNPG assay) indicating that this could be the glucose tolerant isoform (GT-BGL)

This was further proved by fluorescence intensity analysis of the BGL activity bands observed at varying glucose concentrations. BGL produced using wheat bran as C source was run on Native PAGE and activity staining was performed in buffers containing varying concentrations of glucose (0.5-1.5M). Only the low molecular weight band was detected when assayed in presence of glucose and the intensity of this band was negatively correlated to the glucose concentration confirming that this band itself is the glucose tolerant isoform (Figure 3.8).

Figure. 3.8. Glucose tolerance of the BGL proteins secreted by *Aspergillus unguis NII 08123*



Glucose tolerance of the BGL proteins secreted by *A. unguis* when grown in 1% w/v wheat bran as C source. Arrowheads indicate the isoforms. Lane information (Normalized fluorescence intensity and T_g of GT-BGL in parentheses): lanes 1, 2, 3- BGL activity signals in gel incubated without glucose (0.148, 100%). Lanes 4&5 – BGL activity signals in presence of 0.5M glucose (0.118, 80%), Lanes 6&7 – BGL activity signals in presence of 1.0 M glucose (0.117, 79%), Lane 9 – BGL activity signal in presence of 1.5M glucose (0.086, 58%). Image inverted for better clarity.

3.3.3. Optimization of β-glucosidase production

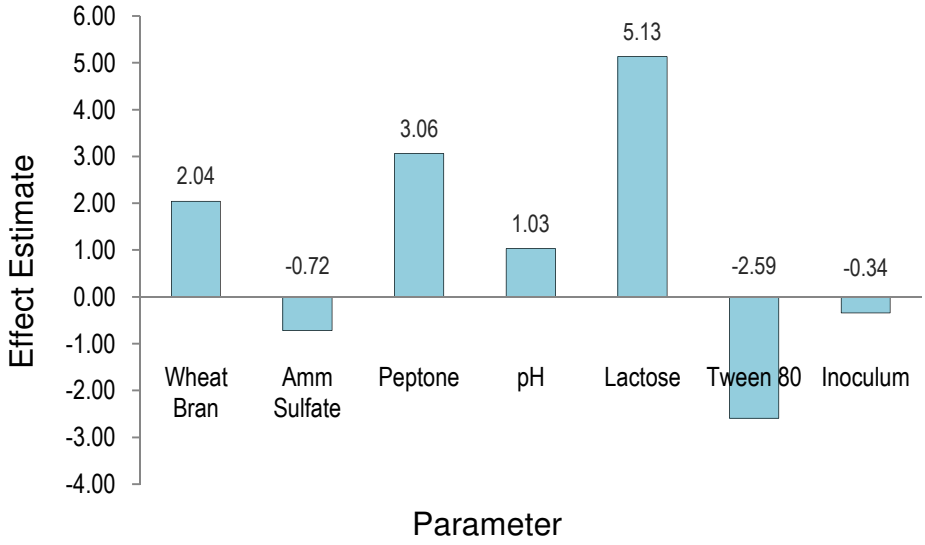
A 2^k fractional factorial experiment design was employed to determine the important parameters that affect β-glucosidase production by *A. unguis*. The results of the 2^k design of experiments showed a wide range of β-glucosidase production ranging from 3.31 IUs/ml to 25.56 IUs/ml (Table 3.4.) This variation showed the importance of optimizing these parameters for improving β-glucosidase production by *A. unguis*.

Table 3.4. β-glucosidase yields for experimental runs performed according to the 2^k design matrix

Std	Wheat Bran (g/L)	Ammonium Sulfate (g/L)	Peptone (g/L)	pH	Lactose (% w/v)	Tween 80 (% w/v)	Inoculum (spore count)	BGL Yield (IUs/ml)
1	2	0.5	0.10	7.5	1.0	0.10	1.0E+05	12.23
2	10	0.5	0.10	4.5	0.0	0.10	1.0E+07	3.31
3	2	2.0	0.10	4.5	1.0	0.05	1.0E+07	13.24
4	10	2.0	0.10	7.5	0.0	0.05	1.0E+05	9.80
5	2	0.5	1.00	7.5	0.0	0.05	1.0E+07	12.60
6	10	0.5	1.00	4.5	1.0	0.05	1.0E+05	25.56
7	2	2.0	1.00	4.5	0.0	0.10	1.0E+05	4.60
8	10	2.0	1.00	7.5	1.0	0.10	1.0E+07	20.32

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 3.9

Figure. 3.9. Effect of process parameters on BGL production by *Aspergillus unguis* NII 08123



The parameters with largest effects were Lactose concentration and the concentration of Peptone in the enzyme production medium followed by concentrations of Tween 80, Wheat bran and medium pH. Lactose and Peptone and Wheat bran concentrations influenced β-glucosidase production positively, while Tween 80 and Ammonium sulfate concentrations had a negative effect on β-glucosidase yield. The data was analyzed by multiple regression analysis using the Design-Expert ® software and the following equation was obtained to represent the relationship between BGL yields to the parameters tested

$$Y = 12.71 + 2.04A + 3.06C + 5.13E - 2.59F \qquad \text{(Equation 3.1)}$$

where Y is the predicted value of β-glucosidase yield, and A, C, E and F are the coded values for Wheat Bran concentration, Peptone concentration, Lactose concentration and Tween 80 concentration respectively.

Analysis of the β-glucosidase 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 the seven process variables indicated that β-glucosidase production by *A. unguis* under SmF can be described by the factorial model (Table 3.5)

Table 3.5. Analysis of Variance (ANOVA) for the factorial model

	Sum of Squares	DF	Mean Square	F Value	p-value
Model	372.64	4	93.16	20.65	0.016
A-WB	33.30	1	33.30	7.38	0.073
C-Peptone	74.98	1	74.98	16.62	0.027
E-Lactose	210.63	1	210.63	46.69	0.006
F-Tween 80	53.73	1	53.73	11.91	0.041
Residual	13.53	3	4.51		
Corr. Total	386.17	7			

The results of the ANOVA indicated that the model is significant and can be used to define the influence of parameters on the β-glucosidase production by *A.unguis* under SmF. Lactose concentration (E) , peptone concentration (C) and Tween 80 concentration (F) with P values 0.006, 0.027 and 0.041 respectively were found to have significant influence on the β-glucosidase production. The variables A (Wheat Bran concentration), C (concentration of peptone), E (inducer/lactose concentration) and F (Tween 80), were included in the model (Eqn. 3.1).

Based on the results obtained for the factorial design, the numerical optimization function in Design Expert ® was used to solve the model equation and predict optimal conditions for maximal production of enzyme. A set of 38 solutions was provided by the software, among which 5 random solutions were selected and validated experimentally (Table 3.6)

Table 3.6. Randomly selected optimal conditions for maximal BGL production by *A unguis* and the predicted yield of enzyme

Solution #	WB	Ammo Sulfate	Peptone	pH	Lactose	Tween 80	Inoculum	BGL Yield (Predicted)	BGL yield (Obtained)
1	10.00	0.50	1	4.50	1	0.05	1.00E+06	25.53	22.38
9	7.23	1.37	1	4.50	1	0.06	1.00E+06	23.36	19.07
16	2.61	1.15	1	5.63	1	0.05	1.00E+06	21.76	21.78
36	2.39	2.00	1	4.51	1	0.08	1.00E+06	18.72	13.32
38	4.89	0.56	0.11	7.50	1	0.07	1.00E+06	14.26	9.58

The actual yields of BGL obtained for the optimal conditions identified by the solving the model equation has an excellent correlation with the predicted yields (Correlation coefficient R = 0.9366). The closer the R value to 1.0, the greater is the correlation and in this case, the obtained and BGL activities correlated well with their predicted values indicating the validity of the model.

3.4. Conclusions

β-glucosidase plays a major role in the conversion of cellulosic biomass to ethanol, being the rate limiting enzyme that determines the action of all cellulase components. A large number of these enzymes from bacteria and plants have been purified and studied, but most microbial β-glucosidases are very sensitive to glucose inhibition. There are only limited reports in publications on glucose tolerant BGLs and most of the reports on filamentous fungi describes *Aspergilli* as the producers of GT-BGLs. To the best of our knowledge, this is the first report on production of glucose tolerant β-glucosidase from *Aspergillus unguis* which belongs to the *Aspergillus nidulans* group.

The level of production of glucose tolerant isoforms was greatly influenced by the carbon source supplied. It is interesting to note that the highest tolerance (32.75%) was observed in cultures grown with lactose as carbon source. From the zymogram analysis, we identified 5 active isoforms of β-glucosidase even though their expression level varies in the presence of different carbon source. Staining the Native as well as SDS PAGE gels using MUG in presence of different concentrations of glucose indicated that the low molecular weight isoform plays a key role in activity retention, even in presence of considerable amount of glucose. It has been reported previously that the glucose tolerant β- glucosidases are low molecular weight proteins expressed at lower levels compared to the highly expressed, high molecular weight glucose sensitive major BGLs in *Candida peltata* (Saha and Bothast 1996), *Aspergillus oryzae* (Riou et al. 1998), A. *foetidus* (Decker et al. 2001), *A. tubingensis* (Decker et al. 2000) and in *A. niger* (Singhania et al. 2011). BGL multiplicity can be attributed to the presence of multiple genes or due to differential post transcriptional modifications (Collins et al. 2007). Differential expression of the various BGL proteins are reported in response to the carbon sources supplied in the medium or the conditions of culture (Nazir et al. 2010; Willick and Seligy 1985) 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. This is apparently useful not only in context of producing BGL for biomass hydrolysis but also for flavour enhancement in wine and fruit juices, provided that the BGL in question has the suitable substrate specificity (Watanabe et al. 1992).

Optimization of environmental parameters and media for fermentation is a significant concern in developing a suitable bioprocess for cellulase production (Lee et al. 2002). There are very few reports on process optimization for production of β- glucosidases despite their importance in various applications. Fungal β- glucosidases are known to be regulated by intricate induction and repression mechanisms and also by feedback control. Glucose tolerant BGL enzymes are produced by relatively very few filamentous fungi and are very important in biomass saccharification. Though a large scale production of BGL without feedback controls may be achieved only by genetic intervention, judicious design of process and the optimization of process parameters can help in maximizing the yield of BGL from fungi. This will also help in designing the process for selective expression of a glucose tolerant BGL protein from a group of BGL proteins probably regulated by different controls from the environment as well as from within. The fungus Aspergillus *unguis* NII08123 isolated at NIIST's Biotechnology division was found to elaborate multiple BGLs among which one was found to be tolerant to elevated glucose concentrations which evoked interest due to the potential applications in biomass saccharification. The present study also addressed SmF production of the crude BGL in flask cultures. Studies on the effect of process variables in controlling the expression of the BGL, and their optimization for fine tuning the fermentation had resulted in a set of conditions that can yield BGL activities in the range of 20-25IUs/ml.

Chapter 4. Purification, Characterization and Kinetic properties of Glucose tolerant β-glucosidase (GT-BGL) from *Aspergillus unguis*

4.1. Introduction

β-glucosidases (EC 3.2.1.21; β- D-glucoside glucohydrolase) are key enzymes in cellulose hydrolysis, being the rate limiting enzyme which is regulated by feedback inhibition from its own product-glucose. These enzymes are therefore of considerable interest as constituents of cellulose-degrading systems to be used for biomass conversion applications (Soewnsen 2010). Inhibition by its product and substrate limits its use, especially in the context of biomass hydrolysis. β-glucosidase (BGL) insensitive to glucose and cellobiose will significantly improve enzymatic conversion of cellulosic biomass to glucose for the subsequent production of fuel ethanol (Saha and Bothast 1996). Glucose tolerant β-glucosidases (GT-BGLs) have been reported from a few filamentous fungi, and differences in their properties were studied including molecular mass, isoelectric points, pH optima and glucose inhibition constants (Decker et al. 2000; Gunata and Vallier 1999; Job et al. 2010; Riou et al. 1998). From the limited number of reports about GT-BGLs it may be speculated that such enzymes are rather uncommon or understudied. However, the impact of addition of GT-BGLs in the cellulase cocktails for biomass hydrolysis can be significant and improvements in the yield and concentration of sugars may be achieved by using such blends.

Aspergillus unguis NII08123 isolated at the CSIR-NIIST Biotechnology division was found to secrete β-glucosidase in the culture filtrate and this crude culture filtrate retained the enzyme activity even in presence of 1.5M glucose. The fungus was found to express at least 5 different β-glucosidases and a low molecular weight protein was identified as the glucose tolerant β-glucosidase (Chapter 3). Purification of this enzyme is essential to study the properties of *Aspergillus unguis*. Hence, in this study the low molecular weight glucose tolerant form of β-glucosidase from *Aspergillus unguis* was purified and its properties were characterized.

4.2. Materials and Methods

4.2.1. Enzyme purification

4.2.1.1 Acetone fractionation of the crude enzyme

Enzyme production was carried out as outlined under section 2.4.1 by submerged fermentation but using only 1% lactose as the carbon source and inducer. Partial purification of the enzyme was carried out using acetone fractionation with increasing volume of the solvent. Approximately 2000ml 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 overnight at 4 °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, 1:3 and 1:4 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 15ml of 0.05M Citrate Buffer (pH 4.8), and used for further studies.

4.2.1.2 Anion exchange chromatography

β-glucosidase precipitated by two volumes acetone (1: 2 culture filtrate: acetone) was purified by ion exchange chromatography on an anion exchange column (High Q support, Biorad, USA). Chromatography experiments were performed on Biorad LP system. The column was equilibrated with Tris-HCl buffer (0.05 M, pH 7.5). Enzyme sample dissolved in citrate buffer (0.05 M, pH 4.8) was loaded on the resin by running it at 0.75 ml/min flow rate through the column. The column was washed by running three column volumes of the loading buffer (0.05M Tris-HCl buffer, pH 7.5) at a flow rate of 0.75 ml/min. Elution was performed using a continuous gradient of NaCl from 0.1-1.0 M and 3 ml fractions were collected. Fractions giving the maximum activity were pooled together, and used for further purification.

4.2.1.3. Electro-Elution

Since multiple β-glucosidase 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 12 % Native PAGE and the BGL activity bands were visualized by activity staining as outlined under section 2.6.1. The fluorescent BGL 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 1h. After 1 hour it was inverted and again run for another 10 min 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.

4.2.2. Characterization of the Glucose tolerant β-glucosidase (BGL5) from Aspergillus unguis

Purified GT-BGL of *Aspergillus unguis* was characterized for its molecular weight by SDS-PAGE. Isoelectric focusing (IEF) was performed using a Biorad Rotophore® system using an Ampholytes having broad pH range (3-10). The optimal pH and temperature for BGL-5 was determined and V_{max} and $_{Km}$ was calculated using pNPG as the substrate. Glucose inhibition kinetics was also determined for the enzyme

4.2.2.1. Determination of the molecular weight of BGL-5

The purified BGL5 protein was run on SDS PAGE (Laemmli 1970) along with standard protein markers (PageRuler™ Pre-stained Protein Ladder (10 – 170 kDa, Fermentas, USA). BGL activity bands were visualized by MUG staining and the zymogram was photographed as outlined under section 2.6.1. 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. The position of BGL isoforms were confirmed by comparing 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 weight.

4.2.2.2. Determination of the optimal temperature for BGL-5 activity

The temperature optimum was analyzed by assaying the purified BGL-5 at different temperatures between 40 and 70 °C. The assay was performed as mentioned under section 2.5.1 and the substrate used was pNPG.

4.2.2.3. Determination of the optimal pH for BGL-5 activity

The optimum pH and the effect of pH on activity were analyzed by assaying the enzyme at different pH. The pH difference was attained by using 0.05M Citrate buffer (pH 4-6); and Tris-HCl buffer (pH 7).

4.2.2.4. Catalytic properties of BGL-5

Michaelis Menton constant ($_{Km}$) and Maximum Velocity (V_{max}) of purified enzyme was determined with different concentrations of pNPG (10-80mM). BGL assays were performed with different incubation time (2.5-15 min). The enzyme activity values were analyzed by Michaelis Menton kinetic graph as well as double reciprocal plot method using Graphpad Prism® software (California, USA)

4.2.2.5. Glucose inhibition kinetics of BGL-5

The glucose inhibition constant (K_i) of the purified BGL-5 was determined graphically using a Dixon Plot (Dixon 1953). BGL assays were performed (as in section 2.5.1.) with different concentrations of glucose (0-1 M glucose) and with pNPG as the substrate, at the optimum temperature and pH for activity. The reciprocal of 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 was determined and the absolute value of this was taken as the K_i for glucose inhibition. The type of inhibition was analyzed with the help of a Cornish –Bowden plot (Cornish-Bowden 1974), where the S/v (Substrate/Velocity) was plotted against I (inhibitor).

4.3. Results and Discussion

4.3.1. Acetone fractionation of BGL-5

All the five isoforms were visible in the crude enzyme sample after activity staining (Figure 3.8, Chapter 3). Maximum glucose tolerance was exhibited by the fraction obtained using 1:2 volume acetone precipitation (Figure 4.1). Maximum activity was obtained in 1:1.5 acetone fraction and this fraction contained two BGL proteins BGL1 and BGL3. The sample from 1:0.5 acetone precipitate showed BGL1 as the major protein and very low amount of BGL-5 while the sample from 1:2 acetone precipitate showed both high and the low molecular weight bands (Figure 4.2). The differences in glucose tolerance of the two bands were also evident from the zymogram analysis and only the low molecular weight band showed activity in presence of 1M glucose. The results indicate that fractional acetone precipitation could be an effective strategy for preliminary separation of the BGL isoforms.

Figure 4.1. Activity and glucose tolerance of acetone fractions of BGL

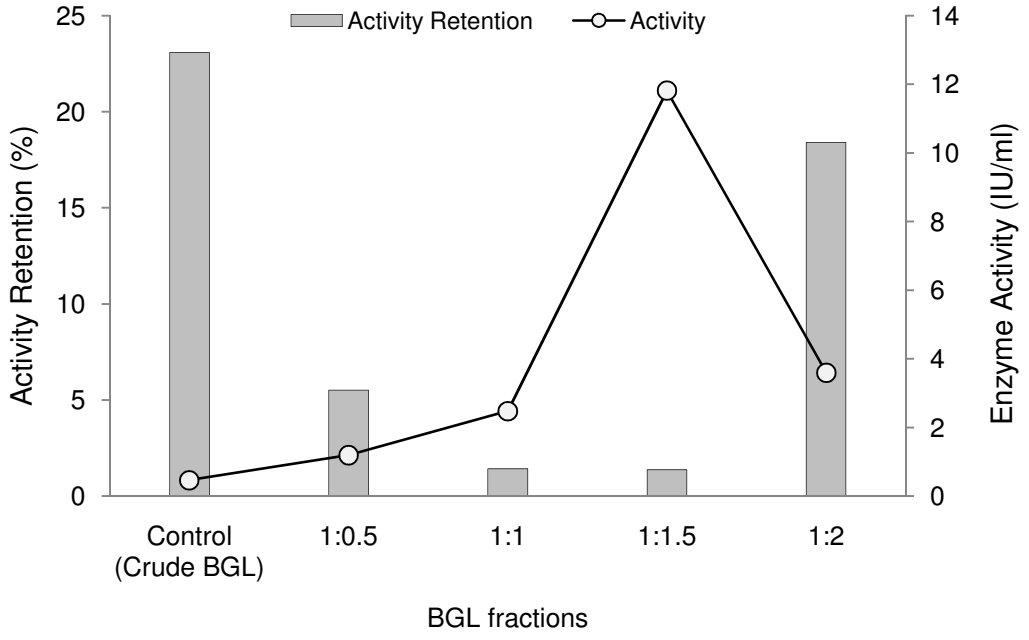
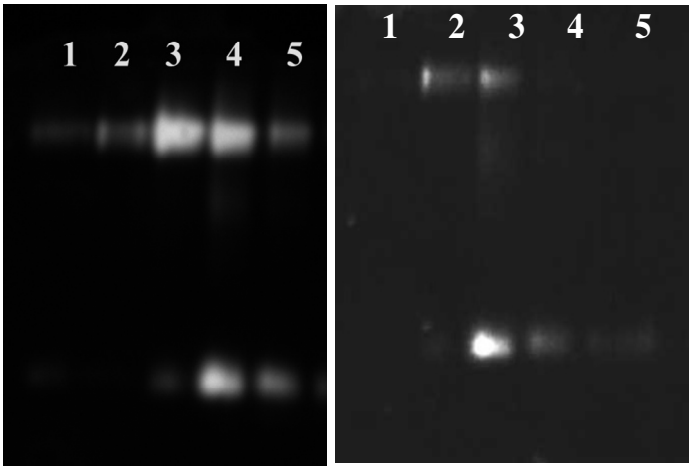


Figure 4.2. Zymogram analysis of the acetone fractions of BGL



Control 0.5M glucose

Lane Information (Control)
Lane 1 Crude enzyme
Lane 2 1: 0.5 acetone precipitate
Lane 3 1:1.0 acetone precipitate
Lane 4 1:1.5 acetone precipitate
Lane 5 1: 2.0 acetone precipitate

Lane Information (0.5M glucose)
Lane 1 1: 0.5 acetone precipitate
Lane 2 1:1.0 acetone precipitate
Lane 3 1:1.5 acetone precipitate
Lane 4 1: 2.0 acetone precipitate
Lane 5 Crude enzyme

Zymogram analysis of crude and acetone precipitated fractions were carried out in presence of 0.5 M glucose and without. Among the different BGL proteins, BGL-5 showed high levels of activity even in presence of 0.5 M glucose. BGL-2, BGL-3 and BGL-4 were inactive in presence of glucose indicated by absence of activity bands in the gel incubated with glucose. However, BGL-1 also showed a low level of activity in the presence of 0.5M glucose with the intensity of the band being very low compared to that of the control.

From the literature, maximum glucose tolerance of the BGL of any microorganism ever reported was observed in *Candida peltata* which had a glucose inhibition constant (K_i) value of 1.4M and a molecular weight of 43kDa (Saha and Bothast 1996). *Aspergillus oryzae* was also reported to produce a glucose tolerant β-glucosidase with a molecular weight of 43kDa and the K_i of this enzyme was 1.36M (Riou et al. 1998). Another strain of *Aspergillus oryzae* produced glucose tolerant β-glucosidase with a molecular mass of 30kDa with a K_i of 953mM (Gunata and Vallier 1999). In *Aspergillus tubingenesis* two BGL proteins that showed glucose tolerance was reported with K_i of 470 and 600mM respectively and both with a molecular weight of 54kDa (Decker et al, 2001). Among the reported BGL proteins, glucose tolerance was always reported for the low molecular weight BGL isoform and the size ranged from 30-54 kDa. In this study also it was observed that the GT-BGL was having a lower molecular weight compared to the other BGL proteins. Acetone fractionation was effective in separating the low molecular weight GT-BGL protein and this required the use of higher volume of acetone (1:2 ratio of enzyme to

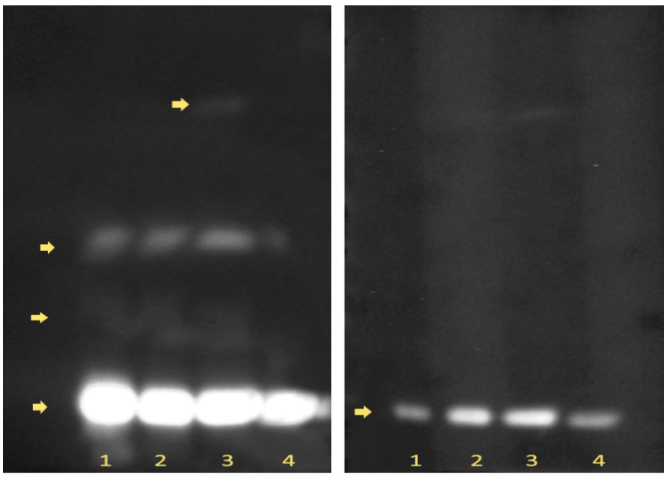
acetone). It is known that a higher amount of solvent is required to precipitate low molecular weight proteins (Simpson 2004).

4.3.2. Purification of GT-BGL from Aspergillus unguis NII 08123

4.3.2.1. Determination of the isoelectric point (pI) of BGL-5

Liquid phase isoelectric focusing was done on the enzyme sample using Biorad Rotophore® system which resulted in the BGL activity with glucose tolerance getting focused in the pH 4 - 5 region of the focusing column. This corresponded to Rotophore® fractions 4-7. The fractions after concentration by vacuum centrifugation were normalized for protein content and were subjected to Zymogram analysis as mentioned under section 2.6.1 and the activity bands were visualized under UV light (Figure 4.3). The pI was found to be between 4-5 from the IEF experiments, and this aided in further purification of the enzyme.

Figure.4.3. Zymogram analysis of the IEF fractions of BGL from Rotaphore®

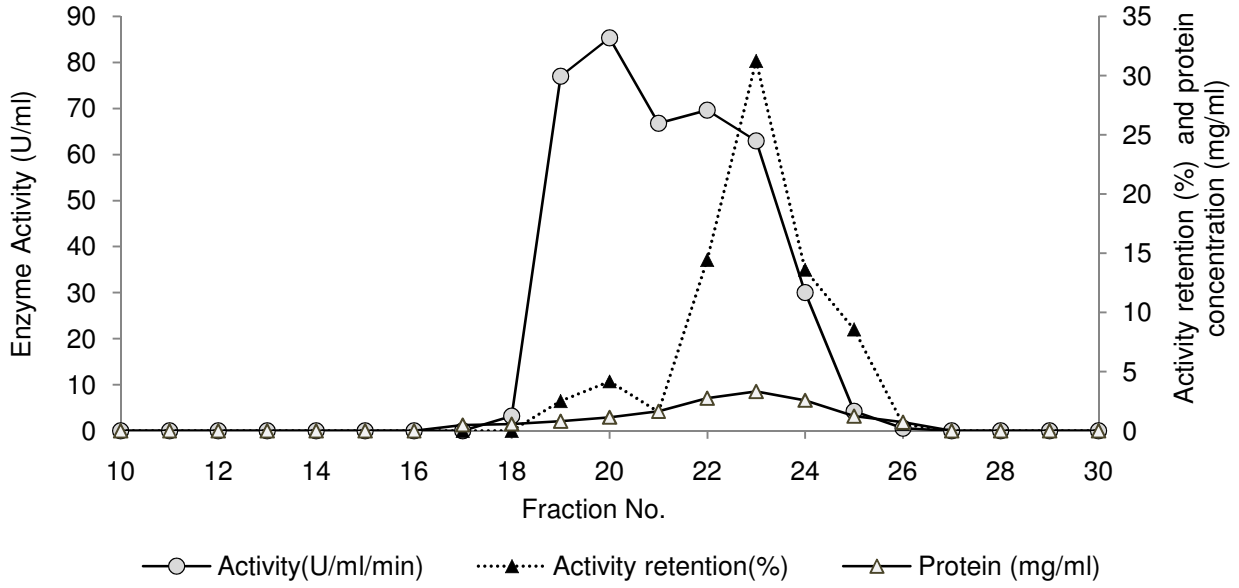


Lane 1 *Fraction 4*
Lane 2 *Fraction 5*
Lane 3 *Fraction 6*
Lane 4 *Fraction 7*

4.3.2.2. Chromatographic separation of BGL-5 from 1:2 volume acetone fractions

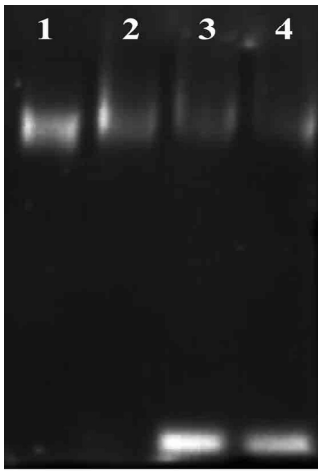
On the basis of the pI, anion exchange chromatography was carried out with strong anion exchanger Q Sepharose. The binding occurred at pH 7 and elution was carried out using a gradient of 0.1-1M NaCl. Two major peaks were obtained and both showed activity as well as glucose tolerance (Retained activity in presence of 1.0M glucose).

Figure 4.4. Chromatographic purification of BGL-5 from *Aspergillus unguis*



Zymogram analysis of the ion exchange fractions indicated that fractions 23 and 24 contained the GT-BGL protein whereas fractions 21 and 22 had only the high molecular weight BGL protein (Figure 4.5)

Figure 4.5. Zymogram analysis of the ion-exchange fractions



Lane 1 Fraction 21
Lane 2 Fraction 22
Lane 3 Fraction 23
Lane 4 Fraction 24

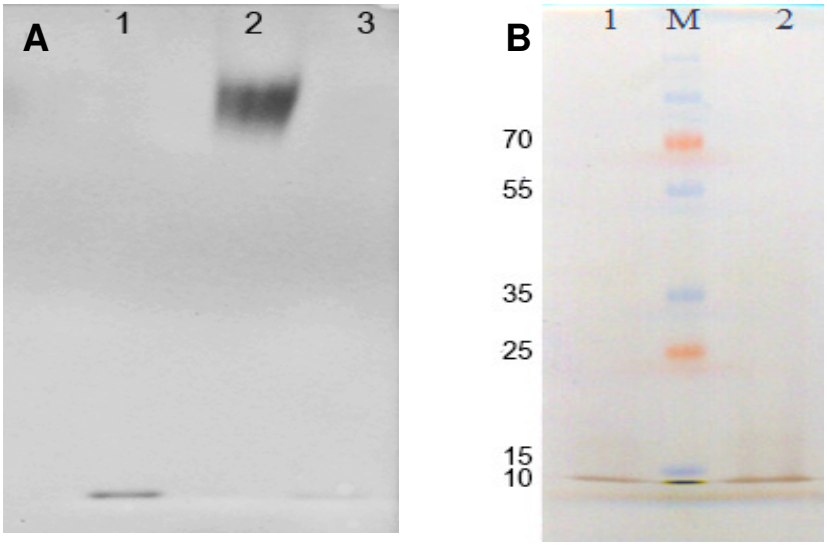
4.3.2.3. Purification of glucose tolerant β-glucosidase using electro-elution

The glucose tolerant β-glucosidase was eluted from the gel and subjected to zymogram analysis by both Native as well as SDS PAGE. From the Zymogram analysis, single protein with a molecular weight of 10kDa was detected. The BGL protein was active in Native as well as SDS PAGE.

Table 4.1 Purification of GT-BGL from *Aspergillus unguis NII 08123*

Purification stage	Total volume (ml)	Total protein (mg)	Total Activity (IU)	Yield (%)	Specific Activity (IU/mg)	Fold Purification
Crude Enzyme	1400	482.7	715.40	100.00	1482	1.00
Acetone PPT	15.00	3.87	12.69	1.77	3275	2.21
Q Sepharose-Peak II	6.00	1.30	11.65	1.63	9182	6.20
Gel Elution of Peak II	2.00	0.10	0.296	0.04	3131	2.11

Figure 4.6. Zymogram analysis of the purified protein (A) and molecular weight determination (B) by SDS PAGE



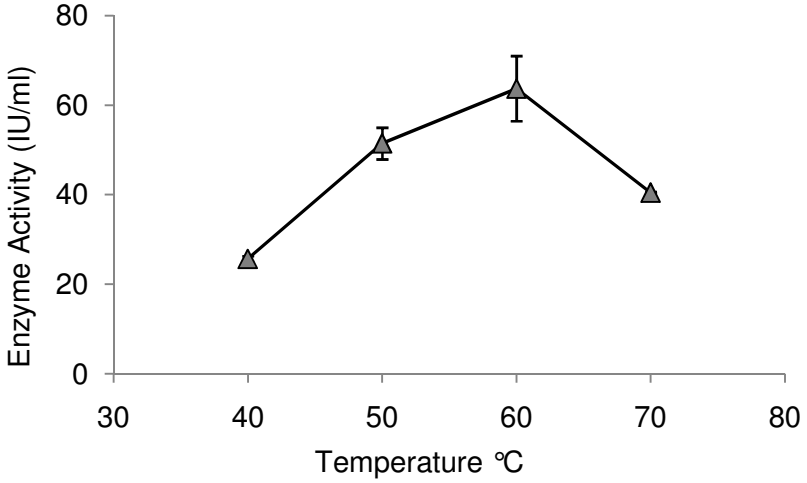
Lane 2	Upper band after gel elution and concentration	Lane M	PAGE ruler® pre-stained protein ladder
Lane 1,3	Lower band after gel elution and concentration	Lane 1,3	Lower band after purification

4.3.3. Biochemical characterization of the glucose tolerant β-glucosidase

4.3.3.1. Optimal temperature

Purified 10kDa GT-BGL protein was characterized for its optimal pH and temperature. BGL assays done at various temperatures (40 , 50 , 60 and 70 °C) showed that the BGL activity increased with an increase in temperature till 60 °C and there was a rapid decline in activity at 70 °C (Figure 4.7). Even though the temperature optimum was 60 °C it could attain 75% of activity even at 50°C. Fungi producing BGL with optimum activity at 60 °C include *Aspergillus niger* (Dekker 1985), *Penicillium verruculosum* (Korotkova et al. 2009), *Trichoderma reesei* (Chen et al. 1992), *Aspergillus sojae* (Iwashita et al. 1999), *Fusarium oxysporum* (Christakopoulos et al. 1994), *Aspergillus pulverulentus* (Mase et al. 2004), *Melanocarpus* sp. (Kaur et al. 2007) and *Aspergillus oryzae* (Langston et al. 2006; Zhang et al. 2007). In *Aspergillus tubingensis*, isoforms BGL III and BGL IV showed an apparent maximum activity at 60 °C (Decker et al. 2001).

Figure 4.7. GT-BGL activity at different temperature

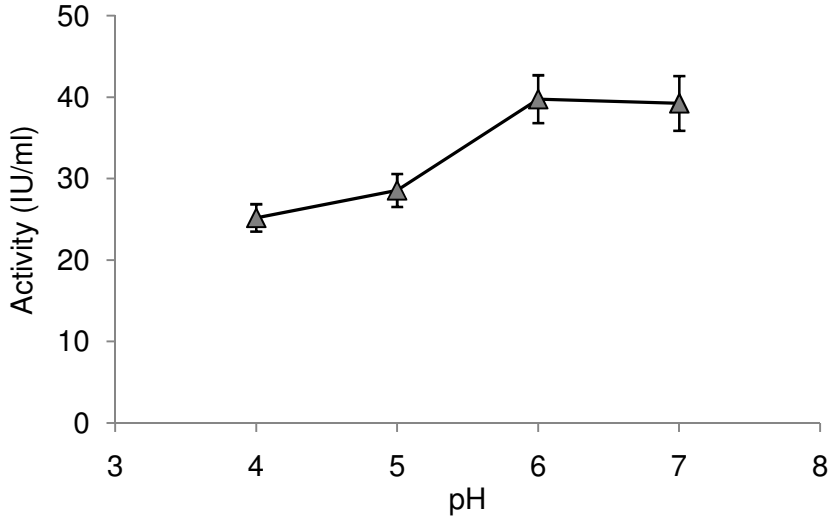


4.3.3.2. Optimal pH

Optimal activity of the glucose tolerant β-glucosidase was at pH 6.0 and it could retain more than 90% activity even at pH 7.0. More than 70 % activity was retained at pH 5.0 and 60% at pH 4.0 (Figure 4.8). Hence, it may be assumed that the GT-BGL can act over a broad range of pH.

Fungi like *Paecilomyces* (Yang et al. 2008) *Candida wickerhamii* (Skory et al. 1996), *Thermomyces lanuginosus* (Petruccioli et al. 1999), *Pichia etchellsii* (Wallecha and Mishra 2003), *Humicola grisea* var. *thermoidea* (Peralta et al. 1997; Souza et al. 2010), *Periconia* sp. (Harnpicharnchai et al. 2009), *Piromyces* sp. (Steenbakkers et al. 2003), *Penicillium aurantiogriseum* (Petruccioli et al. 1999) and *Melanocarpus* (Kaur et al. 2007) were all found to secrete β-glucosidases with optimal pH 6.0. The features of this enzyme probably are helpful in its use as a component in a biomass hydrolyzing enzyme cocktails, since most of the hydrolysis process takes place at 50 °C and pH 5. 0.

Figure 4.8. GT- BGL Activity at different pH

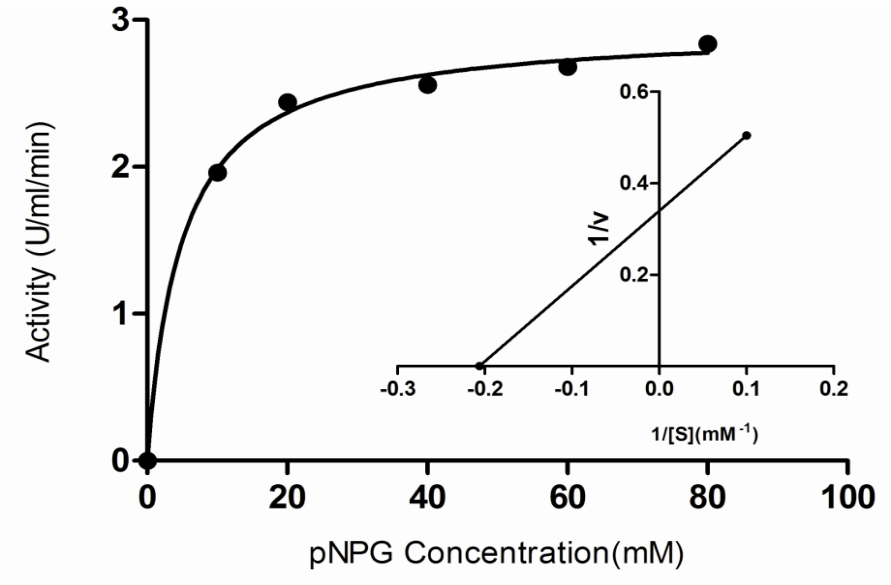


4.3.4. Enzyme kinetics

4.3.4.1. Michelis Menten Kinetics of GT-BGL

The standard assay mixture with pH 6.0 and an incubation temperature of 60 °C was used for the determination of reaction velocities of GT-BGL. K_m and V_{max} for the hydrolysis of pNPG by the enzyme was determined using 10–80 mM substrate. Michaelis–Menten kinetics was fitted and the determination of K_m and V_{max} was performed using the non-linear regression implemented in GraphPad Prism®. K_m and V_{max} were determined to be 4.85 mM and 2.95 mol min^{-1}mg $protein^{-1}$, respectively, for pNPG using the hyperbolic saturation curve (Figure 4.9).

Figure.4.9. Michaelis Menten graph showing the K_m and V_{max} of GT-BGL (BGL-5)



4.3.4.2. Determination of the glucose inhibition constant of BGL 5

End-product inhibition was evaluated using pNPG as substrate and in presence of 0 –1.0 M glucose as the inhibitor. The inhibition kinetics graph was explained based on the Cornish–Bowden plot (Cornish-Bowden 1974), which is a graphical method for determination of the type of enzyme inhibition and the dissociation constant- K_i'. BGL-5 was found to be highly glucose-tolerant with a K_i of 800 mM. Enzyme properties determined by the study are listed in Table.4.2.

Table 4.2 Physicochemical and kinetic properties of *Aspergillus unguis* GT-BGL

Feature	GT-BGL
Molecular weight (SDS-PAGE)	~10 kDa
Optimal pH	6.0
Optimal Temperature (°C)	60
$_{Km}$ (mM)	4.85
V_{max} (U/mg)	2.95
K_i [glucose] (mM)	800
K_i' [Dissociation constant]	100

In the Cornish-Bowden plot, S/V is plotted against [I], where S is the substrate concentration, V is the velocity and I is the inhibitor concentration. If the inhibition constant is greater than the dissociation constant ($K_i > K_i'$), the intersection is above the i-axis in the plot of s/v against I (Cornish-Bowden Plot), and below it in the Dixon Plot (Cornish-Bowden 1974). According to Cornish-Bowden, in the cases of competitive inhibition the $K_i' \rightarrow \infty$ and there is no intersection in the plot of S/V against [I] (Cornish-Bowden plot) whereas in the case of Un-competitive inhibition, the $K_i \rightarrow \infty$ and there is no intersection when 1/V is plotted against [I] (Dixon plot). The mode of inhibition was determined for *Aspergillus unguis* GT-BGL from both the graphs plotted here (Figure 4.10 & 4.11), where the dissociation constant K_i' is 100uM which is less than the inhibition constant K_i which is 800mM. Hence it is clear that GT-BGL (BGL-5) from *A. unguis* exhibited a type of mixed inhibition with a predominating un-competitive inhibition. Previously, it has been reported that β-glucosidase from *Trichoderma viride* showed a type of mixed inhibition with competitive character (Montero and Romeu 1992).

Figure 4.10. Dixon plot for the determination of glucose inhibition kinetics (K_i) of BGL-5

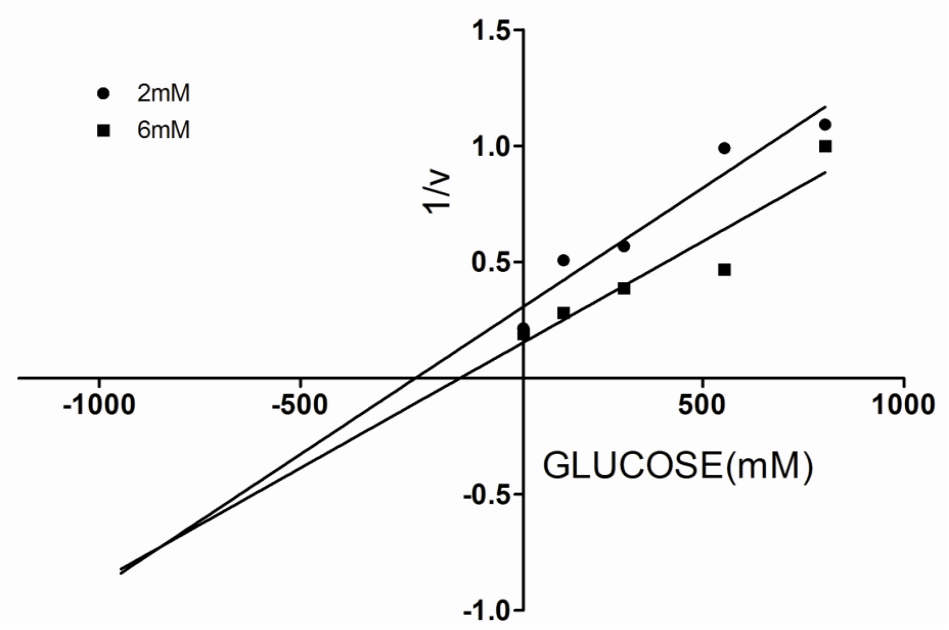
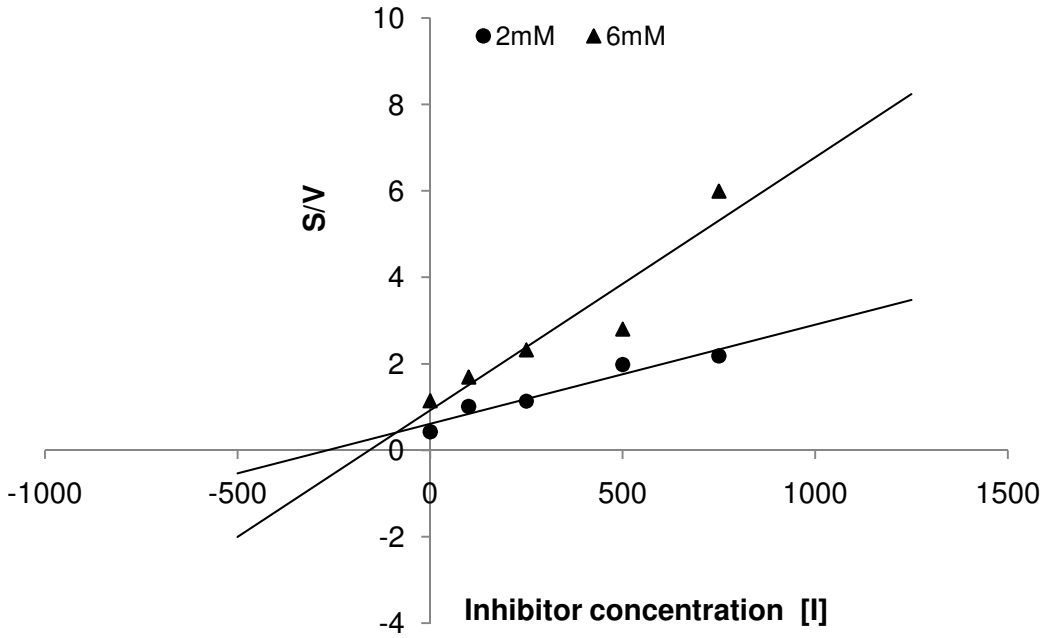


Figure.4.11.Cornish-Bowden plot for the determination of dissociation constant (K_i') of BGL-5



The high resistance of *A. unguis* BGL to glucose inhibition and the advantages due to growth properties of the fungus underlines its importance in biomass hydrolysis. With few exceptions, most microbial β-glucosidase show competitive inhibition kinetics in the presence of glucose (Saha and Bothast 1996). Extracellular β-glucosidases are highly sensitive to glucose and normally have glucose inhibition constants ranging from 0.5 to 100 mM. Noticeable exceptions like *A. oryzae* (Riou et al. 1998) and *C. peltata* (Saha and Bothast 1996), do exist, where the K_i values have been reported to be 1.36 and 1.4 M respectively, though this is very rare. The K_i of 0.8 M (~14% w/v glucose) obtained for *A. unguis* GT-BGL reported here may be considered as the second-highest tolerance ever reported from the *A. nidulans* group.

4.4. Conclusions

The filamentous fungus *Aspergillus unguis* NII 08123 expressed 5 different β-glucosidase proteins. The expression of these BGL isoforms could be altered by altering the carbon source in the medium. Zymogram analysis and chromatographic separation indicated that a low molecular weight isoform retained more than 30% activity in the presence of 1.0 M glucose even after

partial purification. The low molecular weight BGL protein designated as BGL-5 was purified to homogeneity and the properties were characterized. The BGL-5 could be a monomeric protein since retains its activity after SDS PAGE and the size is only ~10kDa. Till now, there are no reports regarding similar sized BGL with glucose tolerant properties and the reported smallest protein having glucose tolerance is 30kDa BGL protein from *Aspergillus oryzae* (Gunata and Vallier 1999). The enzyme had temperature and pH optima of 60 °C and 6.0 respectively. The K_m and V_{max} were determined to be 4.85 mM and 2.95 mol min^{-1}mg $protein^{-1}$, respectively for the substrate 4-Nitrophenyl β-D-glucopyranoside. The inhibition constant for glucose was found to be 0.8 M which qualifies this isoform as a glucose tolerant β-glucosidase. The mode of inhibition was determined from Cornish- Bowden and Dixon plots to be a type of mixed inhibition. These insights may provide some basis for the mechanism of enzyme action, especially in the context of glucose tolerance exhibited by this very low molecular weight protein. From the biochemical characterization and the kinetic studies, the glucose tolerant BGL protein from *A. unguis* could considered as a potent component of enzyme cocktails for biomass hydrolysis.

Chapter 5. Purification and Biochemical characterization of the major β-glucosidase from *Aspergillus unguis* NII 08123

5.1. Introduction

Several studies done on purification of β-glucosidases have stressed their importance in biotechnological applications, especially at the industrial level. It is expected that β-glucosidases are widely distributed among filamentous fungi, including species of *Trichoderma, Penicillium, Fusarium, and Aspergillus* (Singh and Hayashi 1995). Fungi, especially *Aspergilli* excrete a wide range of isoforms of the same enzyme. Various techniques, including size-exclusion chromatography, hydrophobic interaction chromatography, ion-exchange chromatography, isoelectric focusing and electro elution have been applied to the fractionation and purification of the isoforms from the crude enzyme supernatants (Inglin et al. 1980; Ramani et al. 2012; Riou et al. 1998; Saha and Bothast 1996; Wesley and Donal 1982). Even though purification helps to recognize and characterize the enzyme which one is looking for -based on the properties of the purified fraction, only a limited number of reports have addressed the complete purification of the desired isoforms.

Kinetic properties of the different isoforms of β-glucosidase vary within the same species and with the same substrate. In *Aspergillus tubingenesis,* four extracellular isoforms of BGL were purified and an analysis of their kinetic properties proved that a high diversity existed in the properties like pH and temperature optima, substrate specificity and glucose tolerance (Decker et al. 2001). *Aspergillus niger*, the fungus used for commercial production of β-glucosidase also produced two isoforms of BGL with molecular weights 80kDa and 30kDa. Inhibition by glucose was high in the high molecular weight isoform (K_i of 3.5mM) and low in the low molecular weight one, the latter having a K_i of 953mM (Gunata and Vallier 1999).

In *Aspergillus unguis* NII 08123, five different BGL proteins were identified which were designated as BGL 1-5 based on their molecular weights. The low molecular weight isoform BGL-5 was purified and the properties were studied. This BGL isoform was found to be highly glucose tolerant with a K_i of 0.8M (Chapters 3&4). The BGL isoform with highest level of expression was BGL1 (Chapter 3), which was considered as the major BGL of *Aspergillus unguis* based on the levels of protein expression. In the present study, the high molecular weight

major BGL was purified to homogeneity with a combination of steps including ultrafiltration and Ion exchange chromatography. The purified major BGL was characterized for its properties and kinetics.

5.2. Materials and Methods

5.2.1.Enzyme purification

5.2.1.1.Tangential Flow (Ultra) Filtration

Enzyme production was carried out by submerged fermentation as outlined under section 2.4.1, using 1% wheat bran as carbon source in a total volume of 1500ml. Enzyme extraction was carried out by centrifugation of the culture supernatant at 8000 rpm for 15 min at 4 ˚C. Supernatant was clarified by filtration through a 1µm pore size glass microfiber filter (Pall, USA). After filtration,the supernatant was concentrated by ultrafiltration using a 10kD cut off membrane in a Tangential Flow Filtration (TFF) unit (Sartorius ®, USA). Filtrate as well as the retentate was collected separately and the retentate was used for further studies.

5.2.1.2. Ion Exchange Chromatography

After Tangential flow filtration, the retentate was further purified by ion exchange chromatography using an anion exchange column (Q-Sepharose Fast flow, Sigma-Aldrich). Experiments were performed on a Biorad LP system. Column was equilibrated with Tris-HCl buffer (0.1M, pH 7.0). Sample in citrate buffer (0.05M, pH 4.8) was loaded by running it at a flow rate of 1ml/min through the column. The column was washed with three column volumes of the loading buffer (0.1M Tris HCl Buffer, pH 7.0). Elution was performed using step gradient of 0.1 to 1M NaCl and 2 ml fractions were collected. Fractions giving the maximum activity were pooled together and were used for a second stage of ion exchange chromatography. In second ion exchange chromatography, sample loaded on the column as above was eluted with buffers each with a different pH ranging from 4-9. Buffers used were 0.1M acetate buffer (pH 4 & 5) 0.1M Citrate buffer (pH-6 0) and 0.1M Tris-HCl buffer (pH 7, 8 and 9).

5.2.2. Characterization of high molecular weight isoform – BGL1

Purified BGL-1 of *Aspergillus unguis* was characterized for its properties including its molecular weight, optimal pH and temperature and the kinetic properties including V_{max} and K_m with pNPG as the substrate.

5.2.2.1. Determination of the molecular weight of purified BGL1

Crude enzyme as well as the retentate from ultrafiltration was run on Native PAGE along with protein markers (EMerck, India) as outlined under section 2.6.1. BGL activity band was visualized by MUG staining and the Zymogram was photographed using an Imaging system (SyngeneGBox, UK). 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.The position of major BGL isoform was confirmed by overlapping the photographs of the zymogram and silver stained gel and the molecular weights were determined by comparison with the standard protein markers of known molecular weights.

5.2.2.2. Determination of the optimal temperature of BGL1

The optimum temperature for the high molecular weight BGL was determined by measuring the enzyme activity at temperatures ranging from 40-70 ºC. Buffered pNPG was used as substrate and the enzyme assays were performed as mentioned under section 2.5.1.

5.2.2.3. Determination of the optimal pH

The optimum pH was examined by assaying the purified enzyme at different pH ranging from 4-7 using pNPG as the substrate as outlined under section 2.5.1. The different buffer systems used for assay were 0.05M citrate buffer (pH 4-6) and 0.05M Tris buffer (pH 7)

5.2.2.4. Kinetics of high molecular weight β-glucosidase

The V_{max} and K_m value of the purified isoform with pNPG as the substrate was determined using a Michaelis Menton plot. BGL assays were performed with different incubation times (2.5min-15 min) at multiple pNPG concentrations (0.8-10mM). Lineweaver-Burk plot with reciprocal of initial reaction velocities [1/V] were plotted against reciprocal of different substrate concentrations [1/S]. The value of [V] at which the line intersects theY-axis corresponds to V_{max} and the value of [S] at which the lines intersect X-axis corresponds to K_m.

5.3. Results and Discussion

5.3.1. Purification of BGL1

β-Glucosidase from *Aspergillus unguis* was concentrated using ultra filtration. Clarified enzyme supernatant from 4-day old culture grown on 1% wheatbran was subjected to TFF with a cut off limit of 10kDa. Smaller molecules below 10kDa pass as filtrate through the membrane where as larger particles greater than 10kDa are retained. Retentate of the *A.unguis* culture filtrate contained all the five isoforms of β-glucosidase and was used for further studies. The concentrated retentate contained 81% of the initial β-glucosidase activity (Table 5.1). Anion exchange chromatography with Q-sepharose resulted in the separation of BGL as one minor peak and two major peaks (Figure 5.1). Active fractions of the first peak (Fraction No.19-24) were pooled and subjected to a second ion exchange chromatography with buffer elution. The high molecular weight BGL (BGL1) was eluted using pH 5.0 buffer with a final yield of 5.14%. BGL1 was purified to apparent homogeneity as monitored by Native PAGE followed by activity and silver staining. The apparent molecular weight of the native BGL-1 was estimated to be ~240kDa by Native PAGE.

Table.5.1.Purification of the major β-glucosidase -BGL1 from *Aspergillus unguis* NII 08123

Purification stage	Total protein (mg)	Total activity IUs	Yield (%)	Specific activity IU/mg	Fold Purification
Crude Enzyme	690.00	698.00	100	1012	1.00
Ultrafiltration (TFF) retentate	102.90	583.10	83.54	5667	5.60
Ion exchange- NaCl eluate	10.00	61.423	8.79	6142	*1.08
Ion exchange- buffer eluate	0.60	9.00	1.29	15000	2.44

**Fold decrease is due to the separation of the other BGL isoforms.In crude and TFF retentate sample the total activity and total protein was a representative of the entire BGL proteins in that sample.After ion exchange chromatography the activity of the single BGL protein /isoforms was calculated and mentioned in the purification table.*

Figure: 5.1. Ion exchange Chromatogram (NaCl Gradient Elution)

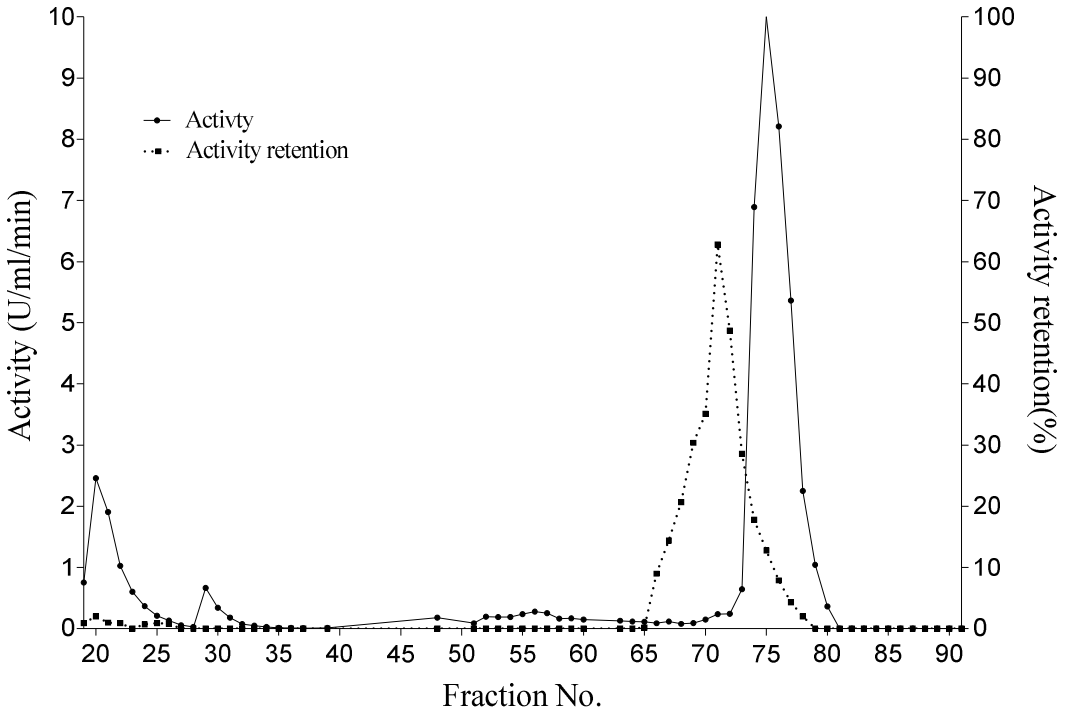
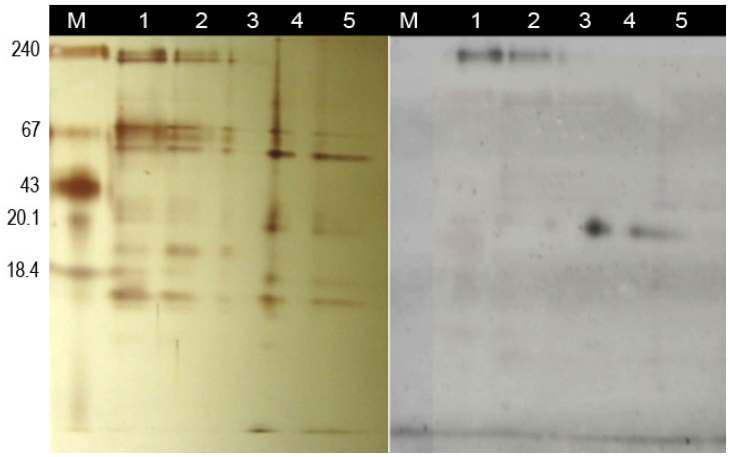


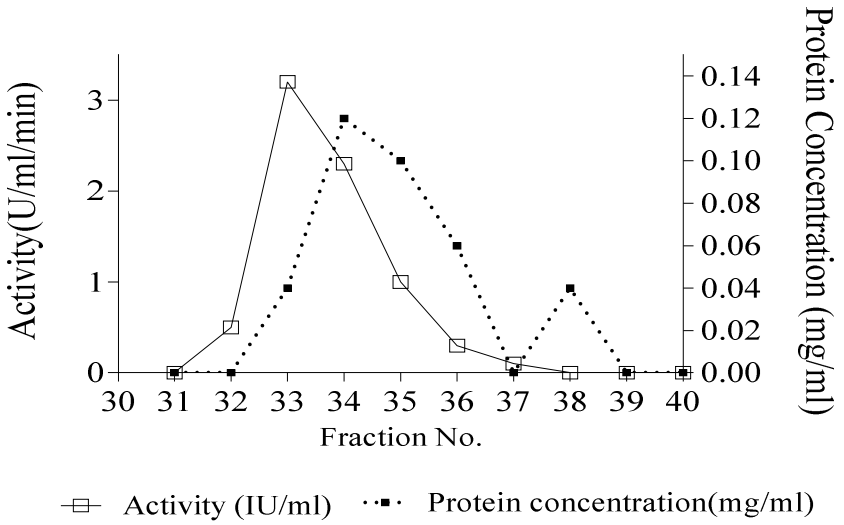
Figure: 5.2. Native PAGE of ion exchange fractions after activity and silver staining



A) Silver stained gel B) Zymogram*

Lane information: M – Native PAGE marker, Lanes 1- Fraction # 19| 2-Fraction # 20| 3-Fraction # 21| 4- Fraction # 69| 5-Fraction # 70.
** Image inverted for clarity*

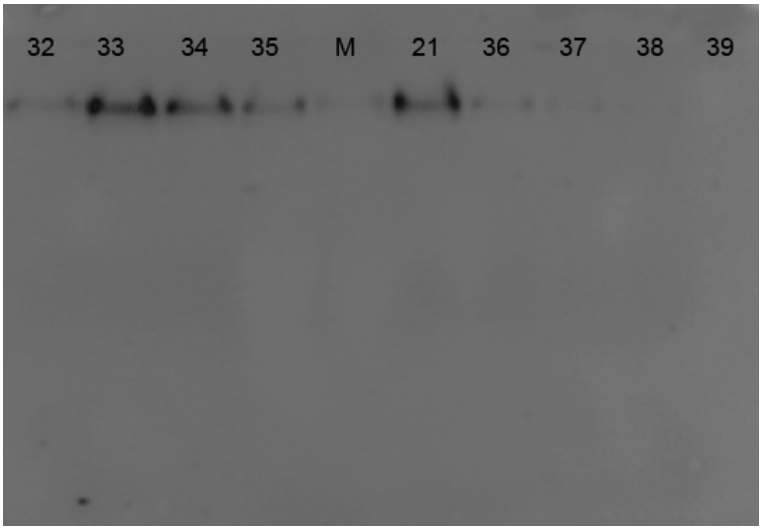
Figure.5.3.Ion exchange chromatogram (Buffer Elution)



Comparison of the overlapped silver stain and zymogram images of the NaCl eluted active fractions with Native PAGE molecular weight markers indicated that the molecular weight of the major β-glucosidase (BGL1) is approximately 240kDa (Figure 5.2). Native PAGE and zymogram analyses of buffer eluted sample showed that the fractions that corresponded to the

activity peak in the ion exchange chromatogram indeed contained only the high molecular weight BGL1 isoform (Figure 5.4) indicating that the two stage ion exchange chromatography is efficient in separating the major BGL protein from other isoforms.

Figure: 5.4. Zymogram of the buffer eluted ion exchange fractions showing BGL1



** Lanes labelled with the numbers of fraction loaded. Image inverted for clarity*
Lane information: M – Native PAGE marker, Lane 32-39 Fraction # 32-39
Lane 21-Fraction # 21 of NaCl gradient elution chromatogram

5.3.2. Effect of pH and Temperature on enzyme activity

pH and Temperature optima of the BGL1 isoform was determined using pNPG as the substrate. Even though the temperature optimum was found to be 60°C, the enzyme could attain 84% activity at 50°C (Figure 5.5). The BGL1 had a sharp pH optimum at pH 5.0. Nevertheless, it retained 94% activity at pH 4.0 and 80% activity at pH 6.0. But at pH 7.0, it was possessed only 56% activity compared to that at pH 5.0. Results of the pH and Temperature optima studies of BGL1 clearly indicated that it could be a suitable candidate for enzymatic hydrolysis of lignocellulosic biomass.

Figure: 5.5. Optimum Temperature of the purified BGL1

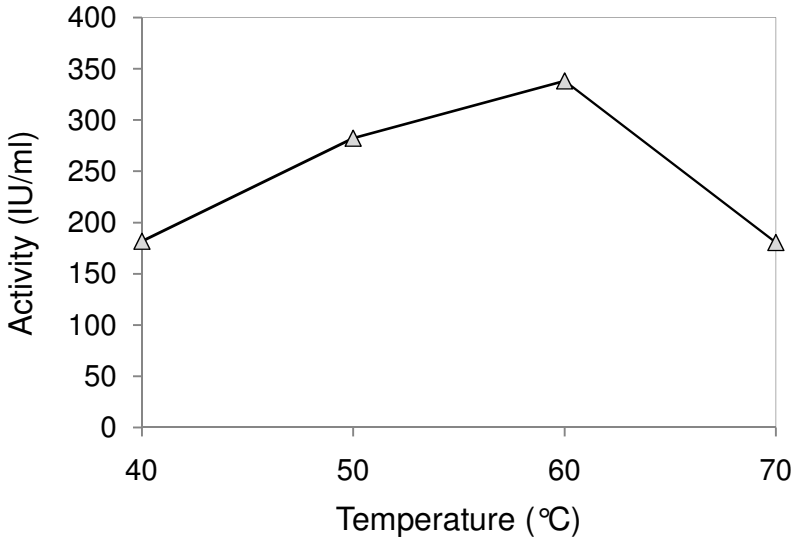
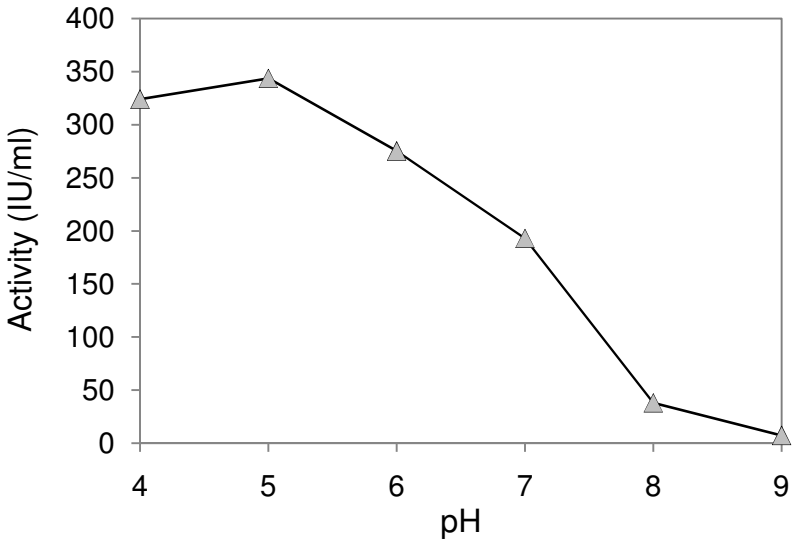


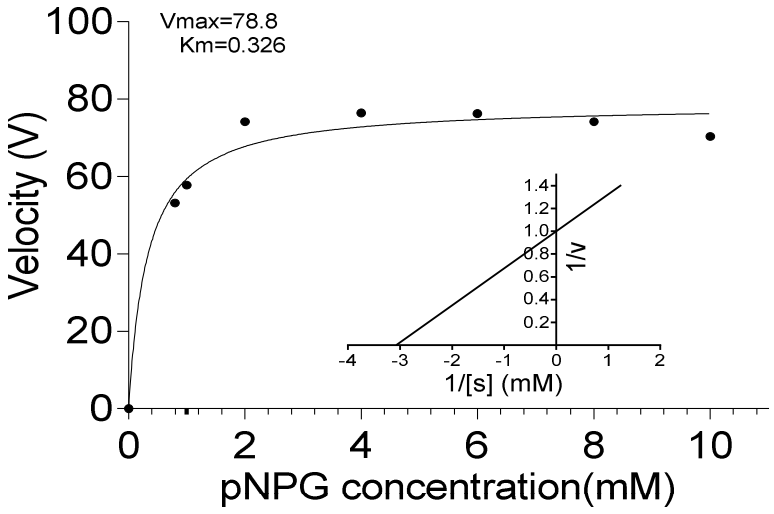
Figure: 5.6. Optimum pH of the purified BGL1



5.3.3. Kinetic studies of BGL-1

Purified β-glucosidase obeyed typical Michaelis Menten kinetics with substrate inhibition at high concentration of pNPG (> 10mM pNPG). *Km* and *Vmax* values were determined by nonlinear regression analyses.*Vmax* and *Km* values were 78.8 mol min^{-1}mg $protein^{-1}$ and 0.326mM respectively (Figure 5.7).The high molecular weight major BGL showed an apparently low *Km* and high *Vmax* compared to the low molecular weight BGL-5 isoform of *Aspergillus unguis*. BGL1 therefore can be considered to have a higher affinity towards pNPG.

Figure 5.7.Michaelis-Menten graph and Line weaver-Burk plot showing V_{max} and K_m



5.4. Conclusions

Aspergillus unguis produced 5 extracellular β-glucosidases after 96 h of submerged fermentation using 1% Wheat bran as the carbon source. Among the five BGL proteins, the major fraction (BGL1) had high molecular weight in its native state. Native PAGE along with standard molecular weight markers followed by zymogram analysis and silver staining confirmed the approximate molecular mass of 240kDa. In the zymogram analysis, BGL1 was had intense fluorescence even with low amount of protein. Even though it was very sensitive to glucose,

BGL1's biochemical properties are suitable for biomass hydrolysis. The purified protein had an optimum temperature of 60 °C, and could retain 84 % of the initial activity at 50 °C, the temperature at which hydrolysis reactions are normally conducted. Optimum pH for BGL1 was 5.0 but the BGL1 retained 94 % activity at pH 4 and 80 % activity at pH 6. Studies on kinetics of the purified BGL1 showed that it obeyed typical Michaelis-Menten kinetics with a low K_m (0.326mM) and high V_{max} 78.8mol min^{-1}mg $protein^{-1}$with pNPG as the substrate. These studies proved that BGL1 was having high affinity towards the substrate and it is efficient in the hydrolysis of the test substrate.

Chapter 6. Molecular cloning and sequencing of β-glucosidase genes from *Aspergillus unguis* NII 08123

6.1. Introduction

β-glucosidase comes under the Glycosyl hydrolase (EC.3.2.1). Family, which are wide group of enzymes that catalyze the hydrolysis of glycosidic bond between two or more carbohydrate moieties or a carbohydrate and non-carbohydrate moiety. The IUBMB nomenclature does not reflect the structural features of these enzymes but rather it was based on their substrate specificity and sometimes based on their molecular mechanism. Later, in 1986, Chothia and Lesk suggested that a direct correlation exists between sequence and folding similarities. Based on this, Henrissat (1991) classified Glycosyl hydrolases into different families and its classification reflects the structural features of the enzymes than their substrate specificity and hence it differs from IUBMB classification. This sequence/structure based classification brings out the evolutionary relationship between the enzymes and can act predict the catalytic mechanism of newly sequenced enzymes. The current classification is available on the CAZy (Carbohydrate Active EnZymes) web site (Henrissat and Coutinho 1999) and the database provides a series of regularly updated sequence based classification that allows reliable prediction of the mechanism (retaining/inverting), active site residues and possible substrates.

Most of the bacterial and fungal β-glucosidases comes under the glycosyl hydrolase Families 1 and 3 (GH1 & GH3). Other glycosyl hydrolase families (GH5 and GH9) also contain β-glucosidases with broad substrate specificities. In GH1, seven β-glucosidases have been characterized and in GH3, 22 β-glucosidases have been characterized (Murphy et al. 2011).

Most common enzymes of glycosyl hydrolase family 1 are β-glucosidase and β-galactosidase. These enzymes have retaining catalytic mechanism with a conserved catalytic nucleophile and were first described in *Agrobacterium* sp (Wang et al. 1995; Withers et al. 1986; Withers et al. 1990). Glycosyl hydrolase family 1 enzymes fold into $(\alpha/\beta)_8$ barrel structures with glutamic acid as the nucleophile where as glycosyl hydrolase family 3 has a two domain structure with an $(\alpha/\beta)_8$ barrel and an $(\alpha/\beta)_6$ sandwich domain, with the active site in between the two. Catalytic carboxylate residue is contributed by each of the two domains and the most commonly found catalytic nucleophile is “aspartate” residue situated in the extended loop of the

β-strand 7 of $(\alpha/\beta)_8$ barrel. The catalytic acid base is a conserved glutamate residue located in the extended loop from $(\alpha/\beta)_6$ sandwich. GH3 catalytic nucleophile was first identified in *Aspergillus wentii* (Bause and Legler 1974) and later confirmed by active site labeling of *Aspergillus niger* β-glucosidase (Dan et al. 2000).

A large number of β-glucosidase genes from bacteria as well as fungi have been sequenced, cloned and transformed into *E.coli* as well as in eukaryotic hosts such as *Saccharomyces*, *Pichia pastoris* and filamentous fungi. Notable examples include the cloning and expression of a 75kDa BGL1 protein from the cellulolytic fungus *Trichoderma reesei* (Barnett et al. 1991), a putative intracellular β-glucosidase with significant similarity to GH1, β-glucosidase from *Taleromyces emersonii* (Collins et al. 2007), Cloning and expression of GH3 BGL from *Aspergillus niger* in *Saccharomyces cerevisiae* and *Pichia pastoris* (Dan et al. 2000), cloning and expression of thermostable GH3 β-glucosidase of *Thermoascus auranticus* in *Pichia pastoris* (Hong et al. 2007), cloning and expression of an ethanol tolerant GH3 β-glucosidase *bgl*3a from *Myceliophthora thermophila* in *Pichia pastoris* (Karnaouri et al. 2013) etc.

In the preliminary experiments, the strain *Aspergillus unguis* NII 08123 was found to produce 5 isoforms of β-glucosidase. This multiplicity of BGL isoforms may be due to the existence of different genes for the enzyme as in *Aspergillus kawachii* (Iwashita et al. 1999) or it could be due to the differences in the post translational modifications (ie from the same gene through differential splicing). The isoforms were distinct with different molecular weight and glucose tolerance and were coded by different genes in *Neurospora crassa*, a genetically well established fungus, which harbored 7 putative β-glucosidase genes (Wu et al. 2013). In order to verify whether the multiplicity of BGL in *Aspergillus unguis* is due to existence of different '*bgl*" genes, BGLs from different glycosyl hydrolase families -GH1, GH3 and GH5 were amplified using degenerate primers for these glycosyl hydrolase families. The amplicons were sequenced and BLAST analyses (Altschul et al. 1990) were conducted to identify the whether the amplified sequences has similarities among themselves or with sequences of the BGLs belonging to the corresponding family of glycosyl hydrolases.

6.2. Materials and Methods

6.2.1. Strain, Plasmids and Media

Aspergillus unguis NII08123 was used for isolation of genomic DNA. *Escherichia coli* JM109 (Fermentas) was used as host for DNA manipulations. Vector pTZ57R/T (Fermentas, USA) was used for the transformation studies. Mandels and Weber medium (1969)was used for the cultivation of *Aspergillus unguis* NII 08123 as described under section 2.4.1. *E.coli* was grown in Luria Bertani (LB) broth and transformants were grown in the same medium supplemented with 100µg/ml Ampicillin.

6.2.2. General DNA Manipulation Techniques

All the DNA manipulation techniques were carried out using the standard procedures as described by Sambrook et al, (1989). Chromosomal DNA isolation was performed as described in section 2.7 and this DNA was used as the template for PCR and for further studies.

6.2.3. Primer Design

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 installation of ClustalW software (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 for the desired glycosyl hydrolase family.

Degenerate primers designed for three major GH families-GH1, GH3, GH5 were used for PCR amplification of β-glucosidase genes from the chromosomal DNA of the fungus. Based on the results from the first set of degenerate primers, another set of degenerate primers as well as specific primers that could amplify a larger stretch of the β-glucosidase gene (s) were designed CODEHOP server (Rose et al. 1998) or the software FastPCR (Kalendar et al. 2009). The

degenerate primers used for amplification of GH1, GH3 and GH5 family β-glucosidases are listed in Table 6.1, and the degenerate and specific primers designed for amplification of larger stretch of GH1 family BGL of *Aspergillus unguis* are given in Table 6.2.

Table 6.1: Degenerate primers used 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	BGL3_DF1	5'-TGC GGT ATA AAC GTA GGG GCN ACN TKB GA -3'
20	BGL3_EF1	5'-TGC GCA AAG CAT TAT ATT TGC WAY GAN MWR GA -3'
21	BGL3_GR1	5'-GGA ATG GTG TGC GAA CCA RTC NSW CAT -3'
22	BGL3_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'

6.2.4. PCR amplification of partial gene sequence of BGL

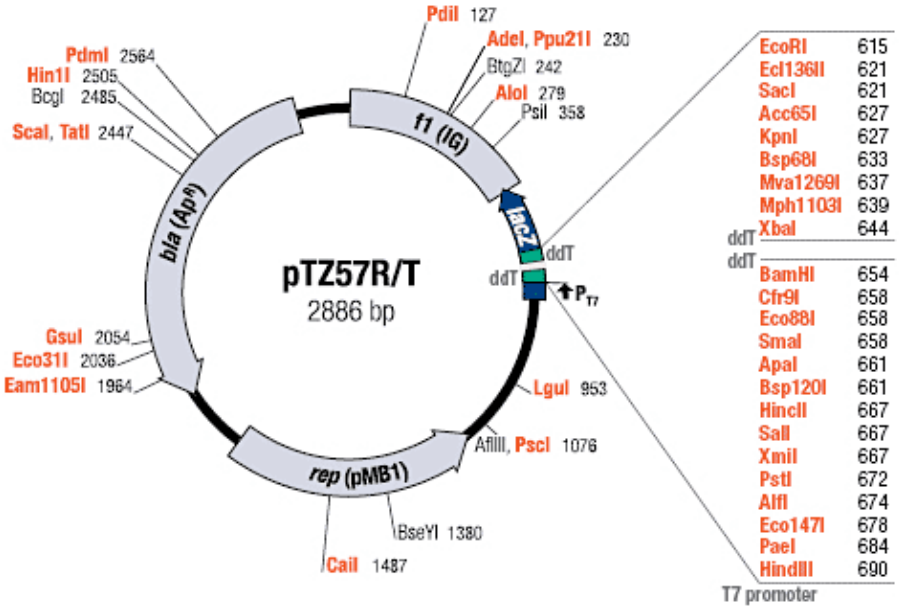
Amplification of partial gene sequences of β-glucosidases were performed by polymerase chain reaction using different sets of the degenerate primers designed for each family. PCR reactions contained 0.5 unit of *Taq* DNA polymerase, 1X buffer, 200 μM of each deoxynucleotide triphosphates, 2.0 μM $MgSO_4$ (All from Fermentas, USA) and 1 μM forward and reverse primers. Reaction conditions for PCR amplification were an initial 94 °C for 3 min, followed by 30 cycles of 94 °C for 30 sec, 50-60 °C (as determined by Tm value of primers) for 30 sec and 72 °C for 1 min; and a final extension step at 72 °C for 10 min. PCR products were separated by electrophoresis on a 1 % agarose gel and products were visualized in long range UV trans-illumination for documentation.

6.2.5. Cloning of PCR products and sequencing of inserts

PCR fragments obtained using degenerate primers were gel purified using QIAquick® Gel Extraction Kit (Qiagen, Germany) and the eluted products were cloned in to pTZ57R/T vector followed by transformation into competent *E. coli* JM109 (Fermentas, USA). Cloning was performed using InsTAclone® PCR Cloning Kit. LB plates containing XGal (40μg/ml), IPTG (0.1Mm), Ampicillin (100μg/ml) were plated with the transformed *E. coli* and were incubated for 12-16h at 37 °C. Colonies were selected based on the blue white screening. White colonies were expected to harbor the plasmid with insert and were used for plasmid isolation and confirmation of inserts. A white colony of *E. coli* was inoculated into LB Broth containing Ampicillin and was cultivated for 12-16h at 37 °C with 200rpm agitation on an incubator shaker. Plasmid DNA was isolated based on the Alkaline Lysis method as outlined under section 2.8 or using the Qiagen Miniprep Plasmid isolation kit when it had to be used for sequencing. Presence and size of the inserts were determined by restriction digestion of the purified plasmids (according to the enzyme manufacturer`s) protocol and also by PCR amplification of the desired fragment using the same set of primers used for generating the amplicons. Sequencing of the insert was outsourced and purified plasmids were sent to Scigenom (Kochi,India) for sequencing using M13 forward and reverse primers which are complimentary to the flanking regions of

pTZ57R/T multiple cloning site. Sequence reads received from the company was analyzed by NCBI-BLAST (Altschul et al. 1990).

Fig.6.1. Map of the pTZ57R/T vector.



Unique restriction sites are indicated

6.2.6. Thermal Asymmetric Interlaced PCR (TAIL PCR)

TAIL PCR was tried to obtain the 5'end of the GH1 BGL gene fragment based on the protocol developed by Liu and Whittier (1995). Seven degenerate primers were designed using CODEHOP server, of which four were based on the N terminal region of GHF1 *bgl*. Specific primers were designed using the partial gene sequence (s) obtained from the amplicons obtained with the degenerate primers for GH1 BGL. FASTPCR (Kalendar et al. 2009) was used for designing these primers. While three or more specific interlaced PCR primers are used for getting the specific products in general, here only one specific primer was used for doing the entire TAIL PCR cycles. The thermal cycling conditions used for TAIL-PCR is given below (Table 6.3) PCR amplicons were separated by electrophoresis on 1% agarose gels, purified from gel and were cloned into the T&A vector. Plasmid was sequenced using the Dye Terminator method using the BigDye® Terminator v3.1 cycle sequencing kit (Life Technologies, USA) as

per manufacturer's protocols using M13F and M13R primer at CSIR-NIIST. Nucleotide sequences were analyzed by NCBI-BLAST (Altschul et al. 1990).

Table 6.2. List of Specific Interlaced PCR primers from the partial gene sequence of GH1 β-glucosidase gene and degenerate primers designed based on the N terminal conserved motifs of GH1 β-glucosidase

SL #	Primer ID	Sequence(5'-3')
Specific primers based on the partial gene sequence		
1	1F1	CCA GTC CAA TCG TCA GCA GCT
2	1F2	TCA GAC AAG GCC CGA GCA TAC
3	1F4	TCG ACG AAG GAC ATC GCG CTC
4	1R1	TTT AGC CAT GGG ACG TTT CCA
5	1R3	ACA AGG ACG CGG CCC AGA AGT
6	1R4	TCG GCT GTT TTG GAG ACC CTG T
GHF1-Degenerate primers based on N-Terminal and C-terminal domains		
1	BGL_D1	GCC CGT CCA TCt ggg aya cnt t
2	BGL_D2	GCG CCT GGA ACG AGg ayg gnm rng g
3	BGL_D3	GCG ACG ACC CCG TCa ayg arv mng g
4	BGL_D4	AGT TCC ACA TCG GCt ggt tyg snr a
5	BGL_D5	GGG CAG GGG CAG Ccr tty tcn gtn a
6	BGL_D6	GGG CAG GGG CAG ccr tty tcn gt
7	BGL_D7	CGC CTG GGC AGG GGs wnc crt tyt c

Table 6.3. Thermal cycling conditions used for TAIL PCR

Temperature Setting and Time	No of cycles
1st PCR reaction cycle	
92 °C (2.0min) > 95 °C (1.0 min)	x 1
94 °C (15s) > 63 °C (1.0 min) > 72 °C (2.0min)	x 5
94 °C (15s) > 30 °C (3.0 min) > 72 °C (2.0min)	x 1
94 °C (5.0s) > 44 °C (1.0 min) > 72 °C (2.0min)	x 10
94 °C (5.0s) > 63 °C (1.0 min) > 72 °C (2.0min)	x 12
94 °C (5.0s) > 63 °C (1.0 min) > 72 °C (2.0min)	
94 °C (5.0s) > 44 °C (1.0 min) > 72 °C (2.0min)	
72 °C (5.0 min)	x 1
4 °C (5.0 min)	-

2nd PCR reaction cycle	
95 °C (1.0min)	x 1
94 °C (30s) > 68 °C (30s) > 68 °C (3.0min 30s)	x 13
94 °C (30s) > 68 °C (30s) > 68 °C (3.0min 30s)	
94 °C (30s) > 44 °C (30s) > 68 °C (3.0min 30s)	
68 °C (5.0min)	x 1
4 °C	-
3rd PCR reaction cycle	
94 °C (1min) > 95 °C (1.0min)	x 1
95 °C (1.0min)	x 1
94 °C (30s) > 68 °C (30s) > 68 °C (3.0min 30s)	x 13
94 °C (30s) > 68 °C (30s) > 68 °C (3.0min 30s)	
94 °C (30s) > 44 °C (30s) > 68 °C (3.0min 30s)	
68 °C (5.0min)	x 1
4 °C	-

6.3. Results and Discussion

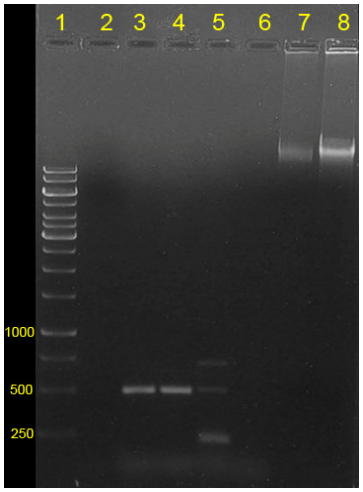
6.3.1. Chromosomal DNA isolation

Fungal genomic DNA was isolated using CTAB method as per the protocol stated in section 2.7 and was visualized in agarose gel. The size of the genomic DNA was >10kb (Fig.6.1)

6.3.2. PCR amplification of β-glucosidase genes

PCR amplification was performed for β-glucosidase genes belonging to glycosyl hydrolase families GH1, GH3 and GH5 using combination of degenerate primers designed to amplify the BGLs from these families. The size of amplicons varied from 0.5-0.75 kb. Among the primer combinations screened for GH1 β-glucosidase, only three combinations gave proper amplification from the genomic DNA. Amplicons from two primer combinations (AF1-DR1 and AF1-FR1) gave clear bands on gel with an approximate size of 500 bp (Fig 6.2). Among the two, primer combination AF1-FR1 was selected for further cloning studies.

Fig.6.2. PCR amplification of GH1 family β-glucosidase from *Aspergillus unguis*

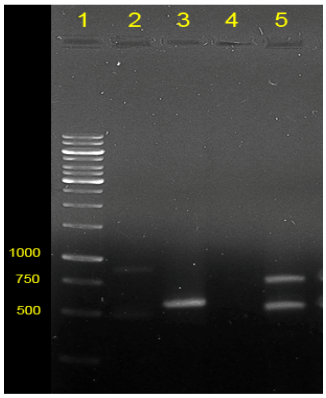


Lane Information

1 GeneRuler ® 1kb ladder (Fermentas)
2 Control
3 Primer pair BGL1_AF1-DR1
4 Primer pair BGL1_AF1-FR1
5 Primer pair BGL1_AF1-IR1
7 Genomic DNA *Aspergillus unguis*
8 Genomic DNA *Aspergillus unguis*

Four pairs of primers gave amplicons with clear bands on gel, in the case of GH3 primer combinations. Among these, the primer pair GF1-KR2 gave an amplicon with a size of ~500bp, while the primer pair GF2 –KR2 gave two amplicons with sizes ~500bp and ~750bp respectively (Fig 6.3). These three amplicons were cloned into pTZ57RT/A vector and was used to transform *E coli*.

Fig.6.3. PCR amplification of GH3 family β-glucosidase from *Aspergillus unguis*

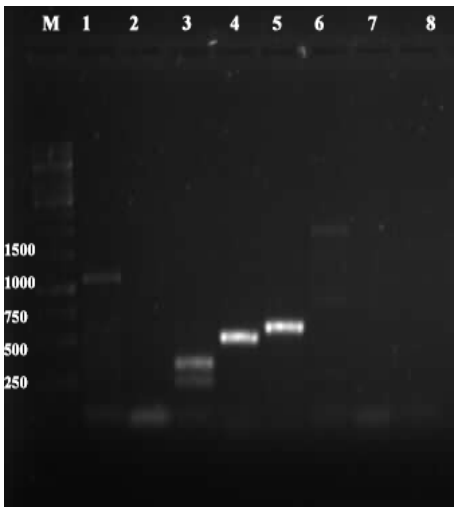


Lane Information

1 GeneRuler® 1kb ladder (Fermentas)
2 Primer pair BGL3_DF1-GR1
3 Primer pair BGL3_GF1-KR2
4 Primer pair BGL3_GF2-KR1
5 Primer pair BGL3_GF2-KR2

In the case of GH5, eight sets of primer pairs were screened and most prominent amplicons were selected for further sequencing. Primers pairs FF1-IR1 and FF1-HR1 gave amplicons with a size of ~600 and 750 bp respectively (Fig 6.4).

Fig.6.4.PCR amplification of GH5 family β-glucosidase from *Aspergillus unguis*



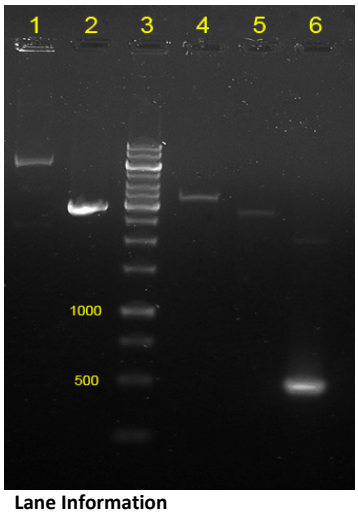
Lane Information

M	GeneRuler ® 1kb ladder (Fermentas)
1	Primer pair BGL5_BF2-JR1
2	Primer pair BGL5_BF2-ER1
3	Primer pair BGL5_FF1-HR1
4	Primer pair BGL5_FF1-IR1
5	Primer pair BGL5_FF2-HR1
7	Primer pair BGL5_FF2-IR1
8	Primer pair BGL5_FF2-JR1

6.3.3. Cloning, sequencing and BLAST analysis of the partial BGL genes of families GH1, GH3 and GH5

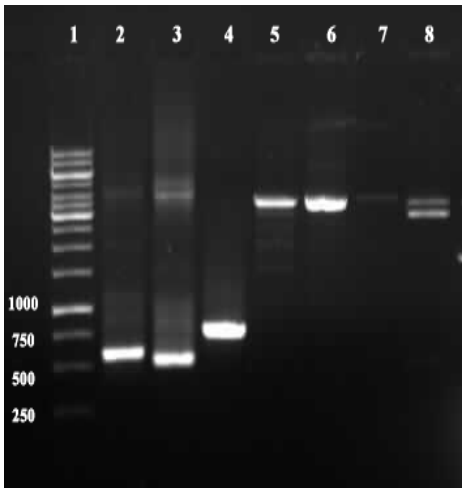
Amplicons of GH1, GH3, and GH5 primer pairs were selected and gel purified and cloned as outlined under section 6.2.5. Plasmids were isolated from the transformants and were purified. The isolated plasmids were used as templates for PCR amplification of the gene using the same set of degenerate primers used for amplifying them from the genomic DNA. Single digestion and double digestion were performed for GH1 using EcoR1 and BamH1 restriction enzymes and as a control linearized vector was used. The presence of amplicons with identical size from genomic DNA and plasmid confirmed the positive clones of partial BGL gene. Fig. 6.5 shows the confirmation of partial gene cloning of β-glucosidase genes belonging to GH1, GH3 and GH5 respectively.

Fig.6.5. Cloning of the partial gene of GHF1, GHF3 and GHF5



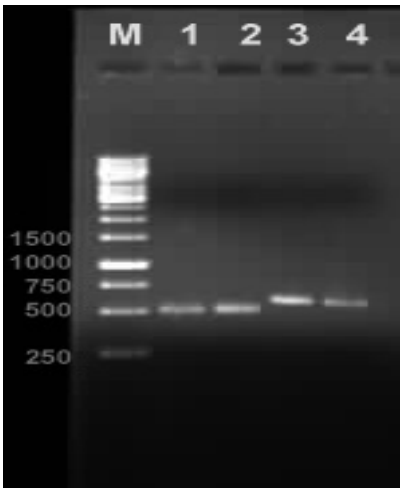
Lane Information

1 pTZ57R/T vector with insert
2 pTZ57R/T linearized vector
3 GeneRuler ® 1kb ladder (Fermentas)
4 Single digestion of plasmid with insert (bglF1 partial gene sequence)
5 Double digestion of the Plasmid with insert (bglF1 partial gene sequence)
6 Partial gene sequence (bglF1) amplification from plasmid DNA of recombinant clones



Lane Information

1 GeneRuler ® 1kb ladder (Fermentas)
2 Plasmid DNA amplicon from Primer pair BGL3_GF1-KR2
3 Plasmid DNA amplicon from Primer pair BGL3_GF2-KR2 (500bp)
4 Plasmid DNA amplicon from Primer pair BGL3_GF2-KR2 (750bp)
5 Single digestion with Bam H1(GF1-KR2)
6 Single digestion with Bam H1(GF2-KR2)
7 Single digestion with Bam H1(GF1-KR2)
8 Control plasmid with vector and vector + Insert



Lane Information

M GeneRuler ® 1kb ladder (Fermentas)
1-2 Primer pair BGL5_FF2-IR1
3-4 Primer pair BGL5_FF2-JR1

6.3.4. Sequencing and Blast analysis of partial genes of GH1, GH3 and GH5

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 sequencing 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

GH-Family 1 BGL Partial gene sequence

```
> Seq_BGLF1
TTGCCAGTCCAATCGTCAGCAGCTGGCGGCTCAGACAAGGCCCGAGCATACTTGGTAGAATAATGGTTCATGCCATG
AAAGCATTTATATGTGCCGTTCGACGAAGGACATCGCGCTCGGCTTCCGTAAAGTAAGGCAGGCGATAACCCAGCGT
AGCTCGCATCTCTGGGGGGTAGTCCTGTCCGAGAAATACAGGGTCTCCAAAACAGCCGATGTAAAAGATCATCCACT
TCTGGGCCGCGTCCTTGTGGGCCTGGCTGGAAACGTCCCATGGCTAAAATATGATCCATTCAACACTATCGAAATTT
CTCCTTTCTG
```

Sequence alignment of the amplicon with *Aspergillus oryzae* BGL (BGL1B) mRNA

```
Query  1     TTGCCAGTCCAATCGTCAGCAGCTGGCGGCTCAGACAAGGCCCGAGCATACTTGGTAGAA  60
             || || || || || ||  | || || || ||||  | ||| || || || || || || |
Sbjct  1307  TTACCGGTGCAGTCATCCTCCGCGGGTGGGTCAGGTAGGGCTCGTGCGTATTTTGTCGTG  1248

Query  61    TAATGGTTCATGCCAT-GAAAGCATTTATATGTGCCGTTCGACGAAGGACATCGCGCTCG  119
             |||||||||||| |||| ||| |  || ||  | || |  || || ||||  ||  ||||
Sbjct  1247  TAATGGTTCATACCATAGAACGAGTTGATTGGGGCAGACCGGCGGAGGAGGTCCAGCTCT  1188

Query  120   GCTTCCGTAAAGTAAGGCAGGCGATAACCCAGCGTAGCTCGCATCTCTGGGGGGTAGTCC  179
               ||   || || | |||||| |||   || |||  |||||||||    |  || || |||
Sbjct  1187  TCTGATGTGAACTCAGGCAGACGACTGCCAAGCTGAGCTCGCATGGGCGCTGGATAATCC  1128

Query  180   TGTCCGAGAAATACAGGGTCTCCAAAACAGCCGATGTAAAAGATCATCCACTTCTGGGCC  239
             | ||| || || |  ||||| || || || || ||||||||   ||  |    ||||||
Sbjct  1127  TTTCCCAGGAAGATGGGGTCACCGAACCAACCAATGTAAAACTCCAAGCGACGCTGGGCA  1068

Query  240   GCGTCCTTGTGGGCCTGGCTGGAAACGTCCCATGGCT-AAAATATGATCCATTCAACACT  298
             ||   |   ||  | | ||||  | | ||||| |||| | ||||    |||||||  ||
Sbjct  1067  GCCAACCGATGTTCTTCGCTGCCAGCATCCCACGGCTCATAATAGTGGCCATTCAGGACG  1008

Query  299   ATCGAAATTTCTCCTTTCTG  318
             |||||||| || ||||||||
Sbjct  1007  ATCGAAATATCCCCTTTCTG  988
```

The sequences that produced significant alignments with the sequence of GH1 amplicon are given in Table 6.4. The homology was with β-glucosidase proteins which belonged to GH1 which probably prove that the primers had indeed amplified a GH1 β- glucosidase.

Table 6.4. Sequences producing significant alignment with the GH1 BGL amplicon

Description	Max score	Total score	Query cover	E value	Identity	Accession
Aspergillus oryzae RIB40 beta-glucosidase 1B, mRNA	82.4	82.4	100%	2e-12	66%	XM_001824278.2
Aspergillus flavus NRRL3357 beta-glucosidase, putative, mRNA	82.4	82.4	100%	2e-12	66%	XM_002381394.1
Aspergillus oryzae RIB40 DNA, SC113	82.4	82.4	100%	2e-12	66%	AP007166.1
Nectria haematococca mpVI 77-13-4 hypothetical protein, mRNA	66.2	66.2	27%	2e-07	76%	XM_003039709.1
Talaromyces stipitatus ATCC 10500 beta-glucosidase, putative, mRNA	59.0	59.0	44%	3e-05	71%	XM_002485735.1

Sequencing of the amplicon produced using GH3 primer pairs gave a 562 bp sequence (seq BGLF3) which produced significant alignment with a putative GH3 β-glucosidase from *Aspergillus nidulans* GH-Family 3 BGL Partial gene sequence

GH-Family 3 BGL Partial gene sequence

```
>Seq_BGLF3
CGTACTGTCTCGCGATGCATCTAGATTAAGTTAGCAGTTCCAGATCCCCACCCCATGGCAAGTGTTCCCTGGGCACA
GCCACGGTCGTCGCAACCGTTGGCACCGTCTTTGCTTGAGCCGGCGTCCTCACCAAGAATGGCGACATTGCGCTCCT
TTCCAGACAAAGGCAAGGAGCCCTCGTTCTTGAGAAGAACAACGCTGTCTGCTGCAACGCGGCGAATGATAGAAGCG
TGGTCGCGCTGGACGTCCACAAACTCGTTGACCGTGTCGTATGCCCCTTCAGAGACAGCAAAGTGCTCGAAGCCCTT
CTCCTGTCGGGTCCAGGAGCTGAAGTTCGGGGGCTGGTACAGACGGTCACGGCCAACCTTGTAGAAACCGGCCATAA
TTCGGACAGCCATATCATCGACGCGCCACTGAGGAATGGTGCCGTTCAGAACAGCAACCGTCAGGTTGGCGCCAAAG
TAAGATTGTCCGTCGTTGAAGGCAATGTCACCAGGCATGGTCATGTCCATTCCAGCAAGAGCAGCACCGACACCGCT
GTGTGTCGCAGACCAATCCGTCATTACGAAGCCTTGGAAAACCGAAATCGGATCCCGGGCCCGTCGACTGCAGAGGC
CTGCATGCAAGCTTTCCCTATAGTGAGTCGTATTAGAGCTTGGCGGTATTCATGGTCATAGCTGTTTCCTGTGTGAA
ATTGTTATCCGCTTCACAATTCCACACACATACGAGCCGGAAGCATAAGGTGTTAAGCCTGGGGTGCCTAATGGAGT
GAGCTAACCTCCACATAATTGGCGTGGCGCTCACTGGCCGGCTTTCAGTCGGAACCTGTCCTGCAGCTGCATTAATG
AATCGGCCAACGCGCGGAGAGCGTTGCGTATTGGAGCTTCGATCTCGTCATGATTCGTGGCTAGCTTCCGATCGTGA
GGTATCGCCACAAGCGTATACGTTATCCAGATCAGGGTAGCTGAAGGACTTGGTCATGTCTCTTAGTCTGATCGGTT
AGCCGATTCGTCTTCCATGTTCGATCTGCACTACTATAAGCATAGCTCAGGCCAA
```

Sequence alignment of the amplicon with *Aspergillus nidulans* FGSC A4 chromosome II Beta-glucosidase

```
Query  28       AAGTTAGCAGTTCCAGATCCCCACCCCATGGCAAGTGTTCCCTGGGCACAGCCACGGTCG  87
                |||||  ||  ||  |||   |||||  ||||||||||| |||||  ||  |||||  ||||||||
Sbjct  2023583  AAGTTGGCTGTACCACTACCCCAACCCATGGCAAGGGTTCCTTGAGCACAACCACGGTCA  2023642

Query  88       TCGCAACCGTTGGCACCGTCTTTGCTTGAGCCGGCGTCCTCACCAAGAATGGCGACATTG  147
                |||||  ||  ||  |||||||      | |||||  ||  ||||||||||  ||  |||||||||
Sbjct  2023643  TCGCAGCCATTTGCACCGTAGGGATTGGAGCCCGCATCCTCACCAAGGATAGCGACATTG  2023702

Query  148      CGCTCCTTTCCAGACAAAGGCAAGGAGCCCTCGTTCTTGAGAAGAACAACGCTGTCTGCT  207
                ||||||||||| |  | ||| |  || ||||||||||| || || || |  ||||||||
Sbjct  2023703  CGCTCCTTTCCGGTGAGAGGAAGCGAACCCTCGTTCTTCAGGAGTACGATACTGTCTGCA  2023762

Query  208      GCAACGCGGCGAATGATAGAAGCGTGGTCGCGCTGGACGTCCACAAACTCGTTGACCGTG  267
                 |||||||  ||  ||||  ||||||  |||||  |||||||  ||  ||||||||  |||||  |||
Sbjct  2023763  CCAACGCGACGGATGAGAGAAGCATGGTCTTGCTGGACATCAACAAACTCATTGACTGTG  2023822

Query  268      TCGTATGCCCCTTCAGAGACAGCAAAGTGCTCGAAGCCCTTCTCCTGTCGGGTCCAGGAG  327
                 ||||  ||  |||  ||   |  | |  |||  |||  |  |||||  ||    |  |||||||||
Sbjct  2023823  CCGTAGGCGCCTCCATCAATCGAAGCGTGTTCGTACCCCTTTTCTGCCCTCGTCCAGGAG  2023882

Query  328      CTGAAGTTCGG-GGGCTGGTACAGACGGTCACGGCCAACCTTGTAGAAACCGGCCATAAT  386
                ||||||||  ||  |||  |||  ||  ||||||||||||||||||||||  |||  |||||||  |
Sbjct  2023883  CTGAAGTTGGGCGGGGTGGC-CAAACGGTCACGGCCAACCTTGTAAAAAGCGGCCATGAC  2023941

Query  387      TCGGACAGCCATATCATCGACGCGCCACTGAGGAATGGTGCCGTTCAGAACAGCAACCGT  446
                ||||||  |||||  ||||||||  |||||  ||  ||||  |||  |||||  ||||||    |  |||
Sbjct  2023942  TCGGACGGCCATGTCATCGACACGCCATTGGGGAACGGTTCCGTTGAGAACACTGATCGT  2024001

Query  447      CAGGTTGGCGCCAAAGTAAGATTGTCCGTCGTTGAAGGCAATGTCACCAGGCATGGTCAT  506
                ||||||||  ||||  |||||||   | ||  ||||||||||  |||||  ||||||||  |||||
Sbjct  2024002  CAGGTTGGGGCCATAGTAAGAGAGCCCATCGTTGAAGGCGATGTCGCCAGGCATTGTCAT  2024061

Query  507      GTCCATTCCAGCAAGAGCAGCACCGACACCGCTGTGTGTCGCAGACCAATCCGTCATTAC  566
                ||||||  |||||  |||||||   ||||||||||||||  ||||||||||  ||   ||||  ||
Sbjct  2024062  GTCCATACCAGCCAGAGCAGAGCCGACACCGCTGTGCGTCGCAGACCAGTCGCTCATGAC  2024121

Query  567      GAAGCCTTGGAAAACCGAAATC  588
                 ||  ||  |  ||||  ||  ||  ||
Sbjct  2024122  AAACCCCT-GAAAGCCTAATTC  2024142
```

Table 6.5. Sequences producing significant alignment with the GH3 BGL amplicon

Description	Max score	Total score	Query cover	E value	Identity	Accession
Aspergillus nidulans FGSC A4 chromosome II	457	457	69%	4e-126	78%	BN001302.1
Aspergillus nidulans FGSC A4 hypothetical protein AN4102.2 partial mRNA	457	457	69%	4e-126	78%	XM_656614.1
Aspergillus aculeatus beta-glucosidase (bgl1) mRNA, partial cds	300	300	67%	6e-79	73%	JN121997.1
Aspergillus aculeatus beta-glucosidase (bgl1) gene, partial cds	300	300	67%	6e-79	73%	JN121996.1
Aspergillus niger isolate ASKU28 beta-glucosidase mRNA, partial cds	279	279	69%	2e-72	71%	JX127252.1

GH-Family 5 BGL Partial gene sequence

```
>Seq_BGLF5
ACGGCGATCGAGGCCATAAACGAGCCCCATATCCCCGGTGGGGTTAACCAGGAACAGCTCAAGGAATACTACACCAACACACTGGC
GACGGTTCGAGCAAATAACCCCGACGCAGCTTTGTTTATAAGTGACGGGTTTTTGGAAACTGAGAGCTGGAACGGTTTCCTGTCCG
GAGATAACGTCTTCATGGATCACCATCACTACGAAGTCTTCGAGGGCGGACAGAACGGTTGGAGCGTACAAAAACATGTGGACGCT
GCCTGTGCACTGGGTCTTCAACACCTCGAAGCTGTCGACAAGCCAGTTGTCGTCGGCGAATGGACTGGCGCCATGACAGACTGCAC
AAGATACCTCAACGGCAGGGGCATTGGGGCTCGTTATGACGGGACGTTGTCTTCGAACGACGCAGTCGGTTCTTGCGGTAATTGGG
CTGATGGCAGCATTTCTCGCTTGAGCTCGGACGAAATTACCAACACCCGTCGCTTCAT
```

Sequence alignment of the amplicon with *Aspergillus nidulans* FGSC A4 hypothetical protein AN4052.2 partial mRNA

```
Query  6     GATCGAGGCCATAAACGAGCCCCATATCCCCGGTGGGGTTAACCAGGAACAGCTCAAGGA  65
             ||| |||||| |||| ||||||||| | || || ||  | |||||||| ||||||||||
Sbjct  819   GATTGAGGCCCTAAATGAGCCCCATGTTCCTGGCGGCATCAACCAGGACCAGCTCAAGGA  878

Query  66    ATACTACACCAACACACTGGCGACGGTTCGAGCAAATAACCCCGACGCAGCTTTGTTTAT  125
              ||||||    | || ||||| | ||| ||    || |  ||||| ||  |  || |  |
Sbjct  879   TTACTACGAAGAGACCCTGGCAAGGGTGCGGAAGAACAGTCCCGAAGCGACCCTGCTCCT  938

Query  126   AAGTGACGGGTTTTTGGAAACTGAGAGCTGGAACGGTTTCCTGTCCGGAGATAACGTCTT  185
                 || |||||  || |||| ||  ||||||| |||||  || | ||||| || ||  |
Sbjct  939   GCACGATGGGTTCGTGCAAACGGAAGGCTGGAATGGTTTTATGACTGGAGAGAATGTTAT  998

Query  186   CATGGATCACCATCACTACGAAGTCTTCGAGGGCGGACAGAACGGTTGGAGCGTACAAAA  245
              |||||   ||| |||||||||| ||||| ||||| ||||| |  |||||| |  ||||
Sbjct  999   GATGGACACCCACCACTACGAAGTTTTCGAAGGCGGCCAGAATGCGTGGAGCATTGAAAA  1058

Query  246   ACATGTGGACGCTGCCTGTGCACTGGGTCTTCAACACCTCGAAGCTGTCGACAAGCCAGT  305
              ||| | || || || ||   ||| || | ||| || ||  | |||| ||||||||| ||
Sbjct  1059  GCATATTGATGCCGCTTGCCAACTCGGACGTCAGCATCTACAGGCTGCCGACAAGCCTGT  1118

Query  306   TGTCGTCGGCGAATGGACTGGCGCCATGACAGACTGCACAAGATACCTCAACGGCAGGGG  365
              | | || ||||| ||||| || ||| || |||| || || ||||| || ||||| | |||
Sbjct  1119  TATAGTGGGCGAGTGGACAGGAGCCCTGTCAGATTGTACTAGATATCTAAACGGAAAGGG  1178

Query  366   CATTGGGGCTCGTTATGACGGGACGTTGTCTTCGAACGACGCAGTCGGTTCTTGCGGTAA  425
              |||||    || || ||||| ||||||  |||||||   ||||||||  ||||||| |
Sbjct  1179  AATTGGAATCCGCTACGACGGTACGTTGGGTTCGAACACAGCAGTCGGCGCTTGCGGAAG  1238

Query  426   TTGGGCTGATGGCAGCATTTCTCGCTTGAGCTCGGACGAAATTACCAACACCCGTCGCTT  485
              |  |  ||| || ||| |  || || ||||| | || || ||  | ||||| ||||||||
Sbjct  1239  TAAGAGTGAGGGTAGCGTGGCTGGCCTGAGCGCAGATGAGATCGCGAACACACGTCGCTT  1298

Query  486   CAT  488
              ||
Sbjct  1299  TAT  1301
```

Table.6.6. Sequences producing significant alignment with the GH5 BGL amplicon

Description	Max score	Total score	Query cover	E value	Identity	Accession
Aspergillus nidulans FGSC A4 chromosome	235	235	98%	1e-59	71%	BN001302.1
Aspergillus nidulans FGSC A4 hypothetical protein AN4052.2 partial mRNA	235	235	98%	1e-59	71%	XM_656564.1
Aspergillus clavatus NRRL 1 exo-beta-1,3-glucanase (Exg1), putative (ACLA_031040), partial mRNA	78.8	78.8	73%	2e-12	66%	XM_001269796.1
Aspergillus niger CBS 513.88 glucan 1,3-beta-glucosidase A, mRNA	71.6	71.6	14%	3e-10	82%	XM_001398831.2
Penicillium marneffei ATCC 18224 exo-beta-1,3glucanase (Exg1), putative, mRNA	71.6	71.6	16%	3e-10	80%	XM_002150320.1

Results from these studies indicated that the β-glucosidase isoforms in *Aspergillus unguis* could be products of different genes since the sequences differed from each other and aligned with sequences of BGLs from different Glycosyl hydrolase families.

6.3.5. N-terminal to partial sequence of the A. unguis GH1 BGL through TAIL PCR

Genomic DNA of *Aspergillus unguis* was used as the template for identifying the 5`end of the GH1 BGL fragment and TAIL PCR was performed. Partial gene sequence information obtained from PCR using degenerate primers was used for generating degenerate primers for TAIL –PCR. A multiple sequence alignment of the GH1 BGL amplicon (using degenerate primers) with matching BGL sequences from NCBI database was generated using ClustalW and this was used for generating degenerate primers for TAILPCR. While the conventional method of doing TAILPCR involves three cycles of PCR amplification, here instead of the tertiary cycle, the amplicons with a size range of 750-1000 bp was eluted from the gel after the second PCR cycle itself. A schematic representation of the method followed is given Fig 6.7. After ligation and transformation the clones were confirmed using the same primers as used for generating the amplicons.

Fig.6.6. Schematic representation of the position of GHF1 N-terminal signatures and partial gene sequence of *Aspergillus unguis GH1 BGL* gene and the location of active site signatures

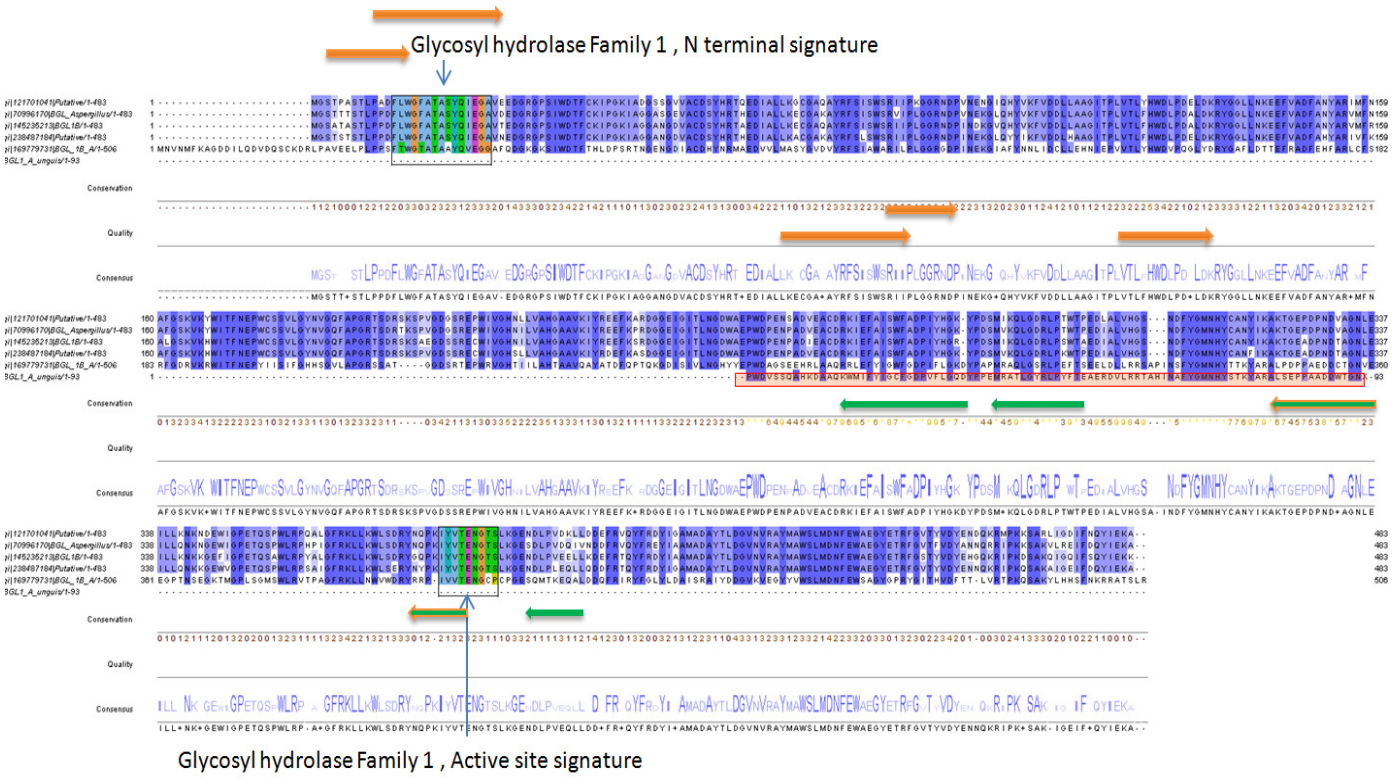
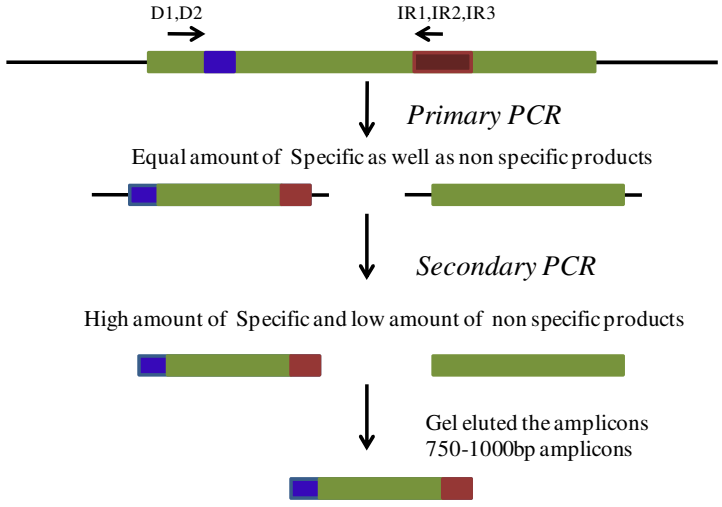
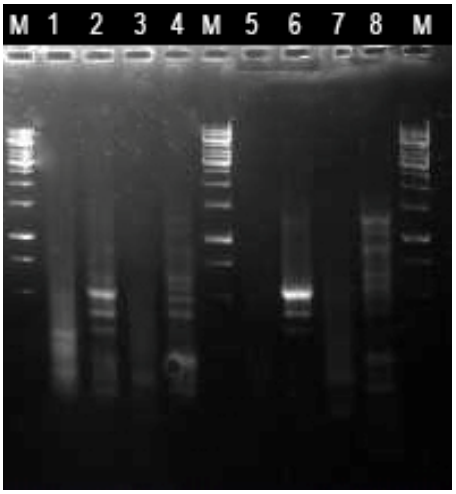


Fig.6.7.Schematic representation of steps followed in TAIL PCR



The blue colored regions in Fig 6.7, indicates the sequence of the gene near the N-terminal region of GH1 and red color indicates the partial gene sequence already obtained using degenerate primers from GH1.The primers selected for amplifying the N-terminal region (Blue color) were D1 and D2 which are degenerate primers and the reverse primers were specific IR1 and IR3 (Table 6.2). The amplicons obtained using these primers were in the size range 0.25-1.5kb. Selected amplicons of size 0.5-1 kb were cloned into suitable vectors. Four clones were selected for plasmid isolation and conformation of the clone was done using PCR amplification. M13 Forward and reverse primers were selected for PCR confirmation.

Fig.6.8. TAIL PCR amplicons and clone confirmation



Lane information

M GeneRuler ® 1kb ladder (Fermentas)

Primary PCR

1 Primer pair BGL D1_IR1

2 Primer pair BGL D1_IR3

3 Primer pair BGL D2_IR1

4 Primer pair BGL D2_IR3

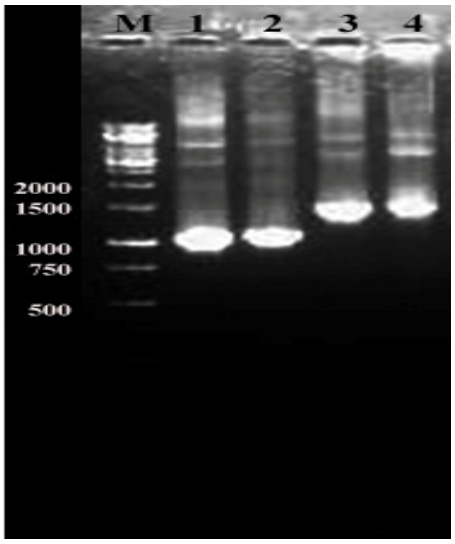
Secondary PCR

5 Primer pair BGL D1_IR1

6 Primer pair BGL D1_IR3

7 Primer pair BGL D2_IR1

8 Primer pair BGL D2_IR3



Lane information

M GeneRuler ® 1kb ladder (Fermentas)

Primary PCR

1-2 Clone Confirmation (Primer pair BGL D1_IR3)

3-4 Clone Confirmation(Plasmid Primer pair BGL D2_IR3)

Assembly of sequences had provided a longer read for the GH1 BGL nucleotide sequence with 840 bp

>Seq_BGLF1

TCTGGGACACGTTCGGTCACACCCCAGGTAAAGTCAAAGACGGCAGCAGTGCAGATGATGGAGTGAGGTCTTACGAC
TTCTATCGCGAAGATGTTGCGCTGATGAAATCGTATGGCGTCAACGCCTATCGATTCTCGCTGTCGTGGTCGCGAAT
CATCCCTCTTGGTGGTGCGGACGATCCAGTCAACGAGCAAGGGATCACATATTACCAAAACTTGATTGATGAGCTGC
TCCAGAATGGGATTACACCATTTGTGACCCTTTTCCATTGGGACGTTCCTCAAGCCCTGGAAGACCGGTATGGAGGC
ATGCTGAACAAGGAGAGGTTTGTTCCTGACTTTATCCGCTATGCGCGTGTCTGCTTCGAACGACTAGGGCCTCGGGT
TCGGCACTGGATTACATTCAACGAGCCAGGGGTATACTCGCTGGCTGGCTACGCAGCGGGGGTTCATGCTCCTGCGC
GATCTTCCTATCGAGAGCGCAACGAAGAGGGAGATTCATCTACGGAGCCTTTCACCGTGGGCCACACGGAGCTGGTG
GCCCACGGACATGTGTCGAAGCTATACCGAGAGGAGTTCCAGCAGCAGCAGAAGGGAACGATTGGGATTACCCTTCA
CGGTAATTGGTCAGAGCCTTGGGATGAGGACGACGTGCGCGACCAGGAGACGGCAGAACGGGCTCGCGAATTTGAGA
TTGCATGGTTTGCAGACCCTTTGTATAGCACTGGAGATTACCCTGCCTCAATGCGGGCTCAACTTGGAGATCGTCTG
CCGCATTTTACACCTGAGGAGTCGAAGCTGGTGCTGGGCTCGTCTGAATTCTACGGAATGAACTCGTACA

There was significant similarity of this BGL1 with a hypothetical GH1 family BGL enzyme from *Aspergillus nidulans*

Sequence alignment of the amplicon with *Aspergillus nidulans* FGSC A4 hypothetical protein AN4052.2 partial mRNA showed that there was 80% identity between the partial sequence of *A unguis* GH1 BGL and the putative BGL from *Aspergillus nidulans* FGSC A4 chromosome VI beta-glucosidase, putative (AFU_orthologue; AFUA_3G12600)

```
Query  1        TCTGGGACACGTTCGGTCACACCCCAGGTAAAGTCAAAGACGGCAGCAGTGCAGATGATG  60
                |||||||||| ||||| || || ||||| ||||||||||   |||| ||| ||||| |
Sbjct  1756036  TCTGGGACACATTCGGGCATACTCCAGGCAAAGTCAAAGACAATAGCAATGCGGATGACG  1755977

Query  61       GAGTGAGGTCTTACGACTTCTATCGCGAAGATGTTGCGCTGATGAAATCGTATGGCGTCA  120
                  |||||||  |||||||| || ||||||||||||||| |||||||| |||||||| ||||
Sbjct  1755976  CTGTGAGGTTCTACGACTTTTACCGCGAAGATGTTGCCCTGATGAAGTCGTATGGTGTCA  1755917

Query  121      ACGCCTATCGATTCTCGCTGTCGTGGTCGCGAATCATCCCTCTTGGTGGTGCGGACGATC  180
                |||| |||||||||||||| || |||||||| || ||||| || || || || ||||| |
Sbjct  1755916  ACGCTTATCGATTCTCGCTATCCTGGTCGCGCATAATCCCACTCGGAGGCGCCGACGACC  1755857

Query  181      CAGTCAACGAGCAAGGGATCACATATTACCAAAACTTGATTGATGAGCTGCTCCAGAATG  240
                | ||||| |||||||||||||  || |||||  || || | || ||||| ||| | ||||
Sbjct  1755856  CGGTCAATGAGCAAGGGATCAAGTACTACCAGGACCTGGTAGACGAGCTACTCAATAATG  1755797

Query  241      GGATTACACCATTTGTGACCCTTTTCCATTGGGACGTTCCTCAAGCCCTGGAAGACCGGT  300
                ||||||| ||||| || ||||| || || ||||| ||||| || ||  |||| || ||||
Sbjct  1755796  GGATTACGCCATTCGTAACCCTCTTTCACTGGGATGTTCCGCAGGCTTTGGAGGATCGGT  1755737

Query  301      ATGGAGGCATGCTGAACAAGGAGAGGTTTGTTCCTGACTTTATCCGCTATGCGCGTGTCT  360
                | || |||||||||||| ||||||||||  |||||||||||| ||||||| ||||||||||
Sbjct  1755736  ACGGGGGCATGCTGAACCAGGAGAGGTTCATTCCTGACTTTGTCCGCTACGCGCGTGTCT  1755677

Query  361      GCTTCGAACGACTAGGGCCTCGGGTTCGGCACTGGATTACATTCAACGAGCCAGGGGTAT  420
                | |||||||| ||||||||   ||| || ||||||||||||||||| || || || || |
Sbjct  1755676  GTTTCGAACGCCTAGGGCCAAAGGTCCGTCACTGGATTACATTCAATGAACCTGGTGTGT  1755617

Query  421      ACTCGCTGGCTGGCTACGCAGCGGGGGTTCATGCTCCTGCGCGATCTTCCTATCGAGAGC  480
                |||||||||| || || || || || ||||||||||||||| || || || | ||| || |
Sbjct  1755616  ACTCGCTGGCAGGTTATGCGGCAGGTGTTCATGCTCCTGCACGGTCATCGTTTCGCGAAC  1755557

Query  481      GCAACGAAGAGGGAGATTCATCTACGGAGCCTTTCACCGTGGGCCACACGGAGCTGGTGG  540
                 ||| |||||||| ||||| || || || || |||| ||| |||||||| || ||||||
Sbjct  1755556  TCAATGAAGAGGGTGATTCCTCGACAGAACCGTTCATCGTCGGCCACACAGAACTGGTGA  1755497

Query  541      CCCACGGACATGTGTCGAAGCTATACCGAGAGGAGTTCCAGCAGCAGCAGAAGGGAACGA  600
                ||||||| ||||| || ||||| |||||||||| |||||| | ||||||||| ||||| |
Sbjct  1755496  CCCACGGCCATGTTTCCAAGCTTTACCGAGAGGTGTTCCAACCGCAGCAGAAAGGAACAA  1755437

Query  601      TTGGGATTACCCTTCACGGTAATTGGTCAGAGCCTTGGGATGAGGACGACGTGCGCGACC  660
                |||| || ||||| || || || ||||| || |||||||||| |||||   ||| ||||
Sbjct  1755436  TTGGCATCACCCTCCATGGCAACTGGTCGGAACCTTGGGATGAAGACGATCCGCGGGACC  1755377

Query  661      AGGAGACGGCAGAACGGGCTCGCGAATTTGAGATTGCATGGTTTGCAGACCCTTTGTATA  720
                ||||  | ||||||||||  | ||||| ||||| || ||||| || ||||| || || |
Sbjct  1755376  AGGAAGCAGCAGAACGGGCCAGGGAATTCGAGATCGCTTGGTTCGCGGACCCCTTATACA  1755317

Query  721      GCACTGGAGATTACCCTGCCTCAATGCGGGCTCAACTTGGAGATCGTCTGCCGCATTTTA  780
                  || || || ||||| |||||||||||||||||| || || ||||| |||| ||| |
Sbjct  1755316  AGACAGGCGACTACCCAGCCTCAATGCGGGCTCAACTCGGGGACCGTCTACCGCGTTTCA  1755257

Query  781      CACCTGAGGAGTCGAAGCTGGTGCTGGGCTCGTCTGAATTCTACGGAATGAACTCGTACA  840
                | || |||||||| || ||||| || ||    || ||||| ||||| |||||||||||||
Sbjct  1755256  CTCCCGAGGAGTCAAAACTGGTACTAGGAAGTTCAGAATTTTACGGTATGAACTCGTACA  1755197
```

Table.6.7. Sequences producing significant alignment with the GH1 BGL amplicon

Description	Max score	Total score	Query cover	E value	Identity	Accession
Aspergillus_nidulans FGSC A4 chromosome VI	776	776	100%	0	80%	BN001306.1
Aspergillus_nidulans FGSC A4 hypothetical protein AN3106.2 partial mRNA	776	776	100%	0	80%	XM_655618.1
Neosartorya fischeri NRRL 181 beta-glucosidase (NFIA_064710) partial mRNA	567	567	100%	3e-159	75%	XM_001263203.1
Aspergillus_fumigatus Af293 beta-glucosidase (AFUA_3G12600), partial mRNA	504	504	100%	3e-140	74%	XM_749268.1
Aspergillus terreeus NIH2624 hypothetical protein (ATEG_04135) partial mRNA	489	489	100%	6e-136	73%	XM_001213313.1
Penicillium_chrysogenum Wisconsin 54-1255 hypothetical protein (Pc13g09130) mRNA, complete cds	477	477	99%	4e-132	73%	XM_002559292.1
Penicillium_chrysogenum Wisconsin 54-1255 complete genome, contig Pc00c13	477	477	99%	4e-132	73%	AM920428.1

Six frame translation of the 840bp gene sequence of GH1 β-glucosidase from *Aspergillus unguis* yielded a 280 amino acid partial protein sequence from the 3rd (5'-3') frame.

>GH1-BGL_A unguis

W D T F G H T P G K V K D G S S A D D G V R S Y D F Y R E D V A
L **M** K S Y G V N A Y R F S L S W S R I I P L G G A D D P V N E Q
G I T Y Y Q N L I D E L L Q N G I T P F V T L F H W D V P Q A L
E D R Y G G **M** L N K E R F V P D F I R Y A R V C F E R L G P R V
R H W I T F N E P G V Y S L A G Y A A G V H A P A R S S Y R E R
N E E G D S S T E P F T V G H T E L V A H G H V S K L Y R E E F
Q Q Q Q K G T I G I T L H G N W S E P W D E D D V R D Q E T A E
R A R E F E I A W F A D P L Y S T G D Y P A S **M** R A Q L G D R L
P H F T P E E S K L V L G S S E F Y G **M** N S Y

6.3.6. Protein-protein BLAST analysis of translated sequences

Protein-protein BLAST (blastp) of peptide sequences was used to search the proteins homologous to *Aspergillus unguis* GH1-BGL. Frame 3 translation 5`-3` (280 amino acids) showed significant similarities with Putative β-1,4-glucosidase of *Aspergillus nidulans* var. (AFU_orthologue; AFUA_3G12600: CBF83397.1) with 100 % query coverage and 90 % identity. Also the sequence had close similarity (100 % query coverage and 84 % identity) to *Neosartorya fischeri NRRL 181* β-glucosidase (XP_001263204.1) and *Aspergillus fumigatus* β-glucosidase (100% query coverage and 83% identity (XP_754361.1).

The matching protein *Aspergillus nidulans* putative β-glucosidase is having 488 amino acid sequence. The N-Terminal signature sequence for glycosyl hydrolase Family 1 is F-x-[FYWM]-[GSTA]-x-[GSTA]-x-[GSTA](2)-[FYNH]-[NQ]-x-E-x-[GSTA] and the active site consensus sequence is [LIVMFSTC]-[LIVFYS]-[LIV]-[LIVMST]-E-N-G-[LIVMFAR]-[CSAGN] where E is the active site residue. The protein sequence of *Aspergillus unguis* BGL and other GHF1 BGL alignment starts from ~ 40 amino acids downstream of the N-Terminal region of most of the significantly similar β-glucosidases and it extends up to 279 amino acids.

The protein showed significant similarity to a putative GH1 β-glucosidase from *Aspergillus nidulans* FGSC A4 and to other GH1 β-glucosidases from filamentous fungi confirming that the sequence obtained by degenerate PCR amplification followed by TAIL-PCR is indeed a Glycosyl hydrolase family 1 β-glucosidase. Major GH1 β-glucosidases producing significant alignments with the *A unguis* GH1 BGL protein are listed in Table 6.7.

Table 6.8. GH1-β-glucosidases producing significant alignments with the *A unguis* GH1 BGL

Description	Max Score	Total Score	Query cover	E-value	Identity	Accession
TPA: beta-glucosidase, putative (AFU_orthologue; AFUA_3G12600) [*Aspergillus nidulans* FGSC A4]	541	541	100%	0.0	92%	CBF83397.1
beta-glucosidase [*Neosartorya fischeri* NRRL 181] >gb|EAW21307.1| beta-glucosidase [*Neosartorya fischeri* NRRL 181]	519	519	100%	0.0	87%	XP_001263204.1
hypothetical protein AN3106.2 [*Aspergillus nidulans* FGSC A4] >gb|EAA63677.1| hypothetical protein AN3106.2 [*Aspergillus nidulans* FGSC A4]	545	545	100%	6e-178	92%	XP_660710.1
beta-glucosidase [*Aspergillus fumigatus* Af293] >gb|EAL92323.1| beta-glucosidase, putative [*Aspergillus fumigatus* Af293]	511	511	100%	1e-177	86%	XP_754361.1
Pc13g09130 [*Penicillium chrysogenum* Wisconsin 54-1255] >emb|CAP91982.1| Pc13g09130 [*Penicillium chrysogenum* Wisconsin 54-1255]	508	508	100%	1e-176	84%	XP_002559338.1
putative beta-glucosidase [*Penicillium oxalicum* 114-2]	500	500	100%	1e-173	84%	EPS29909.1
hypothetical protein ATEG_04135 [*Aspergillus terreus* NIH2624] >gb|EAU35937.1| hypothetical protein ATEG_04135 [*Aspergillus terreus* NIH2624]	496	496	100%	6e-172	84%	XP_001213313.1
Beta-glucosidase, putative [*Penicillium digitatum* PHI26] >gb|EKV17719.1| Beta-glucosidase, putative [*Penicillium digitatum* Pd1]	491	491	100%	8e-170	81%	EKV05985.1

6.4. Conclusions

Filamentous fungi have several *BGL* gene sequences but their cellular location and expression level variations are difficult to find out because some of them directly utilize cellobiose while others may act as inducers. The occurrence of three *BGL* genes has been detected in *Aspergillus unguis* and these sequences are from GH1, GH3 and GH5. Glycosyl hydrolase Family 1 *bgl* gene showed 92% identity to its parental organism *Aspergillus nidulans* GH1- *BGL* and it encodes 488 amino acids. Multiple sequence alignment with similar sequences showed similarity to the N-terminal region of the GH1-BGL sequence. Its catalytic nucleophile is glutamic acid. Glycosyl

hydrolase Family 3 and 5 partial gene sequence from *Aspergillus unguis BGL* also showed significant sequence similarity to the corresponding BGL gene sequence from *Aspergillus nidulans*. Sequence information on these three genes is important because they may aid in the expression of five BGL proteins/isoforms in *Aspergillus unguis*. Using this partial gene sequence, it may become easy to find out the full length gene sequence, which in turn can lead to the characterization of the BGL protein especially the glucose tolerant property.

Chapter 7. Proteomic characterization of β-glucosidases from *Aspergillus unguis* by peptide mass fingerprinting

7.1. Introduction

β-glucosidases are the major group among the glycosyl hydrolyzing enzymes and a number of bacterial, yeast and fungal β-glucosidase have been purified, cloned and characterized. At the sequence level, they are mainly placed either in Family 1 or Family 3 glycosyl hydrolases. Functionally Family 1 and Family 3 β-glucosidases are similar with the selective cleavage of β- 1, 4 glycosidic linkages in di and oligosaccharides. Most of the organisms, especially filamentous fungi express several isoforms of β-glucosidase and contribute to the overall activity of the crude enzyme. Hence sequencing and identification of the individual isoforms from pooled fraction is a very difficult task. Very few studies have been carried out to characterize the cellular location and activity contribution of β-glucosidase genes in filamentous fungi and some of the genes may act as an inducer for the expression of other β-glucosidase genes (Wu et al. 2013). In such cases, proteomic studies are more useful than genomic studies because it reflects the changes in the expression of the protein with respect to the external environment surrounding the organism. Proteome analysis is more difficult than genome analysis due to the high diversity of proteins within the same family and its abundance.

In proteomics one of the most important and widely used protein identification techniques combines separation of the protein using gel electrophoresis and Mass Spectrometry (MS). The two ionization techniques mainly used in Mass Spectrometry are the Electron Spray Ionization (ESI) and Matrix Assisted Laser Desorption Ionization (MALDI). The ESI or MALDI is connected to Time Of Flight (TOF) analyzers. The protein of interest is subjected to proteolysis with specific protease(s) and it results in several unique peptides, which are then compared with the theoretical masses derived from a sequence database. This data comparison and protein identification based on the masses usually done with the help of software (eg. example MASCOT). Main advantage of this technique compared to Edman degradation (Edman et al. 1950) is that only a small amount of protein is required for analysis, and the identification is very fast if the sequence database contains peptides with similar masses.

In this work, proteomic analysis was applied to perform the BGL protein profiling in *Aspergillus unguis NII 08123*. Among the five extracellular isoforms of BGL, BGL1, BGL3 and BGL5 were selected for identification.

7.2. Materials and methods

7.2.1. Production of the crude enzyme and partial purification

Enzyme production was carried out by submerged fermentation as described in chapter 2 (section 2.4.1) in a total volume of 2000 ml. Extraction of the enzyme was performed as outlined under section 2.4.3. The enzyme was concentrated and the BGL1, BGL3 and BGL5 isoforms were partially purified through ion exchange chromatography.

7.2.2. Electro elution of the ion exchange fractions

Electro elution was carried out to separate the three isoforms. Partially purified BGL proteins were run on Native PAGE and the BGL active bands were visualized by activity staining as outlined under section 2.6.1. 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 1h. After 1 hour it was inverted and again run for another 10 minutes to detach the protein from the dialysis bag and then the buffer from the dialysis bag was recovered. This solution was used for further studies.

7.2.3. In-solution digestion of BGL proteins

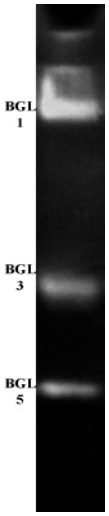
Protein spots were excised from the gel and digested with trypsin according to the protocol of (Trauger et al. 2002). Trypsin digestion was carried out by mixing 2.5µl protein (1µg/µl), 0.4µl trypsin (0.1µg/µl), 1.25µl NH_4HCO_3 (200mM), and 6µl H_2O and allowing the digestion to proceed at 37 °C overnight (12h). The resultant peptides were de-salted, concentrated and fractionated with ZIP TIP® (Millipore USA) as per the manufacturer's protocol. Protein was

eluted with a 60:40 acetonitrile : water mixture containing 0.1% TFA. The peptide solution was mixed with equal volumes of a 10mg/ml solution of α-cyano-4-hydroxycinnamic acid in 50% ACN/0.1% TFA and spotted onto a MALDI sample plate. MS/MS spectra were acquired in the positive ion mode on a MALDI-TOF Mass Spectrometer, (Axima-CFR Plus, Shimadzu, Japan). Database search for protein identification was performed with the mass spectrometry data using MASCOT search engine. The database query was made for microbial species against SwissProt and NCBI nr databases using only mono isotopic masses. MS-BLAST search was performed for sequence homology-based protein identity.

7.3. Results and Discussion

Aspergillus unguis NII 08123 is a producer of multiple β-glucosidases and was found to elaborate five secreted isoforms of the enzyme when grown with wheat bran and/or lactose as the carbon source. Among the five isoforms produced under solid state or submerged fermentation, three were stable and clearly visible in the zymogram analyses. They were named as BGL1, BGL3 and BGL5. BGL1 was the isoform with largest expression among the three and the protein had a high molecular weight. BGL5 was the glucose tolerant isoform which had a very low molecular weight. The expression levels and molecular weight of BGL3 was intermediate compared to the other two isoforms (Fig7.1).

Fig 7.1. Zymogram showing the major BGL isoforms in *Aspergillus unguis*

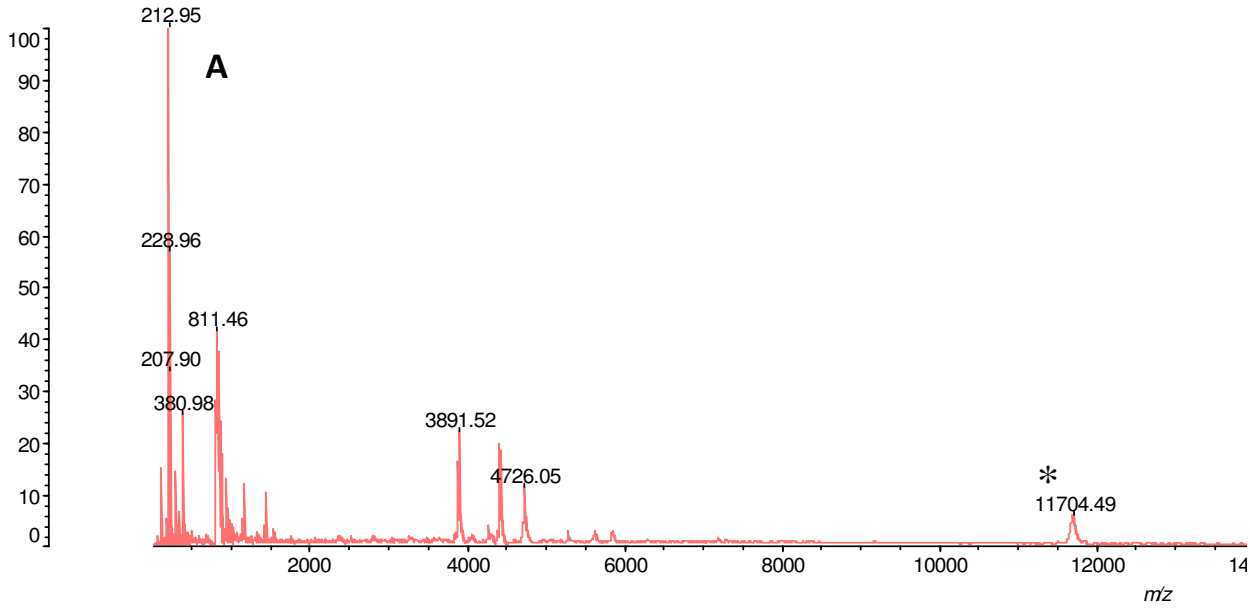


7.3.1. Peptide Mass Fingerprinting through MALDI-TOF and analysis with MASCOT

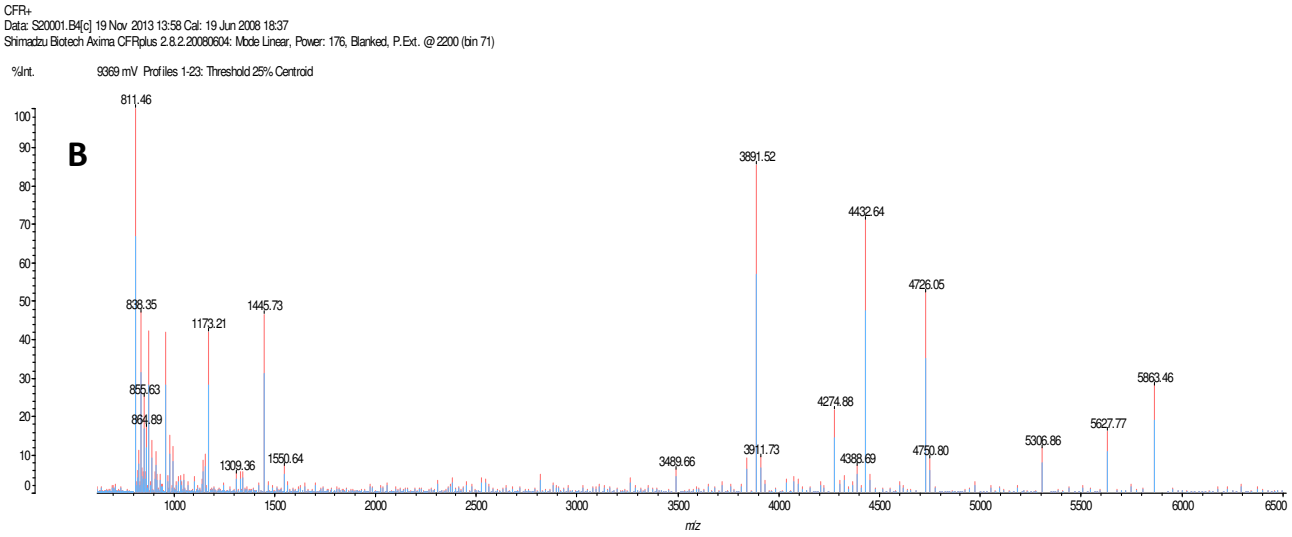
7.3.1.1. BGL1- Peptide mass mapping

The MS experiments were carried out with α-cyano-4-hydroxycinnamic acid as the MALDI matrix solution which is considered to be an excellent matrix for the analysis of peptides. This helps to form homogenous crystals with most of the peptides and ionization process greatly depends on the co-crystallization of both the sample and matrix. The spectra were obtained after in solution digestion and purification with ZIPTIP C_{18}. The sample mixed with the matrix solution were spotted on the plate and dried prior to the MALDI-TOF MS analysis. The MALDI-TOF mass spectra of BGL1 protein is given in Fig 7.2 A-D

Fig 7.2. MALDI-TOF mass spectra of BGL1



**Glycosyl hydrolase Family 3 C-Terminal domain*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%Int.
9369 mV Profiles 1-23: Threshold 25% Centroid
B
811.46
838.35
855.63
864.89
1173.21
1309.36
1445.73
1550.64
3489.66
3891.52
3911.73
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4388.69
4432.64
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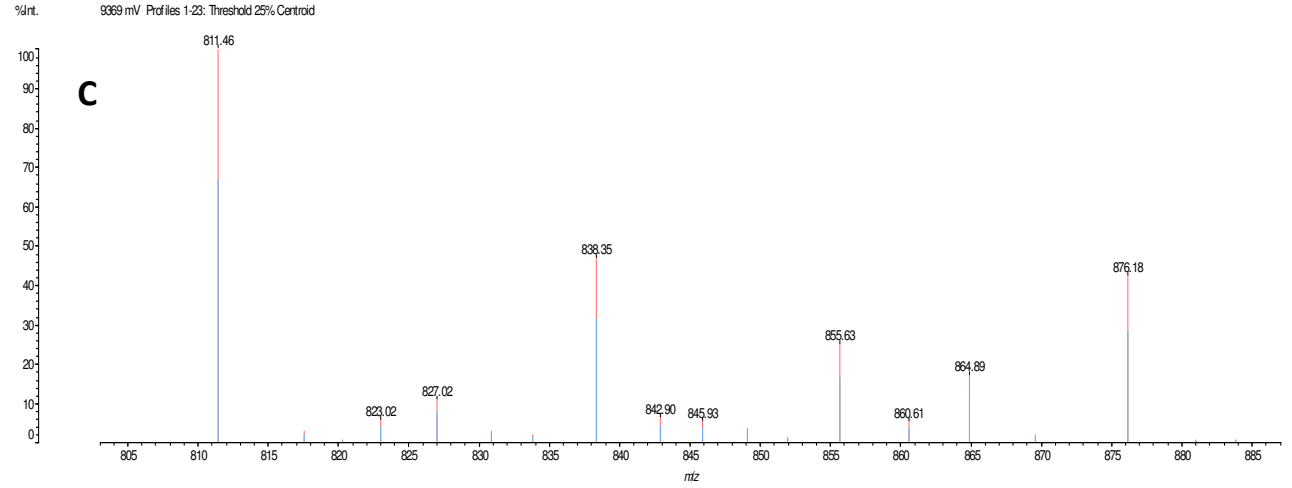
4750.80
5306.86
5627.77
5863.46
m/z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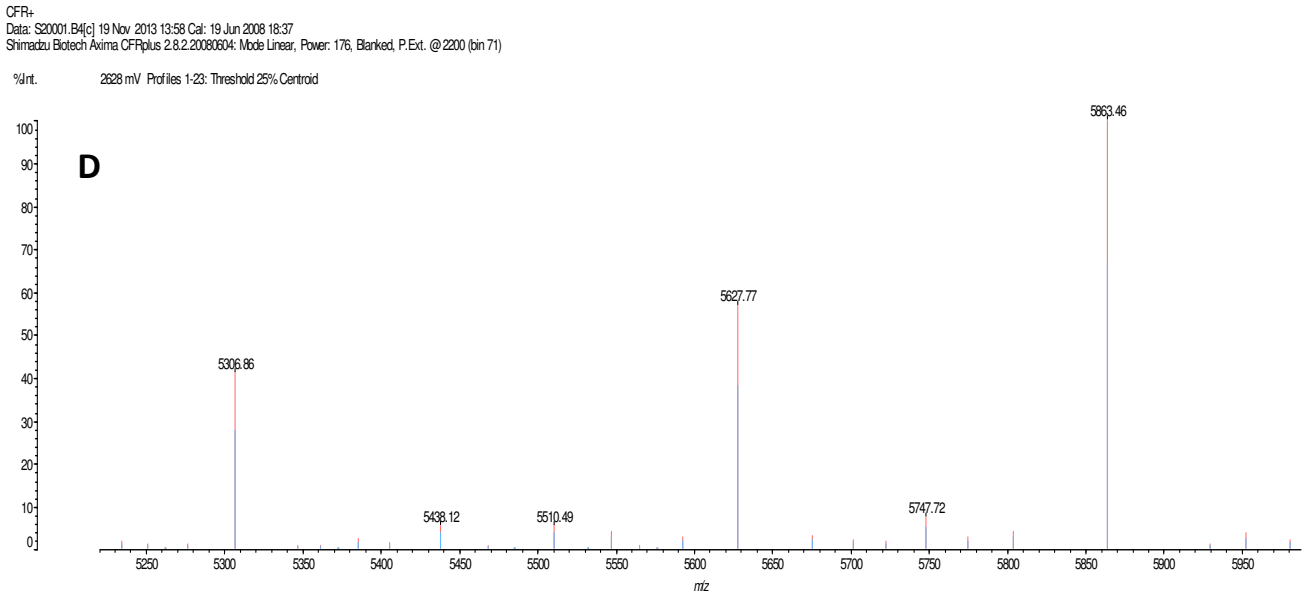
Data: S20001.B4[c] 19 Nov 2013 13:58 Cal: 19 Jun 2008 18:37
Shimadzu Biotech Axima CFRplus 2.8.2.20080604: Mode Linear, Power: 176, Blanked, P.Ext. @2200 (bin 71)
%Int.
9369 mV Profiles 1-23: Threshold 25% Centroid
C
811.46
823.02
827.02
838.35
842.90
845.93
855.63
860.61
864.89
876.18
m/z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Data: S20001.B4[c] 19 Nov 2013 13:58 Cal: 19 Jun 2008 18:37
Shimadzu Biotech Axima CFRplus 2.8.2.20080604: Mode Linear, Power: 176, Blanked, P.Ext. @2200 (bin 71)
%Int.
2628 mV Profiles 1-23: Threshold 25% Centroid
D
5306.86
5438.12
5510.49
5627.77
5747.72
5863.46
m/z

7.3.1.1.1. Mass spectral data analysis using MASCOT software

In-silico digestion (theoretical digestion) of the known proteins deposited in the protein database (Swiss Prot) was performed with the help of MASCOT (Matrix Science, Boston, USA) and the peak values ie theoretically calculated, experimentally observed and expected values were compared. Based on the analysis, the following matching results were obtained. Out of the 36 peaks obtained in the MALDI-TOF mass spectrum, 32 showed identity with β-glucosidase I of *Aspergillus flavus* with 69% protein sequence coverage (Table 7.1). The molecular mass of the protein is 92 kDa and the molecular weight as estimated by SDS-PAGE was 240 kDa, implying that BGL1 is a multimeric protein.

Table 7.1. List of the observed peak value and corresponding peptides from MASCOT search results

Start	End	Observed	Mr(expt)	Mr(calc)	Peptide
1	35	3911.7300	3910.7227	3908.0506	-.MPRLDVEKTIEELSLGEKVALTAGIDFWHTASVPR.L
2	8	849.0586	848.0513	855.4814	M.PRLDVEK.T
2	8	851.9565	850.9492	855.4814	M.PRLDVEK.T
2	8	855.6269	854.6196	855.4814	M.PRLDVEK.T
2	8	855.6300	854.6227	855.4814	M.PRLDVEK.T
2	8	864.8900	863.8827	855.4814	M.PRLDVEK.T
36	42	827.0155	826.0082	825.5072	R.LNIPTLR.M
36	42	830.8458	829.8385	825.5072	R.LNIPTLR.M
36	42	833.7887	832.7814	825.5072	R.LNIPTLR.M
114	157	4750.8000	4749.7927	4748.3657	R.GFESFAEDGVLSGLLAGYISKGIQEKGVAATLKHFVCNDQEHQR.M
158	195	4388.6900	4387.6827	4388.2815	R.MAVDSIVTQRALREIYLLPFQLAMRICRTACVMTAYNK.V
168	213	5306.8600	5305.8527	5305.8076	R.ALREIYLLPFQLAMRICRTACVMTAYNKVNGTHVSQNKEIITDILR.K
168	214	5438.1200	5437.1127	5433.9025	R.ALREIYLLPFQLAMRICRTACVMTAYNKVNGTHVSQNKEIITDILRK.E
296	308	1550.6400	1549.6327	1549.8801	K.ALNRPQDQALLRR.A
328	382	5747.7200	5746.7127	5751.8883	K.KEKSILVIGPNSKVAAYCGGGSASLDAYYTVNPFEGVSAQSKGEVKFSQGVYSHK.D
374	438	7301.1100	7300.1027	7304.7648	K.FSQGVYSHKDLPLLGPLLKTADGKTGFSFKVYNEHPSESNRELIEQLHLVSSSGFLMDYVNPKIK.S

500	511	1309.3600	1308.3527	1308.7037	K.GSKELKAGQTYK.V
506	538	3489.6600	3488.6527	3487.7889	K.AGQTYKVLFQFGTAPTSDLDTRGVVVFGPGGFR.F
544	556	1445.7300	1444.7227	1441.7889	R.RVGQEELISNAVK.L
544	652	11704.4900	11703.4827	11704.7392	R.RVGQEELISNAVKLASEAEQVVVFAGLTSEWETEGYDRDHM DLPPGSDEMISRVLDVNPNAVVVIQSGTPVTMPWANKTKALLH AWFGGNECGNGIADVLYGDVNPSGK.L
624	660	3891.5200	3890.5127	3896.9672	K.ALLHAWFGGNECGNGIADVLYGDVNPSGKLPITFPVR.L
653	688	4274.8800	4273.8727	4274.2025	K.LPITFPVRLQDNPSYVNFRSERGRVLYGEDVYVGYR.Y
693	744	5627.7700	5626.7627	5627.8200	K.VDLAPLFPFGHGLSYTTFTRSDLTLTTTPEKPQYEESGEPITAT VTVTNTGK.V
713	767	5863.4600	5862.4527	5861.0375	R.SDLTLTTTPEKPQYEESGEPITATVTVTNTGKVAGAEIVQLWV APPATEVNRPVR.E
745	784	4432.6400	4431.6327	4429.4638	K.VAGAEIVQLWVAPPATEVNRPVRELKGFTKVFLQPGEQKK.V
768	774	820.2900	819.2827	821.4647	R.ELKGFTK.V
768	774	823.0174	822.0101	821.4647	R.ELKGFTK.V
775	784	1173.2100	1172.2027	1172.6553	K.VFLQPGEQKK.V
784	825	4726.0500	4725.0427	4725.4727	K.KVEIVVEKKLATSWFDEMREKWASEKGEYGVLVTGTGEGV LK.S
785	791	811.4600	810.4527	814.4800	K.VEIVVEK.K
785	791	817.5695	816.5622	814.4800	K.VEIVVEK.K
803	809	876.1831	875.1758	876.4341	R.EKWASEK.G

GH3 BGL from *Aspergillus flavus* showing the regions identified in the *A. unguis* BGL1 (red)

```
  1 MPRLDVEKTI EELSLGEKVA LTAGIDFWHT ASVPRLNIPT LRMSDGPNGV
 51 RGTRFFNGVP AACFPCATAL GATWDTELLH EIGQLMGEES IAKGSHIILG
101 PTINTQRSPL GGRGFESFAE DGVLSGLLAG YISKGIQEKG VAATLKHFVC
151 NDQEHQRMAV DSIVTQRALR EIYLLPFQLA MRICRTACVM TAYNKVNGTH
201 VSQNKEIITD ILRKEWGWDG LVMSDWFGTY STSDAINAGL DLEMPGKTRW
251 RGTALAHAVS SNEVAEFVMD ERVRNVLNLV NFVDGLNIPE NAPEKALNRP
301 QDQALLRRAA AESVVLMKNE EDILPLKKEK SILVIGPNSK VAAYCGGGSA
351 SLDAYYTVNP FEGVSAQSKG EVKFSQGVYS HKDLPLLGPL LKTADGKTGF
401 SFKVYNEHPS ESNRELIEQL HLVSSSGFLM DYVNPKIKSL TYYVDMEGLF
451 TPEEDGVYDF GVTVVGTGQL FIDGELVVDN TKNQRQGSAF FGSATVEEKG
501 SKELKAGQTY KVLFQFGTAP TSDLDTRGVV VFGPGGFRFG ASRRVGQEEL
551 ISNAVKLASE AEQVVVFAGL TSEWETEGYD RDHMDLPPGS DEMISRVLDV
601 NPNAVVVIQS GTPVTMPWAN KTKALLHAWF GGNECGNGIA DVLYGDVNPS
651 GKLPITFPVR LQDNPSYVNF RSERGRVLYG EDVYVGYRYY EKVDLAPLFP
701 FGHGLSYTTF TRSDLTLTTT PEKPQYEESG EPITATVTVT NTGKVAGAEI
751 VQLWVAPPAT EVNRPVRELK GFTKVFLQPG EQKKVEIVVE KKLATSWFDE
801 MREKWASEKG EYGVLVTGTG EGVLKSSFKV EKTRYWLGL
```

The proteins identified as matching to the *A unguis* BGL1 are given below (Table 7.2)

Table 7.2. Proteins identified as matching to *A unguis* BGL1 by MASCOT analysis

Region	Length	Location	Comment
Matured chain length	839	1-839	β-glucosidase I
Bgl X	351	26-376	β-glucosidase related glycosidases
Glyco-Hyrdro-3	243	29-271	Glycosyl hydrolase Family 3 N-Terminal Domain
PA14	71	442-512	PA14 domain
GLYCO-Hydro-3C	160	548-707	Glycosyl hydrolase Family 3 C-Terminal Domain
Fn3-like	61	749-809	Fibronectin type III-like domain

7.3.1.2. BGL5- Peptide mass mapping

The mass spectrum of trypsin digested BGL5 protein showed identity with the glycosyl hydrolase C-terminal domain of the β-glucosidase F of *Emericella nidulans*. Only two peaks showed identity with the conserved region (Fig 7.3 A-C, Table 7.3). BGL-5 is an approximately 10kDa protein with high glucose tolerance. MALDI-MS results also proved that maximum size of the protein is below 15kDa. Almost all BGL proteins deposited in SwissProt and NCBI non-redundant database have a molecular size above 20kDa. BGL-5 being a rare glucose tolerant β-glucosidase with a low molecular weight of ~10kDa, it is difficult to compare the observed values with the theoretical values obtained from the protein sequence present in the database. The peak value (8179.66) from the spectrum showed match with the BGL-F C-terminal conserved domain, which is common to all glycosyl hydrolase family 3 (GH3) members. GH3 C-terminal domain (pfam00933) is involved in catalysis and may also be involved in binding of beta-glucan. However, since the complete sequence of a low molecular weight β-glucosidase protein or gene sequence being not available in the database, the identification of this isoform was inconclusive and it was difficult to assign the type of β-glucosidase family it belongs to. Nevertheless, based on the MALDI –Spectrum, it may be presumed that the BGL5 belongs to the GH3 since it contains the GH3 catalytic domain. Further studies are essential to describe the details of this BGL-5.

Fig 7.3. MALDI-TOF mass spectra of BGL5

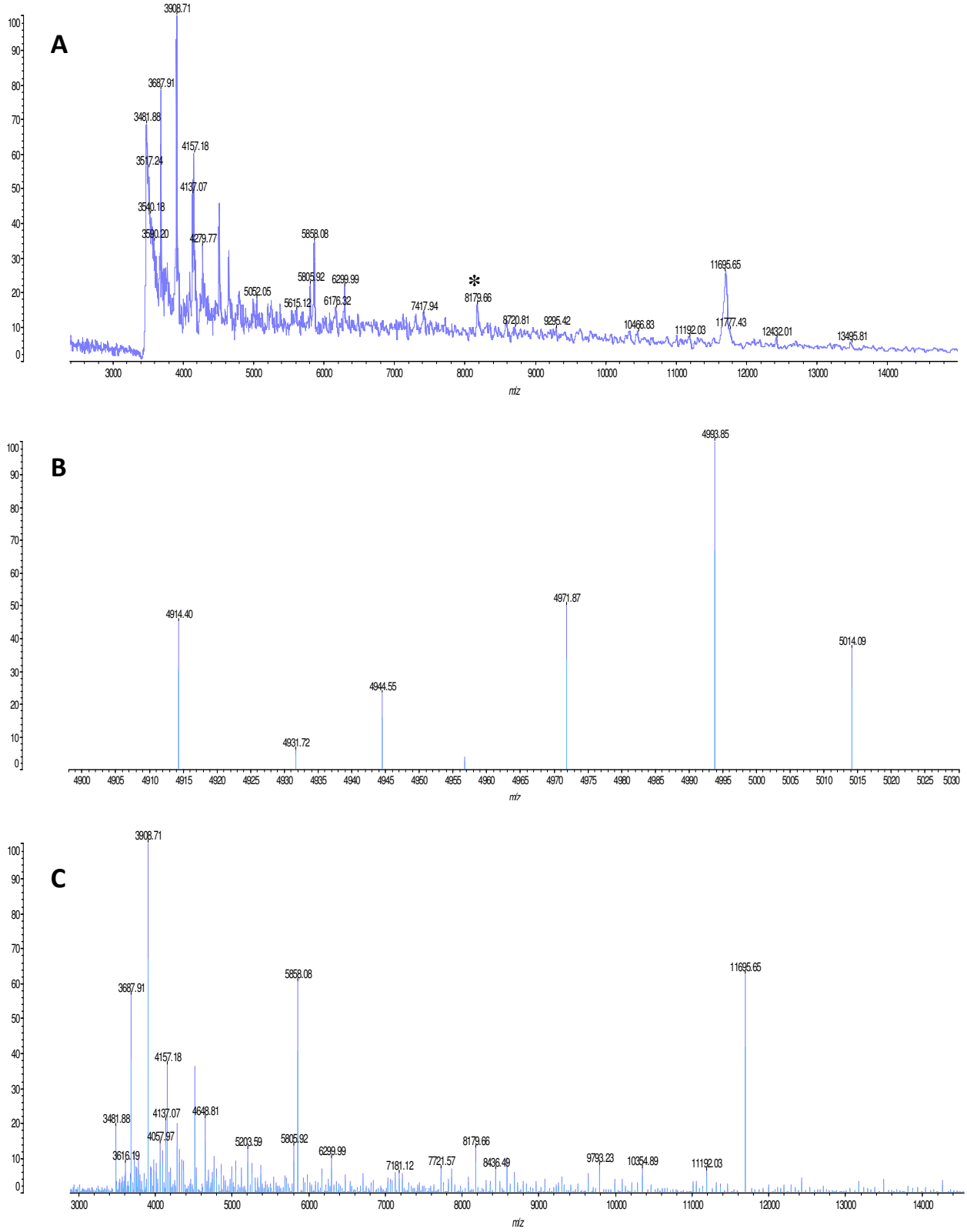


Table 7.3. List of the observed peak value and corresponding peptides from MASCOT analysis of BGL5

Start	End	Observed	Mr(expt)	Mr (calc)	Peptide
445	521	8179.66	8179.6600	8179.8896	K.GCNRGVLTMGWGSGTSKLPYLITPQEAIANI TPTAEFFITDSFPSSVDANDEDIAIVFINSDSGEN YITVDGNPGDR.K
730	777	5014.09	5014.0900	5014.3547	K.TYPYPDGYSTDPQPPPRAGGAEGGNPALWD VAFSVQVTVTNTGQHSGR.A

GH3 BGL from *Emericella nidulans* showing the regions identified in the *A. unguis* BGL5 (red)

```
  1 MAHRWLILAL VAAAAPRALA SPGPSLNERQ SDDEPFSPPY YPAPNGGWVS
 51 TWAEAYEKAH SIVSNLTLAE KVNLTTGTGI FMGPCAGQTG SVPRLGIPNL
101 CLHDSPLGVR NTDHNTAFPP GITVGATFDK SLMYERGVGL GEEARGKGVN
151 VLLGPSVGPL GRKPRGGRNW EGFGFDPVLQ GIGGAETIKG MQSTGLIACI
201 KHFVGNEQEM HRMSSVVTQG YSSNIDDRTL HELYIWPFAE GVRAEVGSVM
251 IAYNDVNKSS CSQNSKLING VLKDELGFQG FVVTDWLAHY GGVSSALAGL
301 DMDMPGDGAV PLFGNSYWGP ELSRSILNGT VPVERLNDMV TRILATWYKM
351 GQDQDYPLPN FSSNTEDEKG LLYPGAVISP IGVVNQYVNV QGNHNITARA
401 IARDAITLLK NEGDLLPLRR NDSLKVFGTD AGPDPQGLNS CADKGCNRGV
451 LTMGWGSGTS KLPYLITPQE AIANITPTAE FFITDSFPSS VDANDEDIAI
501 VFINSDSGEN YITVDGNPGD RKTSGLHAWH NGDELVKAAA ERFSQVVVVI
551 HTVGPIILEE WIDLDSVKAV LIAHLPGQEA GYSLTDVLFG DYSPSGHLPY
601 TIPYQESNYP SSVGLLQQPF GQIQDYYTEG LYIDYRHFLK EDITPRYAFG
651 HGLSYTTFEF SEPALSVVTP LDSAYPPSRP AKGPTPTYPN TIPPASEAAW
701 PAKFNRIWRY IYPYLNNPQA DAAVANSSKT YPYPDGYSTD PQPPPRAGGA
751 EGGNPALWDV AFSVQVTVTN TGQHSGRAVA QLYVELPDSL GLDTPSRQLR
801 QFEKTKVLET GQSETLTLEV TRKDVSVWDV EVQDWKTVVG GEGVKIHIGE
851 SVLDIRTECE  VGGRCVTL
```

Table 7.4. Proteins identified as matching to *A. unguis* BGL5 by MASCOT analysis

Region	Length	Location	Comment
Matured chain length	868	1-868	β-glucosidase F, *Emericella nidulans*.
Bgl X	227	408-635	Glycosyl hydrolase family 3 C-terminal domain

7.3.1.3.. BGL3- Peptide mass mapping

BGL3 matches to *Aspergillus aculateus* β-glucosidase-1 protein. Molecular weight of the protein is 92 kDa with 860 amino acid residues. MALDI-MS spectra peptide masses were unique to BGL1 of *Aspergillus aculeatus*. Matching peptide details are summarized in Table 7.5, and it is a glycosyl hydrolase family 3 β-glucosidase.

Fig 7.4. MALDI-TOF Mass spectra of BGL 3

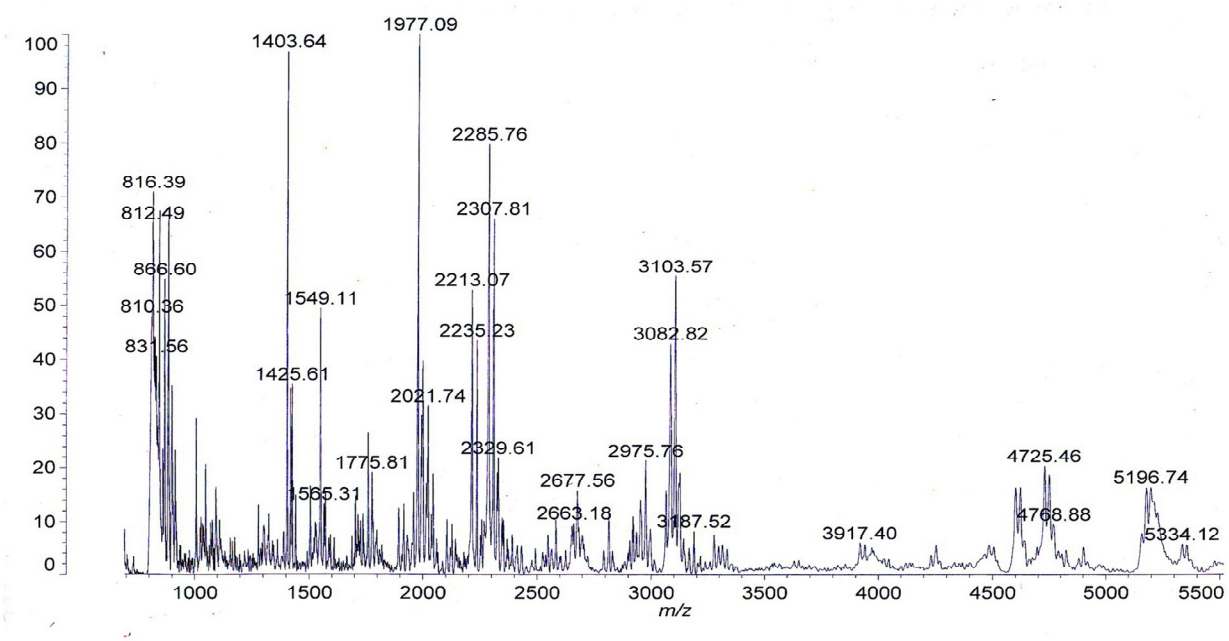


Table 7.5. List of the observed peak value and corresponding peptides from MASCOT analysis of BGL3

Start	End	Observed	Mr(expt)	Mr(calc)	Peptide
47	59	1403.6400	1403.6400	1403.7330	R.AVAIVSQMTLDEK.V
349	366	2235.2300	2235.2300	2234.0433	R.LYQPPNFSSWTRDEYGFK.Y
387	404	1977.0900	1977.0900	1979.1163	R.NHSEVIRKLGADSTVLLK.N
473	521	5196.7400	5196.7400	5196.5276	K.HKGSVYAITDNWALSQVETLAKQASVSLV FVNSDAGEGYISVDGNEGDR.N
718	764	4725.4600	4725.4600	4725.2219	K.ASSGDPYYGVDTAEHVPEGATDGSPQPVLP AGGGSGGNPRLYDELIR.V

GH3 BGL from *Aspergillus aculeatus* showing the regions identified in the *A. unguis* BGL3 (red)

```
  1  MKLSWLEAAA   LTAASVVSAD   ELAFSPPFYP   SPWANGQGEW   AEAYQRAVAI
 51  VSQMTLDEKV   NLTTGTGWEL   EKCVGQTGGV   PRLNIGGMCL   QDSPLGIRDS
101  DYNSAFPAGV   NVAATWDKNL   AYLRGQAMGQ   EFSDKGIDVQ   LGPAAGPLGR
151  SPDGGRNWEG   FSPDPALTGV   LFAETIKGIQ   DAGVVATAKH   YILNEQEHFR
201  QVAEAAGYGF   NISDTISSNV   DDKTIHEMYL   WPFADAVRAG   VGAIMCSYNQ
251  INNSYGCQNS   YTLNKLLKAE   LGFQGFVMSD   WGAHHSGVGS   ALAGLDMSMP
301  GDITFDSATS   FWGTNLTIAV   LNGTVPQWRV   DDMAVRIMAA   YYKVGRDRLY
351  QPPNFSSWTR   DEYGFKYFYP   QEGPYEKVNH   FVNVQRNHSE   VIRKLGADST
401  VLLKNNNALP   LTGKERKVAI   LGEDAGSNSY   GANGCSDRGC   DNGTLAMAWG
451  SGTAEFPYLV   TPEQAIQAEV   LKHKGSVYAI   TDNWALSQVE   TLAKQASVSL
501  VFVNSDAGEG   YISVDGNEGD   RNNLTLWKNG   DNLIKAAANN   CNNTIVVIHS
551  VGPVLVDEWY   DHPNVTAILW   AGLPGQESGN   SLADVLYGRV   NPGAKSPFTW
601  GKTREAYGDY   LVRELNNGNG   APQDDFSEGV   FIDYRGFDKR   NETPIYEFGH
651  GLSYTTFNYS   GLHIQVLNAS   SNAQVATETG   AAPTFGQVGN   ASDYVYPEGL
701  TRISKFIYPW   LNSTDLKASS   GDPYYGVDTA   EHVPEGATDG   SPQPVLPAGG
751  GSGGNPRLYD   ELIRVSVTVK   NTGRVAGDAV   PQLYVSLGGP   NEPKVVLRKF
801  DRLTLKPSEE   TVWTTTLTRR   DLSNWDVAAQ   DWVITSYPKK   VHVGSSSRQL
851  PLHAALPKVQ
```

7.4. Conclusions

Three major BGL proteins from *Aspergillus unguis* NII08123 were subjected to MALDI-MS analysis and the spectrum showed BGL proteins belonging to the glycosyl hydrolase family-3. From the MALDI spectrum, peak values were taken and analyzed with MASCOT software against the SwissProt database. The results showed that BGL1 peptide mass match the β-glucosidase-I of *Aspergillus flavus*. The analysis showed that the peptide was having 69% protein sequence coverage. BGL-3 showed similarity to BGL1 of *Aspergillus aculateus*. Mainly 5 peaks were showing similarity to the corresponding peptide mass of *Aspergillus aculateus* BGL1 protein. The most important among the three proteins was the BGL5 which was the glucose tolerant enzyme with a low molecular weight (~10kDa) which makes this protein different from the already deposited β-glucosidases in the NCBI non-redundant and SwissProt databases. The MS-Data analysis of BGL5 was performed by searching Swiss Prot database and several BGL protein hits were obtained. The best hit was β-glucosidase F from *Emericella nidulans* with a molecular mass of 93.86 kDa and two peptides were matched to this particular protein which were

1) K.GCNRGVLTMGWGSGTSKLPYLITPQEAIANITPTAEFFITDSFPSSVDANDEDIAIVFINSDS GENYITVDGNPGDR.K,
2) K.TYPYPDGYSTDPQPPPRAGGAEGGNPALWDVAFSVQVTVTNTGQHSGR.A.

The first peptide covers 90% of the catalytic domain of the GH3 and this peptide is around 80% of the entire BGL-5 protein. Proteins with very low molecular weights as in this case have never been reported for GH3 β-glucosidases and the observation becomes really important. The occurrence of a fully functional 10kDA protein with glucose tolerant BGL activity has tremendous implications both from the points of understanding the structure function relationships as well as the applications. Further studies are essential to support the data and to understand better about the protein structure.

Chapter 8. Biomass hydrolysis using β-glucosidase from *Aspergillus unguis*

8.1. Introduction

Biofuels have gained worldwide attention because of its potential use as an alternative source of energy and lignocellulosic biomass is the most abundant source on earth for fuel production. Conversion of lignocellulosic biomass involves three major steps ie. pretreatment, hydrolysis and fermentation. One of the major applications of β-glucosidase involves the enzymatic hydrolysis of lignocellulosic biomass to fermentable sugars which is considered to be more efficient than acid hydrolysis. Since most cellulase producing microorganisms are not efficient producers of β-glucosidase, commercial enzyme cocktails require an additional supply of β-glucosidase. The leading cellulase producing companies in the world - Genencor and Novozymes have launched multiple iterations of their biomass hydrolyzing enzymes. Major blends produced by Genencore are Accelerase®1500, Accelerase®XP, Accelerase®XC and Accelerase®BG and Accellerase®Duet. Accelerase®1500 is a cellulases complex (exoglucanase, endoglucanase, hemi-cellulase and β-glucosidase) while the other 3 are accessory enzymes. Accelerase®XP enhances the conversion of both xylan and glucan in the lignocellulosic biomass. Accelerase®XC contains both hemicellulase and cellulase activities so that it can convert both hemicelluloses and cellulose. Accelerase®BG contains β-glucosidase enzymes. In addition to exoglucanase, endoglucanase, β-glucosidase, Accellerase®Duet contains xylanase also (Dupont-Genencor 2012). Novozymes have launched two new commercial mixtures of enzymes for hydrolysis of lignocellulosic biomass: Cellic Ctec and Cellic Htec. Cellic Ctec versions are mainly meant for cellulosic conversion and Cellic Htec for hemicelluloses conversion. So mixtures of these two are capable of converting carbohydrates from a wide variety of pretreated biomass such as corn cob, sugarcane bagasse, corn fiber and wood pulp in to simple sugars (Novozyme 2010). Among the cellulase enzymes, β-glucosidase is the rate limiting enzyme which determine the overall process efficiency for the conversion of cellulose to glucose. β-glucosidase undergoes feedback inhibition when the concentration of product ie.glucose reaches to a particular threshold. Hence glucose tolerant β-glucosidases have remarkable significance in controlling the reaction rate of cellulose to glucose and hence as ingredients in biomass hydrolyzing enzymes

In this study hydrolysis of different feed stock were tried using cellulase from *Penicillium janthinellum* and β-glucosidase from *Aspergillus unguis*. Here three goals aimed were 1) To increase the efficiency of the total sugar yield, 2) Decrease the time required for hydrolysis to yield sugars by increasing the reaction rate, and 3) Reduce the cellulase loading by the supplementation of BGL.

8.2. Materials and Methods

8.2.1. Organism and culture conditions

Penicillium janthinellum was a kind gift from Dr D V Gokhale, from the CSIR-National Chemical Laboratory, Pune, India.

8.2.1.1. Medium preparation

8.2.1.1.1. Solid State Fermentation (SSF) for the production of Cellulase and β-glucosidase

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 500 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 *Penicillium janthinellum* had the following composition in g/l- KH_2PO_4 - 20,$CaCl_2$ – 3, Urea-3g/L,$(NH_4)_2SO_4$- 14, $MgSO_4.7H_2O$ - 3, Peptone –2.5, Yeast extract-1g/L, Tween 80 - 0.5g/L. Trace elements: $FeSO_4.7H_2O$ - 0.5%, $MnSO_4.7H_2O$ –1.6 %, $ZnSO_4.7H_2O$ –1.4% and $CoCl_2.2H_2O$- 0.2%. Cellulose was used as the inducer along with wheat bran for the production of cellulase. 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.

Each flask was inoculated with 2ml of *Aspergillus unguis* spores containing 1 x 10^6 spores/ml or 2ml of 48hr old mycelial suspension in the case of *Penicillium janthinellum*. 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 50ml 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 8000 rpm for 15min at 4 °C to remove debris and was filtered again using glass microfiber filters (Whatman® GF/A). This filtrate was used as the crude enzyme preparation. *Aspergillus niger* β-glucosidase available at CSIR-NIIST was used as control in some studies. Enzyme assays were done as detailed under section 2.5.1 and 2.5.3 for β-glucosidase and cellulase respectively.

8.2.2. Pretreatment of Biomass

Sorghum, sugarcane bagasse, and rice straw were used for analyzing the saccharification efficiency of β-glucosidase supplemented along with cellulase. Sugarcane bagasse was steam exploded and it was a kind gift from Dr AJ Varma, CSIR-National Chemical Laboratory, Pune, India. Sorghum was a kind gift from Directorate of Sorghum Research, Hyderabad and Rice straw was procured locally. These two biomass feed stock were 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 either dilute acid or dilute alkali. In the case of acid pretreatment, 2% H_2SO_4 was used for 1h at 121°C in an autoclave. After cooling, the slurry was neutralized using 10N NaOH. In the case of alkali pretreatment, biomass was pretreated using 2% NaOH for 1 h at 121 °C in an autoclave. After cooling, the slurry was neutralized using 10N H_2SO_4. After neutralization the acid/alkali pretreated biomass was dewatered by filtration using a 140 mesh nylon sieve and washed several times in tap water, followed by a final rinse in distilled

water. The pretreated biomass was air dried at room temperature to remove moisture by spreading on paper sheets. The pretreated feed stock was stored in airtight containers until used.

8.2.3. Hydrolysis of biomass

The hydrolysis was carried out in 150 ml screw capped conical flasks with a working volume of 30ml. The biomass loading was 10% w/w, the cellulase loading was 20 FPUs/g and BGL loadings were 20 or 60 IU/g respectively. Incubation was performed at 50 °C for 48h with sampling at every 12h. Controls without BGL supplementation and with supplementation of 20 IU/g and 60IU/g. Commercial BGL preparations were run in parallel. After 48h, Samples were analyzed for glucose released by the NREL Laboratory Analytical Protocol for sugars using HPLC as well as DNS method as mentioned under section 2.5.4.

Table 8.1. Experiment matrix for study of biomass hydrolysis

Component	Amount Added		
Total reaction volume	30	30	30
Biomass (g –dry weight)	3	3	3
Cellulase (FPUs/g)	20	20	20
BGL (IU/g)	20	60	0
1M citrate buffer pH4.5 (ml)	1.5	1.5	1.5
Tween 80 (%)	0.06	0.06	0.06
Antibiotic mix [Himedia] ml	0.3	0.3	0.3
Distilled H_2O (ml) to make up total volume to 30ml	22.70	21.04	23.54

8.3. Results and Discussions

8.3.1. Preparation of enzyme cocktails for Biomass hydrolysis

Most of the commercial β-glucosidase are produced from *Aspergillus niger* and thus it helps the *T.reesei* cellulase for efficient and complete hydrolysis. Addition of β- glucosidase is necessary since the *T.reesei* lacks sufficient amount of β-glucosidase even though it is a good producer of cellulase (Gusakov et al. 2007). But beyond a certain threshold β-glucosidase undergo product inhibition and this results reduction in the efficiency of the entire hydrolysis process. Insufficient amount of β-glucosidase results the cellobiose accumulation which often leads to inhibition of the other two enzymes ie. exo and endoglucanase (Sukumaran et al. 2005). Hence the proper supplementation of β-glucosidase along with cellulase is essential and the ratio is also important. To overcome the product inhibition, glucose tolerant β-glucosidase can be used and the kinetic analysis also showed that *Aspergillus unguis* produces β-glucosidase with a K_i of 0.8M.

Hydrolysis efficiency also depends on the type of biomass and its pretreatment method. In alkali pretreatment lignin portion is getting removed and the hemicelluloses portion has to be hydrolyzed by hemicellulases where as in acid pretreatment hemicellulose portion is removed (Gray et al. 2006; Hahn-Hägerdal 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. But there is no general method of pretreatment because different types of biomass requires different type pretreatment since their structure and composition vary widely. Similarly there are no common enzyme cocktails for hydrolysis which can give hundred percentage efficiency for all the different types of feedstock. Dependence on the biomass and its pretreatment method, ratio of the enzyme cocktail combination also vary greatly.

8.3.1.1. Hydrolysis of steam exploded bagasse using enzyme blends

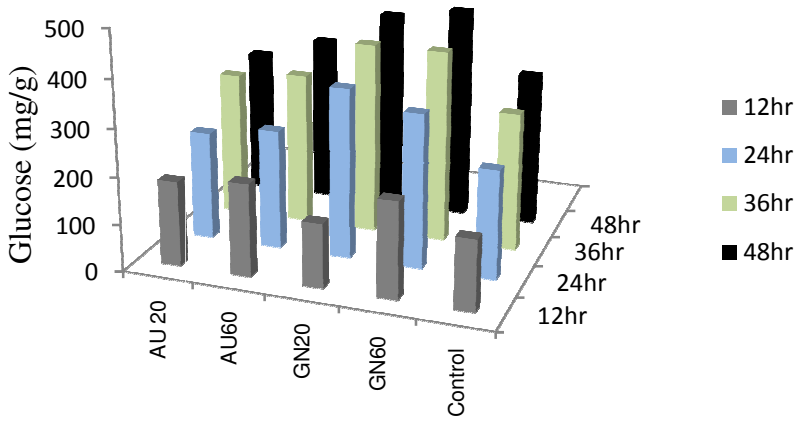
Penicillium janthinellum cellulase and *Aspergillus unguis* BGL were produced under Solid State Fermentation as mentioned in section 8.2. for the preparation of enzyme cocktails.

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; *Penicillium janthinellum enzyme*– Cellulase- 33 FPUs/ml, *Aspergillus unguis* BGL – 16 IU/ml, *Aspergillus niger* BGL - 571 IU/ml.

P. janthinellum cellulase preparation containing ~ 5.5 IU/ml BGL was blended with additional BGL from *Aspergillus unguis* so that the final BGL concentrations were either 20 or 60 IU/g SEB to be hydrolyzed. Hydrolysis experiments showed that there was a considerable improvement in hydrolysis performance when *A. unguis* BGL was blended with the cellulase. With 60 IUs/g additional BGL loading, there was a 20 % improvement inglucose yield even within 12h of hydrolysis. There was a linear improvement in hydrolysis performance for blends containing higher BGL loading, an efficiency of ~ 52.7 % of the theoretical maximum was achieved in 48h in blends containing 60 IU/g additional BGL loading (Table 8.2, Fig 8.1). Hydrolysis performance of blend containing 60IU/g of commercial BGL was significantly better (67.1% efficiency attained in 48h). This could be due to the presence of cellulase and hemicellulase activities in the commercial BGL sample which we had detected. Hydrolysis study done with one of the best biomass hydrolyzing enzyme blends available in the market could yield >75% efficiency with this biomass

Table 8.2. *Aspergillus unguis* β-glucosidase compared with commercial β- glucosidase for hydrolysis of SEB

Time (h)	*A. unguis* 20 IU/g		*A.unguis* 60 IU/g		GN 20 IU/g		GN 60 IU/g		Control –No addnl. BGL	
	Glucose (mg/g)	Efficiency (%)	Glucose (mg/g)	Efficiency (%)	Glucose (mg/g)	Efficiency (%)	Glucose (mg/g)	Efficiency (%)	Glucose (mg/g)	Efficiency (%)
12	179.7	26.8	195.8	29.2	135.5	20.2	203.4	30.4	149.5	22.3
24	228.5	34.0	251.5	38.0	358.4	53.0	324.8	48.0	229.1	34.0
36	306.0	45.7	322.0	48.0	404.0	60.3	405.0	60.4	292.0	43.6
48	308.0	46.0	353.4	52.7	425.4	63.5	449.0	67.1	325.9	48.6



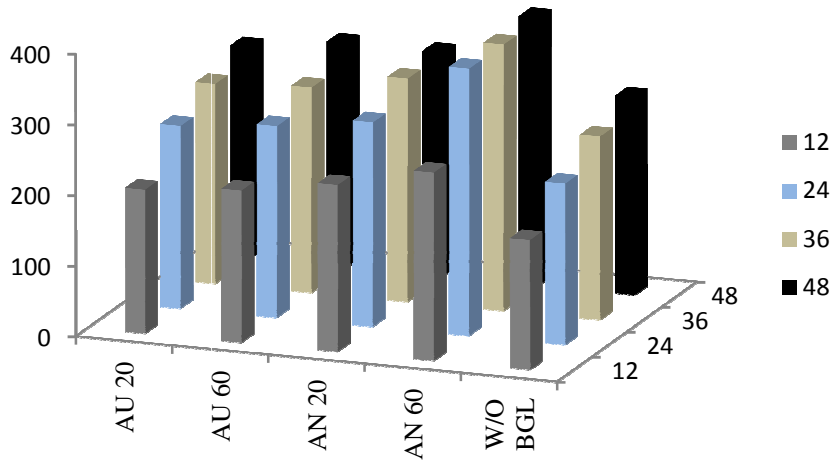
8.3.1.2. Hydrolysis of steam exploded bagasse: A comparative study using β-glucosidase from Aspergillus unguis and Aspergillus niger

Two different combinations of BGL loading were tried for both *Aspergillus unguis* glucose tolerant β-glucosidase and *Aspergillus niger* β-glucosidase along with *Penicillium janthinellum* cellulase. The substrate used here was steam exploded bagasse. From the results it was clear that addition of β- glucosidase increased the hydrolysis efficiency from 39 % to 57 %. The maximum reducing sugar yield obtained was 318 mg/g for *Aspergillus unguis* β-glucosidase where as *Aspergillus niger* β- glucosidase could yield 380mg/g of sugars (Table 8.3, Fig 8.2).

Table.8.3.Comparison of the hydrolysis performance of *Aspergillus unguis* and *Aspergillus niger* β-glucosidase preparations on SEB

Time (h)	*A. unguis* 20 IU/g		*A.unguis* 60 IU/g		*A. niger* 20 IU/g		*A. niger* 60 IU/g		Control –No addnl. BGL	
	Reducing sugar (mg/g)	Efficiency (%)	Reducing sugar (mg/g)	Efficiency (%)	Reducing sugar (mg/g)	Efficiency (%)	Reducing sugar (mg/g)	Efficiency (%)	Reducing sugar (mg/g)	Efficiency (%)
12	204	31	216	32	236	35	267	40	184	27
24	259	39	272	41	290	43	379	56	229	34
36	283	42	291	43	317	48	378	57	260	39
48	300	45	318	48	318	47	380	57	280	42

Fig .8.2.Comparison of the hydrolysis performance of *Aspergillus unguis* and *Aspergillus niger* β-glucosidase preparations on SEB



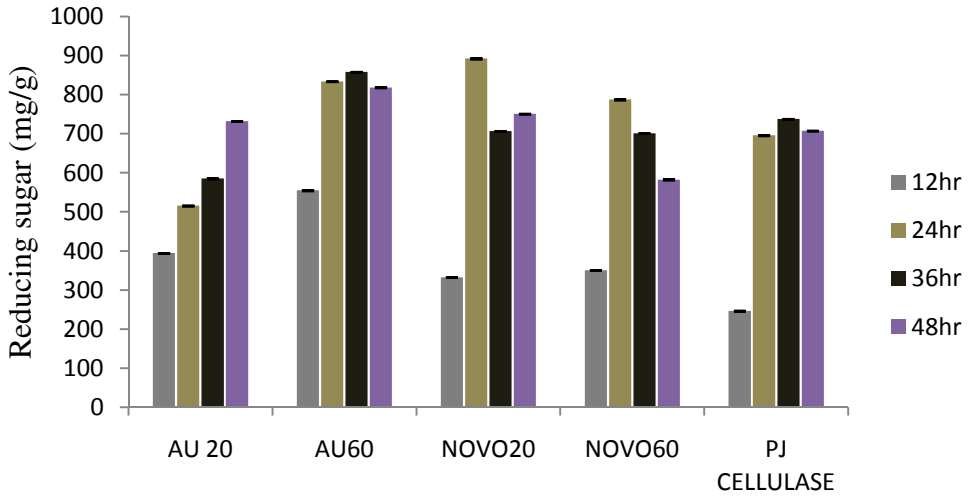
8.3.1.3. Hydrolysis of alkali pretreated rice straw using enzyme blends

Rice straw was alkali pretreated prior to hydrolysis since it contains more amount of lignin. Alkali pretreatment was effective for the removal of lignin. *Aspergillus unguis* glucose tolerant β-glucosidse or commercial β-glucosidase (Novozymes, Denmark) was supplemented along with *Penicillium janthinellum* cellulase. The maximum sugar yield of 555mg/g was obtained within 12h while using 60IU/g *Aspergillus unguis* β-glucosidase whereas 20 IU/g released 394mg/g (Table 8.4, Fig 8.3) Addition of *Aspergillus unguis* β-glucosidase increases the rate of the reaction compared to commercial β-glucosidase. Maximum efficiency (89%) was achieved within 24h while supplementing commercial β-glucosidase whereas; β-glucosidase from *Aspergillus unguis* yielded 83% efficiency with the same time interval. Hence it can be seen that the performance of commercial as well as *A.unguis* β-glucosidase are almost equal.

Table 8.4. Hydrolysis of alkali pretreated rice straw using commercial as well as *Aspergillus unguis* β- glucosidase

Time (h)	*A. unguis* 20 IU/g		*A.unguis* 60 IU/g		*Novozyme* 20 IU/g		*Novozyme* 60 IU/g		Control –No addnl. BGL	
	Reducing sugar (mg/g)	Efficiency (%)	Reducing sugar (mg/g)	Efficiency (%)	Reducing sugar (mg/g)	Efficiency (%)	Reducing sugar (mg/g)	Efficiency (%)	Reducing sugar (mg/g)	Efficiency (%)
12	394	39	555	55	333	33	351	35	247	25
24	516	51	834	83	892	89	787	78	696	69
36	585	58	858	85	707	70	701	70	738	73
48	732	73	819	81	751	75	583	58	708	70

Fig 8.3. Bar chart showing the comparative performance of commercial and *A unguis* BGLs



8.4. Conclusions

The above study explains the importance of BGL supplementation along with cellulase using two different types of biomass (Alkali pretreated rice straw and steam exploded bagasse). Addition of glucose tolerant β glucosidase could improve the rate of hydrolysis in both cases. High efficiency of hydrolysis could be achieved within short duration by use of *Aspergillus unguis* β-glucosidase. The experiments prove that the crude β-glucosidase preparation from Aspergillus unguis enzyme is a potent candidate for the enzyme cocktail preparation for biomass hydrolysis.

Chapter 9. Summary and Conclusions

Lignocellulosic biomass is probably the best alternative resource for biofuel production and it is composed mainly of cellulose, hemicelluloses and lignin. Cellulose is the most abundant among the three and conversion of cellulose to glucose is catalyzed by the enzyme cellulase. Cellulases are groups of enzymes act synergistically upon cellulose to produce glucose and comprise of endoglucanase, cellobiohydrolase and β-glucosidase. β-glucosidase assumes great importance due to the fact that it is the rate limiting enzyme. Endoglucanases (EG) produces nicks in the cellulose polymer exposing reducing and non reducing ends, cellobiohydrolases (CBH) acts upon the reducing or non reducing ends to liberate cellobiose units, and β-glucosidases (BGL) cleaves the cellobiose to liberate glucose completing the hydrolysis. β-glucosidases undergo feedback inhibition by their own product- glucose, and cellobiose which is their substrate. Few filamentous fungi produce glucose tolerant β-glucosidases which can overcome this inhibition by tolerating the product concentration to a particular threshold.

The present study had targeted a filamentous fungus producing glucose tolerant β-glucosidase which was identified by morphological as well as molecular method. The fungus showed 99% similarity to *Aspergillus unguis* strain which comes under the *Aspergillus nidulans* group where most of the glucose tolerant β-glucosidase belongs. The culture was designated the strain number NII 08123 and was deposited in the NII culture collection at CSIR-NIIST.

β-glucosidase multiplicity is a common occurrence in fungal world and in *A.unguis* this was demonstrated using zymogram analysis. A total 5 extracellular isoforms were detected in fungus and the expression levels of these five isoforms varied based on the carbon source available in the medium. Three of these 5 isoforms were expressed in higher levels as identified by the increased fluorescence (due to larger amounts of MUG breakdown by enzyme action) and was speculated to contribute significantly to the total β-glucosidase activity. These isoforms were named as BGL 1, BGL3 and BGL 5. Among the three, BGL5 was demonstrated to be the glucose tolerant β-glucosidase and this was a low molecular weight protein. Major fraction was a high molecular weight protein but with lesser tolerance to glucose. BGL 3 was between the two in both activity and glucose tolerance.

Glucose tolerant β-glucosidase was purified and characterized and kinetic analysis showed that the glucose inhibition constant (K_i) of the protein is 800mM and K_m and V_{max} of the enzyme was found to be 4.854 mM and 2.946 mol min^{-1}mg $protein^{-1}$respectively. The optimum temperature was 60°C and pH 6.0. The molecular weight of the purified protein was ~10kDa in both SDS as well as Native PAGE indicating that the glucose tolerant BGL is a monomeric protein.

The major β-glucosidase, BGL1 had a pH and temperature optima of 5.0 and 60 °C respectively. The apparent molecular weight of the Native protein is 240kDa. The V_{max} and K_m was 78.8 mol min^{-1}mg $protein^{-1}$ and 0.326mM respectively.

Degenerate primers were designed for glycosyl hydrolase families 1, 3 and 5 and the BGL genes were amplified from genomic DNA of *Aspergillus unguis*. The sequence analyses performed on the amplicons results confirmed the presence of all the three genes. Amplicon with a size of ~500bp was sequenced and which matched to a GH1 –BGL from *Aspergillus oryzae*. GH3 degenerate primers producing amplicons were sequenced and the sequences matched to β-glucosidase of GH3 family from *Aspergillus nidula*ns and *Aspergillus acculateus*. GH5 degenerate primers also gave amplification and sequencing results indicated the presence of GH5 family BGL gene in the *Aspergillus unguis* genomic DNA.

From the partial gene sequencing results, specific as well as degenerate primers were designed for TAIL PCR. Sequencing results of the 1.0 Kb amplicon matched *Aspergillus nidulans* β-glucosidase gene which belongs to the GH1 family. The sequence mainly covered the N-Terminal region of the matching peptide.

All the three BGL proteins ie. BGL1, BGL3 and BGL5 were purified by chromatography an electro elution from Native PAGE gels and were subjected to MALDI-TOF mass spectrometric analysis. The results showed that BGL1 peptide mass matched to β-glucosidase-I of *Aspergillus flavus* which is a 92kDa protein with 69% protein coverage. The glucose tolerant β-glucosidase BGL5 mass matched to the catalytic C-terminal domain of β-glucosidase-F from *Emericella nidulans,* but the protein coverage was very low compared to the size of the *Emericella nidulans* protein. While comparing the size of BGL5 from *Aspergillus unguis, the* protein sequence coverage is more than 80%. BGL F is a glycosyl hydrolase family 3 protein. The properties of BGL5 seem to be very unique, in that it is a GH3 β-glucosidase with a very low molecular weight of ~10kDa and at the same time having catalytic activity and glucose

tolerance which is as yet un-described in GH3 β-glucosidases. The occurrence of a fully functional 10kDA protein with glucose tolerant BGL activity has tremendous implications both from the points of understanding the structure function relationships as well as for applications of BGL enzymes. BGL-3 showed similarity to BGL1 of *Aspergillus aculateus* which was another GH3 β-glucosidase. It may be noted that though PCR could detect GH1, GH3 and GH5 β-glucosidases in the fungus, the major isoforms BGL1 BGL3 and BGL5 were all GH3 family enzymes. This would imply that β-glucosidases belonging to other families may also co-exist in the fungus and the other minor isoforms detected in zymograms may account for them.

In biomass hydrolysis, GT-BGL containing BGL enzyme was supplemented to cellulase and the performance of blends were compared with a cocktail where commercial β-glucosidase was supplemented to the biomass hydrolyzing enzyme preparation. The cocktail supplemented with *A unguis* BGL preparation yielded 555mg/g sugar in 12h compared to the commercial enzyme preparation which gave only 333mg/g in the same period and the maximum sugar yield of 858 mg/g was attained in 36h by the cocktail containing *A. unguis* BGL. While the commercial enzyme achieved almost similar sugar yield in 24h, there was rapid drop in sugar concentration after that, indicating probably the conversion of glucose back to di-or oligosaccharides by the transglycosylation activity of the BGl in that preparation. Compared this, the *A.unguis* enzyme containing preparation supported peak yields for longer duration (upto 48h) which is important for biomass conversion to other products since the hydrolysate has to undergo certain unit operations before it goes into the next stage ie – fermentation in any bioprocesses for production of either fuels or chemicals.. Most importantly the *Aspergillus unguis* BGL preparation yields approximately 1.6 fold increase in the sugar release compared to the commercial BGL within 12h of time interval and 2.25 fold increase in the sugar release compared to the control ie. Cellulase without BGL supplementation.

The current study therefore leads to the identification of a potent new isolate producing glucose tolerant β- glucosidase. The organism identified as *Aspergillus unguis* comes under the *Aspergillus nidulans* group where most of the GT-BGL producers belong and the detailed studies showed that the glucose tolerant β-glucosidase was a very low molecular weight protein which probably belongs to the glycosyl hydrolase family 3. Inhibition kinetic studies helped to understand the K_i and it is the second highest among the *nidulans* group of *Aspergilli*. This has promoted us for a detailed study regarding the mechanism of glucose tolerance. The proteomic

analyses clearly indicate the presence of GH3 catalytic domain in the protein. Since the size of the protein is very low and still its active and showed glucose tolerance it is speculated that this could be an entirely new protein or the modification of the existing β-glucosidase with only the catalytic domain present in it. Hydrolysis experiments also qualify this BGL, a suitable candidate for the enzyme cocktail development for biomass hydrolysis.

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APPENDIX 1

LIST OF ABBREVIATIONS

#	Number
%	Percentage
µ	Micron/ micrometer
µg	microgram
µM	micromolar
µl	microlitre
°C	Degree Celsius
ANOVA	Analysis of Variance
BGL	β-glucosidase
BLAST	Basic Local Alignment Search Tool
bp	base pair
CBH	Cellobiohydrolase
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
Da	Dalton
DNA	Deoxy Ribonucleic Acid
dNTP	Deoxyribonucleotide triphosphate
DNS	3, 5 Dinitro Salicylic Acid
DOE	Design of Experiments
EDTA	Ethylene Diamine Tetra Acetic Acid
EG	Endoglucanase
ESI	Electron Spray Ionization
Fig	Figure
FPAase	Filter Paper Hydrolyzing Activity
FPU	Filter paper units
g	gram
g/g	gram per gram
g/L	gram per litre
GBGL	Glucose tolerant β-glucosidase
gds	Grams dry substrate
GH	Glycosyl Hydrolase
GH1	Glycosyl Hydrolase Family 1
GH3	Glycosyl Hydrolase Family 3

GH5	Glycosyl Hydrolase Family 5
GH9	Glycosyl Hydrolase Family 9
GH30	Glycosyl Hydrolase Family 30
h	hour
IU	International Units
IUPAC	International Union for Pure and Applied Chemistry.
kDa	kilo Daltons
Ki	Inhibition constant
Km	Michaelis constant
Ki'	Dissociation constant
L	Liter
LSU	Large Subunit
lbs	Pounds Inch $^{-2}$
M	Molar
MALDI	Matrix Assisted Laser Desorption Ionization
mg	milligram
mg/g	milligram per gram
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	Poly Acrylamide Gel Electrophoresis
PCR	Polymerase chain reaction
PDB	Potato Dextrose Broth
PDA	Potato Dextrose Agar
pI	Isoelectric Point
pNP	para-Nitrophenol
pNPG	para –Nitrophenyl β-D glucopyranoside
RNA	Ribonucleic Acid
rpm	Rotations per minute
rRNA	Ribosomal RNA
SEB	Steam Exploded Sugarcane Bagasse
SDS	Sodium Dodecyl Sulfate

SEM	Scanning Electron Microscope
SmF	Submerged Fermentation
sp	Species
SSF	Solid State Fermentation
SSU	Small Subunit
TAIL PCR	Thermal Asymmetric Interlaced Polymerase Chain Reaction
TFF	Tangential Flow Filtration
TOF	Time of Flight
U	Units
U/gds	Units per gram dry substrate
U/ml	Units/milliliter
UV	Ultra Violet
v/v	Volume /Volume
Vmax	Maximum Velocity
w/v	Weight/Volume
WB	Wheat Bran
YEP broth	Yeast Extract Peptone Broth